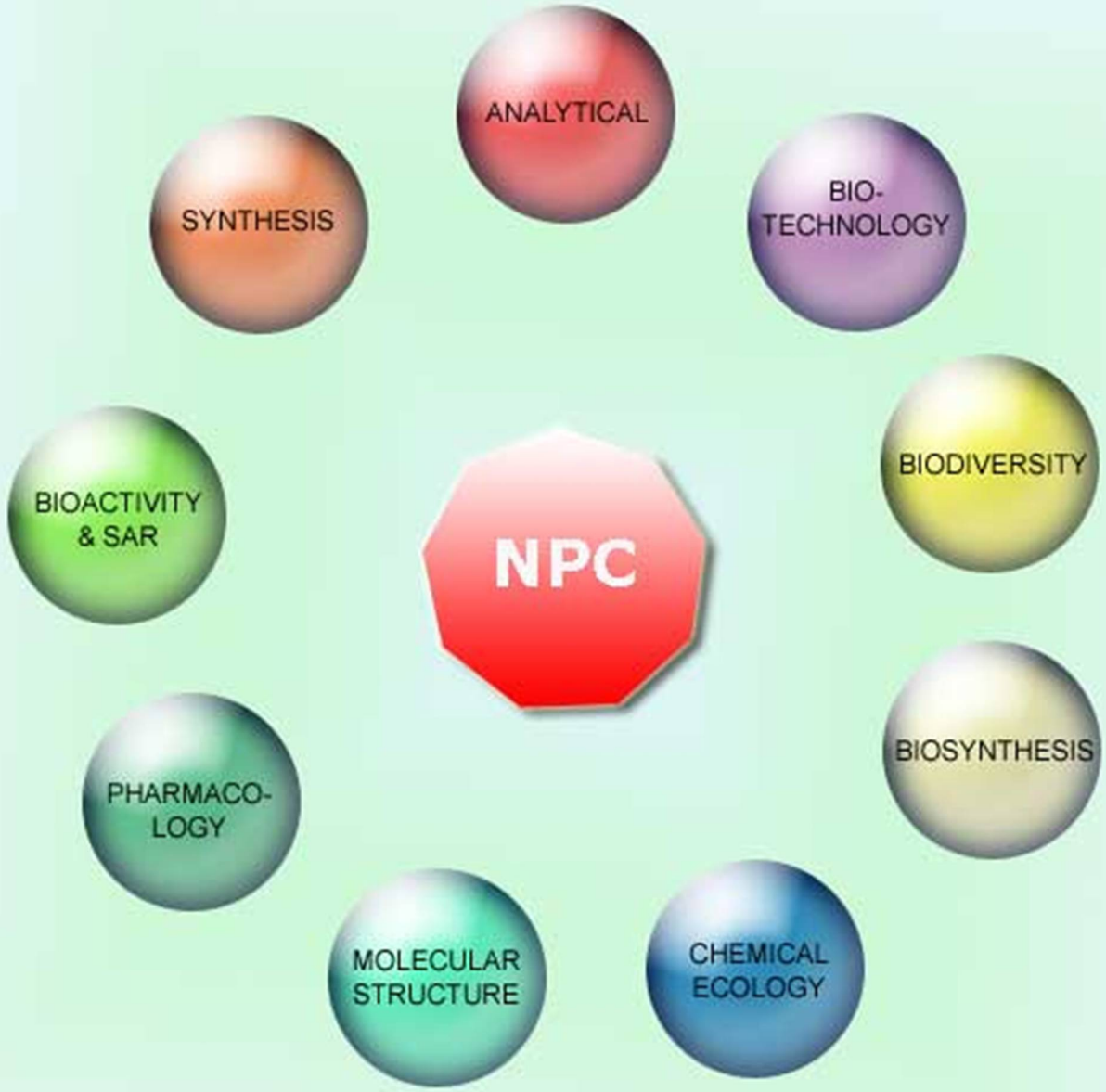
# NATURAL PRODUCT COMMUNICATIONS

**An International Journal for Communications and Reviews Covering all Aspects of Natural Products Research**




Volume 7. Issue 12. Pages 1557-1690. 2012
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

# NPC Natural Product Communications



**EDITOR-IN-CHIEF**

**DR. PAWAN K AGRAWAL**
*Natural Product Inc.*
*7963, Anderson Park Lane,*
*Westerville, Ohio 43081, USA*
*agrawal@naturalproduct.us*

**HONORARY EDITOR**

**PROFESSOR GERALD BLUNDEN**
*The School of Pharmacy & Biomedical Sciences,*
*University of Portsmouth,*
*Portsmouth, PO1 2DT U.K.*
*axuf64@dsl.pipex.com*

**EDITORS**

**PROFESSOR ALEJANDRO F. BARRERO**
*Department of Organic Chemistry,*
*University of Granada,*
*Campus de Fuente Nueva, s/n, 18071, Granada, Spain*
*afbarre@ugr.es*

**PROFESSOR ALESSANDRA BRACA**
*Dipartimento di Chimica Bioorganicae Biofarmacia,*
*Universita di Pisa,*
*via Bonanno 33, 56126 Pisa, Italy*
*braca@farm.unipi.it*

**PROFESSOR DEAN GUO**
*State Key Laboratory of Natural and Biomimetic Drugs,*
*School of Pharmaceutical Sciences,*
*Peking University,*
*Beijing 100083, China*
*gda5958@163.com*

**PROFESSOR YOSHIHIRO MIMAKI**
*School of Pharmacy,*
*Tokyo University of Pharmacy and Life Sciences,*
*Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan*
*mimakiy@ps.toyaku.ac.jp*

**PROFESSOR STEPHEN G. PYNE**
*Department of Chemistry*
*University of Wollongong*
*Wollongong, New South Wales, 2522, Australia*
*spyne@uow.edu.au*

**PROFESSOR MANFRED G. REINECKE**
*Department of Chemistry,*
*Texas Christian University,*
*Forts Worth, TX 76129, USA*
*m.reinecke@tcu.edu*

**PROFESSOR WILLIAM N. SETZER**
*Department of Chemistry*
*The University of Alabama in Huntsville*
*Huntsville, AL 35809, USA*
*wsetzer@chemistry.uah.edu*

**PROFESSOR YASUHIRO TEZUKA**
*Institute of Natural Medicine*
*Institute of Natural Medicine, University of Toyama,*
*2630-Sugitani, Toyama 930-0194, Japan*
*tezuka@inm.u-toyama.ac.jp*

**PROFESSOR DAVID E. THURSTON**
*Department of Pharmaceutical and Biological Chemistry,*
*The School of Pharmacy,*
*University of London, 29-39 Brunswick Square,*
*London WC1N 1AX, UK*
*david.thurston@pharmacy.ac.uk*

**ADVISORY BOARD**

Prof. Berhanu M. Abegaz
*Gaborone, Botswana*

Prof. Viqar Uddin Ahmad
*Karachi, Pakistan*

Prof. Øyvind M. Andersen
*Bergen, Norway*

Prof. Giovanni Appendino
*Novara, Italy*

Prof. Yoshinori Asakawa
*Tokushima, Japan*

Prof. Lee Banting
*Portsmouth, U.K.*

Prof. Julie Banerji
*Kolkata, India*

Prof. Anna R. Bilia
*Florence, Italy*

Prof. Maurizio Bruno
*Palermo, Italy*

Prof. César A. N. Catalán
*Tucumán, Argentina*

Prof. Josep Coll
*Barcelona, Spain*

Prof. Geoffrey Cordell
*Chicago, IL, USA*

Prof. Ana Cristina Figueiredo
*Lisbon, Portugal*

Prof. Cristina Gracia-Viguera
*Murcia, Spain*

Prof. Duvvuru Gunasekar
*Tirupati, India*

Prof. Kurt Hostettmann
*Lausanne, Switzerland*

Prof. Martin A. Iglesias Arteaga
*Mexico, D. F, Mexico*

Prof. Leopold Jirovetz
*Vienna, Austria*

Prof. Karsten Krohn
*Paderborn, Germany*

Prof. Hartmut Laatsch
*Gottingen, Germany*

Prof. Marie Lacaille-Dubois
*Dijon, France*

Prof. Shoei-Sheng Lee
*Taipei, Taiwan*

Prof. Francisco Macias
*Cadiz, Spain*

Prof. Imre Mathe
*Szeged, Hungary*

Prof. Joseph Michael
*Johannesburg, South Africa*

Prof. Ermino Murano
*Trieste, Italy*

Prof. M. Soledade C. Pedras
*Saskatoon, Canada*

Prof. Luc Pieters
*Antwerp, Belgium*

Prof. Peter Proksch
*Düsseldorf, Germany*

Prof. Phila Raharivelomanana
*Tahiti, French Polynesia*

Prof. Luca Rastrelli
*Fisciano, Italy*

Prof. Monique Simmonds
*Richmond, UK*

Prof. John L. Sorensen
*Manitoba, Canada*

Prof. Valentin Stonik
*Vladivostok, Russia*

Prof. Winston F. Tinto
*Barbados, West Indies*

Prof. Sylvia Urban
*Melbourne, Australia*

Prof. Karen Valant-Vetschera
*Vienna, Austria*


## INFORMATION FOR AUTHORS

Full details of how to submit a manuscript for publication in Natural Product Communications are given in Information for Authors on our Web site http://www.naturalproduct.us.


Authors may reproduce/republish portions of their published contribution without seeking permission from NPC, provided that any such republication is accompanied by an acknowledgment (original citation)-Reproduced by permission of Natural Product Communications. Any unauthorized reproduction, transmission or storage may result in either civil or criminal liability.

The publication of each of the articles contained herein is protected by copyright. Except as allowed under national "fair use" laws, copying is not permitted by any means or for any purpose, such as for distribution to any third party (whether by sale, loan, gift, or otherwise); as agent (express or implied) of any third party; for purposes of advertising or promotion; or to create collective or derivative works. Such permission requests, or other inquiries, should be addressed to the Natural Product Inc. (NPI). A photocopy license is available from the NPI for institutional subscribers that need to make multiple copies of single articles for internal study or research purposes.



**To Subscribe**: Natural Product Communications is a journal published monthly. 2012 subscription price: US$1,995 (Print, ISSN# 1934-578X); US$1,995 (Web edition, ISSN# 1555-9475); US$2,495 (Print + single site online); US$595 (Personal online). Orders should be addressed to Subscription Department, Natural Product Communications, Natural Product Inc., 7963 Anderson Park Lane, Westerville, Ohio 43081, USA. Subscriptions are renewed on an annual basis. Claims for nonreceipt of issues will be honored if made within three months of publication of the issue. All issues are dispatched by airmail throughout the world, excluding the USA and Canada.

# Natural Product Communications
# 2012
Volume 7, Number 12

## Contents

***Original Paper*** ***Page***

**A Comparative Study of the Antioxidant/Prooxidant Effects of Carvacrol and Thymol at Various Concentrations on Membrane and DNA of Parental and Drug Resistant H1299 Cells**
Aysun Ozkan and Ayse Erdogan **1557**

**New Secoiridoid Glycosides from the Buds of *Lonicera macranthoides***
Jiang Liu, Jing Zhang, Feng Wang and Xingfu Chen **1561**

**Chemical Constituents from the Aerial Parts of *Gynura bicolor***
Jian Chen, Sven Mangelinckx, An Adams, Wei-lin Li, Zheng-tao Wang and Norbert De Kimpe **1563**

**Chemical Constituents of *Ligularia nelumbifolia* and *L. subspicata* Hybrid Collected in Shangrila County, Yunnan Province of China**
Ryo Hanai, Hiroka Yamada, Yurika Suzuki, Hajime Nagano, Takayuki Kawahara, Jiao-Jun Yu, Xun Gong and Chiaki Kuroda **1565**

**A Practical, Enantiospecific Synthesis of (*S*)-*trans*-γ-Monocyclofarnesol**
Stefano Serra **1569**

**ACAT Inhibitory Activity of Exudates from *Calocedrus macrolepis* var. *formosana***
Yu-Hsin Hsieh, Kuan-Jung Chen, Shih-Chang Chien, Wen-Ling Cheng, Jun-Hong Xiao and Sheng-Yang Wang **1573**

**Cucurbitane-Type Triterpenoids from the Fruit Pulp of *Momordica charantia***
Yun-Wen Liao, Chiy-Rong Chen, Yueh-Hsiung Kuo, Jue-Liang Hsu, Wen-Ling Shih, Hsueh-Ling Cheng, Tzou-Chi Huang and Chi-I Chang **1575**

**Two Novel Phenethylamine Alkaloids from *Streptomyces* sp. YIM10049**
Xueqiong Yang, Guangwei He, Lixing Zhao, Yabin Yang, Yun Liu, Lihua Xu and Zhongtao Ding **1579**

**Alkaloids from *Cinnamomum philippinense***
Hsing-Tan Li, Wei-Jen Li, Hui-Ming Wu and Chung-Yi Chen **1581**

**Conversional Synthesis of Heteratisine**
Ling Wang, Qi-Feng Chen and Feng-Peng Wang **1583**

**Flavonoids and Triterpenes from the leaves of *Artocarpus fulvicortex***
Shajarahtunnur Jamil, Muhammad Taher, Hasnah M. Sirat and Nur Azlin Othman **1587**

**Dihydrospinochalcone-A and Epi-flemistrictin-B, Natural Isocordoin Derivatives from the Root Extract of *Lonchocarpus xuul***
Fabiola Escalante-Erosa, Brenda González-Morales, Ramiro F. Quijano-Quiñones, Gumersindo Miron-López and Luis M. Peña-Rodríguez **1589**

**Phenolic Compounds, Including Novel *C*-glycosylflavone, from the Flowers of the Tall Bearded Iris Cultivar 'Victoria Falls'**
Takayuki Mizuno, Tsutomu Yabuya, Nobuhiro Sasaki and Tsukasa Iwashina **1591**

**Two New Biisoflavonoids from the Roots of *Daphne oleoides***
Shazia Yasmeen, Muhammad Aijaz Anwar, Sadia Ferheen, Nighat Afza, Abdul Malik and Lubna Iqbal **1595**

**Biflavonoids from the Unripe Fruits of *Clusia paralicola* and their Antioxidant Activity**
Rafaela Ferreira Oliveira, Celso Amorim Camara, Maria de Fátima Agra and Tania Maria Sarmento Silva **1597**

**Ochnaflavone and Ochnaflavone 7-*O*-methyl Ether two Antibacterial Biflavonoids from *Ochna pretoriensis* (Ochnaceae)**
Tshepiso J. Makhafola, Babatunde B. Samuel, Esameldin E. Elgorashi and Jacobus N. Eloff **1601**

**Factors Affecting the Separation and Bioactivity of Red Clover (*Trifolium pratense*) Extracts Assayed against *Clostridium sticklandii*, a Ruminal Hyper Ammonia-producing Bacterium**
Isabelle A. Kagan and Michael D. Flythe **1605**

**Exploitation of the Antioxidant Potential of *Geranium macrorrhizum* (Geraniaceae): Hepatoprotective and Antimicrobial Activities**
Niko S. Radulović, Milan B. Stojković, Snežana S. Mitić, Pavle J. Randjelović, Ivan R. Ilić, Nikola M. Stojanović and Zorica Z. Stojanović-Radić **1609**

**Differential Expression of Benzophenone Synthase and Chalcone Synthase in *Hypericum sampsonii***
Lili Huang, Hong Wang, Hechun Ye, Zhigao Du, Yansheng Zhang, Ludger Beerhues and Benye Liu **1615**

**Efficient Preparation of Hangekobokuto (Banxia-Houpo-Tang) Decoction by Adding Perilla Herb before Decoction is Finished**
Megumi Sumino, Yuko Saito, Fumio Ikegami, Yoshiro Hirasaki and Takao Namiki **1619**

**Anti-neutrophilic Inflammatory Secondary Metabolites from the Traditional Chinese Medicine, Tiankuizi**
Chia-Lin Lee, Tsong-Long Hwang, Chieh-Yu Peng, Chao-Jung Chen, Yuan-Shiun Chang and Yang-Chang Wu **1623**

**DART MS Based Chemical Profiling for Therapeutic Potential of *Piper betle* Landraces**
Vikas Bajpai, Renu Pandey, Mahendra Pal Singh Negi, Nikhil Kumar and Brijesh Kumar **1627**

**Total Phenolic Content and Antioxidative Properties of Commercial Tinctures Obtained from Some Lamiaceae Plants**
Adam Kowalczyk, Izabela Biskup and Izabela Fecka **1631**

**Synthesis, Antifungal Activity and Structure-Activity Relationships of Vanillin oxime-*N*-*O*-alkanoates**
Vivek Ahluwalia, Nandini Garg, Birendra Kumar, Suresh Walia and Om P. Sati **1635**

**Cinnamic Acid Derivatives Acting Against *Aspergillus* Fungi. *Taq* Polymerase I a Potential Molecular Target**
Lucas J. Gutierrez, Maria L. Mascotti, Marcela Kurina-Sanz, Carlos R. Pungitore, Ricardo D. Enriz and Fernando A. Giannini **1639**

**Insecticidal Effects of Acetogenins from *Rollinia occidentalis* Seed Extract**
Diego Tolosa, Olga Álvarez Colom, Alicia Bardón and Adriana Neske **1645**

*Continued Overleaf*

**Conservation and Multiplication of Encapsulated Micro Shoots of *Rauvolfia vomitoria* - an Endangered Medicinal Tree: ISSR and RAPD based Evaluation of Genetic Fidelity of Converted Plantlets**
Shakti Mehrotra, Liaq Ur Rahman, Jahnvi Mishra and Arun K. Kukreja **1647**

**Bioactivities of Volatile Components from Nepalese *Artemisia* Species**
Prabodh Satyal, Prajwal Paudel, Ananad Kafle, Suraj K. Pokharel, Bimala Lamichhane, Noura S. Dosoky, Debra M. Moriarity and William N. Setzer **1651**

**Chemical Compositions, Antioxidant and Antimicrobial Activity of the Essential Oils of *Piper officinarum* (Piperaceae)**
Wan Mohd Nuzul Hakimi Wan Salleh, Farediah Ahmad, Khong Heng Yen and Hasnah Mohd Sirat **1659**

**Chemical Composition, Antimicrobial and Antioxidant Properties of the Volatile Oil and Methanol Extract of *Xenophyllum poposum***
Ana M. González, María I. Tracanna, Sara M. Amani, Carola Schuff, Miguel J. Poch, Horacio Bach and César A. N. Catalán **1663**

**Phytochemical and Micromorphological Traits of Endemic *Micromeria pseudocroatica* (Lamiaceae)**
Dario Kremer, Ivna Dragojević Müller, Edith Stabentheiner, Dubravka Vitali, Martina Kopričanec, Mirko Ruščić, Ivan Kosalec, Nada Bezić and Valerija Dunkić **1667**

**Composition and Variability of the Essential Oil of *Salvia nemorosa* (Lamiaceae) from the Vienna Area of Austria**
Remigius Chizzola **1671**

**Essential oil Composition of *Ficus benjamina* (Moraceae) and *Irvingia barteri* (Irvingiaceae)**
Isiaka A. Ogunwande, Razaq Jimoh, Adedoyin A. Ajetunmobi, Nudewhenu O. Avoseh and Guido Flamini **1673**

## *Review/Account*

**Cytotoxic Agents of the Crinane Series of Amaryllidaceae Alkaloids**
Jerald J. Nair, Jaume Bastida, Francesc Viladomat and Johannes van Staden **1677**

## *Cummulative Index*

***Contents*** ***i-xvii***
***Author Index*** ***i-vii***
***Keywords Index*** ***i-vi***

## *Manuscripts in press*

**1689**

## LIST OF AUTHORS

Adams, A ..... 1563
Afza, N ..... 1595
Agra,MF ..... 1597
Ahluwalia, V ..... 1635
Ahmad, F ..... 1659
Ajetunmobi, AA ..... 1673
Amani, SM ..... 1663
Anwar, MA ..... 1595
Avoseh, NO ..... 1673

Bach, H ..... 1663
Bajpai, V ..... 1627
Bardón, A ..... 1645
Bastida, J ..... 1677
Beerhues, L ..... 1615
Bezić, N ..... 1667
Biskup, I ..... 1631

Camara, CA ..... 1597
Catalán, CAN ..... 1663
Chang, CI ..... 1575
Chang, YS ..... 1623
Chen, CJ ..... 1623
Chen, CR ..... 1575
Chen, CY ..... 1581
Chen, J ..... 1563
Chen, KJ ..... 1573
Chen, QF ..... 1583
Chen, X ..... 1561
Cheng, HL ..... 1575
Cheng, WL ..... 1573
Chien, SC ..... 1573
Chizzola, R ..... 1671
Colom, OÁ ..... 1645

De Kimpe, N ..... 1563
Ding, Z ..... 1579
Du, Z ..... 1615
Dunkić, V ..... 1667

Elgorashi, EE ..... 1601
Eloff, JN ..... 1601
Enriz, RD ..... 1639
Erdogan, A ..... 1557
Escalante-Erosa, F ..... 1589

Fecka, I ..... 1631
Ferheen, S ..... 1595
Flamini, G ..... 1673
Flythe, MD ..... 1605

Garg, N ..... 1635
Giannini, FA ..... 1639
Gong, X ..... 1565
González, AM ..... 1663
González-Morales, B ..... 1589
Gutierrez, LJ ..... 1639

Hanai, R ..... 1565
He, G ..... 1575
Hirasaki, Y ..... 1619
Hsieh, YH ..... 1573
Hsu, JL ..... 1575
Huang, L ..... 1615
Huang, TC ..... 1575
Hwang, TL ..... 1623

Ikegami, F ..... 1619
Ilić, IR ..... 1609
Iqbal, L ..... 1595
Iwashina, T ..... 1591

Jamil, S ..... 1587
Jimoh, R ..... 1673

Kagan, IA ..... 1605
Kawahara, T ..... 1565
Kopričanec, M ..... 1667
Kosalec, I ..... 1667
Kowalczyk, A ..... 1631
Kremer, D ..... 1667
Kukreja, AK ..... 1647
Kumar, B ..... 1627,1635
Kumar, N ..... 1627
Kuo, YH ..... 1575
Kurina-Sanz, M ..... 1639
Kuroda, C ..... 1565

Lee, CL ..... 1623
Li, HT ..... 1581
Li, W ..... 1563
Li, WJ ..... 1581
Liao, YW ..... 1575
Liu, B ..... 1615
Liu, J ..... 1561
Liu, Y ..... 1579

Makhafola, TJ ..... 1601
Malik, A ..... 1595
Mangelinckx, S ..... 1563
Mascotti, ML ..... 1639
Mehrotra, S ..... 1647
Miron-López, G ..... 1589
Mishra, J ..... 1647
Mitić, SS ..... 1609
Mizuno, T ..... 1591
Müller, ID ..... 1667

Nagano, H ..... 1565
Nair, JJ ..... 1677
Namiki, T ..... 1619
Negi, MPS ..... 1627
Neske, A ..... 1645

Ogunwande, IA ..... 1673
Oliveira, RF ..... 1597
Othman, NA ..... 1587
Ozkan, A ..... 1557

Pandey, R ..... 1627
Peña-Rodríguez, LM ..... 1589
Peng, CY ..... 1623
Poch, MJ ..... 1663
Pungitore, CR ..... 1639

Quijano-Quiñones, RF ..... 1589

Radulović, NS ..... 1609
Rahman, LU ..... 1647
Randjelović, PJ ..... 1609
Ruščić, M ..... 1667

Saito, Y ..... 1619
Salleh, WMNHW ..... 1659
Samuel, BB ..... 1601
Sasaki, N ..... 1591
Sati, OP ..... 1635
Schuff, C ..... 1663
Serra, S ..... 1569
Setzer, WN ..... 1651
Shih, WL ..... 1575
Silva, TMS ..... 1597
Sirat, HM ..... 1587
Sirat, HM ..... 1659
Stabentheiner, E ..... 1667
Stojanović, NM ..... 1609
Stojanović-Radić, ZZ ..... 1609
Stojković, NB ..... 1609
Sumino, M ..... 1619
Suzuki, Y ..... 1565

Taher, M ..... 1587
Tolosa, D ..... 1645
Tracanna, MI ..... 1663

van Staden, J ..... 1677
Viladomat, F ..... 1677
Vitali, D ..... 1667

Walia, S ..... 1635
Wang, F ..... 1561
Wang, FP ..... 1583
Wang, H ..... 1615
Wang, L ..... 1583
Wang, SY ..... 1573
Wang, Z ..... 1563
Wu, HM ..... 1581
Wu, YC ..... 1623

Xiao, JH ..... 1573
Xu, L ..... 1579

Yabuya, T ..... 1591
Yamada, H ..... 1565
Yang, X ..... 1575
Yang, Y ..... 1575
Yasmeen, S ..... 1595
Ye, H ..... 1615
Yen, KH ..... 1659
Yu, J ..... 1565

Zhang, J ..... 1561
Zhang, Y ..... 1615
Zhao, L ..... 1575

NPC Natural Product Communications

2012
Vol. 7
No. 12
1557 - 1560

# A Comparative Study of the Antioxidant/Prooxidant Effects of Carvacrol and Thymol at Various Concentrations on Membrane and DNA of Parental and Drug Resistant H1299 Cells

**Aysun Ozkan* and Ayse Erdogan**

[a]*Department of Biology*, *Faculty of Science*, *Akdeniz University*, *07058*, *Antalya*, *Turkey*

*aozkan@akdeniz.edu.tr*

**Received: March 8th, 2012; Accepted: October 16th, 2012**

Carvacrol and thymol, both used as flavor agents in cosmetic and food products, have prooxidant and antioxidant activities. To clarify the mechanisms of their cytotoxicity and the factors affecting their antioxidant/prooxidant activities, we investigated cell membrane and DNA damage induced by carvacrol and thymol in parental and drug-resistant human lung cancer cell lines. After 24 and 48 hour incubation periods, the cytotoxicity of carvacrol ($IC_{50}$ 380 and 244 μM) was found to be higher than that of thymol ($IC_{50}$ 497 and 266 μM) in parental cells. However, thymol showed higher cytotoxic effects in drug resistant H1299 cells for three incubation periods. Also, carvacrol and thymol, at higher concentrations, increased malondealdehyde (MDA) levels causing membrane damage and 8-hydroxy deoxyguanozine (8-OHdG) levels, causing DNA damage to both parental and drug resistant cells. On the other hand, carvacrol and thymol protected the cells against $H_2O_2$-induced cytotoxicity, and membrane and DNA damage when the cells were preincubated with these two compounds at lower concentration ($<IC_{50}$) before $H_2O_2$ incubation. These findings suggest that carvacrol and thymol exhibit protective/damaging effects depending on cell resistance, concentration and time.

**Keywords:** Carvacrol, Thymol, DNA, Membrane, Anticancer, Antioxidant.

Monoterpenes are highly hydrophobic substances that exert a wide spectrum of biological actions of great importance in many different areas [1,2]. Thymol (5-methyl-2-(1-methylethyl) phenol) is an isomer of carvacrol (5-isopropyl-2-methyl phenol), having the hydroxyl group at a different location on the phenolic ring. The hydrophobic nature of carvacrol and thymol enables them to react with the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell components [3].

Natural antioxidants are considered useful agents for the prevention of diseases [4-7]. Many studies have shown that phenolic compounds in plant essential oils display antioxidant activity as a result of their capacity to scavenge free radicals [8-10]. On the other hand, effects of antioxidant concentrations on oxidation reactions depend on many factors such as structure of the antioxidant, oxidation conditions and changing of the oxidized structure. Phenolic antioxidants lose their antioxidant effects at higher concentrations and gain a prooxidant structure. They can either protect DNA and membranes against oxidants as an antioxidant at lower concentrations or damage DNA and membranes as a prooxidant at higher concentrations. Recent studies reveal that anti-/pro-oxidant and toxic properties of these molecules change depending on concentration, the kind of cells and organism, and so are not safe for human being [11].

Tumors are heterogeneous in many respects, including chemotherapeutic susceptibility [12]. Resistance to chemo-therapeutic agents is a major problem in the treatment of patients with small cell (SCLC) and non-small cell lung cancer (NSCLC). Acquired multidrug resistance is the main obstacle for the cure of SCLC. A group of drug resistance cells can occur in tumors during chemotherapy. The development of drug resistance and detoxifying mechanisms in human and mice microsome enzymes CYP3A4 used eucalyptol as a substrate [13,14]. We tried to prove the ability of carvacrol and thymol in preventing cytotoxicity, membrane damage and DNA damage induced by the oxidative agent $H_2O_2$, while they also have cytotoxic and damaging effects in parental and drug resistant H1299 cells because many phenolic components have shown various protective/damaging activities in different biological systems dependant on concentration.

Thymol and carvacrol showed cytotoxic effects on parental and drug resistance H1299 cells (Figure 1 and 2). After 24 and 48 hour incubation periods, the cytotoxicity of carvacrol was found to be higher {380 and 244 μM ($IC_{50}$) concentrations} than that of thymol ($IC_{50}$ 497 and 266 μM) in parental cells. However, thymol showed higher cytotoxic effects in drug resistant H1299 cells for three incubation periods. The viability of cells decreased between 25-1000 μM carvacrol and 10-1000 μM thymol concentrations. The cytotoxic effect was close to that of the control until 25 μM concentrations for carvacrol and 5 μM concentrations for thymol. So, neither compound had either an effective cytotoxic or antitumor effect on cancer cells at lower concentrations. Carvacrol and thymol exhibited dose- and incubation time-dependent cytotoxic effects on parental and drug resistant H1299 cells. Also, the effects changed depending on the drug resistance capacity of the target cells. In one study, carvacol and thymol had a dose-dependent antiproliferative effects on Hep G2 cells, which make them potentially interesting for adjuvant experimental cancer treatments.

Both compounds induced membrane damage and cytotoxicity in hepatoma G2 at relatively higher concentrations than those that mediate its anticancer activities. The induction of cytotoxic cell death can be accompanied by membrane damage [2]. Koparal and Zeytinoglu [15] also observed that carvacrol was a very potent inhibitor of cell growth in the A549 cancer cell line. In another study, carvacrol and thymol had dose-dependent antiproliferative effects on human uterine carcinoma cells [16]. Also, carvacrol and thymol differed in their cytotoxic effects on K562 cells [17]. All these studies support our results.

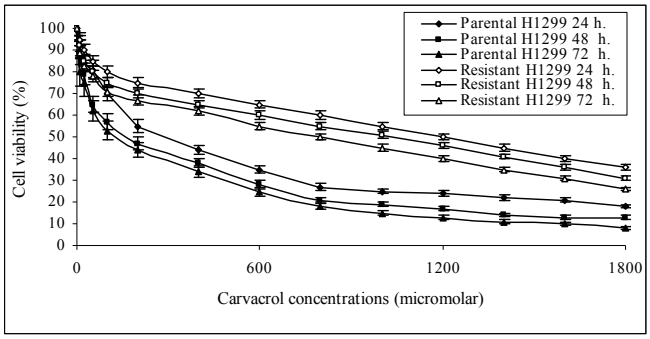



**Figure 1**: Cytotoxic effects of carvacrol on parental and drug-resistant H1299 cells.

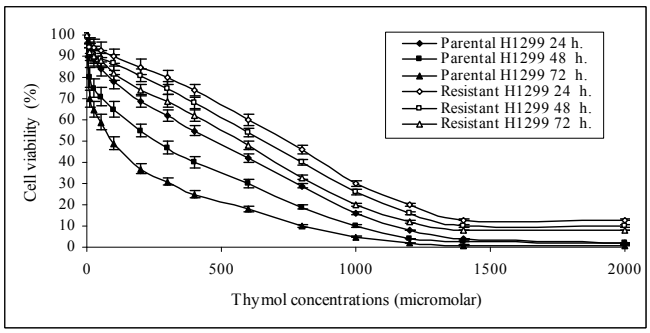



**Figure 2**: Cytotoxic effects of thymol on parental and drug-resistant H1299 cells.

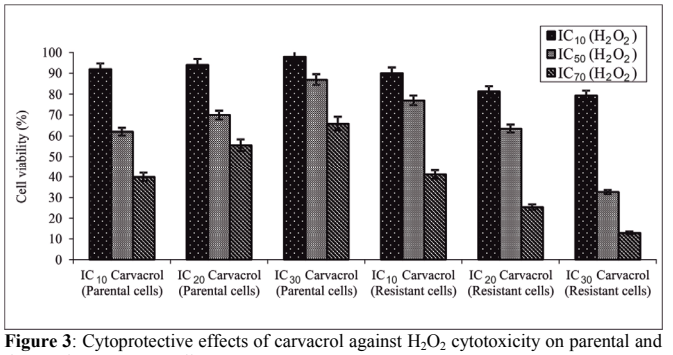



**Figure 3**: Cytoprotective effects of carvacrol against $H_2O_2$ cytotoxicity on parental and drug-resistant H1299 cells.

We measured the cytoprotective (antioxidant) effect of carvacrol and thymol against the cytotoxicity of the strong oxidant $H_2O_2$ in parental and drug-resistant H1299 cells. Figures 3 and 4 show the levels of $H_2O_2$-induced cytotoxicity in the cells pre-incubated with different concentrations of carvacrol and thymol. Carvacrol had a strong antioxidant effect at an $IC_{30}$ concentration for parental cells and an $IC_{10}$ concentration for resistant cells against $H_2O_2$ cytotoxicity (Figure 3).

Also, thymol decreased $H_2O_2$ cytotoxicity in both cells. The maximum antioxidant effect of thymol was found at an $IC_{20}$ concentration for parental cells and $IC_{10}$ concentration for resistant cells (Figure 4). So, resistant cells can protect themselves with lower concentrations of thymol and carvacrol against $H_2O_2$ cytotoxicity than parental cells. We can assume that drug resistant cells show more resistance to cytotoxicity than parental cells.

In this study, carvacrol and thymol increased malondealdehyde (MDA) and 8-OHdG levels in both parental and drug resistant cells at different concentrations (Table 1). While increasing MDA amounts cause membrane damage, increases in 8-OHdG show the DNA damaging effect of carvacrol and thymol on the cells. The membrane and DNA damaging effect of the compounds on both

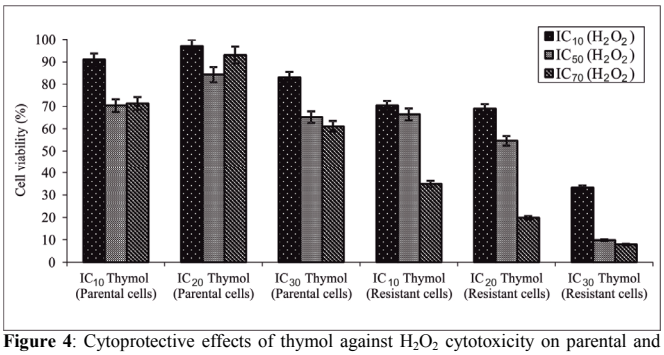



**Figure 4**: Cytoprotective effects of thymol against $H_2O_2$ cytotoxicity on parental and drug-resistant H1299 cells.

**Table 1**: Membrane and DNA damaging effects of carvacrol and thymol on parental and drug resistant H1299 cells

| Concentrations | MDA (nmol/mg protein) X ± SE | 8-OHdG (ng/mL) X ± SE |
|---|---|---|
| $IC_{10}$ Carvacrol (P) | 0.35 ± 0.02 a | 0.09 ± 0.03 a |
| $IC_{50}$ Carvacrol (P) | 0.60 ± 0.03 ab | 0.10 ± 0.04 a |
| $IC_{70}$ Carvacrol (P) | 1.67 ± 0.07 bc | 0.15 ± 0.03 ab |
| $IC_{10}$ Carvacrol (R) | 0.31 ± 0.05 a | 0.08 ± 0.03 a |
| $IC_{50}$ Carvacrol (R) | 0.56 ± 0.08 ab | 0.09 ± 0.02 a |
| $IC_{70}$ Carvacrol (R) | 1.12 ± 0.12 b | 0.12 ± 0.09 ab |
| $IC_{10}$ Thymol (P) | 0.70 ± 0.13 ab | 0.08 ± 0.04 a |
| $IC_{50}$ Thymol (P) | 1.90 ± 0.21 bc | 0.08 ± 0.03 a |
| $IC_{70}$ Thymol (P) | 2.71 ± 0.44 cd | 0.10 ± 0.02 a |
| $IC_{10}$ Thymol (R) | 0.62 ± 0.45 ab | 0.08 ± 0.04 a |
| $IC_{50}$ Thymol (R) | 1.14 ± 0.47 b | 0.09 ± 0.06 a |
| $IC_{70}$ Thymol (R) | 2.70 ± 0.85 cd | 0.15 ± 0.11 ab |
| Control | 0.31 ± 0.02 a | 0.08 ± 0.04 a |
| 0.5% DMSO | 0.33 ± 0.03 a | 0.08 ± 0.03 a |

Results are means of five different experiments. Values that are followed by different letters within each column are significantly different ($p \leq 0.05$). $df_1=2$, $df_2=95$, F=11.96. SE: Standard Error. P; parental, R; resistant cells

cells was found to be close to that of the control at $IC_{10}$ concentrations. The differences between the data are given in Table 1 ($p \leq 0.05$). The higher concentration of carvacrol and thymol caused statistically important membrane and DNA damage to the cells ($p \leq 0.05$). Parental cells were found to be more sensitive than resistant cells to the membrane damaging effects of both compounds. They induced membrane and DNA damage and cytotoxicity in H1299 cells at relatively higher concentrations than those that mediate its anticancer activities.

The induction of cytotoxic cell death can be accompanied by membrane and DNA damage. Koparal and Zeytinoglu [15] also observed that carvacrol was a very potent inhibitor of cell growth in the A549 cancer cell line. In another study, carvacrol and thymol had dose-dependent antiproliferative effects on human uterine carcinoma cells [16].

The present studies show that phenolic compounds have antioxidant/prooxidant properties under different conditions. Carvacrol and thymol significantly decreased membrane and DNA damage in $H_2O_2$ treated H1299 cells (Table 2). The selected protective concentrations are those that showed the highest protective effect against $H_2O_2$ cytotoxicity. In the cytoprotective study, while carvacrol had a strong antioxidant effect at $IC_{30}$ concentration for parental cells and $IC_{10}$ concentration for resistant cell, thymol showed maximum antioxidant effect at $IC_{20}$ concentration for parental cells and $IC_{10}$ concentration for resistant cells against $H_2O_2$ cytotoxicity (Figures 3, 4). Also, at these cytoprotective concentrations, carvacrol and thymol showed different membrane and DNA protective effects against $H_2O_2$ oxidation (Table 2). The most effective membrane protective effect of three concentrations ($IC_{10}$, $IC_{50}$, and $IC_{70}$) was found at $IC_{10}$ for

**Table 2**: Protective effects of carvacrol and thymol against $H_2O_2$ membrane and DNA damaging effects on parental and drug resistant H1299 cells

| Concentrations | MDA (nmol/mg prot.) X ± SE | 8-OHdG (ng/ml) X± SE |
|---|---|---|
| $IC_{20}$ Thymol + $IC_{10}$ $H_2O_2$ (P) | 0.86 ± 0.03 ab | 3.09 ± 0.44 d |
| $IC_{20}$ Thymol + $IC_{50}$ $H_2O_2$ (P) | 0.90 ± 0.08 ab | 8.00 ± 0.56 hi |
| $IC_{20}$ Thymol + $IC_{70}$ $H_2O_2$ (P) | 1.10 ± 0.65 b | 11.70 ± 0.98 lm |
| $IC_{10}$ Thymol + $IC_{10}$ $H_2O_2$ (R) | 0.95 ± 0.13 ab | 3.45 ± 0.22 d |
| $IC_{10}$ Thymol + $IC_{50}$ $H_2O_2$ (R) | 1.14 ± 0.82 b | 7.60 ± 0.32 hi |
| $IC_{10}$ Thymol + $IC_{70}$ $H_2O_2$ (R) | 1.78 ± 0.96 bc | 10.76 ± 0.67 kl |
| $IC_{10}$ Carvacrol+ $IC_{10}$ $H_2O_2$ (R) | 0.56 ± 0.09 ab | 2.35 ± 0.24 c |
| $IC_{10}$ Carvacrol+ $IC_{50}$ $H_2O_2$ (R) | 0.68 ± 0.07 ab | 7.10 ± 0.34 h |
| $IC_{10}$ Carvacrol+ $IC_{70}$ $H_2O_2$ (R) | 0.96 ± 0.08 ab | 10.45 ± 0.99 k |
| $IC_{30}$ Carvacrol + $IC_{10}$ $H_2O_2$ (P) | 0.93 ± 0.08 ab | 2.60 ± 0.14 cd |
| $IC_{30}$ Carvacrol + $IC_{50}$ $H_2O_2$ (P) | 1.10 ± 0.32 b | 8.00 ± 0.36 hi |
| $IC_{30}$ Carvacrol + $IC_{70}$ $H_2O_2$ (P) | 1.70± 0.51 bc | 12.00 ± 0.35 lm |
| $IC_{10}$ $H_2O_2$ (Control P) | 1.30 ± 0.03 b | 2.62 ± 0.03 cd |
| $IC_{50}$ $H_2O_2$ (Control P) | 1.80 ± 0.12 bc | 7.79 ± 0.50 hi |
| $IC_{70}$ $H_2O_2$ (Control P) | 2.40 ± 0.14 c | 11.97 ± 0.41 lm |
| $IC_{10}$ $H_2O_2$ (Control R) | 1.20 ± 0.23 b | 2.51 ± 0.14 cd |
| $IC_{50}$ $H_2O_2$ (Control R) | 1.60 ± 0.34 bc | 6.65 ± 0.31 gh |
| $IC_{70}$ $H_2O_2$ (Control R) | 2.10 ± 0.98 c | 10.55 ± 0.82 kl |
| Control | 0.31 ± 0.05 a | 0.10 ± 0.01 a |
| 0.5% DMSO | 0.32 ± 0.03 a | 0.11 ± 0.01 a |

Results are means of five different experiments. Values that are fallowed by different letters within each column are significantly different ($p \leq 0.05$). $df_1$=2, $df_2$=95, F=11.96. SE: Standard Error. P; parental, R; resistant cells.

carvacrol in resistant cells against $H_2O_2$ damage. Also, the $IC_{10}$ concentration of carvacrol had the highest DNA protective effect in resistant cells against $H_2O_2$ damage. This means that resistant cells have more membrane protective ability than parental cells.

Carvacrol and thymol have effective DNA protective effects in both cells at different concentrations. Aydin *et al*. [18] also observed that these compounds, at concentrations below 0.2 and 0.1 mM, respectively, significantly reduced the oxidative damage in human lymphocytes. In another study, the incubation of Hep G2 and Caco-2 cells in the presence of a range of concentrations of either carvacrol or thymol led, in both cases, to a significant protection of the cells studied from DNA strand breaks induced by the potent oxidant hydrogen peroxide [19]. Epigallocatechin-3-gallate (1 μM), a polyphenol abundant in tea, was shown to significantly reduce MDA production due to $H_2O_2/Fe^{2+}$ exposure, indicating a protection of cells from oxidative stress [20]. The malondialdehyde level increased in $H_2O_2$ exposed ($IC_{50}$ and $IC_{70}$) hepatoma G2 cells, but decreased in these cells preincubated with carvacrol and thymol before $H_2O_2$ exposure [2]. Carvacrol and thymol differed in their cytotoxic and genotoxic effects on K562 cells, and reduced the level of DNA damage induced by the strong oxidation of $H_2O_2$ [17]. Further understanding of the underlying mechanism of their protective effects in reducing intracellular oxygen radicals in H1299 cell death may lead to the development of new therapeutic treatments for cancer, since carvacrol and thymol provide protection against $H_2O_2$ insult. Their lung protective effects against $H_2O_2$ toxicity might be of importance and may contribute in part to their clinical efficacy for the treatment of lung carcinoma. These results suggest that carvacrol and thymol may be potentially valuable sources of natural therapeutic agents. Thus, it is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, have important cancer chemopreventive properties. In the present study, carvacrol and thymol induced DNA and membrane damage, and cytotoxicity in H1299 cells at relatively higher concentrations than those that mediate its anticancer activities. These findings suggest that carvacrol and thymol exhibit anticancer/antioxidant effects dependant on cell resistance, concentration and time.

## Experimental

***Cancer cell culture:*** The H1299 cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI 1640 medium supplemented with10% fetal calf serum, 1% antibiotic-antimycotic solution in a humidified atmosphere containing 5% $CO_2$ at 37°C. For subculturing, cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when monolayer confluence had reached 75%. The drug resistant (Epirubicin-resistant) H1299 tumor cells were derived from the parental line by stepwise selection in increasing concentrations of Epirubicin until the cells were capable of propagating in 220 ng/mL drug, as described previously [21,22].

***Cell viability assay:*** The cancer cells (10,000 cells/well, monolayer) were plated in a 96-well plate. The next day, the cells were treated with different concentrations of carvacrol and thymol in the medium for 24, 48 and 72 h. At the end of the incubation periods, the cytotoxicity of thymol and carvacrol on cancer cells was determined by the the cell titer-blue-cell viability assay. The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal [23]. Following cellular reduction, fluorescence is recorded at 560 nm excitation / 590 nm emission. The data were expressed as average values obtained from 8 wells for each concentration. The $IC_{50}$ value was calculated from the equation of the graph. $H_2O_2$ cytotoxicity on cancer cells was measured in the same way. For measuring the antioxidant effect of carvacrol and thymol against $H_2O_2$ cytotoxicity, the cells were preincubated with them at different concentrations (10–150 μg/mL) for 1 h, before hydrogen peroxide treatment for 24 h.

***Determination of malondealdehyde level:*** The cells were plated at a density 5–10 x $10^5$ cells/100 mm dish. The cells were preincubated with maximum cytoprotective concentrations of carvacrol and thymol for 1 h, before hydrogen peroxide treatment ($IC_{10}$, $IC_{50}$ and $IC_{70}$) for 24 h. Cells were scraped off the culture plates with culture medium and centrifuged at 400 × *g* for 10 min. The cell pellets were washed with PBS and then sonicated (3 × 15 sec) in 50 mM potassium phosphate, pH 7.2, containing 1 mM PMSF (Sigma) and 1 μg/mL of leupeptin (Sigma) and centrifuged at 150,000 × *g* for 1 h. The supernatant was used for the determination of malondealdehyde level, as described by Wasowicz *et al*. [24]. This fluorometric method for measuring thiobarbituric acid-reactive substances (TBARS) in supernatant is based on the reaction between malondialdehyde and thiobarbituric acid. The product of this reaction was extracted into *n*-butanol and measured spectrofluorometrically at 525 nm (excitation) and 547 nm (emission). Protein was determined by the Bradford method [25] with bovine serum albumin as a standard.

***Determination of 8-OHdG level:*** After DNA purification [26] from the cultured cells (Genomic DNA Mini Kit, Invitrogen), the genomic DNA samples were used to determine 8-OHdG with a competitive ELISA kit (Highly sensitive 8-OHdG Check New, Japan Institute for Control of Aging, Fukuroi, Shizuoka, Japan). Micro titer ELISA plates were precoated with 8-OHdG. Fifty μL of the sample and primary antibody were added to each well and incubated at 4°C overnight. The wells were washed 3 times. Then 100 μL secondary antibody was added to each well and incubated for 1 h at room temperature. The wells were again washed 3 times. After that, enzyme substrate solution was added and the wells incubated at room temperature for 15 min. Terminating solution stopped the reaction. The absorbance was read at a wavelength of 450 nm [27,28].

***Data analysis:*** The results of the replicates were pooled and expressed as mean ± standard error. Analysis of variance and Student's t-test were carried out. Significance was accepted at $p \leq 0.05$ [29].

**Acknowledgements -** This work was supported by the Scientific Research Projects of the Administration Unit of Akdeniz University (2008.01.0105.011). The authors wish to thank Akdeniz University Scientific Research Projects Unit for financial support of this work.

## References

[1] Ozbek T, Gulluce M, Sahin F, Ozkan H, Sevsay S, Baris O. (**2008**) Investigation of the antimutagenic potentials of the methanol extract of *Origanum vulgare* L. subsp *vulgare* in the Eastern Anatolia region of Turkey. *Turkish Journal of Biology*, ***32***, 271–276.

[2] Ozkan A, Erdogan A. (**2011**) A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. *Turkish Journal of Biology*, ***35***, 735-742.

[3] Lambert RJV, Skandamis PN, Coote PJ, Nychas GJE. **(2001)** A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, ***91***, 453-462.

[4] Duthie GG, Brown KM. (**1998**) Reducing the risk of cardiovascular disease. In *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Goldberg I. (Ed.), Chapman & Hall, New York, NY, USA, 19-38.

[5] Milner JA. (**1998**) Reducing the risk of cancer. In *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals.* Goldberg I. (Ed.). Chapman & Hall, New York, NY, USA, 39-70 pp.

[6] Gulçin I, Kireçci E, Akkemik E, Topal F, Hisar O. (**2010**) Antioxidant, antibacterial, and anticandidal activities of an aquatic plant: duckweed *(Lemna minor* L., Lemnaceae). *Turkish Journal of Biology*, ***34***, 175-188.

[7] Kutlu T, Durmaz G, Ateş B, Yılmaz I, Cetin MS. (**2011**) Antioxidant properties of different extracts of black mulberry (*Morus nigra* L.). *Turkish Journal of Biology*, ***35***, 103-110.

[8] Seyoum A, Asres K, El-Fiky FK. (**2006**) Structure-radical scavenging activity relationship of flavonoids. *Phytochemistry*, ***67***, 2058-2070.

[9] Ozkan A, Erdogan A, Sokmen M, Tugrulay S, Unal O. (**2010**) Antitumoral and antioxidant effect of essential oils and *in vitro* antioxidant properties of essential oils and aqueous extracts from *Salvia pisidica. Biologia*, ***65***, 990-996.

[10] Ozkan A, Gubbuk H, Gunes E, Erdogan A. (**2011**) Antioxidant capacity of juice from different papaya (*Carica papaya* L.) cultivars grown under greenhouse conditions in Turkey. *Turkish Journal of Biology*, ***35***, 619-625.

[11] Moteki H, Hibasami H, Yamada Y, Katsuzaki H, Imai K, Komiya T. (**2002**) Specific induction of apoptosis by 1,8-cineole in two human leukemia cell lines, but not in human stomach cancer cell line. *Oncology Reports*, ***9***, 757-760.

[12] Rihova B, Strohalm J, Kobackova K. (**2002**) Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs. *Journal of Controlled Release*, ***78***, 97-114.

[13] Miyazawa M, Shindo M. (**2001**) Biotransformation of 1,8-cineole by human liver microsomes. *Natural Product Letters*, ***15***, 49-53.

[14] Duisken M, Sandner F, Blomeke B, Hollender J. (**2005**) Metabolism of 1,8-cineole by human cytochrome P450 enzymes: identification of a new hydroxylated metabolite. *Biochimica et Biophysica Acta*, ***1722***, 304–311.

[15] Koparal AT, Zeytinoglu M. (**2003**) Effects of carvacrol on a human nonsmall cell lung cancer (NSCLC) cell line, A549. *Cytotechnology*, ***43***, 149-154.

[16] Mastelic J, Jerkovic I, Blazevic I, Poljak-Blazi M, Borovic S, Ivancic-Bace I, Smrecki V, Zarkovic N, Brcic-Kostic K, Vikic-Topic D, Muller N. (**2008**) Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. *Journal of Agricultural and Food Chemistry*, ***56***, 3989-3996.

[17] Horvathova E, Turcaniova V, Slamenova D. **(2007**) Comparative study of DNA-damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562. *Neoplasma*, ***54***, 478-483.

[18] Aydin S, Basaran AA, Basaran N. (**2005**) Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. *Journal of Agricultural and Food Chemistry*, ***53***, 1299-1305.

[19] Slamenova D, Horvathova E, Sramkova M, Marsalkova L. (**2007**) DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*. *Neoplasma*, ***54***, 108-112.

[20] Peng IW, Kuo SM. (**2003**) Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. *Journal of Nutrition*, ***133***, 2184-2187.

[21] Jonsson O, Motlagh PB, Persson M, Henriksson R, Grankvist K. (**1999**) Increase in doxorubicin cytotoxicity by carvedilol inhibition of P-glycoprotein activity. *Biochemical Pharmacology*, ***58***, 1801-1806.

[22] Ozkan A. (**2007**) Lymphokine-activated killer cell susceptibility in epirubicin resistant and parental human non-small cell lung cancer (NSCLC). *Biologia*, ***62***, 232-237.

[23] Gloeckner H, Jonuleit T, Lemke HD. (**2001**) Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue (TM). *Journal of Immunological Methods*, ***252***, 131-138.

[24] Wasowicz W, Neve J, Peretz A. (**1993**) Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum; importance of extraction pH and influence of sample preservation and storage. *Clinical Chemistry*, ***39***, 2522-2526.

[25] Bradford MM. (**1976**) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, ***72***, 248-254.

[26] www.invitrogen.com

[27] Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Yoshida A, Uchida K, Hiai H, Oci H, Osawa T. (**1997**) Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45. 1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Laboratory Investigation*, ***76***, 365-374.

[28] Garçon G, Dagher Z, Zerimech F, Ledoux F, Courcot D, Aboukais A, Puskarıc E, Shirali P. **(2006)** Dunkerque city air pollution particulate matter-induced cytotoxicity, oxidative stress and inflammation in human epithelial lung cells (L132) in culture. *Toxicology in Vitro*, ***20***, 519–528.

[29] Kirkman TW. (**1996**) Statistics to use [Online]. Available:http://www.physics.csbsju.edu/stats/1996 [17 August 2008].

NPC Natural Product Communications

2012
Vol. 7
No. 12
1561 - 1562

# New Secoiridoid Glycosides from the Buds of *Lonicera macranthoides*

**Jiang Liu[a], Jing Zhang[b], Feng Wang[a] and Xingfu Chen[a,*]**

[a]*Division of Pharmaceutical Botany*, *College of Agronomy*, *Sichuan Agricultural University*, *Chengdu 611130*, *P. R. China*

[b]*College of Horticulture*, *Sichuan Agricultural University*, *Ya'an 625014*, *P. R. China*

*chenxf64@sohu.com*

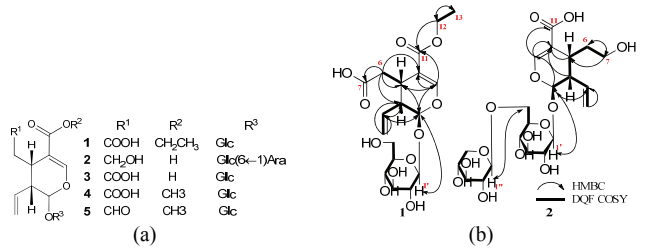
**Received: October 18th, 2012; Accepted: November 6th, 2012**

Two new secoiridoid glycosides, named ethyl secologanoside (**1**) and 6'-*O*-α-L-arabinopyranosyl demethylsecologanol (**2**), together with three known ones, secologanoside (**3**), secoxyloganin (**4**), and loniceroside (**5**), were isolated from the dried buds of *Lonicera macranthoides*. The structures of the new compounds were determined on the basis of detailed spectroscopic analyses and acidic hydrolysis.

**Keywords:** *Lonicera macranthoides*, Caprifoliaceae, Secoiridoid glycosides, Ethyl secologanoside, 6'-*O*-α-L-arabinopyranosyl demethylsecologanol.

*Lonicera* is one of the most important genera in the Caprifoliaceae family. *L. macranthoides* Hand.-Mazz. has long been traditionally used in China and south-east Asia to treat acute fever, headache, pharyngodynia, respiratory infection, pyocutaneous disease and epidemic disease [1]. Earlier chemical studies on *L. macranthoides* led to the isolation of a series of phenolic acids, iridoids and saponins [2]. In this article, we present the isolation and structure elucidation of two new secoiridoid glycosides (**1**) and (**2**), as well as three known compounds: Secologanoside (**3**) [3a], secoxyloganin (**4**) [3a], and loniceroside (**5**) [3b] from the methanol extract of the buds of *L. macranthoides* (Figure 1a)

In the positive-ion FAB-MS of ethyl secologanoside (**1**), quasi-molecular ion peaks were observed at *m/z* 419 $(M+H)^+$ and *m/z* 441 $(M+Na)^+$, consistent with the molecular formula $C_{18}H_{26}O_{11}$. The $^1H$ NMR and $^{13}C$ NMR spectra (Table 1) showed signals that indicated the presence of the olefinic proton of β-alkoxyacrylic acid at δ 7.45 (d, *J*=2.0 Hz, H-3), a set of three protons on the vinyl group at δ 5.63 (ddd, *J*=17.4, 9.7, 9.7 Hz, H-8), 5.23 (d, *J*=9.7 Hz, H-10A) and 5.28 (d, *J*=17.4 Hz, H-10B), an acetal proton at δ 5.47 (d, *J*=4.1 Hz, H-1), and one sugar unit containing a β-linked anomeric proton at δ 4.64 (d, *J*=8.3 Hz, H-1'), which were characteristic of secoiridoid-type monoterpene glycosides. Furthermore, DEPT, HMQC and HMBC experiments (Figure 1b) revealed the 18 carbons, including two carboxyl carbons (δ 176.4, C-7 and δ 168.5, C-11), four alkenyl carbons [δ 154.2 (C-3) and δ 110.4 (C-4) due to the β-alkoxyacrylic acid group, and δ 134.5 (C-8) and δ 120.5 (C-10) due to the vinyl group], one anomeric carbon (δ 99.9, C-1'), one acetal carbon (δ 97.5, C-1), and two hydroxymethyl carbons (δ 61.2, C-12 and δ 62.7, C-6'). The above data indicated that **1** was a lactone open form of iridoid. The $^{13}C$ NMR spectra of **1**, and **3** and **4** were also in good agreement, except for the signal arising from the ethyl group (δ 61.2, C-12 and 14.5, C-13) of the carbethoxy function at C-11. The anomeric proton H-1' (δ 4.64, d, *J*=8.3 Hz) and the splitting pattern of the other sugar protons gave evidence of a *β*-glucose unit. Finally, the absolute configuration of glucose was determined to be of the D-series by HPLC analysis of the hydrolyzate of **1** using an optical rotation detector. On the basis of this evidence, the structure of compound **1** was elucidated to be 2H-pyran-4-acetic acid, 3-ethenyl-2-(β-D-glucopyranosyloxy)-3,4-dihydro-5-(ethoxycarbonyl), and named as ethyl secologanoside.




(a) (b)

**Figure 1**: (a) Structures of compounds **1**-**5**; (b) Selected HMBC and DQF correlations of compounds **1** and **2**.

Table 1: $^1H$ NMR and $^{13}C$ NMR data for **1** in methanol-$d_4$

| | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | 5.47 (d, *J*=4.1) | 97.5 |
| 3 | 7.45 (d, *J*=2.0) | 154.2 |
| 4 | | 110.4 |
| 5 | 3.20 (m) | 28.6 |
| 6 A | 2.25 (dd, *J*=8.9, 16.5) | 35.2 |
| B | 2.92 (dd, *J*=4.8, 16.5) | |
| 7 | | 176.4 |
| 8 | 5.63 (ddd, *J*=17.4, 9.7, 9.7) | 134.5 |
| 9 | 2.80 m | 45.3 |
| 10 A | 5.23 (d, *J*= 9.7) | 120.5 |
| B | 5.28 (d, *J*= 17.4) | |
| 11 | | 168.5 |
| 12 | 4.12 (m) | 61.2 |
| 13 | 1.25 (t, *J*= 6.9) | 14.5 |
| Glu- 1' | 4.64 (d, *J*=8.3) | 99.9 |
| 2' | 3.20 (t, *J*=8.3) | 74.6 |
| 3' | 3.34 (t, *J*=8.3) | 78.0 |
| 4' | 3.28 (overlap) | 71.5 |
| 5' | 3.28 (overlap) | 78.4 |
| 6' A | 3.65 (dd, 5.5, 11.7) | 62.7 |
| B | 3.88 (dd, 2.0, 11.7) | |

6'-*O*-α-L-arabinopyranosyl demethylsecologanol (**2**), an amorphous powder, exhibited quasi-molecular ion peaks at *m/z* 509 $(M+H)^+$ and *m/z* 531 $(M+Na)^+$, consistent with $C_{21}H_{32}O_{14}$. The NMR spectra (Table 2) and HMQC, HMBC, and DQF data (Figure 1b) showed signals that indicated the presence of β-alkoxyacrylic acid, acetal, a vinyl group, and two sugar units, which were also characteristic of secoiridoid-type monoterpene glycosides, like compound **1**. Furthermore, the $^1H$ NMR and $^{13}C$ NMR spectra of **2** were quite similar to those of demethylsecologanol [3c], except for a set of signals due to an additional *α*-arabinopyranoside unit, The downfield shift of the glucosyl C-6' signal (δ 68.9) suggested that the arabino-pyranosyl unit was at the C-6' position of the inner glucose, which was confirmed by the HMBC correlations between

**Table 2**: $^1$H NMR and $^{13}$C NMR data for **2** in methanol-$d_4$.

| No. | **2** | |
|---|---|---|
| | $\delta_H$ | $\delta_C$ |
| 1 | 5.51 (d, *J*=6.2) | 97.6 |
| 3 | 7.42 (brs) | 152.8 |
| 4 | | 110.8 |
| 5 | 2.85 (m) | 31.0 |
| 6 A | 1.75 (m) | 30.9 |
| B | 2.07 (m) | |
| 7 A | 3.66 (dd, *J*=5.5, 11.7) | 62.8 |
| B | 3.89 (overlap) | |
| 8 | 5.76 (ddd, *J*=17.2, 10.3, 10.3) | 136.0 |
| 9 | 2.64 (m) | 45.4 |
| 10 A | 5.23 (d, *J*= 10.3) | 119.3 |
| B | 5.27 (d, *J*= 17.2) | |
| 11 | | 170.2 |
| Glu- 1' | 4.67 (d, *J*=7.6) | 100.0 |
| 2' | 3.19 (t, *J*=7.6) | 74.7 |
| 3' | 3.36 (t, *J*=8.3) | 77.9 |
| 4' | 3.26 (t, *J*=9.6) | 71.6 |
| 5' | 3.29 (overlap) | 78.4 |
| 6' A | 3.50 (overlap) | 68.9 |
| B | 3.89 (overlap) | |
| Ara- 1" | 4.16 (d, *J*=6.9) | 104.9 |
| 2" | 3.50 (overlap) | 72.4 |
| 3" | 3.50 (overlap) | 74.2 |
| 4" | 3.79 (brs) | 69.5 |
| 5" A | 3.50 (overlap) | 66.6 |
| B | 3.82 (dd, 2.8, 12.4) | |

the anomeric proton of the terminal arabinose at δ H 4.16 (H-1") and the C-6' of the inner glucose. Finally, the absolute configuration of glucose and arabinose were determined as D and L, respectively on HPLC analysis of the hydrolyzate of **2** using an optical rotation detector. Accordingly, the structure of compound **2** was elucidated to be 2H-pyran-5-carboxylic acid, 3-ethenyl-2-(α-L-arabinopyranosyl(1-6')-β-D-glucopyranosyloxy)-3,4-dihydro-4-(2-hydroxyethyl), and named as 6'-*O*-α-L-arabinopyranosyl demethylsecologanol.

## Experimental

***General:*** The following instruments were used to obtain physical data: JEOL spectrometer (600 MHz for $^1$H NMR, 150 MHz for $^{13}$C NMR); FAB-MS, JEOL JMS-SX 102A mass spectrometer; CC, Diaion HP-20; TLC: pre–coated silica gel 60F264.

***Plant material:*** The buds of *Lonicera macranthoides* were collected from Suining, Sichuan province, China in 2011, and identified by Prof. Xingfu Chen (Sichuan Agricultural University, China). A voucher specimen of this plant was deposited with the College of Agronomy, Sichuan agricultural University.

***Extraction and isolation*:** The buds of *L. macranthoides* (2 Kg) were extracted 3 times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure provided the MeOH extract (202.1 g), which was fractionated by Diaion HP-20 CC, sequentially eluted with $H_2O$ and MeOH to give $H_2O$-eluted (80.5 g) and MeOH-eluted fractions (120.5 g). The MeOH-eluted fraction (120.5 g) was chromatographed on ODS columns using a gradient of MeOH: $H_2O$ (20: 80→40: 60→60: 40→80: 20)→ MeOH to give 15 fractions (1-15). Fraction 1 (2.0 g) was repeatedly chromatographed on RP-$C_{18}$ [MeOH: $H_2O$ (30: 70)] to give **2** (16.0 mg), **3** (12.3 mg) and **4** (10.3 mg). Fraction 4 (1.0 g) was repeatedly chromatographed on RP-$C_{18}$ [MeOH: $H_2O$ (35: 65)] to give **5** (7.7 mg). Fraction 6 (2.0 g) was repeatedly chromatographed on Sephadex LH-20 columns (MeOH) and RP-$C_{18}$ [MeOH: $H_2O$ (40: 60)] to give **1** (28.9 mg).

**Ethyl secologanoside** (**1**)
White amorphous powder.
$[\alpha]_D^{30}$: –130.83 (*c* 0.29, MeOH).
IR (film, MeOH): 3413, 1684, 1617, 1617, 1268, 1075 cm$^{-1}$.
UV (MeOH) λmax (log ε): 228 (4.17) nm.
$^1$H and $^{13}$C NMR: Table 1.
HR-FAB-MS: 441.1571 ([*M*+Na]$^+$, $C_{18}H_{26}O_{11}Na^+$; calc. 441.1568).

**6'-*O*-α-L-Arabinopyranosyl demethylsecologanol** (**2**)
White amorphous powder.
$[\alpha]_D^{30}$: –79.21 (*c* 0.12, MeOH).
IR (film, MeOH): 3413, 1739, 1693, 1251, 1073, 592 cm$^{-1}$.
UV (MeOH) λmax (log ε): 227 (4.16) nm.
$^1$H and $^{13}$C NMR: Table 2.
HR-FAB-MS: 531.1870 ([*M*+Na]$^+$, $C_{18}H_{26}O_{11}Na^+$; calc. 531.1867).

***Acid hydrolysis of 1 and 2:*** Solutions of **1** (3.0 mg) and **2** (3.0 mg) in 5% $H_2SO_4$–1,4-dioxane (1:1, v/v, 1.0 mL) were heated under reflux for 1 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 ($OH^-$form), and the resin removed by filtration. On removal of the solvent from the filtrate under reduced pressure, the residue was partitioned in an EtOAc–$H_2O$ (1:1, v/v) mixture, and the solvent removed *in vacuo* from the EtOAc-soluble fraction. The aqueous layer was subjected to HPLC analysis under the following conditions: HPLC column, Kaseisorb LC $NH_2$-60-5, 4.6 mm i.d.250 mm (Tokyo Kasei Co., Ltd., Tokyo, Japan); detection, optical rotation [Shodex OR-2 (Showa Denko Co., Ltd., Tokyo, Japan); mobile phase, $CH_3CN$–$H_2O$ (17:3, v/v); flow rate 1.0 mL/min]. Identification of D-glucose from **1** and **2**, and L-arabinose from **2** present in the aqueous layer was carried out by comparison of their retention times and optical rotations with those of authentic samples. *t*R: 7.1 min (L-arabinose, positive optical rotation), 11.6 min (D-glucose, positive optical rotation), respectively.

**Acknowledgments** - This study was funded by a grant from Sichuan Provincial Crop breeding research project Application in the 12$^{th}$ Five-Year Period (No. 2011NZ0098—12—01).

## References

[1] (a) Chen M, Wu W, Shen G., Luo S, Li H. (**1994**) Chemical constituents of *Lonicera macranthoides* Hand.-Mazz part V. Isolation and structures of macranthoin F and G. *Yaoxue Xuebao*, ***29***, 617-620; (b) Mao Q, Cao D, Jia X. (**1993**) Studies on the chemical constituents of *Lonicera macranthoides* Hand.-Mazz. *Yao Xue Xue Bao*, ***28***, 273-281; (c) Shi J, Chen X, Wan L. (**1999**) Hepatoprotective effects of some constituents of *Lonicera fulvotomentosa* Hsu et S.C. Cheng, and *L. macranthoides* Hand.-Mazz. against carbon tetrachloride- and D-galactosamine-induced liver injuries in mice and rats. *Zhongguo Zhongyao Zazhi*, ***24***, 363-364; (d) Wang J, Zhao X, Qi Q, Tao L, Zhao Q. (**2009**) Macranthoside B, a hederagenin saponin extracted from *Lonicera macranthoides* and its anti-tumor activities *in vitro* and *in vivo*. *Food and Chemical Toxicology*, ***47***, 1716-1721.

[2] (a) Chen C, Qi L, Li H, Li P, Yi L, Ma H. (**2007**) Simultaneous determination of iridoids, phenolic acids, flavonoids, and saponins in *Flos Lonicerae* and *Flos Lonicerae* Japonicae by HPLC-DAD-ELSD coupled with principal component analysis. *Journal of Separation Science*, ***30***, 3181-3192; (b) Chen Y, Zhao Y, Wang M, Sun H, Dong Y, Feng X. (**2012**) The first chlorogenic acid ester saponin from *Lonicera macranthoides*. *Chemistry of Natural Compounds*, ***47***, 940-943; (c) Chen Y, Shan Y, Zhao Y, Wang Q, Wang M, Feng X, Liang J. (**2012**) Two new triterpenoid saponins from *Lonicera macranthoides*. *Chinese Chemical Letters*, ***23***, 325-328; (d) Chen Y, Feng X, Wang M, Jia X, Zhao Y, Dong Y. (**2009**) Triterpene glycosides from *Lonicera*. II. Isolation and structural determination of glycosides from flower buds of *Lonicera macranthoides*. *Chemistry of Natural Compounds*, ***45***, 514-518; (e) Chen M, Luo S, Li H. (**1990**) Chemical constituents of *Lonicera macranthoides* Hand.-Mazz. I. Structure of macranthoiside I. *Chinese Chemical Letters*, ***1***, 219-220; (f) Chai X, Li S, Li P. (**2005**) Quality evaluation of *Flos Lonicerae* through a simultaneous determination of seven saponins by HPLC with ELSD. *Journal of Chromatography A*, ***1070***, 43-48.

[3] (a) Calis I, Sticher O. (**1984**) Secoiridoid glucosides from *Lonicera periclymenum*. *Phytochemistry*, ***23***, 2539-2540; (b) Einaggar LJ, Beal JL. (**1980**) Iridoids. A review. *Journal of Natural Products*, ***43***, 649-707; (c) Kitajima M, Fujii N, Yoshino F, Sudo H, Saito K, Aimi N, Takayama H. (**2005**) Camptothecins and two new monoterpene glucosides from *Ophiorrhiza liukiuensis*. *Chemical and Pharmaceutical Bulletin*, ***53***, 1355-1358.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1563 - 1564

# Chemical Constituents from the Aerial Parts of *Gynura bicolor*

**Jian Chen[a,b,c], Sven Mangelinckx[b], An Adams[b], Wei-lin Li[c], Zheng-tao Wang[a*] and Norbert De Kimpe[b*]**

[a]*Department of Pharmacognosy*, *China Pharmaceutical University*, *Nanjing 210009*, *China*
[b]*Department of Sustainable Organic Chemistry and Technology*, *Faculty of Bioscience Engineering*, *Ghent University*, *Coupure links 653*, *B-9000 Ghent*, *Belgium*
[c]*Institute of Botany*, *Jiangsu Province and Chinese Academy of Sciences*, *Nanjing 210014*, *China*

*wangzht@hotmail.com; norbert.dekimpe@ugent.be*

**Received: September 18th, 2012; Accepted: October 3rd, 2012**

*Gynura bicolor* (Willd.) DC., is used in folk recipes for the treatment of diabetes mellitus in Jiangsu, Zhejiang and Sichuan province in the south of China. A previous pharmacological study proved that the plant showed significant hypoglycemic activity on normal and alloxan-diabetic mice. In this study, two terpenes, four megastigmane-type norisoprenoids and two glycosides were isolated from the aqueous ethanolic extract of the aerial parts of *Gynura bicolor* and characterized mainly by NMR spectroscopy and mass spectrometry. These compounds were isolated for the first time from this plant, and no evidence could be found for the previous reported presence of megastigmane-type norisoprenoids in the genus *Gynura*.

**Keywords:** *Gynura bicolor*, Terpenes, Norisoprenoids, Glycosides, Diabetes mellitus.

The genus *Gynura* belongs to the family Asteraceae, comprising approximately 40 species mainly distributed in Asia, Africa and Australia, of which 10 species were recorded in the south of China [1]. In the literature, the presence of some interesting components in plants of the *Gynura* genus, such as volatiles [2,3], phenolics [4a], chromanes [4b], chromanones [4c,d], coumarins [4e], steroids [4d,f] and cerebrosides [5-7a], together with pyrrolizidine alkaloids [7b-10], which are hepatotoxic and could cause hepatic veno-occlusive disease [11a], is reported.

*Gynura bicolor*, which has been cultivated as a popular vegetable, is not only known to be nutritive but is also used for the treatment of diabetes in the south of China. Our previous pharmacological tests proved that the ethyl acetate and *n*-butanol extracts of aerial parts of the plant had significant effects on lowering blood glucose level in normal and alloxan-diabetic mice [11b]. To our knowledge, only three studies investigated the volatiles [3a-c] and one research reported on some anthocyanins [11c] from this plant. In the present study, two terpenes, four megastigmane-type norisoprenoids and two glycosides were obtained by combined chromatographic methods from the aqueous ethanolic extract of the aerial parts of *Gynura bicolor*. By comparison of their $^{1}$H, $^{13}$C NMR and ESI-MS spectral data with those reported, these isolated compounds (**1**-**8**) were unambiguously identified as: ficusic acid (**1**) [12a], loliolide (**2**) [12b], dehydrovomifoliol (**3**) [12c], vomifoliol (**4**) [13a], boscialin (**5**) [13b], (6*S*,9*S*)-roseoside (**6**) [13c], benzyl-β-D-glucopyranoside (**7**) [14a-b], 2-phenylethyl-β-D-glucopyranoside (**8**) [14b]. Noteworthy, the $^{1}$H and $^{13}$C NMR spectral data of compound **2**, which were in correspondence with loliolide [12b], were also nearly identical with those of a compound, named pubinernoid A, which was isolated from *Schisandra pubescens* var. *pubinervis* [14c]. The structure proposal for pubinernoid A, with the same molecular formula as loliolide, was (4*S**,6*R**)-4-hydroxy-4,8,8-trimethyl-9-oxabicyclo[4.2.1]non-1-en-3-one, with an atypical value of 183.2 ppm for the chemical shift of the keto group. However, upon comparing GC-MS spectral data with data available in the WILEY6N database, compound **2** was identified as loliolide.

**Chemotaxonomic significance:** Pyrrolizidine alkaloids are widely found and are characteristic secondary metabolites in the tribe

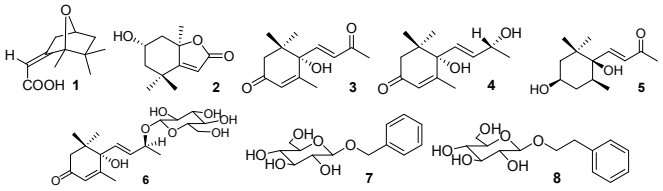


**Figure 1**: Structures of isolated compounds (**1**-**8**).

Senecioneae, belonging to the Asteraceae family [14d]. In this study, chemical constituents of *G. bicolor* from the same tribe are assigned as terpenes (**1**-**2**), megastigmane-type norisoprenoids (**3**-**6**) and glycosides (**6**-**8**). It should be noted that all these compounds were isolated for the first time from this plant, and no evidence could be found of the previous reported presence of megastigmane-type norisoprenoids in the genus *Gynura*. Roseoside (**6**) was firstly isolated from *Vinca rosea* in 1974 [14e], and together with their stereoisomers, they were found in many plant sources. It is interesting to note that (6*S*,9*R*)-roseoside, which is an isomer of (6*S*,9*S*)-roseoside (**6**) currently isolated, has previously been shown to increase insulin secretion from INS-1 β-cells [14f]. Vomifoliol (**4**), the aglycone of an isomer of (6*S*,9*S*)-roseoside (**6**), and its analogue, dehydrovomifoliol (**3**), were identified for the first time in the fruits from *Vitis vinifera* [15]. Boscialin (**5**) can be regarded as a reduced analogue of dehydrovomifoliol (**3**). The occurrence of C13-type norisoprenoids in *Gynura bicolor*, as demonstrated in this paper, is noteworthy.

## Experimental

***Plant material:*** The aerial parts of *Gynura bicolor* (Willd.) DC. were collected in June 2010 in Nanjing Botanical Garden Memorial Sun Yat-Sen, in the south of the Zijin Mountain, Nanjing, China. The plant (voucher specimen No. 510310-1) was identified by Professor Guo Rong-lin of Institute of Botany, Jiangsu Province and Chinese Academy of Sciences.

***Extraction and isolation:*** Dry aerial parts of *G. bicolor* (2.9 kg) were extracted with 80% aqueous ethanol at 70 ˚C twice to afford 295 g of crude extract after evaporation in vacuo of the solvent. The

extract was coated on silica gel, and then successively extracted with *n*-hexane, $CH_2Cl_2$, EtOAc and MeOH to give four fractions. The $CH_2Cl_2$ extract (3.4 g) was further fractionated by column chromatography over silica gel, eluted with $CH_2Cl_2$ containing increasing concentrations of MeOH to obtain seven fractions (Fr. B1-B7). Fr. B2 (600 mg) was fractionated with an automatic flash chromatography system on a reversed-phase column (C-18) eluted by a gradient of water and MeOH (50-100%) to get four fractions (Fr. B2-1~4). Fr. B2-1 (248 mg) and B2-2 (175 mg) were further subjected to column chromatography over MCI gel CHP20P eluted by a gradient of water and MeOH (20-100%). Upon further silica gel column chromatography ($CH_2Cl_2$/MeOH, 100:1 to 10:1) and prep-HPLC ($H_2O$/$CH_3CN$, 75:25), ficusic acid **1** (2.2 mg) was obtained. Following silica gel column chromatography (petroleum ether/EtOAc, 10:1 to 1:1) and prep-HPLC ($H_2O$/$CH_3CN$, 75:25), loliolide **2** (4.0 mg) and dehydrovomifoliol **3** (0.5 mg) were isolated. Fr. B3 (655 mg) was successively fractionated by automatic flash chromatography (C-18, $H_2O$/MeOH, 50:50 to 0:100), MCI gel column chromatography ($H_2O$/MeOH, 80:20 to 0:100), prep-HPLC ($H_2O$/MeCN, 75:25) to give vomifoliol **4** (1.2 mg) and boscialin **5** (0.8 mg). The EtOAc extract (7.7 g) was fractionated by chromatography on a C-18 column, eluted with a gradient of water and MeOH (20-100%), to give five fractions (Fr. C1-C5). Fr. C2 (724 mg) was further separated with automatic flash chromatography on a reverse phase column (C-18) eluted with a gradient of water and MeOH (20-100%). Four fractions (Fr. C2-1~4) were obtained and Fr. C2-2 (510 mg) was subjected to chromatography on a silica gel column eluted with a $CHCl_3$/MeOH gradient (100:1 to 6:1) to give six fractions (C2-2-1~6). The C2-2-3 fraction (12 mg) and C2-2-4 fraction (20 mg) were finally purified by prep-HPLC eluted with MeOH/$H_2O$ (36:64) to yield pure (6*S*,9*S*)-roseoside **6** (2.2 mg) and benzyl-β-D-glucopyranoside **7** (1.3 mg). Fr. C3 (2.1 g) was purified successively via automatic flash chromatography (C-18, $H_2O$/MeOH 80:20 to 0:100), silica gel column chromatography ($CH_2Cl_2$/MeOH 10:1 to 4:1) and prep-HPLC (MeOH/$H_2O$ 35:65) to afford 2-phenylethyl-β-D-glucopyranoside **8** (1.1 mg).

**Acknowledgments** - This study was supported by a grant from the National Natural Science Foundation of China (No. 30900123) and a grant from Jiangsu Center for Research & Development of Medicinal Plants (No. 200902). The authors are also indebted to the Special Research Fund (BOF-UGent) for funding for a joint doctoral study of Jian Chen, and to the Research Foundation-Flanders (FWO-Vlaanderen) for a postdoctoral fellowship for Sven Mangelinckx and An Adams.

## References

[1] Chen YL. (**1999**) Flora of China. Science Press, Beijing, 309.

[2] Rana VS, Blazquez MA. (**2007**) Chemical constituents of *Gynura cusimbua* aerial parts. *Journal of Essential Oil Research*, ***19***, 21-22.

[3] (a) Shimizu Y, Imayoshi Y, Kato M, Maeda K, Iwabuchi H, Shimomura K. (**2009**) Volatiles from leaves of field-grown plants and shoot cultures of *Gynura bicolor* DC. *Flavour and Fragrance Journal*, ***24***, 251-258; (b) Shimizu Y, Imayoshi Y, Kato M, Maeda K, Iwabuchi H, Shimomura K. (**2011**) New eudesmane-type sesquiterpenoids and other volatile constituents from the roots of *Gynura bicolor* DC. *Flavour and Fragrance Journal*, ***26***, 55-64; (c) Chen J, Adams A, Mangelinckx S, Ren BR, Li WL, Wang ZT, De Kimpe N. (**2012**) Investigation of the volatile constituents of different *Gynura* species from two Chinese origins by SPME/GC-MS. *Natural Product Communications*, ***7***, 655-657.

[4] (a) Wan CP, Yu YY, Zhou SR, Tian SG, Cao SW. (**2011**) Isolation and identification of phenolic compounds from *Gynura divaricata* leaves. *Pharmacognosy Magazine*, ***7***, 101-108; (b) Lin WY, Teng CM, Tsai IL, Chen IS. (**2000**) Anti-platelet aggregation constituents from *Gynura elliptica*. *Phytochemistry*, ***53***, 833-836; (c) Jong TT, Chou-Hwang JY. (**1997**) An optically active chromanone from *Gynura formosana*. *Phytochemistry*, ***44***, 553-554; (d) Lin WY, Kuo YH, Chang YL, Teng CM, Wang EC, Ishikawa T. (**2003**) Anti-platelet aggregation and chemical constituents from the rhizome of *Gynura japonica*. *Planta Medica*, ***69***, 757-764; (e) Bohlmann F, Zdero C. (**1977**) Gynuron, ein neues terpen-cumarin-derivat aus *Gynura crepioides*. *Phytochemistry*, ***16***, 494-495; (f) Takahira M, Kondo Y, Kusano G, Nozoe S. (**1977**) Four new 3α-hydroxyspirost-5-ene derivatives from *Gynura japonica* Makino. *Tetrahedron Letters*, ***41***, 3647-3650.

[5] Chen L, Wang JJ, Zhang GG, Song HT, Qin LP. (**2009**) A new cerebroside from *Gynura divaricata*. *Natural Product Research*, ***23***, 1330-1336.

[6] (a) Chen L, Li HQ, Song HT, Zhang GG. (**2009**) A new cerebroside from *Gynura divaricata*. *Fitoterapia*, ***80***, 517-520; (b) Chen L, Wang JJ, Song HT, Zhang GG, Qin LP. (**2009**) New cytotoxic cerebroside from *Gynura divaricata*. *Chinese Chemical Letters*, ***20***, 1091-1093.

[7] (a) Lin WY, Yen MH, Teng CM, Tsai IL, Chen IS. (**2004**) Cerebrosides from the rhizomes of *Gynura japonica*. *Journal of the Chinese Chemical Society*, ***51***, 1429-1434; (b) Liang X T, Roeder E. (**1984**) Senecionine from *Gynura segetum*. *Planta Medica*, ***50***, 362.

[8] Roeder E, Eckert A, Wiedenfeld H. (**1996**) Pyrrolizidine alkaloids from *Gynura divaricata*. *Planta Medica*, ***62***, 386.

[9] Wiedenfeld H. (**1982**) Two new hepatotoxic pyrrolizidine alkaloids from *Gynura scandens*. *Planta Medica*, ***45***, 136-137.

[10] Wiedenfeld H. (**1982**) Two pyrrolizidine alkaloids from *Gynura scandens*. *Phytochemistry*, ***21***, 2767-2768.

[11] (a) Schoental R. (**1968**) Toxicology and carcinogenic action of pyrrolizidine alkaloids. *Cancer Research*, ***28***, 2237-2246; (b) Li WL, Ren BR, Zhuo M, Hu Y, Lu CG, Wu JL, Chen J, Sun S. (**2009**) The anti-hyperglycemic effect of plants in genus *Gynura* Cass. *American Journal of Chinese Medicine*, ***37***, 961-966; (c) Shimizu Y, Imada T, Zhang HL, Tanaka R, Ohno T, Shimomura K. (**2010**) Identification of novel poly-acylated anthocyanins from *Gynura bicolor* leaves and their antioxidative activity. *Food Science and Technology Research*, ***16***, 479-486.

[12] (a) Wang CY, Liu X, Guo LM, Shao CL, Fang YC, Wei YX, Zheng CJ, Gu QQ, Zhu WM, Guan HS. (**2010**) Two new natural keto-acid derivatives from *Sargassum pallidum*. *Chemistry of Natural Compounds*, ***46***, 292-294; (b) Chen BN, Yang GE, Li JK, Du HJ, Li QS, Zhang ZM. (**2009**) Cytotoxic constituents from *Viscum coloratum*. *Chemistry of Natural Compounds*, ***45***, 547-549; (c) Kisiel W, Michalska K, Szneler E. (**2004**) Norisoprenoids from aerial parts of *Cichorium pumilum*. *Biochemical Systematics and Ecology*, ***32***, 343-346.

[13] (a) Hammami S, Jannet HB, Bergaoui A, Ciavatta L, Cimino G, Mighri Z. (**2004**) Isolation and structure elucidation of a flavanone, a flavanone glycoside and vomifoliol from *Echiochilon Fruticosum* growing in Tunisia. *Molecules*, **9**, 602-608; (b) Pauli N, Sequin U, Walter A. (**1990**) Boscialin and boscialin 4′-*O*-glucoside, two new compounds isolated from the leaves of *Boscia salicifolia* OLIV. *Helvetica Chimica Acta*, ***73***, 578-582; (c) Yajima A, Oono Y, Nakagawa R, Nukada T, Yabuta G. (**2009**) A simple synthesis of four stereoisomers of roseoside and their inhibitory activity on leukotriene release from mice bone marrow-derived cultured mast cells. *Bioorganic & Medicinal Chemistry*, ***17***, 189-194.

[14] (a) Deffieux D, Natangelo A, Malik G, Pouysegu L, Charris J, Quideau S. (**2011**) First and biomimetic total synthesis of a member of the C-glucosidic subclass of ellagitannins, 5-*O*-desgalloylepipunicacortein A. *Chemical Communications*, ***47***, 1628-1630; (b) Yoneda Y, Krainz K, Liebner F, Potthast A, Rosenau T, Karakawa M, Nakatsubo F. (**2008**) "Furan endwise peeling" of celluloses: mechanistic studies and application perspectives of a novel reaction. *European Journal of Organic Chemistry*, ***3***, 475-484; (c) Huang SX, Yang J, Xiao WL, Zhu YL, Li RT, Li LM, Pu JX, Li X, Li SH, Sun HD. (**2006**) Three novel terpenoids from *Schisandra pubescens* var. pubinervis. *Helvetica Chimica Acta*, ***89***, 1169-1175; (d) Langel D, Ober D, Pelser PB. (**2011**) The evolution of pyrrolizidine alkaloid biosynthesis and diversity in the Senecioneae. *Phytochemistry Reviews*, ***10***, 3-74; (e) Bhakuni DS, Joshi PP, Uprety H, Kapil RS. (**1974**) Roseoside-A $C_{13}$ glycoside from *Vinca rosea*. *Phytochemistry*, ***13***, 2541-2543; (f) Frankish N, de Sousa Menezes F, Mills C, Sheridan H. (**2010**) Enhancement of insulin release from the beta-cell line INS-1 by an ethanolic extract of *Bauhinia variegata* and its major constituent roseoside. *Planta Medica*, ***76***, 995-997.

[15] Strauss CR, Wilson B, Williams PJ. (**1987**) 3-Oxo-α-ionol, vomifoliol and roseoside in *Vitis vinifera* fruit. *Phytochemistry*, ***26***, 1995-1997.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1565 - 1568

# Chemical Constituents of *Ligularia nelumbifolia* and *L. subspicata* Hybrid Collected in Shangrila County, Yunnan Province of China

**Ryo Hanai*[a], Hiroka Yamada[b], Yurika Suzuki[c], Hajime Nagano[b], Takayuki Kawahara[d], Jiao-Jun Yu[e], Xun Gong[e] and Chiaki Kuroda*[c]**

[a]*Department of Life Science and Research Center for Life Science, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*

[b]*Department of Chemistry, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*

[c]*Department of Chemistry, and Research Center for Smart Molecules, Rikkyo University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan*

[d]*Shikoku Research Center, Forestry and Forest Products Research Institute, Asakuranishi-machi, Kochi, Kochi 780-8077, Japan*

[e]*Kunming Institute of Botany, Chinese Academy of Science, Kunming 650204, China*

*chkkuroda@rikkyo.ac.jp*

**Received: September 11th, 2012; Accepted: October 18th, 2012**

Four *Ligularia* individuals possessing morphological characters of both *L. nelumbifolia* and *L. subspicata* were discovered in Shangrila County, Yunnan Province, China. DNA sequencing showed that the specimens were hybrids of the two species and their maternal parent was *L. subspicata*. The chemical composition of the root extract was examined for three of them as well as *L. nelumbifolia* and *L. subspicata* collected sympatrically. Nelumol was isolated from *L. nelumbifolia*, and furanoeremophilanes from *L. subspicata*. While nelumol and furanoeremophilanes were detected in two of the hybrid samples, only furanoeremophilanes were detected in the others.

**Keywords:** Hybrid, *Ligularia nelumbifolia*, *Ligularia subspicata*, Furanoeremophilanes, Sesquiterpenoids, ITS, *atpB-rbcL*.

The genus *Ligularia* (Asteraceae) is highly diversified in the Hengduan Mountains area of China [1], providing suitable materials for the study of plant evolution. We have been studying the chemical diversity of *Ligularia* by combining two different approaches; analyses of chemical composition in root, and nucleotide sequences of evolutionarily neutral DNA regions. To date, we have found that there are several different modes of intra-specific diversity in *Ligularia*, implying that the mechanism(s) of generation of chemical diversity is complex [2]. For example, some species, such as *L. pleurocaulis* (Franch.) Hand.-Mazz. [3], and *L. virgaurea* (Maxim.) Mattf. [4] were separated into two distinct groups, while *L. dictyoneura* (Franch.) Hand.-Mazz. [5] and *L. kanaitzensis* (Franch.) Hand.-Mazz. [6,7] showed complex diversity. Chemical and genetic analyses of these species suggested that hybridization is one of the major pathways of evolution of *Ligularia* [2], supporting reticulate evolution within and among *Ligularia* and related genera [8]. Some natural *Ligularia* hybrids have actually been found in the Hengduan Mountains area [9].

We previously reported that *L. duciformis* and related species (*L. nelumbifolia* and *L. kongkalingensis*) in the Hengduan Mountains region mostly produced phenylpropenoids [10]. However, other classes of compounds, such as eremophilane- and oplopane-type sesquiterpenoids, were found in a limited number of samples. DNA sequencing strongly suggested hybridization in the plants, implying that the ability to produce sesquiterpenoids may have been brought about by introgression. However, there was no evidence showing that hybridization takes place between *L. duciformis* (or related species) and a plant that produces sesquiterpenoids.

In the course of our continuous field searches, we found several *Ligularia* individuals possessing morphological characters of both *L. nelumbifolia* and *L. subspicata* at Shangrila (Zhongdian) County, Yunnan Province of China [11]. Phylogenetic analyses of these samples indicated that some individuals were possibly F2 or a later generation, although most of them were F1 [11]. Since natural hybridization is an important pathway of evolution, as described above, we analyzed root chemicals of four putative hybrid samples. *L. nelumbifolia* is a dominant species at the place [11]. Chemical constituents of the species have been reported to include 4-*O*-geranylsinapyl alcohol (nelumol; **1**) and related phenylpropenoid derivatives [10,12]. Although major *Ligularia* species in the Hengduan Mountains area produce furanoeremophilanes [2-6], *L. nelumbifolia* and related species do not, whereas *L. subspicata* produces furanoeremophilanes. Subspicatin A (**2**), ligularol (**3**), furanoeremophilan-10β-ol (= tetradymol; **4**), and other furano- and non-furano-eremophilanes were isolated from the roots of *L. subspicata* collected in Yunnan and Sichuan Provinces, China [13]. To the best of our knowledge, no chemical study of a natural hybrid of *Ligularia* has been reported.

Samples were collected at Xiaoxueshan, Shangrila (Zhongdian) County, Yunnan Province of China in August, 2009 and 2011. In 2009, two samples of *L. nelumbifolia* (samples N1 and N2), one of *L. subspicata* (sample S), and three samples of putative hybrids (samples A-C) were collected within 10 m from each other. In 2011, one putative hybrid (sample D) was collected at almost the same place as in 2009.

DNA sequencing was carried out to confirm that samples A-D were hybrids of *L. nelumbifolia* and *L. subspicata* [11]. The results for the ITS1-5.8S-ITS2 region of the nuclear rRNA gene are summarized in Table 1. As can be clearly seen, the sequences of samples A-D are basically a superposition of those of *L. nelumbifolia* (N1 and N2)

**Table 1**: ITS1-5.8S-ITS2 sequences of *L. nelumbifolia*, *L. subspicata*, and putative hybrids[a].

| no. | Species | ITS1 | | | | | | | | | | | | | | | | | | | | | | | | | 5.8S | ITS2 | | | | | | | | | | | | | | | | | | |

$AcOH/H_2SO_4$ as visualizing agents. HPLC was carried out on a Shimadzu LC-20AT pump, SPD-20A Prominence UV/VIS detector, Kanto Mightysil Si60 (10 × 250 mm) ODS column. Purification of DNA from dried leaves, amplification of the ITS1-5.8S-ITS2 and the *atpB-rbcL* intergenic regions by polymerase chain reaction, and DNA sequencing of the regions were carried out as described earlier [13]. Amplification and sequencing of the *rpl32-trnL* intergenic region was carried out with rpL32-F and $trnL^{(UAG)}$ primers [20].

***Plant materials:*** Samples were collected at Xiaoxueshan (Mt. Xiaoxue), Shangrila County, Yunnan Province of China. Each sample was identified by XG (author). Voucher specimens were deposited in the Herbarium of Kunming Institute of Botany (No. 2009-07 (sample N1, *L. nelumbifolia*), 2009-12 (sample N2, *L. nelumbifolia*), 2009-08 (sample S, *L. subspicata*), 2009-09 (hybrid A), 2009-10 (hybrid B), 2009-11 (hybrid C), and 2011-113 (hybrid D)).

***Extraction and purification:*** Dried roots of sample S (*L. subspicata*) (4.6 g) was extracted with EtOH at room temperature and the extract concentrated under reduced pressure to give an oily product (113 mg). Part of this (45.6 mg) was submitted to CC ($SiO_2$ 3 g; eluent: *n*-hexane-EtOAc 20:1 to 5:1) to give subspicatin A (**2**) (6.1 mg, 13%) and ligularol (**3**) (7.3 mg, 16%). From less polar fractions, 6β-ethoxyfuranoeremophilan-10β-ol (**5**) (1.5 mg, 3.3%, $R_f$ 0.80) was isolated after further chromatography ($SiO_2$ 1 g; eluent: *n*-hexane-EtOAc 100:1).

Dried root samples of N1 (*L. nelumbifolia*; 39.1 g) and N2 (*L. nelumbifolia*; 21.3 g) were extracted and the extracts concentrated as described above to give oily products (251 mg and 826 mg, respectively). Part of these (122 mg and 102 mg) were submitted to CC ($SiO_2$ 10 g; eluent: *n*-hexane-EtOAc 20:1 to 5:1) to give nelumol (**1**) (12.1 mg, 9.9%; and 4.7 mg, 4.7%, respectively).

Dried roots of sample A (hybrid) (3.1 g) was also extracted with EtOH at room temperature and the extract concentrated under reduced pressure to give an oily product (99 mg). Isolation of chemical components was attempted using silica gel CC, but was not successful. Although TLC of the extract showed two spots (see text), isolation of the products, presumably ligularol (**3**) and nelumol (**1**), was not successful due to the paucity of the component and severe contamination by some other components.

Dried roots of sample B (hybrid) (12.6 g) was extracted with EtOH at room temperature and the extract concentrated under reduced pressure to give an oily product (257 mg). Part of this (166 mg) was submitted to CC ($SiO_2$ 15 g; eluent: *n*-hexane-EtOAc 20:1 to 1:1) to give tetradymol (**4**) (11.4 mg, 6.9%), subspicatin A (**2**) (9.9 mg, 6.0%), and nelumol (**1**) (4.2 mg, 2.5%).

Dried root samples of C and D (hybrids) (25.5 g, 12.3 g, respectively) were separately extracted and the extracts concentrated as described above to give oily products (582 mg and 1050 mg, respectively). Part of the C extract (182 mg) was submitted to CC ($SiO_2$ 10 g; eluent: *n*-hexane-EtOAc 20:1 to 10:1) to give ligularol (**3**) (19.4 mg, 11%) and subspicatin A (**2**) (5.1 mg, 2.8%). The D extract was submitted to CC ($SiO_2$ 20 g; eluent: *n*-hexane-EtOAc 0:1 to 1:0) to give ligularol (**3**) (2.0 mg, 0.2%) and subspicatin A (**2**) (3.3 mg, 0.3%).

**Acknowledgments** - We thank Mrs Guowen Hu of Kunming Institute of Botany for research coordination. This work was partly supported by a Grant-in-Aid for Scientific Research from JSPS (No. 21404009).

## References

[1] (a) Liu SW. (**1989**) In *Flora Reipublicae Popularis Sinicae*, Yu B, Zeng GF. (Eds). Science Press (Beijing), Vol. ***77***, pp 4-115; (b) Liu S, Illarionova ID (**2011**). In *Flora of China*, Wu ZY, Raven PH, Hong DY. (Eds). Science Press (Beijing) and Missouri Botanical Garden Press (St. Louis), Vol. ***20-21***, pp 376-415.

[2] Kuroda C, Hanai R, Nagano H, Tori M, Gong X. (**2012**) Diversity of furanoeremophilanes in major *Ligularia* species in the Hengduan Mountains. *Natural Product Communications*, ***7***, 539-548.

[3] Nagano H, Iwazaki Y, Gong X, Shen Y, Kuroda C, Hanai R. (**2006**) Chemical and genetic differentiation of *Ligularia pleurocaulis* in northwestern Yunnan and southwestern Sichuan Provinces of China. *Bulletin of the Chemical Society of Japan*, ***79***, 300-304.

[4] Tori M, Honda K, Nakamizo H, Okamoto Y, Sakaoku M, Takaoka S, Gong X, Shen Y, Kuroda C, Hanai R. (**2006**) Chemical constituents of *Ligularia virgaurea* and its diversity in southwestern Sichuan of China. *Tetrahedron*, ***62***, 4988-4995.

[5] Nagano H, Iwazaki Y, Matsushima M, Sato M, Gong X, Shen Y, Hirota H, Kuroda C, Hanai R. (**2007**) High diversity of *Ligularia dictyoneura* in chemical composition and DNA sequence. *Chemistry and Biodiversity*, ***4***, 2874-2888.

[6] Tori M, Watanabe A, Matsuo S, Okamoto Y, Tachikawa K, Takaoka S, Gong X, Kuroda C, Hanai R. (**2008**) Diversity of *Ligularia kanaitzensis* in sesquiterpenoid composition and neutral DNA sequences. *Tetrahedron*, ***64***, 4486-4495.

[7] Shimizu A, Suzuki Y, Torihata A, Hanai R, Saito Y, Tori M, Gong X, Kuroda C. (**2012**) Complex diversity in *Ligularia kanaitzensis*. *Natural Product Communications*, ***7***, 431-434.

[8] (a) Liu JQ, Wang YJ, Wang AL, Ohba H, Abbott RJ. (**2006**) Radiation and diversification within the *Ligularia-Cremanthodium-Parasenecio* complex (Asteraceae) triggered by uplift on the Qinghai-Tibetan plateau. *Molecular Phylogenetics and Evolution*, ***38***, 31-49; (b) Riesberg LH, Carney SE. (**1998**) Plant hybridization. *New Phytologist*, ***140***, 599-624.

[9] (a) Pan Y, Shi S, Gong X, Kuroda C. (**2008**) A natural hybrid between *Ligularia paradoxa* and *L. duciformis* (Asteraceae, Senecioneae) from Yunnan, China. *Annals of the Missouri Botanical Garden*, ***95***, 487-494; (b) Zhou R, Gong X, Boufford D, Wu CI, Shi S. (**2008**) Testing a hypothesis of unidirectional hybridization in plants: Observations on *Sonneratia*, *Bruguiera*, and *Ligularia*. *BMC Evolutionary Biology*, ***8***, 149.

[10] Nagano H, Hanai R, Yamada H, Matsushima M, Miura Y, Hoya T, Ozawa M, Fujiwara M, Kodama H, Torihata A, Onuki H, Nezu Y, Kawai S, Yamazaki M, Hirota H, Saito Y, Tori M, Ohsaki A, Gong X, Kuroda C. (**2012**) Chemical and genetic study of *Ligularia duciformis* and related species in Sichuan and Yunnan Provinces of China. *Chemistry and Biodiversity*, ***9***, 789-805.

[11] Yu JJ, Kuroda C, Gong X. (**2011**) Natural hybridization and introgression in sympatric *Ligularia* species (Asteraceae, Senecioneae). *Journal of Systematics and Evolution*, ***49***, 438-448.

[12] (a) Peng HR, Jia ZJ, Yang L, Zhao Y. (**1995**) A new sesquiterpene from *Ligularia nelumbifolia*. *Chinese Chemical Lett*ers, ***6***, 583-584; (b) Zhao Y, Peng HR, Jia ZJ. (**1995**) Six sinapyl alcohol derivatives from *Ligularia nelumbifolia*. *Chinese Chemical Letters*, ***6***, 387-390; (c) Zhao Y, Jia Z, Yang L. (**1994**) Sinapyl alcohol derivatives and other constituents from *Ligularia nelumbifolia*. *Phytochemistry*, ***37***, 1149-1152; (d) Zhao Y, Jia ZJ, Yang L, Wang JG. (**1993**) Sinapyl alcohol derivatives and other constituents from the roots of *Ligularia nelumbifolia*. *Chinese Chemical Letters*, ***4***, 895-898; (e) Zhao Y, Hao X, Lu W, Cai J, Yu H, Sevénet T, Guéritte F. (**2002**) Synthesis of two cytotoxic sinapyl alcohol derivatives and isolation of four new related compounds from *Ligularia nelumbifolia*. *Journal of Natural Products*, ***65***, 902-908.

[13] (a) Tori M, Okamoto Y, Tachikawa K, Mihara K, Watanabe A, Sakaoku M, Takaoka S, Tanaka M, Gong X, Kuroda C, Hattori M, Hanai R. (**2008**) Isolation of new eremophilane-type sesquiterpenoids, subspicatins A-D and subspicatolide from *Ligularia subspicata*, and chemical and genetic diversity of the species. *Tetrahedron*, ***64***, 9136-9142; (b) Saito Y, Hattori M, Iwamoto Y, Takashima Y, Mihara K, Sasaki Y, Fujiwara M, Sakaoku M, Shimizu A, Chao X, Kuroda C, Gong X, Hanai R, Tori M. (**2011**) Overlapping chemical and genetic diversity in *Ligularia lamarum* and *Ligularia subspicata*. Isolation of ten new eremophilanes and a new seco-bakkane compound. *Tetrahedron*, ***67***, 2220-2231.

[14] Hanai R, Gong X, Tori M, Kondo S, Otose K, Okamoto Y, Nishihama T, Murota A, Shen Y, Wu S, Kuroda C. (**2005**) Chemical and genetic study of *Ligularia tongolensis*, *Ligularia cymbulifera*, and *Ligularia atroviolacea* in the Hengduan Mountains of China. *Bulletin of the Chemical Society of Japan*, ***78***, 1302-1308.

[15] (a) Kuroda C, Ueshino T, Nagano H. (**2004**) Ehrlich's reaction of furanoeremophilanes. *Bulletin of the Chemical Society of Japan*, ***77***, 1737-1740; (b) Kuroda C, Nishio E. (**2007**) Substituent effect in color of Ehrlich's test of tetrahydrobenzofuran, *Natural Product Communications*, ***2***, 581-585.

[16] Yamakawa K, Satoh T. (**1979**) Studies on terpenoids and related alicyclic compounds. XVIII. Stereoselective synthesis of (±)-furanofukinol and (±)-petasalbin. *Chemical and Pharmaceutical Bulletin*, ***27***, 1747-1752.

[17] Bohlmann F, Grenz M, Gupta RK, Dhar AK, Ahmed M, King RM, Robinson H. (**1980**) Eudesmane derivatives from *Verbesina* species. *Phytochemistry*, ***19***, 2391-2397.

[18] Jennings PW, Reeder SK, Hurley JC, Caughlan CN, Smith GD (**1974**) Isolation and structure determination of one of the toxic constituents from *Tetradymia glabrata*. *Journal of Organic Chemistry*, ***39***, 3392-3398.

[19] Harborne JB, Turner BL. (**1984**) *Plant Chemosystematics*. Academic Press, London.

[20] Shaw J, Lickey EB, Schilling EE, Small RL. (**2007**) Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. *American Journal of Botany*, ***94***, 275-288.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1569 - 1572

# A Practical, Enantiospecific Synthesis of (*S*)-*trans*-γ-Monocyclofarnesol

**Stefano Serra**

*C.N.R., Istituto di Chimica del Riconoscimento Molecolare, Via Mancinelli, 7 I-20131 Milano, Italy*

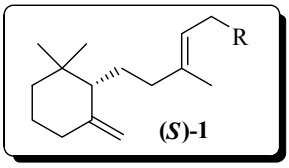
*stefano.serra@cnr.it; stefano.serra@polimi.it*

**Received: August 28th, 2012; Accepted: October 3rd, 2012**

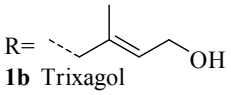
An expedient and concise synthesis of (*S*)-*trans*-γ-monocyclofarnesol is here described. The aforementioned sesquiterpene was prepared starting from enantioenriched (*S*)-γ-dihydroionone, which was in turn obtained from racemic α-ionone through the combined use of two previously developed processes. Key steps of the presented synthesis are the stereoselective Horner-Wadsworth-Emmons reaction between triethyl phosphonoacetate and γ-dihydroionone and the effective fractional crystallization of the γ-monocyclofarnesol-3,5-dinitrobenzoate esters. By these means the target compound was obtained in good yield and with very high stereoisomeric purity.

**Keywords:** Sesquiterpenes, Stereoselective synthesis, Enzyme-mediated resolution, Horner-Wadsworth-Emmons reaction, Fractional crystallization.

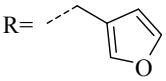
The general monocyclofarnesyl framework **1** (Figure 1) occurs in a large number of terpenic natural products.



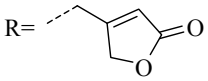

R= OH
**1a** γ−Monocyclofarnesol



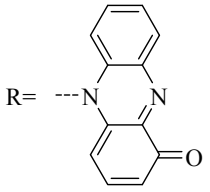

**1b** Trixagol



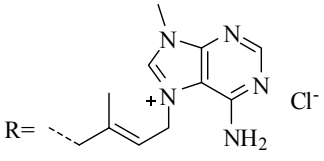

**1c** Dehydroambliol-A




**1d** Luffarin W




**1e** Phenazinomycin




**1f** Agelasine E

**Figure 1**: The (*S*)-*trans*-γ-monocyclofarnesyl framework **1** and some relevant natural products featuring this structure.

The simplest compound belonging to this class is *trans*-γ-monocyclofarnesol **1a** (R=OH), which was first identified [1] among the minor metabolites formed by the mycelia of the fungus *Helminthosporium siccans* in the biosynthetic process that affords the antibiotic siccanin. More than twenty years after, the same alcohol was extracted from the liverwort *Diplophyllum serrulatum* [2], confirming definitively its natural occurrence, albeit from two natural sources only.

Beside the rarity of **1a**, a large number of its homologues (e.g. **1b-f**) were isolated [3-7], and the biological activity of some of these compounds [6-10] greatly increased the synthetic interest for the γ-monocyclofarnesyl structure.

It is noteworthy that all the aforementioned products are usually present in their corresponding natural sources as a single stereoisomeric form. More specifically, they show an enantiocentre of (*S*) configuration and a (*E*)-trisubstituted double bond. Since their biological activity is strictly related to their stereochemistry, chemical syntheses providing a high level of stereocontrol were particularly sought-after. For such purposes, (*S*)-*trans*-γ-monocyclofarnesol is the most suitable chiral building block, having all the steric requirements already present in the molecular scaffold and allowing the preparation of higher homologues by exploiting the chemical reactivity of the hydroxy group.

Such a kind of approach was successfully employed for the synthesis of the alkaloid phenazinomycin **1e** [9]. In this process the key intermediate **1a** was prepared by means of an elegant, highly stereospecific, synthesis. Unfortunately, this alcohol was obtained through a very lengthy path, thus hampering the general use of this approach.

Two different syntheses [11,12] afforded either **1a** or the corresponding alkyl bromide starting from the enantiopure (*S*)-γ-dihydroionone, which was in turn obtained by degradation of the natural triterpenes ambreine and sclareol, respectively. In these procedures the (*E*)-trisubstituted double bond was built up stereoselectively, although close to a minority amount of the (*Z*)-isomer.

Recently, we have described [13] a very useful chemo-enzymatic synthesis of the enantioenriched (4*R*,6*S*)-acetoxy-γ-ionone (**2**) starting from racemic ionone alpha (Figure 2). The following palladium-catalysed reductive elimination of the acetate functional group allows the preparation of (*S*)-γ-ionone (**3**), both in high enantiomeric and stereoisomeric purity. In addition, we have previously demonstrated that enantiopure γ-ionone isomers could be selectively reduced to the corresponding γ-dihydroionone derivatives by means of $Bu_3SnH$ and catalytic $(Ph_3P)_2PdCl_2$ [14]. The combination of these findings offers a reliable synthetic access to (*S*)-γ-dihydroionone (**4**), not strictly depending upon natural sources.

With this chiral building block in hand, we looked for an effective and stereoselective C-2 homologation process.

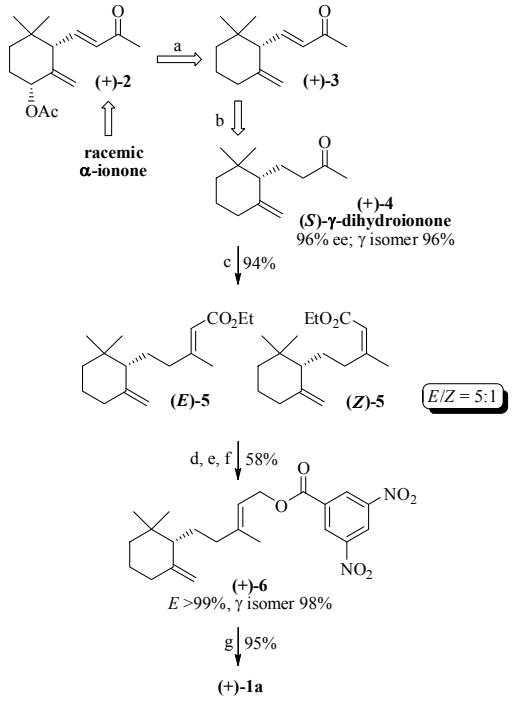



**Figure 2**: Synthesis of (*S*)-*trans*-γ-monocyclofarnesol (+)-**1a** starting from (4*R*,6*S*)-acetoxy-γ-ionone. Reagents and conditions: a) $Et_3N$, $HCO_2H$, $(Ph_3P)_2PdCl_2$ cat., THF reflux; b) $Bu_3SnH$, $NH_4Cl$, $H_2O$, $(Ph_3P)_2PdCl_2$ cat., THF at rt; c) $(EtO)_2POCH_2CO_2Et$, NaH, THF, from 0° to 50° C; d). DIBAH in toluene, THF, –10° C; e) 3,5-dinitrobenzoyl chloride, pyridine, DMAP cat., $CH_2Cl_2$; f) three crystallizations from *n*-hexane; g) NaOH, MeOH, rt.

It was previously demonstrated [11] that Horner-Wadsworth-Emmons reaction between methyl diethylphosphonoacetate and γ-dihydroionone affords a 63:27 mixture of the (*E*)- and (*Z*)-γ-monocyclofarnesyl methyl esters, respectively, which could be reduced to the corresponding γ-monocyclofarnesol isomers. Since neither the latter alcohols nor the starting esters are separable by chromatography, the fractional distillation of the isomeric mixture of the esters was employed in the stereoselective preparation of **1a**. Otherwise, we found that the latter tricky separation can be avoided by combining an improvement in the condensation stereoselectivity with the direct purification of the obtained *E*/*Z* isomeric mixture of alcohols **1a**, through fractional crystallization of their 3,5-dinitrobenzoate esters. More specifically, we observed a considerable increasing in the *E*/*Z* selectivity when the Horner-Wadsworth-Emmons reaction was performed both by using an excess of triethylphosphonoacetate instead of methyl diethylphosphonoacetate and by a carefully control of the reaction temperature (see experimental).

According to our protocol, (+)-**4** was converted into an 83:17 mixture of the γ-monocyclofarnesyl ethyl esters (*E*)-**5** and (*Z*)-**5**, respectively, in 94% overall yield. The latter isomeric mixture was then reduced with DIBAH to give the corresponding allylic alcohols, which were directly treated with 3,5-dinitrobenzoyl chloride and pyridine. Thus, the obtained esters mixture was crystallized three times from *n*-hexane. The collected crystals were obtained in 58% overall yield and turned out to be free of the (*Z*)-isomer. Indeed, neither the NMR analysis of (+)-**6** nor the GC analysis of the alcohol **1a** obtained from the aforementioned dinitrobenzoate ester revealed the presence of the unwanted isomer. Furthermore, the purity of the γ-isomer increased from 96% to 98%, thus indicating that the fractional crystallization is in many respects a very effective purification procedure. As a final point, the basic hydrolysis of the ester (+)-**6** afforded pure alcohol (+)-**1a** whose analytical data agree both with those reported for the natural product and with those described for the synthetic material.

Noteworthy is that the reported overall procedure was tested on a small scale (starting from a few hundred milligrams of γ-dihydroionone) and on a multi-gram scale, and analogous results were obtained. This flexibility corroborates the reliability of the process.

The exploitation of the obtained isomerically pure (*S*)-*trans*-γ-monocyclofarnesol for the synthesis of further natural products is ongoing and the results will be presented in due course.

## Experimental

***General:*** All moisture-sensitive reactions were carried out under a static atmosphere of nitrogen. All reagents were of commercial quality. TLC: Merck silica gel 60 $F_{254}$ plates. Column chromatography: silica gel. GC-MS analyses: HP-6890 gas chromatograph equipped with a 5973 mass detector, using a HP-5MS column (30 m × 0.25 mm, 0.25 μm firm thickness; Hewlett Packard) with the following temp. program: 60° (1 min) – 6°/min – 150° (1 min)– 12°/min – 280° (5 min); carrier gas, He; constant flow 1mL/min; split ratio, 1/30; $t_R$ given in min: $t_R$(**1a**) 21.79, $t_R$(**4**) 16.00, $t_R$(*E*-**5**) 22.71, $t_R$(*Z*-**5**) 21.93; mass spectra: *m/z* (rel.%). Mass spectrum of compound (+)-**6** was recorded on a Bruker ESQUIRE 3000 PLUS spectrometer (ESI detector). Optical rotations: Jasco-DIP-181 digital polarimeter, measured at 20°C. $^1$H and $^{13}$C Spectra: $CDCl_3$ solns. at rt; Bruker-AC-400 spectrometer at 400 and 100 MHz, respectively; chemical shifts in ppm rel to internal $SiMe_4$ (=0 ppm), *J* values in Hz.

**(*S*)-γ-Dihydroionone (+)-4:** Compound (+)-**4** (98% chemical purity; 96% ee; isomers ratio γ:α:β = 96:3.5:0.5) was obtained by reduction of (*S*)-γ-ionone, in turn prepared from (4*R*,6*S*)-acetoxy-γ-ionone, according to our previously reported procedures [13, 14].
$[\alpha]_D$ = +16.5° (*c* = 1, $CHCl_3$); Lit. [14]: $[\alpha]_D$ = +19.4° (*c* = 1.2, $CHCl_3$)
$^1$H NMR, $^{13}$C NMR, MS in accordance with those previously reported [14].

**(*S*)-Ethyl 5-(2,2-dimethyl-6-methylenecyclohexyl)-3-methylpent-2-enoate 5:** Triethyl phosphonoacetate (7 g, 31.2 mmol) was added dropwise under nitrogen over a period of 1 h to a stirred suspension of NaH (60% in mineral oil; 1.25 g, 31.2 mmol) in dry THF (25 mL) at 0°C. To the resulting mixture was slowly added a solution of (+)-**4** (2 g, 10.3 mmol) in dry THF (5 mL). When the addition was complete, the reaction was slowly heated at 50°C, stirring until the starting ketone was no longer detectable by TLC analysis (3 h). After cooling, the reaction was poured onto a mixture of crushed ice and a saturated solution of $NH_4Cl$ aq. (100 mL). The mixture was extracted with diethyl ether (2 × 100 mL). The organic phase was washed with brine, dried ($Na_2SO_4$), and concentrated under reduced pressure. The residue was chromatographed using *n*-hexane/diethyl ether (95:5–9:1) as eluent to afford pure **5** (2.55 g, 94% yield) as an *E*/*Z* mixture of isomers (*E*/*Z* ratio 5:1, by NMR analysis).

(*E*)-**5**: $^1$H NMR (400 MHz, $CDCl_3$): 0.84 (3H, s), 0.92 (3H, s), 1.18-1.27 (1H, m), 1.28 (3H, t, *J* = 7.2 Hz), 1.41-1.58 (4H, m), 1.58-1.69 (1H, m), 1.70 (1H, dd, *J* = 11.4, 3.3 Hz), 1.85-1.96 (1H, m), 1.97-2.16 (3H, m), 2.15 (3H, d, *J* = 1.3 Hz), 4.15 (2H, q, *J* = 7.2 Hz), 4.55 (1H, m), 4.78 (1H, br s), 5.65 (1H, br q, *J* = 1.3 Hz).

$^{13}$C NMR (100 MHz, $CDCl_3$): 14.3 (Me), 18.9 (Me), 23.6 ($CH_2$), 24.4 ($CH_2$), 26.2 (Me), 28.3 (Me), 32.4 ($CH_2$), 34.9 (C), 36.2 ($CH_2$), 39.6 ($CH_2$), 53.7 (CH), 59.4 ($CH_2$), 109.3 ($CH_2$), 115.4 (CH), 148.9 (C), 160.6 (C), 166.9 (C).
GC-MS (EI): *m/z* (%) = 264 [$M^+$] (3), 249 (25), 219 (8), 203 (14), 191 (9), 176 (100), 161 (69), 141 (39), 128 (76), 121 (31), 109 (99), 95 (66), 81 (87), 69 (69), 55 (28).

(*Z*)-**5**: $^1$H NMR (400 MHz, $CDCl_3$): 0.84 (3H, s), 0.93 (3H, s), 1.18-2.52 (11H, m), 1.26 (3H, t, *J* = 7.2 Hz), 1.87 (3H, d, *J* = 1.3 Hz), 4.13 (2H, q, *J* = 7.2 Hz), 4.60 (1H, m), 4.78 (1H, br s), 5.63 (1H, br s).
$^{13}$C NMR (100 MHz, $CDCl_3$): 14.3 (Me), 23.6 ($CH_2$), 25.1 ($CH_2$), 25.3 (Me), 26.4 (Me), 28.3 (Me), 32.2 ($CH_2$), 32.6 ($CH_2$), 34.9 (C), 36.1 ($CH_2$), 54.5 (CH), 59.3 ($CH_2$), 109.2 ($CH_2$), 115.9 (CH), 149.0 (C), 160.8 (C), 166.2 (C).
GC-MS (EI): *m/z* (%) = 264 [$M^+$] (3), 249 (23), 218 (7), 203 (10), 191 (7), 176 (76), 161 (47), 137 (78), 128 (100), 121 (30), 109 (72), 95 (64), 81 (82), 69 (58), 55 (27).

**(*S*)-(*E*)-5-(2,2-dimethyl-6-methylenecyclohexyl)-3-methylpent-2-enyl 3,5-dinitrobenzoate (+)-6:** DIBAH (14.5 mL of 1.7 M solution in toluene) was added dropwise under nitrogen to a stirred solution of ester **5** (*E*/*Z* ratio 5:1, 2.64 g, 10 mmol) in dry THF (40 mL) at −10 °C. The reaction was stirred at 0°C for 1 h, then diluted with diethyl ether (100 mL) and quenched with a saturated solution of $NH_4Cl$ aq. (50 mL). The mixture was acidified with diluted HCl aq. and extracted with diethyl ether (2 × 100 mL). The combined organic phases were washed with brine, dried ($Na_2SO_4$) and concentrated under reduced pressure. The residue was dissolved in $CH_2Cl_2$ (10 mL) and treated with pyridine (2 mL), DMAP (15 mg, 0.1 mmol) and a solution of 3,5-dinitrobenzoyl chloride (3 g, 13 mmol) in $CH_2Cl_2$ (5 mL). After complete transformation of the starting alcohol the mixture was diluted with water (200 mL) and extracted with diethyl ether (2x150 mL). The combined organic phases were washed with aq. $NaHCO_3$ (5% solution), brine and then dried ($Na_2SO_4$). Concentration at reduced pressure gave an oil that was purified by CC (*n*-hexane/$Et_2O$ 9:1) and crystallized 3 times from *n*-hexane. The ester (+)-**6** (2.4 g, 58% yield) was obtained as colorless crystals showing 99% chemical purity, free of *Z*-isomer and showing a γ/α isomeric ratio of 98:2 (by NMR analysis).
MP: 82-83ºC.
$[\alpha]_D$: +10.5 (*c* 2.1, $CHCl_3$).

$^1$H NMR (400 MHz, $CDCl_3$): 0.85 (3H, s), 0.92 (3H, s), 1.18-1.28 (1H, m), 1.42-1.58 (4H, m), 1.57-1.67 (1H, m), 1.71 (1H, dd, *J* = 11.4, 3.3 Hz), 1.80 (3H, s), 1.87 (1H, ddd, *J* = 14.3, 10.5, 6.1 Hz), 1.95-2.14 (3H, m), 4.55 (1H, m), 4.78 (1H, br s), 4.97 (2H, d, *J* = 7.2 Hz), 5.48 (1H, br t, *J* = 7.2 Hz), 9.16 (2H, d, *J* = 2.1 Hz), 9.21 (1H, t, *J* = 2.1 Hz).
$^{13}$C NMR (100 MHz, $CDCl_3$): 16.7 (Me), 23.6 ($CH_2$), 24.3 ($CH_2$), 26.3 (Me), 28.3 (Me), 32.4 ($CH_2$), 34.9 (C), 36.2 ($CH_2$), 38.1 ($CH_2$), 53.6 (CH), 63.7 ($CH_2$), 109.1 ($CH_2$), 116.7 (CH), 122.2 (CH), 129.4 (CH), 134.3 (C), 145.1 (C), 148.6 (C), 149.0 (C), 162.5 (C).
MS (ESI): *m/z* (positive ion) = 439 [$M^+$ + Na].

**(*S*)-*Trans*-γ-monocyclofarnesol = (*S*,*E*)-5-(2,2-dimethyl-6-methylenecyclohexyl)-3-methylpent-2-en-1-ol (+)-1a:** The 3,5-dinitrobenzoate (+)-**6** (0.5 g, 1.2 mmol) was dissolved in methanol (5 mL) and treated with NaOH (1 g, 25 mmol) in methanol (5 mL) stirring at rt until the starting ester was no longer detected by TLC analysis. The mixture was diluted with water (50 mL) and extracted with diethyl ether (2 x 50 mL). The organic phase was washed with brine, dried ($Na_2SO_4$) and concentrated *in vacuo*. The residue was roughly chromatographed (*n*-hexane/$Et_2O$ 9:1) to give (+)-**1a** (255 mg, 95% yield) as a colorless oil showing 97% chemical purity (by GC) and a γ/α isomeric ratio of 98:2 (by NMR analysis).
$[\alpha]_D$: +17.2 (*c* 2.3, $CHCl_3$).
The recorded optical rotation value is slightly inferior to those previously reported: $[\alpha]_D$: +18.2 (*c* 0.3, $CHCl_3$) [2]; $[\alpha]_D$: +21.1 ($CHCl_3$) [11]. Since the (*S*)-α-ionone isomers usually show a higher optical rotation value and opposite sign than their corresponding γ-isomers, the amount of about 2% of the (*S*)-α-monocyclofarnesol might take into account the discrepancy observed.
$^1$H NMR (400 MHz, $CDCl_3$): 0.84 (3H, s), 0.92 (3H, s), 1.17-1.26 (1H, m), 1.36-1.64 (6H, m), 1.67 (3H, s), 1.69 (1H, dd, *J* = 11.4, 3.2 Hz), 1.78 (1H, ddd, *J* = 14.3, 10.8, 6.2 Hz), 1.93-2.12 (3H, m), 4.14 (2H, d, *J* = 6.9 Hz), 4.54 (1H, m), 4.76 (1H, br s), 5.40 (1H, tq, *J* = 6.9, 1.3 Hz).
$^{13}$C NMR (100 MHz, $CDCl_3$): 16.3 (Me), 23.6 ($CH_2$), 24.5 ($CH_2$), 26.2 (Me), 28.3 (Me), 32.4 ($CH_2$), 34.8 (C), 36.2 ($CH_2$), 38.0 ($CH_2$), 53.7 (CH), 59.3 ($CH_2$), 108.9 ($CH_2$), 123.0 (CH), 140.3 (C), 149.1 (C).
GC-MS (EI): *m/z* (%) = 222 [$M^+$] (1), 207 (22), 204 (11), 189 (47), 177 (9), 161 (14), 148 (13), 133 (23), 121 (31), 109 (100), 95 (49), 81 (79), 69 (57), 55 (30).

**Supplementary data:** Copies of the $^1$H NMR, $^{13}$C NMR and DEPT NMR spectra of the (*S*)-*trans*-γ-monocyclofarnesol **1a** are available.

## References


[1] Suzuki KT, Suzuki N, Nozoe S. (**1971**) Isolation and enzymic formation of *trans*-γ-monocyclofarnesol. *Journal of the Chemical Society D: Chemical Communications*, 527.
[2] Toyota M, Ooiso Y, Kusuyama T, Asakawa Y. (**1994**) Drimane-type sesquiterpenoids from the liverwort *Diplophyllum serrulatum*. *Phytochemistry, **35***, 1263-1265.
[3] De Pascual Teresa J, Caballero E, Caballero C, Medarde M, Barrero AF, Grande M. (**1978**) Trixagol, natural γ-cyclogeranyl-geraniol from *Bellardia trixago* (L.) All. *Tetrahedron Letters*, ***19***, 3491-3494.
[4] Walker RP, Faulkner DJ. (**1981**) Diterpenes from the sponge *Dysidea amblia*. *Journal of Organic Chemistry*, ***46***, 1098-1102.
[5] Butler M, Capon R. (**1992**) The Luffarins (A-Z), novel terpenes from an Australian marine sponge, *Luffariella geometrica*. *Australian Journal of Chemistry*, ***45***, 1705-1743.
[6] Funayama S, Eda S, Komiyama K, Ohmura S, Tokunaga T. (**1989**) Structure of phenazinomycin, a novel antitumor antibiotic. *Tetrahedron Letters*, ***30***, 3151-3154.
[7] Wu H, Nakamura H, Kobayashi J, Ohizumi Y, Hirata Y. (**1984**) Agelasine-E and -F, novel monocyclic diterpenoids with 9-methyladeninium unit possessing inhibitory effects on Na,K-ATPase isolated from the Okinawan sea sponge *Agelas nakamurai* Hoshino. *Tetrahedron Letters*, ***25***, 3719-3722.
[8] Bakkestuen AK, Gundersen L-L. (**2003**) Synthesis of (+)-trixagol and its enantiomer, the terpenoid side chain of (−)-agelasine E. *Tetrahedron*, ***59***, 115-121.
[9] Kinoshita Y, Kitahara T. (**1997**) Total synthesis of phenazinomycin and its enantiomer via high-pressure reaction. *Tetrahedron Letters*, ***38***, 4993-4996.

[10] Bakkestuen AK, Gundersen L-L, Petersen D, Utenova BT, Vik A. (**2005**) Synthesis and antimycobacterial activity of agelasine E and analogs. *Organic & Biomolecular Chemistry*, ***3***, 1025-1033.
[11] Schulte-Elte K. Muller BL, Ohloff G. (**1978**) Photosensitized oxygenation of (*E*)- and (*Z*)-(+)-γ-monocyclofarnesol. An access to (*S*)-(+)-ambra-aldehyde. *Nouveau Journal de Chimie*, ***2***, 427-430.
[12] Barrero AF, Alvarez-Manzaneda EJ, Chahboun R, Cuerva JM, Segovia A. (**2000**) Synthetic applications of the thermal rearrangement of ozonides: First enantiospecific synthesis of marine metabolite Luffarin W. *Synlett*, 1269-1272.
[13] Serra S, Fuganti C, Brenna E. (**2006**) Synthesis, olfactory evaluation, and determination of the absolute configuration of the 3,4-didehydroionone stereoisomers. *Helvetica Chimica Acta*, ***89***, 1110-1122.
[14] Fuganti C, Serra S, Zenoni A. (**2000**) Synthesis and olfactory evaluation of (+)- and (−)-gamma-ionone. *Helvetica Chimica Acta*, ***83***, 2761-2768.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1573 - 1574

# ACAT Inhibitory Activity of Exudates from *Calocedrus macrolepis* var. *formosana*

**Yu-Hsin Hsieh[a,c], Kuan-Jung Chen[b], Shih-Chang Chien[f], Wen-Ling Cheng [b], Jun-Hong Xiao[b] and Sheng-Yang Wang[a,b,d,e,f,g,*]**

[a]*Molecular and Biological Agricultural Sciences Program*, *Taiwan International Graduate Program*, *National Chung-Hsing University and Academia Sinica*, *Taiwan*

[b]*Department of Forestry*, *National Chung-Hsing University*, *Taichung*, *Taiwan*

[c]*Graduate Institute of Biotechnology*, *National Chung-Hsing University*, *Taichung*, *Taiwan*

[d]*Agricultural Biotechnology Research Center*, *Academia Sinica*, *Taipei*, *Taiwan*

[e]*Agricultural Biotechnology Center*, *National Chung-Hsing University*, *Taichung*, *Taiwan*

[f]*Experimental Forest Management Office*, *National Chung-Hsing University*, *Taichung*, *Taiwan*

*taiwanfir@dragon.nchu.edu.tw*

**Received: May 28th, 2012; Accepted: October 17th, 2012**

Cholesterol acyltransferase (ACAT) is an enzyme controlling cholesterol esterification in cells. Large amounts of cholesterol esters accumulate in macrophages and smooth muscle cells of blood vessel walls resulting in the initial stages of atherosclerosis. Thus, atherosclerosis might be inhibited through inhibition of the activity of ACAT. In the present study, we identified by spectral analysis and chromatographic quantification that ferruginol was the most abundant component of exudates secreted from the wounding site of *Calocedrus macrolepis* Kurz var. *formosana*. Results obtained from the cholesterol absorption assay revealed that ferruginol exhibited a significant inhibitory activity on cholesterol absorption in mice macrophages (RAW 264.7 cell). Based on the results from analyzing the ratio of cholesterol esterification, ferruginol dose-dependently suppressed cholesterol esterification and the $IC_{50}$ value was 2.0 μg/mL. In conclusion, ferruginol revealed strong inhibitory activities that retarded the absorption and esterification of cholesterol in cells. Our finding indicates that ferruginol might possess a potential for development as a pharmaceutical product for preventing arteriosclerosis.

**Keywords:** Ferruginol, Arteriosclerosis, Acyl-CoA, Cholesterol acyltransferase, *Calocedrus macrolepis* Kurz var. *formosana*.

*Calocedrus macrolepis* Kurz var. *formosana* (Cupressaceae) is an endemic conifer in Taiwan. More than 100 compounds have been isolated from the plant, including monoterpenoids, diterpenoids, lignans and steroids [1-6]. The resin produced by plants, consisting mainly of susquiterpenoids and diterpenoids, is a vital defense system against insect and pathogen attacks [7]. In this study, we collected exudates from the bark surface of 20-year-old *C. macrolepis* and 7 diterpenoids, including ferruginol [8], *trans*-communic acid [9], isopimaric acid [10], isopimarol [11], 6α-hydroxysugiol, sugiol, and secoabietane dialdehyde [5] were identified using chromatographic separation and spectroscopic analyses. Ferruginol (Figure 1) was the most abundant compound identified in the exudates, forming approximately 77%, w/w, of the total.

ACAT is an enzyme which catalyzes the conversion of free cholesterol and fatty acyl CoA to a storage form of cholesterol, cholesteryl ester (CE) [12]. Atherosclerosis results from an excess accumulation of cholesterol and macrophages that deposit on the blood vessel wall and eventually narrow the artery [13]. In the present study, we utilized NBD-cholesterol, a fluorescent sterol analog, to mimic the absorption and esterification of native cholesterol in cultured cells [14].

RAW 264.7 cells pre-treated with ferruginol for 1 h significantly suppressed cholesterol absorption in a dose-dependent manner (Table 1). The inhibitory activity was 62.5% at a concentration of 20 μg/mL and the $IC_{50}$ value was 9.5 μg/mL. Sandoz 58-035, a known inhibitor of cholesterol acyltransferase [15], showed 45.8% inhibition. Our data revealed that ferruginol has a higher potential for suppressing cholesterol absorption than Sandoz 58-035 and the working mechanism is worth further investigation.

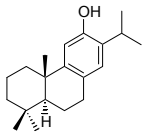



**Figure 1**: The dominant compound, ferruginol, in the exudates secreted from *Calocedrus macrolepis* var. *formosana*.

**Table 1**: Inhibition effect of ferruginol on cholesterol absorption

| Compounds | Concentration (μg/mL) | Inhibition of cholesterol absorption (%) |
|---|---|---|
| Sandoz 58-035 | 10 | 45.8 |
| Ferruginol | 5 | 41.4 |
| Ferruginol | 10 | 52.5 |
| Ferruginol | 20 | 62.5 |

RAW 264.7 cells were incubated in medium containing ferruginol at indicated dosages for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. 10 μg/mL Sandoz 58-035 (Sigma St. Louis MO) was used as a reference compound. The inhibitory effect was compound-treated group compared with vehicle control (DMSO).

To decipher whether the inhibitory effect of cholesterol absorption by ferruginol is correlated with ACAT activity, we isolated the lipid contents from ferruginol treated cells and detected the levels of NDB-cholesterol and NBD-cholesterol ester, respectively, using high performance liquid chromatography (HPLC). Our data showed that ferruginol dose-dependently suppressed the level of NBD-cholesterol ester, which dramatically decreased from 25.3% to 1.4%

at a concentration of 10 μg/mL (Table 2). By comparison with control, cells treated with 10 μg/mL ferruginol suppressed 94.4% of cholesterol esterification. From these findings we suggest that ferruginol efficiently inhibits the ACAT activity of converting free cholesterol to CE by macrophages. In conclusion, ferruginol revealed a strong retarding activity for the absorption and esterification of cholesterol in cells. Our finding indicates that ferruginol might possess the potential to be developed as a pharmaceutical product for preventing arteriosclerosis.

**Table 2**: Inhibitory activity of cholesterol esterification by ferruginol.

| Ferruginol dosages (μg/mL) | Ratio of NBD-cholesteryl esters | Inhibition of cholesterol esterification (%) |
|---|---|---|
| Control | 25.3 | 0.0 |
| 1.0 | 20.2 | 20.4 |
| 2.5 | 9.2 | 63.6 |
| 5.0 | 3.4 | 86.7 |
| 10.0 | 1.4 | 94.4 |

RAW 264.7 cells were incubated in medium containing ferruginol at indicated dosages for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. Isolated lipid contents and detected the levels of NBD-CE and NBD-cholesterol respectively using HPLC. The inhibitory effect was ferruginol-treated group compared with control.

## Experimental

***Materials:*** The exudates secreted from the wounding site of 20-year-old *C. macrolepis* were dissolved in ethyl acetate and separated by HPLC using a Luna silica column (250 × 10 mm; 5 μm, Phenomenex, Torrance, CA) eluted with *n*-hex/EtOAc (*n*-hex/EtOAc = 88/12, flow rate = 2.5 mL/min). Compounds **1** to **7** were obtained at the retention times of 10.8, 16.0, 19.3, 21.6, 24.6, 33.5, and 38.3 min, respectively. The structures of the compounds were elucidated using spectroscopic analysis. The amount of the major compound (ferruginol) in exudates was further analyzed by the same HPLC system. The peak area of ferruginol in the chromatogram of the exudates (with known loading concentration) was then defined and its content in the exudates calculated based on the quantity calibrated from the standard calibration curve.

***Cell culture:*** RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (GIBCO Carlsbad, CA) at 37°C in a 5% $CO_2$ humidified incubator.

***Cholesterol absorption assay:*** RAW 264.7 cells were plated in 24-well culture plates at a density of $1 \times 10^6$ cells/well and allowed to recover for 24 h. Cells were incubated in medium containing ferruginol at various concentrations (5, 10 and 20 μg/mL) for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. 10 μg/mL Sandoz 58-035 (Sigma St. Louis MO) was used as a reference compound. After incubation, the cells were washed thrice with phosphate buffered saline (PBS). Fluorescence was detected using a Chameleon V Multilabel Microplate Reader (Hidex) with 485 nm excitation and 535 nm emission filters [16].

***Cholesterol esterification assay:*** RAW 264.7 cells were plated in 6 cm culture dishes at a density of $5 \times 10^6$ cells/well and allowed to recover for 24 h. Cells were incubated in medium containing ferruginol at various concentrations (1, 2.5, 5 and 10 μg/mL) for 1 h and further treated with 1 μg/mL NBD-cholesterol for 6 h. Cells were collected and the lipid contents isolated using *n*-hex/2-propanol (3:2) solution. The ratio of NDB-cholesterol and NBD-cholesterol ester was determined from the absorbance at 443 nm using HPLC (Luna Silica 250 mm × 10 mm, 5 μm).

$$\text{Inhibition\%} = \left(1 - \frac{\text{ratio of NBD-CE}_{\text{sample}}}{\text{ratio of NBD-CE}_{\text{control}}}\right) 100$$

## References


[1] Fang JM, Hsu KC, Cheng YS. (**1989**) Terpenoids from leaves of *Calocedrus formosana*. *Phytochemistry*, ***28***, 1173-1175.
[2] Fang JM, Hsu KC, Cheng YS. (**1989**) Lignans from leaves of *Calocedrus formosana*. *Phytochemistry*, ***28***, 3553-3555.
[3] Fang JM, Liu MY, Cheng YS. (**1990**) Lignans from wood of *Calocedrus formosana*. *Phytochemistry*, ***29***, 3048-3049.
[4] Chien SC, Liu HK, Kuo YH. (**2004**) Two new compounds from the leaves of *Calocedrus macrolepsis* var. *formosana*. *Chemical and Pharmaceutical Bulletin*, ***52***, 762-763.
[5] Wang SY, Wu JH, Cheng SS, Lo CP, Chang HN, Shyur LF, Chang ST. (**2004**) Antioxidant activity of extracts from *Calocedrus formosana* leaf, bark and heartwood. *Journal of Wood Science*, ***50***, 422-426.
[6] Hsieh CL, Tseng MH, Shao YY, Chang JY, Kuo CC, Chang CY, Kuo YH. (**2006**) C-35 terpenoids from the bark of *Calocedrus macrolepis* var. *formosana* with activity against human cancer cell lines. *Journal of Natural Products*, ***69***, 1611-1613.
[7] Trapp S, Croteau R. (**2001**) Defensive resin biosynthesis in conifers. *Annual Review of Plant Physiology and Plant Molecular Biology*, ***52***, 689-724.
[8] Chang ST, Wang SY, Wu CL, Su YC, Kuo YH. (**1999**) Antifungal compounds in the ethyl acetate soluble fraction of the extractives of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood. *Holzforschung*, ***53***, 487-490.
[9] Hanari N, Yamamoto H, Ooi T, Kusumi T, Kuroda K. (**2001**) A new diterpene dimer from the bark of *Chamaecyparis obtusa*. *Journal of Wood Science*, ***47***, 36-40.
[10] Wenkert E, Buckwalter BL. (**1972**) Carbon-13 nuclear magnetic resonance spectroscopy of naturally occurring substances X. Pimaradienes. *Journal of American Chemical Society*, ***94***, 4367–4369.
[11] Kofujita H, Fujino Y, Ota M, Takahashi K. (**2006**) Antifungal diterpenes from the bark of *Cryptomeria japonica* D. Don. *Holzforschung*, ***60***, 20-23.
[12] Chang TY, Chang CCY, Cheng D. (**1997**) Acyl-coenzyme A: cholesterol acyltransferase. *Annual Review of Biochemistry.* ***66***, 613- 638.
[13] Ross R. (**1999**) Atherosclerosis-an inflammatory disease. *New England Journal of Medicine*, ***340***, 115-126.
[14] Sparrow CP, Patel S, Baffic J, Chao YS, Hernandez M, Lam MH, Montenegro J, Wright SD, Detmers PA. (**1999**) A fluorescent cholesterol analog traces cholesterol absorption in hamsters and is esterified *in vivo* and *in vitro*. *Journal of Lipid Research,* ***40***, 1747–1757.
[15] Ross AC, Go KJ, Heider JG, Rothblat GH. (**1984**) Selective inhibition of acyl coenzyme A: cholesterol acyltransferase by compound 58-035. *Journal of Biological Chemistry*, ***259***, 815-819
[16] Lada AT, Davis M, Kent C, Chapman J, Tomoda H, Omura S, Rudel LL. (**2004**) Identification of ACAT1- and ACAT2-specific inhibitors using a novel, cell-based fluorescence assay: individual ACAT uniqueness. *Journal of Lipid Research*, ***45***, 378-386

NPC Natural Product Communications

2012
Vol. 7
No. 12
1575 - 1578

# Cucurbitane-Type Triterpenoids from the Fruit Pulp of *Momordica charantia*

Yun-Wen Liao[a,#], Chiy-Rong Chen[b,#], Yueh-Hsiung Kuo[c], Jue-Liang Hsu[d], Wen-Ling Shih[d], Hsueh-Ling Cheng[d], Tzou-Chi Huang[a,d,*] and Chi-I Chang[d,*]

[a]*Department of Food Science*, *National Pingtung University of Science and Technology*, *Pingtung 91201*, *Taiwan*

[b]*Department of Life Science*, *National Taitung University*, *Taitung 95002*, *Taiwan*

[c]*Tsuzuki Institute for Traditional Medicine*, *College of Pharmacy*, *China Medical University*, *Taichung 40402*, *Taiwan*

[d]*Department of Biological Science and Technology*, *National Pingtung University of Science and Technology*, *Pingtung 91201*, *Taiwan*

[#]*equal contribution to this article*

*changchii@mail.npust.edu.tw*

**Received: September 26th, 2012; Accepted: October 4th, 2012**

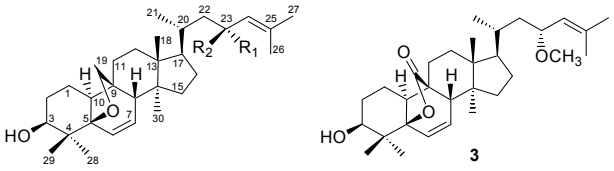
Three new cucurbitane-type triterpenoids, 5*β*,19-epoxy-23(*R*)-methoxycucurbita-6,24-dien-3*β*-ol (**1**), 5*β*,19-epoxy-23(*S*)-methoxycucurbita-6,24-dien-3*β*-ol (**2**), and 3*β*-hydroxy-23(*R*)-methoxycucurbita-6,24-dien-5*β*,19-olide (**3**), were isolated from the fruit pulp of *Momordica charantia*. Their structures were established on the basis of extensive NMR ($^1$H, $^{13}$C, COSY, HMQC, HMBC, and NOESY) and EI-MS studies. Compound **1** exhibited cytotoxic activity against the SK-Hep 1 cell line.

**Keywords:** *Momordica charantia*, Cucurbitaceae, Fruit, Triterpenoid, Cucurbitane.

*Momordica charantia* L. (Cucurbitaceae), commonly known as bitter melon, is a slender-stemmed tendril climber and is cultivated throughout the world as a vegetable crop. Tissues of this plant have extensively been used in folk medicine as a remedy for diabetes in Asia. Previous investigations have shown that the extracts and compounds of tissues of *M. charantia* possess cytotoxic, anti-diabetic, and anti-inflammatory activities [1-4] and more than seventy cucurbitane-type triterpenes have been isolated from the fruits [4-13], seeds [14,15], root [16], and leaves and vines [17,18] of *M. charantia*. In the course of our research for bioactive secondary metabolites from Taiwanese *M. charantia*, we have reported the isolation and structure elucidation of twenty-five cucurbitane-type triterpenoids from the MeOH extract of the stems of this plant [19-23].

In the continuing phytochemical investigation of *M. charantia*, we further identified three new cucurbitane-type triterpenoids, 5*β*,19-epoxy-23(*R*)-methoxycucurbita-6,24-dien-3*β*-ol (**1**), 5*β*,19-epoxy-23(*S*)-methoxycucurbita-6,24-dien-3*β*-ol (**2**), and 3*β*-hydroxy-23(*R*)-methoxycucurbita-6,24-dien-5*β*,19-olide (**3**) from the fruit pulp of *M. charantia*. In this paper, we report the extraction, isolation, purification, and structure elucidation of compounds **1-3** and the cytotoxic activity of compound **1**.

The MeOH extract of the fruit pulp of *M. charantia* was suspended in water and then successively partitioned with EtOAc and *n*-BuOH. The combined *n*-BuOH soluble layer was subjected to Diaion HP-20 and silica gel column chromatography and further purification by normal phase semi-preparative HPLC to yield compounds **1-3**. The structures of the three new cucurbitane-type triterpenoids were elucidated as follows.




**1** $R_1$ = $OCH_3$, $R_2$ = H
**2** $R_1$ = H, $R_2$ = $OCH_3$

**Figure 1: Structures of compounds 1-3 from *M. charantia*.**

The molecular formula of compound **1** was assigned as $C_{31}H_{50}O_3$ based on the molecular ion at *m/z* 470.3767 in the HR-EI-MS. The IR spectrum showed absorption for a hydroxyl group (3463 $cm^{-1}$). The $^1$H and $^{13}$C NMR spectra of **1** (Tables 1 and 2) displayed signals characteristic of the presence of four methyl singlets [$\delta_H$ 0.83, 0.86, 0.87, 1.18 (3H each, s)], one methyl doublet [$\delta_H$ 0.91 (3H, d, *J* = 6.4 Hz)], two vinylic methyls [$\delta_H$ 1.65 (3H, d, *J* = 1.2 Hz), 1.71 (3H, d, *J* = 1.2 Hz)], a methoxy [$\delta_H$ 3.19 (3H, s)], an oxymethylene [$\delta_H$ 3.48 (1H, d, *J* = 8.4 Hz), 3.64 (1H, d, *J* = 8.4 Hz); $\delta_C$ 80.1 (t)], one allylic oxymethine [$\delta_H$ 3.93 (1H, dt, *J* = 3.2, 9.2 Hz)], one olefinic methine [$\delta_H$ 5.00 (1H, dt, *J* = 1.2, 7.6 Hz)], and an allylic ABX system of *cis*-oriented cyclohexene [$\delta_H$ 6.00 (1H, dd, *J* = 2.4, 10.0 Hz, H-6), 5.61 (1H, dd, *J* = 3.6, 10.0 Hz, H-7), 2.32 (1H, brt, *J* = 3.6 Hz, H-8); $\delta_C$ 131.8 (d), 131.9 (d), 52.2 (d)]. The $^{13}$C NMR spectrum of **1** revealed 31 carbon signals, which were assigned by DEPT experiments as seven aliphatic methyl, one methoxy, seven aliphatic methylene, four aliphatic methine, four aliphatic quaternary, three olefinic methine, one quaternary olefinic, one secondary oxygenated, two tertiary oxygenated, and one quaternary oxygenated carbons. The spectroscopic characteristics were similar to the known compound (23*E*)-5*β*,19-epoxycucurbita-6,23-diene-3β,25-diol [19], except for the signals of C-20－C-27.

**Table 1**: $^1$H NMR data for **1-3** (400 MHz in $CDCl_3$).

| No. | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 1.44 m | 1.42 m | 1.26 m, 1.60 m |
| 2 | 1.76 m | 1.76 m | 1.76 m |
| 3 | 3.39 brd (10.0) [a] | 3.38 brd (10.0) | 3.44 brd (8.0) |
| 6 | 6.00 dd (2.4, 10.0) | 6.01 dd (2.4, 10.0) | 6.17 dd (2.4, 10.0) |
| 7 | 5.61 dd (3.6, 10.0) | 5.61 dd (3.6, 10.0) | 5.70 dd (3.6, 10.0) |
| 8 | 2.32 brt (3.6) | 2.30 brt (3.6) | 2.50 brt (3.6) |
| 10 | 2.24 dd (7.6, 11.2) | 2.25 dd (7.2, 11.6) | 2.62 dd (5.6, 12.0) |
| 11 | 1.50 m, 1.72 m | 1.50 m, 1.74 m | 1.70 m, 2.20 m |
| 12 | 1.60 m | 1.60 m | 1.56 m, 1.72 m |
| 15 | 1.28 m | 1.34 m | 1.30 m |
| 16 | 1.34 m, 1.92 m | 1.27 m, 1.94 m | 1.36 m, 1.90 m |
| 17 | 1.38 m | 1.41 m | 1.40 m |
| 18 | 0.86 s | 0.81 s | 0.92 s |
| 19 | 3.48 d (8.4), 3.64 d (8.4) | 3.48 d (8.4), 3.64 d (8.4) | |
| 20 | 1.68 m | 1.24 m | 1.71 m |
| 21 | 0.91 d (6.4) | 0.88 d (7.2) | 0.93 d (7.6) |
| 22 | 0.96 m, 1.66 m | 1.34 m, 1.52 m | 0.94 m, 1.65 m |
| 23 | 3.93 dt (3.2, 9.2) | 3.90 dt (3.6, 9.2) | 3.92 dt (3.6, 9.6) |
| 24 | 5.00 dt (1.2, 9.2) | 4.90 dt (1.2, 9.2) | 5.00 dt (1.2, 9.6) |
| 26 | 1.65 d (1.2) | 1.68 d (1.2) | 1.65 d (1.2) |
| 27 | 1.71 d (1.2) | 1.76 d (1.2) | 1.71 d (1.2) |
| 28 | 1.18 s | 1.18 s | 0.82 s |
| 29 | 0.87 s | 0.87 s | 1.24 s |
| 30 | 0.83 s | 0.84 s | 0.92 s |
| 3-OH | 4.00 d (10.0) | 3.98 d (10.0) | |
| 23-$OCH_3$ | 3.19 s | 3.20 s | 3.19 s |

[a] Coupling constants are presented in Hz.

**Table 2**: $^{13}$C NMR data for **1-3** (100 MHz in $CDCl_3$).

| No. | 1 | 2 | 3 | No. | 1 | 2 | 3 |
|---|---|---|---|---|---|---|---|
| 1 | 17.8 | 17.6 | 18.4 | 17 | 51.2 | 50.9 | 51.1 |
| 2 | 27.6 | 27.3 | 26.5 | 18 | 15.2 | 14.8 | 14.6 |
| 3 | 76.4 | 76.2 | 75.3 | 19 | 80.1 | 79.8 | 181.5 |
| 4 | 37.4 | 37.2 | 37.0 | 20 | 32.7 | 33.1 | 32.5 |
| 5 | 87.8 | 87.5 | 85.4 | 21 | 18.9 | 19.3 | 18.7 |
| 6 | 131.9 | 131.5 | 131.1 | 22 | 43.0 | 42.2 | 42.8 |
| 7 | 131.8 | 131.7 | 133.4 | 23 | 74.9 | 75.9 | 74.6 |
| 8 | 52.2 | 52.0 | 44.4 | 24 | 127.2 | 126.1 | 126.9 |
| 9 | 45.7 | 45.4 | 51.0 | 25 | 134.9 | 136.6 | 134.6 |
| 10 | 39.0 | 38.8 | 39.9 | 26 | 18.4 | 18.5 | 18.1 |
| 11 | 23.8 | 23.6 | 21.6 | 27 | 26.1 | 25.9 | 25.8 |
| 12 | 31.1 | 30.8 | 29.9 | 28 | 20.7 | 20.5 | 19.2 |
| 13 | 45.6 | 45.2 | 45.1 | 29 | 24.8 | 24.5 | 23.5 |
| 14 | 48.8 | 48.6 | 47.7 | 30 | 20.2 | 20.0 | 20.3 |
| 15 | 33.4 | 33.1 | 33.1 | 23-$OCH_3$ | 56.0 | 55.3 | 55.7 |
| 16 | 28.3 | 28.2 | 27.6 | | | | |

Thus, compound **1** was presumed to exhibit a 5*β*,19-epoxycucurbita-6-en-3*β*-ol skeleton. The HMBC spectrum (Figure 2) of **1** showed long-range correlations between H-3 ($\delta_H$ 3.39) and C-1 ($\delta_C$ 17.8), C-4 ($\delta_C$ 37.4), and C-5 ($\delta_C$ 87.8), and this suggested that one hydroxyl group was located at C-3. The HMBC correlations between H-6 ($\delta_H$ 6.00)/C-4 ($\delta_C$ 37.4), C-5 ($\delta_C$ 87.8), C-8 ($\delta_C$ 52.2), and C-10 ($\delta_C$ 39.0); between H-7 ($\delta_H$ 5.61)/C-5 ($\delta_C$ 87.8), C-8 ($\delta_C$ 52.2), C-9 ($\delta_C$ 45.7) and C-14 ($\delta_C$ 48.8); and between H-19 ($\delta_H$ 3.48, 3.64)/C-5, C-8, C-9, C-10, and C-11 ($\delta_C$ 23.8) confirmed that the oxymethylene (C-19) was linked *via* an oxygen atom to C-5. The relative configurations of stereogenic carbon atoms in the tetracyclic rings were determined by significant NOE correlations between H-3 ($\delta_H$ 3.39)/Me-28 ($\delta_H$ 1.18), H-3/Me-29 ($\delta_H$ 0.87), H-8 ($\delta_H$ 2.32)/Me-18 ($\delta_H$ 0.86), H-8/H-19 ($\delta_H$ 3.48), H-10 ($\delta_H$ 2.24)/Me-28, H-10/Me-30 ($\delta_H$ 0.83), Me-18/H-20 ($\delta_H$ 1.68), and H-12 ($\delta_H$ 1.60)/Me-21 ($\delta_H$ 0.91) in the NOESY spectrum (Figure 2). The $^{13}$C chemical shifts from C-20 to C-27 in the side chain were similar to those of cornusalterin G [24]. The $^1$H NMR signals of the side chain include a secondary methyl [$\delta_H$ 0.91 (3H, d, $J$ = 6.4 Hz)], two vinylic methyls [$\delta_H$ 1.65 and 1.71 (3H each, d, $J$ = 1.2 Hz)], one *O*-methyl [$\delta_H$ 3.19 (3H, s)], one allylic oxymethine [$\delta_H$ 3.93 (1H, dt, $J$ = 3.2, 9.2 Hz)], and one olefinic methine [$\delta_H$ 5.00 (1H, dt, $J$ = 1.2, 7.6 Hz)]. The above data as well as the HMBC correlations (Figure 1) between Me-21 ($\delta_H$ 0.91)/C-20 ($\delta_C$ 32.7) and C-22 ($\delta_C$ 43.0); H-20 ($\delta_H$ 1.68)/C-22 ($\delta_C$ 43.0) and C-23 ($\delta_C$ 74.9); 23-OMe ($\delta_H$ 3.19)/C-23; H-23 ($\delta_H$3.93)/C-25 ($\delta_C$ 134.9); Me-26 ($\delta_H$ 1.65)/C-24 ($\delta_C$ 127.2) and C-25 permitted assignment of the side chain structure as a 23-methoxy-$\Delta^{24}$ C8

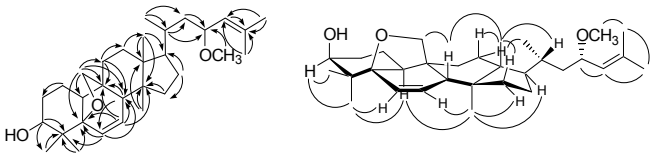



**Figure 2**: Selected HMBC and NOESY correlations of **1**.

moiety [24]. The stereochemistry at C-23 of **1** and its epimer, compound **2**, were determined to be *R* and *S*, respectively, by comparison of the $^{13}$C NMR signals of the side-chain with those of two pairs of 23-epimer compounds, charantosides II/VI [25] and cornusalterin G/H [24]. The $\Delta\delta_C$ values [$\delta_C$ (**1**)−$\delta_C$ (**2**)] for the side-chain signals were calculated as −0.4 (C-20), −0.4 (C-21), +0.8 (C-22), −1.0 (C-23), +1.1 (C-24), and −1.7 (C-25) from the $^{13}$C NMR data of compounds **1** and **2** (Table 2), which were consistent with the $\Delta\delta$ values [$\delta_C$ (23*R*)−$\delta_C$ (23*S*)] between charantosides II/VI and cornusalterin G/H. The results indicated that the absolute configuration of C-23 was *R* in **1** and *S* in **2**. Accordingly, compound **1** was determined to be 5*β*,19-epoxy-23(*R*)-methoxycucurbita-6,24-dien-3*β*-ol.

The HR-EI-MS of **2** showed a molecular ion at *m/z* 470.3762, which corresponded to the molecular formula, $C_{31}H_{50}O_3$, indicating seven degrees of unsaturation. The IR spectrum indicated the presence of a hydroxyl group (3467 cm$^{-1}$). The $^1$H and $^{13}$C NMR spectra of **2** (Tables 1 and 2) showed resonances for four methyl singlets [$\delta_H$ 0.81, 0.84, 0.87, 1.18 (3H each, s) ], one methyl doublet [$\delta_H$ 0.88 (3H, d, $J$ = 7.2 Hz)], two vinylic methyls [$\delta_H$ 1.68 (3H, d, $J$ = 1.2 Hz), 1.76 (3H, d, $J$ = 1.2 Hz)], a methoxy [$\delta_H$ 3.20 (3H, s)], an oxymethylene [$\delta_H$ 3.48 (1H, d, $J$ = 8.4 Hz), 3.64 (1H, d, $J$ = 8.4 Hz); $\delta_C$ 79.8 (t)], one allylic oxymethine [$\delta_H$ 3.90 (1H, dt, $J$ = 3.6, 9.2 Hz)], one olefinic methine [$\delta_H$ 4.90 (1H, dt, $J$ = 1.2, 9.6 Hz)], and an allylic ABX system of *cis*-oriented cyclohexene [$\delta_H$ 6.01 (1H, dd, $J$ = 2.4, 10.0 Hz, H-6), 5.61 (1H, dd, $J$ = 3.6, 10.0 Hz, H-7), 2.30 (1H, brt, $J$ = 3.6 Hz, H-8); $\delta_C$ 131.5 (d), 131.7 (d), 52.0 (d)]. The $^{13}$C NMR spectrum of **2** revealed 31 carbon signals, which were differentiated by DEPT experiments into seven aliphatic methyl, one methoxy, seven aliphatic methylene, four aliphatic methine, four aliphatic quaternary, three olefinic methine, one quaternary olefinic, one secondary oxygenated, two tertiary oxygenated, and one quaternary oxygenated carbons. The $^1$H and $^{13}$C NMR spectra of **2** were similar to those of compound **1**, except for the $^{13}$C NMR signals from C-20 to C-27. As mentioned above, comparison of the side-chain $^{13}$C NMR data with those of 23-epimer compounds, charantosides II/VI and cornusalterin G/H [24,25] permitted us to assign the absolute configuration of C-23 in **2** as *S*. The structure of **2** was consequently characterized as 5*β*,19-epoxy-23(*S*)-methoxycucurbita-6,24-dien-3*β*-ol.

By HR-EI-MS, compound **3** revealed a molecular formula of $C_{31}H_{48}O_4$ from the molecular ion at *m/z* $[M]^+$ 484.3556. The IR spectrum showed a band attributable to a lactone carbonyl group (1742 cm$^{-1}$). The $^1$H and $^{13}$C NMR spectra of **3** (Tables 1 and 2) indicated the presence of four methyl singlets [$\delta_H$ 0.82, 1.24 (3H each, s), 0.92 (3H×2, s)], one methyl doublet [$\delta_H$ 0.93 (3H, d, $J$ = 7.6 Hz)], two vinylic methyls [$\delta_H$ 1.65 (3H, d, $J$ = 1.2 Hz), 1.71 (3H, d, $J$ = 1.2 Hz)], a methoxy [$\delta_H$ 3.19 (3H, s)], an oxymethine [$\delta_H$ 3.44 (1H, brd, $J$ = 8.0 Hz), one allylic oxymethine [$\delta_H$ 3.92 (1H, dt, $J$ = 3.6, 9.6 Hz)], and one olefinic methine [$\delta_H$ 5.00 (1H, dt, $J$ = 1.2, 8.4 Hz)]. Thirty-one carbon signals were observed in the $^{13}$C NMR spectrum of **3**, and were resolved by DEPT experiments as seven aliphatic methyl, one methoxy, seven aliphatic methylene, four aliphatic methine, four aliphatic quaternary, three olefinic methines, one quaternary olefinic, two tertiary oxygenated, one

quaternary oxygenated, and one ketone carbonyl carbons. Comparison of the $^1$H and $^{13}$C NMR spectra of **3** with those of **1** revealed that the two compounds are structurally very similar in rings A, C, and D and the side chain. The only difference was that the oxymethylene [$\delta_H$ 3.48 (1H, d, $J$ = 8.4 Hz), 3.64 (1H, d, $J$ = 8.4 Hz); $\delta_C$ 80.1 (t)] in **1** was replaced by a lactone carbonyl [$\delta_C$ 181.5 (s)] [21]. This proposed structure was further suggested by the fragment ion peak at *m/z* 440 $[M-CO_2]^+$ in the EI-MS of **3** and the HMBC correlations between H-10 ($\delta_H$ 2.62)/C-19 ($\delta_C$ 181.5 s) and H-11 ($\delta_H$ 2.20)/C-19. Thus, compound **3** was formulated as 3*β*-hydroxy-23(*R*)-methoxycucurbita-6,24-dien-5*β*,19-olide.

Compound **1** was evaluated for cytotoxic activity against human hepatoma SK-Hep-1 cells with an $IC_{50}$ value of 26.6 μM. 5-FU (Fluorouracil) was used as positive control ($IC_{50}$ = 1.0 μM).

## Experimental

***General experimental procedures:*** Optical rotations were measured using a JASCO DIP-180 digital spectropolarimeter. The IR spectra were obtained on a Nicolet 510P FT-IR spectrometer. The NMR spectra were recorded in $CDCl_3$ at room temperature on a Varian Mercury plus 400 NMR spectrometer, and the solvent resonance was used as internal shift reference (TMS as standard). The 2D NMR spectra were recorded using standard pulse sequences. EI-MS and HR-EI-MS were recorded on a Finnigan TSQ-700 and a JEOL SX-102A spectrometer, respectively. TLC was performed using silica gel 60 $F_{254}$ plates (200 μm, Merck). Diaion HP-20 (Mitsubishi) and silica gel (230−400 mesh ASTM, Merck) were used for column chromatography. HPLC was performed on a Hitachi L-7000 chromatograph with a Lichrosorb Si gel 60 column (5μm, 250 × 10 mm).

***Plant material:*** The fruits of *Momordica charantia* were collected in Pingtung County, Taiwan, in July 2006. The plant material was identified by Prof. Sheng-Zehn Yang, Curator of the Herbarium, National Pingtung University of Science and Technology. A voucher specimen (no. BT114) was deposited in the Department of Biological Science and Technology, National Pingtung University of Science and Technology, Pingtung, Taiwan.

***Extraction and isolation:*** Oven-dried pieces of fruit pulp of *Momordica charantia* (35.7 kg) were extracted 3 times with methanol (100 L) at room temperature (7 days each). The MeOH extract was evaporated *in vacuo* to afford a black residue, which was suspended in $H_2O$ (6 L), and then partitioned sequentially using EtOAc and *n*-BuOH (4L × 3). The *n*-BuOH fraction (937 g) was chromatographed over Diaion HP-20, using mixtures of water and MeOH of reducing polarity as eluents. Twenty-five fractions were collected as follows: fr. 1 [6000 mL, water−MeOH (40:10)], fr. 2 [4000 mL, water−MeOH (38:12)], fr. 3 [3000 mL, water−MeOH (36:14)], fr. 4 [5000 mL, water−MeOH (34:16)], fr. 5 [5000 mL, water−MeOH (32:18)], fr. 6 [3000 mL, water−MeOH (30:20)], fr. 7 [4000 mL, water−MeOH (28:22)], fr. 8 [6000 mL, water−MeOH (26:24)], fr. 9 [4000 mL, water−MeOH (24:26)], fr. 10 [5000 mL, water−MeOH (22:28)], fr. 11 [3000 mL, water−MeOH (20:30)], fr. 12 [3000 mL, water−MeOH (18:32)], fr. 13 [5000 mL, water−MeOH (16:34)], fr. 14 [4000 mL, water−MeOH (14:36)], fr. 15 [4000 mL, water−MeOH (12:38)], fr. 16 [2000 mL, water−MeOH (10:40)], fr. 17 [3000 mL, water−MeOH (9:41)], fr. 18 [3000 mL, water−MeOH (8:42)], fr. 19 [3000 mL, water−MeOH (7:43)], fr. 20 [2000 mL, water−MeOH (6:44)], fr. 21 [3000 mL, water−MeOH (5:45)], fr. 22 [3000 mL, water−MeOH (4:46)], fr. 23 [4000 mL, water−MeOH (3:47)], fr. 24 [3000 mL, water−MeOH (1:49)], fr. 25 [8000 mL, MeOH]. Fraction 16 was further chromatographed on a silica gel column (5 × 45 cm), eluted with $CH_2Cl_2$-MeOH (20:1 to 0:l), to obtain 5 fractions (each about 800 mL), 16A-16E. Fraction 16D was separated by semi-preparative HPLC on a Merck Lichrosorb Si 60 column (5 μm, 250 × 10 mn) with $CH_2Cl_2$-isopropylalcohol (20:1) as eluent, 2 mL/min, to yield **1** (13.5mg, $t_R$ = 23.8 min) and **2** (1.1 mg, $t_R$ = 24.8 min). Fraction 20 was further purified through a silica gel column (5 × 45 cm), eluted with $CH_2Cl_2$-MeOH (20:1 to 0:1) to obtain 6 fractions (each about 600 mL), 20A-20F. Fraction 20A was separated by a silica gel column (2 × 40 cm), eluted with *n*-hexane-acetone (10:1) to give 5 sub-fractions 20Aa-20Ae. 20Ab was further purified by semi-preparative HPLC on a Merck Lichrosorb Si 60 column (5 μm, 250 × 10 mn) with $CH_2Cl_2$-lsopropylalcohol (20:1) as eluent, 2 mL/min, to yield **3** (1.8 mg, $t_R$ = 21.4 min).

***Cytotoxicity assay:*** The cytotoxicity of compound **1** was evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method based on the described procedures [26]. SK-Hep 1 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, L-glutamine 2 mM, 1% penicillin/streptomycin (penicillin 10000 U/mL and streptomycin 10 mg/mL) in a humidified atmosphere of 5% $CO_2$ at 37 °C. A volume of SK-Hep 1 cells 100 μL at a density of $1 \times 10^5$ cells/mL was incubated under the same conditions for 24 h in a 96-well flat-bottomed microplate. Test samples dissolved in DMSO were added to the cultures. After a 48 h incubation period, the wells were incubated with the MTT (100 μL/well concentrated at 5 mg/mL) at 37°C for 4 h. 200 μL of DMSO was added to redissolve the formazan crystals after removing the supernatant. The absorbance of the resulting formazan was measured by an enzyme-linked immunosorbent assay plate reader at 550 nm. The results were assayed in triplicate. The ratio of cell viability (%) was calculated by using the following formula: [(experimental absorbance - background absorbance)/(control absorbance - background absorbance)] × 100. The activity is shown as $IC_{50}$ value, which the concentration (μM) of the tested sample that results in 50% inhibition of cell growth.

**5β,19-Epoxy-23(*R*)-methoxycucurbita-6,24-dien-3β-ol** (**1**)
Amorphous white powder.
$[\alpha]^{25}_D$: -37.0 (*c* 0.68, $CHCl_3$).
IR (KBr) $\nu_{max}$: 3463, 3026, 2970, 2937, 2873, 2814, 1663, 1645, 1466, 1415, 1376, 1106, 1080, 1055, 999, 948, 911, 771 $cm^{-1}$.
$^1$H NMR: Table 1.
$^{13}$C NMR: Table 2.
EI-MS (70 eV) *m/z* (rel. int.): 470 $[M]^+$ (3), 421 (2), 391 (1), 354 (1), 323 (4), 281 (2), 255 (1), 171 (4), 109 (4), 99 (100), 69 (3).
HR-EI-MS: *m/z* 470.3767 (calcd for $C_{31}H_{50}O_3$ 470.3762, $[M]^+$).

**5*β*,19-Epoxy-23(*S*)-methoxycucurbita-6,24-dien-3*β*-ol** (**2**)
Amorphous white powder.
$[\alpha]^{25}_D$: -48.6 (*c* 0.10, $CHCl_3$).
IR (KBr) $\nu_{max}$: 3467, 3030, 2988, 2962, 2924, 2873, 2809, 1665, 1648, 1449, 1376, 1106, 1084, 940, 915, 775, 741 $cm^{-1}$.
$^1$H NMR: Table 1.
$^{13}$C NMR: Table 2.
EI-MS (70 eV) *m/z* (rel. int.): 470 $[M]^+$ (4), 438 (10), 421 (2), 408 (3), 390 (15), 354 (1), 323 (6), 309 (21), 281 (23), 255 (1), 239 (7), 211 (7), 185 (12), 171 (21), 109 (48), 99 (100), 81 (42), 69 (3), 55 (38).
HR-EI-MS: *m/z* 470.3762 (calcd for $C_{31}H_{50}O_3$ 470.3762, $[M]^+$).

**3*β*-Hydroxy-23(*R*)-methoxycucurbita-6,24-dien-5*β*,19-olide (3)**
Amorphous white powder.
$[\alpha]^{25}_{D}$: -64.5 (*c* 0.12, $CHCl_3$).
IR (KBr) $\nu_{max}$: 3573, 3505, 2965, 2940, 2936, 2877, 2814, 1742, 1470, 1450, 1372, 1280, 1187, 1163, 1090, 1041, 939, 900, 749 $cm^{-1}$.
$^1H$ NMR: Table 1.
$^{13}C$ NMR: Table 2.
EI-MS (70 eV) *m/z* (rel. int.): 484 $[M]^+$ (3), 440 (3), 408 (1), 375 (1), 309 (3), 281 (2), 241 (1), 213 (1), 171 (4), 109 (6), 99 (100), 55 (7).
HR-EI-MS: *m/z* 484.3556 (calcd for $C_{31}H_{48}O_4$ 484.3554, $[M]^+$).

**Acknowledgments** - This research was supported by grants from the National Science Council of the Republic of China (NSC 99-2622-B-020-004-CC1 and NSC 100-2622-B-020-001-CC1) and in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004). We thank Ms Shu-Yun Sun and Ms Lih-Mei Sheu for the EI-MS and HR-EI-MS measurements in the Instrumentation Center of the College of Science, National Taiwan University and National Chung Hsing University. We are also grateful to the National Center for High-performance computing for computer time and facilities.

## References

[1] Wang X, Sun W, Cao J, Qu H, Bi X, Zhao Y. (**2012**) Structures of new triterpenoids and cytotoxicity activities of the isolated major compounds from the fruit of *Momordica charantia* L. *Journal of Agricultural and Food Chemistry*, ***60***, 3927–3933.
[2] Rathi SS, Grover JK, Vats V. (**2002**) The effect of *Momordica charantia* and *Mucuna pruriens* in experimental diabetes and their effect on key metabolic enzymes involved in carbohydrate metabolism. *Phytotherapy Research*, ***16***, 236–243.
[3] Kobori M, Nakayama H, Fukushima K, Ohnishi-Kameyama M, Ono H, Fukushima T, Akimoto Y, Masumoto S, Yukizaki C, Hoshi Y. Deguchi T, Yoshida M. (**2008**) Bitter gourd suppresses lipopolysaccharide-induced inflammatory responses. *Journal of Agricultural and Food Chemistry*, ***56***, 4004–4011.
[4] Harinantenaina L, Tanaka M, Takaoka S, Oda M, Mogami O, Uchida M, Asakawa Y. (**2006**) *Momordica charantia* constituents and antidiabetic screening of the isolated major compounds. *Chemical & Pharmaceutical Bulletin*, ***54***, 1017–1021.
[5] Liu Y, Ali Z, Khan IA. (**2008**) Cucurbitane-type triterpene glycosides from the fruits of *Momordica charantia*. *Planta Medica*, ***74***, 1291–1294.
[6] Kimura Y, Akihisa T, Yuasa N, Ukiya M, Suzuki T, Toriyama M, Motohashi S, Tokuda H. (**2**
[7] **005**) Cucurbitane-type triterpenoids from the fruit of *Momordica charantia*. *Journal of Natural Products*, ***68***, 807–809.
[8] Akihisa T, Higo N, Tokuda H, Ukiya M, Akazawa H, Tochigi Y, Kimura Y, Suzuki T, Nishino H. (**2007**) Cucurbitane-type triterpenoids from the fruits of *Momordica charantia* and their cancer chemopreventive effects. *Journal of Natural Products*, ***70***, 1233–1239.
[9] Li QY, Chen HB, Liu ZM, Wang B, Zhao YY. (**2007**) Cucurbitane triterpenoids from *Momordica charantia*. *Magnetic Resonance in Chemistry*, ***45***, 451–456.
[10] Matsuda H, Nakamura S, Murakami T, Yoshikawa M. (**2007**) Structures of new cucurbitane-type triterpenes and glycosides, karavilagenins D and E, and karavilosides VI, VII, VIII, IX, X, and XI, from the fruit of *Momordica charantia*. *Heterocycles*, ***71***, 331–341.
[11] Nakamura S, Murakami T, Nakamura J, Kobayashi H, Matsuda H, Yoshikawa M. (**2006**) Structures of new cucurbitane-type triterpenes and glycosides, karavilagenins and karavilosides, from the dried fruit of *Momordica charantia* L. in Sri Lanka. *Chemical & Pharmaceutical Bulletin*, ***54***, 1545–1550.
[12] Fatope MO, Takeda Y, Yamashita H, Okabe H, Yamauchi T. (**1990**) New cucurbitane triterpenoids from *Momordica charantia*. *Journal of Natural Products*, ***53***, 1491–1497.
[13] Murakami T, Emoto A, Matsuda H, Yoshikawa M. (**2001**) Medicinal foodstuffs. XXI. Structures of new cucurbitane-type triterpene glycosides, goyaglycosides-a, -b, -c, -d, -e, -f, -g, and -h, and new oleanane-type triterpene saponins, goyasaponins I, II, and III, from the fresh fruit of Japanese *Momordica charantia* L. *Chemical & Pharmaceutical Bulletin*, ***49***, 54–63.
[14] Begum S, Ahmed M, Siddiqui BS, Khan A, Saify ZS, Arif M. (**1997**) Triterpenes, a sterol and a monocyclic alcohol from *Momordica charantia*. *Phytochemistry*, ***44***, 1313–1320.
[15] Miyahara Y, Okabe H, Yamauchi T. (**1981**) Studies on the constituents of *Momordica charantia* L. II. Isolation and characterization of minor seed glycosides, momordicosides C, D and E. *Chemical & Pharmaceutical Bulletin*, ***29***, 1561–1566.
[16] Okabe H, Miyahara Y, Yamauchi T, Miyahara K, Kawasaki T. (**1980**) Studies on the constituents of *Momordica charantia* L. I. Isolation and characterization of momordicosides A and B, glycosides of a pentahydroxy-cucurbitane triterpene. *Chemical & Pharmaceutical Bulletin*, ***28***, 2753–2762.
[17] Chen J, Tian R, Qiu M, Lu L, Zheng Y, Zhang Z. (**2008**) Trinorcucurbitane and cucurbitane triterpenoids from the roots of *Momordica charantia*. *Phytochemistry*, ***69***, 1043–1048.
[18] Yasuda M, Iwamoto M, Okabe H, Yamauchi T. (**1984**) Structures of momordicines I, II and III, the bitter principles in the leaves and vines of *Momordica charantia* L. *Chemical & Pharmaceutical Bulletin*, ***32***, 2044–2047.
[19] Chen JC, Liu WQ, Lu L, Qiu MH, Zheng YT, Yang LM, Zhang XM, Zhou L, Li ZR. (**2009**) Kuguacins F–S, cucurbitane triterpenoids from *Momordica charantia*. *Phytochemistry*, ***70***, 133–140.
[20] Chang CI, Chen CR, Liao YW, Cheng HL, Chen YC, Chou CH. (**2006**) Cucurbitane-type triterpenoids from *Momordica charantia*. *Journal of Natural Products*, ***69***, 1168–1171.
[21] Chang CI, Chen CR, Liao YW, Cheng HL, Chen YC, Chou CH. (**2008**) Cucurbitane-type triterpenoids from the stems of *Momordica charantia*. *Journal of Natural Products*, ***71***, 1327–1330.
[22] Chang CI, Chen CR, Liao YW, Shih WL, Cheng HL, Tzeng CY, Li JW, Kung MT. (**2010**) Octanorcucurbitane triterpenoids protect against *t*-BHP-induced hepatotoxicity from the stems of *Momordica charantia*. *Chemical & Pharmaceutical Bulletin*, ***58***, 225–229.
[23] Chen CR, Liao YW, Shih WL, Tzeng CY, Chang CI. (**2010**) Triterpenoids from the stems of *Momordica charantia*. *Helvetica Chimica Acta*, ***93***, 1355–1361.
[24] Chen CR, Liao YW, Wang L, Kuo YH, Liu HJ, Shih WL, Cheng HL, Chang CI. (**2010**) Cucurbitane triterpenoids from *Momordica charantia* and their cytoprotective activity in *tert*-butyl hydroperoxide-induced hepatotoxicity of HepG2 cells. *Chemical & Pharmaceutical Bulletin*, ***58***, 1639–1642.
[25] Kim KG, Choi SU, Kim YC, Lee KR. (**2011**) Tirucallane triterpenoids from *Cornus walteri*. *Journal of Natural Products*, ***74***, 54–59.
[26] Akihisa T, Higo N, Tokuda H, Ukiya M, Akazawa H, Tochigi Y, Kimura Y, Suzuki T, Nishino H. (**2007**) Cucurbitane-type triterpenoids from the fruits of *Momordica charantia* and their cancer chemopreventive effects. *Journal of Natural Products*, ***70***, 1233–1239.
[27] Kuo YH, Li SY, Shen YC, Huang HC, Hsu YW, Tseng RJ, Ou JC, Chen CF. (**2001**) Sesquiterpene polyol esters and triterpenes from *Celastrus punctatus*. *The Chinese Pharmaceutical Journal*, ***53***, 257–268.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1579 - 1580

# Two Novel Phenethylamine Alkaloids from *Streptomyces* sp. YIM10049

**Xueqiong Yang[a, 1], Guangwei He[a, 1], Lixing Zhao[b], Yabin Yang[a], Yun Liu[a], Lihua Xu[b] and Zhongtao Ding[a, *]**

[a]*Key Laboratory of Medicinal Chemistry for Natural Resource, Ministry of Education, School of Chemical Science and Technology, Yunnan University, Kunming 650091, China*

[b]*Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, China*

[1]The authors contributed equally to this paper

*ztding@ynu.edu.cn*

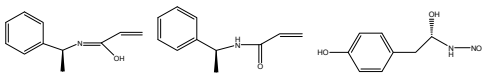
**Received: August 15th, 2012; Accepted: October 17th, 2012**

Two novel phenethylamine alkaloids were isolated from *Streptomyces* sp. YIM 10049. On the basis of spectral data, their structures were determined as (*S*)-*N*-(α-phenylethyl)-2 -hydroxyl-acrylimine (**1**) and (*S*)-*N*-nitroso-1-amino-*p*-hydroxy phenylethanol (**2**). Three known compounds, indole-3-carboxylic acid (**3**), cyclo(L-Ala-L-Tyr)(**4**), and bis(2-ethylhexyl) phthalate (**5**), were also isolated and characterized. Compound **1** is a rare enol tautomer, and compound **2** an unusual phenethylamine alkaloid with a N-NO group.

**Keywords:** *Streptomyces* sp., Alkaloid, Phenethylamine.

The genus *Streptomyces* is recognized as the most important bacterial source of clinically used therapeutic agents, including lifesaving antibiotics and antifungal, anticancer, and immunosuppressive agents [1a]. The alkaloids are reported to possess broad spectrum antimicrobial, antiradical, antioxidant, antiplasmodial, anticancer and antimutagenic activities [1b]. Phenethylamines (PEA) are well known for their psychotropic effects and have been isolated from a variety of organisms, including octocorals, the edible mushroom *Laetiporus sulphureus*, *Streptomyces* sp., *Enterococcus* sp. and other species of cyanobacteria [1c]. *Streptomyces* sp. YIM 10049 was isolated from the excrement of alpaca, and a primary bioactivity screening indicated that this strain showed some cytotoxicity to A549 and Raji cell lines, and antibacterial activity to *Fusarium oxysporum* and *F. solani*. As a part of our persistent search for new and active natural products from actinomycetes, the chemical investigation of *Streptomyces* sp. YIM 10049 led to the isolation of two novel phenethylamines, (*S*)-*N*-(α-phenylethyl)-2-hydroxyl-acrylimine (**1**) and (*S*)-*N*-nitroso-1-amino-*p*-hydroxy phenylethanol (**2**) (Figure 1), together with three known compounds, indole-3-carboxylic acid (**3**) [2a], cyclo(L-Ala-L-Tyr) (**4**) [2b], and bis(2-ethylhexyl) phthalate (**5**) [3].

Compound **1** had a molecular formula of $C_{11}H_{13}NO$ according to HRESIMS analysis. The $^{13}C$ NMR and DEPT spectra of **1** showed 11 carbon signals for one methyl, one olefinic methylene, seven methines (six for an olefinic group), and two quaternary carbons. The 2D NMR spectrum identified monosubstituted phenethyl, allyl units (Figure 2). That C-7′ connected to C-1′ was elucidated by the HMBC correlations from H-7′ and H-8′ to C-1′, and H-7′ to C-6′. The connection of C-2 to N-1 and C-3 was elucidated by the correlations from H-3, H-4 and H-7′ to C-2 in the HMBC. Because of the overlap of signals for H-3, 4, and 7′ in the $^1H$ NMR spectrum in $C_5D_5N$, the 2D NMR spectrum was measured again in MeOD, and the results were consistent. The structure of **1** was similar to that of *N*-(α-phenylethyl)-acrylamide (**1a**), but its IR spectrum showed no obvious CO absorption peak. In order to identify the structure of **1**, **1a** was synthesized (Figure 3). The NMR spectroscopic data of **1** and **1a** were similar, but **1a** showed, in its



**1** **1a** **2**

**Figure 1***:* Structures of compounds **1**, **2** and **1a**

**Table 1**: $^{13}C$ NMR (125M Hz) and $^1H$ NMR (500 MHz) data of compound **1** in $C_5D_5N$.

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| OH | 9.31 (1H, brs) | |
| 2 | | 165.8 |
| 3 | 6.55 (1H, m,) | 133.3 |
| 4 | 5.53 (1H, m), 6.55 (1H, m) | 126.4 |
| 1′ | | 145.9 |
| 2′ | 7.48 (1H, d, 7.6Hz) | 127.6 |
| 3′ | 7.30 (1H, t,7.6Hz) | 129.7 |
| 4′ | 7.22 (1H, d, 7.4Hz) | 128.1 |
| 5′ | 7.30 (1H, t, 7.6Hz) | 129.7 |
| 6′ | 7.48 (1H, d, 7.6Hz) | 127.6 |
| 7′ | 5.53 (1H, m) | 49.8 |
| 8′ | 1.46 (3H, d, 7.0Hz) | 23.1 |

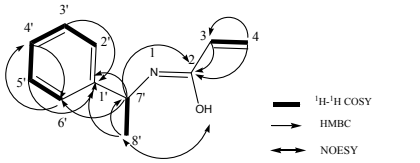



**Figure 2**: $^1H$-$^1H$ COSY, HMBC and NOESY correlations for **1**.

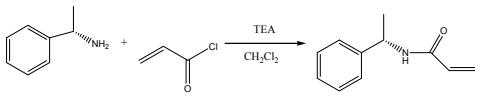



**Figure 3**: Synthesis of compound **1a**.

IR spectrum, carbonyl and amino signals at 1660 and 3286 $cm^{-1}$, but **1** a hydroxyl signal at 3434 $cm^{-1}$. The two compounds also had different TLC chromatographic characteristics [e.g. color development reaction, $R_f$ value: 0.2 for **1** and 0.4 for **1a** with $CHCl_3/CH_3OH$ (3:1) as developing solvent]. Therefore, compound **1** was determined as *N*-(α-phenylethyl)-2-hydroxyl-acrylimine, the enol tautomer of **1a**. The *cis* configuration between N-1 and C-2 was determined by NOESY from 2-OH to H-8′. The stereochemistry of compound **1** was confirmed as *S* ($[\alpha]_D^{25}$ -218.0) by comparison with the optical rotation of **1a** ($[\alpha]_D^{23}$ -207) [4].

Compound **2** had a molecular formula of $C_8H_{10}N_2O_3$. The $^{13}C$ NMR and DEPT spectra of **2** showed eight carbon signals for one methylene, five methines (4 olefinic) and two quaternary carbons, respectively. Four aromatic protons [δ 6.72 (2H, d, 7.6 Hz), 7.10 (2H, d, 7.6 Hz)] were identified, one a *para*-substituted phenyl, which was confirmed by the 2D NMR spectrum (Figure 4). The HMBC correlations from H-2 to C-1′ and COSY correlation between H-1 and H-2 indicated that C-2 was connected to C-1′, and C-1 to C-2. The $^1H$ NMR spectrum in $C_5D_5N$ exhibited a NH signal at δ 7.39, and the IR spectrum showed a nitroso signal at 1567 $cm^{-1}$. So, compound **2** was determined as *N*-nitroso-1-amino-*p*-hydroxy phenylethanol. The configuration of **2** was determined as *S* ($[\alpha]_D$: -4.2) by comparison with the optical rotation of the analogue, (*S*)-2-hydroxy-3-(4-hydroxyphenyl) propionic acid (**2a**) ($[\alpha]_D$: -10) [5]. A novel protein tyrosine phosphatase inhibitor, 2-(*N*-methyl-*N*-nitroso) hydroquinone, with a *N*-nitroso phenyl group like compound **2** was also isolated from a *Streptomyces* [6].

**Table 2**: $^{13}C$ NMR and $^1H$ NMR data of compound **2** in MeOD.

| Position | $\delta_H$ | $\delta_C$ |
|---|---|---|
| 1 | 4.28 (1H, brs) | 73.7 |
| 2 | 2.84, 3.02 (2H, m) | 41.3 |
| 1′ | | 130.2 |
| 2′ | 7.10 (1H, d, 7.6 Hz) | 132.0 |
| 3′ | 6.72 (1H, d, 7.6 Hz) | 116.5 |
| 4′ | | 157.5 |
| 5′ | 6.72 (1H, d, 7.6 Hz) | 116.5 |
| 6′ | 7.10 (1H, d, 7.6 Hz) | 132.0 |

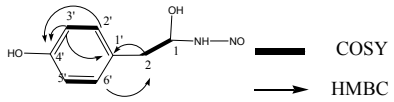



**Figure 4**: $^1H$-$^1H$ COSY, HMBC correlations for **2**

## Experimental

***General experimental procedures:*** Optical rotations, Jasco P-1020; 1D and 2D NMR, Bruker DRX-500 MHz; MS, G3250AA and AutoSpec Premier P776 spectrometer; IR, Nicolet magna-IR 550; silica gel (Qingdao Marine Chemical Group Co.) and Sephadex LH-20 (GE Healthcare Co) were used for column chromatography.

***Biological material and cultivation of actinomycete strain:*** The bacterial strain, isolated from the excrement of alpaca from a zoo in Yunnan province, China, was identified as *Streptomyces* sp. (YIM 10049) by complete 16S rRNA gene sequence. This bacterium was cultivated on a 40 L scale using 1L Erlenmeyer flasks containing 250 mL of seed medium (yeast extract 0.4%, glucose 0.4%, malt extract 0.5%, decavitamin 0.01%, pH 7) and the fermentation medium (soluble starch 0.5%, glucose 2%, soybean powder 0.2%, peptone 0.2%, NaCl 0.4%, $CaCO_3$ 0.2%, $K_2HPO_3$ 0.05%, $MgSO_4$ 0.05%, pH 7) at 28°C for 10 days on rotary shakers (250 rpm).

***Antibacterial and antitumor cell assays:*** The cytotoxicity of this strain towards A549 and Raji cells was determined *in vitro* by the MTT method for an inhibition ratio at 40%−60%, while cisplatin was used as a positive control. The primary antibacterial activity of the culture of this strain to *Fusarium oxysporum* and *F. solani* was measured using the agar diffusion method. Inhibition was measured by the size of zones of inhibition (10 and 12 mm).

***Extraction and isolation of compounds:*** The fermentation broth (40 L) for the *Streptomyces* sp. YIM 10049 was filtered, the filtrate extracted with ethyl acetate, and the solvent removed under vacuum. The resulting extract was separated into 4 fractions by silica gel CC, eluting with $CHCl_3$ /$CH_3OH$ (100%-0%). The first part ($CHCl_3$) was separated by repeated CC on silica gel and Sephadex LH-20, to yield compounds **1** (2.5 mg), **4** (6.7 mg), and **5** (4.7 mg). The third part ($CHCl_3$ /$CH_3OH$ =3:1) was separated by repeated CC on silica gel and Sephadex LH-20, to yield compounds **2** (2.4 mg), and **3** (4.5 mg).

**Compound 1**
White needles ($CH_3OH$).
MP: 99.4 -101.7°C
$[\alpha]_D^{25}$: -218.0 (*c* 0.02, $C_5H_5N$).
IR (KBr): 3434 (OH), 2925 (CH), 2354, 1557 (C=N) $cm^{-1}$;
$^1H$ NMR and $^{13}C$ NMR: Table 1.
HR-ESI *m/z*: $[M+H]^+$ Calcd for $C_{11}H_{14}NO$ 176.1075, Found: *m/z* 176.1054.

**Compound 1a**
White needles ($CH_3OH$).
MP: 92.3-94.6°C
IR (KBr): 3286(NH), 2355, 1660(CO), 1544 $cm^{-1}$.
$^{13}C$ NMR ($CHCl_3$, 75MHz) δ: 164.7, 143.1, 131.0, 128.6, 127.3, 126.4, 126.2, 48.8 , 21.7.
ESI m/z: 176$[M+H]^+$.

**Compound 2**
White needles ($CH_3OH$).
MP: 94.7-96.3°C
$[\alpha]_D^{25}$: -4.2 (*c* 0.05, $CH_3OH$).
IR (KBr): 3409 (OH), 2351, 1567 $cm^{-1}$.
$^1H$ NMR and $^{13}C$ NMR: Table 2.
HR-ESI m/z: $[M-H]^-$ Calcd for $C_8H_9N_2O_3$ 181.0613; found: m/z 181.0616.

**Acknowledgments** - This project was supported by the National Natural Science Foundation of China (No. U0932601; No. 81060263).

## References

[1] (a) Lewis K, Epstein S, D'Onofrio A, Ling L. (**2010**) Uncultured microorganisms as a source of secondary metabolites. *The Journal of Antibiotics*, ***63***, 468-476; (b) Kiplimo JJ, Islam MS, Koorbanally NA. (**2011**) A novel flavonoid and furoquinoline alkaloids from *Vepris glomerata* and their antioxidant activity. *Natural Product Communications*, ***6***, 1847-1850; (c) Malloy KL, Suyama TL, Engene N, Debonsi H, Cao Z, Matainaho T, Spadafora C, Murray TF, Gerwick WH. (**2012**) Credneramides A and B: neuromodulatory phenethylamine and isopentylamine derivatives of a vinyl chloride-containing fatty acid from cf. *Trichodesmium* sp. nov. *Journal of Natural Products*, ***75***, 60−66.

[2] (a) Tan J, Bednarek P, Liu J, Schneider B, Svatoš A, Hahlbrock K. (**2004**) Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected *Arabidopsis thaliana* roots and leaves. *Phytochemistry*, ***65***, 691-699; (b) Chen G, Lin Y, Vrijmoed LLP, Fong WF. (**2006**) A new isochroman from the marine endophytic fungus 1893#. *Chemistry of Natural Compounds*, ***42***, 138-141.

[3] Amade P, Mallea M, Bouaïcha N. (**1994**) Isolation, structural identification and biological activity of two metabolites produced by *Penicillium olsonii* bainier and sartory. *The Journal of Antibiotics*, ***47***, 201-207.

[4] Blaschke G, Donow F. (**1975**) Untersuchung chromatographischer racemattrennungen, IV Trennwirkung optisch aktiven poly [*N*-((*S*)-1-phenyläthyl) acrylamids] in abhängigkeit vom polymerisationsverfahren. *European Journal of Inorganic Chemistry*, ***108***, 1188-1197.

[5] Valls N, Vallribera M, López-Canet M, Bonjoch J. (**2002**) Synthesis of microcin SF608. The *Journal of Organic Chemistry*, ***67***, 4945-4950.

[6] Kakeya H, Imoto M, Takahashi Y, Naganawa H, Takeuchi T, Umezawa K. (**1993**) Dephostatin, a novel protein tyrosine phosphatase inhibitor produced by *Streptomyces* II. structure determination. *The Journal of Antibiotics*, ***46***, 1716-1719.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1581 - 1582

# Alkaloids from *Cinnamomum philippinense*

**Hsing-Tan Li, Wei-Jen Li*, Hui-Ming Wu* and Chung-Yi Chen***

*School of Medical and Health Sciences*, *Fooyin University*, *Kaohsiung 83102*, *Taiwan*

*xx377@mail.fy.edu.tw; mt082@mail.fy.edu.tw; mt019@mail.fy.edu.tw*

**Received: October 5th, 2012; Accepted: October 19th, 2012**

A new pyridine, 2-(4′-hydroxypyridin-3′-yl)-acetic acid (**1**), along with five known alkaloids, cinnaretamine (**2**), crykonisine (**3**), corydaldine (**4**), glaziovine (**5**) and zenkerine (**6**), were isolated from the roots of *Cinnamomum philippinense* (Lauraceae). Their structures were characterized and identified by spectral analysis.

**Keywords:** *Cinnamomum philippinense*, Lauraceae, Pyridine.

*Cinnamomum philippinense* (Merr.) Chang (Lauraceae) is a medium-sized tree distributed widely in the Philippines and the southern part of Taiwan. The chemical constituents of this species have been studied by Wu *et al*. [1]. In the course of screening for biologically and chemically novel agents from Formosan plants in the family Lauraceae [2a-2q], *C. philippinense* was chosen for further phytochemical investigation. In this study, the MeOH extract of its roots was subjected to chromatographic separation to afford six alkaloids, including a pyridine, 2-(4′-hydroxypyridin-3′-yl)-acetic acid (**1**), an amide, cinnaretamine (**2**) [2g], a benzylisoquinoline, crykonisine (**3**) [3a], an isoquinolone, corydaldine (**4**) [3b], a proaporphine, glaziovine (**5**) [3c] and an aporphine, zenkerine (**6**) [3d]. Among them, **1** was a new compound and all of these compounds were isolated for the first time from this species.

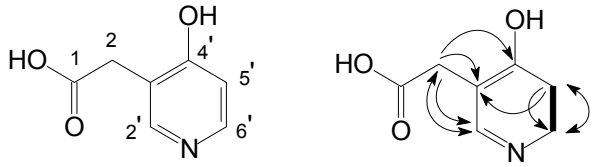



**Figure 1**: COSY (▬), HMBC (→) and NOESY (↔) Correlations of **1**.

2-(4′-Hydroxypyridin-3′-yl)-acetic acid (**1**) was obtained as a white amorphous powder and its molecular formula was deduced as $C_7H_7NO_3$ by HRESIMS (*m/z* 177.0401 $[M + Na + H]^+$; calc. 177.0402). The UV spectrum of **1** showed intense absorption bands at 220, 265 and 280 nm, which were typical of a pyridine skeleton [4]. The IR spectrum of **1** exhibited absorption bands at λ 3100 and 1660 $cm^{-1}$, indicating hydroxyl and carbonyl groups, respectively. The structure was confirmed from the $^1H$ NMR spectrum, which contained signals at δ 6.38 (1H, d, *J* = 6.0 Hz, H-5′), 7.78 (1H, d, *J* = 6.0 Hz, H-6′) and 7.87 (1H, s, H-2′) on the pyridine ring, and δ 3.34 (2H, s, H-2). The $^{13}C$ NMR and DEPT experiments for **1** showed seven resonance lines consisting of one methylene, three methines, and three quaternary carbons (including a carbonyl signal at δ 172.2). The structure of **1** was also confirmed by 2D NMR experiments. A COSY correlation was observed between H-5′ and H-6′ (Figure 1). The HETCOR experiment showed that the carbon signals at δ 116.5 for C-5′, 154.4 for C-2′, 156.0 for C-6′ and 30.9 for C-2 were correlated to the proton signals at δ 6.38 for H-5′, δ 7.87 for H-2′, δ 7.78 for H-6′ and δ 3.34 for H-2, respectively. The NOESY correlations between H-5′ and H-6′ and between H-2′ and H-2 established the connective sites as shown in structure **1** (Figure 1). Thus, **1** is a new pyridine alkaloid, which was further confirmed by HMBC experiment (Figure 1).

## Experimental

***General:*** IR, Hitachi 260-30 spectrophotometer; 1D and 2D NMR, Varian (Unity Plus) NMR spectrometer; Low-resolution ESI-MS, API 3000 (Applied Biosystems); High-resolution ESI-MS, Bruker Daltonics APEX II 30e spectrometer; Silica gel 60 for CC and precoated silica gel plates (Merck) were used for TLC, visualized with 10% $H_2SO_4$.

***Plant material:*** The roots of *C. philippinense* were collected from Taipei County, Taiwan in May 2008 and the plant material was identified by Dr Yen-Ray Hsui (Chungpu Research Center, Taiwan Forestry Research Institute). A voucher specimen (Cinnamo. 8) was deposited at the School of Medical and Health Sciences, Fooyin University, Kaohsiung, Taiwan.

***Extraction and isolation:*** The air-dried roots of *C. philippinense* (3.7 kg) were extracted with MeOH (5 L x 6) at room temperature. A MeOH extract (54.3 g) was obtained by concentration under reduced pressure. The MeOH extract, suspended in $H_2O$ (1 L), was partitioned with $CHCl_3$ (2 L x 5) to give fractions soluble in $CHCl_3$ (34.4 g) and $H_2O$ (8.8 g). The $CHCl_3$-soluble fraction (34.4 g) was chromatographed over silica gel (950 g, 70-230 mesh) using *n*-hexane-EtOAc-MeOH as eluent to produce 6 further fractions. Part of fraction 6 (6.23 g) was subjected to silica gel CC, eluting with $CH_2Cl_2$–MeOH (40:1) and enriched gradually with MeOH, to obtain 5 fractions (6-1-6-5). Fraction 6-1 (0.33 g) was further separated by silica gel CC using the same solvent system and purified by preparative TLC to yield cinnaretamine (**2**) (4 mg). Fraction 6-2 (0.42 g) was further separated by silica gel CC, and purified by preparative TLC ($CH_2Cl_2$–MeOH, 30:1) to gave crykonisine (**3**) (12 mg) and corydaldine (**4**) (17 mg), respectively. Fraction 6-3 (0.29 g) was further separated by silica gel CC using the same solvent system and purified by preparative TLC ($CH_2Cl_2$–MeOH, 50:1) to yield 2-(4′-hydroxypyridin-3′-yl)-acetic acid (**1**) (6 mg). Fraction 6-4 (0.52 g) was further separated by silica gel CC, and purified by preparative TLC ($CH_2Cl_2$–MeOH, 20:1) to give glaziovine (**5**) (5 mg) and zenkerine (**6**) (4 mg), respectively.

**2-(4′-Hydroxypyridin-3′-yl)-acetic acid** (**1**)

White amorphous powder.

IR (neat) $\nu_{max}$: 3100 (br, OH), 1660 (C=O), 1300, 950 $cm^{-1}$.

UV/Vis ($CH_3CN$): $\lambda_{max}$ (log $\varepsilon$): 280 (3.52), 265 (2.54), 220 (2.21) nm.

$^1H$ NMR (500 MHz, $CDCl_3$): 3.34 (2H, s, H-2), 6.38 (1H, d, $J$ = 6.0 Hz, H-5′), 7.78 (1H, d, $J$ = 6.0 Hz, H-6′), 7.87 (1H, s, H-2′).

$^{13}C$ NMR (125 MHz, $CDCl_3$): 30.9 (C-2, $CH_2$), 116.5 (C-3′, CH), 124.0 (C-3′, C), 154.4 (C-2′, CH), 156.0 (C-6′, CH), 172.2 (C-1, C=O), 178.4 (C-4′, C).

MS (ESI): *m/z* (%): 177 $[M + Na + H]^+$

HRMS-ESI: *m/z* $[M + Na + H]^+$ calcd for $C_7H_8O_3NNa$: 177.0402.1422; found: 177.0401.

**Acknowledgments -** This investigation was supported by a grant from the Fooyin University.

## References

[1] Wu TS, Leu YL, Chan YY, Yu SM, Teng CM, Su JD. (**1994**) Lignans and an aromatic acid from *Cinnamomum philippinense*. *Phytochemistry*, ***36***, 785−788.

[2] (a) Chen CY. (**2006**) Butanolides from the stem of *Cinnamomum kotoense*. *Natural Product Communications*, ***1***, 453−455; (b) Hsieh TJ, Chen CH, Lo WL, Chen CY. (**2006**) Lignans from the stem of *Cinnamomum camphora*. *Natural Product Communications*, ***1***, 21−25; (c) Chen CY, Chen CH, Wong CH, Liu YW, Lin YS, Wang YD, Hsui YR. (**2007**) Cytotoxic constituents of the stems of *Cinnamomum subavenium*. *Journal of Natural Products*, ***70***, 103−106; (d) Lin RJ, Lo WL, Wang YD, Chen CY. (**2008**) A novel cytotoxic monoterpenoid from the leaves of *Cinnamomum subavenium*. *Natural Product Research*, ***22***, 1055−1059; (e) Lin RJ, Cheng MJ, Huang JC, Lo WL, Yeh YT, Yeh CM, Lu CM, Chen CY. (**2009**) Cytotoxic compounds from the stems of *Cinnamomum tenuifolium*. *Journal of Natural Products*, ***72***, 1816−1824; (f) Cheng KC, Hsueh MC, Chang HC, Lee AY, Wang HM, Chen CY. (**2010**) Antioxidants from the leaves of *Cinnamomum kotoense*. *Natural Product Communications*, ***5***, 911−912; (g) Chen CY, Yeh HC. (**2011**) A new amide from the stems of *Cinnamomum reticulatum* Hay. *Natural Product Research*, ***25***, 26−30; (h) Cheng MJ, Yeh YT, Wang CJ, Chen CY. (**2011**) Isolation of a nitrobenzoate from the leaves of *Cinnamomum tenuifolium*. *Natural Product Research*, ***25***, 118−122; (i) Hong ZL, Huang JC, Kuo SY, Chen CY. (**2011**) Amides from the stems of *Cinnamomum burmannii*. *Natural Product Communications*, ***6***, 1297−1298; (j) Lin IJ, Yeh HC, Cham TM, Chen CY. (**2011**) A new butanolide from the leaves of *Cinnamomum reticulatum*. *Chemistry of Natural Compounds*, ***47***, 43−45; (k) Chen CY, Wang YD. (**2011**) A novel sesquiterpenoid from the leaves of *Cinnamomum subavenium*. *Chemistry of Natural Compounds*, ***47***, 215−217; (l) Chen CY, Hong ZL. (**2011**) Chemical constituents from the fruits of *Cinnamomum kotoense*. *Chemistry of Natural Compounds*, ***47***, 450−451; (m) Chen CY, Yeh YT. Hsui YR. (**2011**) A new lignin from the roots of *Cinnamomum philippinense*. *Chemistry of Natural Compounds*, ***47***, 519−520; (n) Yang WL, Guo YC. Lin HH, Hong HC, Chen CY. (**2011**) Chemical constituents from the twigs of *Cinnamomum macrostemon*. *Chemistry of Natural Compounds*, ***47***, 1030−1031; (o) Chen CY, Hong ZL, Yang WL, Wu MH, Huang JC, Lee JY. (**2012**) A novel homosesquiterpenoid from the stems of *Cinnamomum burmanii*. *Natural Product Research*, ***26***, 1218−1223; (p) Cheng MJ, Yeh YT, Wang CJ, Chen CY. (**2012**) A new 3,4-dihydronaphthalen-1(2*H*)-one from the leaves of *Cinnamomum philippinense*. *Natural Product Research*, ***26***, 1433−1435; (q) Chen HL, Kuo SY, Li YP, Kang YF, Yeh YT, Huang JC, Chen CY. (**2012**) A new benzodioxocinone from the leaves of *Cinnamomum tenuifolium*. *Natural Product Research*, ***26***, 1881−1886.

[3] (a) Lu ST. (**1967**) Studies on the alkaloids of formosan lauraceous plants. *Yakugaku Zasshi*, ***68***, 1278−1280; (b) Krane BD, Shamma M. (**1982**) The isoquinolone alkaloids. *Journal of Natural Products*, ***45***, 377−384; (c) Sonnet PE, Jacobson M. (**1971**) Tumor inhibitor II. Cytotoxic alkaloids from *Annona purpurea*. *Journal of Pharmaceutical Sciences*, ***60***, 1254−1256; (d) Helene G, Michel L, Ander C. (**1979**) Aporphine alkaloids II. *Journal of Natural Products*, ***42***, 325−360.

[4] Chen CY, Chang FR, Teng CM, Wu CY. (**1999**) Cheritamine, a new *N*-fatty acyl tryptamine and other constituents from the stems of *Annona cherimola*. *Journal of the Chinese Chemical Society*, ***46***, 77−86.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1583 - 1586

# Conversional Synthesis of Heteratisine

**Ling Wang, Qi-Feng Chen and Feng-Peng Wang***

*Department of Chemistry of Medicinal Natural Products, West China College of Pharmacy, Sichuan University, Chengdu, Sichuan, PR China, 610041*

*wfp@scu.edu.cn*

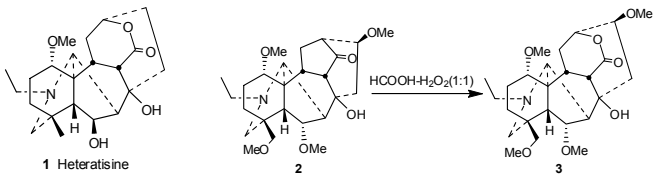
**Received: October 19th, 2012; Accepted: October 27th, 2012**

The first conversional synthesis of heteratisine has been accomplished in 14 steps and 3.2% overall yield from deltaline, mainly including deoxygenation at C-10, removal of the dioxymethylene moiety, *O*-demethylation, as well as Baeyer-Villiger oxidation.

**Keywords:** $C_{19}$ –Diterpenoid Alkaloids, Deltaline, Heteratisine.

Heteratisine (**1**) was first isolated by Jacobs and Craig from *Aconitum heterophyllum* Wall. (Ranunculaeae) in 1942 [1]. The structure and absolute configuration of **1** were established by Pelletier's Group [2a-2b] and Przybylska's Group [2c-2d]. As far as we know, besides *Aconitum heterophyllum* Wall., heteratisine was isolated from only four other plants, *A. tanguticum* (Maxim.) Stapf [3a], *A. palmatum* Don. [3b], *A. zeravschanicum* Steinb.[3c], and *A. pulchellum* Hand.-Mazz. [3d]. In 1996, Dzhakhangirov *et al.* reported that heteratisine and its analogues had pronounced antiarrhythmia activity [4]. However, although the antiarrhythmia index of these alkaloids is quite high, the activity of these phytoagents is insufficient for their use in medicine because of their limited availability [5].

Recently, our research group has successfully completed the conversional synthesis of the lactone-type alkaloid **3** from the aconitine-type $C_{19}$-diterpenoid alkaloid **2** via Baeyer-Villiger oxidation (Scheme 1) [6]. In this paper, we wish to report the conversional synthesis of heteratisine (**1**) , using deltaline (**4**) as starting material, which is abundant in *Delphinium bonvalotii* Franch. (Ranunculaeae).




**1** Heteratisine **2** **3**

**Scheme 1.**

Compound **5** was prepared from deltaline (**4**) by the literature method [7]. Hydrogenation of **5** yielded reduction product **6**. *O*-Demethylation of **6** at C-14 with 6.5% HBr-HOAc produced **7**. Hydrolysis of **7** with 5% NaOH-MeOH generated **8**. Proctection of **8** with TBSOTf, catalyzed by 2,6-lutidine and DMAP in $CH_2Cl_2$ afforded **9**. Compound **10** was obtained by acetylation of **9** with $Ac_2O$/TsOH. Deprotection of **10** with TBAF, followed by oxidation with Jones reagent generated the ketone **12**. Treatment of **12** with formic acid - hydrogen peroxide led to 6-acetylheteratisine (**13)** in 46% yield. Finally, hydrolysis of **13** with ammonium hydroxide afforded the target compoud heteratisine (**1**) (Scheme 2)**.** The NMR spectra for **13** and **1** were identical with those of the natural alkaloids respectively, as reported in the literature [8].

In conclusion, the first conversional synthesis of heteratisine (**1**) is reported in 14 steps and 3.2% overall yield from deltaline (**4**), which makes it a useful, practical method for the synthesis of heteratisine and its analogues.

## Experimental

***General methods:*** Optical rotations, PE-314 polarimeter; IR, Nicolet 200 SXV spectrometer; ESIMS, VG-Autospec-3000 mass spectrometer; HRMS, Bruker BioTOFQ mass spectrometer; NMR, Varian INOVA-400/54 spectrometer; silica gel $GF_{254}$ and H (10–40 μm, Qingdao, China) were used for TLC and CC.

***Preparation of compound 6***: To a solution of compound **5** (140 mg, 0.36 mmol) in 95% EtOH/EtOAc (1:1, 10 mL) were subsequently added acetic acid (3 mL) and 10% Pd/C (50 mg), and the reaction was allowed to proceed with stirring in the presence of hydrogen at 50 °C for 24 h. The insoluble material was filtered off, and the filtrate was concentrated to give a residue, which was diluted with water (5 mL). The subsequent mixture was basified with conc. ammonium hydroxide to pH>9 and extracted with dichloromethane (5 mL×3). The combined extracts were dried over anhydrous sodium sulfate, and concentrated in vacuo to generate a reduced product **6** (white amorphous powder, 138 mg, 98%).

$[\alpha]_D^{20}$ = +6.4 (*c* 0.50, $CHCl_3$).

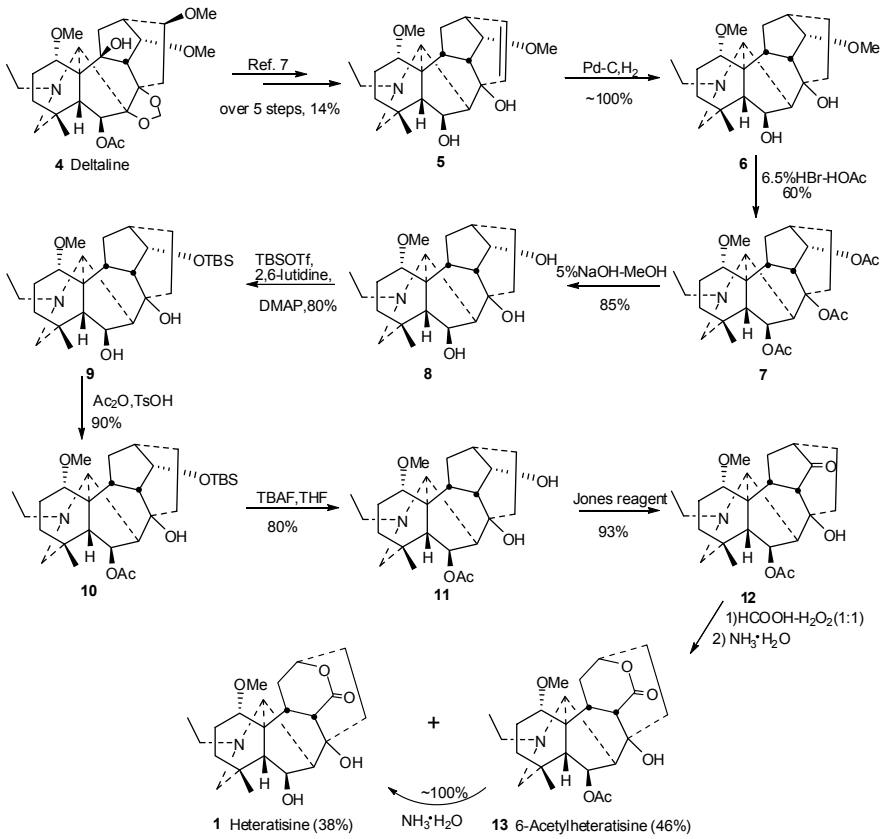
IR (KBr): 3444, 3390, 2934, 2817, 2193, 1459, 1377, 1193, 1164, 1120, 1074, 1030 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.95 (3H, s, H-18), 1.04 (3H, t, *J* = 7.2 Hz, H-22), 3.08 (1H, dd, *J* = 10.8, 6.8 Hz, H-1), 3.30, 3.36 (each 3H, s, $OCH_3$ × 2), 3.57 (1H, d, *J* = 2.0 Hz, H-17), 3.58 (1H, t, *J* = 5.2 Hz, H-14*β*), 4.33 (1H, d, *J* = 7.2 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{23}H_{38}NO_4$: 392.2801; found: 392.2801.

***Preparation of compound 7:*** Compound **6** (300 mg, 0.77 mmol) was dissolved in a solution of 6.5% HBr in acetic acid (10 mL), and the reaction solution was kept stirring at 80 °C for 19 h prior to being poured into ice water (10 mL). The subsequent mixture was basified with conc. ammonium hydroxide to pH > 9, and extracted with dichloromethane (10 mL×3). The combined extracts were dried over anhydrous sodium sulfate, and concentrated under reduced pressure to furnish a residue. Column chromatography of




**Scheme 2.**

**Table 1**: $^{13}C$ NMR data for compounds **1**, **6-13** (100 MHz, $CDCl_3$).

| Position | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **1** |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 86.0 d | 84.5 d | 85.9 d | 86.2 d | 85.8 d | 85.6 d | 85.3 d | 82.2 d | 83.4 d |
| 2 | 22.8 t | 25.2 t | 22.7 t | 23.2 t | 22.8 t | 22.2 t | 25.8 t | 26.7 t | 26.7 t |
| 3 | 37.4 t | 31.0 t | 37.3 t | 37.4 t | 37.2 t | 37.1 t | 36.9 t | 36.3 t | 36.5 t |
| 4 | 37.8 s | 34.2 s | 34.8 s | 34.9 s | 34.8 s | 34.9 s | 34.7 s | 34.7 s | 34.6 s |
| 5 | 58.4 d | 56.6 d | 58.8 d | 58.4 d | 55.6 d | 55.9 d | 55.6 d | 55.4 d | 58.1 d |
| 6 | 73.0 d | 73.9 d | 73.4 d | 73.0 d | 74.2 d | 74.5 d | 74.0 d | 74.0 d | 72.7 d |
| 7 | 49.3 d | 45.6 d | 50.4 d | 49.3 d | 48.0 d | 49.7 d | 49.5 d | 49.6 d | 50.6 d |
| 8 | 77.2 s | 87.3 s | 77.2 s | 77.5 s | 75.2 s | 76.2 s | 84.9 s | 75.1 s | 75.6 s |
| 9 | 44.9 d | 40.5 d | 45.8 d | 45.5 d | 45.2 d | 44.2 d | 44.1 d | 48.5 d | 49.3 d |
| 10 | 31.5 d | 32.4 d | 34.9 d | 35.2 d | 35.1 d | 34.8 d | 42.7 d | 42.7 d | 42.6 d |
| 11 | 49.0 s | 48.4 s | 49.0 s | 49.0 s | 48.6 s | 48.5 s | 48.7 s | 48.6 s | 49.1 s |
| 12 | 29.9 t | 26.3 t | 29.1 t | 30.0 t | 29.5 t | 29.5 t | 30.7 t | 29.3 t | 29.0 t |
| 13 | 45.8 d | 45.2 d | 46.0 d | 46.3 d | 45.7 d | 46.0 d | 54.0 d | 75.2 d | 75.7 d |
| 14 | 83.6 d | 75.7 d | 74.7 d | 75.8 d | 75.9 d | 74.6 d | 221.0 s | 174.0 s | 176.1 s |
| 15 | 30.9 t | 29.5 t | 29.9 t | 31.0 t | 31.1 t | 30.9 t | 33.4 t | 35.7 t | 32.9 t |
| 16 | 25.9 t | 26.1 t | 25.8 t | 26.0 t | 25.9 t | 25.8 t | 26.6 t | 28.8 t | 28.9 t |
| 17 | 64.3 d | 64.7 d | 64.3 d | 64.4 d | 64.6 d | 65.0 d | 65.1d | 62.4 d | 62.1 d |
| 18 | 25.8 q | 25.7 q | 25.9 q | 25.8 q | 25.6 q | 25.7 q | 25.7 q | 25.9 q | 26.1 q |
| 19 | 58.2 t | 57.2 t | 58.0 t | 58.2 t | 57.7 t | 57.7 t | 57.6 t | 57.2 t | 57.7 t |
| 21 | 49.4 t | 49.2 t | 49.4 t | 49.4 t | 49.2 t | 49.3 t | 49.1 t | 48.7 t | 48.9 t |
| 22 | 13.7 q | 13.4 q | 13.6 q | 13.7 q | 13.6 q | 13.6 q | 13.5 q | 13.4 q | 13.5 q |
| 1-$OCH_3$ | 56.4 q | 56.0 q | 56.4 q | 56.4 q | 56.3 q | 56.3 q | 56.1 q | 54.9 q | 55.2 q |
| 14-$OCH_3$ | 57.1 q | — | — | — | — | — | — | — | — |
| 6-OAc | — | 170.9 s<br>22.7 q | — | — | 170.8 s<br>21.6 q | 171.1 s<br>21.7 q | 171.0 s<br>21.7 q | 170.9 s<br>21.7 q | — |
| 8- OAc | — | 170.8 s<br>21.6 q | — | — | — | — | — | — | — |
| 14-OAc | — | 169.4 s<br>21.3 q | — | — | — | — | — | — | — |
| 14-OTBS | — | — | — | 18.0 s<br>25.8 q<br>×3<br>-4.8 q<br>-5.3 q | 17.9 s<br>25.7 q<br>×3<br>-4.9 q<br>-5.3 q | — | — | — | — |

the residue over silica gel, employing petroleum ether/acetone (30:1) as eluent, gave compound **7** (white amorphous powder, 232 mg, 60%).

$[\alpha]_D^{20}$ :-35.0 (*c* 0.50, $CHCl_3$).

IR (KBr): 2936, 2818, 1737, 1370, 1260, 1241, 1086 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.80 (3H, s, H-18), 1.04 (3H, t, *J* = 7.2 Hz, H-22), 1.89, 2.03, 2.05 (each 3H, s, OAc× 3), 3.08 (1H, dd, *J* = 10.0, 6.8 Hz, H-1), 3.27 (3H, s, $OCH_3$), 3.50 (1H, br s, H-17), 3.56 (1H, t, *J* = 5.6 Hz, H-9), 3.61 (1H, d, *J* = 7.2 Hz, H-7), 4.63 (1H, t, *J* = 4.8 Hz, H-14*β*), 5.08 (1H, d, *J* = 7.2 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{28}H_{42}NO_7$: 504.2961; found: 504.2962.

***Preparation of compound 8:*** A solution of compound **7** (232 mg, 0.46 mmol) in of 5% NaOH (7 mL) in methanol was stirred at room temperature for 5h. After removal of methanol under reduced pressure, the residue was suspended with water (10 mL), and the subsequent mixture was extracted with dichloromethane (10 mL×3). The combined extracts were dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a residue. Column chloromatography of the residue, using petroleum ether/acetone (5:1) as eluent, afforded **8** (a white amorphous powder, 147mg, 85%).

$[\alpha]_D^{20}$ : -5.8 (*c* 0.50, $CHCl_3$).

IR (KBr): 3371, 2929, 2809, 1453, 1202, 1164, 1099 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.91 (3H, s, H-18), 1.03 (3H, t, *J* = 7.2 Hz, H-22), 3.06 (1H, overlapped, H-1), 3.28 (3H, s, $OCH_3$), 3.55 (1H, d, *J* = 2.0 Hz, H-17), 3.98 (1H, t, *J* = 5.2 Hz, H-14*β*), 4.38 (1H, d, *J* = 7.2 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{22}H_{36}NO_4$: 378.2644; found: 378.2643.

***Preparation of compound 9:*** To a solution of **8** (100 mg, 0.26 mmol) in $CH_2Cl_2$ (2 mL) under argon, 2,6-lutidine (57 mg, 0.53 mmol), DMAP (7 mg, 0.05 mmol), and TBSOTf (140 mg, 0.53 mmol) were added. The resulting mixture was stirred for 45 min at ambient temperature. The reaction was quenched with methanol (0.5 mL), diluted with water (5 mL), and extracted with $CH_2Cl_2$ (5 mL×3). The combined organic layers were dried ($Na_2SO_4$), and evaporated *in vacuo* to yield a residue. Column chromatography of the residue, using cyclohexane/acetone (20:1) as eluent, afforded **9** (a white amorphous powder, 102 mg, 80%).

$[\alpha]_D^{20}$ : -6.4 (*c* 0.50, $CHCl_3$).

IR (KBr): 3454, 2930, 2860, 2815, 1460, 1254, 1097 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.09, 0.10 (each 3H, s, TBS), 0.91 (9H, s, TBS), 0.95 (3H, s, H-18), 1.04 (3H, t, *J* = 7.2 Hz, H-22), 3.06 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 3.28 (3H, s, $OCH_3$), 3.54 (1H, br s, H-17), 4.02 (1H, t, *J* = 5.2 Hz, H-14*β*), 4.32 (1H, d, *J* = 7.2 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{28}H_{50}NO_4Si$: 492.3509; found: 492.3503.

***Preparation of compound 10:*** A solution of compound **9** (102 mg, 0.21 mmol) and TsOH (19 mg, 0.11 mmol) in $Ac_2O$ (2 mL) was heated at 50 °C for 2 h prior to being poured into ice water (10 mL). The mixture was basified with conc. ammonium hydroxide to pH > 9 and extracted with dichloromethane (10 mL × 3). The extracts were dried over anhydrous sodium sulfate and concentrated. Column chloromatography of the residue, using petroleum ether/acetone (30:1) as eluent, afforded **10** (a white amorphous powder, 100 mg, 90%).

$[\alpha]_D^{20}$ : -5.0 (*c* 0.50, $CHCl_3$).

IR (KBr): 3535, 2931, 2859, 2818, 1741, 1466, 1363, 1251, 1099 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.09, 0.11 (each 3H, s, TBS), 0.83 (3H, s, H-18), 0.91 (9H, s, TBS), 1.05 (3H, t, *J* = 7.6 Hz, H-22), 1.99 (3H, s, OAc), 3.08 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 3.30 (3H, s, $OCH_3$), 3.66 (1H, br s, H-17), 4.00 (1H, t, *J* = 5.2 Hz, H-14*β*), 5.19 (1H, d, *J* = 6.8 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI *m/z* $[M + H]^+$calcd for $C_{30}H_{52}NO_5Si$: 534.3615; found: 534.3619.

***Preparation of compound 11:*** A solution of compound **10** (100 mg, 0.19 mmol) and tetrabutylammonium fluoride trihydrate (151 mg, 0.48 mmol) in THF (2 mL) was heated at 50 °C for 17 h. The mixture was diluted with water (5 mL), basified with conc. ammonium hydroxide to pH > 9 and extracted with dichloromethane (5 mL × 3). The extracts were dried over anhydrous sodium sulfate and concentrated. Column chloromatography of the residue, using cyclohexane/acetone (9:1) as eluent, afforded **11** (a white amorphous powder, 64 mg, 80%).

$[\alpha]_D^{20}$ : -1.5 (*c* 0.50, $CHCl_3$).

IR (KBr): 3418, 2930, 2876, 2823, 1735, 1644, 1256, 1094 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.85 (3H, s, H-18), 1.05 (3H, t, *J* = 7.2 Hz, H-22), 2.05 (3H, s, OAc), 3.09 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 3.29 (3H, s, $OCH_3$), 3.68 (1H, d, *J* = 2.0 Hz, H-17), 3.97 (1H, t, *J* = 4.0 Hz, H-14*β*), 5.24 (1H, d, *J* = 6.8 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{24}H_{38}NO_5$: 420.2750; found: 420.2751.

***Preparation of compound 12:*** To a solution of **11** (37 mg, 0.09 mmol) in acetone (3 mL) was added Jones reagent (0.1 mL) and the mixture was kept stirring at 0 °C for 15 min before being quenched with saturated $Na_2SO_3$ solution. The insoluble material was filtered off, and the acetone was removed from the filtrate under reduced pressure. The concentrated filtrate was diluted with water (5 mL), basified with conc. ammonium hydroxide to pH > 9, and extracted with dichloromethane (3 × 5 mL). The combined extracts were dried over anhydrous sodium sulfate and concentrated to yield **12** (a white amorphous powder, 35 mg, 93%).

$[\alpha]_D^{20}$ : +8.5 (*c* 0.50, $CHCl_3$).

IR (KBr): 3425, 2927, 1738, 1464, 1367, 1248, 1095 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.87 (3H, s, H-18), 1.08 (3H, t, *J* = 7.2 Hz, H-22), 2.08 (3H, s, OAc), 3.20 (1H, dd, *J* = 10.4, 6.4 Hz, H-1), 3.33 (3H, s, $OCH_3$), 3.92 (1H, d, *J* = 2.0 Hz, H-17), 5.32 (1H, d, *J* = 6.8 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{24}H_{36}NO_5$: 418.2593; found: 418.2597.

***Preparation of compounds 13 and 1:*** Compound **13** (26 mg, 0.06 mmol) was dissolved in a solution of $HCOOH\text{-}H_2O_2$ (1:1), and the reaction solution was kept stirring at room temperature for 30 min prior to being poured into ice water (5 mL). The subsequent mixture was basified with conc. ammonium hydroxide to pH > 9, and extracted with dichloromethane (5 mL×3). The combined extracts were rinsed with brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to give a residue. Column chromatography of the residue, using cyclohexane/acetone (10:1) as eluent, gave compounds **13** (a white amorphous powder, 12 mg, 46%) and **1** (a white amorphous powder, 9 mg, 38%).

**Compound 13**

$[\alpha]_D^{20}$ : +8.7 (*c* 0.50, $CHCl_3$).

IR (KBr): 3452, 2928, 2872, 1742, 1715, 1266, 1213, 1085, 1053 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.87 (3H, s, H-18), 1.05 (3H, t, *J* = 7.2 Hz, H-22), 1.44 (1H, br s, H-5), 2.09 (3H, s, OAc), 3.15 (1H, t, *J* = 9.2 Hz, H-1), 3.27 (3H, s, $OCH_3$), 3.58 (1H, br s, H-17), 3.82 (1H, d, *J* = 8.0 Hz, H-9), 4.74 (1H, m, H-13), 5.31 (1H, d, *J* = 7.6 Hz, H-6*α*).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{24}H_{36}NO_6$: 434.2543; found: 434.2541.

**Compound 1**

$[\alpha]_D^{20}$ : +12.8 (*c* 0.50, $CHCl_3$).

IR (KBr): 3456, 3405, 2965, 2917, 2985, 2788, 2559, 2525, 1738, 1443, 1216, 1068, 975 $cm^{-1}$.

$^1H$ NMR (400 MHz, $CDCl_3$): 0.96 (3H, s, H-18), 1.04 (3H, t, *J* = 7.2 Hz, H-22), 1.35 (1H, br s, H-5), 2.09 (3H, s, OAc), 3.14 (1H, dd, *J* = 9.6, 7.6 Hz, H-1), 3.27 (3H, s, $OCH_3$), 3.50 (1H, br s, H-17), 3.97 (1H, d, *J* = 7.6 Hz, H-9), 4.48 (1H, m, H-6*α*), 4.74 (1H, m, H-13).

$^{13}C$ NMR (100 MHz, $CDCl_3$): Table 1.

HRMS-ESI: *m/z* $[M + H]^+$calcd for $C_{22}H_{34}NO_5$: 392.2437; found: 392.2435.

**Acknowledgments** - We are grateful for the National Science Foundation of China (No. 81273387) for financial support of this research.

**References**

[1] Jacobs WA, Craig LC. (**1943**) The aconitine alkaloids: IX. The isolation of two new alkaloids from *Aconitum heterophllyum*, heteratisine and hetisine.*The Journal of Biological Chemistry*, ***143***, 605-609.

[2] (a) Aneja R, Pelletier SW. (**1964**) The diterpene alkaloids. The structure of heteratisine. *Tetrahedron Letters*, ***5***, 669-677. (b) Aneja R, Pelletier SW. (**1965**) The diterpene alkaloids: the pyrolysis and absolute configuration of heteratisine. *Tetrahedron Letters*, ***6***, 215-223. (c) Przybylska M. (**1963**) The molecular structure of heteratisine hydrobromide monohydrate. *Canadian Journal of Chemistry*, ***41***, 2911-2912. (d) Przybylska M. (**1965**) The crystal structure of heteratisine hydrobromide monohydrate. *Acta Crystallographica*, ***18***, 536-540.

[3] (a) Chen DH, Song WL. (**1985**) Alkaloids of tangut monkshood (*Aconitum tanguticum*). *Zhongcaoyao*, ***16***, 338-342. (b) Jiang QP, Pelletier SW. (1988) Four new diterpenoid alkaloids from *Aconitum palmatum* Don. *Tetrahedron Letters*, ***29***, 1875-1878. (c) Salimov BT. (1993) Atidine, and atisine azomethine from *Aconitum zeravschanicum*. *Chemistry of Natural Compounds*, ***29***, 70-72. (d) Chen PQ, He L, Ding LS, Zhang GL, Li BG. (1997) Lactone-type norditerpenoid alkaloids from *Aconitum pulchellum*. *Tianran Chanwu Yanjiu Yu Kaifa*, ***9***, 1-3.

[4] Salimov BT, Kuzibaeva ZK, Dzhakhangirov FN. (**1996**) Study of structure–activity relationships in a series of alkaloids of *Aconitum zeravschanicum* and their analogs. *Chemistry of Natural Compounds*, ***32***, 366-368.

[5] Yunusov MS. (**2011**) Antiarrhythmic agents based on diterpenoid alkaloids. *Russian Chemical Bulletin*, *International Edition*, ***60***, 633-638.

[6] Zhu M, Liu XY, Chen QH, Wang FP. (**2012**) Biomimetic conversion of aconitine-type $C_{19}$ –diterpenoid alkaloids to lactone-type alkaloids. *Journal of Asian Natural Products Research*, ***14***, 441-449.

[7] Wang L, Chen QH, Wang FP. (**2012**) Unusual reactions of a 7,17-*seco*-type $C_{19}$ –diterpenoid alkaloids. *Natural Product Communications*, ***7***, 721-724.

[8] Desai HK, Pelletier SW. (**1993**) $^{13}C$-NMR assignments for lactone-type norditerpenoid alkaloids. *Journal of Natural Products*, ***56***, 2193-2197.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1587 - 1588

# Flavonoids and Triterpenes from the leaves of *Artocarpus fulvicortex*

**Shajarahtunnur Jamil[a,*], Muhammad Taher[b], Hasnah M. Sirat[a] and Nur Azlin Othman[a]**

[a]*Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia*
[b]*Kuliyyah of Pharmacy, P.O. Box 141, International Islamic University Malaysia, Kuantan Campus, Jalan Istana, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia*

*shaja@kimia.fs.utm.my*

**Received: August 3rd, 2012; Accepted: September 18th, 2012**

Five flavonoids, 5-hydroxy-(6:7,3′:4′)-di(2,2-dimethylpyrano)flavone **1**, carpachromene **2**, cycloartocarpesin **3**, norartocarpetin **4** and 2′-hydroxy-4,4′,6′-trimethoxychalcone **5**, along with three triterpenes, friedelin **6**, lupeol **7** and β-sitosterol **8** were isolated for the first time from the leaves of *Artocarpus fulvicortex* F.M. Jarrett. The structures of these compounds were established by analysis of their spectroscopic (1D and 2D NMR) and spectrometric (MS) data, as well as by comparison of these with those reported in the literature.

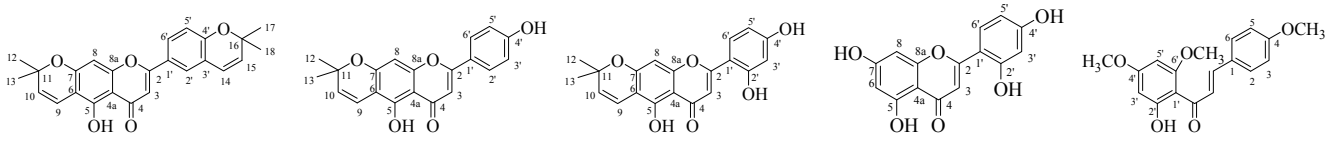
**Keywords:** *Artocarpus fulvicortex*, Moraceae, Flavonoids, Triterpenes.

The genus *Artocarpus* of the family Moraceae consists of approximately 50 species of which 11 are known to produce edible fruits and most species are indigenous to the tropical rain forests of Malaysia, Indonesia and Philippines [1a]. *Artocarpus fulvicortex* F.M. Jarret is one of Malaysia's rare *Artocarpus* species and can be found only in the rainforests of Besut situated at the East Coast of Peninsular Malaysia. This plant is locally known as 'keledang tampang gajah'. It is a medium-sized tree with orange brown or reddish brown bark [1b]. Previous phytochemical work on the genus *Artocarpus* has resulted in the isolation of numerous phenolic compounds especially flavonoids [2a-2d]. Flavonoids isolated from *Artocarpus* species showed interesting biological activities including, antiplatelet [2e, 2f], antimicrobial [2g], antioxidant [2h], antiinflammatory [2i] and also exhibited cytotoxic effects [2j]. In continuing research on Malaysian *Artocarpus* plants, we investigated the leaves of *A. fulvicortex* F.M. Jarrett. Here, we wish to report for the first time the chemical constituents of this species.

The powdered leaves of *A. fulvicortex* were sequentially extracted with petroleum ether (PE), $CH_2Cl_2$, EtOAc and MeOH at room temperature. The MeOH crude extract was further suspended in water before partitioned with $CH_2Cl_2$ and EtOAc to yield $CH_2Cl_2$-soluble extract and EtOAc-soluble extract. The chromatographic separations using vacuum liquid chromatography (VLC) and column chromatography (CC) on silica gel of the PE extract, followed by recrystallization afforded **1**, **5**, **6** and **7**. Purification by VLC and CC of the $CH_2Cl_2$ extract furnished **2** and **8**. Purification using VLC and repeated CC of EtOAc and EtOAc-soluble extracts yielded **3** and **4**. The structures of **1-8** were elucidated by comparison of the spectroscopic data with respective literature [2a-7c]. Compound **1** was found to be a new pyranoflavone.

Compound **1** (2.0 mg, 0.01%) was obtained as a pale yellow solid with m.p. 215-217ºC. The TLC of **1** gave a yellow spot after spraying with vanillin sulphuric acid reagent suggestive of a flavone-type structure. The molecular formula was determined to be $C_{25}H_{22}O_5$ from its HREIMS. The $^1$H NMR of **1** showed signals at δ 6.41 (1H, d, *J* = 10.0 Hz, H-14), δ 5.73 (1H, d, *J* = 10.0 Hz, H-15) and δ 1.49 (6H, s, H-17 and H-18); δ 6.74 (1H, d, *J* = 10.0 Hz, H-9), δ 5.64 (1H, d, *J* = 10.0 Hz, H-10) and δ 1.48 (6H, s, H-12 and H-13), characteristic of two sets of 2,2-dimethylpyrano moieties, as well as three signals at δ 7.65 (1H, dd, *J* = 8.8 and 2.4 Hz, H-6′), δ 7.49 (1H, d, *J* = 2.4 Hz, H-2′) and δ 6.89 (1H, d, *J* = 8.8 Hz, H-5′) corresponding to an ABX spin system of ring B. Other signals observed were at δ 13.14 (OH, s), characteristic of a chelated hydroxyl group, as well as at δ 6.53 (1H, s, H-3) and δ 6.41 (1H, s, H-8) for isolated aromatic protons. In the $^{13}$C NMR spectrum, a resonance for a carbonyl group at δ 182.4 was observed together with resonances for four methyl groups (δ 28.2, δ 28.3, δ 29.6 and δ 30.9). NMR data comparison with carpachromene (**2**) [3a,b] and other pyranoflavonoids [3c] was carried out to confirm the position of both 2,2-dimethylpyrano moieties in the structure. Thus, compound **1** was deduced as 5-hydroxy-(6:7,3′:4′)-di(2,2-dimethylpyrano)flavones. To the best of our knowledge, the isolation of **1** from *A. fulvicortex* or other plant has not been reported elsewhere.

This is also the first report on the presence of 2′-hydroxy-4,4′,6′-trimethoxychalcone **5** in *Artocarpus* species. Previously, **5** has been encountered from *Goniothalamus gardneri* (Annonaceae) [4a] and *Andrographis lineate* (Acanthaceae) [4b]. Carpachromene **2** has been previously isolated from *A. bracteata* [2b] and *A. heterophyllus* [3a] while cycloartocarpesin **3** has been previously identified from *A. heterophyllus* [3a] and *A. elasticus* [5a]. The occurrence of **3** was also reported in *Maclura pomifera*, one of the Moraceae species [5b]. Norartocarpetin **4** was previously isolated from *A. champeden* [6a], *A. scortechinii*, *A. kemando*, and *A. gomezianus* [2b], *A. heterophyllus* [6b], and *A. dadah* [6c]. Friedelin **6**, lupeol **7** and β-sitosterol **8** are commonly found in plants [7a-7c].



(**1**) (**2**) (**3**) (**4**) (**5**)

## Experimental

***Plant material:*** The leaves of *Artocarpus fulvicortex* J.M. Jarrett were collected from Besut, Terengganu in September 2007. A voucher specimen (HTBP 962) has been deposited at Putrajaya Botanical Garden Herbarium, Malaysia.

***Isolation procedure:*** Sequential extraction of the dried powdered leaves (1.5 kg) of *A. fulvicortex* at room temperature using different polarity of organic solvents for 48 h each afforded the PE extract (16.1 g, 1.1%), the $CH_2Cl_2$ extract (18.0 g, 1.2%), the EtOAc extract (17.9 g, 1.2%), and the MeOH extract (78.5 g, 4.2%). The MeOH extract was further suspended in water, then sequentially partitioned with $CH_2Cl_2$ and then EtOAc to yield the $CH_2Cl_2$-soluble extract (AFLMD) (1.1 g, 1.4%) and the EtOAc-soluble extract (AFLME) (1.2 g, 1.5%). Fractionation of the leaves PE extract (15.0 g) by silica gel VLC afforded twenty five fractions of 100 mL each. Fractions with a similar TLC profile were combined to give six major fractions AFLP 1 - AFLP 6. Repeated CC of AFLP 3 (1.7 g), followed by PLC with hexane/$Et_2O$ (7:3) as the solvent system gave **1** (2.0 mg, 0.01%) as a pale yellow solid. Successive purification of AFLP 4 (3.4 g) using silica gel CC yielded **6** (115.8 mg, 0.72%) as white needles; **7** (23.6 mg, 0.15%) as a white powder and **5** (9.3 mg, 0.06%) as a yellow solid. Fractionation of the leaves $CH_2Cl_2$ extract (12.0 g) by VLC yielded seven major fractions. Purification of fraction 5 (1.6 g) by silica gel CC gave 446 fractions. Fractions 187-264 were combined and washed with hexane to afford **8** (112.2 mg, 0.62%) as white crystalline needles. Purification of fraction 6 (2.8 g) by silica gel CC yielded carpachromene **2** (99.2 mg, 0.55%) as pale yellow needles. Fractionation of the EtOAc extract (10.0 g) by silica gel VLC afforded twenty fractions which were combined to yield seven major fractions (AFLE 1 - AFLE 7). Purification of AFLE 3 (0.9 g) by silica gel CC afforded **3** (21.2 mg, 0.12%) as pale yellow needles. Purification of the EtOAc-soluble fraction of the MeOH extract (1.0 g) using silica gel CC and hexane/EtOAc (7:3) as the solvent yielded **5** (13.6 mg, 0.14%) as a yellow solid.

**5-Hydroxy-(6:7,3′:4′)-di(2,2-dimethylpyrano)flavone** (**1**)
MP: 215-217ºC.
IR (KBr): 3441, 2923, 1655, 1606, 1127 $cm^{-1}$.
$^1$H NMR (400 MHz, $CDCl_3$): 1.49 (6H, s, H-17 and H-18), 1.48 (6H, s, H-12 and H-13), 5.64 (1H, d, *J* = 10.0 Hz, H-10), 5.73 (1H, d, *J* = 10.0Hz, H-15), 6.41 (1H, d, *J* = 10.0 Hz, H-14), 6.41 (1H, s, H-8), 6.53 (1H, s, H-3), 6.74 (1H, d, *J* = 10.0 Hz, H-9), 6.89 (1H, d, *J* = 8.8 Hz, H-5'), 7.49 (1H, d, *J* = 2.4 Hz, H-2'), 7.65 (1H, dd, *J* = 8.8 Hz, 2.4 Hz, H-6'), 13.14 (1H, s, 5-OH).
$^{13}$C NMR ($CDCl_3$, 100 MHz): δ 28.2 ($CH_3$), 28.3 ($CH_3$), 29.6 ($CH_3$), 30.9 ($CH_3$), 72.6 (C), 77.9 (C), 94.9 (CH), 104.0 (CH), 105.4 (C), 105.5 (C), 109.3 (C), 115.5 (CH), 116.9 (CH), 121.5 (CH), 123.5 (C), 124.3 (CH), 127.4 (CH), 128.0 (CH), 131.6 (CH), 157.4 (C), 156.4 (C), 159.7 (C), 161.5 (C), 163.8 (C), 182.4 (C=O).
MS (EI, 70 eV): *m/z* (%) 402 (27) [$M^+$], 387 (100), 219 (29), 203 (13), 186 (32), 131 (12); HREIMS: *m/z* [M+] calcd. for $C_{25}H_{22}O_5$: 402.14574; found 402.14618.

**Acknowledgments** - The authors wish to thank the Ministry of Science, Technology and Innovations (MOSTI) for the financial support and the Dept. of Chemistry, Faculty of Science, Universiti Teknologi Malaysia for the facilities.

## References

[1] (a) Burkhill IH. (**1966**) A dictionary of the economic products of the Malay Peninsula, Ministry of Agriculture and Co-operatives, Kuala Lumpur, 2nd. Edn., vol. ***1***, 249–260. (b) Whitmore TC. (**1978**) Tree flora of Malaya, Longman, Kuala Lumpur, vol. ***3***, 119–167.

[2] (a) Nomura T, Hano Y, Aida M. (**1998**) Isoprenoid-substituted flavonoids from *Artocarpus* plants (Moraceae). *Heterocycles*, ***47***, 1179–1205; (b) Hakim EH, Achmad SA, Juliawaty LD, Makmur L, Syah YM, Aimi N, Kitajima M, Takayama H, Ghisalberti EL. (**2006**) Prenylated flavonoids and related compounds of the Indonesian *Artocarpus* (Moraceae). *Journal of Natural Medicine*, ***60***, 161–184; (c) Jamil S, Sirat HM, Jantan I, Aimi N, Kitajima M. (**2008**) A new prenylated dihydrochalcone from the leaves of *Artocarpus lowii*. *Journal of Natural Medicine*, ***62***, 321–324; (d) Musthapa I, Latip J, Takayama H, Juliawaty LD, Hakim EH, Syah YM. (**2009**) Prenylated flavones from *Artocarpus lanceifolius* and their cytotoxic properties against P-388 cells. *Natural Product Communications*, ***4***, 927-930; (e) Weng JR, Chan SC, Lu YH, Lin HC, Ko HH, Lin CN (**2006**) Antiplatelet prenylflavonoids from *Artocarpus communis*. *Phytochemistry*, ***67***:824–829; (f) Jantan I, Mohd Yasin YH, Jamil S, Sirat HM, Basar N. (**2010**) Effect of prenylated flavonoids and chalcones isolated from *Artocarpus* species on platelet aggregation in human whole blood. *Journal of Natural Medicine*, ***64***, 365–369; (g) Kuete V, Ango PY, Fotso GW, Kapche GDWF, Dzoyem JP, Wouking AG, Ngadjui BT, Abegaz BM (**2011**) Antimicrobial activities of the methanol extract and compounds from *Artocarpus communis* (Moraceae). *BMC Complementary and Alternative Medicine*, ***11***, 42–46; (h) Jayasinghe L, Balasooriya BAIS, Padmini WC, Hara N, Fujimoto Y. (**2004**) Geranyl chalcone derivatives with antifungal and radical scavenging properties from the leaves of *Artocarpus nobilis*. *Phytochemistry*, ***65***, 1287–1290. (i) Wei BL, Weng JR, Chiu PH, Hung CF, Wang JP, Lin CN (**2005**) Anti-inflammatory flavonoids from *Artocarpus heterophyllus* and *Artocarpus communis*. *Journal of Agricultural and Food Chemistry*, ***53***, 3867–3871. (j) Hsu C-Lin, Shyu M-H, Lin J-A, Yen G-C, Fang S-C (**2011**) Cytotoxic effects of geranyl flavonoid derivatives from the fruit of *Artocarpus communis* in SK-Hep-1 human hepatocellular carcinoma cells. *Food Chemistry*, ***127***, 127–134.

[3] (a) Zheng ZP, Cheng K-W, To JT-K, Li H, Wang M (**2008**) Isolation of tyrosinase Inhibitors from *Artocarpus heterophyllus* and use of its extract as antibrowning agent. *Molecular Nutrition and Food Research*, ***52***, 1530–1538; (b) Jain AC, Khazanchi R, Kumar A (**1978**) Synthesis of Carpachromene. *Chemistry Letters*, ***9***, 991–994; (c) Hano Y, Itoh N, Hanaoka A, Nomura T (**1995**) Paratocarpins F-L, seven new isoprenoid-substituted flavonoids from *Paratocarpus venenosa* Zoll. *Heterocycles*, ***41***, 2313–2326.

[4] (a) Seidel V, Bailleu, F, Waterman PG. (**2000**) (*Rel*)-1b,2a-di-(2,4-dihydroxy-6-methoxybenzoyl)-3b,4a-di-(4-methoxyphenyl)-cyclobutane and other flavonoids from the aerial parts of *Goniothalamus gardneri* and *Goniothalamus thwaitesii*. *Phytochemistry*, ***55***, 439–446. (b) Kishore PH, Reddy MVB, Reddy MK, Gunasekar D, Caux C, Bodo B. (**2003**) Flavonoids from *Andrographis lineate Phytochemistry*, ***63***, 457–461.

[5] (a) Kijjoa A, Cidade HM, Pinto MMM, Gonzalez MJTG, Anantachoke C, Gedris TE, Herz W. (**1996**) Prenylflavonoids from *Artocarpus elasticus Phytochemistry*, ***43***, 691–694. (b) Monache DM, Rosa MCD, Scurria R, Vitali A, Cuteri A, Monacelli B, Pasqua G, Botta B (**1995**) Comparison between metabolite productions in cell culture and in whole plant of *Maclura pomifera*. *Phytochemistry*, ***39***, 575–580.

[6] (a) Hakim EH, Juliawaty LD, Syah YM, Achmad SA (**2005**) Molecular diversity of *Artocarpus champeden* (Moraceae): A species endemic to Indonesia. *Molecular Diversity*, ***9***, 149–158; (b) Lin C-N, Lu C-M, Huang P-L (**1995**) Flavonoids from *Artocarpus heterophyllus*. *Phytochemistry*, ***39***, 1447–1451. (c) Su B-N, Cuendet M, Hawthorne ME, Kardono LBS, Riswan S, Fong HHS, Mehta RG, Pezzuto JM, Kinghorn AD (**2002**) Constituents of the barks and twigs of *Artocarpus dadah* with cyclooxygenase inhibitory activity. *Journal of Natural Products*, ***65***, 163–169.

[7] (a) Agarwal RB, Rangari VD (**2003**) Antiinflammatory and antiarthritic activities of lupeol isolated from *Strobilanthus callosus* and *Strobilanthus ixiocephala* Roots. *Indian Journal of Pharmacology*, ***35***, 384–387. (b) Queiroga CL, Silva GF, Dias PC, Possenti A, Carvalho JE (**2000**) Evaluation on the antiulcerogenic activity of friedelin-3β-ol and friedelin isolated from *Maytenus ilicifolia* (Celastraceae). *Journal of Ethnopharmacology*, ***72***, 465–468. (c) Dhakal RC, Rajbhandari M, Kalauni, SK, Awale S, Gewali MB (**2008**) Phytochemical constituents of the barks of *Vitex negundo* L. *Journal of Nepal Chemical Society*, ***23***, 89–92.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1589 - 1590

# Dihydrospinochalcone-A and Epi-flemistrictin-B, Natural Isocordoin Derivatives from the Root Extract of *Lonchocarpus xuul*

**Fabiola Escalante-Erosa[a], Brenda González-Morales[a], Ramiro F. Quijano-Quiñones[b], Gumersindo Miron-López[c] and Luis M. Peña-Rodríguez*[,a]**

[a]*Laboratorio de Química Orgánica*, *Unidad de Biotecnología*, *Centro de Investigación Científica de Yucatán*, *Calle 43 No. 130*, *Colonia Chuburná*, *Mérida*, *Yucatán*, *México*

[b]*Laboratorio de Química Farmacéutica and* [c] *Laboratorio de Resonancia Magnética Nuclear, Facultad de Química*, *Universidad Autónoma de Yucatán*, *Calle 41 No. 421 Col. Industrial*, *C.P. 97150, Mérida*, *Yucatán*, *México*

*lmanuel@cicy.mx*

**Received: December 26th, 2011; Accepted: July 15th, 2012**

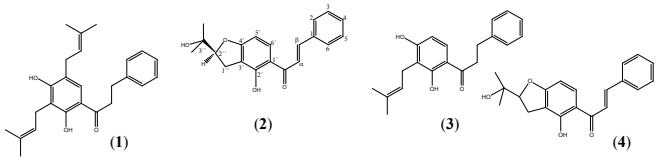
Purification of the root extract of *Lonchocarpus xuul* resulted in the isolation and identification of dihydrospinochalcone A (**1**) and epi-flemistrictin B (**2**) as additional natural isocordoin derivatives. Identification was based on the analysis of their spectroscopic data and by comparing these with those of previously reported metabolites.

**Keywords:** *Lonchocarpus xuul*, Leguminosae, Chalcone, Isocordoin, Dihydroisocordoin, Dihydrospinochalcone A, Epi-flemistrictin B.

*Lonchocarpus xuul* Lundell (Leguminosae) is a tree endemic to the Yucatan Peninsula where it is known as "xuul", "kanxuul" or "yaax-xuul" [1]. Previous phytochemical investigations of the root extract resulted in the isolation and identification of a number of flavonoids [2a-2c], some with antiprotozoal and cytotoxic activity [3]. In continuing our search for bioactive metabolites from the Yucatan native flora, we report herein the isolation and identification of dihydroespinochalcone A (**1**), and epi-flemistrictin B (**2**), as two additional natural isocordoin derivatives from the root extract of *L. xuul.*

Concentration of the *n*-hexane extract of the roots of *L. xuul* yielded a pure metabolite whose $^1$H and $^{13}$C NMR spectra showed a number of similarities with those of dihydroisorcordoin (**3**), a previously reported chalcone from *L. xuul* [1]; the similarities included signals for a monosubstituted aromatic ring B and for a saturated ketone. However, a two vinylic-proton multiplet at δ 5.26, together with four signals for methyl groups bonded to quaternary sp$^2$ carbons (δ 1.69, 1.77, 1.78, and 1.82), and an isolated aromatic proton singlet at δ 7.35, indicated the presence of two isoprenyl groups in a pentasubstituted ring A. These findings were confirmed by both the parent ion peak (*m/z* 378) and the suggested molecular formula ($C_{25}H_{30}O_3$) obtained from the mass spectrum of the purified metabolite. On the basis of these data, and by comparison with those reported in the literature, the isolated metabolite was identified as dihydrospinochalcone A (**1**), a new natural chalcone whose unsaturated relative, spinochalcone A, has been previously reported from the roots of *Tephrosia spinosa* (Leguminosae) [4].

The second metabolite showed a protonated parent ion peak at *m/z* 325 in its mass spectrum, indicating a molecular formula of $C_{20}H_{20}O_4$ for the parent metabolite, and suggesting it having a structure with eleven unsaturation sites. The $^1$H and $^{13}$C NMR data of the purified metabolite proved to be very similar to those reported for flemistrictin B (**4**), a natural derivative of isocordoin first isolated from the leaves of *Flemingia stricta* [5] and recently reported from the roots of *L. xuul* [1]. However, while the $^1$H NMR spectrum of **4** shows the H -2" signal as a doublet of doublets




(*J*= 8.4, 9.6 Hz) at δ 4.78 [1], the same proton appears as an AB quartet (*J*= 5.2 and 17.4 Hz) centered at δ 2.98 in the $^1$H NMR spectrum of the new metabolite. These differences in both chemical shift and coupling pattern suggested that the two metabolites were epimeric in their C-2" stereochemistry and, on the basis of these results, we propose the name of epi-flemistrictin B for the new metabolite.

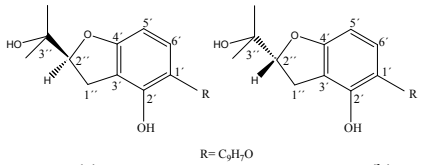



**Figure 1:** Suggested stereochemistry at C-2'' for epiflemistrictin B (a) and flemistrictin B (b).

The fact that the stereochemistry at the C-2''position of flemistrictin B (**3**) has not yet been established has been attributed to the possibility of its occurring naturally as a racemic mixture, resulting from the non-stereospecific cyclization between the phenolic hydroxyl group at C-4' and the epoxidated prenylated chain at C-3' [1]. In order to define the stereochemistry at the C-2'' position of the epimeric chalcones, a theoretical calculation of the H-2'' chemical shift was carried out using DFT [B3LYP/6-311+G(d,p)] and GIAO methodologies. The results obtained confirmed the significant differences in the chemical shift value for H-2'' in each of the epimeric structures (Figures 1a and 1b), and showed that the H -2" in the alpha orientation (Figure 1a) appears at a significantly higher field (δ 3.41) when compared to that of the H-2'' in the beta orientation (δ 4.72; Figure 1b). On the basis of

these calculations, the stereochemistry at the C-2'' position of the epimeric chalcones could be assigned as those shown in Figures 1a and 1b for epi-flemistrictin B and flemistrictin B, respectively, and the structure of epi-flemistrictin B could be established as that depicted in **2**.

## Experimental

***General:*** IR, FT-IR Nicolet Magna Protégé 460 instrument; NMR, Bruker AMX-400 NMR spectrometer; GC-MS, Agilent Technologies 6890 N gas chromatograph coupled to a 5975B mass selective detector.

***Theoretical calculations:*** All quantum chemical calculations were performed using the Spartan'06 program [6]. Calculations were carried out and geometries were optimized without geometry constraints within the DFT [7] methodology using the B3LYP [8] functional. The triplet split valence polarized and diffuse 6-311+G(d,p) basis set was used for geometry optimization and frequency calculations. In addition, the stationary points were characterized with frequency calculations [8]; no imaginary frequencies were present in final geometries. Calculations of the isotropic shielding constant (σ) for the corresponding equilibrium geometries were carried out at the same level of calculation within the GIAO [9] methodology. Finally, the *J* couplings were calculated using the PC model software [10].

***Plant material***: Roots of *L. xuul* were collected in December 2008 from plants growing in a field located at Sierra Papacal in Yucatán, Mexico. The plant material was washed with tap water and dried, first for a week at room temperature, and then for 72 h in an oven at 55°C.

***Extraction and isolation:*** Dry and ground roots (4 Kg) of *L. xuul* were extracted 3 times first with *n*-hexane (each 14 L) and then with ethanol (each 14 L). Evaporation of the combined *n*-hexane extracts under reduced pressure produced 18.34 g (0.46 %) of crude extract and 1.6 g (0.04 %) of a yellow precipitate identified as dihydrospinochalcone A (**1**). Similarly, evaporation of the combined ethanol extracts yielded 166.95 g (5.22%) of crude extract, which was suspended in 1.25 L of a 3:2 water-methanol mixture and subjected to a liquid-liquid partition procedure with *n*-hexane (3 times; 2:1, 1:1, 1:1; v:v) to produce 5.1 g of a low-polarity fraction. Successive purifications of the low-polarity fraction using VLC (column diameter: 7 cm; height: 7 cm), eluting with increasing amounts of acetone in *n*-hexane, followed by open column chromatography eluting with a mixture of *n*-hexane/acetone 8:2, resulted in the isolation of epi-flemistrictin B (**2**, 47.4 mg) in pure form.

**Dihydrospinochalcone A** (**1**)
Yellow powder.
R*f*: 0.34 (*n*-hexane-ethyl acetate 8:2); GC: $t_R$ 20.78 min.
IR $\nu_{max}$ (film): 3395 (OH), 2929 (C-H), and 1618 (C=O) $cm^{-1}$.
$^1$H NMR (400 MHz, $CDCl_3$): 6.77 (2H, d, H-2/H-6), 7.10 (3H, m, H-3,4,5), 7.35 (1H, s, H-6'), 3.42 (2H, d, *J*= 7.2 Hz, H-1''), 3.25 (2H, d, *J*= 6.8 Hz, H-1'''), 5.20 (2H, m, H-2''/H-2'''), 1.69 (3H, s, $CH_3$), 1.78 (3H, s, $CH_3$), 1.82 (3H, s, $CH_3$), 1.77 (3H, s, $CH_3$), 3.17 (2H, t, *J*= 7.6 Hz ), 2.97 (2H, t, *J*= 7.8 Hz), 6.20 (1H, s, OH-4'), 12.99 (1H, s, OH-2').
$^{13}$C NMR (100 MHz, $CDCl_3$): 133.14 (C-1), 115.35 (C-2/C-6), 129.50 (C-3/C-4/C-5), 112.88 (C-1'), 161.17 (C-2'), 118.90 (C-3'), 159.95 (C-4'), 114.26 (C-5'), 128.70 (C-6'), 21.80 (C-1''), 121.74 (C-2''), 135.14 (C-3''), 28.90 (C-1'''), 121.30 (C-2'''), 134.70 (C-3'''), 17.88 ($CH_3$), 17.90 ($CH_3$), 25.80 ($CH_3$/$CH_3$) 40.01 (C-α), 29.80 (C-β), 203.91 (C=O).
MS: *m/z* 378 [M+].

**Epi-flemistrictin B** (**3**)
Yellow oil.
R*f*: 0.34 (*n*-hexane-ethyl acetate 8:2); GC: $t_R$ 22.81 min.
$^1$H NMR (400 MHz, $CDCl_3$): 7.65 (2H, m, H-2/H-6), 7.42 (3H, m, H-3,4,5), 6.44 (1H, d, *J*= 8.9 Hz, H-5'), 7.73 (1H,d, *J*= 9 Hz, H-6'), 3.89 (1H, t, H-2''), 2.98 (2H,qAB, *J*= 5.2 Hz, 17.36, H-1''), 1.35 (3H, s, $CH_3$),1.49 (3H, s, $CH_3$), 7.59 (1H, d, *J*= 15.48 Hz), 7.88 (1H, d, *J*= 15.48 Hz), 13.81 (1H, s, OH-2').
$^{13}$C NMR (100 MHz, $CDCl_3$): 134.94 (C-1), 128.6 (C-2/C-6), 129.1 (C-3/C-5), 130.7 (C-4), 120.4 (C-1'), 164.5 (C-2'), 107.3 (C-3'), 159.7 (C-4'), 109.14 (C-5'), 129.1 ( C-6'), 69.01 (C-2''), 25.8 (C-1''), 78.3 (C-3''), 22.2 ($CH_3$), 24.9 ($CH_3$), 120.4 (C-α), 144.3 (C-β), 192.0 (C=O).
MS: m/z 324 [M+].

**Acknowledgments** - We wish to thank Carlos A. Cruz-Cruz, J. Alejandro Yam-Puc and Paulino Simá-Polanco for assistance in collecting the plant material.

## References

[1] Yam-Puc A, Peña-Rodríguez LM. (**2009**) Isocordoin derivaties from the root exract of *Lonchocarpus xuul*. *Journal of the Mexican Chemical Society*, ***53***, 12-14.
[2] (a) Borges-Argáez R, Peña-Rodríguez LM, Waterman PG. (**2002**) Flavonoids from two *Lonchocarpus* species of the Yucatán Península. *Phytochemistry*, ***60***, 533-540; (b) Borges-Argáez R, Peña-Rodríguez LM, Waterman PG. (**2000**) Flavonoids from the stem bark of *Lonchocarpus xuul*. *Phytochemistry*, ***54***, 611-614; (c) Borges-Argáe R, Poot-Díaz ME, Waterman PG, Peña-Rodríguez LM. (**2005**) Additional flavonoids from *Lonchocarpus xuul*. *Journalof the Brazilian Chemical Society*, ***16***, 1078-1081.
[3] Borges-Argáez R, Balnbury L, Flowers A, Giménez-Turba A, Ruiz E, Waterman PG, Peña-Rodríguez LM. (**2007**) Cytotoxic and antiprotozoal activity of flavonoids from *Lonchocarpus xuul*. .*Phytomedicine*, ***14***, 530-533.
[4] Venkata Rao, Prasad R. (**1992**) The chalcones from *Tephrosia spinosa*. *Phytochemistry*, ***31***, 2121-2122
[5] Subrahmanyam K, Madhusudhana-Rao J, Vemuri VSS, Sivaram-Bacu S, Roy CP, Jagannadha Rao KV. (**1982**) New chalcones from leaves of *Flemingia stricta*. *Indian Journal of Chemistry*, ***21***, 895-897.
[6] Wavefunction, Inc. Irvine, CA. *Spartan'06*
[7] Kohn W, Sham LJ. (**1965**) Self-consistent equations including exchange and correlation effects. *Physical Review*, ***140A***, 1133-1138.
[8] Lee C, Yang W, Parr RG. (**1988**) Development of the Colle-Salvetti conelation energy formula into a functional of the electron density. *Physical Review*, ***37B***, 785-789.
[9] Wolinski K, Hinton JF. Pulay P. (**1990**) Efficient implementation of the gauge-independent atomic orbital method for NMR chemical shift calculations. *Journal of the American Chemical Soc*iety, ***112***, 8251-8260.
[10] PC model for Windows V7.00, Serena Software.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1591 - 1594

# Phenolic Compounds, Including Novel *C*-glycosylflavone, from the Flowers of the Tall Bearded Iris Cultivar 'Victoria Falls'

**Takayuki Mizuno[a]*, Tsutomu Yabuya[b], Nobuhiro Sasaki[c] and Tsukasa Iwashina[a,d]**

[a]*United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Saiwai-cho, Fuchu, Tokyo 183−8509, Japan*

[b]*Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, University of Miyazaki, Miyazaki 889−2192, Japan*

[c]*Department of Biotechnology and Life Science, Faculty of Engineering, Tokyo University of Agriculture and Technology, Naka-cho, Koganei, Tokyo 184−8588, Japan*

[d]*Department of Botany, National Museum of Nature and Science, Tsukuba, Ibaraki 305−0005, Japan*

*tmizuno@kahaku.go.jp*

**Received: October 18th, 2012; Accepted: November 7th, 2012**

A new *C*-glycosylflavone, luteolin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] (swertiajaponin 2″-*O*-arabinoside) (**1**) was isolated and identified from the violet blue colored flowers of the tall bearded iris (*Iris* hybrid) cultivar 'Victoria Falls', together with five known flavonoids, swertisin 2″-*O*-arabinoside (**2**), schaftoside (**3**), isoschaftoside (**4**), swertiajaponin (**5**), swertisin 2″-*O*-glucoside (**6**) and a *C*-glycosylxanthone, mangiferin (**7**). Of these compounds, **1** and **2** were elucidated from NMR spectroscopic data, which revealed the rotameric pairs. *C*-Glycosylflavone *O*-arabinosides were isolated from the genus *Iris* for the first time.

**Keywords:** Iridaceae, Bearded iris, *C*-glycosylflavones, Mangiferin, Rotamers.

Tall bearded irises are ornamental plants belonging to sect. *Iris* of the genus *Iris* (Iridaceae). Bearded iris plants (subgenus *Iris*) grow to more than 70 cm tall and are the largest and most glamorous of the *Iris* taxa, and the last group to bloom. Bearded irises have been bred by interspecific crossing using such species as *Iris pallida* Lam., *I. variegata* L. and *I. kashimiriana* Baker. [1]. Phytochemical investigations of *Iris* species have resulted in the isolation of compounds including flavonoids, isoflavonoids and their glycosides, benzoquinone, terpenoids and stilbene glycosides [2, 3]. Flavonols, quercetin and kaempferol glycosides and the xanthone, mangiferin, have been reported from the flowers of the bearded iris group [4]. However, further chemical investigation of the flowers of this iris group has not been performed. The tall bearded iris cultivar 'Victoria Falls', which has violet blue flowers, was the Dykes memorial medal winner, USA in 1984 [1]. In this paper, we describe the isolation and identification of six *C*-glycosylflavones, including a novel flavone, and a *C*-glycosylxanthone from the flowers of the tall bearded iris cultivar 'Victoria Falls' and discuss the phytochemical significance of these findings in the genus *Iris*.

Compounds **1**−**7** were isolated from the flowers of the iris cultivar 'Victoria Falls' as pale yellow powders. The chemical structures of **1** (*ca.* 15 mg) and **2** (*ca.* 40 mg) were elucidated by UV spectra, LC-MS, HR-ESI-MS, acid hydrolysis, and $^{1}$H and $^{13}$C NMR spectroscopy. A mixture of schaftoside (**3**) and isoschaftoside (**4**) (small amount), swertiajaponin (**5**, 6 mg), swertisin 2″-*O*-glucoside (**6**, 8 mg) and mangiferin (**7**, *ca.* 30 mg) were identified by UV spectroscopy, LC-MS, acid hydrolysis, and direct TLC and HPLC comparisons with authentic samples.

UV spectral properties of **1** were measured according to reference [5]. Absorption maxima were observed at $\lambda_{max}$ 347 nm (Band I) and 271 nm (Band II) in MeOH, showing that **1** is a luteolin glycoside. In addition to shift reagents (NaOMe, $AlCl_3$, $AlCl_3$/HCl, NaOAc and NaOAc/$H_3BO_3$), it was shown that **1** is a luteolin glycoside having free 5-, 3′- and 4′-hydroxyl groups (see compound data of **1**). Acid hydrolysis of **1** liberated swertiajaponin (luteolin 7-methyl ether 6-*C*-glucoside) and arabinose. Attachment of 1 mol arabinose to swertiajaponin was determined by LC-MS (molecular ion peak at *m/z* 595 $[M+H]^+$) and HR-ESI-MS (*m/z* 617.1471 $[M+Na]^+$). Proton and carbon signals of **1** occurred as rotameric pairs by $^{1}$H and $^{13}$C NMR analysis in DMSO-$d_6$ (Table 1). Rotamers had previously been found for several X″-acetylated and *O*-glycosylated *C*-glycosylflavones [6]. The signal duplication reveals two conformational isomers created by rotational hindrance at the *C* ($sp^3$)-*C* ($sp^2$) glycosylflavone linkage [7]. The proton and carbon signals were assigned by $^{1}$H-$^{1}$H COSY, NOESY, HMQC and HMBC. The proton signals of **1a** and **1b** appeared at $\delta_H$ 7.47/7.47 (1H, d, H-2′), 6.89/6.89 (1H, d, H-5′), 7.46/7.46 (1H, d, H-6′), 6.74/6.74 (1H, s, H-3) and 6.75/6.76 (1H, s, H-8). The carbon signals were revealed at $\delta_C$ 181.95/182.20 (C-4), 159.80/160.95 (C-5), 145.95/145.95 (C-3′) and 150.04/150.04(C-4′), showing that either carbonyl (C-4) or hydroxy groups are attached to the

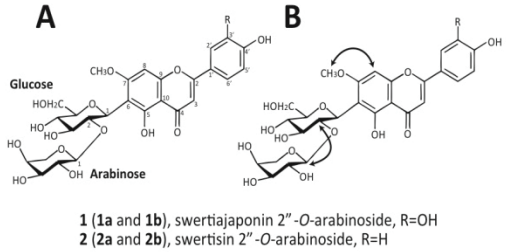



**Figure 1:** (A) Chemical structures of **1** (**1a** and **1b**) and **2** (**2a** and **2b**) from the flowers of the tall bearded iris cultivar 'Victoria Falls'. (B) Observed important NOESY correlations of **1** (**1a** and **1b**) and **2** (**2a** and **2b**) are indicated by arrows.

**Table 1**: $^{1}$H (500 MHz) and $^{13}$C NMR (125 MHz) spectral data (DMSO-$d_6$) of **1a**, **1b***, **2a** and **2b*** from the flowers of the tall bearded iris cultivar 'Victoria Falls'.

| Position | **1a** | | **1b*** | | **2a** | | **2b*** | |
|---|---|---|---|---|---|---|---|---|
| | δ $^{1}$H (*J* in Hz) | δ $^{13}$C | δ $^{1}$H (*J* in Hz) | δ $^{13}$C | δ $^{1}$H (*J* in Hz) | δ $^{13}$C | δ $^{1}$H (*J* in Hz) | δ $^{13}$C |
| *Aglycone* | | | | | | | | |
| 2 | | 164.18 | | 165.46 | | 165.59 | | 164.09 |
| 3 | 6.74 (s) | 103.18 | 6.74 (s) | 103.12 | 6.87 (s) | 103.28 | 6.85 (s) | 103.22 |
| 4 | | 181.95 | | 182.20 | | 182.18 | | 182.43 |
| 5 | | 159.80 | | 160.95 | | 159.90 | | 161.05 |
| 6 | | 108.71 | | 108.76 | | 108.82 | | 108.88 |
| 7 | | 164.03 | | 163.90 | | 164.03 | | 163.96 |
| 8 | 6.75 (s) | 90.66 | 6.76 (s) | 90.12 | 6.80 (s) | 90.91 | 6.81 (s) | 90.36 |
| 9 | | 157.10 | | 156.99 | | 157.24 | | 157.12 |
| 10 | | 104.15 | | 104.43 | | 104.27 | | 104.56 |
| 1′ | | 121.37 | | 121.37 | | 121.93 | | 121.93 |
| 2′ | 7.47 (d, 2.3) | 113.64 | 7.47 (d, 2.3) | 113.59 | 7.97 (d, 9.2) | 128.81 | 7.97 (d, 9.2) | 128.78 |
| 3′ | | 145.95 | | 145.95 | 6.90 (d, 9.2) | 116.21 | 6.90 (d, 9.2) | 116.21 |
| 4′ | | 150.04 | | 150.04 | | 161.53 | | 161.53 |
| 5′ | 6.89 (d, 8.5) | 116.11 | 6.85 (d, 8.5) | 116.11 | 6.90 (d, 9.2) | 116.12 | 6.90 (d, 9.2) | 116.12 |
| 6′ | 7.46 (d, 8.5) | 119.16 | 7.46 (d, 8.5) | 119.21 | 7.97 (d, 9.2) | 128.81 | 7.97 (d, 9.2) | 128.78 |
| 7-OMe | 3.87 (s) | 56.29 | 3.88 (s) | 56.61 | 3.87 (s) | 56.43 | 3.88 (s) | 56.74 |
| *6-C-Glucose* | | | | | | | | |
| 1 | 4.67 (d, 11.0) | 70.81 | 4.64 (d, 10.0) | 71.19 | 4.67 (d, 9.7) | 70.93 | 4.65 (d, 9.9) | 71.31 |
| 2 | 4.43 (t, 19.5) | 81.39 | 4.25 (t, 19.5) | 80.89 | 4.41 (t, 18.5) | 81.50 | 4.25 (t, 18.0) | 81.00 |
| 3 | 3.40 (m) | 78.84 | 3.40 (m) | 78.39 | 3.40 (m) | 78.96 | 3.40 (m) | 78.51 |
| 4 | 3.13 (m) | 70.49 | 3.13 (m) | 70.46 | 3.14 (m) | 70.56 | 3.14 (m) | 70.59 |
| 5 | 3.14 (m) | 82.15 | 3.14 (m) | 81.83 | 3.14 (m) | 82.28 | 3.14 (m) | 81.95 |
| 6 | 3.67 (d, 11.5) | 61.55 | 3.67 (d, 11.5) | 61.60 | 3.68 (d, 12.2) | 61.66 | 3.68 (d, 12.2) | 61.69 |
| | 3.33 (m) | | 3.33 (m) | | 3.35 (m) | | 3.35 (m) | |
| *2″-O-Arabinose* | | | | | | | | |
| 1 | 4.15 (d, 7.5) | 106.10 | 4.10 (d, 8.0) | 106.16 | 4.15 (d, 7.6) | 106.21 | 4.10 (d, 7.6) | 106.28 |
| 2 | 2.82 (m) | 74.22 | 2.82 (m) | 74.17 | 2.83 (m) | 74.29 | 2.83 (m) | 74.34 |
| 3 | 2.98 (m) | 76.44 | 2.98 (m) | 76.27 | 2.98 (m) | 76.56 | 2.98 (m) | 76.39 |
| 4 | 3.01 (m) | 69.35 | 3.01 (m) | 69.41 | 3.00 (m) | 69.51 | 3.00 (m) | 69.47 |
| 5 | 3.22 (m) | 65.91 | 3.22 (m) | 65.74 | 3.22 (dd, 4.5, 11.5) | 66.03 | 3.15 (m) | 65.86 |
| | 3.01 (m) | | 3.01 (m) | | 2.73 (m) | | 2.51 (m) | |

* Rotameric forms of **1** and **2**.

respective position. The attachment of a methoxyl group to the 7-position was shown by the occurrence of crosspeaks at 3.87/3.88 (3H, s, OMe) and 164.03/163.90 (C-7) in the long-range $^{1}$H-$^{13}$C HMBC spectrum. Thus, **1a** and **1b** were shown to be swertiajaponin, glycosylated with arabinose. The C-C linkage between the aglycone and glucose at the 6-position was confirmed by the occurrence of crosspeaks at $\delta_H$ 4.67/4.64 (1H, d, Glc-1) and $\delta_C$ 108.71/108.76 (C-6), 164.03/163.90 (C-7) and 159.80/160.95 (C-5) in the HMBC spectrum. Since the anomeric protons of C-glucose at $\delta_H$ 4.67/4.64 exhibited large coupling constants ($J$ = 11.0/10.0 Hz), the sugars were of the *β*-pyranose type. The $^{3}J_{H,C}$ correlations between $\delta_H$ 4.67/4.64 (Glc-1) and $\delta_C$ 82.15/81.83 (Glc-5) supported the pyranose structure of the *C*-glucosyl group. The *β*-pyranose structures of arabinose were shown by the $^{3}J_{H,C}$ correlation of $\delta_H$ 4.15/4.10 (1H, d, Ara-1) with $\delta_C$ 65.91/65.74 (Ara-5) and the large coupling constants ($J$ = 7.5/8.0 Hz) of the anomeric protons of **1a** and **1b**, respectively. Moreover, HMBC correlation between the Ara-1 proton and Glc-2 carbon at $\delta_C$ 81.39/80.89 showed that the glucose-arabinose linkages are 1→2. Thus, **1** (**1a** and **1b)** was identified as luteolin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] (swertiajaponin 2″-*O*-arabinoside), which is a new natural flavonoid [8]. The chemical structure of **1** (**1a** and **1b**) and significant NOESY signals are shown in Figure 1.

The absorption maxima of **2** at $\lambda_{max}$ 334 nm (Band I) and 272 nm (Band II) in MeOH, showed that **2** is an apigenin glycoside. In addition to shift reagents, it was shown that **2** is an apigenin glycoside with free 5- and 4′-hydroxyl groups (see compound data of **2**). Furthermore, swertisin (apigenin 7-methyl ether 6-*C*-glucoside) and arabinose were liberated by acid hydrolysis. Attachment of 1 mol arabinose to swertisin was confirmed by LC-MS (molecular ion peak at *m/z* 579 $[M+H]^{+}$) and HR-ESI-MS (*m/z* 601.1535 $[M+Na]^{+}$). These data suggested that **2** is swertisin X″-*O*-arabinoside. The proton and carbon signals of **2** (**2a** and **2b**) appeared as rotameric pairs and were assigned by $^{1}$H–$^{1}$H COSY, NOESY, HMQC and HMBC. Since the anomeric protons of *C*-glucose in the axial position occurred as a doublet with a large coupling constant ($J$ = 9.7/9.9 Hz), the *C*-glucosyl groups were determined to be in the *β*-pyranose form. The $^{3}J_{H,C}$ correlations between $\delta_H$ 4.67/4.65 (Glc-1) and $\delta_C$ 82.28/81.95 (Glc-5) supported the pyranose structure of the *C*-glucosyl group of **2a** and **2b**. The $^{3}J_{H,C}$ correlations of $\delta_H$ 4.15/4.10 (1H, *d*, Ara-1) with $\delta_C$ 66.03/65.86 (Ara-5) and the large coupling constant ($J$ = 7.6/7.6 Hz) of the Ara-1 protons of **2a** and **2b** showed that the arabinose had *β*-pyranose structures. HMBC correlations of $\delta_H$ 4.15/4.10 (Ara-1) to $\delta_C$ 81.50/81.00 (Glc-2) confirmed the arabinosyl-(1→2)-glucosyl linkages. Finally, the chemical structure of **2** (**2a** and **2b**) was determined as apigenin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] (swertisin 2″-*O*-arabinoside), which has only been reported from the leaves of *Achillea fragrantissima* (Forssk) Sch. Bip (Asteraceae) [9]. However, since chemical data of the compound have not been reported, $^{1}$H and $^{13}$C NMR spectroscopic characteristics are recorded in Table 1. The chemical structure of **2** (**2a** and **2b**) and significant NOESY signals are shown in Figure 1.

Four known *C*-glycosylflavones and a *C*-glycosylxanthone were also isolated from the flowers. They were identified as schaftoside (apigenin 6-*C*-glucosyl-8-*C*-arabinoside, **3**), isoschaftoside (apigenin 6-*C*-arabinosyl-8-*C*-glucoside, **4**), swertiajaponin (**5**), swertisin 2″-*O*-glucoside (**6**) and mangiferin (**7**). Characterization was achieved by UV spectroscopy, LC-MS, acid hydrolysis and direct TLC and HPLC comparisons with authentic samples. Although another two xanthones, xanthone *C*-glycoside and mangiferin X″-*O*-glucoside, were also isolated, they could not be characterized because of the very small amounts obtained.

To the best our knowledge, this is the first report of the isolation of luteolin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] in nature. Five known *C*-glycosylflavones, swertisin 2″-*O*-arabinoside, schaftoside, isoschaftoside, swertiajaponin and swertisin 2″-*O*-glucoside and a xanthone, mangiferin, were also found in the flowers of the tall bearded iris cultivar 'Victoria Falls'. Flavones and xanthones are known as co-pigment substances with anthocyanins [10]. We presumed that the isolated flavones and xanthone contribute to the flower color of the tall bearded iris cultivar 'Victoria Falls'.

The distribution of flavonoids in the genus *Iris* has been reviewed [11]. More recently, flavonoids, including new ones, were reported from a few *Iris* species [2,12]. However, *C*-glycosylflavone *O*-arabinosides have not been found previously in the genus *Iris*. In this study, we isolated and identified two *C*-glycosylflavone *O*-arabinosides (**1** and **2**) from the flowers of the tall bearded iris for the first time. These *C*-glycosylflavone *O*-arabinosides are presumed to be contained only in the bearded iris group, e.g. *Iris pallida* and *I. variegata*, but further flavonoid investigations are necessary.

## Experimental

***General experimental procedures:*** The isolated flavones and xanthone were identified by UV spectral survey, LC-MS, HR-ESI-MS, acid hydrolysis and direct TLC using: BAW (*n*−BuOH/HOAc/$H_2O$ = 4:1:5, upper phase), 15% HOAc, and BEW (*n*−BuOH/EtOH/$H_2O$ = 4:1:2.2), and HPLC comparisons with authentic samples. UV spectra were measured on a Shimadzu MPS−2000 multipurpose recording spectrophotometer (220−500 nm) according to Mabry *et al*. [5]. LC-MS were measured by a Shimadzu LC-MS system using a *L*−column 2 ODS (I.D. 2.1 × 100 mm, Chemical Evaluation and Research Institute, Japan), at a flow-rate of 0.2 mL $min^{-1}$, a detection wavelength of 350 and 300 nm, and FMW (HCOOH/MeCN/$H_2O$ = 1:12:87) as eluant, $ESI^+$ 4.5 kV, $ESI^-$ 3.5 kV, 250°C. Compounds **1** and **2** were analyzed by HR-MS using a microTOF-Q system (Bruker Daltonic, Bremen, Germany) in electrospray ionization (ESI) mode. Acid hydrolysis was performed in 12% aq. HCl for 30 min at 100°C. After cooling in water, the solution was shaken with diethyl ether. The aglycone and sugars were identified by HPLC (flavones) and PC (sugars) by comparison with authentic samples using BBPW (*n*−BuOH/benzene/pyridine/$H_2O$ = 5:1:3:3) and BTPW (*n*−BuOH/toluene/pyridine/$H_2O$ = 5:1:3:3) for sugars; **3**−**5** and **7** could not be hydrolyzed. Authentic samples of schaftoside, isoschaftoside, swertiajaponin, swertisin and mangiferin were obtained from the flowers and leaves of *Iris rossii* Baker [12] and swertisin 2″-*O*-glucoside (flavoayamenin) from the flowers of *I. setosa* Pallas var. *nasuensis* Hara [13]. HPLC comparisons with authentic specimens were performed using a Shimadzu HPLC system with a *L*−column2 ODS (I.D. 6.0×150 mm, Chemical Evaluation and Research Institute), at a flow-rate of 1.0 mL $min^{-1}$, detection: 190−400 nm and eluent: MeCN/$H_2O$/$H_3PO_4$ (12:88:0.2). NMR spectra ($^1H$ and $^{13}C$ NMR, $^1H$-$^1H$ COSY, NOESY, HMBC and HMQC) were measured on a JEOL JNM−ECA500 spectrometer (JEOL Ltd., Tokyo, Japan). Chemical shifts (δ) of $^1H$ (500 MHz) and $^{13}C$ (125 MHz) NMR are given in parts per million (ppm) relative to $\delta_H$ 2.49 /$\delta_C$ 39.6 for DMSO-$d_6$.

***Plant material:*** The tall bearded iris (*Iris* hybrid) cultivar 'Victoria Falls' was cultivated at the nursery of the Faculty of Agriculture, University of Miyazaki, Miyazaki Pref., Japan. Voucher specimen was deposited in the herbarium of the National Museum of Nature and Science, Japan (TNS).

***Extraction and isolation:*** The dried perianths were extracted with 0.1% HCl in MeOH. After concentration, the extracts were separated by preparative paper chromatography using as development systems: BAW, 15% HOAc and BEW. The isolated flavonoids and xanthone were purified by Sephadex LH-20 CC using 70% MeOH, and were further purified by preparative HPLC using Inertsil ODS-4 (I.D. 10×250 mm, GL-science Inc., Japan) at a flow-rate of 3.0 mL/min, injection of 300−350 μL, detection wavelength 340 nm, and an eluent of FMW2 (HCOOH/MeCN/$H_2O$=1:18:81).

**Luteolin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] (swertiajaponin 2″-*O*-arabinoside) (1)**

TLC: $R_f$ 0.59 (BAW), 0.72 (15% HOAc), 0.51 (BEW); Color UV (365 nm)−dark purple, UV/$NH_3$−yellow.
HPLC: $R_t$ 25.1 min.
UV $\lambda_{max}$ (nm): MeOH 244, 258, 271, 347; +NaOMe 271, 396 (inc.); +$AlCl_3$ 277, 425; +$AlCl_3$/HCl 260sh, 279, 295sh, 363, 390sh; +NaOAc 268, 402; +NaOAc/$H_3BO_3$ 265, 374.
$^1H$ and $^{13}C$ NMR: Table1.
LC-MS: *m/z* 595 $[M+H]^+$, 593 $[M-H]^-$.
HR-ESI-MS: *m/z* 617.1471 $[M+Na]^+$ (Calcd for $C_{27}H_{30}O_{15}Na$ = 617.1477).

**Apigenin 7-methyl ether 6-*C*-[*β*-arabinopyranosyl-(1→2)-*β*-glucopyranoside] (swertisin 2″-*O*-arabinoside) (2)**

TLC: $R_f$ 0.49 (BAW), 0.54 (15% HOAc), 0.40 (BEW); Color UV (365 nm)−dark purple, UV/$NH_3$−dark yellow.
HPLC: $R_t$ 49.7 min.
UV $\lambda_{max}$ (nm): MeOH 272, 334; +NaOMe 275, 387 (inc.); +$AlCl_3$ 280, 303, 354, 380sh; +$AlCl_3$/HCl 281, 302, 350, 370sh; +NaOAc 270, 390; +NaOAc/$H_3BO_3$ 272, 340.
$^1H$ and $^{13}C$ NMR: Table 1.
LC-MS: *m/z* 579 $[M+H]^+$ and 577 $[M-H]^-$.
HR-ESI-MS: *m/z* 601.1535 $[M+Na]^+$ (Calcd for $C_{27}H_{30}O_{14}Na$ = 601.1528).

**Mixture of schaftoside and isoschaftoside (3 and 4)**

TLC: $R_f$ 0.29 (BAW), 0.33 and 0.44 (15% HOAc), 0.23 (BEW); Color UV (365 nm)−dark purple, UV/$NH_3$−dark yellow.
HPLC: $R_t$ 19.8 (schaftoside) and 20.9 (isoschaftoside) min.
UV $\lambda_{max}$ (nm): MeOH 273, 332; +NaOMe 283, 333, 400 (inc.); +$AlCl_3$ 278, 305, 353, 380sh; +$AlCl_3$/HCl 278, 305, 351, 375sh; +NaOAc 282, 396; +NaOAc/$H_3BO_3$ 275sh, 285, 321, 350sh, 405.
LC-MS: *m/z* 565 $[M+H]^+$ and 563 $[M-H]^-$.

**Swertiajaponin (5)**

TLC: $R_f$ 0.47 (BAW), 0.41 (15% HOAc), 0.51 (BEW); Color UV (365 nm)−dark purple, UV/$NH_3$−yellow.
HPLC: $R_t$ 23.7 min.
UV $\lambda_{max}$ (nm): MeOH 243, 260sh, 270, 347; +NaOMe 274, 399 (inc.); +$AlCl_3$ 274, 422; +$AlCl_3$/HCl 260sh, 277, 295sh, 364, 384sh; +NaOAc 266, 401; +NaOAc/$H_3BO_3$ 266, 374.
LC-MS: *m/z* 463 $[M+H]^+$ and 461 $[M-H]^-$.

**Swertisin 2″-*O*-glucoside** (**6**)

TLC: $R_f$ 0.41 (BAW), 0.85 (15% HOAc), 0.54 (BEW); Color UV (365 nm)−dark purple, UV/$NH_3$−dark yellow.
HPLC: $R_t$ 39.9 min.
UV $\lambda_{max}$ (nm): MeOH 272, 334; +NaOMe 275, 384 (inc.); +$AlCl_3$ 260sh, 280, 301, 351, 380sh; +$AlCl_3$/HCl 260sh, 282, 302, 350, 380sh; +NaOAc 269, 392; +NaOAc/$H_3BO_3$ 272, 340.
LC-MS: *m/z* 609 $[M+H]^+$ and 607 $[M-H]^-$.

**Mangiferin** (**7**)

TLC: $R_f$ 0.36 (BAW), 0.37 (15% HOAc), 0.54 (BEW); Color UV (365 nm)−orange, UV/$NH_3$−bright yellow.
HPLC: $R_t$ 10.0 min.
UV $\lambda_{max}$ (nm): MeOH 241, 258, 316, 365; +NaOMe 273, 300sh, 350sh, 394; +$AlCl_3$ 268, 285sh, 352, 407; +$AlCl_3$/HCl 263, 310sh, 336, 400; +NaOAc 271, 304, 387; +NaOAc/$H_3BO_3$ 263, 285sh, 323, 359, 414.
LC-MS: *m/z* 423 $[M+H]^+$ and 421 $[M-H]^-$.

**Acknowledgments** - The authors thank Prof. Dr. Yoshihiro Ozeki, Dr Keiichi Noguchi, Mr Hiroshi Sekiguchi and Mr Motoki Yasunaga (Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology) for NMR and HR-ESI-MS research support and precious information.

## References

[1] Austin C. (**2005**) *Irises: A Gardener's Encyclopedia*. Timber Press, Inc., Oregon.
[2] Fang R, Veitch NC, Kite GC, Howes MR, Porter HE, Simmonds MSJ. (**2011**) Glycosylated constituents of *Iris fulva* and *Iris brevicaulis*. *Chemical and Pharmaceutical Bulletin*, ***59***, 124-128.
[3] (a) Rahman AU, Nasim S, Baig I, Jahan IA, Sener B, Orhanand I, Choudhary MI. (**2002**) Isoflavonoid glycosides from the rhizomes of *Iris germanica*. *Chemical and Pharmaceutical Bulletin*, ***50***, 1100-1102; (b) Singh N, Mahmood U, Kaul VK, Gupta AP, Jirovetz L. (**2006**) A new alkylated benzoquinone from rhizomes of *Iris kumaonensis*. *Natural Product Research*, ***20***, 75-78; (c) Farag SF, Takaya Y, Niwa M. (**2009**) Stilbene glucosides from the bulbs of *Iris tingitana*. *Phytochemistry Letters*, ***2***, 148-151.
[4] (a) Bate-Smith EC, Harborne JB. (**1963**) Mangiferin and other glycophenolics in *Iris* species. *Nature*, ***198***, 1307-1308; (b) Ashtakala SS, Forward DF. (**1971**) Pigmentation in iris hybrids: occurrence of flavonoid pigments and six cultivars of *Iris germanica*. *Canadian Journal of Botany*, ***49***, 1975-1979.
[5] Mabry TJ, Markham KR, Thomas MB. (**1970**) *The Systematic Identification of Flavonoids*. Springer, Berlin.
[6] (a) Jay M. (**1993**) *C*-Glycosylflavonoids. In *The Flavonoids: Advances in Research since 1986*. Harborne JB. (Ed.). Chapman and Hall, London. 57-93; (b) Rayyan S, Fossen T, Nateland HS, Andersen ØM. (**2005**) Isolation and identification of flavonoids, including flavone rotamers, from the herbal drug 'crataegi folium cum flore' (hawthorn). *Phytochemical Analysis,* ***16***, 334-341; (c) Bjorøy Ø, Rayyan S, Fossen T, Kalberg K, Andersen ØM. (**2009**) *C*-glycosylanthocyanidins synthesized from *C*-glycosylflavones. *Phytochemistry*, ***70***, 278-287.
[7] Rayyan S, Fossen T, Andersen ØM. (**2010**) Flavone *C*-glycosides from seeds of fenugreek, *Trigonella foenum-graecum* L. *Journal of Agricultural and Food Chemistry*, ***58***, 7211-7217.
[8] (a) Harborne JB, Baxter H. (**1999**) *Handbook of Natural Flavonoids.* Vol. ***1***. Wiley, Chichester; (b) Andersen ØM, Markham KR. (**2006**) *Flavonoids: Chemistry, Biochemistry, and Applications*. Taylor & Francis, Boca Raton; (c) Veitch NC, Grayer RJ. (**2008**) Flavonoids and their glycosides, including anthocyanins. *Natural Product Reports*, ***25***, 555-611; (d) Veitch NC, Grayer RJ. (**2011**) Flavonoids and their glycosides, including anthocyanins. *Natural Product Reports*, ***28***, 1625-1695.
[9] Ahmed AA, Shalaby AM, Melek FR, Mabry TJ. (**1988**) Swertisin 2″-*O*-arabinoside, a new *C*-glycosylflavone from *Achillea fragrantissima. Journal of Natural Products*, ***51***, 971-972.
[10] Asen S, Stewart RN, Norris KH. (**1972**) Co-pigmentation of anthocyanins in plant tissues and its effect on color. *Phytochemistry*, ***11***, 1139-1144.
[11] (a) Iwashina T, Ootani S. (**1998**) Flavonoids of the genus *Iris*: structures, distribution and function (review). *Annals of the Tsukuba Botanical Garden*, ***17***, 147-183; (b) Wang H, Cui Y, Zhao C. (**2010**) Flavonoids of genus *Iris* (Iridaceae). *Mini Reviews in Medicinal Chemistry*, ***10***, 643-661.
[12] Mizuno T, Okuyama Y, Iwashina T. (**2012**) Phenolic compounds from *Iris rossii*, and their chemotaxonomic and systematic significance. *Biochemical Systematics and Ecology*, ***44***, 157-160.
[13] Hayashi K, Ootani S, Iwashina T. (**1989**) Comparative analysis of the flavonoids and related compounds occurring in three varieties of *Iris setosa* in the flora of Japan. *Science Report of the Research Institute of Evolutionary Biology*, ***6***, 30-60.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1595 - 1596

# Two New Biisoflavonoids from the Roots of *Daphne oleoides*

**Shazia Yasmeen[a], Muhammad Aijaz Anwar[a], Sadia Ferheen[a], Nighat Afza[a*], Abdul Malik[b] and Lubna Iqbal[a]**

[a]*Pharmaceutical Research Centre*, *PCSIR Labs. Complex Karachi*, *Karachi-75280*, *Pakistan*
[b]*International Center for Chemical and Biological Sciences*, *HEJ Research Institute of Chemistry*, *University of Karachi*, *Karachi-75270*, *Pakistan*

*hocprc@yahoo.com, abdul.malik@iccs.edu*

**Received: August 20th, 2012; Accepted: October 10th, 2012**

Phytochemical investigation of the ethyl acetate soluble fraction of the methanol soluble extract of the roots of *Daphne oleoides* resulted in isolation and identification of two new isomeric biisoflavonoids characterized as 8,8''-bi-6-hydroxyorobol (**1**) and 8,8''-bi-6, 2'-dihydroxygenistein (**2**). The structures of these compounds were established by analysis of their 1D and 2D NMR and HRMS data.

**Key words:** *Daphne oleoides*, Biisoflavonoids, 8,8''-Bi-6-hydroxyorobol, 8,8''-Bi-6, 2'-dihydroxygenistein.

*Daphne oleoides* Linn. is a medicinally important xerophytic shrub belonging to the family *Thymelaeaceae* [1a]. It grows in the Western Himalaya, from Garhwal westward to Murree, occurring at an altitude of 3000 to 9000 feet. The roots are used as a purgative. An infusion of its leaves is used in the treatment of gonorrhea and applied to abscesses, while the bark and leaves are applied to skin diseases [1b]. Previous phytochemical studies on the genus *Daphne* have resulted in the isolation of different classes of compounds, including coumarins [2a,2b], lignans [2c], flavonoids [2d] and terpenes [2b, 2e]. The chemotaxanomic and ethnopharmacological importance of *D. oleoides* prompted us to carry out further studies on this species. Herein we report the isolation and structure of two new isomeric biisoflavonoids characterized as 8,8''-bi-6-hydroxyorobol (**1**) and 8,8''-bi-6, 2'-dihydroxygenistein (**2**), from the ethyl acetate soluble fraction of the methanolic extract. The structures of these compounds were established by analysis of their 1D and 2D NMR and HRMS data.

Compound **1**, obtained as a yellow amorphous powder, m.p 266-268°C, gave a violet coloration for a phenol with $FeCl_3$. The IR spectrum revealed the presence of hydroxyl (3490 $cm^{-1}$), conjugated ketone (1670 $cm^{-1}$), and an aromatic moiety (1616, 1530, 1505 $cm^{-1}$). The UV spectrum showed characteristic absorptions at 273 and 343 nm for an isoflavone [3a,3b]. On addition of $AlCl_3$ and $AlCl_3$/HCl, a bathochromic shift of 28 nm in band II, revealed the presence of a chelated hydroxyl group at C-5. The positive mode fast atom bombardment mass spectrum (FAB-MS) of **1** showed a quasimolecular ion peak at *m/z* 603, while the negative mode FAB-MS showed a quasimolecular ion peak at *m/z* 601. The molecular formula was established as $C_{30}H_{18}O_{14}$ on the basis of elemental analysis and HREI-MS showing a molecular ion peak $[M]^+$ at *m/z* 602.0691 (Calcd. for $C_{30}H_{18}O_{14}$: 602.0697). The broad band decoupled (BB) and distortionless enhancement by polarization transfer (DEPT) $^{13}C$ NMR spectra showed 15 signals attributed to four methine and eleven quaternary carbons, each assigned to two carbons, indicating that compound **1** is a symmetrical biisoflavonoid derivative. The signals at δ 152.4, 122.3, 179.7, 165.5 and 102.7 were characteristic of C-2/2'', C-3/3'', C-4/4'', C-9/9'' and C-10/10'' of a symmetric biisoflavone derivative. Five other oxygenated carbon signals (Table 1), each attributed to two carbons, were due to ten hydroxyl bearing aromatic carbons in **1**.

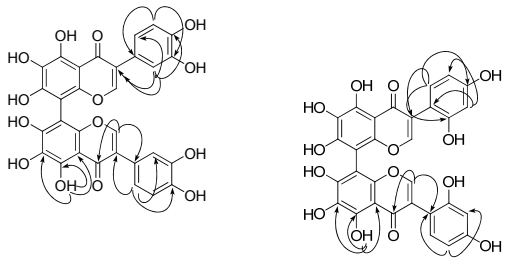


**Figure 1**: HMBC correlations of compounds **1** and **2**.

In the $^1H$ NMR spectrum the chelated hydroxyl group appearing as a singlet at δ 12.09 and corresponding to two protons was assigned to C-5/5'' hydroxyl groups as they showed $^2J$ correlation with C-5/5'' (δ 149.2), $^3J$ correlations with C-10/10'' (δ 102.7) and C-6/6'' (δ 127.7). A two-proton downfield singlet at δ 8.43, which showed HMQC correlation with the carbon at δ 152.4 and HMBC correlation with C-4 (Figure 1), was assigned to H-2/2'' of the bisisoflavonoid moiety [4]. The 1,3,4-trisubstituted ring B gave signals at δ 7.01 (1H, d, $J$ = 2.7 Hz, H-2'), 6.50 (1H, d, $J$ = 9.0 Hz, H-5') and 6.77 (1H, dd, $J$ = 9.0, 2.7 Hz, H-6'), respectively. The $^1H$-$^1H$ COSY and HMBC correlations (Figure 1) were in complete agreement with those of 5,6,7,3',4'-pentahydroxy isoflavone (6-hydroxyorobol) [5a,5b]. The absence of a proton at C-8 indicated that compound **1** is an 8-8'' linked biisoflavonoid. The signal for C-8 showed a downfield shift of 6 ppm compared with the monomer, allowing us to characterize compound **1** (Figure 1) as 8,8'' linked bi [4H-1-benzopyran-4-one,5,6,7-trihydroxy-3-(3,4 dihydroxyphenyl)] or 8,8''-bi-6-hydroxyorobol.

Compound **2** was obtained as a yellow amorphous powder, m.p 280-282°C, which gave a violet coloration with $FeCl_3$ for a phenol. The UV and IR spectra were very similar to those of **1**. The FAB-MS in positive and negative modes showed quasimolecular ion peaks at *m/z* 603 and 601, respectively. Its molecular formula was established as $C_{30}H_{18}O_{14}$ on the basis of elemental analysis and HREI-MS showing a molecular ion peak $[M]^+$ at *m/z* 602.0693 (Calcd. for $C_{30}H_{18}O_{14}$ : 602.0697). The BB and DEPT $^{13}C$ NMR spectra exhibited 15 signals, attributed to four methine and eleven quaternary carbons, each assigned to two carbons, which indicated

**Table 1**: $^1$H- and $^{13}$C-NMR (DMSO-$d_6$) data of compounds **1** and **2** at 400 and 100 MHz, respectively; δ in ppm, *J* in Hz.

| position | **1** | | **2** | |
|---|---|---|---|---|
| | $\delta_H$ ( *J* = Hz) | $\delta_C$ | $\delta_H$ ( *J* = Hz) | $\delta_C$ |
| 2/2'' | 8.43 (s) | 152.4 | 8.59 (s) | 151.5 |
| 3/3'' | - | 122.3 | - | 123.1 |
| 4/4'' | - | 179.7 | - | 180.7 |
| 5/5'' | - | 149.2 | - | 148.7 |
| 6/6'' | - | 121.7 | - | 122.5 |
| 7/7'' | - | 156.1 | - | 155.7 |
| 8/8'' | - | 98.3 | - | 98.4 |
| 9/9'' | - | 165.5 | - | 166.3 |
| 10/10'' | - | 102.7 | - | 103.1 |
| 1'/1''' | - | 124.3 | - | 113.6 |
| 2'/2''' | 7.01 (d, *J* = 2.7) | 115.3 | - | 156.2 |
| 3'/3''' | - | 145.5 | 7.16 (d, *J* = 2.1) | 106.3 |
| 4'/4''' | - | 143.2 | - | 160.8 |
| 5'/5''' | 6.50 (d, *J* = 9.0) | 117.5 | 7.09 (dd, *J* = 8.7, 2.1) | 109.2 |
| 6'/6''' | 6.77 (dd, *J* = 9.0, 2.7) | 120.6 | 7.81 (d, *J* = 8.7) | 131.4 |
| 5/5''-OH | 12.09 (s) | - | 12.15 (s) | |

that compound **2** is a symmetrical biisoflavonoid derivative. It showed close similarity to those of **1**, except for minor differences in the chemical shifts of the carbons of ring B/ring E. The $^1$H NMR spectrum of **2** was also very similar to that of **1** except for the notable differences in the chemical shifts of ring B/ring E, as well as HMBC correlations [δ 7.81 (1H, d, *J* = 8.7 Hz), 7.16 (1H, d, *J* = 2.1 Hz) and 7.09 (1H, dd, *J* = 8.7, 2.1 Hz)]. The $^1$H-$^1$H COSY and HMBC correlations (Figure 1) were in conformity to a 1,2,4-trisubtituted ring B. The spectral data showed close agreement to those of 6-hydroxygenestein and 5,6,7,2',4'-pentahydroxy isoflavone (6, 2'-dihydroxygenistein) [6] thereby indicating that compound **2** is a regioisomer of compound **1**. Compound **2** could therefore be characterized as 8,8'' linked bi [4H-1-benzopyran-4-one,5,6,7-trihydroxy-3-(2,4-dihydroxyphenyl)] or 8,8''-bi-6,2'-dihydroxygenistein.

## Experimental

***General:*** UV measurements were obtained on a Hitachi-UV-3200 spectrophotometer, and IR spectra on a Jasco-302-A spectrophotometer. Aluminum sheets 4.5 x 10 cm, coated with silica gel 60 $F_{254}$ were used for analytical TLC and compounds were visualized under UV light and detections were made at 254 nm and by spraying with ceric sulfate reagent. Column and flash chromatography were carried out on silica gel (230-400 mesh E. Merck). HPLC analyses were carried out on a JAIGEL using an ODS-M 80 column. Melting points (m.p.) were recorded on a Gallenkamp apparatus and were uncorrected. NMR experiments were performed on a Bruker AMX 400 MHz spectrometer. Chemical shifts (δ in ppm) are referenced to the carbon ($\delta_C$ 39.52) and residual proton ($\delta_H$ 2.50) signals of $(CD_3)_2SO$. FAB-MS were recorded on a Jeol-JMS HX-110 mass spectrometer. EI-MS and HREI-MS were conducted on a Jeol JMS-DA-500 mass spectrometer. Elemental analyses were performed on a Carlo Elba MOD-1106 Elemental Analyzer.

***Plant material:*** The roots of *Daphne oleoides* (15 kg) were collected from Peshawar (Pakistan) in May 2010 and identified by Prof. Manzoor Husain, Plant Taxonomist, Govt. Postgraduate College-1, Abbotabad, KPK, Pakistan, where a voucher specimen has been deposited.

***Extraction and isolation:*** Dried and ground roots (15 kg) of *Daphne oleoides* were extracted with MeOH (3 × 50 L) at room temperature. The combined MeOH extracts were filtered and concentrated under reduced pressure to yield 800 g of gummy residue, which was suspended in $H_2O$ and fractionated into *n*-hexane (40 g), ethyl acetate (EtOAc) (60 g), and *n*-BuOH (80 g) soluble fractions. The EtOAc soluble fraction was subjected to column chromatography (CC) over silica gel eluting with mixtures of *n*-hexane and $CHCl_3$ in increasing order of polarity. The fraction obtained from $CHCl_3$-MeOH (9:1) was subjected to recycling HPLC (ODS-M 80 column, 2 mL/min) and eluted with $H_2O$/MeOH (1:1) resulting in the isolation of compounds **1** (10 mg) and **2**, (13 mg).

**8,8''-Bi-6-hydroxyorobol** (**1**)
Yellow amorphous powder.
MP: 266-268°C.
IR (KBr) $\nu_{max}$ cm$^{-1}$: 3490 (OH), 1670 (conjugated ketone), 1616, 1530, 1505 (aromatic C=C).
UV (MeOH) ($\lambda_{max}$) (log ε): 273 (4.17), 343 (3.92) (sh) nm
$^1$H and $^{13}$C NMR: Table 1
(+)-FAB-MS *m/z*: 603 $[M+H]^+$; (-)-FAB-MS *m/z*: 601 $[M-H]^-$
HREI-MS *m/z*: $[M]^+$ calcd. for $C_{30}H_{18}O_{14}$: 602.0697; found: 602.0691
Elemental analysis: Calcd. C 59.81, H 3.01, O 37.18; found C 59.79, H 3.04, O 37.09

**8,8''-Bi-6, 2'-dihydroxygenistein** (**2**)
Yellow amorphous powder.
MP: 280-282°C.
IR (KBr) $\nu_{max}$ cm$^{-1}$: 3510 (OH), 1675 (conjugated ketone), 1610, 1530, 1510 (aromatic C=C).
UV (MeOH) ($\lambda_{max}$) (log ε): 275 (4.29), 347 (3.52) (sh) nm
$^1$H and $^{13}$C NMR: Table 1
(+)-FAB-MS *m/z*: 603 $[M+H]^+$; (-)-FAB-MS *m/z*: 601 $[M-H]^-$
HREI-MS *m/z*: $[M]^+$ calcd. for $C_{30}H_{18}O_{14}$: 602.0697; found: 602.0693
Elemental analysis: Calcd. C 59.81, H 3.01, O 37.18; found C 59.76, H 3.07, O 37.15.

## References


[1] (a) Baquar SR. (**1989**) *Medicinal and Poisonous Plants of Pakistan*, Printas Press Karachi, Pakistan. 161; (b) Ullah N, Ahmed S, Ahmed Z, Khan PM, Malik A. (**1999**) Dimeric guaianolides from *Daphne oleoides*. *Phytochemistry*, ***51***, 559-562.

[2] (a) Kreher B, Neszmelyi A, Hildebert W (**1990**) Triumbellin, a tricoumarin rhamnopyranoside from *Daphne mezereum*. *Phytochemistry*, ***29***, 3633-3637; (b) Ullah N, Ahmed S, Malik A. (**1999**) A dicoumarin glycoside from *Daphne oleoides*. *Phytochemistry*, ***51***, 99-101; (c) Riaz M, Ullah N, Mehmood A, Rabnawaz H, Malik A, Afza N. (**2000**) Furanoid and furofuranide lignans from *Daphne oleoides*. *Zeitschrift für Naturforschung*, ***55b***, 1216-1220; (d) Taniguchi M, Baba K. (**1996**) Three biflavonoids from *Daphne odora*. *Phytochemistry*, ***42***, 1447-1453; (e) Ullah N, Ahmed Z. Ahmed S, Khan PM, Malik A. (**1999**) A pentacyclic triterpene from *Daphne oleoides*. *Phytochemistry*, ***50***, 839-841.

[3] (a) Markham KR. (**1982**) *Techniques of Flavonoids Identification,* Academic Press, New York; (b) Anouar EH, Gierschner J, Duroux JL, Trouillas P. (**2012**) UV/Visible spectra of natural polyphenols: A time dependant density functional theory study. *Food Chemistry*, ***131***, 79-89.

[4] Wong SM, Konno C, Oshima Y, Pezzuto JM, Fong HHS, Farnsworth NR. (**1987**) Irisones A and B: Two new isoflavones from *Iris missouriensis*, *Journal of Natural Products*, ***50***, 178-180.

[5] (a) Horie T, Sasagawa M, Torii F, Kawamura Y, Yamashita K. (**1996**) Studies of the selective *O*-alkylation and dealkylation of flavonoids. XX. A convenient method for synthesizing 5,6,7-trihydroxyflavones and 5,6-dihydroxy-7-methoxyisoflavones. *Chemical and Pharmaceutical Bulletin*, ***44***, 486-491; (b) Kulling SE, Honig DM, Simat TJ, Metzler M. (**2000**) Oxidative *in vitro* metabolism of the soy phytoestrogens daidzein and genistein, *Journal of Agricultural and Food Chem*istry, ***48***, 4963-4972.

[6] Markham KR, Mabry TJ, Swift TW. (**1968**) New isoflavones from the genus *Baptisia* (*Leguminosae*). *Phytochemistry*, ***7***, 803-808.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1597 - 1600

# Biflavonoids from the Unripe Fruits of *Clusia paralicola* and their Antioxidant Activity

**Rafaela Ferreira Oliveira[a], Celso Amorim Camara[a], Maria de Fátima Agra[b] and Tania Maria Sarmento Silva[a*]**

[a]*Departamento de Ciencias Moleculares*, *Universidade Federal Rural de Pernambuco*, *Recife, Pernambuco*, *CEP 52171-900*, *Brazil*

[b]*Laboratório de Tecnologia Farmacêutica*, *Universidade Federal da Paraíba*, *João Pessoa*, *Paraiba*, *CEP 58051-970*, *Brazil*

*taniasarmento@dcm.ufrpe.br*

**Received: October 27th, 2011; Accepted: October 6th, 2012**

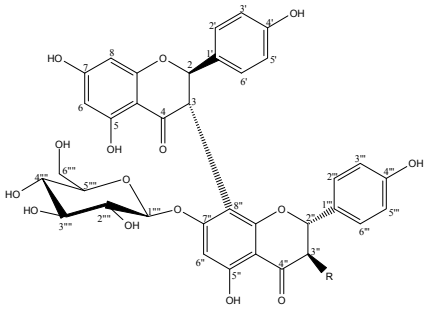
Investigation of the green fruits of *Clusia paralicola* (Clusiaceae) led to the isolation and characterization of two 3,8"-biflavonoids, 2*R*, 3*S*, 2"*R*, 3"*R*-GB1-7"-*O*-β-glucoside (**1**) and 2*R*, 3*S*, 2"*R*, 3,8"-binaringenin-7"-*O*-β-glucoside (**2**), together with four known compounds: β-sitosterol, stigmasterol, β-amyrin, and epicatechin. The structures were established from the IR, LC-ESI-MS and NMR spectral data, including 2D-NMR experiments. The absolute configurations of **1** and **2** were determined by CD spectra. The total extract and the biflavonoids demonstrated significant antioxidant activity in DPPH, ABTS, and β-carotene/linoleic acid tests.

**Keywords:** Clusiaceae, *Clusia paralicola*, Biflavonoid glucosides, Antioxidant activity.

The genus *Clusia* (Clusiaceae) occurs in the neotropical region and comprises around 250 species of semi-epiphytes, vines, shrubs and trees [1, 2]. *C. paralicola* G. Mariz Cunha is native and endemic to northeastern Brazil, such as in the states of Paraiba, Pernambuco, Bahia and Alagoas, in areas of Caatinga and Atlantic Forest [3], and is popularly known as pororoca. Previous studies of *C. paralicola* showed the presence of xanthone, an uncommon prenylated dihydrophenanthrene, paralycoline A [4, 5] and new biphenyls with DNA strand-scission activity [6].

In the present investigation, fractionation of an extract of the unripe fruits of *C. paralicola* led to the isolation of compounds **1-6**. We report herein the isolation and structure elucidation of biflavonoids **1** and **2**. Although a wide variety of compounds, both aromatic and nonaromatic, including biflavonoids of the *Garcinia* type [4] have been isolated from *Clusia* species earlier, to our knowledge this is the first report of these biflavonoids from this source. In our continuing search for potent antioxidants from natural sources [7,8], we found that the ethanolic extract, the fractions thereof, and compounds **1** and **2** displayed antioxidant activity.

The UV spectra of compounds **1** and **2** were typical of biflavonones with an absorption maximum at 285 nm, followed by a shoulder at 330 nm [9], and showed an $[M+H]^+$ ion at *m/z* 721 and 705 in its ESI-MS, corresponding to the molecular formula, $C_{36}H_{32}O_{16}$ and $C_{36}H_{32}O_{15}$, respectively. In addition, the ESI-MS fragments at *m/z* 559 and 543 for **1** and **2**, revealed the loss of glucose in each case. The structure of **1** was deduced from detailed analysis of $^1H$ and $^{13}C$ NMR data aided by 2D NMR experiments ($^1H$-$^1H$ COSY, HSQC, and HMBC) (Table 1). Duplication of signals (in a ratio 1:0.6) in the $^1H$ NMR spectrum of the optically active **1** in DMSO-$d_6$ suggests the existence of two conformers at room temperature. This observation has been previously reported for the biflavanone GB-1 and could be attributed to the hindered rotation between the flavanone and the flavanonol moieties around the C-3/C-8 axis [10]. For this type of molecules, the existence of rotational isomers at ambient temperature is explained by the strong intra- and intermolecular H-bonding that occurs due to the many OH groups present [11].



**1** R=OH
**2** R=H

**Figure 1**: Structures of biflavonoids **1** and **2** from fruits of *Clusia paralicola*.

Biflavonoid **1** (Figure 1) showed singlets for OH protons, two at low fields at $\delta_H$ 12.12 and 11.66, attributed to the H-bonded OH-(C5) and OH-(C5"), respectively (Table1). The doublets at $\delta_H$ 7.23, 7.18, and 6.68, 6.60 (*J*=8.5 Hz) could be assigned to the *AA'BB'* spin system of the *para*-substituted B rings H-2'''/6''', H-2'/6', H3'''/5''', and H-3'/5', respectively. Two doublets for two protons at $\delta_H$ 5.89 and 5.75 (*J*=2.0 Hz) were accordingly located at H-6 and H-8, respectively. H-6" exhibited a singlet at $\delta_H$ 6.21. In the same way, the remaining three doublets at $\delta_H$ 5.45 (*J*=12 Hz), 5.06 (*J*=12 Hz) and 5.03 (*J*=11 Hz), and one doublet of doublets at $\delta_H$ 4.30 (*J*=11 and 6 Hz) for one proton each were assigned to H-2, H2", H-3, and H-3", respectively. The vicinal coupling constants (11-12 Hz) *J* [H-C(2), H-C(3)] and *J* [H-C(2"), H-C(3")] established the *trans*-diaxial arrangements of these pairs. The resonances of the β-glucose moiety were observed as follows: an anomeric proton H-1"" at $\delta_H$ 4.73 (*d*, *J*=7.5 Hz), with methylene protons H-6"" at $\delta_H$ 3.44 (m) and 3.70 (*d*, *J*=11 Hz) and the other

**Table 1**: $^{1}$H (500 MHz) and $^{13}$C NMR (125 MHz) spectroscopic data for **1** and **2** (major conformer) (DMSO-$d_6$, δ in ppm).

| | **1** | | | | **2** | |
|---|---|---|---|---|---|---|
| Position | $\delta_C$ | $\delta_H$ | $^{2}J_{CH}$ | $^{3}J_{CH}$ | $\delta_C$ | $\delta_H$ |
| 2 | 82.1 | 5.45 (d, 12.0) | | | 81.8 | 5.55 (d, 12) |
| 3 | 46.7 | 5.06 (d, 12.0) | | | 46.6 | 5.09 (d, 12) |
| 4 | 196.9 | | H-3 | H-2 | 196.9 | |
| 5 | 163.6 | | | H-6 | 163.5 | |
| 6 | 95.9 | 5.89 (d, 2.0) | | H-8 | 95.9 | 6.18 (s) |
| 7 | 168.9 | | H-6, H-8 | | 166.5 | |
| 8 | 94.8 | 5.75 (d, 2.0) | | | 94.9 | 5.79 (d, 2.0) |
| 9 | 163.2 | | H-8 | | 163.1 | |
| 10 | 101.1 | | | H-6, H-8 | 101.3 | |
| 1’ | 127.0 | | H-3’, H-5’ | | 128.4 | |
| 2’, 6’ | 129.8 | 7.18 (d, 8.5) | | H-2 | 128.5 | 7.25 (d, 8.5) |
| 3’, 5’ | 114.8 | 6.60 (d, 8.5) | | | 115.1 | 6.60 (d, 8.5) |
| 4’ | 157.6 | | H-3’, H-5’ | H-2’, H-6’ | 157.9 | |
| 2” | 82.7 | 5.06 (d, 11.0) | | | 78.4 | 5.48 (dd; 12.5, 3.0) |
| 3” | 72.4 | 4.30 (dd; 11.0, 6.0) | | | 42.1 | 3.05 (dd; 17.5, 13.0) 2.75 (dd; 17.5, 3.0) |
| 4” | 198.2 | | H-3 | H-2 | 197.6 | |
| 5” | 161.8 | | H-6 | | 162.0 | |
| 6” | 96.0 | 6.21 (s) | | | 95.9 | 6.18 (s) |
| 7” | 162.8 | | H-6 | H-1””, H-3 | 162.8 | |
| 8” | 102.7 | | H-3 | H-6” | 103.3 | |
| 9” | 158.6 | | | H-3 | 160.1 | |
| 10” | | | | | 102.7 | |
| 1”’ | 127.4 | H-2” | H-3”, H-3”’, H-5”’ | | 128.1 | |
| 2”’, 6”’ | 128.3 | 7.23 (d, 8.5) | | | 128.0 | 7.19 (d, 8.5) |
| 3”’, 5”’ | 114.9 | 6.68 (d, 8.5) | | | 115.1 | 6.68 (d, 8.5) |
| 4”’ | 157.5 | | H-3”’, H-5”’ | H-2”’, H-6”’ | 157.6 | |
| 1”” | 99.9 | 4.73 (d, 7.5) | | | 99.9 | 4.72 (d, 7.0) |
| 2”” | 73.0 | 3.18 (m) | | | 73.0 | 3.17 (m) |
| 3”” | 77.1 | 3.66 (m) | | | 77.1 | 3.37 (m) |
| 4”” | 69.6 | 3.09 (m) | | | 69.6 | 3.11 (m) |
| 5”” | 76.1 | 3.15 (m) | | | 76.8 | 3.16 (m) |
| 6”” | 60.6 | 3.70 (m) 3.44 (m) | | | 60.5 | 3.66 (m) 3.44 (m) |
| OH-C(5)/C(5”) | | 12.12 (s) 11.66 (s) | | | | 12.14 (s) |
| OH-C(3) | | 5.85 (d, 6.5) | | | | |

protons absorbing at $\delta_H$ 3.15-3.66. The APT spectrum displayed low field signals at $\delta_C$ 198.2 and 196.9, typical of carbonyl groups of flavanone and flavanonol. The signals at $\delta_C$ 129.8, 128.3, 114.8, and 114.9 could be assigned to the carbons 2’, 6’, 2”’, 6”’, 3’, 5’, 3”’, and 5”’ to IB and IIB rings in the AA’BB’ system, respectively. The signals at $\delta_C$ 72.4 and 46.7 represented the aliphatic C-atoms of the 3-hydroxyflavanone and flavanone moieties, respectively. Similarly, the signals at $\delta_C$ 82.7 and 82.1 were assigned to C-2” and C-2, respectively. By comparison with $^{13}$C NMR data of other related 3,8”-biflavonoids, I-3,II-8-binaringenin or GB-1a (*Garcinia* biflavonoid) [10], the measured resonances of **1** could be attributed to a 3,8”-biflavonoid consisting of 3-hydroxyflavonone and flavanone moieties. The connectivity in the molecule, particularly the 3,8”-linkage of the flavonoid, was confirmed by HMBC correlations (Table 1). The glycosidic linkage site of β-D-glucose at C-7 was further verified by the HMBC experiments in which the anomeric proton at $\delta_H$ 4.73 showed a cross peak with the $\delta_C$ C-7 at 168.9. Detailed analyses of the HSQC and HMBC spectra allowed for the assignments of all the $^{1}$H- and $^{13}$C-NMR signals for both the glucosyl and aglycone moieties. The $^{1}$H- and $^{13}$C-NMR data for compound **2** (Figure 1) were similar to those of **1**, except for the signals at $\delta_H$ 5.48 (H-2”; dd, *J*=12.5 and 3.0 Hz), $\delta_H$ 3.05 (Hα-3"; *dd*, *J*=17.5 and 13.0 Hz), $\delta_H$ 2.75 (Hβ-3”; *dd, J*=17.5 and 3.0 Hz) and $\delta_C$ 42.1 (C-3”). The absolute configurations of the two biflavonoids were determined by CD spectra. The CD spectrum of biflavonoid **1** revealed Cotton effects due to the electronic n→p* and π→p* transitions of the two constituent structural moieties: the 3-substituted flavanone upper unit (I) and the flavanonol lower unit (II) (Figure 2). The observed Cotton effects were similar to those observed for the flavonoids *ent*-naringeninyl-(I-3α,II-8)-4’-*O*-methylnaringenin [12] and GB1 [13]. The positive Cotton effect near 330 nm for the n→p* transition and the negative Cotton effect near 300 nm for the π→p* transition may be assigned to the lower flavanonol unit. Similarly, the negative Cotton effect (shoulder) near 325 nm for the n→p* transition and the positive Cotton effect near 280 nm for the π→p* transition may be assigned to the upper flavanone unit. Application of Gaffield’s rule then permits assignment of (2*R*, 3S) and (2”*R*, 3”*R*) absolute configurations for the flavanone and flavanonol moieties, respectively [14]. Therefore, the structure of compound **1** could be established unambiguously as 2*R*, 3*S*, 2”*R*, 3”*R*-GB1-7”-*O*-β-glucoside. The CD spectrum of **2** was similar to biflavanoid **1** (Figure 2) and the absolute configuration was established as 2*R*, 3*S*, 2”*R*, 3,8”-binaringenin-7”-*O*-β-glucoside. GB1-7”-*O*-β-glucoside was reported to exist in *Garcinia kola*, but no data for structural identification was published previously. Although 3,8”-binaringenin-7”-*O*-β-glucoside was earlier isolated from *Garcinia multiflora* [16], the $^{13}$C NMR and CD presented here are, to our knowledge, new data. 3,8”-Biflavanoids have been isolated mainly from *Garcinia*, *Rheedia*, and *Allanblakia* species (Clusiaceae), and have recently been isolated from *Clusia columnaris* [17]. Compounds **3** (epicatechin) [18], **4** (β-sitosterol) [19], **5** (stigmasterol) [19], and **6 (**β-amyrin) [20] were identified by comparison with published spectroscopic data.

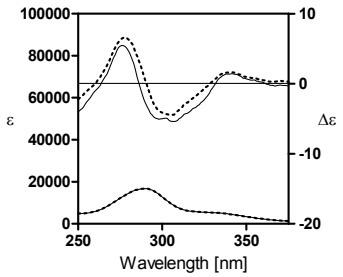



**Figure 2**: Upper curves: CD spectra of **1** (dotted line) and **2** (solid line). Lower curves: UV spectra of **1** (dotted line) and **2** (solid line).

**Table 2**: Total phenolics and antioxidant activity of extracts, fractions and biflavonoids from *Clusia paralicola*.

| Sample | Total phenolic content (mg GAE/g ± SD) | DPPH ($EC_{50}$)[a] | ABTS ($EC_{50}$) | β-Carotene bleaching (% O.I.)[b] |
|---|---|---|---|---|
| EtOH extract | 132.1±1.3 | 12.7±0.1 | 7.4±0.4 | 65.6±3.8 |
| *n*-Hexane fraction | 42.9±3.2 | 17.4±0.1 | 18.6±0.1 | 68.3±9.8 |
| AcOEt fraction | 135.4±3.2 | 10.8±0.1 | 6.2±0.3 | 70.6±3.2 |
| MeOH:$H_2O$ fraction | 94.8±3.3 | 13.9±0.2 | 13.9±0.2 | 82.4±2.3 |
| Biflavonoids | | 60.2±1.6 | 26.7±3.3 | 53.2±2.7 |
| Ascorbic acid | | 2.1±0.0 | 2.8±0.2 | |
| Trolox | | | | 77.4±1.8 |

Mean value ± standard deviation: n=3
[a]Concentration of antioxidant required to reduce the original amount of radicals by 50 %. [b]Oxidation inhibition at 60 min.

The total phenolic content and the antioxidant activity of the EtOH extract, *n*-hexane, EtOAc, and MeOH: $H_2O$ fractions were evaluated by DPPH and ABTS radical scavenging, as well as β-carotene/linoleic acid system activity assays and the data were compared with those of the reference antioxidants, ascorbic acid and Trolox (Table 2). The extract and fractions tested displayed antioxidant activity in all of the test methods. Biflavonoids **1** and **2** displayed moderate antioxidant activity in all the assays. The

EtOAc fraction showed activity similar to that of the EtOH extract in all tests. The EtOH extract and fractions showed correlation (Pearson correlation, *r*, data not shown) with the total phenolic content (Table 2).

## Experimental

***General:*** Melting points were determined on a Koefler hot stage and are uncorrected. The infrared absorption spectra were recorded in KBr pellets, using a Varian 640 FT-IR spectrophotometer with a PIKE ATR accessory operating in the 4000-400 $cm^{-1}$ range. The LC-ESI-MS was obtained in positive electrospray mode using an Esquire 3000 Plus (Bruker). Silica gel 60 was used for CC, and silica gel 60 $F_{254}$ (E. Merck) for TLC plates. Sephadex®LH-20 (Sigma) was employed for gel permeation chromatography. $^1H$ and $^{13}C$ NMR spectra were obtained using a Bruker DRX 500 (500 MHz for $^1H$ and 125 MHz for $^{13}C$) and Bruker DPX300 (300 MHz for $^1H$ and 75 MHz for $^{13}C$) in DMSO-$d_6$. The CD was recorded with a Jasco J-515 CD spectrometer. The optical rotation was determined in a KRUESS OPTRONIC spectrometer. All solvents used were of commercial HPLC grade.

***Plant material:*** The unripe fruits of *Clusia paralicola* were collected in Santa Rita city, State of Paraiba, Brazil in May 2010. The plant was identified by Maria de Fátima Agra. Voucher specimen (M. F. Agra *et al.* 7455) is deposited at the Herbarium Lauro Pires Xavier, Universidade Federal da Paraiba.

***Extraction and isolation:*** The plant material (280.0 g) was extracted successively with EtOH to give 40.0 g of dry extract. This was dissolved in MeOH: $H_2O$ (1:1) and successively fractionated with *n*-hexane and AcOEt. The AcOEt fraction (8.0 g) was successively submitted to CC using Sephadex® LH-20 and silica gel to yield **1** (50.0 mg), **2** (20.0 mg) and **3** (25.0 mg), as yellow amorphous solids. The *n*-hexane fraction (10.0 g) was subjected to CC using Sephadex® LH-20. Fractions 4 and 5 were submitted to CC on silica gel using *n*-hexane, EtOAc and MeOH to furnish the mixture of **4** and **5** (11.0 mg) and **6** (35.0 mg).

**2*R*, 3*S*, 2"*R*, 3"*R*-GB1-7"-*O*-β-glucoside** (**1**)
MP: 223-225ºC.
$[\alpha]_D$: -11.9 (*c* 0.23, MeOH).
IR (KBr) $\nu_{max}$: 3400 (OH), 1640 (C=O), 1600, 1520 (C=C from aromatic rings).
$^1H$ and $^{13}C$ NMR (DMSO-$d_6$): Table 1.
LC-ESI-MS (positive mode) *m/z*: 721 $[M+H]^+$ ($C_{36}H_{32}O_{16}$).

**2*R*, 3*S*, 2"*R*, 3,8"-binaringenin-7"-*O*-β-glucoside** (**2**)
MP: 218-220ºC;
$[\alpha]_D$: -35.1 (*c* 0.67, MeOH).
IR (KBr) $\nu_{max}$: 3400 (OH), 1640 (C=O), 1600, 1520 (C=C from aromatic rings).
$^1H$ and $^{13}C$ NMR (DMSO-$d_6$): Table 1.
LC-ESI-MS (positive mode) *m/z*: 705 $[M+H]^+$ ($C_{36}H_{32}O_{15}$).

***Determination of total phenolic content:*** Total soluble phenolic contents of the EtOH extract, and the *n*-hexane, AcOEt, and MeOH: $H_2O$ fractions (1 mg/mL) was determined with the Folin-Ciocalteu reagent according to the method of Slinkard & Singleton [21], with modification by using gallic acid as a standard phenolic compound.

***DPPH• radical scavenging assay, ABTS+• radical cation decolorisation assay, and antioxidant activity in linoleic acid oxidation:*** The free radical-scavenger activity (DPPH) [7], the radical cation decolorization assay (ABTS) [22], and antioxidant activity in linoleic acid oxidation [23] of the EtOH extract, and *n*-hexane, AcOEt, MeOH:$H_2O$ fractions and the biflavonoids was determined as described previously.

***Statistical analysis:*** All samples were analyzed in triplicate unless stated otherwise and the results are expressed as average ± standard deviation. All statistical analyses were carried out using the Microsoft Excel software package (Microsoft Corp., Redmond, WA). Correlation analysis of antioxidant activity versus the total phenolic content was carried out using the correlation and regression program in Graphpad Prism 5.0 DEMO (GraphPad Software, San Diego, California USA).

**Acknowledgements -** The authors thank CNPq (TMSS, CAC and MFA research fellowships), CAPES (ROF graduate fellowship) and FACEPE (PRONEM APQ-1232.1.06/10 and APQ-0158-1.06/08 grant numbers) for financial support, CENAPESQ and CETENE analytical centers for the recorded data kindly provided by them. ROF also thanks PRPPG-UFRPE for their kind fellowship support. The authors also thank Dr J. Bhattacharyya for his kind suggestions.

## References

[1] Bittrich V, Amaral MCE. (**1996**) Flower morphology and pollination biology of some *Clusia* species from the Gran Sabana (Venezuela). *Kew Bulletin*, ***51***, 681-694.
[2] Bittrich V, Amaral MCE. (**1997**) Floral biology of some *Clusia* species from Central Amazonia. *Kew Bulletin*, ***52***, 617-635.
[3] Forzza RC, Baumgratz JFA, Costa A, Hopkins M, Leitman PM, Lohmann LG, Martinelli G, Morim MP, Coelho MAN, Peixoto AI, Pirani JR, Queiroz LP, Stehmann JR, Walter BMT, Zappi D. (**2010**) In *Catálogo de plantas e fungos do Brasil: As angiospermas do Brasil*. Forza RC. Forza RC, Leitman PM, Costa A, Carvalho JrAA, Peixoto AL, Walter BMT, Bicudo C, Zappi D, Costa DP, Lleras E, Martinelli G, Lima HC, Prado J, Stehmann JR, Baumgratz JFA, Pirani JR, Sylvestre LS, Maia LC (Eds). Jardim Botânico do Rio de Janeiro, Rio de Janeiro. pp 857.
[4] Delle Monache F, Delle Monache G, Botta B, Gacs-Baitz E. (**2002**) A biphenyl, a dihydrophenanthrene and a xanthone from *Clusia paralycola*. *Heterocycles*, ***56***, 589-597.
[5] Delle Monache F, Delle Monache G, Cavalcanti JF, Pinheiro RM. (**1987**) An unexpected dihydrophenanthrene from *Clusia paralycola*. *Tetrahedron Letters*, ***28***, 563-566.
[6] Seo EK, Huang L, Wall ME, Wani MC, Navarro H, Mukherjee R, Farnsworth NR, Kinghorn AD. (**1999**) New biphenyl compounds with DNA strand-scission activity from *Clusia paralicola*. *Journal of Natural Products*, ***62***, 1484-1487.
[7] Silva TMS, Camara CA, Lins ACS, Barbosa JM, Silva EMS, Freitas BM, Santos FAR. (**2006**) Chemical composition and free radical scavenging activity of pollen loads from stingless bee *Melipona subnitida* Ducke. *Journal of Food Composition and Analysis*, ***19***, 507-511.
[8] Silva TMS, Camara CA, Lins ACS, Agra MF, Silva SEM, Reis IT, Freitas BM. (**2009**) Chemical composition, botanical evaluation and screening of radical scavenging activity of collected pollen by the stingless bees *Melipona rufiventris* (Uruçu-amarela). *Anais da Academia Brasileira de Ciências*, ***81***, 173-178.
[9] Mabry TJ, Markhan KR, Thomas MB. (**1970**) *The Systematic Identification of Flavonoids*. Springer-Verlag, New York.
[10] Han QB, Lee SF, Qiao CF, He ZD, Song JZ, Sun HD, Xu HX. (**2005**) Complete NMR assignments of the antibacterial biflavonoid GB1 from *Garcinia kola*. *Chemical & Pharmaceutical Bulletin*, ***53***, 1034-1036.

[11] Jackson B, Locksley HD, Scheinmann F, Wolstenholme WA. (**1971**) Extractives from Guttiferae. Part XXII. The isolation and structure of four novel biflavanones from the heartwoods of *Garcinia buchananii* Baker and *G. eugeniifolia* Wall. *Journal of the Chemical Society C*, 3791-3804.
[12] Mbwambo ZH, Kapingu MC, Moshi MJ, Machumi F, Apers S, Cos P, Ferreira D, Marais JPJ, Berghe DV, Maes L, Vlietinck A, Pieters L. (**2006**) Antiparasitic activity of some xanthones and biflavonoids from the root bark of *Garcinia livingstonei*. *Journal of Natural Products*, ***69***, 369-372.
[13] Ferrari J, Terreaux C, Kurtan T, Szikszai-Kiss A, Antus S, Msonthi JD, Hostettmann K. (**2003**) Isolation and online LC/CD analysis of 3,8″-linked biflavonoids from *Gnidia* involucrata. *Helvetica Chimica Acta*, ***86***, 2768-2778.
[14] Gaffield W. (**1970**) Circular dichroism, optical rotatory dispersion and absolute configuration of flavanones, 3-hydroxyflavanones and their glycosides - determination of aglycone chirality in flavanone glycosides. *Tetrahedron*, ***26***, 4093-4108.
[15] Okunji CO, Ware TA, Hicks RP, Iwu MM, Skanchy DJ. (**2002**) Capillary electrophoresis determination of biflavanones from *Garcinia kola* in three traditional African medicinal formulations. *Planta Medica*, ***68***, 440-444.
[16] Chen FC, Lin YM, Hung, JG. (**1975**) A new biflavanone glucoside from *Garcinia multiflora*. *Phytochemistry*, ***14***, 818-820.
[17] Compagnone RS, Suarez AC, Leitao SG, Monache FD. (**2008**) Flavonoids, benzophenones and a new euphane derivative from *Clusia columnaris* Engl. *Revista Brasileira de Farmacognosia*, ***18***, 6-10.
[18] *Agrawal* PK. (***1989***) *Carbon-13 NMR of Flavonoids.* Elsevier, New York.
[19] Goulart MOF, Sant'ana AEG, de Lima RA, Cavalcante SH, de Carvalho MG, Filho RB. (**1993**) Fitoconstituintes químicos isolados de *Jatropha elliptica*. Atribuição dos deslocamentos químicos dos átomos de carbono e hidrogênio dos diterpenos jatrofolanos A e B. *Quimica Nova*, ***16***, 95-100.
[20] Maia RM, Barbosa PR, Cruz FG, Roque NF, Fascio M. (**2000**) Triterpenos da resina de *Protium heptaphyllum* March (Bourseraceae): caracterização em misturas binárias. *Quimica Nova*, **23**, 623-626.
[21] Slinkard K, Singleton VL (**1977**) Total phenol analyses: automation and comparison with manual methods. *American Journal of Enology and Viticulture*, ***28***, 49-55.
[22] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (**1999**) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology & Medicine*, ***26***, 1231-1237.
[23] Emmons CL, Peterson DM, Paul GL. (**1999**) Antioxidant capacity of oat (*Avena sativa* L.) extracts. 2. *In vitro* antioxidant activity and contents of phenolic and tocol antioxidants. *Journal of Agricultural and Food Chemistry*, ***47***, 4894-4898.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1601 - 1604

# Ochnaflavone and Ochnaflavone 7-*O*-methyl Ether two Antibacterial Biflavonoids from *Ochna pretoriensis* (Ochnaceae)

**Tshepiso J. Makhafola, Babatunde B. Samuel, Esameldin E. Elgorashi and Jacobus N. Eloff***

*Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, Private Bag x04, Onderstepoort, 0110, South Africa*

*kobus.eloff@up.ac.za*

**Received: August 18th, 2012; Accepted: October 10th, 2012**

The acetone extract of *Ochna pretoriensis* was evaluated for antibacterial activity using bioautography and serial microplate dilution methods against four nosocomial bacterial pathogens namely: *Escherichia coli*, *Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa*. A bioassay-guided fractionation of the crude extract led to the isolation of two antibacterial biflavonoids, ochnaflavone and ochnaflavone 7-*O*-methyl ether. Gram-negative bacteria were more sensitive to the isolated compounds than the Gram-positive bacteria (MIC values: 31.3 µg/mL for *P. aeruginosa* and 62.5 µg/mL for *S. aureus*). In addition, the isolated compounds were assessed for their potential toxic effects in the MTT toxicity assay using monkey kidney vero cells and Ames genotoxicity test using *Salmonella typhimurium* strain TA98. $LC_{50}$ values were 125.9 µg/mL for ochnaflavone and 162.0 µg/mL for ochnaflavone 7-*O*-methyl ether. The isolated compounds have selectivity index values ranging from 1.29 to 4.03. Selectivity index values higher than one indicate that test samples are less toxic to the host cells than to the pathogens. The biflavonoids did not have any mutagenic effects in the *Salmonella*/microsome assay without metabolic activation.

**Keywords:** *Ochna pretoriensis*, Antibacterial activity, Ochnaflavone, Ochnaflavone 7-*O*-methyl ether.

Antibiotic resistance and the ever increasing development of multi-drug resistant by pathogenic microbes are of concern. This resistance has resulted in an increased incidence of infectious diseases leading to high morbidity and mortality [1]. As a result the search for new antimicrobial/anti-infective drugs (from plants) is imperative to counter resistance development and to avert or delay the arrival of a post-antibiotic era [2]. Plants have been used throughout history to cure and prevent infectious diseases and thus present an obvious source of new antimicrobial compounds [3].

The genus *Ochna,* (Ochnaceae) comprises 86 species of evergreen trees and shrubs and occurs widely in Africa, Asia and Madagascar. Biflavonoids isolated from this genus have diverse biological and pharmacological properties, which include antiviral, antihepatotoxic, anticancer and immune suppressive activities [4-6]. While several pharmacological activities have been reported for members of the genus *Ochna*, there has been no previous report of the antibacterial activity of members of this genus. In a previous study, we investigated the antibacterial potential of several *Ochna* species from which we identified the most active ones [7]. This study was an attempt, to isolate the antibacterial constituents of *O. pretoriensis*. Two main antibacterial compounds were isolated using bioassay-guided fractionation and chemically characterised. Moreover, the potential toxic effects (cytotoxic and genotoxic effects) of the isolated compounds were evaluated using a tetrazolium based colorimetric assay (MTT assay) on Vero monkey kidney cells, and a bacterial based mutagenicity test in *S. typhimurium* (Ames tests), respectively.

Bioassay-guided fractionation of the crude extract of the dried leaves of *O. pretoriensis*, followed by column chromatography, yielded two compounds identified as ochnaflavone (**1**) and ochnaflavone 7-*O*-methyl ether (**2**) using $^1H$ and $^{13}C$ NMR spectroscopic and mass spectrometric analysis. The structures were confirmed by comparison of the NMR data with that in the literature [8].

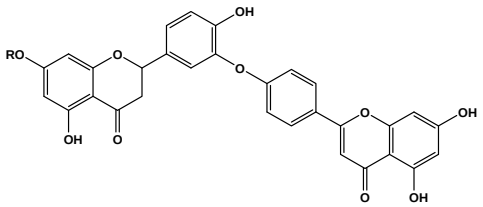



**Figure 1**: Chemical structures of the two compounds isolated from *O. pretoriensis*. 1: R = H= ochnaflavone, 2: R = $CH_3$ = ochnaflavone 7-*O*-methyl ether.

The MIC of the tested crude extracts, different solvent-solvent fractions and the biflavonoids ochnaflavone and ochnaflavone 7-*O*-methyl ether are presented in Table 1. The most sensitive organism to both biflavonoids was *P. aeruginosa* with an MIC value of 31.3 µg/mL. *E. faecalis* was more sensitive to ochnaflavone. Ochnaflavone had higher activity than its methoxy derivative.

The cytotoxic potential of ochnaflavone (**1**) and ochnaflavone 7-*O*-methyl ether (**2**) was tested in the MTT assay using Vero monkey kidney cells. Ochnaflavone and ochnaflavone 7-*O*-methyl ether had low cytotoxicity compared with berberine. The $LC_{50}$ values were 125.9 µg/mL for ochnaflavone and 162.0 µg/mL for ochnaflavone 7-*O*-methyl ether. Berberine, the positive control, had a $LC_{50}$ of 39.0 µg/mL. To evaluate potential efficacy, tolerability and therapeutic antibacterial activity of the two compounds, selectivity index (SI) was calculated by dividing the $LC_{50}$ in mg/mL by MIC in mg/mL. The selectivity Index (SI) values of the isolated compounds are presented in Table 2. The isolated compounds had positive, but low, selectivity indices ranging from 1.29 to 4.03.

Results obtained from the mutagenicity and antimutagenicity test of ochnaflavone and ochnaflavone 7-*O*-methyl ether using the *Salmonella typhimurium* strain TA98, based on the number of induced revertant colonies, are presented in Table 4. Test samples are considered mutagenic when the number of induced revertant

**Table 1**: Minimal inhibitory concentrations (MIC) (μg/mL) of the crude extract, solvent-solvent fractions and compounds isolated from *O. pretoriensis*.

| Bacteria | Crude extract | Chloroform | Ethyl acetate | 1 | 2 | Gentamycin |
|---|---|---|---|---|---|---|
| *E. coli* | 65 ± 0.002 | 156 ± 0.00 | 39 ± 0.00 | 41.6 ± 0.02 | 125 ± 0.00 | 7 |
| *P. aeruginosa* | 104 ± 0.004 | 78 ± 0.00 | 39 ± 0.00 | 31.3 ± 0.00 | 31.3 ± 0.00 | 8 |
| *S. aureus* | 104 ± 0.004 | 39 ± 0.00 | 39 ± 0.00 | 62.5 ± 0.00 | 125 ± 0.00 | 8.3 |
| *E. faecalis* | 39 ± 0.00 | 39 ± 0.00 | 39 ± 0.00 | 31.3 ± 0.00 | 62.5 ± 0.00 | 10 |

**Table 2**: Cytotoxic effects against vero cells $LC_{50}$ (μg/mL) and selectivity index values of compounds isolated from *O. pretoriensis*.

| Compounds | $LC_{50}$ | *E. coli* | *P. aeruginosa* | *S. aureus* | *E. faecalis* | Average |
|---|---|---|---|---|---|---|
| 1 | 125.9 ± 0.00 | 3.02 | 4.03 | 2.10 | 4.02 | 3.27 |
| 2 | 162.0 ± 0.00 | 1.29 | 5.18 | 1.29 | 2.59 | 2.28 |

**Table 3**: Mutagenic and antimutagenic activity of compounds isolated from *O. pretoriensis* in the frame shift of *Salmonella typhimurium* TA98 ± standard deviation.

| | MUTAGENICITY | | | ANTIMUTAGENICITY | | |
|---|---|---|---|---|---|---|
| Compound | 1000 μg/mL | 500 μg/mL | 50 μg/mL | 1000 μg/mL | 500 μg/mL | 50 μg/mL |
| 1 | 32.0 ± 0.00 | 37.3 ± 4.9 | 32.7 ± 3.0 | 182.7 ± 5.8 | 167.3 ± 4.9 | 178.7 ± 4.2 |
| 2 | 30.7 ± 0.00 | 40.7 ± 2.1 | 29.0 ± 4.0 | 166.7 ± 0.0 | 181.0 ± 3.2 | 178.3 ± 1.5 |
| Solvent blank | 28.8 ± 0.00 | | | 4-NQO (2 μg/mL) | 210.0± 12.3 | |

colonies is at least double that of the negative control. Both ochnaflavone and ochnaflavone 7-*O*-methyl ether had no potential mutagenic effect. Neither of the biflavonoids tested significantly modified the mutagenicity of the known mutagen 4-NQO. However, the slight reduction in the number of revertants was not dose dependent and indicative of toxicity.

Ochnaflavone had been isolated previously from several members of the Ochnaceae family including *O. squarrosa* [9], *O. pumila* [10], and *O. obtusata* [8] among others while its methyl ether ochnaflavone 7-*O*-methyl ether had been isolated from *O. squarrosa* [9]. Ochnaflavones have a wide range of biological activities including inhibition of cyclooxygenase-2 and 5-lipoxygenase in mouse bone marrow derived mast cells [11] and inhibition of arachidonate release by phospholipase $A_2$ from rat peritoneal macrophage [12]. The flavonoid has also antioxidant activity, inhibits lipopolysaccharide-induced iNOS expression lipopolysaccharide-induced nitric oxide production and inhibits TNF-α [6,13].

Although many biflavonoids have been isolated from the family Ochnaceae, this is the first report of the isolation of ochnaflavone and ochnaflavone 7-*O*-methyl ether from *Ochna pretoriensis* and it is also the first report of their antibacterial activity. The compounds isolated in this study may have synergistic and/or combined antibacterial activity since on average, the chloroform and ethylacetate fractions in some cases had a higher activity than the pure compounds. The isolated compounds had better antibacterial activity than the crude extract.

The assumption that herbal medicine and pure phytocompounds are safer than synthetic compounds is certainly an overstatement, as many natural products are cytotoxic, mutagenic and carcinogenic. Ochnaflavone and ochnaflavone 7-*O*-methyl ether did not have substantial toxicity towards vero cells. However, they had low specific/selective antibacterial activity with selectivity indices ranging from 1.29 to 4.03. A selectivity index of higher than 10 is preferred to ensure that an overdose does not harm the host. A high selectivity index is an indication of a large safety margin between a beneficial and toxic dose. At these relatively low selectivity indices the compounds would only be useful in topical applications. The first assumption is that cellular toxicity approximates *in vivo* toxicity, but this may not necessarily be true.

Even though the numbers of revertant colonies obtained in the Ames test for each compound at differing concentrations were more than that in the negative control, the two compounds tested are considered to be non-mutagenic. Test substances are only considered mutagenic when the number of revertant colonies in the test sample is more than twice that in the negative control. Moreover, a dose-response should be evident for the various concentrations of the mutagen [14]. At the highest concentration tested for each compound, the number of revertants seemed to decrease as compared with the lower concentrations tested. This may be a result of toxicity to the bacteria since the compounds being tested have proven to have antibacterial activity.

Antimutagenic effects of the compounds were less than 25% in each case, thus all the compounds may be considered to have weak or no antimutagenic activity. Verschaeve and Van Staden [14] explained that when the antimutagenic effects of a substance is more than 25%, then the test sample may be considered to have moderate antimutagenic effects and that it can be concluded that a test sample has a strong antimutagenic effect when the percentage of antimutagenicity is higher than 40%. Since the compounds tested in this experiment have demonstrated antibacterial activity, then their toxicity towards the test strain used may mask the genotoxic response.

In conclusion, the potential antibacterial activity of *O. pretoriensis* was demonstrated for the first time and its most active constituents shown to be ochnaflavone and ochnaflavone 7-*O*- methyl ether. The isolated compounds were not mutagenic in the Ames test and did not have substantial toxicity towards Vero cells. However, given the low selectivity index values of the isolated compounds, herbal preparations from this plant should probably be used only as topical applications for treating external microbial infections.

## Experimental

***Plant material collection:*** Leaves of *Ochna pretoriensis* E. Phillips were collected from the National Botanical Garden in Pretoria, South Africa. A voucher specimen (HGWJ Schweichkerdt Herbarium, UP 114801/2) was deposited at the Herbarium of the University of Pretoria. The leaves were dried in the dark at room temperature, pulverized into fine powder and stored in closed glass bottles until use.

***Extraction and bioassay-guided fractionation of biflavonoids:*** The powdered leaves (100 g) were extracted with 1 L of acetone overnight. The extract was filtered through Whatman No.1 filter paper and concentrated under reduced pressure to yield 10.46 g

crude extract, which was subjected to solvent-solvent fractionation using *n*-hexane, carbon tetrachloride, chloroform, ethyl acetate, 70% water in methanol, water and *n*-butanol [15]. Aliquots of the fractions were separated by TLC and tested in the bioautographic antibacterial assay described below. The most active ethyl acetate fraction (250 mg) was subjected to CC using silica gel 60 (Merck) and eluted with an isocratic system of chloroform and methanol [90:10→0:100] at 10% increments. Fractions were collected and combined based on TLC. All fractions were analysed again for bioactivity using the antibacterial bioautographic method. Fractions 2&3 were the most active (with 6 yellow lanes of bacterial growth inhibition) and were subjected to further purification using a Sephadex LH20 column with ethyl acetate as eluent to give compounds **1** (30 mg) and **2** (10 mg).

***Qualitative antibacterial assay by bioautography:*** The antibacterial bioautographic assay was carried out according to the method of Begue and Kline [16]. Briefly, overnight cultures of *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922), which were grown in Müeller-Hinton broth at 37°C, were centrifuged at 3000 x *g* for 10 min. The pellets were resuspended 10 mL of fresh MH broth and sprayed onto a TLC plate spotted with 100 µg of each test sample. The plate was then incubated under 100% humidity conditions at 37°C overnight. Thereafter, the plates were sprayed with 2 mg/mL aqueous solution of *p*-iodonotrotetrazolium violet (INT, Sigma) solution and incubated for a further 30 min. Clear zones against a pinkish background on the plates indicated inhibition of bacterial growth by antibacterial compounds present in the fractions.

***Quantitative antibacterial assay by serial microdilution:*** The serial dilution assay described [17] was used to determine the minimum inhibitory concentrations of the crude extract and the isolated biflavonoids against *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 25922). Each compound was dissolved in acetone (1 mg/mL) and serially diluted two-fold in a 96-well microplate with equal volumes of water for each of the 4 bacteria used. One hundred µL of each bacterial culture were added to each well. Acetone was used as negative control and gentamicin as the positive control. The plates were then incubated overnight at 37°C. To indicate bacterial growth, 40 µL of 0.2 mg/mL INT was added to each well after incubation and the plates incubated further at 37°C for 30 min. The MIC was recorded as the lowest concentration of the compounds at which the bacterial growth was inhibited. The MIC values were also determined after 12 h and 24-36 h incubation with the compounds to determine if they possess either bacteristatic or bactericidal activity. The experiment was performed in triplicate and repeated twice.

***Tetrazolium-based cytotoxicity test:*** Cytotoxic effects of the isolated compounds were determined using the tetrazolium-based colorimetric assay (MTT assay) against Vero monkey kidney cells using the methods described by Mosmann, [18]. Briefly, the cells were maintained in minimal essential medium supplemented with 5% fetal calf serum and 0.1% gentamycin. Cell suspensions were prepared from confluent monolayer cultures and plated at a density of $0.5 \times 10^3$ cells into each well of a 96-well microtiter plate. After incubation at 37°C in a 5% $CO_2$ incubator, the subconfluent cells in the microtiter plate were used in the cytotoxicity assay. The test

compounds were assayed at concentrations ranging from 10 to 200 µg/mL. Berberine chloride was used as positive control. The intensity of color is directly proportional to the number of surviving cells. Tests were carried out in quadruplicate and each experiment was repeated 3 times.

***Genotoxicity testing (Ames test):*** The potential genotoxic effect of **1** and **2** was investigated using the Ames test [19], performed with *Salmonella typhimurium* test strain TA98. 4-Nitroquinoline 1-oxide (4-NQO) was used as a positive control at a concentration of 2 µg/mL.

**Acknowledgments** - The National Research Foundation (SA) and the University of Pretoria provided financial support. The Pretoria National Botanical Garden allowed the collection of the plant material and Dr Victor Bagla assisted with the cytotoxicity assay.

## References

[1] Walsh FM, Amyes SG. (**2004**) Microbiology and drug resistance mechanisms of fully resistant pathogens. *Current Opinion in Microbiology*, ***7***, 439–444.
[2] Leggadrio RJ. (**1995**) Emerging drug resistant bacteria: the wake-up call has come. *Southern Medical Journal*, ***88***, 884-885.
[3] Cowan MM. (**1999**) Plant products as antimicrobial agents. *Clinical Microbiology*, ***12***, 564-582.
[4] Kim HP, Son KH, Chang HW, Kang SS. (**2004**) Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of Pharmacology and Science*, ***96***, 229-245.
[5] Kang SS, Lee JY, Choi YK, Song SS, Kim JS, Jeon SJ, Han YN, Son KH, Han BH. (**2005**) Neuroprotective effects of naturally occurring biflavonoids. *Bioorganic and. Medical Chemistry Letters*, ***15***, 3588-3591.
[6] Suh SJ, Chung TW, Son MJ, Kim SH, Moon TC, Son KH, Kim HP, Chang HW, Kim CH. (**2006**) The naturally occurring biflavonoid, ochnaflavone, inhibits LPS induced iNOS expression, which is mediated by ERK1/2 via NF-κB regulation, in RAW 264.7 cells. *Archives of Biochemistry and. Biophysics*, ***447***, 136-146.
[7] Makhafola TJ, Eloff JN. (**2012**) Five *Ochna* species have high antibacterial activity and more than 10 antibacterial compounds. *South African Journal of Science, **108***, Art. #689, 6 pages.. http:// dx.doi.org/10.4102/sajs. v108i1/2.689.
[8] Rao KV, Sreeramulu K, Venkata-Rao C, Gunasekar D. (**1997**) Two new biflavonoids from *Ochna obtusata*. *Journal of Natural Products*, ***60***, 632-634.
[9] Okigawa M, Kawano N, Aqil M, Rahman W. (**1976**) Ochnaflavone and its derivative: A new series of diflavonyl ethers from *Ochna squarrosa* Linn. *Journal of the Chemical Society, Perkin Transactions*, ***1***, 580-583.
[10] Kamil M, Khan NA, Alam MS, Ilyas M. (**1987**) A biflavone from *Ochna pumila*. *Phytochemistry*, ***26***, 1171-1173.
[11] Min JS, Tae CM, Eun KL, Kun HS, Hyun PK, Sam SK, Jong KS, Seung HL, Hyeun WC. (**2006**) Naturally occurring biflavonoids, ochnaflavone inhibits cyclooxygenase-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells. *Archives of Pharmacal Research*, ***29***, 282-286.
[12] Lee SJ, Son RH, Chang HW, Kang SS, Kim HP. (**1997**) Inhibition of arachidonate release from rat peritoneal macrophage by biflavonoids. *Archives of Pharmacal Research*, ***20***, 533-538.
[13] Cheon BS, Kim YH, Son KH, Chang HW, Kang SS, Kim HP. (**2000**) Effects of prenylated flavonoids and biflavonoids on lipopolysaccharide-induced nitric oxide production from the mouse macrophage cell line RAW 264.7. *Planta Medica*, ***66***, 596-600.
[14] Verschaeve L, van Staden J. (**2008**) Mutagenic and antimutagenic properties of extracts from South African traditional medicinal plants. *Journal of Ethnopharmacology*, ***119***, 575-587.

[15] Suffness M, Douros J. (**1979**) Drugs of plant origin. *Methods in Cancer Research*, ***26***, 73-126.
[16] Begue WJ, Kline RM. (**1972**) The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography*, ***64***, 182-184.
[17] Eloff JN. (**1998**) A sensitive and quick microplate method to determine the minimum inhibitory concentration of plants extracts for bacteria. *Planta Medica*, ***64***, 711-713.
[18] Mosmann T. (**1983**) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay. *Journal of Immunological Methods*, ***65***, 263-271.
[19] Maron DM, Ames BN. (**1983**) Revised methods for the *Salmonella* mutagenicity test. *Mutation Research*, ***113***, 173–215.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1605 - 1608

# Factors Affecting the Separation and Bioactivity of Red Clover (*Trifolium pratense*) Extracts Assayed against *Clostridium sticklandii*, a Ruminal Hyper Ammonia-producing Bacterium

**Isabelle A. Kagan[a,b] and Michael D. Flythe[a,c]**

[a]*USDA-ARS Forage-Animal Production Research Unit, Lexington, KY 40546, USA*
[b]*Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546, USA*
[c]*Department of Animal and Food Sciences, University of Kentucky, Lexington, KY 40546, USA*

*isabelle.kagan@ars.usda.gov*

**Received: October 5th, 2012; Accepted: November 7th, 2012**

Red clover (*Trifolium pratense*) is rich in phenolic compounds. Both the crude phenolic extract and biochanin A, an isoflavonoid component of the extract, suppress growth of *Clostridium sticklandii*, a bovine, Gram-positive, ruminal hyper-ammonia producing bacterium (HAB). The purpose of this study was to determine if other components of red clover extract contributed to its anti-HAB activity. Extracts of the Kenland cultivar of red clover, collected 0 h and 24 h after cutting, were separated by normal-phase thin-layer chromatography (TLC) in either ethyl acetate-hexanes (9:1, v/v) or ethyl acetate-methanol (4:1, v/v). Bands on TLC plates were assayed by either overlaying the plates with agar seeded with *C. sticklandii*, or setting the excised bands face-down onto plates of bacteria-seeded agar. Biochanin A inhibited *C. sticklandii* growth on TLC plates when as little as 8 nmol was present in the extract. An antimicrobial band, seen in a previous bioassay, was not found, suggesting that this second compound may be more labile than biochanin A.

**Keywords:** *Trifolium pratense*, Phenolic, Isoflavonoid, Thin-layer chromatography, HAB.

The phenolic compounds of red clover (*Trifolium pratense* L.) have been extensively studied because of their varied roles in human [1,2], plant [3], and animal health. In the last area, some red clover isoflavones have adverse effects on reproduction in sheep [4,5]. However, at least one red clover isoflavone, biochanin A, may promote growth in cattle by decreasing loss of protein nitrogen as ammonia, thus improving assimilation of protein from forages. Both crude red clover phenolic extracts and biochanin A inhibited growth of *Clostridium sticklandii* SR, a bovine, Gram-positive, ruminal hyper ammonia-producing bacterium, or HAB [6]. Because red clover extracts differing 10-fold in biochanin A concentrations inhibited *C. sticklandii*, and about 10 peaks were consistently present in high-performance liquid chromatographic (HPLC) separations of extracts [6], it seemed possible that other red clover phenolics might contribute to the observed anti-HAB activity. The purpose of this study was to test that hypothesis.

Red clover phenolic extracts from plants collected 24 h after cutting (Figure 1) had an HPLC profile similar to that of red clover collected immediately after cutting: the same peaks were present at both times, as could be ascertained from the minimal shifts in peak retention times ($t_R$; Table 1). However, concentrations of phenolics differed by as much as 15-fold between the 0-h and 24-h sampling periods (Table 1). These differences may reflect the changes occurring in tissue over the 24-h wilting period. In dying plant cells, organelle integrity is destroyed, allowing the soluble phenolics (normally in the vacuole) to be exposed to oxidases and hydrolytic enzymes [7]. The 14-fold decrease in phaselic acid content between 0 and 24 h (Table 1) may indicate oxidation by polyphenol oxidase (PPO) [8]. The decreases in glucoside malonates of formononetin and biochanin A (FGM and BGM) may indicate that they were hydrolyzed to aglycones during the 24-h wilting period, given the 9- to 15-fold increases, respectively, in formononetin and biochanin A (Table 1). Such reactions have been found to occur readily in damaged clover tissue, due to endogenous glucosidases [9].

**Table 1**: Quantification of compounds a-i (see Figure 1) in crude extracts of red clover tissue collected 0 and 24 h after cutting. Amounts are means ± average absolute deviations of duplicate extractions. Mean retention times are the averages of the four injections (two per timepoint).

| Compound | $t_R$ on HPLC (min), mean ± std deviation (N=4) | nmol/gdw in total sample, mean ± avg. absolute deviation | |
|---|---|---|---|
| | | 0 h after cutting (N=2) | 24 h after cutting (N=2) |
| a (phaselic acid) | 30.8 ± 0.01 | 7800 ± 760 | 540 ± 49 |
| b (clovamide) | 32.0 ± 0.01 | 18000 ± 2200 | 3200 ± 110 |
| c (*p*-coumaroyl malate) | 35.0 ± 0.02 | 1500 ± 160 | 790 ± 20 |
| d (ononin) | 43.4 ± 0.01 | 590 ± 120 | 482 ± 36 |
| e (FGM) | 47.0 ± 0.00 | 10200 ± 1200 | 5600 ± 210 |
| f (sissotrin) | 48.1 ± 0.00 | 2400 ± 120 | 1600 ± 49 |
| g (BGM) | 50.8 ± 0.01 | 10300 ± 1100 | 2400 ± 86 |
| h (formononetin) | 53.6 ± 0.01 | 810 ± 23 | 7400 ± 230 |
| i (biochanin A) | 57.4 ± 0.01 | 480 ± 34 | 7400 ± 220 |

Abbreviations: $t_R$, retention time; avg., average; FGM, formononetin glucoside malonate; BGM, biochanin A glucoside malonate.

Extracts of clover from both timepoints were separated by normal-phase thin-layer chromatography (TLC) and bioassayed, in case antibacterial activity might exist in compounds more abundant at one timepoint than at the other. Separations in EtOAc-MeOH (4:1, v/v) revealed a few prominent clusters of bands on TLC plates viewed under short-wave UV light (254 nm, Figures 2A and 2B). Because bands on TLC plates were eluted in zones, HPLC retention times of most compounds could not be matched to exact retention factor ($R_f$) values. HPLC analysis of eluates demonstrated that the most polar compounds (phaselic acid, clovamide, *p*-coumaroyl malate, FGM, and BGM) eluted in a zone ranging from $R_f$=0 to $R_f$=0.096 in EtOAc-MeOH (4:1, v/v; zone 6 on Figure 2B) and in a zone from $R_f$=0 to $R_f$=0.029 in EtOAc-hexanes (9:1, v/v). The 7-*O*-glucosides of formononetin and biochanin A (ononin and sissotrin, respectively) migrated to an $R_f$ of 0.37-0.43 in EtOAc-MeOH (4:1, v/v; zone 3 on Figure 2B), although small amounts were detected in eluates from the most polar zone. In EtOAc-hexanes (9:1, v/v), ononin and sissotrin were at highest concentrations in the zone with

an $R_f$ value of 0-0.029, although small amounts were found in a zone with an $R_f$ value of 0.08-0.31. Other compounds also were eluted from more than one zone: biochanin A ($R_f$=0.67) and formononetin ($R_f$=0.63), which formed somewhat diffuse bands in EtOAc-MeOH (4:1, v/v; zone 1 on Figure 2B), formed distinct, well-resolved bands in EtOAc-hexanes (9:1, v/v), with $R_f$ values at 0.48 and 0.44, respectively. However, both were present in eluates expected to contain only one or the other. This result suggests that even on a 0.2-mm layer of silica, compounds may diffuse beyond the zone immediately detectable under UV light.

**Table 2:** Amounts of compounds a-i (see Figure 1) on bioassayed TLC plates, based on concentrations determined in crude extracts (Table 1).

| Compound | nmol compound on bioassayed TLC plates | | |
|---|---|---|---|
| | extract 1 (0 h post cutting) 14% loaded | extract 2 (24 h post cutting) 5% loaded | extract 3 (24 h post cutting) 10% loaded |
| a (phaselic acid) | 120 | 3.2 | 5.9 |
| b (clovamide) | 280 | 19 | 33 |
| c (*p*-coumaroyl malate) | 23 | 4.7 | 8.0 |
| d (ononin) | 9.2 | 2.9 | 4.4 |
| e (FGM) | 160 | 33 | 57 |
| f (sissotrin) | 37 | 9.4 | 16 |
| g (BGM) | 160 | 14 | 25 |
| h (formononetin) | 12 | 44 | 76 |
| i (biochanin A) | 7.5 | 44 | 75 |

The only TLC plate zones consistently eliciting an antimicrobial response (Figure 2C) were those containing biochanin A, found in zone 1 on Figure 2B. HPLC quantification of peaks in unseparated extracts indicated that antimicrobial zones were formed in response to 7.5-75 nmol biochanin A (Table 2). HPLC analysis of most eluates encompassing all or part of this zone revealed three peaks with retention times of 49.4, 50.4, and 53.2 min, respectively (data not shown). Because these peaks represented 2% to 11% of the biochanin A concentrations, their potential role in bacterial inhibition seemed unlikely. The peak at 49.4 min had a $t_R$ very similar to that of genistein, an isoflavone of red clover [10], but 20 nmol genistein had no effect on *C. sticklandii* growth (data not shown). Formononetin, which was poorly resolved from biochanin A in the TLC plate bioassayed in Figure 2C, had been found previously to lack activity against *C. sticklandii* [6].

In bioassays of the TLC plate shown in Figure 2B, zone 2 also elicited a small zone of inhibition, which was not consistently present, even in extracts of tissue in which it had been observed previously [6] (data not shown). This second antimicrobial zone may have been due to the presence of an unstable compound, or to diffusion of biochanin A into that zone, as mentioned previously.

Despite high concentrations of conjugates of caffeic acid, formononetin and biochanin A (Table 1), none of these inhibited *C. sticklandii*. One of the reasons for using EtOAc-MeOH (4:1, v/v) as a TLC solvent was to see if slightly separating the most polar compounds might reveal an active compound previously masked by high concentrations of co-eluting compounds. Incubation of bands face-down onto agar was expected to maximize diffusion of compounds into the medium. The lack of observed activity indicates that *C. sticklandii* may be relatively insensitive to glycosylated phenolics. The antimicrobial properties of isoflavones are generally associated with aglycones instead of glycosides [11], and the same may be true of other phenolics as well.

In summary, the activity of red clover phenolic extracts against *C. sticklandii* may be due almost entirely to biochanin A, with occasional contributions from less stable compounds. The fact that biochanin A was active over a 10-fold range of concentrations indicates that it could account entirely for the activity against *C. sticklandii* observed in this study and in previous work [6]. This conclusion is in contrast with effects of biochanin A on other bacteria. Biochanin A inhibited the efflux of ethidium bromide from *Mycobacterium smegmatis*, which indicates inhibition of one or more drug-efflux pumps [12]. This latter result leads to the hypothesis that biochanin A potentiates the antimicrobial activity of other drugs, and does not necessarily have antimicrobial activity in the absence of other inhibitors. However, it is important to note that mycobacteria and clostridia have distinctly different cell envelopes. Recent research demonstrates that different Gram-positive bacteria, including clostridia, are sensitive to biochanin A, even when other inhibitors are not present [2,13]. Our results support the conclusions of these latter research groups, and indicate that biochanin A has an additional mechanism of action against *Firmicutes* that have the classical Gram-positive cell envelope, such as clostridia.

## Experimental

***Plant material:*** Seed from the Kenland cultivar of red clover, an older cultivar that is still grown widely in Kentucky [14], was planted on April 16, 2004, at the University of Kentucky's Spindletop Farm in Lexington, KY. Plants were cut to a 5-cm

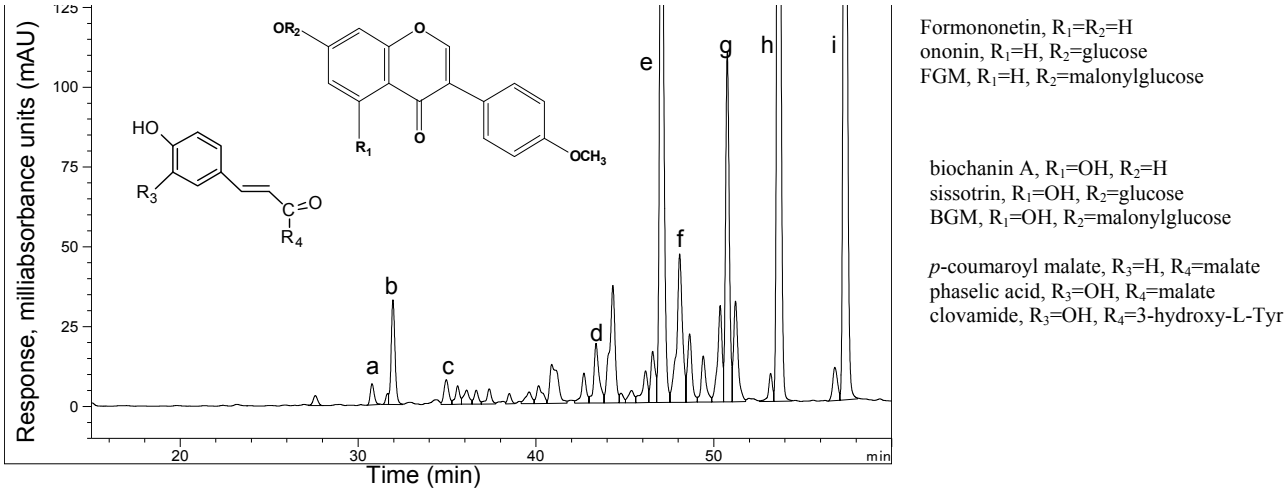



**Figure 1**: HPLC chromatogram of an extract of Kenland red clover collected 24 h after cutting. The compounds indicated by lowercase letters (a: phaselic acid, b: clovamide, c: *p*-coumaroyl malate, d: ononin, e: FGM, f: sissotrin, g: BGM, h: formononetin, and i: biochanin A) were found in eluates of zones from TLC plates. General structures are shown within the chromatogram, and relationships of general structures to individual compounds are shown at right.

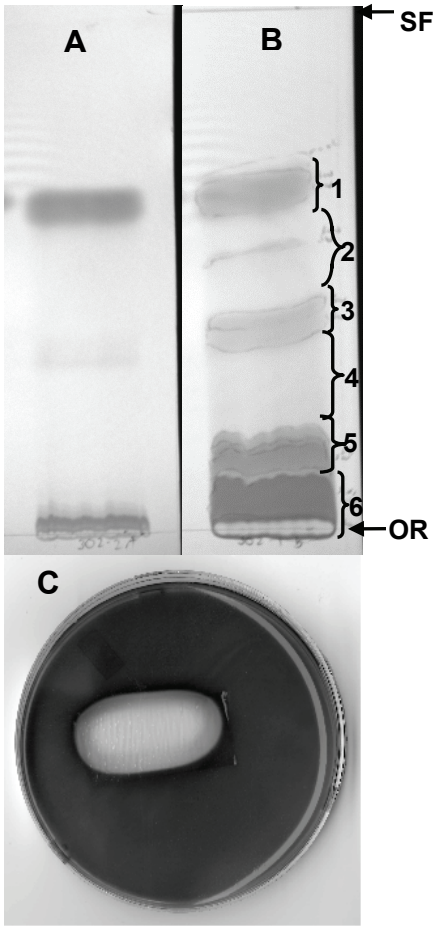



**Figure 2**: TLC plates of red clover extract collected 24 h (A) and 0 h (B) after cutting and developed in EtOAc-MeOH (4:1, v/v). Plates, photographed under short-wave ultraviolet light (254 nm), are aligned at the origin (OR) and solvent front (SF). Five percent and 14% of the 24-h and 0-h extracts were loaded, respectively. Brackets indicate zones that were cut out from both plates and assayed by laying face-down onto agar seeded with *C. sticklandii*. The zone of inhibition shown (C) was from zone 1 of the 0-h extract, comprising both formononetin (Rf=0.63, 12 nmol) and biochanin A (Rf=0.67, 7.5 nmol). Zone 2 in the 0-h extract gave a small, inconsistent zone of inhibition. No other antimicrobial zones were found in bioassays.

stubble on July 13, 2004. Plants used in the hydrolysis studies described below (part of a separate study in preparation) were cut on September 20, 2004. Some leaves and stems were put on dry ice immediately in order to assess the phenolic profiles at the time of harvest. Other plants were collected 24 h after harvest, in order to assess changes in the phenolic profiles during wilting. All samples were transported on dry ice to freezer storage (-20°C). Frozen material was lyophilized (Model DX48SA freeze-dryer, Botanique Preservation Equipment, Peoria, AZ, USA) and ground to pass a 1-mm mesh (Wiley mill, Thomas Scientific, Swedesboro, NJ, USA).

***Reagents and chemicals:*** Biochanin A, formononetin, sissotrin (biochanin A 7-*O*-glucoside), ononin (formononetin 7-*O*-glucoside), and genistein were purchased from Indofine Chemical Company (Hillsboro, NJ, USA). Clovamide (caffeoyl-3-hydroxy-L-tyrosine) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Phaselic acid (caffeoyl malic acid) and *p*-coumaroyl malic acid were generously provided by Drs Wayne Zeller and Paul Schatz (USDA-ARS Dairy Forage Research Center, Madison, WI, USA). The synthesis of phaselic acid has been described by Zeller [15], and the synthesis of *p*-coumaroyl malate by Sullivan and Zarnowski [8]. Tetrazolium red and ethyl ether ($Et_2O$) were purchased from Sigma-Aldrich. Ethyl acetate (EtOAc), acetic acid (HOAc), and hydrochloric acid (HCl) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Methanol was purchased from EMD Chemicals (Gibbstown, NJ, USA).

***Phenolic extractions:*** The extractions were made as described by Kagan [16], with the following changes. After filtration of 6.5 mL of extract (in MeOH-HOAc-water 60:0.35:40), all filtrates were diluted with an equal volume of water and acidified to pH 1-2 with 3 M HCl before being partitioned with 3 × 0.5 volume EtOAc-$Et_2O$ (1:1, v/v). Organic phases, after being dried over $MgSO_4$, were evaporated to dryness under reduced pressure at ambient temperature, resuspended in EtOAc-$Et_2O$ (1:1, v/v), dried in vials under nitrogen, and stored at -20°C.

***Separation of phenolic compounds by TLC:*** Aluminum-backed silica $F_{254}$ plates 0.2 mm thick (EMD Chemicals, Gibbstown, NJ, USA) were used. These were washed in EtOAc-MeOH (3:1, v/v) and activated for 10-15 min at 100°C prior to use. Extracts were resuspended in 0.25-0.30 mL MeOH, and 5% to 14% was loaded for bioassays. For isolation of bands, 30% to 60% of the extract was loaded, depending on plate size. Plates were developed either once in EtOAc-MeOH (4:1, v/v), or once in EtOAc-hexanes (9:1, v/v). Bands were visualized under ultraviolet light (254 and 365 nm) using a portable 4-Watt lamp (UVP, Upland, CA, USA) in a viewing cabinet (Chromato-Vue model C-10E, UVP).

***Elution and HPLC of bands:*** To minimize sample loss from chipped-off silica, TLC plates were cut into zones corresponding to one or more of the bands visualized under long-wave or short-wave UV light. These zones were covered with either MeOH or EtOAc-$Et_2O$ (1:1, v/v) and stirred several times while incubating for 1.5 h to overnight (16-23 h). The eluates were gravity-filtered through #1 filter paper (Whatman, Piscataway, NJ, USA), concentrated under reduced pressure (25-35°C for MeOH eluates, ambient for EtOAc-$Et_2O$ eluates), dried under nitrogen for long-term storage, and resuspended in 0.2 to 0.5 mL for HPLC. Eluates were mixed with MeOH and 0.86% aqueous acetic acid (3:2, final proportions). A 15 µL aliquot was separated on an HPLC system (model 1100, Agilent Technologies, Wilmington, DE, USA), equipped with a $C_{18}$ column (LiChrospher RP-18 endcapped, 250 mm length × 4.6 mm i.d.) at 28°C and 0.8 mL/min. Samples were separated by the following program (A=1.5% HOAc in water, B=MeOH): 0-3 min, 8% B; 3-18 min, linear gradient to 21% B; 18-40 min, linear gradient to 54% B; 40-55 min, linear gradient to 79% B; 55-61 min, held at 79% B; 62-75 min, wash at 100% B; 76-104 min, reequilibration at 8% B. Separations were monitored with a single-wavelength detector at 260 or 300 nm (the absorbance maxima of most eluates). For quantification, separations were monitored at 270 nm, the wavelength used for standard curves in concurrent work. Peaks whose spectra resembled those of phenolic acids (maxima at ~260 and ~300 nm) were quantified as equivalents of caffeic acid, and peaks whose spectra resembled those of isoflavones (maximum at 260 nm) were quantified as equivalents of biochanin A. Peak spectra in *T. pratense* extracts had been obtained previously with a photodiode array detector [16].

***Compound identification:*** Phaselic acid, *p*-coumaroyl malate, clovamide, ononin, sissotrin, formononetin, and biochanin A were identified by spiking red clover extracts with standards and looking for increases in the corresponding peaks. The glucoside malonates of formononetin and biochanin A were identified by a mild hydrolysis patterned after the method of de Rijke *et al*. [17]. Extracts, diluted five-fold (0.7 mL volume; 60% MeOH in 0.35% acetic acid), were heated for 75 min at 80-87°C before HPLC

analysis. Peak areas were compared in heated and unheated samples, to verify that the glucoside malonates had decreased after hydrolysis, and that the glucosides (ononin and sissotrin) had increased by a similar magnitude.

***Bacterial strains and growth conditions:*** The strain of *Clostridium sticklandii* SR, and the basal medium, were the same as those used by Flythe and Kagan [6] and described by Chen and Russell [18]. The basal medium was prepared by adding the following to 1 L deionized water: 600 mg cysteine hydrochloride, 292 mg $K_2HPO_4$, 292 mg $KH_2PO_4$, 480 mg $Na_2SO_4$, 480 mg NaCl, 100 mg $MgSO_4 \cdot 7H_20$, and 64 mg $CaCl_2.2H_20$. The pH was adjusted to 6.7 with 20% NaOH. The mixture was autoclaved (121°C, 15 min), and cooled under $O_2$-free $CO_2$. When the solution was at room temperature the following additions were made: 4g $Na_2CO_3$, 2 mg pyridoxamine dihydrochloride, 2 mg riboflavin, 2 mg thiamine hydrochloride, 2 mg nicotinamide, 2 mg calcium pantothenate, 2 mg lipoic acid, 0.1 mg *p*-aminobenzoic acid, 0.5 mg folic acid, 0.5 mg biotin, and 0.5 mg coenzyme $B_{l2}$. The broth was anaerobically dispensed into serum bottles with butyl rubber stoppers and aluminum crimp seals. The bottles were autoclaved for sterility (121°C, 15 min).

***Bioassays for anti-HAB activity:*** Bioassays with *C. sticklandii* were conducted in an anaerobic chamber (95% $CO_2$, 5% $H_2$; Coy, Grass Lake, MI, USA). The bioassays consisted of cutting bands, or clusters of bands, from TLC plates, and laying them face-down onto Petri plates containing basal medium with 0.75% agar and seeded with *C. sticklandii.* Bacteria were added to molten agar while in early stationary phase or late in exponential phase. After a 24 h anaerobic incubation at 39°C, the TLC bands were removed and the agar was stained with 1% tetrazolium red, which stains living cells [19].

**Disclaimer:** Mention of trade names or commercial products in the article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA.

**Acknowledgments** We thank the late Dr Norman L. Taylor (Department of Plant and Soil Sciences, University of Kentucky) for access to his field plots of Kenland red clover, and for many helpful discussions. This study was funded by the U.S. Department of Agriculture.

## References

[1] Lipovac M, Chedraui P, Gruenhut C, Gocan A, Stammler M, Imhof M. (**2010**) Improvement of postmenopausal depressive and anxiety symptoms after treatment with isoflavones derived from red clover extracts. *Maturitas*, ***65***, 258-261.

[2] Liu G, Liang J-C, Wang X-L, Li Z-H, Wang W, Guo N, Wu X-P, Shen F-G, Xing M-X, Liu L-H, Li L, Liu M-Y, Yu L. (**2011**) *In vitro* synergy of biochanin A and ciprofloxacin against clinical isolates of *Staphylococcus aureus*. *Molecules*, ***16***, 6656-6666.

[3] Higgins VJ, Smith DG. (**1972**) Separation and identification of two pterocarpanoid phytoalexins produced by red clover leaves. *Phytopathology*, ***62***, 235-238.

[4] Braden AWH, Hart NK, Lamberton JA. (**1967**) The oestrogenic activity and metabolism of certain isoflavones in sheep. *Australian Journal of Agricultural Research*, ***18***, 335-348.

[5] Lundh T J-O, Pettersson HI, Martinsson KA. (**1990**) Comparative levels of free and conjugated plant estrogens in blood plasma of sheep and cattle fed estrogenic silage. *Journal of Agricultural and Food Chemistry*, ***38***, 1530-1534.

[6] Flythe M, Kagan I. (**2010**) Antimicrobial effect of red clover (*Trifolium pratense*) phenolic extract on the ruminal hyper ammonia-producing bacterium, *Clostridium sticklandii*. *Current Microbiology*, ***61***, 125-131.

[7] Bittner S. (**2006**) When quinones meet amino acids: chemical, physical and biological consequences. *Amino Acids*, ***30***, 205-224.

[8] Sullivan ML, Zarnowski R. (**2011**) Red clover HCT2, a hydroxycinnamoyl-coenzyme A:malate hydroxycinnamoyl transferase, plays a crucial role in biosynthesis of phaselic acid and other hydroxycinnamoyl-malate esters *in vivo*. *Plant Physiology*, ***155***, 1060-1067.

[9] Toebes AHW, de Boer V, Verkleij JAC, Lingeman H, Ernst WHO. (**2005**) Extraction of isoflavone malonylglucosides from *Trifolium pratense* L. *Journal of Agricultural and Food Chemistry*, ***53***, 4660-4666.

[10] Krenn L, Unterrieder I, Ruprechter R. (**2002**) Quantification of isoflavones in red clover by high-performance liquid chromatography. *Journal of Chromatography B*, ***777***, 123-128

[11] Naim M, Gestetner B, Zilkah S, Birk Y, Bondi A. (**1974**) Soybean isoflavones. Characterization, determination, and antifungal activity. *Journal of Agricultural and Food Chemistry*, ***22***, 806-810.

[12] Lechner D, Gibbons S, Bucar F. (**2008**) Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*. *Journal of Antimicrobial Chemotherapy*, ***62***, 345-348.

[13] Sklenickova O, Flesar J, Kokoska L, Vlkova E, Halamova K, Malik J. (**2010**) Selective growth inhibitory effect of biochanin A against intestinal tract colonizing bacteria. *Molecules*, ***15***, 1270-1279.

[14] Taylor NL. (**2008**) A century of clover breeding developments in the United States. *Crop Science*, ***48***, 1-13.

[15] Zeller WE. (**2012**) Synthesis of (+) and (-) phaselic acid. *Synthetic Communications*, doi 10.1080/00397911.2011.633252

[16] Kagan IA. (**2011**) Effects of pH, sample size, and solvent partitioning on recovery of soluble phenolic acids and isoflavonoids in red clover (*Trifolium pratense* cv. Kenland). *Natural Product Communications*, ***6***, 1657-1660.

[17] De Rijke E, Zafra-Gómez A, Ariese F, Brinkman UA Th, Gooijer C. (**2001**) Determination of isoflavone glucoside malonates in *Trifolium pratense* L. (red clover) extracts: quantification and stability studies. *Journal of Chromatography* A, ***932***, 55-64.

[18] Chen G, Russell JB. (**1989**) More monensin-sensitive, ammonia-producing bacteria from the rumen. *Applied and Environmental Microbiology*, ***55***, 1052-1057.

[19] Lund BM, Lyon GD. (**1975**) Detection of inhibitors of *Erwinia carotovora* and *E. herbicola* on thin-layer chromatograms. *Journal of Chromatography*, ***110***, 193-196.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1609 - 1614

# Exploitation of the Antioxidant Potential of *Geranium macrorrhizum* (Geraniaceae): Hepatoprotective and Antimicrobial Activities

**Niko S. Radulović[a,*], Milan B. Stojković[a], Snežana S. Mitić[a], Pavle J. Randjelović[b], Ivan R. Ilić[c], Nikola M. Stojanović[d] and Zorica Z. Stojanović-Radić[e]**

[a]*Department of Chemistry*, *Faculty of Science and Mathematics*, *University of Niš, Višegradska 33*, *18000 Niš*, *Serbia*
[b]*Department of Physiology*, *Faculty of Medicine*, *University of Niš*, *Zorana Đinđića 81*, *18000 Niš*, *Serbia*
[c]*Institute of Pathology*, *Faculty of Medicine*, *University of Niš*, *Zorana Đinđića 81*, *18000 Niš*, *Serbia*
[d]*Faculty of Medicine*, *University of Niš*, *Zorana Đinđića 81*, *18000 Niš*, *Serbia*
[e]*Department of Biology and Ecology*, *Faculty of Science and Mathematics*, *University of Niš*, *Višegradska 33*, *18000 Niš*, *Serbia*

*nikoradulovic@yahoo.com*

**Received: October 14th, 2012; Accepted: November 1st, 2012**

In this study we evaluated *in vitro* (radical scavenging) and *in vivo* (hepatoprotective effect) antioxidant activities and antimicrobial properties of the extracts of the above- and underground parts of *Geranium macrorrhizum* L. (Geraniaceae), an ethnopharmacologically renowned plant species. The antioxidant activity and total phenol and flavonoid contents of four different solvent extracts were evaluated by seven different methods. The methanol extracts, administered *i.p.* to rats (120-480 mg/kg), were evaluated for hepatoprotective activity in a $CCl_4$-induced hepatotoxicity model. The same extracts were tested for antimicrobial activity against seven bacterial and two fungal species. The administered methanol extracts with the highest antioxidant potential showed a significant dose-dependent hepatoprotective action against $CCl_4$-induced liver damage in both decreasing the levels of liver transaminases and bilirubin and in reducing the extent of morphological malformations of the liver. The leaf methanol extract displayed a very strong antibacterial activity, especially against *Staphylococcus aureus*, with low minimal inhibitory and bactericidal concentrations. These results justify the frequent use of this plant species in folk medicine. Besides the known astringent effect, one can expect that the observed antimicrobial activity against several human pathogens contributes to the wound healing properties of this plant.

**Keywords:** *Geranium macrorrhizum* L., Radical scavenging, $CCl_4$-induced liver damage, Antimicrobial activity, Methanol extract.

*Geranium* species (Geraniaceae L.), commonly known as cranesbills, are flowering annual to perennial plants that are common throughout temperate regions and used in many different places as popular folk remedies [1]. To a great extent, it is believed that the content of hydrolysable and condensed tannins determines the diuretic, antidiarrheal and haemostatic effects of a number of species of this genus [2]. More recently, various *Geranium* species have been used as antiinfective agents [1,3], and antioxidant activity has also been demonstrated [4,5].

In Serbia, *G. macrorrhizum* L. (*Zdravac*, in Serbian, a word that can be translated into English as 'giver of health') is a common plant, possessing a long and large rhizome, which occurs sporadically on calcareous soil, in shaded locations, in mountainous terrain [6]. Both the rhizome and the herb have been used since antiquity for medicinal purposes to treat diarrhea, and externally for wounds, excessive bleeding, and sores [2,7]. Additionally, an ethnobotanical study conducted in Montenegro [8] revealed that *G. macrorrhizum* is utilized for treating inflammatory conditions of gallbladder and its ducts, kidney, bladder and liver. Similarly, in the Kopaonik region of Serbia, *zdravac* is used for the treatment of intestinal ailments in animals [9]. The hepatoprotective effect of a related plant species, *G. thunberghii* Sieb et Zucc., used traditionally in Japan as a remedy for liver disorders, is well known and corroborated in a number of experimental models of hepatic injury in rats [10]. A considerable amount of research has been undertaken on a tannin, geraniin, isolated from this and other *Geranium* species, which is believed to exert this protective effect [10,11].

Phytochemical studies of *G. macrorrhizum* have concentrated mostly on volatile constituents (essential oil) and polyphenolic compounds. The oils were shown to possess a very high and selective antibacterial activity against *Bacillus subtilis* with minimum inhibitory concentrations (MIC) of 0.4–1.0 μg/mL [1]. Polyphenolic compounds such as gallic, ellagic and 4-galloyl quinic acids, the flavonoid quercetin and three of its glycosides, were identified in the extracts of *G. macrorrhizum* [5]. Due to the antioxidant properties of these compounds, they suggested a new possible use of *zdravac* extracts in food (sausage) preservation [12].

Bearing all of the above in mind, the aim of this study was to evaluate the possible protective effect of *G. macrorrhizum* extractable matter in a $CCl_4$-induced hepatotoxicity model in rats by biochemical assays and histopathological observations in the hope of providing a possible explanation for the use of this plant in the traditional medicine of Balkan peoples. To minimize the number of animals used in the *in vivo* assays, we decided to perform a number of different *in vitro* antioxidant experiments prior to the *in vivo* ones to pinpoint the best candidate extract expected to show the highest hepatoprotective effect. HPLC profiles of the best radical scavenger extracts were additionally acquired and the total phenol and flavonoid contents of all extracts were correlated to their antioxidant potential in order to try to corroborate the starting assumption that the polyphenolic compounds are the ones responsible for the observed activities. An additional goal of this work was to verify whether a possible antimicrobial effect of *G. macrorrhizum* non-volatile extracts on wound healing exists and not only the above mentioned astringent properties.

**Table 1**: Yields, total phenolic and flavonoid contents, and results of antioxidant assays of different solvent extracts of *G. macrorrhizum*.

| solvent | % extraction | TP[a] | TF[b] | DPPH[c] | ABTS[c] | IRA[a] | FRAP[d] | CUPRAC[c] |
|---|---|---|---|---|---|---|---|---|
| methanol (leaf) | 51.2 ± 0.3 | 160.2 ± 3.1 | 44.9 ± 1.1 | 178.7 ± 1.8 | 323.3 ± 1.2 | 84.2 ± 0.2 | 1347.9 ± 46.7 | 466.0 ± 4.1 |
| ethanol (leaf) | 42.7 ± 0.3 | 109.5 ± 3.8[e] | 30.1 ± 0.2[e] | 71.0 ± 0.5[e] | 205.9 ± 1.0[e] | 17.83 ± 0.3[e] | 936.6 ± 26.3[e] | 355.0 ± 6.5[e] |
| acetone (leaf) | 9.9 ± 0.2 | 13.8 ± 0.5[e] | 9.0 ± 0.02[e] | 9.50± 0.09[e] | 11.3 ± 0.09[e] | 4.0 ± 0.3[e] | 79.5 ± 3.7[e] | 30.5 ± 0.4[e] |
| ethyl acetate (leaf) | 7.9 ± 0.2 | 6.2 ± 0.4[e] | 4.1 ± 0.05[e] | 4.2 ± 0.09[e] | 5.7 ± 0.08[e] | 2.1 ± 0.03[e] | 30.4 ± 1.3[e] | 21.1 ± 0.3[e] |
| methanol (rhizome) | 45.8 ± 0.2 | 85.7 ± 1.3[e] | 27.5 ± 0.7[e] | 106.4 ± 1.8[e] | 169. 5 ± 1.1[e] | 42.4 ± 0.2[e] | 632.1 ± 9.0[e] | 268.9 ± 2.2[e] |
| ethanol (rhizome) | 21.3 ± 0.2 | 50.6 ± 2.0[e] | 14.9 ± 0.3[e] | 50.0 ± 0.5[e] | 72.2 ± 1.0[e] | 11.6 ± 0.3[e] | 355.3 ± 4.0[e] | 207.5 ± 3.2[e] |
| acetone (rhizome) | 6.0 ± 0.1 | 22.4 ± 0.8[e] | 5.8 ± 0.2[e] | 20.4 ± 0.7[e] | 20.3 ± 0.1[e] | 8.5 ± 0.4[e] | 108.3 ± 6.0[e] | 49.8 ± 0.6[e] |
| ethyl acetate (rhizome) | 4.8 ± 0.1 | 5.5 ± 0.06[e] | 2.5 ± 0.03[e] | 4.1 ± 0.2[e] | 4.4 ± 0.05[e] | 2.1 ± 0.07[e] | 24.6 ± 0.8[e] | 14.8 ± 0.08[e] |

[a]mg gallic acid equivalents per g dry weight (mg GAE/g d.w.); [b]mg catechin equivalents per g dry weight (mg CE/g d.w.); [c]mg Trolox equivalents per g dry weight (mg TE/g d.w.); [d]μmol $Fe^{2+}$ equivalents per g dry weight (μmol $Fe^{2+}$/g d.w.); [e]significantly different from the value for the leaf methanol extract, at $p<0.001$.

**Table 2**: Correlation coefficients between the values of antioxidant equivalents obtained in different *in vitro* assays.

| | TF | DPPH | ABTS | IRA | FRAP | CUPRAC |
|---|---|---|---|---|---|---|
| TP | 0.9922 | 0.9572 | 0.9965 | 0.8928 | 0.9973 | 0.9905 |
| TF | 1 | 0.9634 | 0.9928 | 0.9045 | 0.9881 | 0.9812 |
| DPPH | | 1 | 0.9632 | 0.9761 | 0.9425 | 0.9397 |
| ABTS | | | 1 | 0.9095 | 0.9958 | 0.9813 |
| IRA | | | | 1 | 0.8802 | 0.8530 |
| FRAP | | | | | 1 | 0.9855 |

Thus, in the continuation of our work on the antibacterial and antifungal activity of *Geranium* spp. [1,13,14], we screened the mentioned extracts in a microdilution assay against 7 bacterial and 2 fungal species.

Of the four solvent extracts prepared by ultrasound-aided exhaustive maceration, the best one in turns of yield was the methanol one, followed closely by ethanol, whereas acetone and ethyl acetate gave low extractible values for *G. macrorrhizum* (Table 1). The extraction efficiency of methanol in combination with ultrasound was extraordinary: 45.8 and 51.2%, w/w, for the rhizome and aboveground parts (mostly leaves), respectively. The leaves proved to be a better source of total extractible matter in comparison with the rhizome with, in some cases, double the yield (Table 1).

The total phenolic (TP) content of *G. macrorrhizum* methanol extracts (Table 1), with 160.2 ± 3.1 and 85.7 ± 1.3 mg of gallic acid equivalents per g of dry plant material (for leaves and rhizomes, respectively), make this plant and these extracts a very rich resource of natural phenolics. Similar results, though not as good as the ones presented here, were reported for *G. macrorrhizum* by Miliauskas *et al.* [15]. Ethanol extracts contained a lower, but still significant, amount of phenolics. Extraction with the other solvents, however, did not result in such high values of TP (Table 1). In addition to TP content, the total flavonoid (TF) content was also the highest for the methanol extracts (44.9 ± 1.1 and 27.5 ± 0.7 mg of catechin equivalents per g dry plant material for leaves and rhizomes, respectively). In general, the leaf extracts had a higher TF content than the corresponding rhizome extracts (Table 1). The very high correlation coefficient ($r^2$=0.9922, Table 2) between TP and TF contents suggests that the flavonoid-type compounds are the ones responsible for the total antioxidant capacity (chemical species capable of reducing Mo(VI) to Mo(V) heteropolyacids) of the mentioned extracts.

The methanol extracts showed higher scavenging properties towards the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (Table 1) compared with other solvent extracts, which can be related to the previous two assays (TP and TF). The reductive capacity of the methanol extracts towards DPPH, compared with Trolox, is shown in Figure 1. The leaf extracts had a higher DPPH scavenging capacity than the corresponding rhizome extracts (except for the acetone extract). Again, the methanol extracts had the strongest 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging capacity: 323.3 ± 1.2 (leaf) and 169. 5 ± 1.1 (rhizome) mg TE per g dry weight (Table 1). Both the rhizome and leaf extracts possessed a strong activity, as presented in Figure 1. As expected, the value of Trolox equivalents obtained in this assay per g of the same plant material compared with the one from the DPPH test were much higher due to the less stable nature of the ABTS cation radical. These results also agree with those of Miliauskas *et al*. [15].

The iron(III) to iron(II) reduction assay (IRA) provides further data on the reducing capacity of a sample and may be a significant indicator of its potential antioxidant activity [16]. The reducing power ability of *G. macrorrhizum* extracts were compared with the gallic acid standard (the methanol extract activity presented in Figure 1). Once again, the leaf extracts had a higher reducing power than the corresponding rhizome extracts (acetone was once more the exception). This assay confirmed that methanol presents the best extraction medium (Table 1). As expected from the notion that the flavonoid-like compounds are responsible for the observed activities, the reducing power of the extracts decreased in order of their polarity decrease: methanol > ethanol > acetone > ethyl acetate.

The results of a related method to the previous one, the ferric reducing antioxidant power (FRAP), for the methanol, ethanol, acetone and ethyl acetate extracts of *G. macrorrhizum* follow a similar trend of this capacity ($r^2$=0.8802, Table 2), and these are presented in Table 1. The methanol extracts yet again had the highest reduction efficiency, 1347.9 ± 46.7 (leaf) and 632.1 ± 9.0 (rhizome) μmol of $Fe^{2+}$ per g dry weight (Figure 1). As was the case with the previous full spectrum of *in vitro* antioxidant tests, the final one performed in this work, the cupric ion reducing antioxidant capacity (CUPRAC) method (466.0 ± 4.1 and 268.9 ± 2.2 mg TE per g dry weight, for leaf and rhizome, respectively), also demonstrated the strongest reducing capacity of the methanol extracts (Table 1, Figure 1). The same order of reducing power was observed: methanol > ethanol > acetone > ethyl acetate. High correlation was observed between total phenol content and individual antioxidant assays. The highest correlation of TP was noted with FRAP ($r^2$=0.9973), followed by ABTS ($r^2$=0.9966), CUPRAC ($r^2$=0.9906), and DPPH ($r^2$=0.9572), whereas the lowest one (but still significant) was between TP and IRP ($r^2$=0.8928).

This demonstrates that the expected high total phenolic content is directly linked to the *in vitro* and most probably *in vivo* antioxidant activities. Since there are high values of $r^2$ (Table 2) between all the performed antioxidant tests covering almost the whole span of radical scavenging activities (stable and reactive radicals) and redox potentials (ferro/ferric and cuprous/cupric, fine tuned by ligation) and the total phenol and flavonoid contents, one can assume that no other compounds but those that are phenolic in nature are responsible for the activity. All the *in vitro* tests performed point to the methanol extract as the best (showing several fold higher activity than the other ones) in its radical scavenging ability, total content of phenols and flavonoids, and also having the highest yield of extractible matter per g of dry plant material.

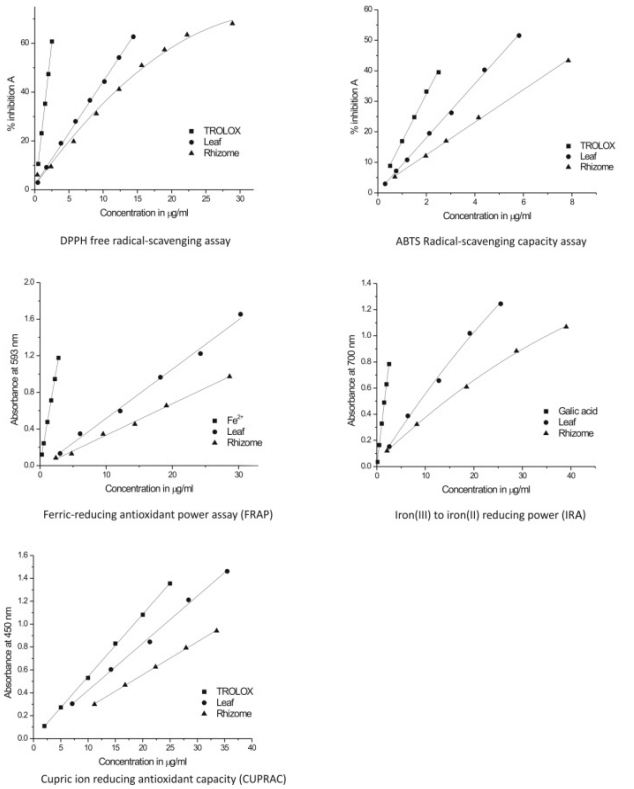



**Figure 1**: *Geranium macrorrhizum* methanol extracts antioxidant activity vs. concentration plots from five different *in vitro* antioxidant assays.

For these reasons, we decided to make detailed compositional analyses, as well as determine hepatoprotective and antimicrobial activities of the methanol extracts of the leaf and rhizome of *G. macrorrhizum*.

HPLC analysis of the leaf and rhizome methanol extracts revealed that they mostly consist of glycosidically bound phenolics, most probably possessing gallic and ferulic acid moieties (from UV spectra gathered during these analyses). In order to corroborate this, a portion of the extracts was acid hydrolyzed and analyzed (the chromatographic conditions were optimized for free phenolic acids determination). Both gallic and ferulic acids were identified by co-injection of the pure substances and quantified. Free gallic acid was also present in the rhizome extract, but only in minute amount (*c.a.* 3 mg per g dry weight, whereas the leaf extract was completely devoid of the free acid). This indicates that the determined total phenolics are for the most part bound.

In the present study, the hepatoprotective activity of *G. macrorrhizum* leaf and rhizome methanol extracts was assessed in a $CCl_4$-induced hepatotoxicity model in rats [17]. The administration of $CCl_4$ significantly increased the levels of AST, ALT, ALP, γ – GT, PCHE and TB in the rat serum when compared with the control group. The increase of enzyme activity is a characteristic of liver injury, especially the rise in ALT levels [18]. Although the serum levels of these enzymes and TB in the groups of animals that were treated with either of the methanol extracts and $CCl_4$ were significantly elevated when compared with the control group, these parameters were significantly decreased when compared with the $CCl_4$ group (Table 3, Figure 2). Increase in total serum bilirubin concentration after $CCl_4$ administration might be attributed to the failure of normal uptake, conjugation and excretion by the damaged hepatic parenchyma and is a very sensitive test to substantiate the

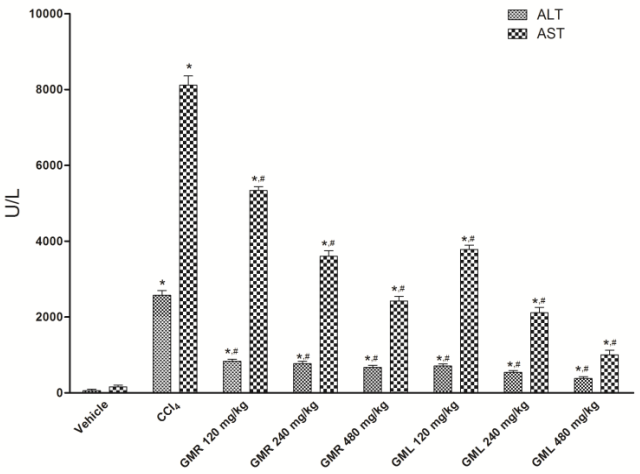



**Figure 2**: Effect of *G. macrorrhizum* rhizome (GMR) and leaf (GML) extracts on plasma ALT and AST levels. $^{*}p<0.001$ vs. Control; $^{\#}p<0.001$ vs. $CCl_4$.

functional integrity of the liver and severity of necrosis [19,20]. Thus, the observed dose-dependent normalization of bilirubin levels in the groups treated with both methanol extracts indicates that these extracts have the potential of restoring liver synthetic function (Table 3). These biochemical analysis results suggest a positive effect of the extracts on the reduction of liver damage.

The obtained histological results were in agreement with the measured serum parameters. Microscopic examination of the control group livers (Fig. 3A) showed a normal morphology with the central vein in the centre of the lobule. The liver cells are radially arranged to form sheets. The venous sinusoids converge upon the central vein. Administration of $CCl_4$ induced severe histopathological deformations of the liver. Normal architecture of the liver was completely lost with the appearance of centrilobular necrosis with tiny vacuoles, lymphocytes infiltration of the periportal area and around the central vein, also fatty changes and sinusoidal congestion were observed. Additionally, the histopathological examinations showed diffuse, numerous, ballooning degenerations. These ballooned hepatocytes were of different sizes and much larger than normal hepatocytes and occasionally appeared as confluent areas. Liver histology of the animals treated with $CCl_4$ + leaf or rhizome methanol extract (Figure 3C and 3D) shows a normalization of the hepatic cells, central vein and portal triad. The extract treatments decreased focal necrosis, vacuolation and reduced the lymphocytic infiltration in the liver and presented regenerative effects. This can be considered as an expression of the functional improvement of hepatocytes, which might be due to accelerated regeneration of parenchymal cells or limited damage in the presence of the two *G. macrorrhizum* extracts.

The trichloromethyl radical, which is generated in the presence of $CCl_4$, binds to tissue macromolecules and thus induces peroxidative degradation of membrane lipids and disturbs $Ca^{2+}$ homeostasis, which leads to hepatocellular injury and/or depletes antioxidant defenses. Thus, the antioxidant activity or the inhibition of free radical formation must be important in the protection against $CCl_4$-induced hepatopathy [21]. However, the reversal of increased serum enzyme levels in $CCl_4$-induced liver damage by these substances may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes [22].

**Table 3**: Effect of *G. macrorrhizum* rhizome and leaf extracts on plasma ALP, γ – GT, PCHE and TB levels.

| Groups | | Dose (mg/kg) | ALP (U/L) | γ-GT (U/dl) | PCHE (KU/dl) | TB (μmol/L) |
|---|---|---|---|---|---|---|
| Vehicle 10 mL/kg | | - | 101.0 ± 8.4 | 90.1 ± 4.2 | 24.3 ± 2.8 | 2.3 ± 0.4 |
| $CCl_4$[a] | | - | 446.4 ± 12.5[b] | 215.3 ± 10.7[b] | 72.1 ± 5.8[b] | 23.2 ± 1.2[b] |
| *G. macrorrhizum* | root extract | 120 | 305.3 ± 9.9[b,c] | 182.5 ± 9.3[b,c] | 54.0 ± 2.7[b,c] | 13.1 ± 0.9[b,c] |
| | | 240 | 256.7 ± 9.6[b,c] | 156.8 ± 7.4[b,c] | 39.5 ± 3.3[b,c] | 8.3 ± 0.7[b,c] |
| | | 480 | 201.2 ± 9.5[b,c] | 122.7 ± 8.6[b,c] | 35.5 ± 1.4[b,c] | 7.9 ± 0.6[b,c] |
| | leaf extract | 120 | 246.6 ± 9.2[b,c] | 148.4 ± 8.5[b,c] | 49.6 ± 3.7[b,c] | 10.5 ± 0.8[b,c] |
| | | 240 | 189.8 ± 5.6[b,c] | 131.7 ± 9.7[b,c] | 33.5 ± 4.1[b,c] | 8.1 ± 0.7[b,c] |
| | | 480 | 123.4 ± 8.1[b,c] | 102.5 ± 7.3[c] | 25.8 ± 2.1[c] | 6.2 ± 0.5[b,c] |

[a] 2.5 ml/kg of 50% $CCl_4$ solution in olive oil (v/v). [b] $p<0.001$ vs. Control. [c] $p<0.001$ vs. $CCl_4$

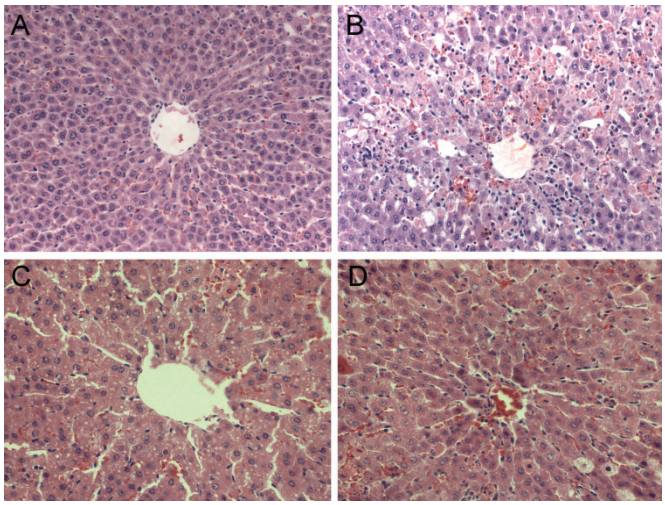



**Figure 3**: Effect of *G. macrorrhizum* rhizome and leaf extracts on the histological changes in the liver of the $CCl_4$-treated rats (HE×200). A: Control group: normal lobular architecture and cell structure; B: $CCl_4$-treated group: extensive hepatocellular damage with the presence of portal inflammation, centrizonal necrosis, and Kupffer cell hyperplasia; C: Liver section of rats treated with $CCl_4$ and 480 mg/kg of *G. macrorrhizum* leaf extract showing almost normal liver architecture. Regeneration of hepatocytes around the central vein; D: Liver section of rats treated with $CCl_4$ and 480 mg/kg of *G. macrorrhizum* rhizome extract showing: minimal inflammatory cellular infiltration, congestion of blood vessels.

The efficacy of any hepatoprotective drug is essentially dependent on its capability to either reduce the harmful effects or maintain the normal hepatic physiological mechanisms that have been altered by a hepatotoxin [23]. On the other hand, there is growing evidence that activation of the antioxidant system is required to attenuate the inflammatory response in several diseases, including $CCl_4$-induced hepatotoxicity [24]. Hepatotoxins, such as $CCl_4$ or endotoxin, rapidly induce proinflammatory cytokines, such as TNF-α and IL-1β by Kupffer cells, and recruit stromal cells of the liver (endothelial cells, stellate cells and hepatocytes) to participate in this inflammatory response via paracrine production of cytokines, as well as chemokines to attract circulating immune cells, further amplifying an inflammatory response [25].

The antimicrobial activity of the two methanol extracts (leaf and rhizome) was evaluated against seven bacterial and two fungal strains. The results obtained by a microdilution assay (Table 4) showed a significant inhibiting activity of the extracts, for which the MIC values ranged from 15.6 to 500 μg/mL (leaves) and from 15.6 to 5000 μg/mL (rhizomes). The leaf extract exhibited inhibitory activity against six bacterial and one fungal strain.

**Table 4**: Antimicrobial activity of *G. macrorrhizum* leaf and rhizome extracts (μg /mL).

| Bacterial/fungal strain | | ATCC | Leaf extract MIC | Leaf extract MBC | Rhizome extract MIC | Rhizome extract MBC | Positive control |
|---|---|---|---|---|---|---|---|
| $G^+$ | *Staphylococcus aureus* | 25923 | 15.6 | 62.5 | 620.5 | 1250 | 0.09[a] |
| | *Bacillus subtilis* | 6633 | 250 | >500 | 150.6 | 2500 | 0.39[a] |
| | *Listeria innocua* | 51742 | 500 | >500 | 5000 | 5000 | 0.39[a] |
| $G^-$ | *Proteus vulgaris* | 8427 | 500 | >500 | 620.5 | 5000 | 0.19[a] |
| | *Escherichia coli* | 8739 | >500 | >500 | 5000 | >5000 | 1.56[a] |
| | *Salmonella enteritidis* | 13076 | 125 | >500 | 310.2 | >5000 | 0.19[a] |
| | *Klebsiella pneumoniae* | 10031 | 500 | >500 | 620.5 | >5000 | 0.39[a] |
| *Fungi* | *Candida albicans* | 10231 | >500 | >500 | 310.2 | 1250 | 0.78[b] |
| | *Aspergillus niger* | 16404 | 500 | >500 | 2500 | >5000 | 6.25[b] |

[a] tetracycline, [b] nystatin

The most resistant bacterial strain, whose growth was not inhibited by the extracts in the tested concentration range, was the Gram-negative *Escherichia coli*.

Additionally, confirmation of this higher resistance of all tested Gram-negative strains can be found in the fact that all strains, except *Salmonella enteritidis*, were inhibited only by the extracts' highest tested concentration - 500 μg/mL. On the other hand, Gram-positive strains showed a higher sensitivity with *Staphylococcus aureus* being inhibited by only 15.6 μg/mL of the extracts (Table 4). Although having a good inhibitory effect against bacteria, a microbicidal one was not observed, even at the highest tested concentrations, except in the case of the most sensitive strain, *S. aureus*, with a minimum bactericidal concentration of 62.5 μg/mL. Antifungal activity of the leaf extract of *G. macrorrhizum* was only detected against *Aspergillus niger*, with an inhibitory effect displayed at 500 μg/mL. The yeast *Candida albicans* was not susceptible to this extract in the tested concentration range (Table 4).

The rhizome extract of *G. macrorrhizum*, with ten times higher MIC values, showed lower activity than the one obtained from the leaves extract. This extract also showed an inhibitory effect against all the tested strains, whereas microbicidal activity was exhibited against five strains. In accordance with the results for the leaf extract, the more resistant strains belonged to the Gram-negative bacteria. The results for the rhizome extract indicated the highest sensitivity of the Gram-positive *Bacillus subtilis*, while the most resistant one was, once again, *E. coli*. The rhizome extract showed an antifungal effect against both tested fungal strains, but this time the yeast *C. albicans* showed a much higher susceptibility than the tested mold, *A. niger*.

Generally speaking, the obtained activity of the two tested extracts isolated from *G. macrorrhizum* is very significant bearing in mind that in most cases, essential oils are the main carriers of the plant's antimicrobial activity, while extracts usually possess lower activity. The present leaf extract and essential oil [1] of the aerial parts showed very similar active concentrations, except in the case of *B. subtilis*. Since both studies utilized four strains with the same ATCC number (*S. aureus, E. coli, B. subtilis* and *C. albicans*), closer comparison of these results is discussed herein. The extracts from the present study showed higher activity against *C. albicans* (rhizome extract in comparison with the rhizome essential oil) and *S. aureus* (both extracts in comparison with the oil from the same plant parts). On the other hand, activity of the essential oil was slightly higher in the case of *E. coli* and much higher in the case of *B. subtilis*, as mentioned above. In total, we must highlight very similar active ranges of concentrations of the extracts in comparison with the essential oils isolated from the same part of *G. macrorrhizum*.

The *in vitro* analyses performed in this work included a wide variety of antioxidant activity methods: TP, TF, DPPH, ABTS, IRA, FRAP

and CUPRAC. The antioxidant activities of *G. macrorrhizum* extracts presented here were significantly higher than those determined by other authors [5]. Whether this is a consequence of a different extraction method or climatic conditions favoring *G. macrorrhizum* development is a question that needs further consideration. This study showed that among the methanol, ethanol, acetone and ethyl acetate extracts of *G. macrorrhizum*, the methanol one of both the leaves and rhizomes demonstrated the highest antioxidant activities. The antioxidant potency in all performed assays decreased in the order of decreasing solvent polarity: methanol > ethanol > acetone > ethyl acetate. Overall, the methanol extracts of *G. macrorrhizum* were the most potent in scavenging the DPPH and ABTS radicals, as well as in reducing ferric and cupric ions and were for that reason chosen for the *in vivo* test (hepatoprotective activity) and antimicrobial assays. The last two can be connected to the ethnopharmacological uses of this medicinal plant. The presence of high levels of phenolic compounds in the methanol extracts significantly contributed to the observed antioxidant activities and dose dependent hepatoprotective effect of the extracts in the $CCl_4$-induced liver damage model. There are at least three possible mechanisms of action that can be envisaged: 1) the radical scavenging effect (the formed $CCl_3^{\cdot}$ radicals are quenched by the phenolic groups of *G. macrorrhizum* constituents), 2) inhibitory action of the extract constituents on the enzymes producing the before mentioned radical from $CCl_4$, and 3) cell membrane strengthening effect and the prevention of cell lysis and leakage of cellular content that can induce further liver damage. These *modi operandi* have all been established for a compound named geranin isolated from a Japanese *Geranium* species (*G. thunbergii*) and may well be operational here, but need confirmation [10,11]. All of the above provides a justification for the popular use of this plant species in folk medicine (the stimulative properties and positive general health effects) and especially for the liver restoring action. One more usage of this plant species is in wound healing, and besides the known astringent effect, we have demonstrated that it also possesses antimicrobial activity against human pathogens that is likewise beneficial in this respect. In addition to the activity of the polar (methanol) extracts of *G. macrorrhizum* with a high phenol content, the essential oil of this species was also found to be active against a number of bacterial and fungal strains [1]. This gives another argument for the total antibacterial and antifungal effect observed for this plant species extracts.

## Experimental

***General:*** Extractions were made using an ultrasonic bath (Bandelin SONOREX® Digital 10 P, Sigma, USA). UV measurements were obtained on an Agilent 8453 (Agilent Technologies, USA) spectrophotometer. An Agilent Technologies 1200 HPLC system (Agilent Technologies, USA) equipped with a quaternary pump, autosampler and diode array detector was used for the identification and quantification of phenolic acids and flavonoids. All chromatographic separations were performed on an analytical column ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 µm, Agilent Technologies, USA). A gradient of acetonitrile (A) and deionized water containing 0.1%, v/v, of formic acid (B) mixtures of decreasing polarity were used for elution: 0-15 min 10% A, 15-35 min 10-30% A. The flow rate was set at 0.8 mL/min, column temperature at 30°C, and 20 µL of the above prepared solutions was injected. Data were acquired in the spectral range of 200-600 nm and integrated at 260, 280 and 320 nm.

***Plant material:*** Aerial and underground parts of *G. macrorrhizum* L. (Geraniaceae) were collected from the mountain range Svrljiške planine, in the vicinity of the city of Svrljig, south-eastern Serbia.

***Preparation of the extracts for* in vivo *experiments and microbiological assays:*** Two batches of 100 g of the aerial and underground parts were exhaustively extracted with methanol to afford *c.a.* 5.0 g and 4.5 g of extractive, respectively, after evaporation of the solvent in a gentle stream of nitrogen. The resulting oily residues were dissolved in physiological saline at a concentration of 300 mg/mL for evaluation of the hepatoprotective effect, and another portion was dissolved in dimethylsulfoxide to give a solution containing 100 mg/mL of extractible matter for the *in vitro* microdilution microbiological tests.

***Phytochemical and* in vitro *antioxidant studies:*** The total phenolic content of the extracts was determined using Folin-Ciocalteau assay [26a], whereas the total flavonoid content was determined by a colorimetric method [26b]. The antioxidant capacity was studied through the evaluation of the extracts' free radical-scavenging effect on DPPH [26b] and ABTS [26c] radicals. Iron(III) to iron(II) reduction assay [26d], ferric-reducing antioxidant power assay [26e] and cupric ion reducing antioxidant capacity [26f] were also performed.

***Evaluation of the hepatoprotective effect:*** Male Wistar rats weighing 200–250 g, obtained from our own animal facilities, were housed in large spacious cages at 27±2°C with 12 h light and dark cycle throughout the period of the experiment. The animals were provided with a standard laboratory diet and given tap water *ad libitum*. On the day of the experiment, the animals were divided into 8 experimental groups consisting of 6 animals each. Two groups served as the positive and negative controls and received $CCl_4$ (2.5 mL/kg of 50%, v/v, $CCl_4$ solution in olive oil) and olive oil (10 mL/kg) by intraperitoneal injection (*i.p.*), respectively. Test groups of rats, 3 groups per extract, were *i.p.* treated with *G. macrorrhizum* extracts (leaf and rhizome) prepared as previously mentioned in doses of 120, 240 and 480 mg/kg (body weight). Sixty min after the application of the 2 extracts all animals received 2.5 mL/kg of the 50% $CCl_4$ solution in olive oil. Twenty-four h after the toxin administration, blood samples were withdrawn by cardiac puncture and rats sacrificed with an overdose of ketamine (Ketamidor 10%, Richter Pharma AG, Wels, Austria). Additionally, the liver of each rat was promptly removed and small sections were collected for histological analysis. All experimental procedures with animals were conducted in compliance with The European Council Directive of November 24$^{th}$, 1986 (86/609/EEC) and approved by the local Ethics Committee (number 01-2857-3) given on 27$^{th}$ April, 2012.

***Biochemical measurements:*** The blood was centrifuged at 1500 rpm at 4°C for 15 min to obtain the serum. Aspartate transferase (AST), alanine transaminase (ALT), serum alkaline phosphatase (ALP), γ-glutamate transpeptidase (γ-GT), pseudocholineesterase (PCHE) and total bilirubin (TB) activities were assayed using an Olympus AU680® Chemistry-Immuno Analyzer (Olympus America Inc, USA).

***Histopathological observation:*** The liver tissue specimens separated for histopathological examination were fixed in buffered formaldehyde solution (10%, w/w). The fixed tissue was then dehydrated with different aqueous ethanol solutions from 50–100%, v/v, embedded in paraffin, cut into 4–5 µm thick sections, stained with hematoxylin and eosin, and examined with an Olympus BH2 light microscope (Olympus America INC, USA).

***Evaluation of antimicrobial activity:*** *G. macrorrhizum* extracts (leaf and rhizome) were tested against a panel of microorganisms, see Table 4.

***Microdilution antimicrobial assay*:** Antimicrobial activity was evaluated using a broth microdilution method [1]. Stock solutions of the extracts were made in DMSO (100%) and then serially diluted (the diluting factor 2). The final concentrations of *G. macrorrhizum* extracts (rhizome and leaf) in the media were in the ranges 2.00-5000 μg/mL and 0.200-500 μg/mL, respectively. All experiments were conducted in triplicate and repeated twice.

***Statistical analysis*:** Results were expressed as the mean ± standard deviation. Statistically significant differences were determined by one-way analysis of variance (ANOVA) followed by Tukey´s post hoc test for multiple comparison (Graph pad Prism version 5.03, San Diego, CA, USA). Probability values ($p$) less than 0.05 were considered to be statistically significant.

**Acknowledgments** - This research has been supported by the Ministry of Education, Science and Technological Development of Serbia (Project 172061).

## References

[1] Radulović N, Dekić M, Stojanović-Radić Z, Zoranić S. (**2010**) *Geranium macrorrhizum* L. (Geraniaceae) essential oil: a potent agent against *Bacillus subtilis*. *Chemistry & Biodiversity*, ***7***, 2783–2800.
[2] Genova E, Ivancheva S. (**1995**) Quantitative analysis of tannin content in *Geranium macrorrhizum* L. (*Geraniaceae*) growing in Bulgaria. *Phytologia Balcanica*, ***1***, 93–99.
[3] Serkedjieva J, Hay AJ. (**1998**) *In vitro* antiinfluenza virus activity of a plant preparation from *Geranium sanguineum* L. *Antiviral Research*, ***37***, 221–230.
[4] Lamaison JL, Petitjean-Freytet C, Carnat A. (**1993**) Teneurs en polyphenols et activities antioxydantes chez les Geraniaceae françaises. *Plantes Medicinales et Phytotherapie*, ***26***, 130–134.
[5] Miliauskas G, Van Beek T, Venskutonis P, Linssen J, Waard P. (**2004**) Antioxidative activity of *Geranium macrorrhizum*. *European Food Research and Technology*, ***218***, 253–261.
[6] Janković MM. (**1973**) *Flora of Serbia*. Srpska akademija nauke i umetnosti, Belgrade.
[7] Tucakov J. (**1990**) *Phytotherapy*. Rad, Belgrade.
[8] Menković N, Savikin K, Tasić S, Zdunić G, Stesević D, Milosavljević S, Vincek D. (**2011**) Ethnobotanical study on traditional uses of wild medicinal plants in Prokletije Mountains (Montenegro). *Journal of Ethnopharmacology*, ***133***, 97–107.
[9] Jarić, S, Popović Z, Mačukanović-Jocić M., Djurdjević L, Mijatović M, Karadžić B, Mitrović M, Pavlović P. (**2007**) An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *Journal of Ethnopharmacology*, ***111***, 160–175.
[10] Nakanishi Y, Orita M, Okuda T, Abe H. (**1998**) Effects of geraniin on the liver in rats I - effects of geraniin compared to ellagic acid, and gallic acid on hepatic injuries induced by $CCl_4$, D-galactosamine, and thioacetamide. *Natural Medicines*, ***52***, 396–403.
[11] Nakanishi Y, Okuda T, Abe H. (**1999**) Effects of geraniin on the liver in rats III - correlation between lipid accumulations and liver damage in $CCl_4$-treated rats. *Natural Medicines*, ***53*,** 22–26.
[12] Miliauskas G, Mulder E, Linssen J, Houben J, Van Beek T, Venskutonis P. (**2007**) Evaluation of antioxidative properties of *Geranium macrorrhizum* and *Potentilla fruticosa* extracts in Dutch style fermented sausages. *Meat Science*, ***77***, 703–708.
[13] Radulović N, Dekić M, Stojanović-Radić Z, Palić R. (**2011**) Chemical composition and antimicrobial activity of the essential oils of *Geranium columbinum* L. and *G. lucidum* L. (Geraniaceae). *Turkish Journal of Chemistry*, ***35***, 499–512.
[14] Radulović N, Dekić M., Stojanović-Radić Z. (**2012**) Chemical composition and antimicrobial activity of the volatile oils of *Geranium sanguineum* L. and *G. robertianum* L. (Geraniaceae). *Medicinal Chemistry Research*, ***21***, 601–615.
[15] Miliauskas G, Venskutonis P, Van Beek T. (**2004**) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, ***85***, 231–237.
[16] Sacan O, Yanardag R. (**2010**) Antioxidant and antiacetylcholinesterase activities of chard (*Beta vulgaris* L. var. *cicla*). *Food and Chemical Toxicology*, ***48***, 1275–1280.
[17] Rao M, Rao C, Pushpangadan P, Shirwaikar A. (**2006**) Hepatoprotective effects of rubiadin, a major constituent of *Rubia cordifolia* Linn. *Journal of Ethnopharmacology*, ***103***, 484–490.
[18] Lin J, Wang C. (**1986**) Protection of crocin dyes in the acute hepatic damage induced by aflatoxin B and dimethylnitrosamine in rats. *Carcinogenis*, ***7***, 595–599.
[19] Rajesh M, Latha M. (**2001**) Hepatoprotection by *Elephantopus scaber* Linn. in $CCl_4$-induced liver injury. *Indian Journal of Physiology and Pharmacology*, ***45***, 481–486.
[20] Klaassen C, Watkins J. (**1984**) Mechanisms of bile formation, hepatic uptake, and biliary excretion. *Pharmacological Reviews*, ***36***, 1–67.
[21] Castro J, Ferrya G, Castro C, Sasame H, Fenos O, Gillete J. (**1974**) Prevention of carbon tetrachloride induced necrosis by inhibitors of drug metabolism. Further studies on the mechanism of their action. *Biochemical Pharmacology*, ***23***, 295–302.
[22] Thabrew M, Joice P. (**1987**) A comparative study of the efficacy of *Pavetta indica* and *Osbeckia octanda* in the treatment of liver dysfunction. *Planta Medica*, ***53***, 239–241.
[23] Raghavendran B, Sathivel A, Devaki T. (**2003)** Hepatoprotective nature of seaweed alcoholic extract on acetaminophen induced oxidative stress. *Journal of Health Science*, ***50***, 42–46.
[24] Campo G, Avenoso A, Campo S, Nastasi G, Traina P, D'Ascola A, Rugolo C, Calatroni A. (**2008**) The antioxidant activity of chondroitin-4-sulphate, in carbon tetrachloride-induced acute hepatitis in mice, involves NF-kappaB and caspase activation. *British Journal of Pharmacology*, ***155***, 945–956.
[25] Simpson K, Lukacs N, Colletti L, Strieter R, Kunkel S. (**1997**) Cytokines and the liver. *Journal of Hepatology*, ***27***, 1120–1132.
[26] (a) Mitic M, Obradovic M, Kostic D, Naskovic D, Micic R. (**2011**) Phenolics content and antioxidant capacity of commercial red fruit juices, *Hemijska industrija*, ***5***, 611–619; (b) Mitic M, Obradovic M, Kostic D, Micic R, Paunovic D. (**2011**) Phenolic profile and antioxidant capacities of dried red currant from Serbia, extracted with different solvent. *Food Science and Biotechnology*, ***6***, 1625–1631; (c) Zhou S, Fang Z, Lü Y, Chen Y, Liu D, Ye XI. (**2009**) Phenolics and antioxidant properties of bayberry (*Myrica rubra* Sieb. et Zucc.) pomace. *Food Chemistry*, ***112***, 394–399; (d) Dorman H, Peltoketo A, Hiltunen R, Tikkanen M. (**2003**) Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Laminaceae herbs. *Food Chemistry*, ***83***, 255–262; (e) Maksimovic Z. (**2008**) *In vitro* antioxidant activity of ragweed (*Ambrosia artemisiifolia* L., Asteraceae) herb. *Industrial Crops and Products*, ***28***, 356–360; (f) Yıldız L, Baskan K, Tutem E, Apak R. (**2008**) Combined HPLC-CUPRAC (cupric ion reducing antioxidant capacity) assay of parsley, celery leaves and nettle. *Talanta*, ***77***, 304–313.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1615 - 1618

# Differential Expression of Benzophenone Synthase and Chalcone Synthase in *Hypericum sampsonii*

**Lili Huang[a,d], Hong Wang[d], Hechun Ye[a], Zhigao Du[a], Yansheng Zhang[c,*], Ludger Beerhues[b] and Benye Liu[a,b,*]**

[a]*Key Laboratory of Plant Molecular Physiology*, *Institute of Botany*, *The Chinese Academy of Sciences*, *Beijing 100093*, *China*

[b]*Institute of Pharmaceutical Biology*, *Technische Universität Braunschweig*, *Braunschweig 38106*, *Germany*

[c]*CAS Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture*, *Wuhan Botanical Garden*, *The Chinese Academy of Science*, *Wuhan 430074*, *China*

[d]*Graduate University of the Chinese Academy of Sciences*, *19A Yuquanlu*, *Beijing 100049*, *China*

*benyel@ibcas.ac.cn; zhangys@wbgcas.cn*

**Received: September 26th, 2012; Accepted: October 16th, 2012**

cDNAs encoding *Hypericum sampsonii* benzophenone synthase (HsBPS) and chalcone synthase (HsCHS) were isolated and functionally characterized. Differential expressions of *HsBPS* and *HsCHS* were monitored using quantitative polymerase chain reaction (PCR). In the vegetative stage, *HsBPS* was highly expressed in the roots; its transcript level was approx. 100 times higher than that of *HsCHS*. Relatively high transcript amounts of *HsBPS* were also detected in older leaves, whereas the youngest leaves contained higher transcript amounts of *HsCHS*. In the reproductive stage, maximum *HsCHS* expression was detected in flowers, the transcript level being approx. 5 times higher than that of *HsBPS*. The inversed situation with a 10-fold difference in the expression levels was observed with fruits. High transcript amounts for both proteins were found in roots.

**Keywords:** Benzophenone synthase, Chalcone synthase, Gene expression, *Hypericum sampsonii*, Quantitative PCR, Sampsoniones.

*Hypericum sampsonii* Hance is used as a traditional Chinese herbal medicine in the treatment of numerous disorders such as hematemesis, epistaxis, menstrual irregularity, external traumatic injury, snakebite and swellings [1]. In recent years, the plant has aroused further scientific interest for its anticancer activity [2]. Extensive phytochemical studies demonstrated that *H. sampsonii* contains an array of sampsoniones (A-Q), which are polyprenylated benzophenones characterized by complex caged tetracyclic skeletons (Figure 1). These compounds possess profound cytotoxic activity [3]. Despite their pharmaceutical importance, biosynthesis of these benzophenone derivatives remains poorly understood. The nucleus of sampsoniones is benzophenone, which is biosynthesized by benzophenone synthase (BPS; EC 2.3.1.151), a new member of the type III polyketide synthase (PKS) [4]. The first published *BPS* (*HaBPS*) cDNA was from elicitor-treated *Hypericum androsaemum* cell cultures, which served as a model system for studying benzophenone metabolism [5]. Recently, the second BPS cDNA was cloned and characterized from *Garcinia mangostana* [6]. However, no information about expression of *BPS in planta* is so far available. Here we have cloned and functionally characterized a cDNA encoding *H. sampsonii* benzophenone synthase (HsBPS). The organ-specific expression pattern of *HsBPS* was analyzed by quantitative polymerase chain reaction (PCR). For comparison, the same studies were carried out for chalcone synthase (*HsCHS*), which is a well studied ubiquitous type III PKS in all higher plants.

The cDNAs for *HsBPS* and *HsCHS* were cloned by the homology-based cloning strategy. The ORF of the *HsBPS* cDNA was 1188 bp long and encoded a 42.7 kDa protein (395 amino acids) with a calculated p*I* of 5.91. The *HsCHS* cDNA contained a 1173 bp ORF encoding a 42.7 kDa protein (390 amino acids) with a calculated p*I* of 6.55. *HsBPS* and *HsCHS* shared around 57.0% identity at the nucleotide as well as at the amino acid sequence level. HsBPS and HsCHS were expressed as *N*-terminally $His_6$-tagged proteins in *Escherichia coli* and purified by affinity chromatography. Protein bands of approximately 43 kDa each were observed after SDS-PAGE (Figure 2).

**Table 1**: Substrate specificities of recombinant HsBPS and HsCHS [a]

| Substrate | Enzyme activity (% of max) | |
|---|---|---|
| | HaBPS | HaCHS |
| Benzoyl-CoA | 100 | 11 |
| 2-Hydroxybenzoyl-CoA | 5 | 0 |
| 3-Hydroxybenzoyl-CoA | 66 | 0 |
| 4-Hydroxybenzoyl-CoA | 0 | 0 |
| Cinnamoyl-CoA | 0 | 65 |
| 2-Coumaroyl-CoA | 0 | 0 |
| 3-Coumaroyl-CoA | 0 | 0 |
| 4-Coumaroyl-CoA | 0 | 100 |
| Acetyl-CoA | 0 | 0 |

[a] Data are means of two independent experiments.

HsBPS preferred benzoyl-CoA as a starter substrate (Table 1). The enzymatic product was identified as 2,4,6-trihydroxybenzophenone by liquid chromatography-UV spectroscopy (LC-UV) and liquid chromatography-mass spectrometry (LC-MS) in comparison with a sample of authentic reference compound. The pH and temperature optima were 6.5-7.0 and 40°C, respectively. There were also traces of a side product, 6-phenyl-4-hydroxy-2-pyrone. Beside benzoyl-CoA, HsBPS also accepted 3-hydroxybenzoyl-CoA as starter substrate to form 2,3',4,6-tetrahydroxybenzophenone and a small amount of 6-(3'-hydroxyphenyl)-4-hydroxy-2-pyrone as a derailment product. 4-Hydroxybenzoyl-CoA, acetyl-CoA, and CoA esters of cinnamic acids were not accepted by HsBPS as starter molecules (Table 1).

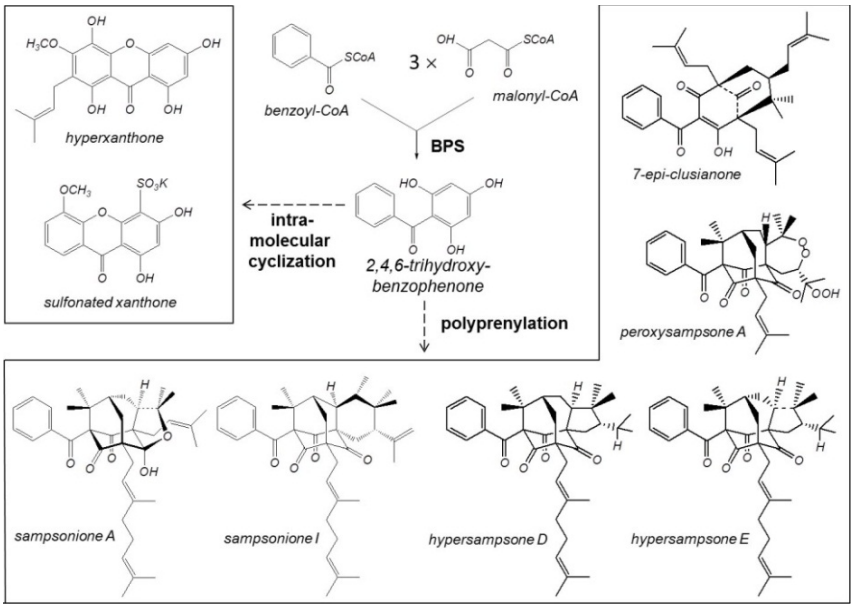



**Figure 1**: Examples and biosynthetic origin of polyprenylated polycyclic benzophenone derivatives isolated from *Hypericum sampsonii*. BPS, benzophenone synthase.

**Table 2**: Steady-state kinetic parameters of HsBPS and HsCHS[a]

| | Benzoyl-CoA | | | 4-Coumaroyl-CoA | | | Malonyl-CoA |
|---|---|---|---|---|---|---|---|
| | $k_{cat}$ ($min^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) | $k_{cat}$ ($min^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) | $K_m$ (μM) |
| *HsBPS* | 23.7 | 15.8 | 25028 | - | - | - | 92.5 |
| *HsCHS* | - | - | - | 1.4 | 5.4 | 4365 | 12.7 |

[a] Data are means of two independent experiments

The $k_{cat}$ and $K_m$ values of HsBPS were about seven times and twice, respectively, higher than those of HaBPS as *N*-terminally $His_6$-tagged protein [7]. The catalytic efficiency ($k_{cat}/K_m$) of HsBPS was around 4 times higher than that of HaBPS and recently published BPS from *Garcinia mangostana* [6]. The substrate preference of HsBPS for benzoyl-CoA well correlates with the observation that *H. sampsonii* contains an array of polyprenylated benzophenone derivatives that share an unsubstituted benzoyl moiety (Figure 1).

HsCHS exhibited highest activity with 4-coumaroyl-CoA (Table 1). The enzymatic product was naringenin, as identified by LC-UV and LC-MS in comparison with a sample of authentic reference compound. Cinnamoyl-CoA was also a starter substrate for HsCHS and the product formed was pinocembrin. The optimum pH and temperature values for HsCHS were 7.0 and 40°C, respectively. Side products were not detected. HsCHS also accepted benzoyl-CoA as a starter molecule with around 10% relative activity, and produced, like HsBPS, 2,4,6-trihydroxybenzophenone (Table 1). The kinetic data of HsCHS are indicated in Table 2.

The expression patterns of *HsBPS* and *HsCHS* were analyzed in various organs harvested at both the vegetative and reproductive stages. Quantitative PCR was used to monitor the transcript levels of *HsBPS* and *HsCHS* (Figure 3). *HsBPS* and *HsCHS* were differentially regulated. In the vegetative stage (Figure 3a), the expression level of *HsBPS* in roots was approx. 100 times higher than that of *HsCHS*. In stems, both transcript levels were low, but similar. In leaves, expression of both genes was developmentally regulated. The *HsCHS* transcript level was high in the youngest leaves (L1) and decreased upon aging. In contrast, the *HsBPS* expression level was low in the youngest leaves, but significantly increased in older leaves (L3 and L4). The *HsBPS* expression level in leaves was, however, only around one third of that in roots.

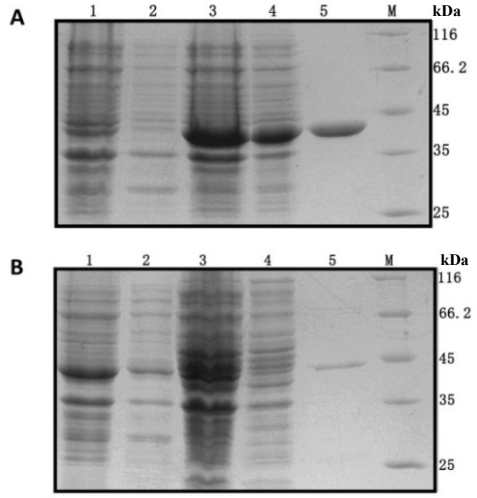



**Figure 2**: SDS-PAGE analysis of recombinant HsBPS (**A**) and HsCHS (**B**) after staining with coomassie brilliant blue. Lane 1, total protein isolated from *Escherichia coli* strain BL21 (DE3) harboring the corresponding plasmid prior to IPTG treatment; Lane 2, soluble proteins from *E. coli* strain BL21(DE3) harboring the corresponding plasmid prior to IPTG treatment; Lane 3, total protein from BL21 (DE3) cells harboring the corresponding plasmid after induction with IPTG for 8 h at 28°C. Lane 4, soluble proteins from BL21 (DE3) cells harboring the corresponding plasmid after induction with IPTG for 8 h at 28°C; Lane 5, recombinant HsBPS or HsCHS protein after $Ni^{2+}$-chelating chromatography; M, molecular mass markers with masses indicated in kDa.

In the reproductive stage (Figure 3b), the expression level of *HsBPS* in roots was comparable with that in the vegetative stage. However, the expression level of *HsCHS* was markedly increased and reached almost that of *HsBPS*. Highest expression of *HsCHS* was observed in flowers, where the transcript level was approx. five times higher than that of *HsBPS*. The inverse situation was found in fruits, where expression of *HsBPS* was approx. ten times higher than that of *HsCHS*. The transcript amounts for both genes were relatively low in stems and leaves.

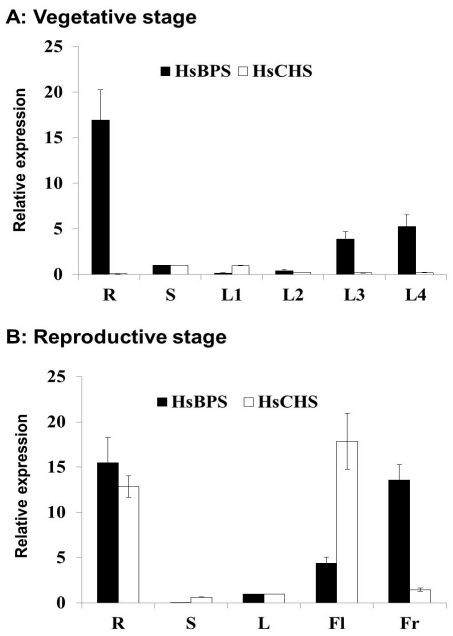



**Figure 3**: Quantitative PCR analysis of the transcript levels of *HsBPS* and *HsCHS* in various organs of *H. sampsonii* at the vegetative stage (**A**) and the reproductive stage (**B**). Primers specific for *HsBPS* and *HsCHS* were used to measure the transcript levels of the two genes. The *H. sampsonii* 18s rRNA gene (AY573011.1) was used as an internal standard. Relative quantification used the stem values in the vegetative stage and the leaf values in the reproductive stage as calibrators, which were set to a nominal value of 1. Error bars represent the standard deviations (SD, n=3). R, roots; S, stems; L, the first leaf pair under the inflorescence; L1-4, the first, second, third and fourth leaf pairs from the shoot tip in the vegetative stage; Fl, flowers; Fr, fruits.

In most organs, the expression level of *HsBPS* was higher than that of *HsCHS*, which is consistent with the finding that polyprenylated benzophenones and xanthones are typical and dominant secondary products of Hypericaceae plants, whereas the concentration of flavonoids in these plants is lower [3, 8]. *HsBPS* was most highly expressed in the roots at both the vegetative and reproductive stages, while its expression in the upper parts of the plant was lower. This expression pattern well correlated with the distribution of polyprenylated benzophenones in the plant. The concentration of prenylated benzophenones in the roots is much higher than that in the aerial parts. For example, the 7-*epi*-clusianone content in the roots of *H. sampsonii* is about 0.1% of dry weight, which is around 100 times higher than the maximum concentration of sampsoniones in the aerial parts [3]. Another reason for the high *HsBPS* expression level in roots may be that xanthones also appear to mainly accumulate in the underground organs of many *Hypericum* species [9]. The *HsBPS* expression results suggested that roots of *H. sampsonii* may be the better parts to be used as a herbal remedy because of the high amounts of benzophenone derivatives.

The differential expression pattern of *HsBPS* and *HsCHS* in *H. sampsonii* is interesting and should be related to the function of the corresponding end products, benzophenones and flavonoids, respectively. It has been proved that flavonoids serve as flower pigments, UV protectants and signal molecules in plants [10]. The expression pattern of *HsCHS* can be well explained by these functions, especially, the high transcript level of *HsCHS* in flowers is likely to be related to pigmentation for insect attraction. Constituents that are widely distributed in species of Hypericaceae are polyprenylated benzophenones and xanthones and their antimicrobial properties suggest that they may also have a defensive role against pathogens. In *H. calycinum*, polyprenylated acyl and benzoyl phloroglucinols made up to approx. 20% of the dry weight of anthers and ovarian walls and were presumed to protect the developing seeds against herbivores and microorganisms [11]. In *H. perforatum* cell cultures, *Agrobacterium tumefaciens* as a biotic stress factor activated xanthone biosynthesis, which resulted in a rapid up-regulation of *BPS* expression and a 12-fold increase in the total xanthone concentration [12]. These compounds were supposed to act as both phytoalexins and antioxidants to help the host prevent disease development. It is reasonable to assume a similar function of benzophenone derivatives in *H. sampsonii*. The differential expression patterns of *HsBPS* and *HsCHS* might indicate that benzophenone derivatives serve in a complementary way, in addition to flavonoids, to protect the plant from biotic stress.

The results of this work provided the first insight into the *in planta* expression pattern of *HaBPS*, which was clearly different from that of *HaCHS* in *H. sampsonii*. These data are the solid basis for further deep investigations of the biosynthesis of these polyprenylated benzophenone derivatives.

## Experimental

***Chemicals:*** All the CoA esters of cinnamic acids and benzoic acids, apart from benzoyl-CoA, were synthesized as described in the literature [13]. Malonyl-CoA, acetyl-CoA, benzoyl-CoA and naringenin were purchased from Sigma-Aldrich (St Louis, MO, USA), 2,4,6-trihydroxybenzophenone from ICN (Meckenheim, Germany), and 6-phenyl-4-hydroxy-*2H*-pyran-2-one (benzoyl-diacetic acid lactone) from Aurora Fine Chemicals (Graz, Austria).

***Plant materials:*** *Hypericum sampsonii* Hance (Hypericaceae) seeds were kindly provided by Professor Hongfei Lv (Zhejiang Normal University, Jinhua, China). A voucher specimen of this plant (No. 01262860) was deposited in the Herbarium of the Institute of Botany, the Chinese Academy of Sciences, Beijing, China. The seeds were germinated in petri dishes covered with filter paper and grown at 25°C with a photoperiod of 16/8 h (light/dark). Three-week-old seedlings were transferred to 1.5-L pots filled with a mixture of mineral soil and humus in a greenhouse with the same temperature and photoperiod. When plants were 6-8 weeks old (vegetative stage), roots, stems, and leaves were harvested. The leaves were divided into unopened leaf pair (L1), the second leaf pair (L2), the third leaf pair (L3) and the fourth leaf pair (L4). In the reproductive stage, leaf (L) was the first leaf pair under the florescence, the fully opened flower heads (Fl) and green fruits (Fr) were harvested. All the samples were divided into 0.2 g-samples and immediately frozen in liquid nitrogen and stored at -80°C.

***Cloning of HsBPS and HsCHS cDNAs:*** Total RNA was isolated from roots and leaves of *H. sampsonii* using the Universal Plant Total RNA Extraction Kit (Bioteke, Beijing, China). Reverse transcription was carried out at 42°C with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), and the oligo (dT)-adaptor primer RRP (primer sequences and PCR conditions are provided in supplementary data). Gene specific primers were designed from the 5' untranslated region (5' UTR) of *HaBPS* (Pr-1) and *HaCHS* (Pr-2) of *H. androsaemum* [5]. For *HsCHS*, the full-length cDNA sequence was amplified using primer pair Pr-2 and the adaptor of RRP and the reverse-transcription product of leaf mRNA as template. For *HsBPS*, we were not able to amplify the full-length *HsBPS* with primer pair Pr-1 and the adaptor of RRP, but the core cDNA fragment was amplified by PCR using primers Pr-F and Pr-R derived from the ORF of *HaBPS* [5]. Full-length cDNA was obtained by rapid amplification of cDNA ends (RACE) using the protocol of the SMARTer$^{TM}$ RACE cDNA Amplification kit

(Clontech, Beijing, China). The PCR product was purified, cloned into pMD18-T vector (Takara) and sequenced by a commercial sequencing company. Sequence data of this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *HsBPS*, JQ670939; *HsCHS*, JQ670940.

***Heterologous expression and enzyme purification:*** The ORFs encoding HsBPS and HsCHS were reamplified by PCR using Pfu DNA polymerase. The amplified DNA was cloned to pRSET B (Invitrogen, Carlsbad, CA, USA) with *Nhe* I/*Kpn* I site. After sequencing of the ORFs on both strands, the recombinant plasmids were introduced into *E. coli* BL21-CodonPlus (DE3)-RIL (Stratagene, Amsterdam, Netherlands) for overexpression as *N*-terminally $His_6$-tagged proteins. HsBPS and HsCHS were purified to homogeneity using Ni-NTA agarose according to the manufacturer's instructions (Qiagen, Hilden, Germany). The purification efficiency was monitored by SDS-PAGE (Figure 2).

***Enzyme assay and kinetic data determiation:*** The standard assay (250 µL) contained 54 µM starter CoA, 324 µM malonyl-CoA, 0.1 M potassium phosphate (pH 7.0) and 2 µg protein. After incubation at 30°C for 10 min, the enzymatic products were extracted and detected as described previously [5]. Kinetic constants were determined using 8 substrate concentrations covering the range of 0.2-12 $K_m$, the concentration at which the second substrate was saturated. The amount of enzyme added to the assay was 2 µg. The incubation time was limited to 5 min for HsBPS and 10 min for HsCHS at the optimum reaction conditions.

***Quantitative polymerase chain reaction:*** Total RNA was isolated from 100 mg of the above mentioned materials using Plant Total RNA Isolation Kit (BioTeck) according to the manufacturer's instructions. All of the total RNA preparations were treated with DNase I and reverse-transcribed using the M-MLV Reverse Transcriptase (Invitrogen) and the oligo d(T) primers. The quantitative PCRs were performed with the Applied Biosystems StepOne Real-time PCR System using FastStart Universal SYBR Green Master (Roche, Mannheim, Germany) in 3 technical replicates (primer sequences and PCR conditions are provided in supplementary data). Relative quantification used the stem value in the vegetative stages and the leaf value in the reproductive stages as calibrators, which were set to a nominal value of 1.

**Acknowledgments** - The work was financially supported by the Starting Funding of Talent Recruitment of Institute of Botany, The Chinese Academy of Sciences (1103000114), the Grant for One Hundred Talents Program of the Chinese Academy of Sciences, China (Y129441R01) and National Sciences Foundation of China (30870217).

## References

[1] Jiangsu New Medical College College. (**1997**) *Dictionary of Chinese Crude Drugs*. Shanghai Scientific Technological Publishers. 345-346.

[2] Zeng JZ, Sun DF, Wang L, Cao XH, Qi JB, Yang T, Hu CQ, Liu W, Zhang XK. (**2006**) *Hypericum sampsonii* induces apoptosis and nuclear export of retinoid X receptor-alpha. *Carcinogenesis*, ***27***, 1991-2000.

[3] (a) Xin WB, Jin GL, Mao ZJ, Qin LP. (**2011**) Two unusual phenolic substances and one new xanthone from *Hypericum sampsonii. Helvetica Chimica Acta*, ***94***, 686-692; (b) Xiao ZY, Mu Q, Shiu WKP, Zeng YH, Gibbons S. (**2007**) Polyisoprenylated benzoylphloroglucinol derivatives from *Hypericum sampsonii. Journal of Natural Products*, ***70***, 1779-1782; (c) Hu LH, Sim KY. (**2000**) Sampsoniones A-M, a unique family of caged polyprenylated benzoylphloroglucinol derivatives, from *Hypericum sampsonii. Tetrahedron*, ***56***, 1379-1386; (d) Xiao ZY, Shiu WKP, Zeng YH, Mu Q, Gibbons S. (**2008**) A naturally occurring inhibitory agent from *Hypericum sampsonii* with activity against multidrug-resistant *Staphylococcus aureus*. *Pharmaceutical Biology*, ***46***, 250-253.

[4] Beerhues L, Liu B. (**2009**) Biosynthesis of biphenyls and benzophenones - Evolution of benzoic acid-specific type III polyketide synthases in plants. *Phytochemistry*, ***70***, 1719-1727.

[5] Liu BY, Falkenstein-Paul H, Schmidt W, Beerhues L. (**2003**) Benzophenone synthase and chalcone synthase from *Hypericum androsaemum* cell cultures: cDNA cloning, functional expression, and site-directed mutagenesis of two polyketide synthases. *The Plant Journal*, ***34***, 847-855.

[6] Nualkaew N, Morita H, Shimokawa Y, Kinjo K, Kushiro T, De-Eknamkul W, Ebizuka Y, Abe I. (**2012**) Benzophenone synthase from *Garcinia mangostana* L. pericarps. *Phytochemistry*, ***77***, 60-69.

[7] Klundt T, Bocola M, Lutge M, Beuerle T, Liu B, Beerhues L. (**2009**) A single amino acid substitution converts benzophenone synthase into phenylpyrone synthase. *Journal of Biological Chemistry*, ***284***, 30957-30964.

[8] (a) Don MJ, Huang YJ, Huang RL, Lin YL. (**2004**) New phenolic principles from *Hypericum sampsonii. Chemical and Pharmaceutical Bulletin*, ***52***, 866-869; (b) Chen MT, Chen CM. (**1985**) Constituents of Formosan antitumor folk medicine. Part I. Xanthones from *Hypericum sampsonii. Heterocycles*, ***23*****,** 2543-2548; (c) Hong D, Yin F, Hu LH, Lu P. (**2004**) Sulfonated xanthones from *Hypericum sampsonii. Phytochemistry*, ***65***, 2595-2598.

[9] (a) Sparenberg B. (**1993**) *MAO-inhibitierende Eigenschaften von Hypericum inhaltsstoffen und Untersuchungen zur Analytik und Isolierung von Xanthonen aus Hypericum perforatum L*., University of Marburg, Marburg; (b) Nahrstedt A, Butterweck V. (**1997**) Biologically active and other chemical constituents of the herb of *Hypericum perforaturn* L. *Pharmacopsychiatry*, ***30***, 129-134.

[10] (a) Dhawale S, Souciet G, Kuhn DN. (**1989**) Increase of chalcone synthase mRNA in pathogen-inoculated soybeans with race-specific resistance is different in leaves and roots. *Plant Physiology*, ***91***, 911-916; (b) Ormrod DP, Landry LG, Conklin PL. (**1995**) Short-term UV-B radiation and ozone exposure effects on aromatic secondary metabolite accumulation and shoot growth of flavonoid-deficient *Arabidopsis* mutants. *Physiologia Plantarum*, ***93***, 602-610; (c) Vanderkrol AR, Lenting PE, Veenstra J, Vandermeer IM, Koes RE, Gerats AGM, Mol JNM, Stuitje AR. (**1988**) An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature*, ***333***, 866-869.

[11] Gronquist M, Bezzerides A, Attygalle A, Meinwald J, Eisner M, Eisner T. (**2001**) Attractive and defensive functions of the ultraviolet pigments of a flower (*Hypericum calycinum*). *Proceedings of the National Academy of Sciences of the United States of America*, ***98***, 13745-13750.

[12] Franklin G, Conceicao LFR, Kombrink E, Dias ACP. (**2009**) Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. *Phytochemistry*, ***70***, 60-68.

[13] Beuerle T, Pichersky E. (**2002**) Enzymatic synthesis and purification of aromatic coenzyme A esters. *Analytical Biochemistry*, ***302***, 305-312.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1619 - 1622

# Efficient Preparation of Hangekobokuto (Banxia-Houpo-Tang) Decoction by Adding Perilla Herb before Decoction is Finished

**Megumi Sumino[a,*], Yuko Saito[b], Fumio Ikegami[a], Yoshiro Hirasaki[b] and Takao Namiki[b]**

[a]*Center for Environment, Health and Field Sciences, Chiba University, 6-2-1 Kashiwanoha, Kashiwa 277-0882, Japan*

[b]*Department of Japanese Oriental (Kampo) Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan*

*sumino@faculty.chiba-u.jp*

**Received: October 5th, 2012; Accepted: November 7th, 2012**

Hangekobokuto (banxia-houpo-tang) is a Kampo (traditional Japanese) medicine used mostly for anxiety disorder and consists of Pinellia Tuber, Poria Sclerotium, Magnolia Bark, Perilla Herb and Ginger. Perilla Herb contains an essential oil rich in perillaldehyde, which has a pleasant flavor, but this is easily lost due to heating. We therefore investigated how the major constituents of hangekobokuto, namely perillaldehyde, rosmarinic acid, magnolol and [6]-gingerol, varied with time during decoction and approached an optimal condition for decoction. Rosmarinic acid at 15 min after boiling, and magnolol and [6]-gingerol at 30 min were mostly extracted, while 0.09 mg of perillaldehyde remained at 15 min, but was not detected at 30 min. From these results, a decoction was prepared by adding Perilla Herb 1, 2, 5 and 10 min prior to the termination time of decoction at 30 min. When Perilla Herb was added 1 min beforehand, perillaldehyde was abundant (1.58 mg) and the amount of rosmarinic acid was already the same as that in the conventional decoction at 30 min, but the amount of total extract was inadequate. When Perilla Herb was added 5 min beforehand, perillaldehyde remained to some extent (0.61 mg) and rosmarinic acid was significantly increased compared with that in the conventional decoction at 30 min. From these results, we can conclude that the decoction should be prepared by boiling for not more than 30 min and, if possible, Perilla Herb should be added 5 min prior to the termination time. Considering the antidepressant-like activity of perillaldehyde and rosmarinic acid, the suggested method will not only achieve better treatment for anxiety, but also provide an effective use of crude drugs in the resource-limited environment.

**Keywords:** Kampo medicine, Hangekobokuto, Perilla Herb, Perillaldehyde, Rosmarinic acid.

Hangekobokuto (banxia-houpo-tang) is a Kampo (traditional Japanese) medicine composed of five crude drugs: Pinellia Tuber, Poria Sclerotium, Magnolia Bark, Perilla Herb and Ginger. It was described in Jinkuiyaolue, the Chinese traditional literature established in the 3rd century that describes major chronic diseases and treatments. This text suggests that when having a foreign-body sensation such as a lump in the throat, called Plum-pit Qi in China, hangekobokuto is suitable. According to Kampo medicine, Pinellia Tuber and Poria Sclerotium remove stagnating water in the stomach to improve nausea, Ginger improves gastrointestinal activity, Magnolia Bark relaxes muscle tension to improve vitality stasis, and Perilla Herb also improves vitality stasis. Hangekobokuto is widely used for anxiety disorder, dyspepsia, asthma and foreign-body sensation in the throat. Hangekobokuto Extract is newly listed in the Japanese Pharmacopoeia (JP), 16th edition, and is regulated by three compounds: rosmarinic acid from Perilla Herb, magnolol from Magnolia Bark and [6]-gingerol from Ginger (Figure 1) [1]. Several case reports and research papers about the effectiveness of this treatment for anxiety [2], deglutition disorder [3, 4] and throat dysesthesia disease [5] have been previously published. Perilla Herb contains perillaldehyde, shisonin, apigenin, luteolin and rosmarinic acid [6]. JP Perilla Herb should contain not less than 0.08% perillaldehyde [6]. Perillaldehyde has a variety of biological properties such as antibacterial [7], antimicrobial [8] and antidepressant-like activity [9]. It is also reported that the flavors of crude drugs themselves have biological activities like essential oils [10, 11]. So, the pleasant flavor of Perilla Herb, derived from perillaldehyde, may contribute to the effectiveness of hangekobokuto in this regard. Perillaldehyde is easily volatilized during decoction, but its behavior has not been studied. Thus, the aim of our study was to clarify the optimal decoction time of hangekobokuto by varying the chemical constituents with time and determining the effect of adding Perilla Herb 1, 2, 5 or 10 min prior to the termination time of decoction to maintain a high level of perillaldehyde, along with non-volatile rosmarinic acid.

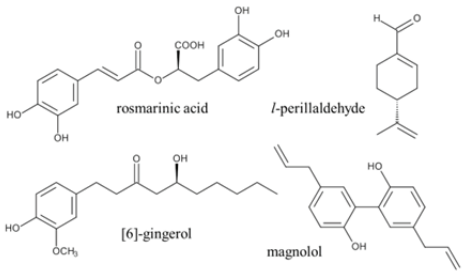



**Figure 1**: Structure of main principle compounds in hangekobokuto.

In the first experiment, we investigated how the quantities of major constituents of hangekobokuto varied with time during decoction. A daily dose of hangekobokuto was decocted with 400 mL of water. About 13 min passed until boiling occurred, and each decoction was prepared by continuous boiling for 0, 15, 30, 45 and 60 min, respectively. Perillaldehyde, rosmarinic acid, magnolol and [6]-gingerol from Ginger in each decoction were measured by HPLC (Table 1). Perillaldehyde was detected in the decoction at 0 min (1.55±0.35 mg), but its level greatly decreased until 15 min (0.09±0.04 mg) and was not detected after 30 min. On the other hand, rosmarinic acid was mostly extracted by 15 min, and the amount did not show any significant difference from that in the decoctions at 30, 45 and 60 min. Magnolol and [6]-gingerol contents did not change in the decoction after 30 min. Thus, it is

**Table 1**: Comparison of constituents and extract of hangekobokuto during decoction.

| decoction time (min) | 0 | | 15 | | 30 | | 45 | | 60 | |
|---|---|---|---|---|---|---|---|---|---|---|
| extract (g) | 1.43±0.21 | c* | 1.88±0.06 | b | 2.09±0.13 | ab | 2.12±0.11 | ab | 2.26±0.18 | a |
| decoction volume (mL) | 361±6 | | 318±6 | | 287±1 | | 262±8 | | 228±2 | |
| magnolol (mg) | 2.81±0.60 | c | 6.84±1.09 | b | 7.79±0.80 | ab | 9.50±0.63 | a | 8.63±1.20 | ab |
| [6]-gingerol (mg) | 1.10±0.06 | c | 2.19±0.15 | b | 2.36±0.31 | ab | 2.61±0.10 | a | 2.56±0.09 | a |
| rosmarinic acid (mg) | 11.3±1.0 | b | 14.8±1.2 | a | 14.4±1.6 | ab | 14.9±0.9 | a | 13.6±0.4 | ab |
| perillaldehyde (mg) | 1.55 ±0.35 | | 0.09±0.04 | | n.d. | | n.d. | | n.d. | |

Each value represents mean±SD. n.d.: not detected. * The results of the Tukey-Kramer test (within lines) are indicated by different letters where values differ significantly at $p<0.05$. n=4, 5 (constituents at 0 and 15 min).

**Table 2**: Comparison of constituents and extract of hangekobokuto when Perilla Herb was added before the termination time of decoction.

| | perillaldehyde (mg) | rosmarinic acid (mg) | | extract (g) | | magnolol (mg) | | [6]-gingerol (mg) | |
|---|---|---|---|---|---|---|---|---|---|
| Pe1* | 1.58±0.07 | 14.9±0.6 | b[x] | 1.63±0.12 | b | - | | - | |
| Pe2* | 1.18±0.04 | 15.4±0.7 | b | 1.65±0.17 | b | - | | - | |
| Pe5* | 0.61±0.04 | 18.3±0.8 | a | 2.25±0.11 | a | 7.69±0.71 | a | 2.60±0.34 | a |
| Pe10* | 0.15±0.01 | 20.3±1.5 | a | 1.95±0.22 | ab | 8.90±1.23 | a | 2.22±0.25 | a |
| decoction at 30 min | n.d. | 14.4±1.6 | b | 2.09±0.13 | a | 7.79±0.80 | a | 2.36±0.31 | a |

* Pe1, Pe2, Pe5, Pe10: decoction when Perilla Herb was added 1, 2, 5 and 10 min prior to the termination time of decoction, i.e., 30 min, respectively (n=4). Each value represents mean±SD. n.d.: not detected. -: no data. x The results of the Tukey-Kramer test (within columns) are indicated by different letters where values differ significantly at $p<0.05$.

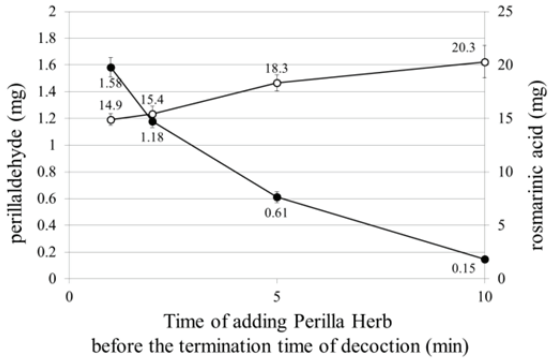



**Figure 2**: Perillaldehyde decrease and rosmarinic acid increase when Perilla Herb was added before the termination time of decoction. Each value represents mean±SD (n=4). Filled circles: perillaldehyde, open circles: rosmarinic acid.

sufficient to prepare the hangekobokuto decoction with continuous boiling for 30 min, and there seems to be no justification to decoct it for more than that time if we are not concerned with the amount of perillaldehyde in the decoction.

Considering the biological activities of perillaldehyde in Perilla Herb, the crude drugs in hangekobokuto other than Perilla Herb were decocted, and Perilla Herb was added either 5 min (decoction : Pe5) or 10 min (Pe10) prior to the termination time of decoction, i.e., at 30 min, to maintain a high level of perillaldehyde content in the decoction. The constituents and total extract were compared with those in the conventional decoction at 30 min (Table 2). Perillaldehyde in Pe5 was observed at an amount of 0.61±0.04 mg, but had almost disappeared in Pe10 (0.15±0.01 mg). The rosmarinic acid levels in Pe5 and Pe10 were 18.3±0.8 mg and 20.3±1.5 mg, respectively. They were significantly higher than that in the decoction at 30 min (14.4±1.6 mg). The total extract, magnolol and [6]-gingerol contents were the same as those in the conventional decoction at 30 min.

To determine the changes in greater detail, Perilla Herb was added 1 min (decoction: Pe1) and 2 min (Pe2) prior to the termination time of the decoction and the total extract, perillaldehyde and rosmarinic acid contents were measured (Figure 2, Table 2). Perillaldehyde was abundant (1.58±0.07 mg) in Pe1, but decreased to 1.18±0.04 mg in Pe2. On the other hand, rosmarinic acid was extracted sufficiently in Pe1 (14.9±0.6 mg) and Pe2 (15.4±0.7 mg). Each amount of rosmarinic acid was the same as that in the decoction at 30 min (14.4±1.6 mg), though each amount of total extract was significantly less than that in the decoction at 30 min.

To clarify the increase of rosmarinic acid in Pe5 and Pe10, only Perilla Herb was decocted for 30 min, and the results were compared with those obtained when Perilla Herb was added to boiling water prior to the termination time of decoction. When Perilla Herb was added 5 and 10 min beforehand, the amounts of rosmarinic acid were 20.2±0.7 mg (n=4) and 22.1±0.9 mg (n=4), respectively. Both amounts were significantly large compared with that in the Perilla Herb decoction at 30 min (16.0±1.0 mg, n=4), as determined by the Tukey-Kramer test.

In summary, when Perilla Herb was added 1, 2, 5 and 10 min beforehand, perillaldehyde decreased within 15 min, but rosmarinic acid had been extracted sufficiently after 1 min. In Pe1 and Pe2, perillaldehyde was abundant and the amount of rosmarinic acid was the same as that in the decoction at 30 min, but the total extract amount was inadequate compared with that obtained in the conventional way. In Pe5, perillaldehyde remained to some extent, but rosmarinic acid was significantly increased compared with the amount obtained in the conventional way, and the amount of total extract was adequate. Since rosmarinic acid has a number of interesting biological activities such as antiviral, antibacterial, anti-inflammatory, antioxidant [12] and antidepressant-like properties [13], our study sought to increase it by adding Perilla Herb before finishing the decoction. When Perilla Herb itself was decocted, the rosmarinic acid level was significantly increased when Perilla Herb was added prior to the termination time of decoction, as is the case with the hangekobokuto decoction. From these results, it is suspected that the increase in rosmarinic acid is not influenced by other constituents and boiling water may cause more cellular destruction than gradually warming water and may promote the release of chemical constituents in the case of Perilla Herb. We also found that rosmarinic acid linearly increased from 0 to 15 min by checking the amount in each conventional decoction at 5 and 10 min, respectively (data not shown).

In conclusion, considering its active constituents, Perilla Herb should be added 5 min prior to the termination time of the decoction. If it is not possible to separate Perilla Herb from the crude drugs of hangekobokuto when prescribed as a mixture, a decoction time of not more than 30 min is sufficient. The decoction method in which Perilla Herb is added before the decoction is finished could increase the constituents, i.e. perillaldehyde and rosmarinic acid, and may lead to a better treatment for anxiety in particular. This method is worth trying if the medicine does not work well or if greater effectiveness is desired. The effective dosage of perillaldehyde when administered orally or nasally to humans is not clear, but it is known that the antidepressant-like

activity is greater with nasal than with oral administration in mice [9]. Since perillaldehyde easily evaporates during decoction, better treatment can also be expected if patients themselves prepare the decoction and then inhale the vapor or take in the warm decoction by smelling it. In this study, the empirical effect of adding a specific crude drug such as fragrant leaves or flowers before the decoction was finished was experimentally clarified in the case of hangekobokuto.

## Experimental

***Plant materials:*** The hangekobokuto (daily dose) used in this experiment was composed of 5 crude drugs: Pinellia Tuber (*Pinellia ternata* Breitenbach, 6 g), Poria Sclerotium (*Wolfiporia cocos* Ryvarden et Gilbertson, 5 g), Magnolia Bark (*Magnolia obovata* Thunberg, 3 g), Perilla Herb (*Perilla frutescens* Britton var. *acuta* Kudo, 3 g) and Ginger (*Zingiber officinale* Roscoe, 1 g). Crude drugs were sealed and kept in the refrigerator at 6°C. Pinellia Tuber (Sichuan, China, Lot. 009110012), Poria Sclerotium (Yanbian, China, Lot. 009511014) and Perilla Herb (Zhejiang, China, Lot. 006910004) were purchased from Tochimoto Tenkaido (Osaka, Japan). Magnolia Bark (Nagano, Japan, Lot. A5H0140) and Ginger (Yunnan, China, Lot. OBK0227) were purchased from Uchida Wakanyaku (Tokyo, Japan). The Perilla Herb used in this experiment contained 0.14% perillaldehyde according to the analysis sheet from Tochimoto Tenkaido; that is, 4.2 mg perillaldehyde was contained in 3 g. All crude drugs met the criteria of the JP.

***Reagents:*** Authentic specimens of *l*-perillaldehyde, rosmarinic acid, magnolol, and [6]-gingerol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphoric acid from Wako and acetic acid from Kanto Chemical Co., Inc. (Tokyo, Japan) were used. Acetonitrile of high-performance liquid chromatography (HPLC) grade was purchased from Sigma-Aldrich (Tokyo, Japan). Methanol of HPLC grade was purchased from Wako. Distilled water was prepared by the purification system of Millipore. Tap water was used for preparing decoctions (pH 7.2, $Ca^{2+}$: 24.7 mg/L, $Mg^{2+}$: 6.3 mg/L, measured using a polarized Zeeman atomic absorption spectrophotometer, Z-5300, HITACHI).

***Preparation of decoction:*** In the first experiment, a daily dose of hangekobokuto and 400 mL of tap water were added to a heat-resistant glass pot with a lid. The pot was heated on a heater (350W, EK-SA10, Tochimoto Tenkaido, Osaka, Japan). The decoction time (time after boiling) was set at 0, 15, 30, 45 and 60 min. The decoction was filtered through paper, and a portion was freeze-dried and weighed. Another portion was mixed with an equal amount of methanol and filtered through a Millipore filter for HPLC analysis. In the second experiment, the crude drugs other than Perilla Herb were decocted with 400 mL of tap water for 30 min with Perilla Herb then added 1, 2, 5 and 10 min prior to the termination time.

***HPLC analysis:*** HPLC analysis was conducted using the following instruments. Column: TSK gel ODS-80TM (4.6 i.d. x 150 mm, TOSOH), Column oven: L-5025 (HITACHI), Pump: L-2130 (HITACHI), UV detector: L-4000 (HITACHI), Integrator: D-2500 Chromato-Integrator (HITACHI).

***HPLC conditions:*** The conditions were based on the method of hangekobokuto described in the JP. Rosmarinic acid, magnolol and [6]-gingerol are listed as principal compounds for the quality control of hangekobokuto. Perillaldehyde is a guiding component of Perilla Herb. Rosmarinic acid (mobile phase, flow rate, wave length, column temperature, observed retention time): $H_2O/CH_3CN/H_3PO_4$=800/200/1, 1 mL/min, 330 nm, 30°C, 13.6 min; magnolol: $H_2O/CH_3CN$/acetic acid =50/50/1, 1 mL/min, 289 nm, 40°C, 17.0 min; [6]-gingerol : $H_2O/CH_3CN/H_3PO_4$=620/380/1, 1 mL/min, 282 nm, 30°C, 15.9 min; perillaldehyde: $H_2O/CH_3CN$=40/60, 1 mL/min, 230 nm, 40°C, 17.6 min. Injection volume was 10 μL. All calibration curves showed good linearity ($r^2$>0.999) within the test ranges (rosmarinic acid and magnolol: 1-50 μg/mL, [6]-gingerol: 1-20 μg/mL, perillaldehyde: 0.1-10 μg/mL). Limit of detection (LOD) of perillaldehyde (0.06 μg/mL) was calculated according to the formula: 3.3SD/S, based on the standard deviation (SD) of the response and the slope (S) of the calibration curve at a level approximating to the LOD.

***Statistical analysis:*** Each value represents the mean ± SD, n=4-5. Differences were considered to be statistically significant when the *p* value was less than 0.05 according to the Tukey-Kramer test.

**Acknowledgments** - This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The authors declare that they have no competing interests.

## References

[1] Ministry of Health, Labour and Welfare. (**2011**) Hangekobokuto Extract. *Commentary of the Japanese Pharmacopoeia Sixteenth Edition.* Hirokawa Publishing Co., Tokyo, Japan, D-688-692.
[2] Luo L, Wang JN, Kong LD, Jiang QG, Tan RX. (**2000**) Antidepressant effects of Banxia Houpu decoction, a traditional Chinese medicinal empirical formula. *Journal of Ethnopharmacology*, ***73***, 277–281.
[3] Iwasaki K, Tei M, Sasaki H. (**2002**) Effects of Banxia Houpo Tang (Hange-Koboku To) for the treatment of the aspiration pneumonia. *Igaku no Ayumi*, ***203***, 1005-1007.
[4] Iwasaki K, Wang Q, Nakagawa T, Suzuki T, Sasaki H. (**1999**) The Traditional Chinese Medicine Banxia Houpo Tang improves swallowing reflex. *Phytomedicine*, ***6***, 103-106.
[5] Inoue Y, Kano K, Matsumoto Y, Shimodaira H, Uchida H, Nishizato Y. (**1995**) Comparative effect of Hange-koboku-tou and Sai-hoku-tou for patients with esophageal abnormal sensation. *Journal of New Remedies & Clinic*, ***44***, 1896-1902.
[6] Ministry of Health, Labour and Welfare. (**2011**) Perilla Herb. *Commentary of the Japanese Pharmacopoeia Sixteenth Edition.* Hirokawa Publishing Co., Tokyo, Japan, D-507-512.
[7] Burt S. (**2004**) Essential oils: their antibacterial properties and potential applications in foods-a review. *International Journal of Food Microbiology*, ***94***, 223-253.
[8] Sato K, Krist S, Buchbauer G. (**2006**) Antimicrobial effect of *trans*-cinnamaldehyde, (-)-perillaldehyde, (-)-citronellal, citral, eugenol and carvacrol on airborne microbes using an airwasher. *Biological & Pharmaceutical Bulletin*, ***29***, 2292-2294.
[9] Ito N, Nagai T, Oikawa T, Yamada H, Hanawa T. (**2011**) Antidepressant-like effect of l-perillaldehyde in stress-induced depression-like model mice through regulation of the olfactory nervous system. *Evidence-Based Complementary and Alternative Medicine*, ***2011***, Article ID 512697, 5 pages doi:10.1093/ecam/nen045.
[10] Ito M. (**2009**) Relaxing with fragrant vapor of natural medicine: its sedative effects on mice. *Aroma Research*, ***10***, 234-241.

[11] Niijima A, Hashimoto K, Sakakibara I, Amagaya S, Aburada M. (**2003**) Effects of olfactory and gustatory stimulation of Zingiberis rhizoma on the efferent nerve activity of the gastric branch of the vagus nerve and adrenal branch of the sympathetic nerve. *Japanese Journal of Taste and Smell Research*, ***10***, 97-102.
[12] Petersen M, Simmonds MSJ. (**2003**) Rosmarinic acid. *Phytochemistry*, ***62***, 121-125.
[13] Ito N, Yabe T, Gamo Y, Nagai T, Oikawa T, Yamada H, Hanawa T. (**2008**) Rosmarinic acid from Perillae Herba produces an antidepressant-like effect in mice through cell proliferation in the hippocampus. *Biological & Pharmaceutical Bulletin*, ***31***, 1376-1380.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1623 - 1626

# Anti-neutrophilic Inflammatory Secondary Metabolites from the Traditional Chinese Medicine, Tiankuizi

**Chia-Lin Lee[a,b], Tsong-Long Hwang[c], Chieh-Yu Peng[a,b], Chao-Jung Chen[d,e], Yuan-Shiun Chang[f] and Yang-Chang Wu[a,b,g,*]**

[a]*School of Chinese Medicine*, *College of Chinese Medicine*, *China Medical University*, *Taichung 404*, *Taiwan*

[b]*Natural Medicinal Products Research Center*, *China Medical University Hospital*, *Taichung 404*, *Taiwan*

[c]*Graduate Institute of Natural Products*, *College of Medicine*, *Chang Gung University*, *Taoyuan 333*, *Taiwan*

[d]Graduate Institute of Integrated Medicine, *College of Chinese Medicine*, *China Medical University*, *Taichung 404*, *Taiwan*

[e]*Proteomics Core Laboratory*, *Department of Medical Research*, *China Medical University Hospital*, *Taichung 404*, *Taiwan*

[f]*School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources*, *College of Pharmacy*, *China Medical University*, *Taichung 404*, *Taiwan*

[g]*Center for Molecular Medicine*, *China Medical University Hospital*, *Taichung 404*, *Taiwan*

*yachwu@mail.cmu.edu.tw*

**Received: September 25th, 2012; Accepted: October 18th, 2012**

Through bioassay-guided fractionation, thirteen compounds (**1**-**13**) were isolated from the dry root of *Semiaquilegia adoxoides*, known as Tiankuizi in traditional Chinese medicine (TCM). Among these, four benzoic acid derivatives (**1**, **2**, **4**, **5**), one 4,6-dimethoxy-5-methyl-2*H*-pyran-2-one (**10**) and one 1,2,3-propanetriol (**13**) were found for the first time in *S. adoxoides*. This is the first record of compound **10** from a natural source. 4-Hydroxybenzoic acid (**1**) and 3,4-dihyroxybenzoic acid (**2**) showed selective inhibition against elastase release and superoxide anion generation, with $IC_{50}$ values of 3.20 and 6.21 μg/mL, respectively. Compound **1** had 7-fold better activity than the positive control against elastase release induced by human neutrophils. Overall, our studies demonstrated Tiankuizi (*S. adoxoides*) as a potential TCM and isolates **1** and **2** as promising lead compounds for neutrophilic inflammatory diseases.

**Keywords:** *Semiaquilegia adoxoides*, Tiankuizi, Ranunculaceae, Anti-neutrophilic inflammatory activity.

*Semiaquilegia adoxoides* (DC.) Makino, family Ranunculaceae, distributed mainly in mainland China, Japan and Korea, is the only species in the genus *Semiaquilegia*. The dry roots of this plant, called Tiankuizi in traditional Chinese medicine (TCM), have been used for their anti-inflammatory, anti-neoplastic, and antibacterial activities, and for the treatment of snakebite in folk medicine [1-6]. Thirty compounds have been reported from *S. adoxoides* to date, including six cyano-compounds [1,6,7], three alkaloids [1,4,8], three benzoic acid derivatives [4,8], three benzofuranones [1,7], three diterpenes [5,6], three steroids [1,2], three fatty acids [8], two simple benzene derivatives [8], one nitro-compound [2], one flavonoid [1], one amide [9] and one furan [9]. However, it is interesting that the bioactive constituents from this plant have not been evaluated in a systematic manner.

Tiankuizi, especially, is often included in many multiple TCM formulas to cure cancer patients. In our preliminary study, a MeOH extract of Tiankuizi was separated into EtOAc-, *n*-BuOH- and water-soluble fractions, respectively. The three extracts were screened for cytotoxic activity against human lung (A549, H1299), breast (MDA-MB-231), ovarian (SKOV3), colon (HCT116, HT29), pancreas (AsPC-1) and oral (Ca922) cancer cell lines. All were inactive, with $IC_{50}$ values of > 100 μg/mL. However, during the course of an anti-neutrophilic inflammatory screening program on natural products, it was found that the EtOAc- and *n*-BuOH-extracts of Tiankuizi were active against superoxide anion generation and elastase release induced by human neutrophils.

**Table 1**: Effects of Tiankuizi extract on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB.

| Extract | Superoxide anion Inh % | | | | Elastase release Inh % | | | |
|---|---|---|---|---|---|---|---|---|
| SAE | 84.8 | ± | 1.0 | *** | 106.4 | ± | 0.2 | *** |
| SAB | 54.4 | ± | 1.4 | *** | 15.9 | ± | 3.2 | * |
| SAW | 26.8 | ± | 1.5 | ** | 5.5 | ± | 2.0 | |

SAE: EtOAc-soluble fraction; SAB: *n*-BuOH-soluble fraction; SAW: water-soluble fraction. Percentage of inhibition (Inh %) at 10 μg/mL concentration. Results are presented as mean ± S.E.M. (n = 2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$compared with the control value.

Respiratory system diseases always have the severe problem of mucus hypersecretion, in which superoxide anion and elastase are produced in large amounts by activated neutrophils. In such a situation, neutrophil inflammation will lead to deterioration of the disease and a high mortality rate. Therefore, finding potential new drugs to treat neutrophilic inflammatory diseases has become a necessity. Therefore, the TCM, Tiankuizi, was first selected as a lead to be investigated for anti-neutrophilic inflammatory activity through bioassay-guided fractionation.

A MeOH extract of Tiankuizi was partitioned between EtOAc-$H_2O$ (1:1), and then the aqueous layer between *n*-BuOH and $H_2O$ (1:1) to obtain EtOAc (SAE), *n*-BuOH (SAB) and water (SAW) fractions, respectively. As shown in Table 1, the EtOAc- and *n*-BuOH-soluble fractions possessed inhibitory effects of 84.8% and 54.4% against superoxide anion generation, respectively at the concentration of 10 μg/mL. In addition, only the EtOAc-soluble fraction produced significant inhibition of elastase release. Fractionation of the EtOAc

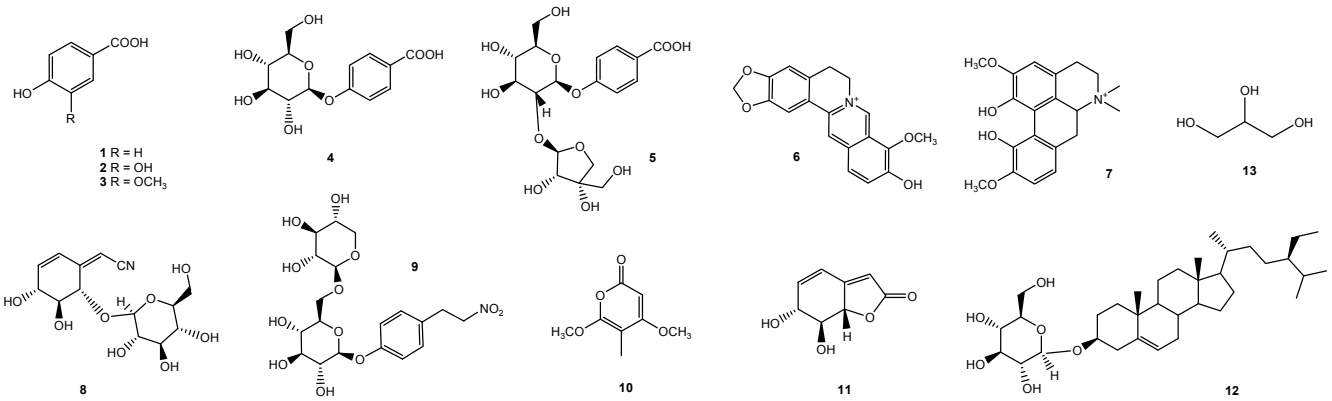



**Figure 1**: All structures of compounds **1**-**13** isolated from Tiankuizi.

and *n*-BuOH extracts led to the isolation of thirteen compounds, including five benzoic acid derivatives, 4-hydroxybenzoic acid (**1**), 3,4-dihyroxybenzoic acid (**2**), 3-methoxy-4-hydroxybenzoic acid (**3**), *p*-*β*-D-glucosyloxybenzoic acid (**4**) [10], monordicophenoide A (**5**) [11], three alkaloids, thalifendine (**6**) [8], magnoflorine (**7**) [1], and 4-hydroxy-1-(2-nitroethyl) benzene 4-*O*-(6′-*O*-*β*-D-xylopyranosyl)-*β*-D-glucopyranoside (**9**) [2], one cyano-compound, lithospermoside (**8**) [1], one 4,6-dimethoxy-5-methyl-2*H*-pyran-2-one (**10**) [12,13], one benzofuranone, griffonilide (**11**) [1], sitosterol-3-*β*-D-glucose (**12**) [1], and 1,2,3-propanetriol (**13**). All structures were identified by NMR spectroscopic and MS data interpretation.

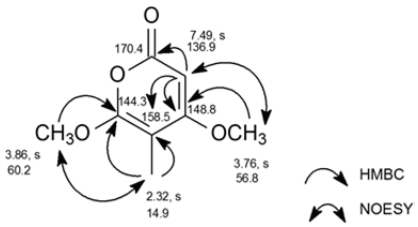



**Figure 1**: Key correlations of HMBC and NOESY, along with $^{1}$H and $^{13}$C NMR data for compound **10**.

$^{1}$H, $^{13}$C and HSQC NMR spectra of compound **10** indicated three singlet methyl groups ($\delta_H$ 2.32/$\delta_C$ 14.9, $\delta_H$ 3.76/$\delta_C$ 56.8 and $\delta_H$ 3.86/$\delta_C$ 60.2) and one olefinic proton ($\delta_H$ 7.49, s/$\delta_C$ 136.9). In the $^{13}$C NMR spectrum, there are four quaternary carbons (144.3, 148.8, 158.5, 170.4) in addition. As Figure 1 shows, the HMBC and NOESY spectra suggested the structure of **10** as 2*H*-pyran-2-one, 4,6-dimethoxy-5-methyl. This was synthesized in 1989 by Venkataraman and Cha [12,13], but clear spectral data and biological properties of **10** were never reported. Compound **10** is reported by us for the first time as a naturally occurring substance.

Of the thirteen compounds isolated from Tiankuizi, the four benzoic acid derivatives (**1**, **2**, **4**, **5**), and compounds **10** and **13** are reported for the first time for *S. adoxoides*. Although, the crude extracts of Tiankuizi did not have cytotoxicity, all isolates were tested for cytotoxicity toward eight human cancer cell lines (A549, H1299, MDA-MB-231, SKOV3, HCT116, HT29, AsPC-1 and Ca922), but all were inactive, with $IC_{50}$ values of > 100 μg/mL.

All the isolates, excluding **10**, were tested in the anti-neutrophilic inflammation assay based on their effects against superoxide anion generation and elastase release by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)/cytochalasin B (CB) (Table 2). Diphenyleneiodonium (DPI) and phenylmethyl-sulfonyl fluoride (PMSF) were used as the positive controls, respectively. Compounds **1** and **2** showed selective inhibition

**Table 2**: Effects of compounds isolated from Tiankuizi on superoxide anion generation and elastase release in FMLP/CB-induced human neutrophils.

| compd | Superoxide anion | | | | Elastase release | | | |
|---|---|---|---|---|---|---|---|---|
| | $IC_{50}$ (μg/mL)[a)] | Inh % | | | $IC_{50}$ (μg/mL)[a)] | Inh % | | |
| **1** | >10 | 32.4 ± 2.1 | | *** | 3.2 ± 0.7 | 75.1 ± 2.1 | | *** |
| **2** | 6.2 ± 1.9 | 59.2 ± 5.5 | | *** | >10 | 25.4 ± 4.0 | | ** |
| **3** | >10 | 23.7 ± 3.6 | | ** | >10 | 19.1 ± 2.4 | | ** |
| **4** | >10 | 13.9 ± 1.6 | | *** | >10 | 2.4 ± 1.0 | | |
| **5** | >10 | 2.3 ± 0.8 | | * | >10 | 2.0 ± 1.3 | | |
| **6** | >10 | 34.4 ± 2.0 | | *** | >10 | 20.1 ± 5.9 | | * |
| **7** | >10 | 18.6 ± 5.5 | | * | >10 | 15.8 ± 5.4 | | * |
| **8** | >10 | 5.0 ± 3.3 | | | >10 | 7.0 ± 4.7 | | |
| **9** | >10 | 10.7 ± 2.8 | | ** | >10 | 3.3 ± 0.9 | | * |
| **10** | NT[b)] | NT[b)] | | | NT[b)] | NT[b)] | | |
| **11** | >10 | 6.9 ± 3.9 | | | >10 | 1.7 ± 2.8 | | |
| **12** | >10 | 29.0 ± 6.9 | | * | >10 | 3.7 ± 4.8 | | |
| **13** | >10 | 7.9 ± 3.1 | | | >10 | 8.7 ± 3.1 | | |
| DPI[c)] | 0.22±0.13 | | | | | | | |
| PMSF[c)] | | | | | 22.8±5.1 | | | |

Percentage of inhibition (Inh %) at 10 μg/mL concentration. Results are presented as mean±S.E.M. (n=3 or 4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control value . [a)] Concentration necessary for 50% inhibition ($IC_{50}$). [b)] NT means not tested. [c)] Diphenyleneiodonium (DPI) and phenylmethylsulfonyl fluoride (PMSF) were used as positive controls for superoxide anion generation and elastase release, respectively.

against elastase release and superoxide anion generation with $IC_{50}$ values of 3.2 and 6.2 μg/mL, respectively. Compound **1** had a 7-fold better activity than the positive control, PMSF, against elastase release by human neutrophils. Among the benzoic acid derivatives **1**-**5**, the main differences in their structures are the *para*-(OH, glucopyranosyl, furanosyl-glucopyranosyl) and *meta*-substituents (H, OH, OMe). The data for compounds **1**, **4**, and **5** in Table 2, suggest that the free OH *para*-substituent of benzoic acid is more favorable. In addition, the potency of a *meta*-substituent H or OH is better than that of OMe among the *p*-hydroxy benzoic acids **1**-**3**. The alkaloids and other compounds showed no anti-neutrophilic inflammatory property. Overall, our studies indicated Tiankuizi (*S. adoxoides*) as a potential natural source and 4-hydroxybenzoic acid (**1**) and 3,4-dihyroxybenzoic acid (**2**) as promising compounds for further study of neutrophilic inflammatory diseases, like asthma.

## Experimental

***General:*** 1D ($^{1}$H, $^{13}$C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra were measured on Bruker ULTRASHIELD™ 500 and 600 PLUS instruments. Chemical shift (δ) values are given in ppm with $C_5D_5N$, $CDCl_3$, $CD_3OD$, and DMSO-$d_6$ as internal standards, and coupling constants (*J*) are in

Hz. Low-resolution ESIMS were measured on a Bruker Daltonics EsquireHCT ultra high capacity trap mass spectrometer. TLC was performed on either Kieselgel 60 $F_{254}$ (0.25 mm, Merck) or RP-18 $F_{254}S$ (0.25 mm, Merck) plates, and compounds were detected under UV light at 254 nm and 356 nm before being stained by spraying with 10% $H_2SO_4$ and heating on a hot plate. For CC, silica gel (SiliCycle® 70-230 and 230-400 mesh), RP-18 (LiChroprep® 40-63 μm, Merk), Sephadex™ LH-20 (GE Healthcare, Sweden) and Diaion® HP-20 (SUPELCO™) were used.

***Plant material:*** TCM Tiankuizi, the dry root of *Semiaquilegia adoxoides*, was purchased from a Chinese medicine shop in Tainan, Taiwan, in February, 2011 and identified by one author, Prof. Y.-S. Chang. A voucher specimen (SA201102) was deposited at the Natural Medicinal Products Research Center, CMUH, Taiwan.

***Extraction and isolation:*** Tiankuizi (260.5 g) was extracted 3 times with MeOH (700 mL each) at room temperature to obtain a crude extract. The MeOH extract (94.7 g) was partitioned between EtOAc and $H_2O$ (400 mL/400 mL, 5 times) to give an EtOAc-soluble fraction (4.4 g) and the aqueous phase (89.3 g), which was further partitioned with *n*-BuOH-$H_2O$ (500 mL/500 mL, 3 times) to give *n*-BuOH- (16.7 g) and $H_2O$-soluble fractions (69.7 g).The EtOAc-soluble fraction (4.4 g) was subjected to open CC on silica gel (70-230 mesh, column: 5 × 19 cm), using gradients of *n*-hexane-$CHCl_3$-MeOH to give 12 subfractions (SAE1~SAE12). Fraction SAE94 (27.6 mg) was further purified by preparative TLC to obtain **1** (2.4 mg). SAE9 (98.8 mg) was fractionated by Sephadex LH-20 CC (column diameter: 3 cm, length: 29 cm; $CHCl_3$-MeOH, 1:1) to yield 5 fractions (SAE91~ SAE95). Subfraction SAE10 (872.9 mg) was divided into 5 fractions by silica gel CC (230-400 mesh, column: 2.5 × 25 cm) using gradients of $CHCl_3$-MeOH (10:1; 8:1; 6:1; 4:1; 1:1). A precipitate, obtained from subfraction SAE103 (293.3 mg), using MeOH, afforded **12** (60.4 mg). The residue (232.9 mg) of SAE103 was subjected to Sephadex LH-20 CC (column diameter: 3 cm, length: 28.5 cm; $CHCl_3$-MeOH, 1:1) to give 6 subfractions. Subfraction SAE1034 (109.7 mg) was chromatographed on a silica gel column (230-400 mesh, column: 2 × 26 cm), using EtOAc-MeOH (100:0; 50:1; 30:1; 5:1; 0:100). Subfraction SAE10344 (28.8 mg) was purified subsequently by preparative TLC with $CHCl_3$-MeOH (10:1) to obtain **11** (10.5 mg). Subfraction SAE1035 (39.8 mg) was purified by preparative TLC ($CHCl_3$-MeOH, 8:1) to give **2** (8.5 mg). Fraction SAE7 (104.9 mg) was subjected to Sephadex LH-20 CC (column diameter: 3 cm, length: 28 cm; $CHCl_3$-MeOH, 1:1) and subfraction SAE73 (47.9 mg) to preparative TLC (*n*-Hexane-EtOAc, 1:2) to give **3** (4.6 mg). Fraction SAE3 (48.2 mg) was purified by preparative TLC (*n*-Hexane-EtOAc, 1:1) to give **10** (2.0 mg). The *n*-BuOH-soluble fraction (16.7 g) was chromatographed over Diaion® HP-20 (column: 5 × 25.5 cm; $H_2O$-MeOH-Acetone, 100:0:0; 75:25:0; 50:50:0; 25:75:0; 0:100:0; 0:0:100) to give 6 subfractions (SAB1~SAB6). Subfractions SAB4 (110.0 mg) and SAB5 (140.0 mg) were combined and subjected to open CC on silica gel (230-400 mesh, column: 2.5 × 25 cm), using gradients of $CHCl_3$-MeOH (8:1; 6:1; 4:1; 2:1; 0:1) to obtain 8 subfractions (SAB41~SAB48). Subfraction SAB48 (68.0 mg) was separated by RP-18 chromatography (column: 2.5 × 26 cm; MeOH) and silica chromatography (230-400 mesh, column: 2.5 × 27 cm; $CHCl_3$-MeOH, 8:1; 6:1) to obtain **6** (8.0 mg). Fraction SAB2 (1.4 g) was separated into 7 subfractions by CC on silica gel (column: 2.5 × 25 cm; EtOAc-MeOH, 5:1). Subfraction SAB22 (168.0 mg) was subjected to silica gel chromatography (230-400 mesh, column: 2 × 25 cm; EtOAc-MeOH, 100:0; 10:1; 5:1; 1:1; 0:100) and subfraction SAB229 (30.9 mg) was further purified by preparative TLC ($CHCl_3$-MeOH, 3:1) to give **4** (11.6 mg). Fraction SAB23 (235.0 mg) was purified by Sephadex LH-20 (column diameter: 3 cm, length: 24 cm; MeOH) and silica gel CC (230-400 mesh, column: 2 × 22 cm; $CHCl_3$-MeOH, 3:1) to give **5** (18.7 mg). Compound **8** (3.9 mg) was precipitated and purified from subfraction SAB24 (120.0 mg) using MeOH. Fraction SAB27 (106.0 mg) was divided by RP-18 CC (column: 2.5 × 28 cm; MeOH-$H_2O$, 80:20) into **7** fractions (56.0 mg). Subfraction SAB3 (413.0 mg) was separated by silica gel (230-400 mesh, column: 2.5 × 7 cm; $CHCl_3$-MeOH, 8:1; 1:1; 0:1) and Sephadex LH-20 CC (column diameter: 2.5 cm, length: 26 cm; MeOH) to give subfraction SAB313 (69.7 mg), which was further purified by preparative TLC ($CHCl_3$-MeOH, 3:1; EtOAc-MeOH, 4:1) to give **9** (10.9 mg).

**4,6-dimethoxy-5-methyl-2*H*-Pyran-2-one** (**10**)

$^1$H NMR (600 MHz, $CDCl_3$) δ: 2.32 (3H, s), 3.76 (3H, s), 3.86 (3H, s), 7.49 (1H, s).

$^{13}$C NMR (150 MHz, $CDCl_3$) δ: 14.9, 56.8, 60.2, 136.9, 144.3, 148.8, 158.5, 170.4.

ESI-MS *m/z*: 170.9 $[M+H]^+$.

***Cytotoxicity:*** Compounds were tested against human lung (A549, H1299), breast (MDA-MB-231), ovarian (SKOV3), colon (HCT116, HT29), pancreas (AsPC-1) and oral (Ca922) cancer cell lines using an established colorimetric MTT assay protocol [14]. The absorbance was measured at 550 nm using a microplate reader. The $IC_{50}$ is the concentration of agent that reduced cell viability by 50% under the experimental conditions.

***Measurement of superoxide anion generation:*** The assay of the generation of superoxide anions was carried out according to established protocols by monitoring the reduction of ferricytochrome *c* that could be inhibited by superoxide dismutase [15]. *N*-Formyl-Met-Leu-Phe (FMLP, 100 nM)/cytochalasin B (CB, 1 μg/mL) was used as a stimulant to activate neutrophils to produce superoxide anions. The positive control was diphenyleneiodonium (DPI), which is a NADPH oxidase inhibitor.

***Elastase release assays:*** Elastase release was measured by degranulation of azurophilic granules, as described previously [15]. Neutrophils were activated by 100 nM FMLP and 0.5 μg/mL CB to release elastase. Phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, was used as the positive control.

**Acknowledgments** - This work was supported by a grant from the China Medical University Hospital, Taiwan awarded to C.L. Lee (DMR-100-174).

## References

[1] Han QB, Jiang B, Mei SX, Ding G, Sun HD, Xie JX, Liu YZ. (**2001**) Constituents from the roots of *Semiaquilegia adoxoides*. *Fitoterapia*, ***72***, 86−88.

[2] Su YF, Zhang ZX, Guo CY. (**2004**) A new nitroethylphenolic glycoside from *Semiaquilegia adoxoides*. *Fitoterapia*, ***75***, 420−422.

[3] Zhang H, Liao ZX, Yue JM. (**2004**) Cyano- and nitro-containing compounds from the roots of *Semiaquilegia adoxoides*. *Chinese Journal of Chemistry*, ***22***, 1200−1203.

[4] Niu F, Cui Z, Chang HT, Jiang Y, Chen FK, Tu PF. (**2006**) Constituents from the roots of *Semiaquilegia adoxoides*. *Chinese Journal of Chemistry*, ***24***, 1788−1791.

[5] Niu F, Chang HT, Jiang Y, Cui Z, Chen FK, Yuan JZ, Tu PF. (**2006**) New diterpenoids from *Semiaquilegia adoxoides*. *Journal of Asian Natural Products Research*, ***8***, 87−91.
[6] Niu F, Cui Z, Li Q, Chang H, Jiang Y, Qiao L, Tu PF. (**2006**) Complete assignments of $^{1}H$ and $^{13}C$ NMR spectral data for a novel diterpenoid from *Semiaquilegia adoxoides*. *Magnetic Resonance in Chemistry*, ***44***, 724−726.
[7] Niu F, Niu Z, Xie G, Meng F, Zhang G, Cui Z, Tu PF. (**2006**) Development of an HPLC fingerprint for quality control of Radix Semiaquilegiae. *Chromatographia*, ***64***, 593−597.
[8] Niu F, Xie GB, Cui Z, Chen FK, Tu PF. (**2006**) Chemical constituents from roots of *Semiaquilegia adoxoides*. *Journal of Chinese Pharmaceutical Sciences*, ***15***, 251−254.
[9] Zou J, Yang J. (**2004**) Study on chemical constituents isolated from *Semiaquilegia adoxoides*. *Zhongguo Yaoxue Zazhi (Beijing, China)*, ***39***, 256−257.
[10] Gaind KN, Chopra KS. (**1976**) Phytochemical investigation of *Abutilon indicum*. *Planta Medica*, ***30***, 174−185.
[11] Li Q, Liang H, Wang, Zhao Y. (**2009**) Chemical constituents of *Momordica charantia* L. *Yaoxue Xuebao*, ***44***, 1014−1018.
[12] Cha JK, Harris TM, Ray JA, Venkataraman H. (**1989**) Nucleophilic attack on 4,6-dimethoxy-2-pyrones; discovery of a remarkable facile rearrangement of the pyrones. *Tetrahedron Letters*, ***30***, 3505−3508.
[13] Venkataraman H, Cha JK. (**1989**) The scope and mechanism of rearrangement of 4,6-dialkoxy-2-pyrones. *Tetrahedron Letters*, ***30***, 3509−3512.
[14] Mosmann T. (**1983**) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, ***65***, 55–63.
[15] Hwang TL, Wang CC, Kuo YH, Huang HC, Wu YC, Kuo LM, Wu YH. (**2010**) The hederagenin saponin SMG-1 is a natural FMLP receptor inhibitor that suppresses human neutrophil activation. *Biochemical Pharmacology*, ***80***, 1190−1200.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1627 - 1629

# DART MS Based Chemical Profiling for Therapeutic Potential of *Piper betle* Landraces

**Vikas Bajpai[a], Renu Pandey[a], Mahendra Pal Singh Negi[b], Nikhil Kumar[c] and Brijesh Kumar[a]***

[a]*Sophisticated Analytical Instrument Facility*, *Central Drug Research Institute (CSIR)*, *Lucknow-226001*, *India*
[b]*Biometry and Statistics Division*, *Central Drug Research Institute (CSIR)*, *Lucknow-226001*, *India*
[c]*National Botanical Research Institute (CSIR)*, *Lucknow-226001*, *India*

*brijesh_kumar@cdri.res.in*

**Received: September 26th, 2012; Accepted: November 9th, 2012**

*Piper betle* Linn.leaves are traditionally used as a folk medicine in India and other Asiatic countries. Twenty-one *P. betle* landraces were analyzed using a Direct Analysis in Real Time (DART) mass spectral technique and evaluated on the basis of molecules detected in the leaves. Clustering of landraces based on three well known biologically active phenols (*m/z* 151,165,193) showed two broad groups with high and low phenol contents suggesting differences in their therapeutic potential. Findings of this study could be useful in rapid screening of the landraces for determining their medicinal potential and optimum utilization of the bioresource.

**Keywords:** DART-MS, *Piper betle*, Landraces, Profiling.

*Piper betle* Linn. (PB) is a pan-Asiatic cultural plant and, according to estimates, nearly 600 million people consume it daily in one form or another [1]. In terms of consumption it ranks next only to coffee and tea. The plant is a native of tropical Asia and grows wild in these regions. Traditional uses of this plant are well known in India and many others countries of South East and Far East Asia. It is widely used in the treatment of coughs and colds, wound healing, oral hygiene and many other ailments. It is also known to have digestive, carminative, anti-inflammatory, and cardiotonic properties. The Indian system of medicine, Ayurveda, recognized the importance of PB and some thirteen properties were ascribed to it [2]. In recent years several properties have been demonstrated in PB like pro-apoptotic, anti-amoebic, anti-oxidant, larvicidal, anti-allergic, anti-fungal, anti-bacterial, anti-inflammatory, hepatoprotective, anti-diabetic, wound healing and suppression of halitosis [3 and references therein].

PB is a shade loving climber and cultivated widely in tropical and some subtropical regions of India. In India more than a hundred PB landraces have been recognized [3]. Biodiversity within a species is represented by landrace and/ or variety. No extensive systematic studies on characterizing these landraces have been made in the past. Some work on the essential oil has shown that most of the PB landraces fall into five broad groups, Bangla, Kapoori, Khasi, Sanchi and Meetha [4]. Studies conducted on molecular taxonomy using random amplified polymorphic DNA (RAPD) using seventy landraces have also shown the existence of major groups as Bangla, Kapoori and others [5,6]. Recently DART-MS was used for studying some PB landraces and the method was found to be useful for screening [7]. Since PB is an important cultural and medicinal plant it is important to explore the available biodiversity for its therapeutic potential. In this communication we have screened twenty-one PB landraces using DART-MS and shown its utility in predicting their therapeutic potential.

DART-MS profiles of twenty-one *P. betle* landraces showed characteristic differences, as illustrated by the spectra shown in Figure 1. The representative spectra of three landraces, namely Jagarnathi Green, Bangla Mahoba and MeethaPatta, showed intraspecific variations within the landraces (spectra of other landraces are given as supplementary data). The peaks at *m/z* 193, 207, 235 and 252 were common to all the landraces, which makes these peaks constitutive for PB. Other major peaks seen in the spectra at *m/z*104, 118,132,135,151,163,165,166,175,177 and 205 were not constitutive making them useful as markers for landraces. The DART mass spectra did not show peaks attributable to terpenes, except for a small peak at *m/z* 205 corresponding to sesquiterpenes. Peaks were observed at *m/z* values that corresponded to several reported phenols and their acetates in PB leaf. The peaks at *m/z* 135, 151, 165, 177, 193, 207 and 235 could be due, respectively, to chavicol, allylpyrocatechol (APC) and/or carvacrol, eugenol and/or chavibetol (CHV), chavicol acetate, allylpyrocatechol acetate, chavibetol acetate and allylpyrocatechol diacetate. Since some of the phenols have the same molecular weight it was not possible to distinguish them from DART mass spectra alone, as in the case of the peaks at *m/z* 151 and 165. A peak at *m/z* 135, corresponding to chavicol, was detected only in landraces Saufia and MeethaPatta. From the spectra it can be seen that APC and/or carvacrol was present in all landraces, except Desawari, whereas eugenol and/or CHV was absent in Desawari, Mahoba and Sanchi*. Chavicol acetate was present only in Bangla, MeethaPatta and Saufia, as it could not be detected in other landraces. The structures of the bioactive molecules giving the ions at *m/z* 151 and 165 are shown in Figure 2.

The chemical constituents of betel essential oil consist mainly of terpenes and phenols [4], and the characteristic flavor of betel is due to phenols. The terpenoids include 1,8-cineole,cadinene, camphene, caryophyllene, limonene, and pinene. Chavicol, allylpyrocatechol, carvacrol, safrole, eugenol and chavibetol and their acetates are commonly found in PB. Bioactive molecules reported in the literature are mainly eugenol, methyl eugenol, chavibetol, APC, APC acetate, carvacrol and methyl chavicol. However, APC is the most active constituent [3 and references there in], and its concentration is correlated with biological activity [3,8]. The importance of chavicol [8], and total phenol content has also been

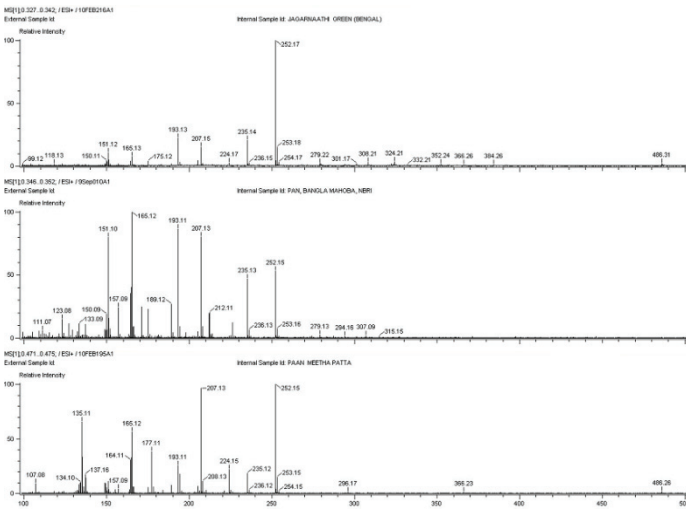


**Figure 1**: Leaf DART MS of *Piper betle* landraces Jalesar Green, Bangla Mahoba and MeethaPatta.

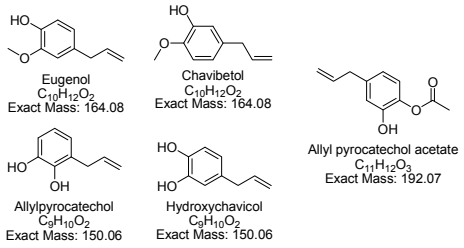



**Figure 2**: Structures of purified active molecules reported from *Piper betle* leaf.

reported [10]. Thus the biological activity in PB is mainly due to various phenols [3].The DART mass spectra of PB showed peaks at *m/z* values corresponding to several of the reported phenols and their acetates in PB leaf; these are in agreement with earlier findings [7]. Peaks corresponding to chavicol, APC, chavibetol, chavicol acetate, APC acetate, chavibetol acetate and allylpyrocatecholdiacetate were detected in PB landraces. Due to the same molecular weight of some of the phenols it was not possible to distinguish them from the DART mass spectra alone, as in the case of the peaks at *m/z* 151 and 165, which could be respectively due to APC and /or carvacrol and eugenol and /or chavibetol.

The percent ionization of the peaks at *m/z* 151, 165 and 193 is shown in Table 1. Based on total phenolic content (sum of these three peaks) the top four landraces in descending order were Jalesar Green>Kalkatiya>Sirugamani>Deshi. Most of the earlier findings suggested that the biological activity of PB was due to APC [3 and references therein] and possibly its acetate, which was detected unambiguously in the present study. The level of APC and /or carvacrol varied in landraces and the highest value was observed in landrace Mahoba; other notable landraces with high contents were Sirugamani>Jalesar Green >Deshi Bangla. Based on eugenol and /or CHV, the landraces may be graded as Saufia> Bangla Mahoba>MeethaPatta>Deshi Bangla >Deshi>the rest.

Most of the studies of PB were made using a crude extract of one or two landraces [3]. Significant differences in total phenol content and antioxidant activity in five PB landraces, mainly BanglaMahoba, Deshi Bangla, Kaker, KapooriVellaikodi and Madras PanKapoori were reported by Tripathi [10]. Our findings based onDART-MS also show marked differences in phenols, which are in agreement with the reported findings [10].

**Table 1**: Percent ionization of three biologically active phenols allylpyrocatechol /carvacrol (*m/z* 151), eugenol/chavibetol (*m/z* 165), andallylpyrocatechol acetate (*m/z* 193) in the leaf of *Piper betle* landraces obtained from different locations in India. (Mean ± SD, n=5).

| *S.No. P. betle* landraces | Allylpyrocatechol /Carvacrol (*m/z* 151) | Eugenol/ Chavibetol (*m/z* 165) | Allylpyrocatechol acetate (*m/z* 193) |
|---|---|---|---|
| 1 Bangla | 11.8 ± 1.5 | 7.2 ± 1.4 | 16.5 ± 3.3 |
| 2 Bangla Meetha | 7.7 ± 1.0 | 6.5 ± 2.1 | 12.3 ± 1.5 |
| 3Bangla Mahoba | 13.3 ± 1.7 | 13.1 ± 1.0 | 17.2 ± 0.8 |
| 4Deshi Bangla | 16.3 ± 1.8 | 10.4 ± 1.1 | 18.6 ± 1.08 |
| 5Desawari | nd | nd | 20.8 ± 0.9 |
| 6 Deshi | 14.4 ± 1.6 | 10.4 ± 1.6 | 21.1 ± 0.7 |
| 7Ganzaam | 6.2 ± 1.5 | 1.6 ± 0.5 | 10.3 ± 2.0 |
| 8Jalesar Green | 17.2 ± 0.7 | 8.1 ± 0.5 | 23.1 ± 0.6 |
| 9Jalesar White | 13.2 ± 0.7 | 4.8 ± 1.4 | 21.6 ± 1.0 |
| 10Jagarnathi Green | 6.7 ± 4.0 | 5.2 ± 0.8 | 15.0 ± 2.6 |
| 11Jagarnathi White | 6.5 ± 1.2 | 3.0 ± 0.5 | 11.3 ± 1.3 |
| 12Kalkatiya | 15.5 ± 3.3 | 7.2 ± 0.6 | 24.7 ± 0.8 |
| 13Maghi White | 9.5 ± 2.2 | 5.2 ± 2.3 | 15.2 ± 1.6 |
| 14Maghi | 5.1 ± 2.7 | 4.6 ± 1.8 | 8.7 ± 0.6 |
| 15Mahoba | 20.1 ± 1.5 | nd | 22.6 ± 1.4 |
| 16MaiharDeshi | 9.8 ± 1.3 | 4.8 ± 2.0 | 12.8 ± 0.3 |
| 17MeethaPatta | 4.9 ± 1.0 | 12.7 ± 2.6 | 6.2 ± 1.1 |
| 18 Sanchi * | 5.0 ± 0.8 | 2.5 ± 0.9 | 15.7 ± 2.7 |
| 19 Sanchi** | 7.0 ± 2.1 | 1.9 ± 1.1 | 16.6 ± 3.2 |
| 20Saufia | 1.2 ± 0.4 | 18.2 ± 1.4 | 6.5 ± 0.8 |
| 21Sirugamani | 19.7 ± 2.5 | 5.0 ± 0.2 | 22.2 ± 0.5 |

* (V) Varanasi, **(WB) West Bengal; nd- not detected

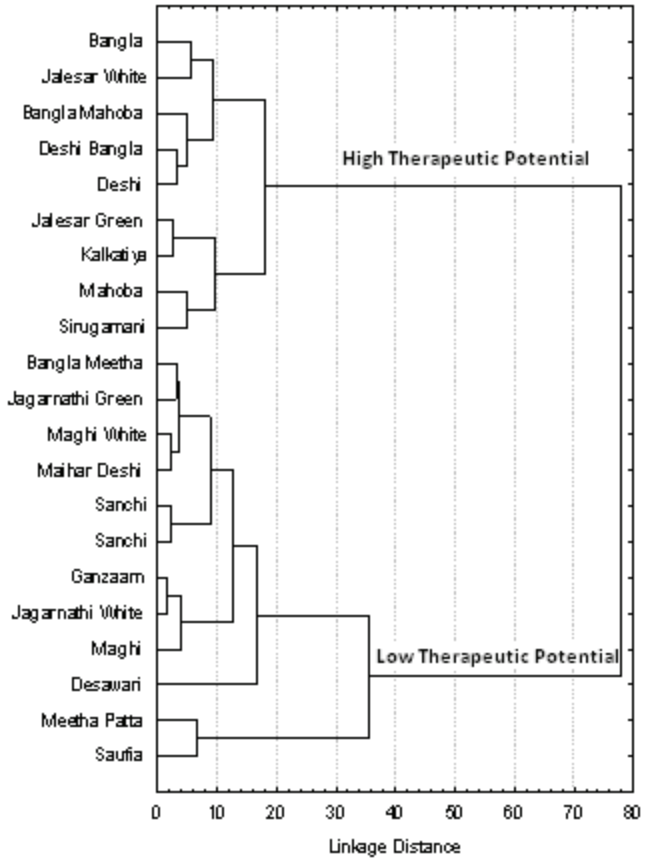



**Figure 3**: Tree view of *P. betle* landraces according to average of three biologically active ions (*m/z* 151,165 and 193) estimated by DART MS. Upper cluster from Bangla to Sirugamani and the lower cluster from Bangla Meetha to Saufia, respectively, represent higher and lower therapeutic potential of the landraces.

The clustering of twenty-one PB landraces was based on three ions (Table 1) and summarised in Figure 3. The average of the three ions clustered twenty-one landraces into two broad groups. Landraces Bangla, Bangla Mahoba, Deshi Bangla, Deshi, Jalesar Green, Jalesar White, Kalkatiya, Mahoba, and Sirugamani, with higher

APC and/or carvacrol contents, clustered in one group. The other group comprised landraces Bangla Meetha, Desawari, GanzaamUdisa, Jagarnathi Green, Jagarnathi White, Maghi white, Maghi, MaiharDeshi, MeethaPatta, Sanchi*, Sanchi**, and Saufia, with low APC and/or carvacrol or higher eugenol and/or CHV content. Considering the importance of APC in biological activity, groups with high and low APC were, respectively, designated as high and low therapeutic potential groups. Thus, the DART-MS profiles for APC and CHV is a good predictor of the therapeutic potential of *P. betle* landraces. Percent ionization of bioactive molecules can be taken as a parameter to compare the relative biological activity of landraces based on the assumption that ions are directly related with molecules. Further studies on quantification and biological activity are required to authenticate these findings. It is estimated that in India nearly one hundred PB landraces are in cultivation [3]. Thus, in order to select the most suitable ones for medicinal uses, it is important to screen the available biodiversity by generating their chemical profile. Thus screening of the landraces will not only validate existing information, but will also yield new data on their chemical constituents for predicting biological activity. It will also help in optimizing mass cultivation of the best suited landraces for obtaining drug(s) leading to the best utilization of natural resource.

**Statistical analysis:** Neighbor joining (NJ) tree for PB landraces was generated from the *m/z* peak data such that only the polymorphic peaks are included in the analysis. The binary state criterion (present/ absent) was used for generating the NJ tree by FREETREE software using the Jaccard coefficient and the NJ method. Four peaks present in all PB landraces screened were not included in this analysis. Cluster analysis was based on three known biologically active ions (*m/z* 151, *m/z* 165 and *m/z* 193) after standardization (mean=0, SD=1). All statistical analyses were performed on STATISTICA windows version 7.0 (StatSoft, Inc., USA).

## Experimental

Twenty-one PB Indian landraces, namely Bangla, Bangla Mahoba, Deshi Bangla, Desawari, Deshi, Jalesar Green, Jalesar White, Kalkatiya, Mahoba and Sirugamani (from Lucknow); GanzaamUdisa, Jagarnathi Green, Jagarnathi White, Maghi White, MaiharDeshi, and Sanchi* (from Varanasi); and Bangla Meetha, Maghi, MeethaPatta, Sanchi** and Saufia (from Kolkata); were used in the study. The leaves were collected from different markets and institutions in India and were washed and wiped dry before use. The mass spectrometer (JMS-T100 LC; AccuTof, atmospheric pressure ionization time-of-flight mass spectrometer, Jeol, Tokyo, Japan) fitted with a DART ion source was used in the study. The mass spectrometer was operated in positive-ion mode with a resolving power of 6000 (full-width at half-maximum). The orifice 1 potential was set to 28 V, resulting in minimal fragmentation. The ring lens and orifice 2 potentials were set to 13 and 5 V, respectively. Orifice 1 was set at 100°C. The RF ion guide potential was 300 V. The DART ion source was operated with helium gas flowing at approximately 4.0 L/min. The gas heater was set to 300°C. The potential on the discharge needle electrode of the DART source was set to 3000 V; electrode 1 was 100 V and the grid was at 250 V. Freshly cut pieces of betel leaf were positioned in the gap between the DART source and mass spectrometer for measurements. Data acquisition was from *m/z* 10 to 1050. Exact mass calibration was accomplished by including a mass spectrum of neat polyethylene glycol (PEG), as well as a 1:1 mixture of PEG 200 and PEG 600 in the data file. *m*-Nitrobenzyl alcohol was also used for calibration. The mass calibration was accurate to within ± 0.002 *m/z* and the elemental composition was determined on selected peaks using the Mass Center software.

**Acknowledgments** – Grateful acknowledgement is made to SAIF, CDRI (CSIR), Lucknow, where the mass spectrometric studies were carried out. V B is thankful to CSIR, New Delhi for research grant NWP0045. Thanks are given to Dr SA Ranade, Scientist, National Botanical Research Institute Lucknow for helpful discussion. CDRI Communication No 8361

## References

[1] Richard R. (**1999**)*The Lost Civilizations of the Stone Age*. The Free Press, New York. USA.
[2] Thakur BS, Chunekar,KC. (**1972**) *Glossary of Vegetable Drugs in Brahattrayi*.Chowkhamba Sanskrit Series, Varanasi, India.
[3] Kumar N, Misra P, Dube A, Bhattacharya S, Dikshit M, Ranade SA. (**2010**) *Piper betle* Linn. a maligned Pan-Asiatic plant with an array of pharmacological activities and prospects for drug discovery. *Current Science*, ***99***, 922-932.
[4] Balasubrahmanyam VR, Johri JK, Rawat AKS, Tripathi RD, Chaurasia RS. (**1995**) Betelvine (*Piper betle* L.), NBRI, Lucknow, India.
[5] Ranade SA, Verma A, Gupta M, Kumar N. (**2002**) RAPD profile analysis of betel vine cultivars. *Biologia Plantarum*, ***45***, 523–527.
[6] Verma A, Kumar N, Ranade SA. (**2004**) Genetic diversity amongst landraces of a dioeciousvegetatively propagated plant, betelvine (*Piper betle* L.). *Journal of Biosciences*, ***29***, 319-328.
[7] Bajpai,V, Sharma D, Kumar B, Madhusudanan KP. (**2010**) Profiling of *Piper betle* Linn. cultivars by direct analysis in real time mass spectrometric technique. *Biomedical Chromatography*, ***24***, 1283-1286.
[8] Mula S, Banerjee D, Patro BS, Bhattacharya S, Barik A, Bandopadhyay SK, Chattopadhyay S. (**2008**) Inhibitory property of the *Piper betle*phenolics against photosensitization-induced biological damages. *Bioorganic and Medicinal Chemistry*, ***16***, 2932-2938.
[9] Maisuthisakul P, Suttajit M, Pongsawatmanit R. (**2007**) Assessment of phenolic content and free radical scavenging capacity of some Thai indigenous plants. *Food Chemistry*, ***100***, 1409-1418.
[10] Tripathi S. (**2008**) Chemical investigation of betel vine (*Piper betle* L.) as antioxidant agent. PhD Thesis,Lucknow University, Lucknow, India.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1631 - 1634

# Total Phenolic Content and Antioxidative Properties of Commercial Tinctures Obtained from Some Lamiaceae Plants

**Adam Kowalczyk[a*], Izabela Biskup[a] and Izabela Fecka[a]**

[a]*Department of Pharmacognosy*, *Wrocław Medical University*, *Nankiera 1*, *50-140 Wrocław*, *Poland*

*akow2@poczta.onet.pl*

**Received: September 3rd, 2012; Accepted: October 17th, 2012**

The antioxidant level of commercial tinctures from three Lamiaceae plants, *Salvia officinalis*, *Mentha piperita*, and *Melissa officinalis*, have been determined by the Folin-Ciocalteu method, the 2.2-diphenyl-2-picrylhydracyl hydrate (DPPH) radical scavenging technique and ABTS assay. Total phenolic content was expressed as GAE (gallic acid equivalent) and ranged from 0.24 to 3.99 mg/mL. Antioxidant activity in the ABTS assay, calculated as TEAC (Trolox equivalent antioxidant capacity), ranged from 23.5 to 35.6 μmol Trolox/mL, while in the DPPH method, the $EC_{50}$ value ranged from 0.04 to 0.07 mL/assay. Radical scavenging activity was correlated with total phenolic content. Correlations between ABTS and F-C methods, DPPH and F-C methods and ABTS and DPPH methods were calculated. The obtained results can be useful as additional information about the antioxidant activity of galenical preparations.

**Keywords:** Antioxidant activity, DPPH, ABTS, Total phenolic content, S*alvia officinalis*, *Mentha piperita*, *Melissa officinalis*, Galenical preparations.

Natural antioxidants play a very important role in the prevention of different diseases such as cancer, arteriosclerosis and neurodegenerative diseases [1-3]. Therefore, there is an increasing interest in the antioxidant effects of natural compounds from medicinal plants and pharmaceutical products for health [4-8]. In particular, a great number of different species and aromatic herbs have been investigated for their antioxidant activity [9,10]. Lamiaceae plants are widely cultivated and mainly used in medicine and as culinary herbs. *Salvia officinalis*, one of the best known plants from the family, is rich in flavonoids (luteolin and apigenin glycoside), tannins (e.g., rosmarinic acid, caffeate oligomers) phenolic glycosides and triterpenes [11-18]. Sage is frequently used by people suffering from gastrointestinal problems and excessive perspiration, as well as a gargle for inflammation in the mouth or throat [19]. Peppermint (*Mentha* × *piperita*) contains, for example, caffeic acid derivatives, flavonoids, phenolic acids, volatile oil, and terpenoids. Peppermint leaves have spasmolytic, analgesic and carminative activities [20,21]. The leaves of lemon balm (*Melissa officinalis*), a herb found in southern Europe and the Mediterranean region, contain caffeate oligomers, flavonoids, terpenoids and volatile oil. Lemon balm has spasmolytic and sedative properties and is commonly used in the form of tea infusions [22-24], and in extract form given orally to treat gastrointestinal troubles and nervous disorders

One of the easiest forms of pharmaceutical formulation is either an alcoholic extract of leaves or other plant material. It is easy for production and usage by patients, and this, therefore, is a reason of its popularity. The pharmaceutical formulations, e.g. extracts or tinctures, have an impact on the pharmacokinetics, pharmacodynamics and the safety profile of medicaments.

The objective of this work was to evaluate the antioxidant activity of commercial alcoholic extracts of *Salvia officinalis*, *Mentha piperita* and *Melissa officinalis* and to correlate these results with their total phenolic content. All products were produced by pharmaceutical companies and can be obtained as OTC preparations in a dispensing pharmacy or herbal shop. This kind of galenical formulation is easily available on the market and is very popular among patients. Because of this it is worth confirming if it has antioxidative properties and in this way whether it can be of added benefit for health.

**Total phenolic content and antioxidant activity:** The total phenolic content (TPC), determined by the Folin-Ciocalteau method, expressed in gallic acid equivalents (GAE), ranged from 2.12 (±0.03) to 3.00 (±0.03) for lemon balm samples, from 2.02 (±0.02) to 3.99 (±0.03) for sage samples, and from 0.24 (±0.01) to 0.80 (±0.02) for peppermint samples (Table 1).

**Table 1**: Total phenolic contents (TPC) of analyzed samples (n=3). All values are expressed as mean ±SD for three determinations.

| Lemon balm samples | TPC,GAE (mg /mL) | Sage samples | TPC, GAE (mg /mL) | Peppermint samples | TPC, GAE (mg /mL) |
|---|---|---|---|---|---|
| MoIntr1 | 3.00±0.03 | SoTinc1 | 3.69±0.05 | MpGtt1 | 0.73±0.02 |
| MoIntr2 | 2.46±0.04 | SoTinc2 | 2.08±0.05 | MpGtt2 | 0.64±0.01 |
| MoIntr3 | 2.57±0.04 | SoTinc3 | 2.02±0.02 | MpGtt3 | 0.24±0.01 |
| MoIntr4 | 2.12±0.03 | SoTinc4 | 3.48±0.01 | MpGtt4 | 0.35±0.01 |
| MoIntr5 | 2.53±0.02 | SoTinc5 | 3.01±0.03 | MpGtt5 | 0.80±0.02 |

The average lowest phenol concentration was estimated for extracts from peppermint, while the highest was for sage preparations. Differences in total phenolic content between samples are due to the differences in the plant materials, and also might be caused by the method of production. *Intractum* is a galenical preparation obtained by extraction of fresh plant material with 70% ethanol in water after its stabilization, while tincture is a 70% ethanol extract produced from the dried plant material.

Two methods were used for antioxidant activity estimation. The results from the ABTS method are expressed as TEAC (Trolox equivalents antioxidant capacity) (Table 2). According to this assay, the best antioxidant of all the tested preparations was the lemon balm samples. The lowest antioxidant properties were obtained for peppermint preparations.

The antioxidant properties of the tested preparations were correlated with total phenolic content (Figure 1). The highest correlation was obtained for lemon balm ($R^2 = 0.973$), and the lowest for sage(R2 = 0.543).

**Table 2**: Antioxidant capacity of analyzed samples measured in the ABTS radical scavenging assay (n=3).

| TEAC (µmol TROLOX/mL) | | | | | |
|---|---|---|---|---|---|
| **Sage samples** | | **Peppermint samples** | | **Lemon balm samples** | |
| So Tinc 1 | 46.13 | MpGtt 1 | 5.50 | Mo Intr 1 | 35.59 |
| S oTinc 2 | 24.09 | MpGtt 2 | 7.83 | Mo Intr2 | 27.47 |
| S oTinc 3 | 23.87 | MpGtt 3 | 1.91 | Mo Intr 3 | 30.45 |
| S oTinc 4 | 36.36 | MpGtt 4 | 2.88 | Mo Intr4 | 24.75 |
| S oTinc 5 | 10.60 | MpGtt 5 | 8.49 | Mo Intr 5 | 28.35 |
| S oTinc 6 | 24.93 | MpGtt65 | 3.37 | Mo Intr 6 | 23.56 |

Positive correlations between antioxidant activity and phenol content were found suggesting that the antioxidant capacity of the analyzed products was due, to a great extent, to their polyphenols.

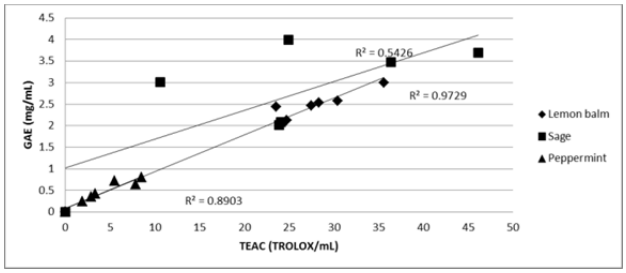



**Figure 1**: Total phenolic content and ABTS methods correlation

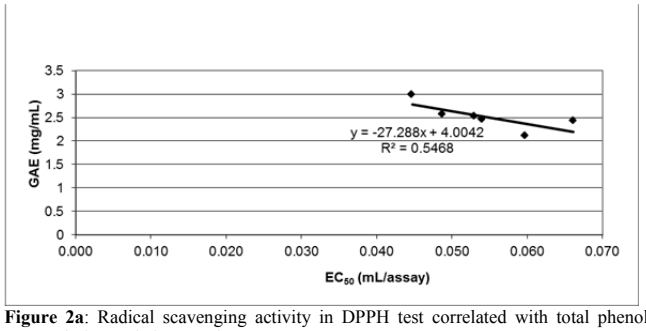



**Figure 2a**: Radical scavenging activity in DPPH test correlated with total phenolic content in lemon balm samples.

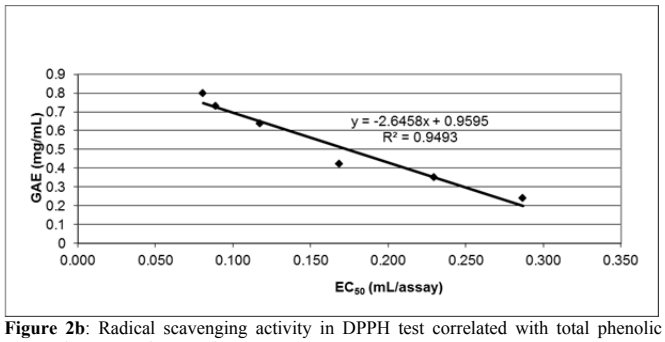



**Figure 2b**: Radical scavenging activity in DPPH test correlated with total phenolic content in peppermint samples.

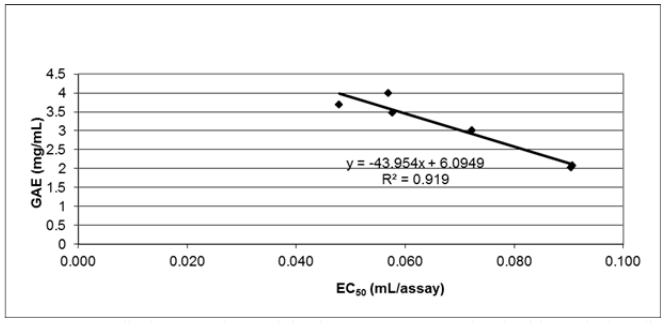



**Figure 2c**: Radical scavenging activity in DPPH test correlated with total phenolic content in sage samples.

**Table 3**: Antioxidant capacity in analyzed samples measured in DPPH radical scavenging assay (n=3).

| $EC_{50}$ (mL/assay) | | | | | |
|---|---|---|---|---|---|
| **Lemon balm samples** | | **Sage samples** | | **Peppermint samples** | |
| Mo Intr 1 | 0.04 | S oTinc 1 | 0.05 | MpGutt 1 | 0.09 |
| Mo Intr 2 | 0.05 | S oTinc 2 | 0.09 | MpGutt2 | 0.12 |
| Mo Intr 3 | 0.05 | So Tinc 3 | 0.09 | MpGutt3 | 0.29 |
| Mo Intr 4 | 0.06 | S oTinc 4 | 0.06 | MpGutt4 | 0.23 |
| Mo Intr 5 | 0.05 | S oTinc 5 | 0.07 | MpGutt5 | 0.08 |
| Mo Intr 6 | 0.07 | S oTinc 6 | 0.06 | MpGutt6 | 0.17 |

The second method used for the estimation of antioxidant properties of the tested preparations was the DPPH radical scavenging assay. The results are expressed as $EC_{50}$ (mL/assay) (Table 3). The lowest values were obtained for lemon balm and the highest for peppermint, which means that lemon balm preparations were stronger antioxidants in this test compared with the sage and peppermint samples.

Radical scavenging activity from this test was also correlated with total phenolic content (Figures 2a,b,c). The highest correlation was observed for peppermint preparations ($R^2 = 0.9493$).

Additionally, the correlation between ABTS and DPPH methods was estimated (Table 4). ABTS and DPPH radical scavenging activities correlated very well for lemon balm samples ($R^2$=0.897), while for sage samples this correlation was the lowest ($R^2$=0.364).

**Table 4**: Correlation between two radical scavenging assays: ABTS and DPPH assays.

| **Sample** | **y** | **$R^2$** |
|---|---|---|
| Lemon balm | y = -532.93x + 57.35 | R² = 0.897 |
| Peppermint | y = -28.908x + 9.69 | R² = 0.767 |
| Sage | y = -403.75x + 55.69 | R² = 0.364 |

Galenical preparations, based on traditional recipes, are produced in many countries. The pharmacological activity connected with the plant material can be extended also by their antioxidant properties, which are closely linked with the way of production – either extracts or tinctures.

The results obtained in the present study exhibit that high antioxidant and free radical scavenging activities depend both on the plant material and the form of the product. Therefore, studies on pharmaceutical preparations would be of great importance. Pharmaceutical products from Lamiaceae plants are widely used in medicine.Therefore, they seem to have additional benefits for health as antioxidants.

## Experimental

***Chemicals and reagents:*** Organic solvents and reagents used were of analytical grade. Water was glass-distilled and deionised. Methanol (MeOH) was obtained from Merck (Germany), Folin-Ciocalteu (F-C) reagent (POCH) from Sigma Aldrich, and ABTS (2.2'-azinobis(3-ethylbenzothiazoline-6 sulfonic acid) diammonium salt) and potassium persulfate (di-potassium peroxdisulfate) from Sigma-Aldrich.

***Standards:*** Gallic acid was purchased from Koch-Light Laboratories, UK, and Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman- 2-carboxylic acid) from Hoffman-La Roche.

***Plant material:*** The following commercial methanolic extracts, obtained from a dispensing pharmacy as over the counter products, were analyzed: five different extracts (*intractum*) of lemon balm - **MoIntr1, MoIntr2, MoIntr3, MoIntr4, MoIntr5, MoIntr6 -** (PhytoPharmKlękaSA,Poland), 10 different tinctures (*tinctura*)

from peppermint – **MpGtt1, MpGtt2, MpGtt3, MpGtt4, MpGtt5, MpGtt6** (Hasco-Lek SA, Wrocław Poland, Aflofarm Sp. z o.o., Poland, "Gemi" Karczew, Poland), and sage **SoTinc1, SoTinc2, SoTinc3, SoTinc4, SoTinc5, SoTinc6 (**HerbapolKraków S.A., Poland, PhytoPharm, Klęka S.A., Poland). All products differed from one another by serial number and expiry date.

***Total phenols determination:*** Total phenolic content was determined spectrophotometrically according to the method of Singleton & Rossi [25]. Briefly, 250 µL of the galenical preparation as methanolic solutions were added to tubes. Next, 250 µL F-C reagent, 500 µL of saturated sodium carbonate and 4 mL of distilled water were added immediately. Samples were vortexed and left for 30 min. incubation at room temperature away from light. After 20 min incubation, the samples were centrifuged for 5 min at 4000 rpm. Supernatants were used for spectrophotometric determination at 725 nm. The assay was calibrated against gallic acid standard solutions of different concentrations (0.025 – 0.15 mg/mL) and the results were expressed as GAE (gallic acid equivalents). Data presented are average of 3 measurements.

***DPPH radical scavenging activity:*** The experimental method followed that of Brand-Williams *et al*. [26]. A methanolic solution of DPPH (concentration 0.25 mg/mL) was prepared 2 h before the analysis and left at room temperature in the dark. 0, 20, 40, 60, 80, 100 µL of methanolic samples of each galenical preparation were added to tubes and filled with methanol to 100 µL. Next, 2 mL of methanol and 0.25 mL DPPH solution were added to all tubes, vortexed and left for 20 min in a dark place; the absorbance was then taken at 517 nm. Methanol was used as a blank solution. Results are the average of 3 measurements.

***ABTS radical scavenging assay:*** Experiments were conducted according to Re *at al.* [27], with small modifications. Trolox (2.5 mM) was prepared in methanol for use as a stock standard. Working standards were prepared daily on the dilution with methanol. ABTS and potassium persulfate were dissolved in distilled water to a final concentration: 7 mM and 2.45 mM, respectively. These 2 solutions were mixed and kept in the dark at room temperature for 16 h before use in order to produce the ABTS radical ($ABTS^{\bullet+}$). For the study of phenolic compounds, the ABTS radical solution was diluted with distilled water to an absorbance of 0.700 at 734 nm. 20 µL of methanolic samples of the galenical preparations (diluted 10, 20 or 30 times) or Trolox standards (final concentration 0.2 – 1.757 µmol/mL) were added to 2 mL diluted ABTS•+ solution and the absorbance reading taken after mixing and a 6 min incubation at room temperature. Distilled water was used as blank solution. All determinations were carried out in triplicate. The percentage of inhibition of absorbance at 734 nm was calculated and plotted as a function of concentration of either antioxidants or Trolox. The results were expressed as TEAC (Trolox equivalents).

***Statistical analysis****:* All data were the average of triplicate analyses. Mean values, standard deviations (SD), medians, and both minimum and maximum contents of all achieved results have also been determined. Correlation analysis of antioxidant activity was carried out using the correlation and regression program in the Microsoft EXCEL program.

## References


[1] Chu YF, Chen Y, Brown PH, Lyle BJ, Black RM, Cheng IH, Ou B, Prior RL. (**2012**) Bioactivities of crude caffeine: Antioxidant activity, cyclooxygenase-2 inhibition, and enhanced glucose uptake. *Food Chemistry*, ***131***, 564-568.

[2] Fernández-Mar MI, Mateos R, García-Parrilla MC, Puertas B, Cantos-Villar E. (**2012**) Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Food Chemistry*, ***130***, 797-813.

[3] Kontou N, PsaltopoulouT, Panagiotakos D, Dimopoulos MA, Linos A. (**2011**) The Mediterranean diet in cancer prevention: A review. *Journal of Medicinal Food*, ***14***, 1065-1078.

[4] Katz DL, Doughty K, Ali A. (**2011**) Cocoa and chocolate in human health and disease. *Antioxidants and Redox Signaling*, ***15***, 2779-2811.

[5] Vertuani S, Beghelli E, Scalambra E, Malisardi G, Copetti S, Toso RD, Baldisserotto A, Manfredini S. (**2011**) Activity and stability studies of verbascoside, a novel antioxidant, in dermo-cosmetic and pharmaceutical topical formulations. *Molecules*, ***16***, 7068-7080.

[6] Ramful D, Aumjaud B, Neergheen VS, Soobrattee MA, Googoolye K, Aruoma OI, Bahorun T. (**2011**) Polyphenolic content and antioxidant activity of *Eugenia pollicina* leaf extract *in vitro* and in model emulsion systems. *Food Research International*, ***44***, 1190-1196.

[7] Fecka I, Turek S. (**2007**) Determination of water-soluble polyphenolic compounds in commercial herbal teas from Lamiaceae: peppermint, melissa, and sage. *Journal of Agriculture and Food Chemistry*, ***55***, 10908-10917.

[8] Fecka I, Turek S. (**2008**) Determination of polyphenolic compounds in commercial herbal drugs and spices from Lamiaceae: thyme, wild thyme and sweet marjoram by chromatographic techniques. *Food Chemistry*, ***108***, 1039-1053.

[9] Lu Y, Foo LY. (**2000**) Flavonoid and phenolic glycosides from *Salvia officinalis*. *Phytochemistry*, ***55***, 263-267.

[10] Cuvelier ME, Berset C, Richard H. (**1994**) Antioxidant constituents in sage (*Salvia officinalis*). *Journal of Agriculture and Food Chemistry,* ***42***, 665-669.

[11] Lu Y, Foo LY, Wong H. (**1999**) Sagecoumarin, a novel caffeic acid trimer from *Salvia officinalis*. *Phytochemistry*, ***52***, 1149-1152.

[12] Wang M, Shao Y, Li J, Zhu N, Rangarajan M, LaVoie EJ, Ho CT. (**1999**) Antioxidative phenolic glycosides from sage (*Salvia officinalis*), *Journal of Natural Products*, ***62***, 454-456.

[13] Lu Y, Foo LY. (**1999**) Rosmarinic acid derivatives from *Salvia officinalis*. *Phytochemistry,* ***51***, 91-94.

[14] (a) LuY, Foo LY. (**2001**) Antioxidant activities of polyphenols from sage (*Salvia officinalis*). *Food Chemistry*, ***75***, 197-202; (b) LuY, Foo LY. (**2000**) Flavonoid and phenolic glycosides from *Salvia officinalis. Phytochemistry*, ***55***, 263-267.

[15] LuY, Foo LY. (**2002**) Polyphenolics of *Salvia*—a review. *Phytochemistry*, ***59***, 117–140.

[16] Koşar M, Damien Dorman HJ, Hüsnü Can Başer K, Hiltunen R. (**2010**) *Salvia officinalis* L.: Composition and antioxidant-related activities of a crude extract and selected sub-fractions. *Natural Product Communications*, ***5***, 1453-1456.

[17] Areias F, Valentão P, Andrade PB, Ferreres F, Seabra RM. (**2000**) Flavonoids and phenolic acids of sage: Influence of some agricultural factors. *Journal of Agriculture and Food Chemistry*, ***48***, 6081-6084.

[18] Miguel G, Cruz C, Faleiro ML, Simões MTF, Figueiredo AC, Barroso JG, Pedro LG. (**2011**) *Salvia officinalis* L. essential oils: effect of hydrodistillation time on the chemical composition, antioxidant and antimicrobial activities. *Natural Product Research*, ***25***, 526-541.

[19] Stanojević D, Čomić L, Stefanović O, Solujić-Sukdolak S. (**2010**) *In vitro* synergistic antibacterial activity of *Salvia officinalis* L. and some preservatives. *Archives of Biological Sciences*, ***62***, 167-174.

[20] Tyagi AK, Malik A. (**2011**) Antimicrobial potential and chemical composition of *Mentha piperita* oil in liquid and vapour phase against food spoiling microorganisms. *Food Control*, ***22***, 1707-1714.

[21] Abdolmaleki M, Bahraminejad S, Salari M, Abbasi S, Panjeke N. (**2011**) Antifungal activity of peppermint (*Mentha piperita* L.) on phytopathogenic fungi. *Journal of Medicinal Plants*, ***10***, 26-34.

[22] (a) Azar PA, Porgham-Daryasari A, Sharifan A, Saber-Tehrani M, Soleimani M.(**2011**) Antibacterial activity and chemical constitutions of *Melissa officinalis* L. *Asian Journal of Chemistry*, ***23***,1381-1383; (b) Koksal E, Bursal E, Dikici E, Tozoglu F, Gulcin I. (**2011**) Antioxidant activity of *Melissa officinalis* leaves. *Journal of Medicinal Plant Research*, ***5***, 217-222.

[23] (a) Suschke U, Sporer F, Schneele J, Heinrich Konrad Geiss HK, Reichling J. (**2007**) Antibacterial and cytotoxic activity of *Nepeta cataria* L., *N. cataria* var. *citriodora* (Beck.) Balb. and *Melissa officinalis* L. essential oils. *Natural Product Communications*, ***2***, 1277-1286; (b) Uysal B, Sozmen F, Buyuktas BS. (**2010**) Solvent–free microwave extraction of essential oils from *Laurus nobilis* and *Melissa officinalis*: Comparison with conventional hydro-distillation and ultrasound extraction. *Natural Product Communications*, ***5***, 111-114.

[24] Moradkhani H, Sargsyan E, Bibak H, Naseri B, Sadat-Hosseini M, Fayazi-Barjin A, Meftahizade H. (**2010**) *Melissa officinalis* L., a valuable medicine plant: A review. *Journal of Medicinal Plant Research*, ***4***, 2753-2759.

[25] Singleton VL, Rossi JA Jr. (**1965**) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, ***16***, 144-158.

[26] Brand-Williams W, Cuvelier ME, Berset C. (**1995**) Use of free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft & Technologie*, ***28***, 28-30.

[27] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (**1999**) Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, ***26***, 1231-1237.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1635 - 1638

# Synthesis, Antifungal Activity and Structure-Activity Relationships of Vanillin oxime-*N*-*O*-alkanoates

**Vivek Ahluwalia[a,b], Nandini Garg[a,c], Birendra Kumar[a], Suresh Walia[a*] and Om P. Sati[b]**

[a]*Division of Agricultural Chemicals*, *Indian Agricultural Research Institute*, *New Delhi 110 012*, *India*
[b]*Department of Chemistry*, *HNB Garhwal University*, *Srinagar (Garhwal)*, *Uttarakhand 246174*, *India*
[c]*Chem-Master International Inc.*, *State University of New York*, *Stony Brook NY*, *11794-3400*, *USA*

*suresh_walia@yahoo.com*

**Received: March 31st, 2012: Accepted: October 22nd, 2012**

Vanillin oxime-*N*-*O*-alkanoates were synthesized following reaction of vanillin with hydroxylamine hydrochloride, followed by reaction of the resultant oxime with acyl chlorides. The structures of the compounds were confirmed by IR, $^{1}H$, $^{13}C$ NMR and mass spectral data. The test compounds were evaluated for their *in vitro* antifungal activity against three phytopathogenic fungi *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* by the poisoned food technique. The moderate antifungal activity of vanillin was slightly increased following its conversion to vanillin oxime, but significantly increased after conversion of the oxime to oxime-*N*-*O*-alkanoates. While vanillin oxime-*N*-*O*-dodecanoate with an $EC_{50}$ value 73.1 µg/mL was most active against *M. phaseolina,* vanillin oxime-*N*-*O*-nonanoate with $EC_{50}$ of value 66.7 µg/mL was most active against *R. solani*. The activity increased with increases in the acyl chain length and was maximal with an acyl chain length of nine carbons.

**Keywords:** Vanillin, Oxime esters, Structure activity relationships, Antifungal activity, *Macrophomina phaseolina*, *Rhizoctonia solani*, *Sclerotium rolfsii*.

Naturally occurring vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most abundant aroma ingredients of the *Vanilla* plant. It contributes a great deal to the typical vanilla flavor and is widely used in the perfume, fragrance and food industry. Vanillin is produced by a multistep curing process of the green pods of *V. planifolia, V. pompona* and *V. tahitiensis* [1]. The ethyl acetate extract of leaves and stem of *V. fragrans* has been reported to be toxic to mosquito larvae [2]. Vanillin has been shown to exhibit moderate antifungal [3,4] and antioxidant activity [5]. It showed minimal inhibitory concentrations (MICs) of 1250 and 738 µg/mL and minimal fungicidal concentrations (MFCs) of 5000 and 1761 µg/mL against *Candida albicans* and *C. neoformans*, respectively [6]. Structure activity relationship studies on vanillin and related aldehydes revealed that the aldehyde moiety plays a significant role in imparting antifungal activity [7].

Oxime ether groups of compounds are known for their insecticidal synergistic and insecticidal activity [8]. Among the large number of oximine compounds, piperonal oxime *N*-*O*-alkyl ether exhibited insecticide synergistic activity far greater than that of piperonyl butoxide [9]. Several of these also exhibited anti-insect properties against *Dysdercus kocnigii* [10] and *Schistocerca gregaria* [11]. Chowdhury *et al.* synthesized alkoxy-substituted benzaldoxime ethers and evaluated their IGR activity against the desert locust (*S. gregaria*) [11]. On topical application (at 20 µg/nymph), the most active 3-methoxy-4-ethoxybenzadoxime-*N*-methyl ether, caused 100% deformity. Very recently, the activity of benzoylphenylureas (BPUs) containing oxime ether and oxime ester groups were found effective against larvae of oriental armyworm and mosquito [12]. In another study, oxime esters of 2-tridecanone exhibited significant nematicidal activity against the root knot nematode *Meloidogyne incognita* [13]. Essential oil based citral oxime esters also exhibited significant antifungal activity against *Rhizoctonia solani* and *Sclerotium rolfsii* [14].

*Macrophomina phaseolina*, *Sclerotium rolfsii* and *Rhizoctonia solani* are major soilborne pathogens of many important crops worldwide. They cause damping-off of seedlings, and rot and collar rot on young and mature plants [15]. Control of these fungi is rather difficult in soil because of the extensive host range, their fast growing capacity, and the production of sclerotia that may persist in the soil for many years.

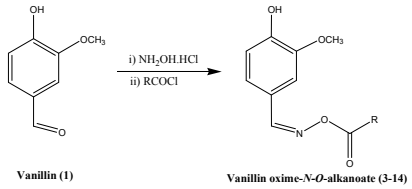
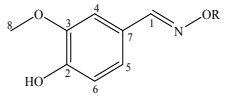



**Figure 1:** Synthesis of vanillin oxime-*N*-*O*-alkanoates

**Table 1**: % age yield of synthesized compounds.



| S. No. | Compounds | % Yield |
|---|---|---|
| **2** | R = H | 98.6 |
| **3** | R = $COCH_3$ | 88.5 |
| **4** | R = $COCH_2CH_3$ | 87.4 |
| **5** | R = $COCH_2CH_2CH_3$ | 84.4 |
| **6** | R = $COCH(CH_3)_2$ | 82.3 |
| **7** | R = $COCH_2(CH_2)_2CH_3$ | 65.7 |
| **8** | R = $COCH_2CH(CH_3)_2$ | 83.7 |
| **9** | R = $COC(CH_3)_3$ | 79.7 |
| **10** | R = $COCH_2(CH_2)_3CH_3$ | 79.2 |
| **11** | R = $COCH_2(CH_2)_4CH_3$ | 80.6 |
| **12** | R = $COCH_2(CH_2)_6CH_3$ | 89.6 |
| **13** | R = $COCH_2(CH_2)_7CH_3$ | 90.3 |
| **14** | R = $COCH_2(CH_2)_9CH_3$ | 80.2 |

Esters of vanillin and vanillin oxime are also known for their fragrance properties [16]. Since naturally occurring vanillin exhibits moderate antifungal activity, efforts were made to enhance the activity by chemical modifications of the lead molecule. The present paper reports the synthesis, antifungal activity and structural

activity relationship (SAR) of naturally occurring vanillin and its oxime esters against *M. phaseolina*, *R. solani* and *S. rolfsii.*

Vanillin, on reaction with hydroxylamine hydrochloride, yielded vanillin oxime (**2**), which is usually a 1:1 mixture of *E* and *Z* isomers. The structure of vanillin oxime was confirmed by IR, NMR and mass spectral data. The IR spectra of the compound showed presence of absorption bands at 3600, 1665 and 945 $cm^{-1}$ confirming formation of oxime moiety. In the $^1H$ NMR spectrum, the aldehyde peak in vanillin recorded at δ 9.8 was absent in vanillin oxime. Appearance of an additional peak at δ 8.10 due to the H-1 proton indicated the presence of the aldehyde oxime moiety. Vanillin oxime also showed an additional $D_2O$ exchangeable proton singlet at δ 2.03 corresponding to the oxime proton. The $^{13}C$ NMR spectra of vanillin and vanillin oxime exhibited almost similar patterns of carbon resonances except that the oxime carbon (C-1) appeared downfield at δ 150.39 and the peak appearing at δ191.02 for the aldehydic group was absent. The mass spectrum of vanillin oxime showed protonated and sodiated molecular adduct ion peaks at *m/z* 168.0 $[M+H]^+$ and 189.9 $[M+Na]^+$.

Vanillin oxime-*N*-*O*-alkanoates were prepared following the reaction of compound **2** with different acid chlorides (Figure 1**)**. The compounds were purified by either column chromatography or preparative TLC. The percentage yield of the different products is given in Table 1. The IR spectra of the compounds showed sharp band at 1760 $cm^{-1}$ confirming formation of oxime ester. The $^1H$ NMR spectra of the synthesized compounds **(3-14)** exhibited peaks characteristic of the vanillin oxime ester moiety. The pattern of the proton peaks was almost the same in a homologous series, but differed from each other with respect to the number of peaks corresponding to the methylene or methyl protons constituting the alkanoyl moiety. The $^1H$ NMR spectrum of the representative vanillin oxime-*N*-*O*-heptanoate (**11**) showed two doublets at δ 6.93 and δ 7.45, and one double doublet at δ 7.04 corresponding to aromatic protons. Compound **2** has two hydroxyl functions and either of them could have been acylated. Since a peak at δ 6.03, corresponding to a phenolic OH in vanillin, remained intact and an oxime proton detected at δ 2.03 in compound **2** was absent in the oxime ester molecule, it was inferred that only the oxime function had been acylated. Beside the oximine proton (H-1) at δ 8.26, triplet and multiplet peaks at δ 2.45 (H-2′) and δ 1.71 (H-3′), respectively, were characteristic of the molecules. Compound **11**, in its $^{13}C$ NMR spectrum, exhibited 15 peaks. The characteristic carbon resonances at δ171.52, 156.11 and 56.22 correspond to acyl carbon (C-3′), oxime carbon (C-1) and a methoxy group (C-8) respectively while peaks at δ149.24, 147.02, 124.40, 122.38, 114.40 and 107.57 were attributed to aromatic carbons, those at δ 31.44, 24.89, 32.91, 22.49 and 14.05 corresponded to carbons of the heptanoyl chain.

The mass fragmentation pattern of compound **11** is depicted in Figure 2. It showed a sodiated molecular adduct ion peak at *m/z* 302 along with another potassiated adduct ion peak at m/z 319. MS-MS of the sodiated ion peak resulted in fragment ion peaks at *m/z* 189.1 and 171.9 due to sequential loss of $CH_3(CH_2)_5CO$- and $CH_3(CH_2)_5COOH$, and at *m/z* 150.0 due to the loss of $CH_3(CH_2)_5COOH$ from the parent ion. On the basis of NMR and mass spectral data, the compound was identified as vanillin oxime-*N*-heptanoate. The NMR and mass spectra of other oxime alkanoates exhibited patterns of peaks characteristic of vanillin oxime-*N*-*O*-alkanoates.

The test compounds were assayed for their antifungal activity against the phytopathogenic fungi *M. phaseolina*, *R. solani* and *S. rolfsii* (Table 2). The test compounds exhibited significant antifungal activity. The activity was slightly increased following conversion of vanillin to vanillin oxime, but significantly increased after conversion of the oxime to oxime esters. Among the various oxime esters, vanillin oxime-*N*-*O*-dodecanoate (**14**) and vanillin oxime-*N*-*O*-decanoate (**13**), with respective $EC_{50}$ values of 73.1 µg/mL and 79.9 µg/mL were the most active. However, compared with the standard reference, Bavistin® ($EC_{50}$ 12.2 µg/mL against *M. phaseolina*), the oxime esters were less active.

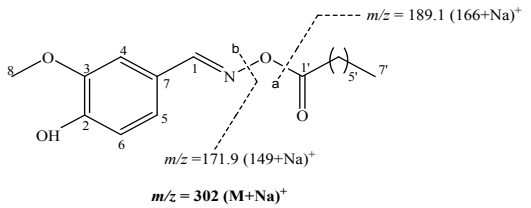



**Figure 2**: Mass fragmentation pattern of compound **11**.

**Table 2**: Antifungal activity of test compounds.

| Compounds | $EC_{50}$ (µg/mL) | | |
|---|---|---|---|
| | *M. phaseolina* | *R. solani* | *S. rolfsii* |
| **1** | 566.0 | 382.0 | 633.6 |
| **2** | 401.6 | 346.1 | 495.1 |
| **3** | 179.8 | 207.7 | 384.4 |
| **4** | 101.6 | 199.6 | 375.5 |
| **5** | 199.9 | 143.0 | 345.9 |
| **6** | 174.7 | 129.8 | 321.2 |
| **7** | 142.6 | 94.6 | 227.7 |
| **8** | 105.4 | 74.9 | 210.7 |
| **9** | 139.3 | 91.4 | 190.1 |
| **10** | 119.2 | 86.9 | 188.2 |
| **11** | 91.8 | 80.7 | 171.4 |
| **12** | 85.5 | 66.7 | 161.5 |
| **13** | 79.9 | 97.5 | 205.7 |
| **14** | 73.1 | 205.6 | 218.0 |
| **Bavistin®** | 12.2 | 10.4 | 15.5 |

A similar trend was evident with *R. solani.* Vanillin oxime-*N*-*O*-nonanoate (**12**) and vanillin oxime-*N*-*O*-isovalerate (**08**) with respective $EC_{50}$ value of 66.7 µg/mL and 74.9 µg/mL were most effective*.* Vanillin oxime-*N*-*O*-dodecanoate (**14**) and vanillin oxime-*N*-*O*-acetate (**03**), with respective long and short acyl chains, were moderately active ($EC_{50}$ 205-208 µg/mL).

Vanillin oxime-*N*-*O*-nonanoate (**12**) was found to be the most active ($EC_{50}$ = 161.5 µg/mL) against *S. rolfsii*. Other oxime esters (heptyl, hexyl, pivolyl and decanoyl) with $EC_{50}$ values ranging from 171.4 µg/mL to 205.7 µg/mL, were moderately active. On the basis of structure-activity relationships, it was revealed that the activity was dose dependent and increased with increase in acid chain length up to C-9 or C-10 and decreased thereafter for *R. solani* and *S. rolfsii*. However, in the case of *M. phaseolina*, the activity increased till the acyl chain length reached C-12. It was thus concluded that vanillin and related compounds bearing carbonyl functions can be a good template for developing ecologically sound pest control chemicals. Because of their environmentally benign nature, such compounds could gain considerable importance.

## Experimental

***Chromatography:*** TLC was performed on 200 µm thick plastic or aluminum TLC plates (silica gel 60 $F_{254}$). Purification of compounds was achieved either by Prep-TLC or CC over silica gel (60-120 mesh) preactivated at 110°C.

***Spectroscopy:*** $^1H$ NMR and $^{13}C$ NMR spectra were recorded on a Bruker Avance$^{II}$ 400 Spectrometer (400 MHz). Samples were dissolved in either $CDCl_3$ or DMSO (D-6). Tetramethyl silane (TMS) was used as internal standard. Chemical shifts were recorded

in δ (ppm) values relative to TMS and *J* values are expressed in Hertz. MS were recorded using a Thermo LC-MS-MS Spectrometer (Thermo Electron Corporation USA) equipped with a P-2000 binary pump. Mass was determined using an ESI source with a Finnigan LCQ tune plus program fitted with a MAX-detector. Xcalibur Software was used for the purpose of identification, quantification and fragmentation of the required masses. IR spectra were recorded in Nujol with a Nicolet Impact 700 FT-infra-red-spectrophotometer.

***Preparation of vanillin oxime:*** An equimolecular quantity of vanillin (4 g) in methanol (50 mL), hydroxylamine hydrochloride (1.35 g) and anhydrous potassium carbonate (4 g) was placed in a 250 mL round bottomed flask and the contents refluxed for 1 h. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was cooled, filtered and the solvent removed by distillation under vacuum. Ice chilled water (100 mL) was then added and the resultant oxime was extracted with ethyl acetate (3 × 50 mL). The organic phase was passed through anhydrous sodium sulfate to remove traces of water and the solvent evaporated under vacuum to obtain solid vanillin oxime (**2**). The product crystallized from methanol as fine powder.

**Compound 2**

$R_f$: 0.4 (*n*-hexane-acetone, 7:3);
IR: 3600 (OH), 3310, 3167, 2890, 1423, 1460, 1665 (C=N), 1250, 945 (N-O), 780 $cm^{-1}$.
$^1H$ NMR (400 MHz, $CDCl_3$): δ 2.03 (s, 1H, NOH-1), 3.90 (s, 3H, H-8), 6.03 (s, 1H, OH-2), 6.99 (d, 1H, H-6, *J* = 8 Hz), 7.01 (dd, 1H, H-4, *J* = 8 Hz and 1.8 Hz), 7.21 (d, 1H, H-5, *J* =1.8 Hz), 8.10 (s, 1H, H-1).
$^{13}C$ NMR (100 MHz, $CDCl_3$): δ 55.98 (C-8), 107.57 (C-7), 114.40 (C-6), 122.38 (C-5), 124.4 (C-4), 146.9 (C-3), 147.63 (C-2), 150.39 (C-1).
ESI-MS: *m/z* 168.0 [$(M+H)^+$, 100%], 189.9 [$(M+Na)^+$, 20%],.
MS-MS: *m/z* 151[168-OH, 100%].

***General procedure for the preparation of vanillin oxime-N-O-alkanoates:*** To a solution of vanillin oxime (**2**) (0.05 mM) in dichloromethane (30 mL) in a 100 mL round bottomed flask, 2-3 drops of triethylamine (TEA) was added. An equimolar quantity of the respective acid chloride (0.05 mM), dissolved in dichloromethane (1:2), was added drop wise. The reaction mixture was stirred for 3 h with a magnetic stirrer at 0-5°C and the progress of the reaction was monitored by TLC. After completion, cold water (100 mL) was added and the mixture extracted with dichloromethane (3 × 50 mL). The organic phase was then washed with water (2 × 50 mL) and the resultant organic phase passed through anhydrous sodium sulfate to remove traces of water. The solvent was removed under vacuum at a temperature not exceeding 40°C to obtain the desired oxime-*N*-*O*-alkanoate. The products were further purified by either CC over silica gel using *n*-hexane and *n*-hexane/acetone as the eluting solvent or by prep-TLC.

**Compound 11**

$R_f$: 0.6 (*n*-hexane-acetone, 7:3).
IR: 3310 (OH), 3167, 2890, 1760 (C=O), 1650 (C=N), 1515, 1460, 1423, 780 $cm^{-1}$.
$^1H$ NMR (400 MHz, $CDCl_3$): δ 0.88 (t, 3H, H-7′, *J* = 7.2 Hz), 1.30-1.41 (m, 6H, H-4′, 5′, 6′), 1.71 (m, 2H, H-3′), 2.45 (t, 2H, H-2′, *J* = 7.6 Hz), 3.95 (s, 3H, H-8), 6.03 (s, 1H, OH-2), 6.93 (d, 1H, H-6, *J* = 8Hz), 7.04 (dd, 1H, H-4, *J* = 8 Hz and 1.8 Hz), 7.45 (d, 1H, H-5, *J* = 1.6 Hz), 8.26 (s, 1H, H-1).
$^{13}C$ NMR (100 MHz, $CDCl_3$): δ 14.05 (C-7′), 22.49 (C-6′) 32.91 (C-5′), 28.82 (C-4′), 24.89 (C-3′), 31.44 (C-2′), 56.22 (C-8), 107.57 (C-7), 114.40 (C-6), 122.38 (C-5), 124.4 (C-4), 147.02 (C-3), 149.24 (C-2), 156.11 (C-1), 171.52 (C-1′).
ESI-MS: *m/z* 302.0 [$(M+Na)^+$, 100%], 319.0 [$(M+H+K)^+$, 8%].
MS-MS: *m/z* 189.6 [302.0-$CO(CH_2)_5CH_3$, 75%], 171.9 [302-$CH_3(CH_2)_5COOH$, 30%], 150 [280.0- $CH_3(CH_2)_5COOH$, 20%]

**Bioassay**

***Fungicidal activity:*** *Macrophomina phaseolina* (ITCC 6267), *Rhizoctonia solani* (ITCC 4502), and *Sclerotium rolfsii* (ITCC 6263) were procured from the Indian Type Culture Collection (ITCC) center, Division of Mycology and Plant Pathology, IARI, New Delhi, India. The cultures were maintained on potato dextrose agar (PDA) slants at 27±1°C and were subcultured prior to testing. Antifungal activity of the compounds was carried out employing the poisoned food technique using 4% PDA medium by the macrodilution method [17]. A stock solution of each compound was prepared by dissolving 130 mg in 2 mL acetone. An appropriate quantity of the test compound in acetone was added to molten PDA medium in order to obtain the desired concentration. The medium of each concentration and control was poured into a set of 3 Petri-dishes (3-replications) under aseptic conditions in a laminar flow chamber. The dishes were then kept under UV light in the laminar flow chamber till the medium partially solidified. A 5 mm thick disc of fungus (spore and mycelium) cut from earlier sub-cultured Petri-dishes was put at the centre of the semi solid medium in the test Petri-dishes and the lids of the dishes were fixed. Both treated and control dishes were kept in a BOD incubator at 28±1°C till the fungal growth in the control dishes was complete (4-6 days), after which the readings were taken. The mycelial growth (cm) in both treated (T) and control (C) Petri-dishes was measured diametrically in 3 different directions. From the mean results of the above readings, the percentage inhibition of growth (I) and corrected inhibition (IC) were calculated using Abbott's formula:

$$I\ (\%) = [(C-T)/C] \times 100$$

Percent inhibition (I) was converted to corrected per cent inhibition (IC) using the following formula.

$$IC = \{[I(\%)-C.F.] / (100-C.F.)\} \times 100$$

IC = corrected percent inhibition, CF = correction factor

$$C.F.\ (Correction\ Factor) = [(90-C)/C] \times 100$$

Where, 90 mm is the diameter of the Petri-dishes and C is the growth of the fungus in mm in control.

***Statistical analysis:*** $EC_{50}$ (μg/mL) (effective concentration for 50 percent inhibition of mycelial growth) was calculated from the concentration (μg/mL) and corresponding IC data of each compound, with the help of Statistical Package (GW BASIC) using a personal computer (PC) [18].

**Supplementary data:** The spectral data of all compounds is included in a "Supplementary Data" section.

**Acknowledgments-** Authors are thankful to Head, Division of Agricultural Chemicals, IARI, New Delhi, India for providing the necessary facilities.

*IARI contribution No. 1108.

**References**

[1] Walton NJ, Mayer MJ, Narbad A. (**2003**) Molecules of interest vanillin. *Phytochemistry*, ***63***, 505-515.
[2] Sun R, Sacalis JN, Chin CK, Still CC. (**2001**) Bioactive aromatic compounds from leaves and stem of *Vanilla fragrance. Journal of Agricultural and Food Chemistry*, ***49***, 5161-5164.

[3] Cerrutti P, Alzamora SM. (**1996**) Inhibitory effect of vanillin in some food spoilage yeasts in laboratory media and fruit purees. *International Journal of Food Microbiology*, ***29***, 379-386.
[4] Fitzgerald DJ, Stratford M, Gasson MJ, Narbad A. (**2004**) The potential application of vanillin in preventing yeast spoilage of ready-to-drink beverages. *Journal of Food Protection*, ***67***, 391-395.
[5] Burri J, Graf M, Lambelet P, Loliger J. **(1989)** Vanillin: more than a flavouring agent- a potential antioxidant. *Journal of the Science of Food and Agriculture*, ***48***, 49-56.
[6] Boonchird C, Flegel TW. (**1982**) *In vitro* antifungal activity of eugenol and vanillin against *Candida albicans* and *Cryptococcus neoformans*. *Canadian Journal of Microbiology*, ***28***, 1235-1241.
[7] Fitzgerald DJ, Stratford M, Gasson MJ, Narbad A. (**2005**) Structure-function analysis of the vanillin molecules and its antifungal properties. *Journal of Agricultural and Food Chemistry*, ***53***, 1769-1775.
[8] Bull MJ, Davies HJ, Robert JG, Henry AC. (**1980**) Alkyl aryl ketone oxime-*O*-ethers, a novel group of pyrethroids. *Pesticide Science,* ***44,*** 249-256.
[9] Walia S, Saxena VS, Mukerjee SK. (**1985**) Synthesis and synergistic activity of oxime ethers containing a benzo-1,3-dioxole group. *Journal of Agricultural and Food Chemistry*, ***33***, 308-310.
[10] Walia S, Gujar GT. (**1993**) Bioactivity of some substituted benzo-1-3-dioxole oxime ethers against *Dysdercus koenigii. Pesticide Research Journal*, ***5***, 205-208.
[11] Chowdhury H, Saxena VS, Walia S. (**1998**) Synthesis and insect growth regulatory activity of alkoxy-substituted benzaldoxime ethers. *Journal of Agricultural and Food Chemistry*, ***46***, 731-736.
[12] Sun R, Li Y, Lu M, Xiong L, Wang Q. (**2010**) Synthesis, larvicidal activity, and SAR studies of new benzoylphenylureas containing oxime ether and oxime ester group. *Bioorganic & Medicinal Chemistry Letters*, ***20***, 4693-4699.
[13] Paul R, Walia S, Mondal A, Kumar J, Pankaj. (**2009**) Synthesis and nematicidal activity of 2-tridecanone and its oxime esters against *Meloidogyne incognita***.** *Annals of Plant Protection Sciences*, ***17***, 453-455.
[14] Choudhary G, Walia S, Kumar J. (**2007**) Synthesis and antifungal activity of citral oxime esters against two phytopathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii*. *Pesticide Research Journal*, ***19***, 15-19.
[15] Abeysinghe A. (**2009**) Efficacy of combined use of biocontrol agents on control of *Sclerotium rolfsii* and *Rhizoctonia solani* of *Capsicum annuum*. *Archieves of Phytopathology and Plant Protection*, ***42***, 221–227.
[16] Dikusar EA, Kozlov NG, Zhukovskaya NA, Moiseichuk KL. (**2004**) Esters of vanillin and vanilla oximes. *Chemistry of Natural Compounds*, ***40***, 180-183.
[17] Shukla P, Walia S, Ahluwalia V, Parmar BS, Nair MG. (**2012**). Activity of alkanediol alkanoates against pathogenic plant fungi *Rhizoctonia solani* and *Sclerotium rolfsii*. *Natural Product Communications*, ***7***, 1219-1222
[18] Trevors JT. (**1986**) A basic programme for estimating $LD_{50}$ values using the IBM-PC. *Bulletin of Environmental Contamination and Toxicology*, ***37***, 18-26.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1639 - 1644

# Cinnamic Acid Derivatives Acting Against *Aspergillus* Fungi. *Taq* Polymerase I a Potential Molecular Target

**Lucas J. Gutierrez[a,b], Maria L. Mascotti[a,c], Marcela Kurina-Sanz[a,c], Carlos R. Pungitore[a,c], Ricardo D. Enriz[a,b] and Fernando A. Giannini[a*]**

[a]*Departamento de Química, Facultad de Química, Bioquímica y Farmacia. Universidad Nacional de San Luis (UNSL), Chacabuco 917-5700-San Luis, Argentina*

[b]*Instituto Multidisciplinario de Investigaciones Biológicas de San Luis (UNSL, CONICET), Ejército de Los Andes 950, 5700 San Luis, Argentina*

[c]*Instituto de Tecnología Química de San Luis (UNSL, CONICET), Chacabuco 917-5700-San Luis, Argentina*

*fagian3@gmail.com*

**Received: September 19th, 2012; Accepted: October 16th, 2012**

Some members of a series of cinnamic acid derivatives possess promising inhibitory activities in cellular assays against fungi of the *Aspergillus* genus. In order to search for a possible molecular target of such compounds, their role as *Taq* polymerase I inhibitors was studied. Four of the compounds studied displayed $IC_{50}$ values within the range of those considered active as DNA polymerase inhibitors when searching for new cytotoxic molecules. The results obtained in our molecular modeling study appear to show that the inhibitory activity depends on the presence of a stabilizing interaction between the phenylpropanoid derivatives and the residues Asp610, Thr664, Phe667, Tyr671, and Asp785 located in the active site of *Taq* polymerase I. Also, it is possible to assert that the polymerization of DNA would be the molecular target of cinnamic acid derivatives with antifungal activity, which correlates with the inhibition of *Taq* polymerase I and the quantitative descriptor for the lipophilia (ClogP).

**Keywords:** Drupamine, *Aspergillus*, *Taq* polymerase I, Molecular dynamics.

Over the last twenty years the interest in natural products as sources of lead structures has resurfaced. Of the various reasons for this fact the uniqueness of their structures may be considered as one of the most important. Natural compounds and their derivatives could potentially serve as effective alternatives to conventional antifungal agents. Several phenolic compounds and derivatives have been reported to possess antifungal activity. Among others, gallic acid has been reported to prevent aflatoxin biosynthesis by *A. flavus* [1]. Two new natural products {1-(3′-methoxypropanoyl)-2-4-5-trimethoxybenzene and 2-(2*Z*)-(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol} isolated from the root bark of *Cordelia alliodora* have been reported as antifungal and larvicidal compounds [2]. More recently, Kim *et al.* [3] reported antifungal effects of several phenolic compounds, including cinnamic acid (**1**), *m*-coumaric acid (**12**), *p*-coumaric acid (**3**) and caffeic acid (**6**). Antimicotic effects towards the dermatophytes *Microsporum canis*, *Epidermophyton floccosum*, *Trichophyton mentagraphytes* and *Trichophyton rubrum* were reported for 3-(3,3-dimethylallyl)-*p*-coumaric acid isolated from the aerial parts of *Baccharis grisebachii* collected in the Province of Mendoza, Argentina [4]. However, this compound did not show antifungal activity against *Aspergillus fumigatus, A. flavus* and *A. niger* [5].

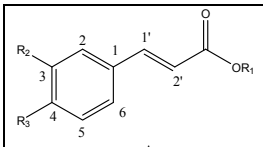
*Aspergillus* was first reported in 1729 by the Italian biologist Micheli. Nowadays, more than 189 species of this genus have been described; they are among the most abundant fungi in natural environments, such as soils, plants and decomposing organic waste [6]. Several species are phytopathogenic and cause, among other kinds of damages, severe losses to cereal crops. Moreover, this genus is one of the leading producers of aflatoxins, which are highly toxic and carcinogenic compounds [7].

The *Aspergillus* genus includes saprophytic and opportunistic species; the latter may cause important infections that have become relevant in recent years. In fact, along with *Candida* and *Fusarium* they are considered as the major opportunistic pathogens in humans [8]. The number of persons at risk has increased in the last two decades. Thus, AIDS and transplant patients, and those who receive chemotherapy with cytotoxic drugs, corticosteroids and other immunosuppressive agents are the most likely to develop such infections. In spite of the increasing knowledge about *Aspergillus* infection epidemiology, mortality caused by invasive aspergillosis still remains very high [9]. Spore inhalation significantly increases the risk of lung infections in immunocompromised patients and/or in persons who are particularly exposed, such as horticulturists or gardeners [10]. Besides, invasive aspergillosis is frequent in cancer patients [11,12]. Although *A. fumigatus* is the species most frequently isolated in clinical cases, *A. flavus*, *A. terreus* and *A. niger* are also frequent [13].

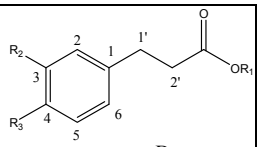
Currently, there are four classes of bioactive compounds against *Aspergillus* spp: polyenes, which include amphotericin B and their lipidic formulations; azoles and triazoles, such as fluconazole, voriconazole, itraconazole and posaconazole; aminocandines, such as caspofungin and micofungine, and allyl amines, such as terbinafin [14]. Acute toxicity and development of resistance are two drawbacks shared by these four groups which significantly restrict their applicability and fully justify the search and design of new antifungal drugs [15].

In a previous work [16] we studied the antifungal activity of a series of cinnamic acid-derived phenylpropanoids against *A. flavus*, *A. terreus* and *A. niger* (Table 1). In that study two compounds were observed to have the most significant antifungal activity: the methoxylated derivatives of the natural product drupanin (**15**) and the ubiquitous metabolite, ferulic acid (**6**). The first presented a Minimal Inhibition Concentration (MIC) similar to that of miconazol against the three tested fungal strains, while compound **6**

**Table 1**: Structural profile of the cinnamic acid derivatives studied.



A



B

| Compound | Type | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|---|
| **1** | A | H | H | H |
| **2** | A | $CH_3$ | H | H |
| **3** | A | H | H | OH |
| **4** | A | $CH_3$ | H | OH |
| **5** | A | $CH_3$ | H | $OCH_3$ |
| **6** | A | H | OH | OH |
| **7** | A | $CH_3$ | OH | OH |
| **8** | A | $CH_3$ | $OCH_3$ | $OCH_3$ |
| **9** | A | H | $OCH_3$ | $OCH_3$ |
| **10** | A | $CH_3$ | $OCH_3$ | OH |
| **11** | A | H | OH | H |
| **12** | A | $CH_3$ | OH | H |
| **13** | A | H | γ,γ-dimethylallyl | OH |
| **14** | A | $CH_3$ | γ,γ-dimethylallyl | $OCH_3$ |
| **15** | A | H | γ,γ-dimethylallyl | $OCH_3$ |
| **16** | A | $CH_3$ | γ,γ-dimethylallyl | OH |
| **17** | A | H | γ,γ-dimethylallyl | OAcOH |
| **18** | B | $CH_3$ | isopentyl | OH |
| **19** | B | H | $OCH_3$ | OH |
| **20** | B | $CH_3$ | H | OH |
| **21** | B | H | OH | H |

was active mostly against *A. niger* and *A. terreus*. However, the possible action mechanism at a molecular level of these phenylpropanoids was not addressed in that study.

Enzymes involved in the metabolism of nucleic acids, such as polymerases, topoisomerases and girases, are molecular targets in the search for new antimicrobial drugs [17]. This strategy could be considered analogous to the screening of DNA polymerase inhibitors in the search of new potential anticancer compounds, which is now widely accepted. Plant natural products of different biogenetic origins, such as terpenes, fatty acids, and phenolics have been reported as promising inhibitors. Among them, the iridoid catalpol, isolated from *Budleja cordobensis*, and catechins, phenolic compounds from *Camellia sinensis*, are good inhibitors of DNA polymerase from the thermophilic bacillus *Thermus aquaticus* (Taq) [18-20]. This enzyme is currently one of the most extensively used in PCR applications because of its ability to replicate DNA at high temperatures. DNA polymerases are highly conserved proteins in respect of their active sites throughout different phylogenies [21]. On the basis of such concepts, it would be reasonable to hypothesize that if a compound inhibits a bacterial polymerase activity, it might also have a similar effect against a fungal one. In this context, the aim of our study was to determine a possible molecular target for phenylpropanoids that showed antifungal activities in cellular assays by assessing their inhibitory activity toward *Taq* DNA polymerase. Moreover, an approach to such putative molecular mechanisms was simulated using molecular modeling techniques.

Previously reported phenylpropanoids [16] were evaluated as possible *Taq* polymerase I inhibitors at a concentration of 500 μM (Table 2). Compounds **2**, **3**, **4**, **5**, **9**, **10**, **11**, **12**, **17**, **18**, **20** and **21** did not show any activity at such concentration. In contrast, compounds **1**, **8**, **13**, **14** and **19** displayed inhibitory effects lower than 100%

**Table 2**: Antifungal activity toward *Aspergillus* [12], inhibition of *Taq* polymerase I and ClogP of selected compound.

| Compound | MIC [a] (μg/mL) | | | Inhibition of *Taq* polymerase I | | ClogP |
|---|---|---|---|---|---|---|
| | *A. terreus* | *A. niger* | *A. flavus* | % 500 μM | $IC_{50}$ μM | |
| **1** | 250 | 125 | 250 | 27.6 | nd | 2.115 |
| **6** | >250 | >250 | >250 | 100 | 1.57 | -1.207 |
| **7** | 125 | 125 | >250 | 100 | 1.28 | -0.415 |
| **8** | >250 | >250 | >250 | 59.2 | nd | 1.111 |
| **13** | >250 | 125 | >250 | 68.1 | nd | 2.533 |
| **14** | 125 | >250 | >250 | 62.6 | nd | 4.04 |
| **15** | 31.25 | 1.95 | 62.5 | 100 | 184.2 | 3.249 |
| **16** | 125 | >250 | 250 | 100 | 193.1 | 3.324 |
| **19** | >250 | >250 | >250 | 97.5 | nd | -0.444 |

[a] Minimum inhibitory concentration according to reference [12].
nd = not determined

(Figure 1, supplementary data). Interestingly, compounds **6**, **7**, **15** and **16** showed 100% inhibitory activity. $IC_{50}$ values of those compounds displaying a 100% inhibitory effect (**6**, **7**, **15** and **16**) were then evaluated. Compounds **15** and **16** displayed $IC_{50}$ values of 184.2 +/- 2.5 μM and 193.1 +/- 1.9 μM, respectively. $IC_{50}$ values obtained for caffeic acid (**6**) and its methylester (**7**) were 1.57 +/- 0.31 μM and 1.28 +/- 0.27 μM, respectively. These results are particularly interesting considering that compounds previously reported as DNA polymerase inhibitors displayed $IC_{50}$ values ranging from 400 to 0.7 μM [14].

By comparing the results of *Taq* polymerase I inhibition with the MIC values previously reported for antifungal activity (Table 2), interesting conclusions can be drawn. Compound **15** displayed inhibitory activity against *Taq* polymerase I, as well as significant antifungal activity against *Aspergillus* fungi. This result suggests that the molecular mechanism of action of this compound could be the inhibition of that enzyme. Compound **16** displayed an enzymatic inhibitory effect similar to **15**, but did not show antifungal activity. However, we have previously demonstrated that this phenolic compound is metabolized into 2-(2-hydroxypropan-2-yl)-2,3-dihydrobenzofuran-5-carboxylic acid by A. *terreus* [16], which could explain the lack of antifungal activity.

A more striking observation is that compound **7**, which had the strongest inhibitory activity against *Taq* polymerase I, displayed just a marginal antifungal activity. A similar result was observed for compounds **6** and **19**, which did not show any antifungal effect. In order to find an explanation for the discrepancy between the results of the cellular and the molecular assays, ClogP, a lipophilic quantitative molecular descriptor, was calculated for each compound {Table 2 and Table 1 (supplementary data)}. Compound **6** displayed a ClogP value of -1.207; this molecule possesses two phenolic groups and a carboxylic moiety in the side chain and, therefore, it is reasonable to assume that it is unable to pass through the cellular membrane by simple diffusion. A similar result was obtained for compounds **7** and **19**, which displayed Clog P values of -0.415 and -0.444, respectively. On the contrary, drupanine derivatives (**14**-**16**) (all substituted by isoprenoid moieties in the aromatic ring) displayed ClogP values ranging from 4.041 to 3.249.

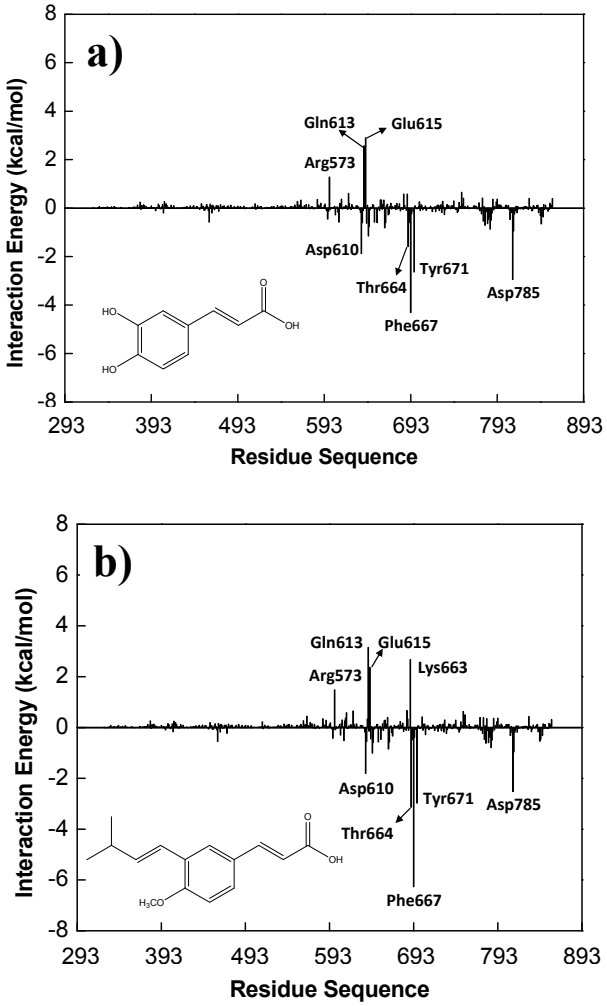



**Figure 1**: Inhibitor-residue interaction spectra of (a) *Taq*-**6** and (b) *Taq*-**15**, according to the MM-GBSA method. The x-axis denotes the residue number of *Taq* Polymerase I and the y-axis denotes the interaction energy between the inhibitor and specific residues.

To acquire a more-detailed insight into the mechanisms driving the bindings of cinnamic acid derivatives to the active site of *Taq* polymerase I, the structure-affinity relationship was analysed to complement the experimental results. The information obtained from these calculations is very important for quantitative analyses and is highly useful to the understanding of the binding mechanism.

Figure 1 shows the inhibitor-residue interaction spectra calculated by free energy decomposition, which suggests that the interaction spectra of compounds **6** and **15** with *Taq*-polymerase are almost the same and reflect their similar binding modes (note that the disfavoured interaction of Lys 663 with compound **15** is the only difference obtained when comparing both spectra). Closely related spectra were obtained for compounds **7** and **16** (data not shown), indicating that all the compounds possessing a significant inhibitory effect displayed very similar interaction spectra. For these compounds, the favourable residues can be divided into five groups around Asp610, Thr664, Phe667, Tyr671, and Asp785. The contribution to binding of an individual residue varies in the range of 3.1 to -6.2 kcal/mol. The dominant favourable interactions come from residues Thr664 and Asp785 performing stabilizing hydrogen bonds, as well as from residues Phe667 and Tyr671 making pi stacking interactions with the aromatic ring of the ligand. Figure 2 displays the relative spatial positions of inhibitors **6** and **15** in their

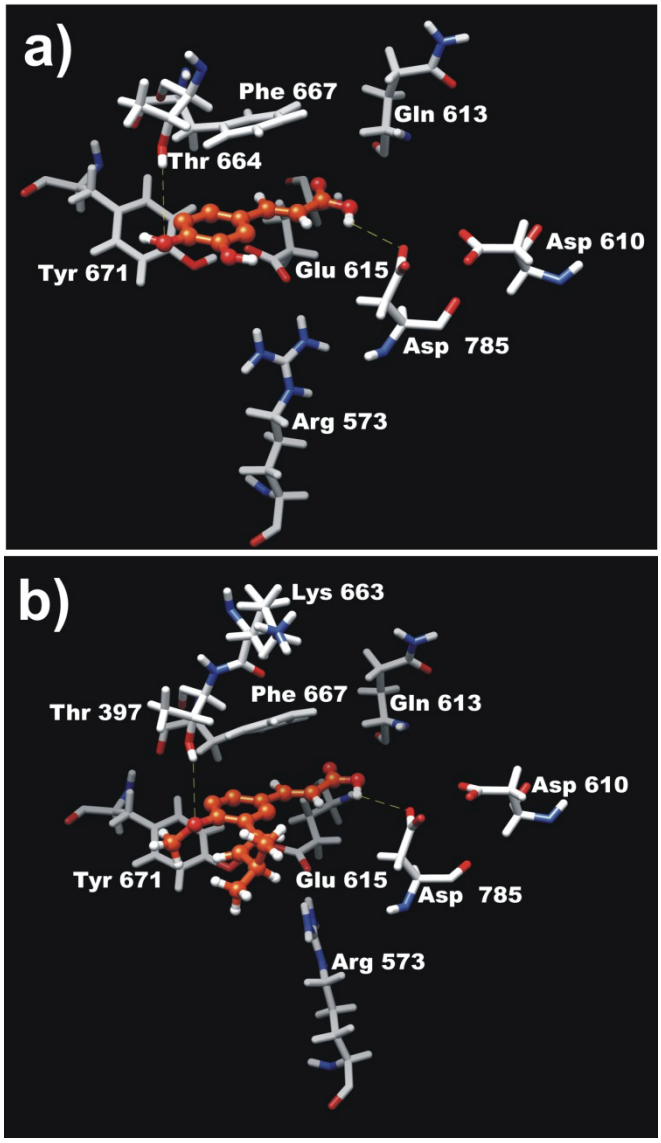



**Figure 2**: Spatial view of the stabilizing and destabilizing interactions obtained for compounds **6** (a) and **15** (b) interacting with *Taq* polymerase I. The different amino acids involved in this complex are shown.

lowest-energy structures at the binding complexes extracted from MD simulations. Figure 2 also shows the different stabilizing and destabilizing interactions.

It is interesting to note that the interaction spectra obtained for compounds **17** and **18** are different from those observed for compounds possessing an inhibitory effect (Figure 3). In the case of compound **17** (Figure 3a), there are three strong destabilizing interactions, namely with residues Arg573 (5.4 kcal/mol), Lys663 (7.2 kcal/mol) and Gln754 (7.4 kcal/mol). These destabilizing contributions make the binding energy obtained for compound **17** markedly higher than that obtained for compound **6**. Figure 4a displays the relative spatial positions of compound **17** in the lowest-energy structure of the binding complexes extracted from MD simulations. The main stabilizing and destabilizing interactions can be well observed in this figure. Although the residues stabilizing the complex are very similar to those observed in the complexes obtained for compounds **6** and **15**, in this case the distances between compound **17** and the stabilizing residues are larger and, therefore,

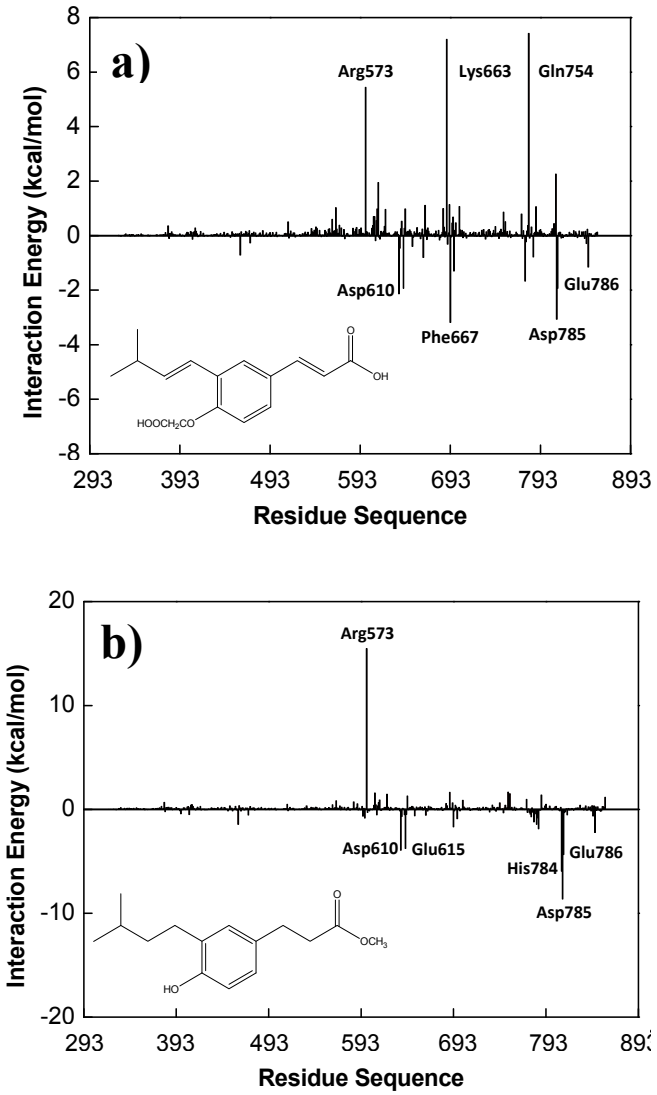



**Figure 3**: Inhibitor-residue interaction spectra of (a) *Taq*-**17** and (b) *Taq*-**18** according to the MM-GBSA method. The x-axis denotes the residue number of *Taq* Polymerase I and the y-axis denotes the interaction energy between the inhibitor and specific residues.

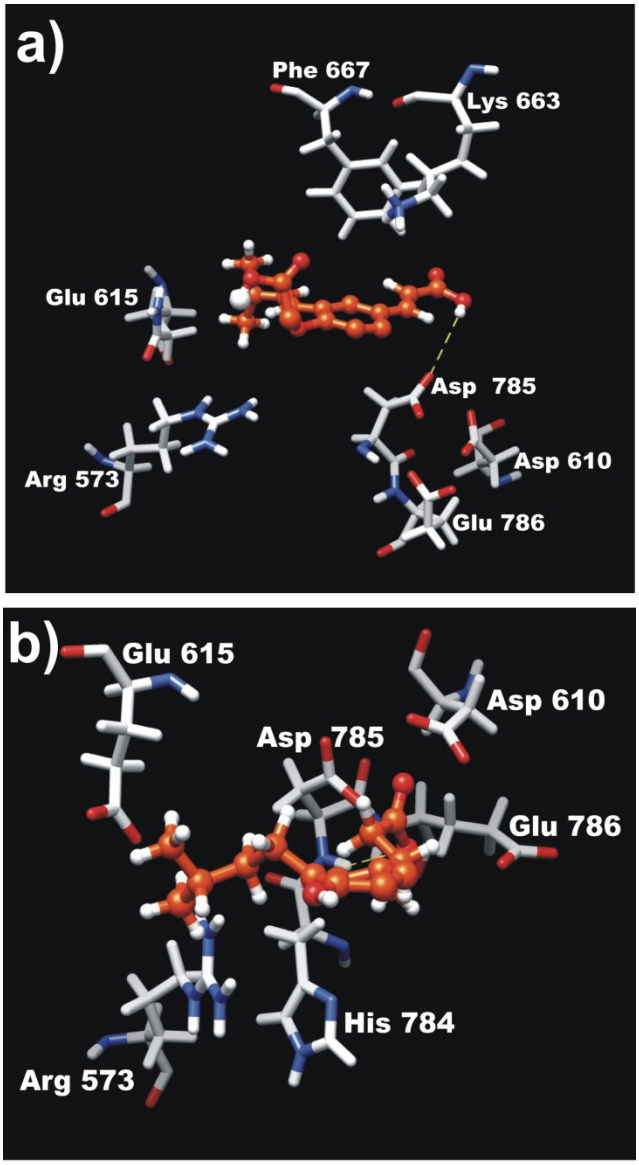



**Figure 4**: Spatial view of the stabilizing and destabilizing interactions obtained for compounds **17** (a) and **18** (b) interacting with *Taq* polymerase.

the stabilizing energies are weaker (compare Figures 2a and 4a). In addition, it should be noted that in this complex the stabilizing interaction of Thr664 disappears. The strongest destabilizing interaction was obtained for compound **18**, namely the interaction between compound **18** and Arg573, which displays about 15.4 kcal/mol (Figures 3b and 4b). This important destabilizing interaction, together with other minor ones, is responsible for the high binding energy obtained for compound **18** in comparison with those for compounds **6** and **15**, which possess inhibitory effects.

These theoretical results are in a complete agreement with our experimental data. Thus the results obtained in our molecular modelling study make it clear that it is possible to discriminate between compounds possessing significant inhibitory effects (**6**, **7**, **15** and **16**) from those which are inactive toward *Taq* polymerase I (**17** and **18**).

On the basis of our experimental and theoretical results, it is reasonable to assume that the cinnamic acid derivatives here reported are candidates to be leading structures in the design of new *Taq* polymerase I inhibitors.

*Taq* polymerase I may be the molecular target for the antifungal activity displayed by the studied derivatives, but it is evident that a ClogP value of about 3 is also necessary to produce the antifungal effect at a cellular level.

The strong antifungal activity, the relatively low acute toxicity previously reported, and the molecular mechanism here reported indicate that the methoxylated derivative of drupanin (**15**) is a very good candidate on which to perform further studies in the search for new agents to control *Aspergillus* infections.

## Experimental

***Test compounds:*** Compounds **1**, **2**, **3**, **6**, and **11** were purchased from Sigma-Aldrich Co. Drupanin (**13**) was isolated as colorless crystals (mp 147-148°C) from aerial parts of *B. grisebachii* Hieron. Its chemical identity was confirmed by spectroscopic data, compared with previous reports [4]. Compounds **4**, **5**, **6**, **7**, **8**, **9**, **10**, **12**, **14**-**21** were prepared as previously described by Bisogno and coworkers [16].

***Taq polymerase I inhibition assays:*** Inhibition of *Taq* polymerase I from *Thermusaquaticus* (Sigma-Aldrich) was studied by PCR. The assayed compounds were dissolved in DMSO (1 mg/mL) and inhibition studies were carried out at a screening concentration of 500 μM. The PCR master mixture consisted of 1.8 μM $MgCl_2$, 0.2 μMdNTPs, 1 μM primer sense (5′GGATCCATGAGCGGGGACCATCTCCA), and 1 μM primer antisense (5′TCAAAACTCGTAGTCCTCATAG). DNA concentration was 0.013 mg/mL and all PCR reactions were made in 20 μL. Thermocycling conditions consisted of 35 cycles of denaturation at 94°C for 30s followed by primer annealing at 53°C for 30s and primer extension at 72°C for 1 min 30s. Catapol 100 μM was used as positive control. Assays were made in triplicate.

Compounds that displayed 100% inhibition at the screening concentration were further evaluated at lower concentrations by serial dilution (500 μM to 0.95 μM) in order to obtain the $IC_{50}$ value.

***Analysis of PCR products:*** Relative intensities of ethidium bromide stained PCR products were analyzed using an optical scanner. The digitized band images were processed using the Image processing software Scion Image, a public domain program. $IC_{50}$ values were determined by the GraphPad Prism software.

***Molecular modeling:*** The coordinate of *Taq* polymerase I was downloaded from Brookhaven Protein Data Bank (www.rcsb.org). The PDB code entry is 3KTQ. To explore the dynamic stability of the *Taq*-inhibitor complexes reported here, root mean square deviation (RMSD) values for the protease $C_\alpha$ atoms during the MD simulations (relative to the docking structure) were calculated. The RMSD values of the *Taq*-**6**, *Taq*-**15**, *Taq*-**17** and *Taq*-**18** complexes are 1.60±.031, 2.13±0.59, 1.96±0.34 and 1.80±0.31 Å, respectively, showing a deviation lower than 0.6 Å from the mean. These results suggest that the stability of the dynamic equilibriums of these complexes is reliable.

***Molecular docking:*** In order to search for the preferable conformation, AutoDock 4.0 software [22] was used for docking calculations, combined with Lamarckian genetic algorithm (LGA). The center of the grid box was the active site and the size of the box was 50 x 45 x 55 grid points, with grid spacing of 0.375 Å. During the docking experiment, the receptor was kept rigid and the ligand was set flexible. The maximum number of energy evaluations was set to 25 x $10^6$; the initial population of trial ligands consisted of 250 individuals; for the rest of the docking parameters the default values were used. Fifty docking runs were performed to find the preferable conformation. The docking results were clustered according to the RMSD of 2Å. Structures with relative lower binding free energy and most cluster members were chosen, which were then subjected to molecular dynamics simulations (MD).

***Molecular dynamics simulations:*** To relax the binding complexes and investigate their dynamics, three-nanosecond MD simulations were performed for the different *Taq*-inhibitor systems using Amber software [23]. The protein force field was taken from ff99SBilnd [24]. The general Amber force field (GAFF) [25] was used to handle small organic molecules and the force field parameters of the inhibitors were produced by the antechamber program in Amber. Each *Taq*-inhibitor complex was soaked in a truncated octahedral periodic box of TIP3P water molecules. The distance between the edges of the water box and the closest atom of the solutes was at least 10Å. $Na^+$ ions were added to neutralize the systems charge. The entire system was subjected to energy minimization in two stages to remove bad contacts between the complex and the solvent molecules. First, the water molecules were minimized by holding the solute fixed with harmonic constraints of 100 kcal/molÅ$^2$ strength. Secondly, conjugate gradient energy minimizations were performed repeatedly 4 times using positional restraints to all heavy atoms of the receptor with 15, 10, 5 and 0 kcal/molÅ$^2$. The system was then heated from 0 to 300 K in 300 ps and equilibrated at 300 K for another 200 ps. After minimization and heating, 2.5 ns dynamics simulations were performed at a constant temperature of 300 K and a constant pressure of 1 atm. During minimization and MD simulations, the particle mesh Ewald (PMD) method was employed to treat the long-range electrostatic interactions in a periodic boundary condition. The SHAKE method was used to constrain hydrogen atoms. The time step for all MD is 2 fs, with a direct-space, non-bonded cutoff of 8Å. Initial velocities were assigned from a Maxwellian distribution at the initial temperature.

***The MM-GBSA method:*** In MM-GBSA [26], the binding free energy ($\Delta G_{bind}$) resulting from the formation of a RL complex between a ligand (L) and a receptor (R) is calculated as

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{sol} - T\Delta S \quad (1)$$

$$\Delta E_{MM} = \Delta E_{internal} + \Delta E_{electrostatic} + \Delta E_{vdw} \quad (2)$$

$$\Delta G_{sol} = \Delta G_{GB} + \Delta G_{SA} \quad (3)$$

where $\Delta E_{MM}$, $\Delta G_{sol}$ and -TΔS are the changes of the gas phase MM energy, the solvation free energy, and the conformational entropy upon binding, respectively. $\Delta E_{MM}$ includes $\Delta E_{internal}$ (bond, angle, and dihedral energies), $\Delta E_{electrostatic}$ (electrostatic), and $\Delta E_{vdw}$ (van der Waals) energies. $\Delta G_{solv}$ is the sum of electrostatic solvation energy (polar contribution), $\Delta G_{GB}$, and the non-electrostatic solvation component (nonpolar contribution), $\Delta G_{SA}$. Polar contribution is calculated using the GB model, while the nonpolar energy is estimated by solvent accessible surface area (SASA). The conformational entropy change, -TΔS, is usually computed by normal-mode analysis, but in this study the entropy contributions were not calculated due to the computational cost involved in such calculations.

***ClogP calculations:*** All geometries of the compounds reported here were optimized from DFT calculations using the Becke3-Lee-Yang-Parr [27-29] (B3LYP) functional with the 6-31G(d) basis set. These calculations were carried out using the Gaussian 03 [30] program. Once the different optimized geometries were obtained, ClogP values were calculated using Chem Office Ultra 4.5 (Cambridgesoft. Corp 1988) software.

**Supplementary data:** Theoretical ClogP values for compounds **1-21**(Table1). Image of the *Taq*polymerase I inhibition assay (Figure1).

**Acknowledgments** - This work was supported by grants from Universidad Nacional de San Luis: PROICO 1412 and 22/Q027. Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP 6228 and 0357. Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT 352, 1416 and 1832. LJG and MLM are doctoral CONICET fellows. MKS, CRP and RDE are members of the Research Career of CONICET, Argentina.

## References

[1] Mahoney N, Molyneux RJ. (**2004**) Phytochemical inhibition of aflatoxigenicity in *Aspergillusflavus* by constituents of walnut (*Juglansregia*). *Journal of Agricultural and Food Chemistry*, ***52***, 1882–1889.

[2] Ioset JR, Marston A, Gupta MP, Hostettmann K. (**2000**) Antifungal and larvicidal compounds from the root bark of *Cordia alliodora*. *Journal of Natural Products*, ***63***, 424–426.
[3] Kim JH, CampbellBC, Mahoney NE, Chan KL, Molyneux RJ. (**2004**) Identification of phenolics for control of *Aspergillus flavus* using *Saccharomyces cerevisiae* in a model target-gene bioassay. *Journal of Agricultural and Food Chemistry*, ***52***, 7814–7821.
[4] Gianello JC, Giordano OS. (**1987**) Constituents from *Bacccharis grisebachii*. *Anales de la Asociación Química Argentina*, ***75***, 1–3.
[5] Feresin GE, Tapia A, Gimenez A, Ravelo AG, Zacchino S, Sortino M, Schmeda-Hirschmann G. (**2003**) Constituents of the Argentinian medicinal plant *Baccharis grisebachii* and their antimicrobial activity. *Journal of Ethnopharmacology*, ***89***, 73–80.
[6] Lass-Flörl C, Perkhofer S. (**2008**) *In vitro* susceptibility-testing in *Aspergillus* species. *Mycoses*, ***51***, 437–446.
[7] Lu FC. (**2003**) Assessment of safety/risk vs. public health concerns: Aflatoxins and hepatocarcinoma. *Environmental Health and Preventive Medicine*, *7*, 235–238.
[8] Solé A, Salavert M. (**2008**) Fungal infections after lung transplantation. *Transplantation Reviews (Orlando, Fla.)*, ***22***(2), 89–104.
[9] Gangneux JP, Camus C, Philippe B. (**2010**) Epidemiology of invasive aspergillosis and risk factors in non neutropaenic patients. *Revue des Maladies Respiratoires*, ***27***, e34–46.
[10] Summerbell RC. (**1998**) Taxonomy and ecology of *Aspergillus* species associated with colonizing infections of the respiratory tract. *Immunology and Allergy Clinics of North America*, ***18***, 549–573.
[11] Lee LD, Hachem RY, Berkheiser M, Hackett B, Jiang Y, Raad II. (**2012**) Hospital environment and invasive aspergillosis in patients with hematologic malignancy. *American Journal of Infection Control*, ***40***, 247–249.
[12] Nilsson JR, Restrepo CS, Jagirdar J. (**2011**) Two cases of endobronchial carcinoid masked by superimposed aspergillosis: a review of the literature of primary lung cancers associated with *Aspergillus*. *Annals of Diagnostic Pathology*, in press.
[13] Bodey G, Bueltmann B, Duguid W, Gibbs D, Hanak H, Hotchi M, Mall G, Martino P, Meunier F, Milliken S. (**1992**) Fungal infections in cancer patients: an international autopsy survey. *European Journal of Clinical Microbiology & Infectious Diseases : Official Publication of the European Society of Clinical Microbiology*, ***11***, 99–109.
[14] Qiao J, Liu W, Li R. (**2008**) Antifungal resistance mechanisms of *Aspergillus*. *Nihon Ishinkin Gakkaizasshi* (*Japanese Journal of Medical Mycology*), ***49***, 157–63.
[15] Chamilos G, Kontoyiannis DP. (**2005**) Update on antifungal drug resistance mechanisms of *Aspergillus fumigatus*. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy*, ***8***, 344–358.
[16] Bisogno F, Mascoti L, Sanchez C, Garibotto F, Giannini F, Kurina-Sanz M, Enriz R. (**2007**) Structure-antifungal activity relationship of cinnamic acid derivatives. *Journal of Agricultural and Food Chemistry*, ***55***, 10635-10640.
[17] Pungitore CR. (**2008**) Natural products as inhibitors of DNA related enzymes. *Current Enzyme Inhibition*, ***4***, 194-215.
[18] Pungitore CR, Ayub MJ, Borkowski EJ, Tonn CE, Ciuffo GM. (**2004**) Inhibition of *Taq* DNA polymerase by catalpol. *Cellular and Molecular Biology*, ***50***, 767–772.
[19] Jaziri I, Ben Slama M, Mhadhbi H, Urdaci MC, Hamdi M. (**2009**) Effect of green and black teas (*Camellia sinensis* L.) on the characteristic microflora of yogurt during fermentation and refrigerated storage. *Food Chemistry*, ***112***, 614–620.
[20] Pungitore CR, Ayub MJ, García M, Borkowski EJ, Sosa ME, Ciuffo G, Giordano OS, Tonn CE. (**2004**) Iridoids as allelochemicals and DNA polymerase inhibitors. *Journal of Natural Products*, ***67***, 357–361.
[21] Joyce CM, Steitz T A. (**1994**) Function and structure relationships in DNA polymerases. *Annual Review of Biochemistry*, ***63***, 777–822.
[22] Morris GM, Goodsell DS, Huey R, Olson AJ. (**1996**) Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4. *Journal of Computer-aided Molecular Design*, ***10***, 293–304.
[23] Case DA, Cheatham TE, Darden T, GohlkeH, Luo R, Merz KM, Onufriev A, Simmerling C, Wang B, Woods RJ. (**2005**) The Amber biomolecular simulation programs. *Journal of Computational Chemistry*, ***26***, 1668–1688.
[24] Lindorff-Larsen K, Piana S, Palmo K, Maragakis P, Klepeis JL, Dror RO, Shaw DE. (**2010**) Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins*, ***78***, 1950–1958.
[25] Cornell W, Cieplak P, Bayly C, Gould I, Merz, K, Ferguson D, Spellmeyer D, Fox T, Caldwell J, Kollman P. (**1995**) A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *Journal of the American Chemical Society*, ***117***, 5179–5197.
[26] Kollman PA, Massova I, Reyes C, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y, Wang W, Donini O, Cieplak P, Srinivasan J, Case DA, Cheatham TE. (**2000**) Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Accounts of Chemical Research*, ***33***, 889–897.
[27] Becke AD. (**1988**) Density-functional exchange-energy approximation with correct asymptotic behavior. *Physical Review A*, ***38***, 3098–3100.
[28] Becke AD. (**1993**) A new mixing of Hartree–Fock and local density-functional theories. *The Journal of Chemical Physics*, ***98***, 1372-1377.
[29] Lee C, Yang W, Parr RG. (**1988**) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Physical Review B*, ***37***, 785–789.
[30] Frisch MJ, Trucks GW, Schlege HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery JA, Vreven JrT, Kudin KN, Burant JC, Millam JM, Iyengar SS, Tomasi J, Barone V, Mennucci B, Cossi M, Scalmani G, Rega N, Petersson GA, Nakatsuji H, Hada M, Ehara M, Toyota K, Fukuda R, Hasegawa J, Ishida M, Nakajima T, Honda Y, Kitao O, Nakai H, Klene M, Li X, Knox JE, Hratchian HP, Cross JB, Adamo C, Jaramillo J, Gomperts R, Stratmann RE, Yazyev O, Austin A J, Cammi R, Pomelli C, Ochterski JW, Ayala PY, Morokuma K, Voth GA, Salvador P, Dannenberg JJ, Zakrzewski VG, Dapprich S, Daniels AD, Strain MC, Farkas O, Malick DK, Rabuck AD, Raghavachari K, Foresman JB, Ortiz JV, Cui Q, Baboul AG, Clifford S, Cioslowski J, Stefanov BB, Liu G, Liashenko A, Piskorz P, Komaromi I, Martin RL, Fox DJ, Keith T, Al-Laham MA, Peng CY, Nanayakkara A, Challacombe M, Gill PM W, Johnson B, Chen W, Wong MW, Gonzalez C, Pople JA. Gaussian 2003 (**2003**).

NPC Natural Product Communications

2012
Vol. 7
No. 12
1645 - 1646

# Insecticidal Effects of Acetogenins from *Rollinia occidentalis* Seed Extract

**Diego Tolosa[a], Olga Álvarez Colom[a], Alicia Bardón[a,b] and Adriana Neske*[a]**

*[a]Instituto de QuímicaOrgánica, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 471, Tucumán 4000, Argentina; [b]INQUINOA-CONICET, Tucumán, Argentina*

*aneske@fbqf.unt.edu.ar*

**Received: September 14th, 2012; Accepted: November 5th, 2012**

From the methanol seed extract of *Rollinia occidentalis* five acetogenins were isolated, sylvaticin (**1**), rolliniastatin-1 (**2**), rolliniastatin-2 (**3**), motrilin (**4**) and desacetyluvaricin (**5**), and evaluated for their toxicity and nutritional alterations on *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae). All but one of the compounds produced more than 90% mortality on early larval instars at 50 and 100 µg per g of diet. Sylvaticin caused 15 and 60% mortality at 50 and 100 µg/g, respectively, while the methanol seed extract reached 35 and 50% at 100 and 250 µg/g, respectively. The addition of *R. occidentalis* methanolic seed extract (100 and 250 µg/g) and sylvaticin (100 µg/g) to the larval diet brought about significant changes to the nutritional indices in connection with larvae fed the control diet. This is the first report on the isolation of acetogenins from *R. occidentalis* and their insecticidal effects on *S. frugiperda.*

**Keywords:** Plant metabolites, Polyphagous lepidopteran, Toxicity, Nutritional alterations.

*Rollinia occidentalis* Fries (Annonaceae) is a tree commonly found in northwest Argentina. Characteristic constituents of these plants are the annonaceous acetogenins that possess unique structures and anticancer [1a,b], cytotoxic [2], insecticidal [3a,b], and antiparasitic activities. Previous results from our laboratory indicated that the acetogenins from *Annona cherimolia* and *A. montana* have larvicidal effects on the *Spodoptera frugiperda* (Lepidoptera) corn pest [4a,b], nymphal and adult mortality on the *Oncopeltus fasciatus* (Hemiptera) cotton pest[4c],and insecticidal activity against *Ceratitis capitata* Wiedemann (Diptera) [4d].

This is the first report on the isolation and identification of 5 known and structurally relatedbistetrahydrofuran acetogenins from the methanolic seed extract of *R. occidentalis*: sylvaticin (**1**) [5], rolliniastatin-1 (**2**) [6], rolliniastatin-2 (**3**) [7a], motrilin (**4**) [7b]and desacetyluvaricin (**5**)[7c].Continuing with our search for natural insecticides from Annonaceae plants, we evaluated the toxic effects produced by the methanol seed extract and annonaceous acetogenins (ACG) isolated from *R. occidentalis* on *S. frugiperda*. Additionally, Consumption Index (CI), Growth (GR), and Efficiency in the Consumption Index (ECI) were also assessed. The "fall armyworm", *S. frugiperda* Smith (Lepidoptera: Noctuidae), is a widely distributed pest in America. It causes serious economic damage to many crops even though its host preference is corn. Toxicity was determined by evaluating *S. frugiperda* larval and pupal mortality and malformations of larvae, pupa and adults. They were fed an artificial diet treated with different ACG at a final concentration of 50 and 100 mg ACG/g of diet. An untreated diet was used as control.

The methanolic seed extract of *R. occidentalis* at 50 µg/mL did not have a significant toxic effect under the experimental conditions. However, at 100 and 250 µg/mL it had a toxic effect on *S. frugiperda* with a larval mortality of 45 and 50%, respectively. Sylvaticin (100 µg/mL) produced a significant toxic effect on *S. frugiperda* with 70% mortality while rolliniastatin-1, rolliniastatin-2, motrilin and desacetyluvaricin caused 100% larval mortality under the experimental conditions. Most of the tested compoundsproduced significant larval and pupal mortality with malformations of larvae, pupa and adults.

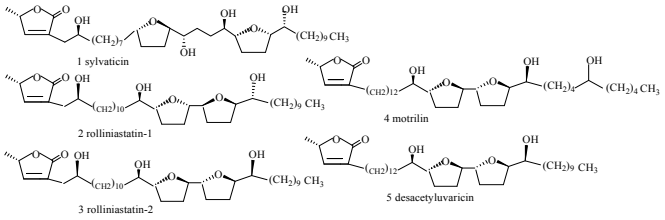



**Figure 1**: Annonaceous acetogenins from *R. occidentalis* methanolic seed extract.

The addition of methanolic seed extract and sylvaticin to the diet affecting the early instar larvae in their growth and nutrition could not be quantified in the remaining rolliniastatin-1, rolliniastatin-2, motrilin and desacetyluvaricin as larvae mortality occurred within 10 days.

***Toxicity of annonaceous acetogenins*:** *R. occidentalis* methanolic seed extract (50 µg/mL) did not have a significant toxic effect under the experimental conditions; 100 and 250 µg/mL had a slight toxic effect on *S. frugiperda* with larval mortality of 45 and 50%, respectively. However, sylvaticin (100 µg/mL) produced significant toxic effects on *S. frugiperda* with 70% mortality and malformed adults (20%), while rolliniastatin-1, rolliniastatin-2, motrilin and desacetyluvaricin were the most toxic (100% larval mortality) under the experimental conditions, as shown in Table 1. Pupal mortality was apparently related to deficient melanization and malformation in wings, legs and antenna cover. Remarkably, every surviving adults were abnormal in abdomen and wings. We analyzed the nutritional effects produced by the addition of the above mentioned extract and ACG to the 2nd instar larval diet of *S. frugiperda* to assess how treatment induced mortality. Larvae fed with *R. occidentalis* (50 µg/g) and sylvaticin (50 µg/g) methanolic seed extract presented physiological indices similar to those of control larvae. The addition of *R. occidentalis* (100 and 250 µg/g) and sylvaticin (100 µg/g) methanolic seed extract to the larval diet caused significant changes to the nutritional indices compared with those of larvae fed the control diet (Table 2).This resulted in an important larval growth decrease and subsequent 100% larval mortality at very early stages in their life cycle and would reveal the

Sylvaticin (50 µg/g) had the lowest intake percentage and the poorest nutrient absorbed conversion. Nutritional indices for rolliniastatin-1, rolliniastatin-2, motrilin and desacetyluvaricin could not be measured as mortality occurred at an early stage.

As shown in Figure 1, all the tested acetogenins carry two THF rings and the THF are adjacent in four of them. Our results indicated that acetogenins**2**, **3**, **4** and **5**had killed more than 90% of *S. frugiperda* larvae at 120 h after the start of the assay, while the remaining acetogenin**1**, had a lower effect at the doses tested. The most important toxic action was observed on larval instar. In fact, all acetogenins produced more than 70% larval mortality with no dependence on the position of the THF rings or the number and location of the OH groups. The evaluation of food consumption indicated that the tested acetogenins were not antifeedant agents because there were no significant differences related to the control. The high ECI values for sylvaticin (100 µg/g) are due to the very low larval weight even when larvae consume the diet regularly, suggesting that sylvaticin reduces the efficiency to convert food into biomass. The influence of chemical agents and their toxic effects on insects and their nutritional behaviour can be used in the development of environmentally friendly pest control agents.

## Experimental

***Plant material:*** *R. occidentalis* fruits were collected in Tucumán, Argentina, in March 2005. A voucher sample (No. 604639) was deposited at the Herbarium of Instituto Lillo of Tucumán.

***Acetogenin extraction and purification:*** Dried and powdered seeds of *R. occidentalis* were percolated with methanol. Evaporation of the solvent yielded a crude MeOH extract which was further partitioned between $CHCl_3$ and $H_2O$. The solvent was then evaporated from the chloroform extract under reduced pressure and the residue was chromatographed on a silica gel column by using chloroform and increasing amounts of ethyl acetate (0-100%) and finally methanol as eluents. Chromatographic fractions containing acetogenins (TLC detection by Kedde's reagent) were further processed by high-performance liquid chromatography (HPLC) using a Beckman C 18 column (25 cm x 1 cm i.d., 5 µm particle size) and mixtures of methanol and water to yield pure acetogenins. Acetogenins represented around 0.05% of the seed weight.

**Table 1**: Toxicity produced by *R. occidentalis* methanolic seed extract and ACG on larvae of *S. frugiperda*.

| Compounds | (%) Larval Mortality | (%) Pupal Mortality | Malformed Adults (%) |
|---|---|---|---|
| Extract Ro 50 ppm | 5 | 30 | - |
| Extract Ro 100 ppm | 35 | 45 | 20 |
| Extract Ro 250 ppm | 50 | 50 | - |
| Sylvaticin 50 ppm | 15 | 70 | 15 |
| Sylvaticin 100 ppm | 70 | 10 | 20 |
| Rolliniastatin-1 50 ppm | 90 | 10 | - |
| Rolliniastatin-1 100 ppm | 100 | - | - |
| Rolliniastatin-2 50 ppm | 100 | - | - |
| Rolliniastatin-2 100 ppm | 100 | - | - |
| Motrilin 50 ppm | 100 | - | - |
| Motrilin 100 ppm | 100 | - | - |
| Desacetyluvaricin 50 ppm | 100 | - | - |
| Desacetyluvaricin 100 ppm | 100 | - | - |

***Test insects:*** *S. frugiperda* larvae were obtained from our laboratory population and maintained with an artificial diet.Toxicity tests were recorded for treatments with all acetogenins (50 and 100 $\mu$g/mL) and control experiments [7d].Determination of Consumption (CI), Growth (GR), and Efficiency in the Consumption Index (ECI) were carried out. For comparison purposes, rates are expressed as a relationship between treatment and control; the latter are considered 100%. Values are expressed as ($GR_T/GR_C$) 100%, ($CI_T/CI_C$) 100% and ($ECI_T/ECI_C$) 100% in Table 2 [7d],

**Table 2**: Nutritional alterations produced by *R. occidentalis* methanolic seed extract and ACG on larvae of *S. frugiperda*.

| Compounds | $GR_T/GR_C$% | $CI_T/CI_C$% | $ECI_T/ECI_C$% |
|---|---|---|---|
| Extract Ro 50 ppm | 88 | 103 | 102 |
| Extract Ro 100 ppm | 62 | 54 | 51 |
| Extract Ro 250 ppm | 47 | 50 | 43 |
| Sylvaticin 50 ppm | 89 | 97 | 97 |
| Sylvaticin 100 ppm | 57 | 78 | 78 |

**Acknowledgements** – This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT) and Concejo Nacional de Investigaciones Científicas y Técnicas (CONICET), República Argentina.

## References

[1] (a) Alali F, Liu X, Mc Laughlin JL. (**1999**) Annonaceous acetogenins: recent progress. *Journal of Natural Products*, ***62***, 504-540; (b) Murray B, Leatemia J. (**2004**) Toxicity and antifeedant activity of crude seed extracts of *Annona squamosa* (Annonaceae) against lepidopteran pests and natural enemies. *International Journal of Tropical Insect Science*, ***24***, 150-158.

[2] Cortes D, Myint S, Dupont B, Davoust D. (**1993**) Bioactive acetogenins from seeds of *Annona cherimolia*. *Phytochemistry*, ***32***, 1475-1482.

[3] (a) Alkofahi A, Rupprecht JK, Anderson JE, Mc Laughlin JL, Mikolajczak K, Scott B. (**1989**) Search for new pesticides from higher plants. In *Insecticides of Plant Origin*. Arnason JT, Philogene B, Morand, A.(Eds).American Chemical Society, Washington DC, 25-43; (b) Cole MD. (**1994**) Key antifungal, antibacterial and anti-insect assays-a critical review. *Biochemical Systematics and Ecology*, ***22***, 837-856.

[4] (a) Álvarez Colom O, Neske A, Popich S, Bardón A. (**2007**) Toxic effects of annonaceous acetogenins from *Annona cherimolia* (Magnoliales: Annonaceae) on *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Journal of Pest Science*, ***80***, 63-67; (b) Di Toto Blessing L, Álvarez Colom O, Popich S, Neske A, Bardón A. (**2010**) Antifeedant and toxic effects of acetogenins from *Annona montana* on *Spodoptera frugiperda*. *Journal of Pest Science*, ***83***, 307-310; (c) Álvarez Colom O, Barrachina I, Ayala Mingol I, Gonzalez Mas MC, MoyaSanz P, Neske A, Bardón A. (**2008**) Toxic effects of annonaceous acetogenins on *Oncopeltus fasciatus*. *Journal of Pest Science*, ***81***, 85-89; (d) Álvarez Colom O, Salvatore A, Willink E, Ordóñez R, Isla MI, Neske A, Bardón A. (**2010**) Insecticidal, mutagenic and genotoxic evaluation of annonaceous acetogenins. *Natural Product Communications*, ***5***, 391-394.

[5] Shi G, Zeng L, Gu Z, MacDougal JM, Mc Laughlin JL. (**1995**) Absolute stereochemistries of sylvaticin and 12,15-*cis*-sylvaticin, bioactive C20, 23-*cis* non-adjacent bis-tetrahydrofuran annonaceous acetogenins, from *Rollinia mucosa*. *Heterocycles*, ***41***, 1785-1796.

[6] Pettit GR, Cragg GM, Polonsky J, Herald DL, Goswami A, Smith CR, Moretti C, Schmidt JM, Weisleder D. (**1987**) Isolation and structure of rolliniastatin-1 from the South American tree *Rollinia mucosa*. *Canadian Journal of Chemical Engineering*, ***65***, 1433-1435.

[7] (a) Pettit GR, Riensen R, Leet JE, Polonsky J, Smith CR, Schmidt JM, Dufresne C, Schaufelberger D, Moretti C. (**1989**) Isolation and structure of rolliniastatin-2, a new cell growth inhibitory acetogenin from *Rollinia mucosa*. *Heterocycles*,***28***, 213-217; (b) Cortes D, Myint S, Hocquemiller R. (**1991**) Molvizarin and motrilin: two novel cytotoxic bis-tetrahydro-furanic$\gamma$-lactone acetogenins from *Annona cherimolia*. *Tetrahedron*, ***47***, 8195-8202; (c) Jolad SHD, Hoffmann JJ, Cole JR, Barry CE, Bates RB, Linz GS. (**1985**) Desacetyluvaricin from *Uvaria accuminata*, configuration of uvaricin AT C-36.*Journal of Natural Products*, ***48***, 644-645; (d) Villafañe E, Tolosa D, Bardón A, Neske A. (**2011**) Toxic effects of *Citrus aurantium* and *C. lemon* essential oils on *Spodoptera frugiperda* (Lepidoptera: Noctuidae). *Natural Product Communications*, ***6***, 1389-1392.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1647 - 1650

# Conservation and Multiplication of Encapsulated Micro Shoots of *Rauvolfia vomitoria* - an Endangered Medicinal Tree: ISSR and RAPD based Evaluation of Genetic Fidelity of Converted Plantlets

**Shakti Mehrotra*, Liaq Ur Rahman, Jahnvi Mishra and Arun K. Kukreja**

*Plant Biotechnology Department, Central Institute of Medicinal and Aromatic Plants, (Council of Scientific and Industrial Research), PO; CIMAP, Picnic Spot Road, Lucknow-226015, India*

*shaktimehrotra22@gmail.com*

**Received: August 21st, 2012; Accepted: October 3rd, 2012**

The *in vitro* grown axillary micro shoots of *Rauvolfia vomitoria* were encapsulated in alginate beads. Following 6 months of normal storage at 25 ± 2ºC the re-growth of encapsulated micro shoots, reached 95.2% within 40 days of incubation on MS medium containing 1.0 mg/L BAP and 0.1 mg/L NAA. Among the responding encapsulated explants 69.6% showed emergence of multiple shoots. The developing shoots showed rhizogenesis in two weeks following their transfer to rooting medium. Healthy plants were established in a glass house with 95% survival. Of the 50 RAPD primers tested, 10 produced 23 clear and reproducible amplicons, with an average of 2.3 bands per primer. Eleven ISSR primers produced a total of 42 bands, with a size range of 0.1-1.9 kb. The number of scorable bands for each primer varied from 2 to 6, with an average of 3.81. The similarity matrix, calculated individually from the results obtained from ISSR and RAPD analysis, showed similarity coefficients ranging from 1.0 for RAPD and 0.85 to 1.0 for ISSR.

**Keywords:** Encapsulation, *Rauvolfia vomitoria*, Germplasm conservation, Genetic fidelity, ISSR, RAPD analysis.

*Rauvolfia vomitoria* Afzelius, a tree species native to the African continent, is found in wet Guineo-Congolian forests in association with the palms, *Trema* and *Combretum*. The medicinal properties of *R. vomitoria* are attributed to compounds that are predominantly present in the underground parts of the plant [1]. Roots, the natural source of reserpine, are used in the treatment of hypertension, high blood pressure and illness related to the central nervous system. Besides reports showing sedative, aphrodisiac and antispasmodic properties of the roots, a US patent also exists for antidiabetic properties of *R. vomitoria* root extract [2]. The plant belongs to the endangered category due to unsystematic collection for medicinal uses, as well as the growth of other invasive plant species. In spite of the extensive exploitation of *R. vomitoria* for the extraction of pharmaceutically active compounds, the biodiversity and conservation aspect of this species have not been passably noticed, and no conventional or unconventional attempt has been made for multiplication and conservation of this important species. Recognizing the loss of the natural germ resource due to hasty and reckless exploitation, biotechnology based conservation and multiplication of *R. vomitoria* germplasm has drawn the attention of this current research.

Synthetic seed technology or alginate encapsulation of explants (embryogenic / non-embryogenic) is a well established practice and has been employed in conjunction with micropropagation to establish *in vitro* gene banks of various pharmaceutically important species [3-9]. The efficiency of using alginate encapsulated propagules lies in their small size and relative ease of handling and transportation. Besides, increased productive potential and ease of long or short term storage gives additional significance. The main idea behind utilizing synthetic seed technology in the present study is that the technique is endowed with the shared advantages of clonal multiplication and germplasm storage and conservation.

However, in response to normal / cryogenic storage, the occurrence of somaclonal variation as a possible snag necessitates assurance about genetic stability of the upcoming progeny plants. This has drawn attention to PCR based techniques to assess the molecular conformity of plants obtained from encapsulated explants. Though there are many reports available on utilization of synthetic seed technology for mass micro multiplication, along with short and long term storage, only a few recent studies are available on confirmation of the genetic integrity of syn-seed derived plantlets [11-14]. This paper reports, for the first time, that alginate encapsulation provides a secure and stable *in vitro* clonal repository for the germplasm of *R. vomitoria* to ensure multiplication, conservation and year round supply of genetically uniform quality plant material. Besides, the genetic integrity of re-grown plants was assessed by RAPD and ISSR based markers.

Synthetic seeds of *R. vomitoria* stored under moist conditions and at 25 ± 2ºC for 6 months showed re-growth within 10 days upon transferring to solidified MS medium supplemented with 0.1 mg/L NAA and 1.0 mg/L BAP (Fig.1a-b). Encapsulated explants exhibited 95.3% re growth frequency in terms of both single and multiple shoots during a 40 day culture period (Table 1). This result was in concurrence with previous studies of *Cineraria maritima* and *Glycyrrhiza glabra*, where alginate encapsulated micro shoots showed about 82% and 98% re-growth after six months of storage at room temperature and under moist conditions [14-15]. Among the responding encapsulated micro shoots 69.6% showed emergence of multiple shoots while only 30.4% explants exhibited the formation of a single shoot after 40 days of culture (Figure 1b). No major difference in shoot regeneration frequency was observed in cultures incubated for 20 or 40 days. Multiple shoots from encapsulated single nodal explants may develop due to the formation of more than one lateral primordium from actively dividing meristematic cells after obtaining the desired nutrition from the MS alginate complex of the bead. This is desirable, especially when the intention is not only to preserve the plant material, but also to mass multiply it after storage.

**Figure 1(a-f)**: Encapsulated micro shoots of *R. vomitoria* and its response. Micro shoots in calcium alginate beads (a); Single and multiple shoot formation from alginate beads on MS medium containing 0.1 mg/L NAA and 1.0 mg/L BAP (b); Multiple shoot growth from encapsulated beads (c); Rooting in multiple shoots derived from alginate encapsulated micro shoots (d); Complete rooted plant (e); Healthy plants derived from encapsulated micro shoots growing in glass house (f).

**Table 1**: Percentage re-growth response of encapsulated explants after six months of storage

| Experimental set | Number of explants encapsulated | Number of explants re-grown | % Re-growth frequency | |
|---|---|---|---|---|
| | | | Multiple shoot | Single shoot |
| I | 100 | 96 | 68.0±2.0 | 32.0±1.8 |
| II | 100 | 95 | 69.7±1.9 | 30.2±2.0 |
| III | 100 | 95 | 71.1±2.2 | 28.9±2.1 |

Emergence of multiple shoots with increased number of nodes from alginate encapsulated axillary micro cutting after storage could serve as the best option for an integrated conservation procedure. The results also substantiate earlier reports in which alginate-encapsulated shoot tips of *Spilanthe sacmella* and *Eclipta alba* showed noteworthy re-growth potential, with multiple shoots after 60 days storage at 4ºC [16,17]. The present findings also corroborate a previous report of encapsulation of shoot tips of *R. serpentina* and their regeneration followed by storage at 4 ºC for up to 14 weeks [18]. Reports of encapsulation, cryopreservation and germination of micro shoots of some shrubs [19, 20], medicinal herbs [8,21], and trees [22-24] also validate the present results. However, in the current study, encapsulated beads stored under sterile moist conditions at 25 ± 2ºC reduced the overall cost of this protocol and made it easy to handle. This kind of simple low cost method would be especially useful when developing a low temperature or cryostorage method that requires species-based complex protocols and when the test plant is perennial and woody for which *in vitro* multiplication and rooting is difficult.

The developing shoots showed rhizogenesis in two weeks following their transfer to rooting medium (half strength MS containing 2% sucrose and 1.0 mg/L NAA; Fig 1c-e). Approximately 95% plant survival was observed during acclimatization of syn-seed derived plants of *R. vomitoria*, which again correlates with previous reports [13,15] (Figure 1f). It is relevant to mention that, in the present work, axillary micro cuttings were taken as explants as, of the various methods for plant micropropagation and conservation, these are described as being particularly useful due to their simplicity and the relatively high propagation rates. Furthermore, axillary meristem culture is normally believed to have a low risk of genetic precariousness due to the existence of organized meristems, which are less sensitive to genetic variations that might occur during cell division or differentiation under *in vitro* conditions. Moreover, the utilization of stem micro cuttings obtained from aseptically established cultures provides an excellent source of plant material during storage as they are free from infection.

**Table 2**: Total number and size range of amplified fragments and number of polymorphic fragments generated by RAPD and ISSR analysis of *R.vomitoria*.

| Primer | Total no. of amplified products | No. of polymorphic bands | Size range (kb) | % polymorphic bands |
|---|---|---|---|---|
| OPJ-07 | 2 | 0 | 0.1-1.4 | 0 |
| OPJ-10 | 3 | 0 | 0.1-1.9 | 0 |
| OPT-12 | 2 | 0 | 0.5-1.6 | 0 |
| OPT-13 | 2 | 0 | 0.1-1.6 | 0 |
| OPT-15 | 4 | 0 | 0.5-1.4 | 0 |
| OPT-17 | 4 | 1 | 0.6-1.8 | 25 |
| OPO-06 | 1 | 0 | 1.7-1.8 | 0 |
| OPO-09 | 2 | 0 | 0.5-1.8 | 0 |
| OPO-12 | 1 | 0 | 1.0-1.3 | 0 |
| OPO-17 | 2 | 0 | 0.5-1.8 | 0 |
| UBC 810 | 5 | 0 | 0.5-1.7 | 0 |
| UBC 826 | 3 | 0 | 0.5-1.7 | 0 |
| UBC 828 | 5 | 1 | 0.6-1.9 | 20 |
| UBC 841 | 4 | 1 | 0.1-1.1 | 25 |
| UBC 848 | 2 | 0 | 0.6-1.9 | 0 |
| UBC 855 | 4 | 0 | 0.6-1.5 | 0 |
| UBC 845 | 5 | 0 | 0.1-1.6 | 0 |
| UBC 843 | 2 | 0 | 0.6-1.9 | 0 |
| UBC807 | 2 | 0 | 0.1-1.5 | 0 |
| UBC844 | 6 | 1 | 0.6-1.9 | 16 |
| UBC823 | 4 | 0 | 0.4-1.4 | 0 |

**Table 3**: Summary of RAPD and ISSR amplified products from *R. vomitoria*.

| Description | RAPD | ISSR |
|---|---|---|
| Total bands scored | 23 | 42 |
| Number of monomorphic bands | 22 | 39 |
| Number of polymorphic bands | 1 | 3 |
| Percentage of polymorphism | 4.34 | 7.14 |
| Number of primers used | 10 | 11 |
| Average polymorphism per primer | 0.1 | 0.27 |
| Average number of fragments per primer | 2.3 | 3.81 |
| Size range of amplified fragments (kb) | 0.1-1.9 | 0.1-1.9 |

A total of 50 RAPD primers from OPO (01-20), OPT (01-20) and OPJ (1-10) series were tested for the assessment of genetic integrity among syn-seed derived plants, *in vitro* established shoot cultures, and source plants of *R. vomitoria*. Amongst the 50, a total of 10 primers produced 23 clear and reproducible bands from genomic DNA (Table 2), out of which 95.7% were monomorphic in nature, whereas only 4.3 were polymorphic obtained from primer OPT 17. A low degree of occurrence of polymorphic bands indicated a higher similarity scale amongst the sampled plants. An average of 2.3 fragments per primer was amplified with 10 RAPD primers, which were in a size range of 0.1-1.9 kb. On the other hand, after an initial screening of 13 ISSR primers, eleven primers amplified and produced a total of 42 amplification bands, out of which 39 monomorphic bands were obtained revealing 92.8% similarities among the tested plants.

The number of scorable bands for each primer varied from 2 (UBC 848, 843 and 807) to 6 (UBC 844), with an average of 3.81 bands per primer. The number of bands per primer was greater in ISSR (3.81) than RAPD (2.3) (Table 3).The possible reason for this difference could be the high melting temperature for ISSR primers, which allows much more rigorous annealing conditions and, consequently, more precise and repeatable amplification. Moreover, the ISSRs are widely distributed throughout the genome and make amplification of genomic DNA possible in much larger numbers of fragments per primer.

Research reports are available which pragmatically support that ISSR fingerprints sense more polymorphic loci in comparison with RAPD fingerprinting [14,25,26]. A monomorphic pattern was observed in the overall banding profile obtained from RAPD and ISSR. The similarity matrix calculated individually from the results obtained from ISSR and RAPD analysis showed similarity

coefficients of 1.0 for RAPD and 0.85 to 1.0 for ISSR. This strongly indicated a similar genetic stature among sampled and source plants and revealed that the plants derived from the alginate encapsulated micro shoots stored for six months under moist conditions were not affected during storage.

The result is in agreement with recent studies where RAPD based markers revealed a higher similarity scale in syn-seed derived plants after moist storage [9-12]. However, in the present study, two PCR-based techniques, RAPD and ISSR, were chosen because of their simplicity and cost-effectiveness. Having the ability to amplify different regions of the genome, both ISSR and RAPD markers allow better analysis of genetic affinity/diversity among the samples. Besides the advantage of higher reproducibility and relatively low cost, the higher amplification efficiency of ISSRs makes the genetic inferences much clearer at inter and even intra specific levels.

This paper also signifies the use of ISSR markers for the first time for genetic profiling in *Rauvolfia* species. In a recent report, the clonal fidelity assessment of synthetic seed derived plants of *Glycyrrhiza glabra* was also assessed by the use of two types of DNA markers [14]. ISSRs and RAPD assisted assessment of genetic similarity was also reported in the long term for*in vitro* maintained shoot cultures of *Gerbera jamesonii* [26], *Vanilla planifolia* [27] and banana [28].

In conclusion, the reported protocol involving axillary micro shoots of *R. vomitoria* for alginate encapsulation proves to be a simple, cost effective method for standard storage and further recovery of genetically stable and high quality plants. In addition, it ensures year round supply of genetically uniform germplasm for commercial cultivation without any climatic and geographical limitation.

## Experimental

***Plant material:*** Nodal segments of *Rauvolfia vomitoria* Afzelius (field book number 7202 as per CIMAP herbarium) obtained from a 10 year old tree growing in CIMAP farm (26.5° N latitude, 80.5° E longitude and 120 M altitude) at Lucknow, Uttar Pradesh, (India) were used as explants to initiate and establish the aseptic cultures according to the protocol described earlier for *R. serpentina* [29]. For encapsulation purposes, 3-5 mm micro cuttings were used having apical or axillary buds dissected from 2 month old *in vitro* shoot cultures maintained on MS medium containing 1.0 mg/L BAP and 0.1 mg/L NAA.

***Encapsulation and storage of axillary/apical micro cuttings:*** Encapsulation of micro cuttings having apical or axillary buds was performed by using 4% sodium alginate in MS medium as gelling mixture and 100mM $CaCl_2$ as solution for ion exchange, autoclaved at 120ºC for 18 min under 15 lb pressure. Micro shoots of *R. vomitoria* were suspended in the gelling mixture and drop wise dispensed to ion exchange solution under continuous shaking with a magnetic stirrer. The resultant beads were allowed to remain in $CaCl_2$ for 20-30 min for ion exchange and the formation of the Ca-alginate complex. Beads were then thoroughly washed with pre sterilized distilled water and immediately kept for storage in sterile Petridishes (10 beads / Petridishes) under moist conditions maintained by placing sterile filter paper lining soaked with sterile distilled water at 25 ± 2ºC. To maintain moist conditions, the filter paper lining was frequently (20 d interval) sprayed with sterile distilled water. Data on re-growth (single or multiple shoot) response of the stored encapsulated explants was recorded after 20 and 40 d of incubation on solidified MS medium supplemented with 0.1 mg/L NAA and 1.0 mg/L BAP. Developing shoots were further transferred to half strength MS medium containing 2% sucrose and 1.0 mg/L NAA for rhizogenesis. The experiment was repeated thrice with 10 Petridishes each time.

***Hardening of regenerated plants:*** Synthetic seed derived plantlets were transplanted to earthen pots containing a mixture of sand: soil: FYM in the ratio of 1:2:1. Initially the plants were covered with transparent polythene to maintain humidity (80-90%). Covers were temporarily withdrawn for 2 to 3 h every day after the second week for further acclimatization, and were completely withdrawn after the fourth week. Then these plants were kept in a green house for 1 month to check the survival rate.

***Assessment of genetic stature of in vitro regenerated plantlets:*** Three randomly-selected plants, derived from encapsulated micro shoots from each of the 3 sets, 2 month old *in vitro* established shoot cultures and field grown mother plants were analyzed for their genetic stature through RAPD and ISSR analysis. Approximately 200 mg of leaves was ground and powdered in liquid nitrogen and subjected to DNA isolation. Quantity and quality of DNA was measured using a Nanodrop spectrophotometer (Nanodrop, ND-1000, Nanodrop Technologies, USA), as well as by electrophoresis on 0.8%, w/v, Agarose gel. Fifty RAPD decamer primers from OPJ (01-10), OPO (01-20) and OPT (01-20) series from Operon Tech., Alameda, CA, USA and 13 ISSR primers (UBC-807, UBC-810, UBC-823, UBC-826, UBC-828, UBC-834, UBC-835, UBC-841, UBC-843, UBC-844, UBC-845, UBC-848, and UBC-855) from the University of British Columbia, Vancouver, Canada were used for DNA amplification. Amplification was performed using 25 µL PCR mixture comprised of 2.5 µL buffer, 1.0 µL dNTPs (10 µM: 2.5 µM each of the dNTPs viz. dCTP, dATP, dGTP, and dTTP), 0.2 µL Taq polymerase (all procured from GeNei™, Bangalore, India), 1.0 µL DNA (approximate concentration 25 ng/µL), 1.0 µL (5 pM) primer, and 19.2 µL Mili Q water (Millipore, India). Amplifications were carried out in a thermal Cycler (*i*- cycler™; model 4.006, Bio-Rad, USA). The initial denaturation of DNA was at 94ºC for 5 min, followed by 1 min denaturation at 94ºC, 1 min annealing at 35ºC, and 2 min extension at 72ºC. The reaction continued for 35 cycles, followed by a final extension of 3 min at 72ºC.

Similar amplification conditions were maintained for ISSR primers, except that the annealing temperature was 52ºC. PCR products were loaded with 5 µL of bromophenol blue, and separated on a 1.2% agarose gel slab (Hi-Media, India) in 1.0% TAE buffer (40 mMTris acetate, pH 8.2; 1 mM EDTA) using a Minipack-250 electrophoresis system (GeNeiTM,) at 50 V for 3 h. A 1 kb λ DNA marker, double-digested with *EcoRI* and *HindIII* was used as a molecular standard. Gels were documented using an Image Master VDS, Thermal Imaging System, FTI- 500 (Amersham Pharmacia Biotech., USA).

***Statistical analysis data scoring:*** The data obtained on re-growth frequencies of encapsulated micro shoots from repeated experiments were analyzed using the statistical analysis tool box MAT LAB 7.7 version through mean, standard error and one way ANOVA. The significance of results was checked for $p< 0.05$. The RAPD profiles were analyzed by recording the presence and absence of bands in sampled plants. Similarity indices were generated using Nei and Li's coefficient [30]. The average similarity matrix was used to generate a tree for cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic average) using the NTSYS 2.0j software package.

**Acknowledgement -** The authors wish to express their sincere thanks to the Director, CIMAP (CSIR), for providing the facilities to carry out this research. The financial assistance to the authors Shakti Mehrotra and Janhvi Mishra from Department of Science and Technology, India is gratefully acknowledged.

**References**

[1] Eluwa MA, Udoaffah MT, Vulley MBG, Ekanem TB, Akpantah AO, Asuquo OA, Ekong MB.(**2010**) Comparative study of teratogenic potentials of crude ethanolic root bark and leaf extract of *Rauwolfia vomitoria* (Apocynaceae) on the fetal heart. *North American Journal of Medical Science*, ***2***, 592-595.

[2] Joan CT. (**2006**) Anti-diabetic extract isolated from *Rauvolfia vomitoria* and *Citrus aurantium*, and methods of using same. US patent: WO/2006/046156.

[3] Ara H, Jaiswal U, Jaiswal VS. (**2000**) Synthetic seed: prospects and limitations. *Current Science*, ***78***, 1438-1444.

[4] Rao NK.(**2004**) Plant genetic resources: advancing conservation and use through biotechnology. *African Journal of Biotechnology*, ***3***, 136–145.

[5] Borner A.(**2006**) Preservation of plant genetic resources in the biotechnology era. *Biotechnology Journal*, ***1***, 1393–1404.

[6] Singh AK, Sharma M, Varshney R, Agarwal SS, Bansal KC. (**2006**) Plant regeneration from alginate-encapsulated shoot tips of *Phyllanthus amarus* Schum and Thonn, a medicinally important plant species. *In vitro Cell Developmental Biology Plant*, ***42***, 109–113.

[7] Singh AK, Sharma M, Varshney R, Agarwal SS, Bansal KC. (**2006**) Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *Journal of Plant Physiology*, ***163***, 220–223.

[8] Lata H, Chandra S, Khan IA, ElSohly MA. (**2009**) Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L.—an important medicinal plant. *Physiology and Molecular Biology of Plants*, ***15***, 79–86.

[9] Banerjee S, Singh S, Pandey H, Pandey P, Rahman LU. (**2011**) Conservation and storage of *Curcuma amada* Roxb.syn-seeds on Luffa sponge matrix and RAPD analysis of the converted plantlets. *Industrial Crops and Products*, ***36***, 383–388.

[10] Ray A, Bhattacharya S. (**2010**) Storage and conversion of *Eclipta alba* syn-seeds and RAPD analysis of the converted plantlets. *Plant Cell Tissue and Organ Culture*, ***54***, 547–550.

[11] Lata H, Chandra S, Techen N, Khan IA, ElSohly MA. (**2011**) Molecular analysis of genetic fidelity in *Cannabis sativa* L. plants grown from synthetic (encapsulated) seeds following *in vitro* storage. *Biotechnology Letters*, ***33***, 2503-2508.

[12] Ramesh M, Vijaya kumar KP, Vijaya kumar A, Pandian SK.(**2011**) RAPD based genetic stability analysis among micropropagated synthetic seed derived and hardened plants of *Bacopa monnieri* (L.): a threatened Indian medicinal herb. *Acta Physiologia Plantarum*, ***33***, 163–171.

[13] Mishra J, Singh M, Palni LMS, Nandi SK. (**2011**) Assessment of genetic fidelity of encapsulated micro shoots of *Picrorrhiza kurroa*. *Plant Cell Tissue and Organ Culture*, ***104***, 181-186.

[14] Mehrotra S, Khwaja O, Kukreja AK, Rehman L. (**2012**) ISSR and RAPD based evaluation of genetic stability of encapsulated micro shoots of *Glycyrrhiza glabra* following six months of storage.*Molecular Biotechnology*. In press DOI 10.1007/s12033-011-9491-6

[15] Srivastava V, Khan SA, Banerjee S. (**2009**) An evaluation of genetic fidelity of encapsulated microshoots of the medicinal plant: *Cineraria maritima* following six months of storage. *Plant Cell Tissue and Organ Culture*, ***99***, 193-198.

[16] Singh SK, Rai MK, Asthana P, Pandey S, Jaiswal VS, Jaiswal U. (**2009**) Plant regeneration from alginate-encapsulated shoot tips of *Spilanthe sacmella* (L.) Murr, a medicinally important and herbal pesticidal plant species. *Acta Physiologia Plantarum*, ***31***, 649-653.

[17] Singh SK, Rai MK, Asthana P, Sahoo L. (**2010**) Alginate-encapsulation of nodal segments for propagation, short-term conservation and germplasm exchange and distribution of *Eclipta alba* L. *Acta Physiologia Plantarum*, ***32***, 607-610.

[18] Ray A, Bhattacharyaa S. (**2008**) Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentina* - an effective way of conservation and mass propagation. *South African Journal of Botany*, ***74***, 776–779.

[19] Gangopadhyay G, Bandyopadhyay T, Poddar R, Gangopadhyay SB, Mukherjee KK. (**2005**) Encapsulation of pineapple micro shoots in alginate beads for temporary storage. *Current Science*, ***88***, 972–977.

[20] Todd PW, John EP. (**2006**) Bulk alginate encapsulation of *Hibiscus moscheutos* nodal segments. *Plant Cell Tissue and Organ Culture*, ***97***, 345-351.

[21] Mandal J, Pattnaik S, Chand PK.(**2001**) Growth response of the alginate-encapsulated nodal segments of *in vitro*-cultured basils. *Journal of Herbs, Spices & Medicinal Plants*, ***8***, 65-74.

[22] Anand Y, Bansal YK. (**2002**) Synthetic seeds: a novel approach of *in vitro* plantlet formation in Vasaka (*Adhatoda vasica*. Nees.)*Plant Biotechnology*, ***19***, 159–162.

[23] Scocchi A, Faloci M, Medina R, Olmos S, Mroginski L. (**2004**) Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. *Euphytica*, ***135***, 29–38.

**[24] Tsvetkov I, Hausman JF. (2005)** *In vitro* regeneration from alginate-encapsulated microcuttings of *Quercus* sp. *Scientia Horticulturae*, ***103***, 503-507.

[25] Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N.(**2002**) RAPD, ISSR and RFLP finger printing as useful markers to evaluate genetic integrity of microprpagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. Assamica (Assam India type). *Plant Cell Rep*ort, ***21***, 166-173.

[26] Bhatia R, Singh KP, Sharma TR, Jhang T. (**2011**) Evaluation of the genetic fidelity of *in vitro* propagated gerbera (*Gerbera jamesonii* Bolus) using DNA based markers. *Plant Cell Tissue and Organ Culture*, ***104***, 131-135.

[27] Lakshamanan V, Venkataramareddy SR, Neelwarne B. (**2007**) Molecular analysis of genetic stability in long term microshoots of banana using RAPD and ISSR markers. *Electronic Journal of Biotechnology*, ***10***, 1-8.

[28] Sreedhar RV, Venkatachalam L, Bhagyalakshmi N.(**2007**) Genetic fidelity of long term micropropagated shoot cultures of vanilla (*Vanilla palvifolia* Andrews) as assessed by molecular markers. *Biotechnology Journal*, ***2***, 1007-1013.

[29] Goel MK, Mehrotra S, Kukreja AK, Shanker K, Khanuja SP.(**2009**) *In vitro* propagation of *Rauwolfia serpentina* using liquid medium, assessment of genetic fidelity of micropropagated plants, and simultaneous quantitation of reserpine, ajmaline, and ajmalicine. In: Jain SM, Saxena PK (Eds.) *Methods in Molecular Biology. Protocols for In vitro Cultures and Secondary Metabolite Analysis of Aromatic Plants (Vol)* ***547***, Humana Press Inc.,Springer,USA,pp. 17-33.

[30] Nei M, Li W. (**1979**) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Science, USA*, ***76***, 5269–5273.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1651 - 1658

# Bioactivities of Volatile Components from Nepalese *Artemisia* Species

**Prabodh Satyal[a], Prajwal Paudel[a], Ananad Kafle[b], Suraj K. Pokharel[c], Bimala Lamichhane[d], Noura S. Dosoky[e], Debra M. Moriarity[e] and William N. Setzer[a,*]**

[a]*Department of Chemistry*, *University of Alabama in Huntsville*, *Huntsville*, *AL 35899*, *USA*
[b]*Department of Natural Sciences*, *Kathmandu University*, *Dhulikhel*, *Kavre*, *Nepal*
[c]*Laboratory High School*, *Kirtipur*, *Kathmandu*, *Nepal*
[d]*Tribhuvan University*, *Central Department of Chemistry*, *Kirtipur*, *Nepal*
[e]*Department of Biological Sciences*, *University of Alabama in Huntsville*, *Huntsville*, *AL 35899*, *USA*

*wsetzer@chemistry.uah.edu*

**Received: September 24th, 2012; Accepted: October 18th, 2012**

The essential oils from the leaves of *Artemisia dubia*, *A. indica*, and *A. vulgaris* growing wild in Nepal were obtained by hydrodistillation and analyzed by GC-MS. The major components in *A. dubia* oil were chrysanthenone (29.0%), coumarin (18.3%), and camphor (16.4%). *A. indica* oil was dominated by ascaridole (15.4%), isoascaridole (9.9%), *trans*-*p*-mentha-2,8-dien-1-ol (9.7%), and *trans*-verbenol (8.4%). The essential oil of Nepalese *A. vulgaris* was rich in α-thujone (30.5%), 1,8-cineole (12.4%), and camphor (10.3%). The essential oils were screened for phytotoxic activity against *Lactuca sativa* (lettuce) and *Lolium perenne* (perennial ryegrass) using both seed germination and seedling growth, and all three *Artemisia* oils exhibited notable allelopathic activity. *A. dubia* oil showed *in-vitro* cytotoxic activity on MCF-7 cells (100% kill at 100 μg/mL) and was also marginally antifungal against *Aspergillus niger* (MIC = 313 μg/mL). DFT calculations (B3LYP/6-31G*) revealed thermal decomposition of ascaridole to be energetically accessible at hydrodistillation and GC conditions, but these are spin-forbidden processes. If decomposition does occur, it likely proceeds by way of homolytic peroxide bond cleavage rather than retro-Diels-Alder elimination of molecular oxygen.

**Keywords:** *Artemisia dubia*, *Artemisia indica*, *Artemisia vulgaris*, Allelopathy, Antimicrobial, Cytotoxicity, α-Thujone, 1,8-Cineole, Camphor, Ascaridole, Density functional theory.

The genus *Artemisia* (Asteraceae) is made up of around 350 species of aromatic shrubs and herbs distributed over the northern hemisphere, many of which are used as traditional herbal medicines [1]. In this work we have examined the essential oil compositions of three species of *Artemisia* growing wild in Nepal: *A. dubia* Wall. ex Besser, *A. indica* Willd. and *A. vulgaris* L. In Nepal, the leaf juice of *A. dubia* is used to treat cuts and wounds while the plant paste is used against fever; *A. indica* leaf juice is used to treat ringworm [2], and *A. vulgaris* for digestive problems, intestinal worms [3], nose bleeds, neurological disorders [4], and as incense [5].

The composition of the essential oil of *A. dubia* from China has been previously studied [6]. The major components were 1,8-cineole, α-bergamotene, (*E*)-caryophyllene, and τ-cadinol. *A. dubia* extract [7] and essential oil [8] from Nepal have shown antimicrobial activity. *A. indica* essential oil from China was found to be largely composed of 1,8-cineole, camphor, borneol, germacrene D, (*E*)-caryophyllene, and caryophyllene oxide [9]. *A. vulgaris* oils have been extensively studied [10] and show a wide variation in chemical composition. *A. vulgaris* oils have demonstrated allelopathic [11], insecticidal, repellent [12], antioxidant [13], and antimicrobial activities [10r,14].

**Essential oil compositions:** *Artemisia* essential oils were obtained in 0.04%, 0.05%, and 0.8% yield from *A. dubia*, *A. indica*, and *A. vulgaris*, respectively. The chemical compositions of the oils are summarized in Table 1. *A. dubia* oil from Nepal was dominated by chrysanthenone (29.0%), coumarin (18.3%), and camphor (16.4%), with lesser amounts of verbenone (5.2%) and borneol (3.1%), in marked contrast to *A. dubia* essential oil from China [6]. *A. herba-alba* essential oils from Algeria [15] and Morocco [16] have been characterized as having high concentrations of both chrysanthenone and camphor. Chrysanthenone is known to be a photoisomerization product of verbenone [17] and filifolone has been shown to be a thermal decomposition product of chrysanthenone [18].

Likewise, Nepalese *A. indica* essential oil was notably different from that from China [9]. The *A. indica* oil in this present study showed high concentrations of ascaridole (15.4%) and isoascaridole (9.9%), along with *trans*-*p*-mentha-2,8-dien-1-ol (9.7%), *trans*-verbenol (8.4%), *cis*-*p*-mentha-1(7),8-dien-2-ol (6.0%), α-terpineol (5.6%), and menthol (5.4%). The essential oil of *A. molinieri* from France has been reported to contain high levels of ascaridole [19], as did the essential oil of *A. persica* from Iran [20]. Ascaridole is the active component of *Chenopodium ambrosioides* oil and is responsible for the antiparasitic effects of that oil [21]. The compound is known to be thermally labile, however, and isomerizes to isoascaridole [22]. Because of the reported heat-sensitivity of ascaridole, possible thermal decomposition reactions were investigated using density functional theory, DFT (see below).

Because of the great degree of chemical variability in *A. vulgaris* oils [23], a numerical cluster analysis based on the 36 most abundant components was carried out (Figure 1). *A. vulgaris* oils are generally dominated by the monoterpenoids camphor, 1,8-cineole, borneol, α-thujone and β-thujone, as well as the sesquiterpene hydrocarbons α-humulene and (*E*)-caryophyllene, but the relative abundance of these constituents and the presence and abundance of other components is variable and likely depends on geographical, ecological, and phenological factors. The cluster analysis indicates that the sample from Nepal in this study is more

**Table 1**: Chemical compositions of *Artemisia* essential oils from Nepal.

| RI[a] | Compound | Percent Composition | | |
|---|---|---|---|---|
| | | *A. dubia* | *A. indica* | *A. vulgaris* |
| 869 | 1-Hexanol | tr[b] | --- | --- |
| 931 | Tricyclene | --- | --- | tr |
| 935 | α-Thujene | --- | --- | tr |
| 941 | α-Pinene | --- | --- | 0.4 |
| 952 | α Fenchene | --- | --- | 0.6 |
| 953 | Camphene | --- | --- | 0.9 |
| 964 | Benzaldehyde | tr | --- | --- |
| 976 | Sabinene | --- | --- | 0.6 |
| 978 | β-Pinene | 0.1 | --- | 0.5 |
| 981 | 1-Octen-3-ol | 1.9 | --- | 0.3 |
| 988 | 3-Octanone | --- | --- | tr |
| 989 | 6-Methyl-5-hepten-2-one | --- | 1.7 | --- |
| 992 | Dehydro-1,8-cineole | --- | --- | tr |
| 994 | Mesitylene | --- | --- | tr |
| 996 | 3-Octanol | 0.1 | --- | 0.1 |
| 1004 | α-Phellandrene | tr | --- | --- |
| 1016 | α-Terpinene | --- | --- | tr |
| 1021 | 1,2,4-Trimethylbenzene | --- | --- | tr |
| 1024 | *p*-Cymene | 0.3 | --- | 3.7 |
| 1028 | Limonene | tr | --- | 0.2 |
| 1030 | 1,8-Cineole | 1.1 | 1.8 | **12.4** |
| 1032 | Benzyl alcohol | tr | --- | --- |
| 1038 | (*Z*)-β-Ocimene | 0.1 | --- | --- |
| 1048 | (*E*)-β-Ocimene | tr | --- | --- |
| 1058 | γ-Terpinene | 0.1 | --- | 0.1 |
| 1062 | Artemisia ketone | --- | --- | tr |
| 1066 | *cis*-Sabinene hydrate | 1.2 | 2.1 | 0.4 |
| 1071 | 1-Octanol | tr | --- | --- |
| 1098 | *trans*-Sabinene hydrate | 0.6 | 1.9 | 0.4 |
| 1101 | Linalool | 0.2 | --- | --- |
| 1103 | Filifolone | 0.6 | --- | --- |
| 1107 | α-Thujone | **---** | **3.6** | **30.5** |
| 1110 | Isochrysanthenone[c] | 0.8 | --- | --- |
| 1114 | 2-Phenethanol | 0.1 | --- | --- |
| 1117 | β-Thujone | **---** | 1.1 | **7.1** |
| 1120 | Isophorone | --- | --- | 0.5 |
| 1121 | *cis-p*-Menth-2-en-1-ol | --- | --- | 0.1 |
| 1121 | *trans-p*-Mentha-2,8-dien-1-ol | --- | **9.7** | --- |
| 1123 | Unidentified | --- | 0.9 | --- |
| 1126 | Chrysanthenone | **29.0** | --- | **3.7** |
| 1134 | *cis-p*-Mentha-2,8-dien-1-ol | --- | 2.7 | --- |
| 1135 | *iso*-3-Thujanol | --- | --- | 0.6 |
| 1138 | *trans*-Pinocarveol | 0.2 | 1.6 | 0.5 |
| 1139 | *trans*-Sabinol | --- | 1.0 | --- |
| 1140 | *trans-p*-Menth-2-en-1-ol | --- | --- | 0.2 |
| 1144 | *trans*-Verbenol | --- | **8.4** | --- |
| 1144 | Camphor | **16.4** | --- | **10.3** |
| 1155 | Isoborneol | 0.3 | --- | 0.2 |
| 1157 | Sabina ketone | --- | --- | 0.1 |
| 1162 | Pinocarvone | 0.5 | 0.8 | 0.1 |
| 1164 | Borneol | **3.1** | --- | **2.7** |
| 1166 | δ-Terpineol | --- | --- | 0.1 |
| 1167 | Lavandulol | --- | --- | 0.1 |
| 1172 | Menthol | --- | **5.4** | --- |
| 1173 | *cis*-Pinocamphone | --- | --- | 0.1 |
| 1176 | Terpinen-4-ol | 0.5 | 0.9 | 0.8 |
| 1183 | *p*-Methylacetophenone | --- | --- | 0.1 |
| 1184 | *p*-Cymen-8-ol | --- | --- | 0.3 |
| 1186 | *trans-p*-Mentha-1(7),8-dien-2-ol | --- | 3.5 | --- |
| 1189 | α-Terpineol | 1.0 | **5.6** | 0.4 |
| 1195 | Myrtenol | 0.8 | 0.7 | 0.4 |
| 1197 | Unidentified | --- | --- | 0.6 |
| 1207 | Verbenone | **5.2** | **4.3** | **0.7** |
| 1217 | *trans*-Carveol | 0.3 | 1.9 | 0.2 |
| 1218 | *cis*-Sabinene hydrate acetate | --- | --- | 0.1 |
| 1225 | Bornyl formate | --- | --- | 0.3 |
| 1225 | *m*-Cumenol | 0.3 | --- | --- |
| 1225 | *cis-p*-Mentha-1(7),8-dien-2-ol | --- | **6.0** | --- |
| 1228 | *cis*-Carveol | 0.2 | --- | --- |
| 1233 | *trans*-Chrysanthenyl acetate | --- | --- | 0.2 |
| 1235 | Ascaridole | --- | **15.4** | --- |
| 1236 | Cuminaldehyde | --- | --- | 0.1 |
| 1238 | Unidentified | 0.9 | --- | --- |
| 1240 | Carvone | --- | 2.1 | 0.2 |
| 1246 | Carvotanacetone | --- | --- | 0.1 |
| 1250 | *cis*-Myrtanol | 0.1 | --- | --- |
| 1252 | *trans*-Myrtanol | 0.3 | --- | --- |
| 1252 | Piperitone | --- | --- | tr |
| 1254 | Chavicol | 0.1 | --- | --- |
| 1261 | *cis*-Chrysanthenyl acetate | --- | --- | tr |
| 1265 | *iso*-3-Thujanol acetate | --- | --- | tr |
| 1267 | *trans*-Ascaridol glycol | --- | --- | 0.1 |
| 1270 | Unidentified | 0.9 | --- | tr |
| 1272 | Perilla aldehyde | 0.4 | --- | tr |
| 1281 | neoiso-3-Thujanol acetate | --- | --- | 0.1 |
| 1285 | Bornyl acetate | 0.2 | --- | 0.7 |
| 1286 | Unidentified | --- | 0.8 | --- |
| 1289 | *p*-Cymen-7-ol | 0.1 | --- | 0.2 |
| 1291 | Lavandulyl acetate | 0.8 | --- | 1.0 |
| 1296 | Perilla alcohol | 0.3 | --- | tr |
| 1301 | Isoascaridole | --- | **9.9** | --- |
| 1301 | Carvacrol | 0.1 | --- | 0.1 |
| 1317 | Unidentified | 0.1 | --- | 0.9 |
| 1343 | Unidentified | 0.5 | --- | --- |
| 1356 | Eugenol | 1.2 | --- | --- |
| 1366 | Piperitenone oxide | --- | 1.1 | --- |
| 1375 | α-Copaene | --- | --- | 0.1 |
| 1379 | *trans*-Soberol | --- | --- | tr |
| 1396 | 4-Methyl-2-prenylfuran[c] | 0.9 | --- | --- |
| 1412 | *cis*-3-Hydroxy-*p*-menth-1-en-6-one[c] | 0.3 | --- | --- |
| 1419 | (*E*)- β-Caryophyllene | 0.9 | 0.9 | 0.1 |
| 1436 | Coumarin | **18.3** | --- | --- |
| 1443 | (*Z*)-β-Farnesene | --- | --- | 0.2 |
| 1453 | α-Humulene | 0.2 | --- | --- |
| 1460 | *allo*-Aromadendrene | 0.1 | --- | 0.1 |
| 1482 | Germacrene D | 0.3 | --- | 0.8 |
| 1486 | β-Selinene | --- | --- | 0.2 |
| 1495 | *epi*-Cubebol | --- | --- | 0.1 |
| 1501 | α-Muurolene | --- | --- | 0.1 |
| 1514 | γ-Cadinene | --- | --- | 0.2 |
| 1524 | δ-Cadinene | 0.1 | --- | 0.1 |
| 1553 | (*Z*)-Caryophyllene oxide[c] | --- | --- | 0.5 |
| 1568 | Palustrol | --- | --- | 0.2 |
| 1576 | Germacrene D-4-ol | 1.6 | 0.7 | --- |
| 1578 | Spathulenol | --- | --- | 0.6 |
| 1583 | Caryophyllene oxide | **1.2** | --- | **3.5** |
| 1590 | Unidentified | --- | 1.4 | --- |
| 1603 | Ledol | 0.1 | --- | 0.4 |
| 1609 | Humulene epoxide II | --- | --- | 0.5 |
| 1628 | 1-*epi*-Cubenol | --- | --- | tr |
| 1629 | Eremoligenol | 0.4 | --- | --- |
| 1631 | γ-Eudesmol + (*E*)-Sesquilavandulol | 1.4 | --- | --- |
| 1635 | Caryophylla-4(12),8(13)-dien-5-ol | 0.6 | 0.8 | tr |
| 1642 | τ-Muurolol | tr | --- | 0.2 |
| 1645 | α-Muurolol | 0.1 | --- | 0.1 |
| 1650 | β-Eudesmol | 0.2 | --- | 0.6 |
| 1654 | α-Cadinol | 0.4 | --- | 0.4 |
| 1669 | 14-Hydroxy-9-*epi*-(*E*)- β-caryophyllene | --- | --- | 0.5 |
| 1676 | (*Z*)-α-Santalol | --- | --- | 0.2 |
| 1684 | *epi*-α-Bisabolol | --- | 1.4 | --- |
| 1685 | Germacra-4(15),5,10(14)-trien-1α-ol | tr | --- | --- |
| 1741 | Oplopanone | --- | --- | 0.3 |
| 1751 | (6*S*,7*R*)-Bisabolone | --- | --- | 0.2 |
| 1809 | Cryptomeridiol | 0.1 | --- | --- |
| 2108 | (*E*)-Phytol | 0.1 | --- | --- |
| | Total Identified | 95.9 | 96.8 | 93.9 |

[a] RI = "Retention Index" with respect to a series of normal alkanes on a HP-5ms column. [b] tr = "trace" (< 0.05%). [c] Identification based on MS only.

closely related, chemically, to a sample from Turkey [10r] and a sample from India [10d] and represents an α-thujone-rich chemotype (Figure 1). In addition to α-thujone (30.5%), the Nepalese *A. vulgaris* oil was also rich in 1,8-cineole (12.4%) and camphor (10.3%).

**Allelopathic activity:** The allelopathic potentials of *Artemisia* essential oils from Nepal have been assessed in terms of inhibition of seed germination as well as inhibition of seedling growth against a representative dicot (lettuce, *Lactuca sativa*) and a representative monocot (perennial ryegrass, *Lolium perenne*). The allelopathic activities of *A. dubia*, *A. indica*, and *A. vulgaris* essential oils are summarized in Table 2. *Lactuca sativa* seed germination is notably inhibited by all three essential oils: *A. dubia* $IC_{50}$ = 160 μg/mL, *A. indica* $IC_{50}$ = 250 μg/mL, and *A. vulgaris* $IC_{50}$ < 125 μg/mL. *Lolium perenne* was less susceptible to germination inhibition ($IC_{50}$ = 657, 1015, and 1000 μg/mL, respectively, for *A. dubia*, *A. indica*, and *A. vulgaris*).

Both root (radicle) and shoot (hypocotyl) growth of *L. sativa* and *L. perenne* were notably inhibited by the three *Artemisia* essential oils. *A. dubia* and *A. indica* oils significantly inhibited growth of both seedlings at concentrations of 250 μg/mL or higher. *A. vulgaris* oil, however, was somewhat less active in terms of growth inhibition, with significant activity at 500 μg/mL or higher.

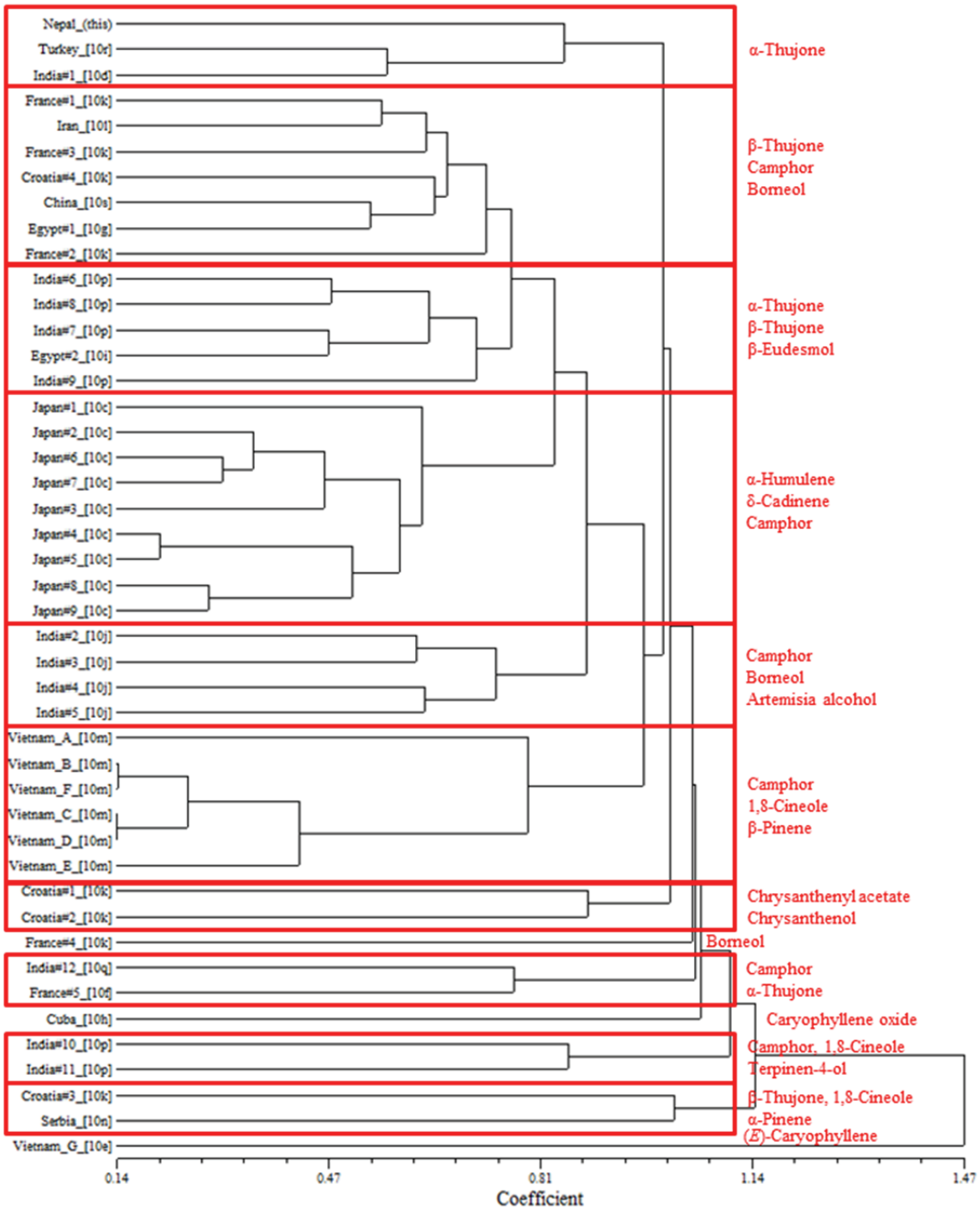



**Figure 1**: Dendrogram obtained by cluster analysis of the percentage composition of essential oils from *A. vulgaris* samples, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA). Chemotypes are indicated in red.

The allelopathic activity of *A. dubia* essential oil can be attributed to the high concentrations of coumarin and camphor. Coumarin has previously shown allelopathic potential against *Zoysia japonica* (seedling growth inhibition) [24], *Daucus carota* (*in-vitro* cell culture) [25], and *Zea mays* (root growth and morphology) [26]. In this present work, we have found that coumarin inhibits both seed germination of *L. sativa* and *L. perenne* ($IC_{50}$ = 51.4 and 265 µg/mL, respectively), as well as seedling growth (significant inhibition of radicle and hypocotyl elongation of both species at 62.5 µg/mL). Additionally, camphor has demonstrated allelopathic activity on *Brassica campestris* seedlings [27] and *Oryza sativa* seedlings [28], as well as germination inhibition and seedling growth of *L. sativa* and *L. perenne* [29].

The allelopathic activity of *A. indica* essential oil from Nepal is likely due to the high concentration of ascaridole (15.4%), a compound that has demonstrated broad bioactivity [30], as well as allelopathic activity [67]. α-Terpineol (5.6%) is also allelopathic [29,32] and may contribute to the activity of *A. indica* oil.

**Table 2**: Allelopathic activity of Nepalese *Artemisia* essential oils on lettuce (*Lactuca sativa*) and perennial ryegrass (*Lolium perenne*).

| Concentration (µg/mL) | Germination Inhibition (%) | | Seedling Growth (% of Controls) | | | |
|---|---|---|---|---|---|---|
| | | | *Lactuca sativa* | | *Lolium perenne* | |
| | *Lactuca sativa* | *Lolium perenne* | radicle | hypocotyl | radicle | hypocotyl |
| *A. dubia* | | | | | | |
| 1000 | 100 | 100 | --- | --- | --- | --- |
| 500 | 76.7 | 13.3 | 18.3[a] | 55.2[a] | 30.4[a] | 71.3[b] |
| 250 | 65.0 | 15.0 | 41.4[a] | 79.2[a] | 56.2[a] | 74.0[c] |
| 125 | 46.7 | 15.0 | 70.0[a] | > 100[e] | 96.8[e] | > 100[e] |
| *A. indica* | | | | | | |
| 1000 | 96.7 | 48.3 | 41.2[a] | 0.0[a] | 16.8[a] | 3.0[a] |
| 500 | 53.3 | 20 | 73.4[b] | 74.2[c] | 48.3[a] | 38.7[a] |
| 250 | 50.0 | 13.3 | 81.2[c] | 77.3[c] | 53.2[a] | 29.1[a] |
| *A. vulgaris* | | | | | | |
| 2000 | 100 | 60.0 | --- | --- | 47.1[a] | 15.9[a] |
| 1000 | 96.7 | 50.0 | 74.4[b] | 36.8[a] | 51.1[a] | 18.7[a] |
| 500 | 58.3 | 43.3 | 96.3[e] | 64.0[b] | 70.2[c] | 56.8[c] |
| 250 | 58.3 | 36.7 | > 100[e] | > 100[e] | >100[e] | 67.9[d] |
| 125 | 63.3 | nt[f] | > 100[e] | > 100[e] | --- | --- |
| Coumarin | | | | | | |
| 500 | 100 | 100 | --- | --- | --- | --- |
| 250 | 93.3 | 43.3 | 21.8[a] | 42.0[a] | 41.7[a] | 24.7[a] |
| 125 | 85.0 | 30.0 | 29.1[a] | 48.1[a] | 59.9[a] | 33.7[a] |
| 62.5 | 73.3 | 21.7 | 52.5[a] | 88.6[d] | 78.5[c] | 58.1[a] |
| α/β-Thujones | | | | | | |
| 3000 | 98.3 | 78.3 | 15.3[a] | 0.0[a] | 44.0[a] | 0.0[a] |
| 1500 | 93.3 | 60.0 | 62.4[a] | 22.2[a] | 67.4[a] | 12.3[a] |
| 750 | 90.0 | 40.0 | 96.3[e] | 71.1[b] | 76.3[c] | 20.5[a] |
| 375 | 35.0 | 35.0 | > 100[e] | 97.1[e] | > 100[e] | 65.1[c] |

[a] Significantly inhibited compared with controls ($P < 0.001$). [b] Significantly inhibited compared with controls ($0.001 < P < 0.01$). [c] Significantly inhibited compared with controls ($0.01 < P < 0.05$). [d] Significantly inhibited compared with controls ($0.5 < P < 0.1$). [e] Not significantly inhibited.[f] "nt" = not tested.

**Table 3:** Cytotoxic and antimicrobial activity of Nepalese *Artemisia* essential oils.

| Sample | MCF-7 Cytotoxicity (% kill at 100 µg/mL) | Antimicrobial Activity (MIC, µg/mL) | | | | |
|---|---|---|---|---|---|---|
| | | *B. cereus* | *S. aureus* | *E. coli* | *P. aeruginosa* | *A. niger* |
| *A. dubia* | 100 | 1250 | 625 | 1250 | 2500 | 313 |
| *A. indica* | 15.0 (2.8)[a] | 625 | 1250 | 625 | 625 | 625 |
| *A. vulgaris* | 33.6 (2.8) | 2500 | 2500 | 2500 | 2500 | 2500 |

[a] Standard deviations in parentheses.

The allelopathy of *A. vulgaris* has been reported previously [11] and this current work complements that previously reported. The phytotoxic activity of *A. vulgaris* oil against *L. sativa* and *L. perenne* in this study can be attributed to relatively high concentrations of the known allelopathic agents 1,8-cineole [27,29,33], α- and β-thujone [34], and camphor [27-29,32]. In the present work, we found that a mixture of α- and β-thujones weakly inhibited *L. sativa* and *L. perenne* germination with $IC_{50}$ values of 448 and 1060 µg/mL, respectively.

**Cytotoxic and antimicrobial activities:** Of the three *Artemisia* oils examined in this study, only *A. dubia* showed notable *in vitro* cytotoxic activity with 100% killing of MCF-7 human breast adenocarcinoma cells at a concentration of 100 µg/mL (Table 3). Neither *A. dubia* nor *A. vulgaris* essential oils showed notable antimicrobial activity in our assays (Table 3). *A. dubia* was marginally active against *Staphylococcus aureus* (MIC = 625 µg/mL) and *Aspergillus niger* (MIC = 313 µg/mL), while *A. indica* oil was marginally active against *B. cereus*, *E. coli*, *P. aeruginosa*, and *A. niger* (MIC = 625 µg/mL). The crude ethanol extract of *A. dubia* had shown weak antibacterial activity against *S. aureus* and *S. epidermidis* (MIC = 800 µg/mL) [7], while *A. dubia* essential oil showed weak antifungal activity against *Alternaria brassicicola* (64% growth inhibition at 10 µL/mL) [8], consistent with our present results. A thujones-rich *A. vulgaris* oil from Turkey had shown antibacterial and antifungal activity in a zone-of-inhibition assay, but minimum inhibitory concentrations were not determined [10r].

**Thermal decomposition of ascaridole:** Thermal decomposition reactions of ascaridole were investigated using density functional theory at the B3LYP/6-31G* level. The retro-Diels-Alder reaction of ascaridole (singlet ground state) to give α-terpinene and molecular oxygen (triplet ground state) had a calculated free energy of reaction ($\Delta G°$) of 8.8 kJ/mol. Decomposition reactions of ascaridole, either retro-Diels-Alder reaction or homolytic peroxide O-O bond cleavage, are both spin-forbidden processes. That is, on the first step of either reaction, ascaridole is a singlet ground state but dissociation by retro-Diels-Alder reaction would ultimately lead to triplet-ground-state $O_2$ as a product. Likewise, homolytic cleavage of singlet ascaridole would lead to a triplet diradical intermediate (Fig. 2). In either of these decomposition processes, high-energy transition structures could be avoided by crossing from the singlet to the triplet potential energy surface. The activation energies are estimated to be where the energies of the two spin-state processes are equal in energy (*i.e.*, where the energies cross with concomitant singlet-to-triplet crossing). It is assumed that the rate of intersystem crossing and the rate of bond cleavage must be similar, and calculating the transition probabilities between the singlet and triplet surfaces can presumably be estimated using the Landau-Zener formula [35], but are beyond the scope of this present study. The free energy of activation ($\Delta G^{‡}$) for the retro-Diels-Alder reaction was estimated to be the point where the energies for the singlet reaction profile and the triplet reaction profile are the same (Fig. 3). This point corresponds to an average C-O distance of 2.184 Å with a free energy, $G°$ = -540.757851 au, corresponding to an activation energy, $\Delta G^{‡}$ = 170.1 kJ/mol. Homolytic bond cleavage of the ascaridole peroxide bond with subsequent ring closure to give the diepoxide isoascaridole was calculated to be very exergonic ($\Delta G°$ = -130.1 kJ/mol), but with activation energies of 111.9 and 90.2 kJ/mol for the O-O bond cleavage and the epoxide ring closure, respectively (see Figs. 2 and 4). There is another triplet-singlet crossing in the final epoxide ring closing, corresponding to $\Delta G^{‡}$ = 93.8 kJ/mol, to give finally isoascaridole. The free energy of activation for the retro-Diels-Alder reaction is much higher than that of homolytic O-O bond cleavage, suggesting the retro-Diels-Alder reaction to be a less likely pathway for decomposition of ascaridole.

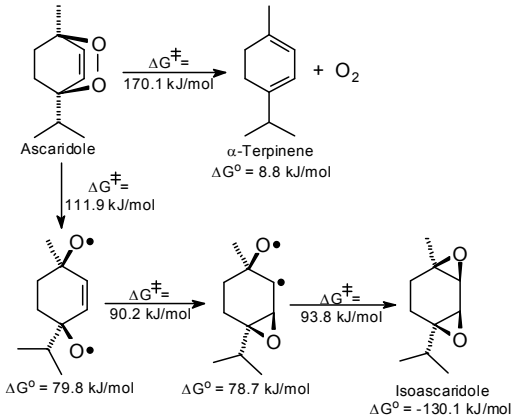



**Figure 2**: B3LYP/6-31G* free energy differences for decomposition reactions of ascaridole. All energies are relative to the free energy of ascaridole.

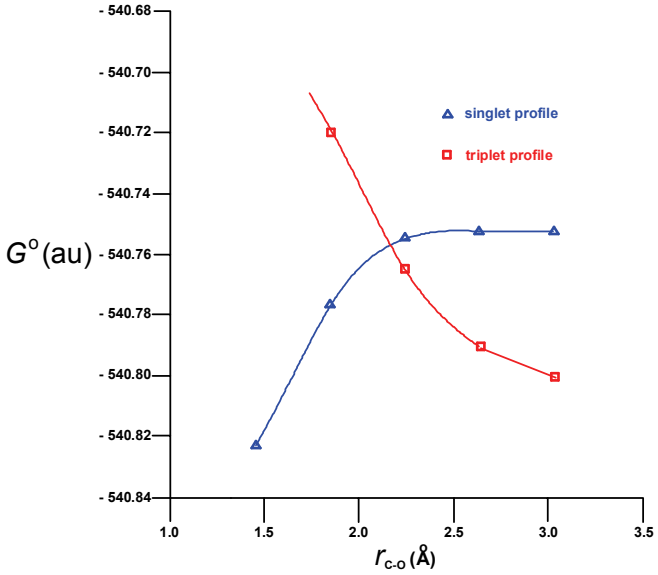



**Figure 3**: Energy profile for retro-Diels-Alder elimination of $O_2$. The singlet-triplet potential surface crossing point corresponds to an average C-O distance of 2.184 Å with a free energy, $G°$ = -540.757851 au.

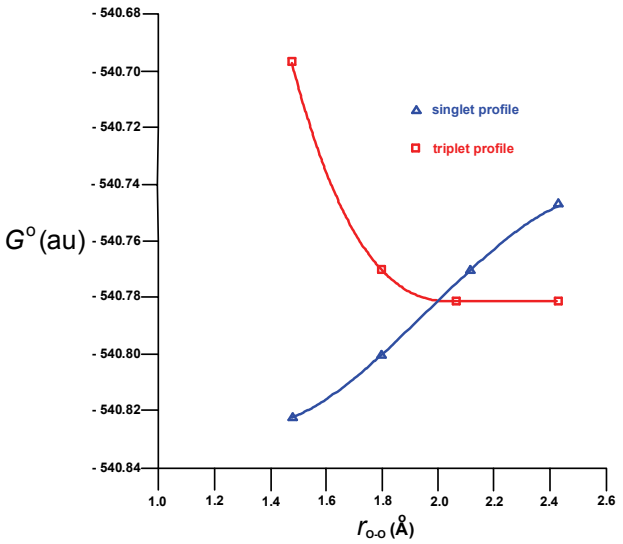



**Figure 4**: Energy profile for homolytic O—O bond cleavage. The transition structure (singlet-triplet potential surface crossing) corresponds to a free energy, $G°$ = -540.780011 au and O-O bond length of 2.010 Å.

In general, essential oils rich in ascaridole are also rich in both α-terpinene and isoascaridole [19b,30b-e,36], consistent with the reported thermal lability of ascaridole [22,37]. In contrast, neither the essential oil of *Artemisia indica* (this work) nor the essential oil of *Achillea biebersteinii* [38] had detectible quantities of α-terpinene, suggesting that thermal decomposition by way of retro-Diels-Alder reaction either during hydrodistillation or GC injection is not an important decomposition pathway. Additionally, Toncer and co-workers did not detect isoascaridole in their ascaridole-rich sample of *Achillea biebersteinii* [38], suggesting also that decomposition of ascaridole to form isoascaridole is not an important pathway during either hydrodistillation or gas chromatography. It seems reasonable, then, that the presence of α-terpinene and isoascaridole in essential oils rich in ascaridole are not due to thermal decomposition during essential oil isolation or analysis.

## Experimental

***Plant material:*** *A. dubia* was collected on 16th May, 2011, from the Kirtipur Municipality (27.67° N, 85.28° E, 1360 m above sea level), Kathmandu, Nepal. The plant was identified by Dr. Krishna Shrestha and a voucher specimen (number TUCH 49) has been deposited in the Tribhuvan University Central Herbarium. Fresh leaves (60.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.025 g essential oil.

*A. indica* was collected on 1st July, 2011, from Dhulikhel (27.61° N, 85.55° E, 1550 m above sea level), Kavre, Nepal. The plant was identified by Sameer Thapa and a voucher specimen (number NH5401) has been deposited in the National Herbarium and Plant Laboratories. Fresh leaves (80.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.042 g essential oil.

*A. vulgaris* was collected on 21st May, 2011, from Hetauda Makwanpur (27.42° N, 85.03° E, 1550 m above sea level), Nepal. The plant was identified by Tilak Gautam and a voucher specimen (number 1204) has been deposited in the Botany Department, Tribhuvan University, Post-Graduate Campus, Biratnagar. Fresh leaves (100.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.8 g essential oil.

***Gas chromatographic/mass spectral analysis:*** The *Artemisia* essential oils were analyzed by GC-MS using an Agilent 6890 GC with an Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was a HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 µm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Injector temperature was 200°C and interface temperature 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, held for 10 min; increased at 3°C/min to 200°C; increased 2°/min to 220°C. A 1% w/v solution of the sample in $CH_2Cl_2$ was prepared and 1 µL was injected using a splitless injection technique.

Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature [39] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version

C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

***Allelopathic activity assays:*** An allelopathic bioassay based on lettuce (*Lactuca sativa*) and perennial rye grass (*Lolium perenne*) germination and subsequent radicle and hypocotyl growth was used to study the effects of the *Artemisia* essential oils. Stock solutions of each essential oil (2.0 g/L essential oil and 1.0 g/L Tween-80 in water) were prepared and used for the assays. Two-fold serial dilutions of stock test solutions were prepared to give test concentrations of 4000, 2000, 1000, 500, and 250 µg/mL with the control being 1.0 g/L aqueous Tween-80. Seeds were placed in 6-well test plates (10 seeds per well) each well lined with two layers of Whatman No. 1 filter paper moistened with test solution and the test plates were sealed with Parafilm®. The test plates were incubated at room temperature in the dark for 5 days, after which the number of germinated seeds was determined and the root (radicle) and shoot (hypocotyl) lengths were measured. Student's *t*-test [40] was used to compare radicle and hypocotyl test means with controls. Seed germination $IC_{50}$ values were determined using the Reed-Muench method [41].

***Antimicrobial screening:*** The essential oils were screened for antimicrobial activity against Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579), *Staphylococcus aureus* (ATCC No. 29213); and Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MIC) were determined using the microbroth dilution technique [42]. Dilutions of the essential oil were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 µL of 1% w/w solutions of essential oil in DMSO plus 50 µL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately $1.5 \times 10^8$ colony forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 h; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Geneticin was used as a positive antibiotic control; DMSO was used as a negative control. Antifungal activity against *Aspergillus niger* (ATCC No. 16888) was determined as above using potato dextrose broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

***Cytotoxicity screening:*** Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22) [43] were grown in a 3% $CO_2$ environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per L of medium, 15 mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at $2.5 \times 10^4$ cells per well. The volume in each well was 100 µL. After 48 h, supernatant fluid was removed by suction and replaced with 100 µL growth medium containing 1.0 µL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 µg/mL for each well. Solutions were added to wells in 4 replicates. Medium and DMSO controls (10 µL DMSO/mL) were used. Tingenone [44] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% $CO_2$; medium was then removed by suction, and 100 µL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out [45]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated.

***Numerical cluster analysis:*** A total of 45 *Artemisia vulgaris* essential oil compositions from this work and the published literature were treated as operational taxonomic units (OTUs). The percentage composition of 36 principle essential oil components (camphor, 1,8-cineole, α-thujone, α-humulene, borneol, β-thujone, δ-cadinene, (*E*)-caryophyllene, α-pinene, β-pinene, terpinen-4-ol, caryophyllene oxide, α-muurolene, sabinene, *trans*-chrysanthenyl acetate, α-terpineol, germacrene D, artemisia alcohol, *p*-cymene, camphene, α-phellandrene, chrysanthenol, spathulenol, γ-cadinene, β-eudesmol, β-elemene, γ-terpinene, *trans*-sabinene hydrate, chrysanthenone, α-copaene, methyl eugenol, bornyl acetate, linalool, β-selinene, lyratol, and *trans*-isoelemicin) was used to determine the chemical relationship between the different *A. vulgaris* essential oil samples by cluster analysis using the STSYSpc software, version 2.2 [46]. Correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

***Density functional theory calculations:*** All calculations were carried out using Spartan '10 for Windows [47]. The hybrid B3LYP functional [48] and the 6-31G* basis set [49] were used for the optimization of all stationary points in the gas phase. Frequency calculations were employed to characterize stationary points as minima or first-order saddle points. All reaction and activation enthalpies reported are zero-point (ZPE) corrected and thermally corrected. Entropies were calculated using the linear harmonic oscillator approximation. Two reaction profiles were modeled: A retro-Diels-Alder elimination of dioxygen and a homolytic O-O peroxide cleavage with subsequent diepoxide ring closure. In order to model these decomposition reactions, the reaction profiles were calculated stepwise for both the singlet and triplet spin states at the B3LYP/6-31G* level.

**Acknowledgments** – PS and PP are grateful to Dr. Krishna Shrestha, Tilak Gautam, Kiran Pokharel and Sameer Shrestha for identifying the plant samples. We thank Bernhard Vogler for technical assistance with the GC-MS measurements and James Baird for helpful discussions on singlet-triplet interconversions.

## References

[1] Mabberly DJ. (**1997**) *The Plant Book*, 2nd Ed. Cambridge University Press, Cambridge, UK, 57.
[2] Gewali MB. (**2008**) *Aspects of Traditional Medicine in Nepal*. Institute of Natural Medicine, University of Toyama, Japan, 17-20.
[3] Tamang G. (**2003**) An ethnobiological study of the Tamang people. *Our Nature*, ***1***, 37-41.
[4] Hussain S, Hore DK. (**2007**) Collection and conservation of major medicinal plants of Darjeeling and Sikkim Himalayas. *Indian Journal of Traditional Knowledge*, ***6***, 352-357.
[5] Turin M. (**2003**) Ethnobotanical notes on Thangmi plant names and their medicinal and ritual uses. *Contributions to Nepalese* Studies, ***30***, 19-52.
[6] Zheng W, Tan R, Liu Z. (**1996**) Chemical constituents of essential oil of two varieties of *Artemisia dubia* Wall. ex Bess. *Acta Phytotaxonomica Sinica*, ***34***, 410-414.
[7] Bhatt LR, Lim JA, Baek SH. (**2005**) Antimicrobial activity of Nepalese medicinal plants on skin microbial inflammation pathogens. *Journal of Cosmetic and Public Health*, ***1***, 6-9.
[8] Parajuli RR, Tiwari RD, Chaudhary RP, Gupta VN. (**2005**) Fungitoxicity of the essential oils of some aromatic plants of Manang against *Alternaria brassicicola*. *Scientific World*, ***3***, 39-43.

[9] Wu H, Wei Z, Li Y, Liang C, Liang H. (**2009**) Analysis of the constituents of volatile oil in *Artemisia indica* from Guangxi. *China Pharmacy* **2009-09** doi: CNKI:SUN:ZGYA.0.2009.09.024.

[10] (a) Nano GM, Bicchi C, Frattini C, Gallino M. (**1976**) On the composition of some oils from *Artemisia vulgaris*. *Planta Medica*, ***30***, 211-215; (b) Michaelis K, Vostrowsky O, Paulini H, Zintl R, Knobloch K. (**1982**) Das ätherische Öl aus Blüten von *Artemisia vulgaris* L. *Zeitschrift für Naturforschung*, ***37c***, 152-158; (c) Aoki T, Shigetaka H. (**1985**) Seasonal variations and geographical differences of volatile constituents from *Artemisia vulgaris* L. var. *indica* Maxim. *Bulletin of Suzugamine Women's College of Natural Science*, ***26***, 9-14; (d) Misra LN, Singh SP. (**1986**) α-Thujone, the major component of the essential oil from *Artemisia vulgaris* growing wild in Nilgiri hills. *Journal of Natural Products*, ***49***, 941-941; (e) Dũng NX, Nam VV, Huóng HT, Leclercq PA. (**1992**) Chemical composition of the essential oil of *Artemisia vulgaris* L. var. *indica* Maxim. from Vietnam. *Journal of Essential Oil Research*, ***4***, 433-434; (f) Milhau G, Valentin A, Benoit F, Mallié M, Bastide JM, Pélissier Y, Bessière JM. (**1997**) *In vitro* antimalarial activity of eight essential oils. *Journal of Essential Oil Research*, ***9***, 329-333; (g) Aboutabl EA, El-Azzouny A, El Dahmy SI. (**1998**) Composition of the essential oil of *Artemisia vulgaris* L. grown in Egypt. *Journal of Essential Oil-Bearing Plants*, ***1***, 21-27; (h) Pino JA, Rosado A, Fuentes V. (**1999**) Composition of the essential oil of *Artemisia vulgaris* L. herb from Cuba. *Journal of Essential Oil Research*, ***11***, 477-478; (i) Haggag MY, El Tantawy ME, Ahmed FI, Shams MM. (**2000**) Study of chemical composition, antimicrobial and anthelmintic activities of the essential oils of *Artemisia vulgaris* L. and *Santolina chamaecyparissus* L. cultivated in Egypt. *Azerbaijan Journal of Pharmaceutical Sciences*, ***26***, 23-39; (j) Haider F, Dwivedi PD, Naqvi AA, Bagchi GD. (**2003**) Essential oil composition of *Artemisia vulgaris* harvested at different growth periods under Indo-Gangetic Plain conditions. *Journal of Essential Oil Research*, ***15***, 376-378; (k) Jerkovic I, Mastelic J, Milos M, Juteau F, Masotti V, Vlano J. (**2003**) Chemical variability of *Artemisia vulgaris* L. essential oils originated from the Mediterranean are of France and Croatia. *Flavour and Fragrance Journal*, ***28***, 436-440; (l) Sefidkon F, Jahili A, Rabie M, Hamzehee B, Asri Y. (**2003**) Chemical composition of essential oil of five *Artemisia* species from Iran. *Journal of Essential Oil-Bearing Plants*, ***6***, 41-45; (m) Thao NTP, Thuy NT, Hoi TM, Thai TH, Muselli A, Bighelli A, Castola V, Casanova J. (**2004**) *Artemisia vulgaris* L. from Vietnam: Chemical variability and composition of the oil along the vegetative life of the plant. *Journal of Essential Oil Research*, ***16***, 358-361; (n) Blagojević P, Radulović N, Palić R, Stojanović G. (**2006**) Chemical composition of the essential oils of Serbian wild-growing *Artemisia absinthium* and *Artemisia vulgaris*. *Journal of Agricultural and Food Chemistry*, ***54***, 4780-4789; (o) Judžentienė A, Buzelytė J. (**2006**) Chemical composition of essential oils of *Artemisia vulgaris* L. (mugwort) from north Lithuania. *Chemija*, ***17***, 12-15; (p) Lohani H, Sah S, Singh A, Sati M, Yadav RK. (**2007**) Chemical composition of essential oil of *Artemisia vulgaris* L. from three different areas of Uttaranchal. *Fafai Journal*, ***9***, 69-72; (q) Govindaraj S, Kumari BDR, Cioni PL, Flamini G. (**2008**) Mass propagation and essential oil analysis of *Artemisia vulgaris*. *Journal of Bioscience and Bioengineering*, ***105***, 176-183; (r) Baykan Erel S, Reznicek G, Şenol SG, Karabay Yavaşoğul NU, Konyalioğlu S, Zeybek AU. (**2011**) Antimicrobial and antioxidant properties of *Artemisia* L. species from western Anatolia. *Turkish Journal of Biology*, ***35***, doi:10.3906/biy-0912-27; (s) Zhu L, Li Y, Li B, Lu B, Zhang W. (**1995**) *Artemisia vulgaris* Linn. (Compositae). In *Aromatic Plants and Essential Constituents* (Supplement). Hai Feng Publishing Co., Hong Kong, 135.

[11] Barney JN, Hay AG, Weston LA. (**2005**) Isolation and characterization of allelopathic volatiles from mugwort (*Artemisia vulgaris*). *Journal of Chemical Ecology*, ***31***, 247-265.

[12] Wang J, Zhu F, Zhou XM, Niu CY Lei CL. (**2006**) Repellent and fumigant activity of essential oil from *Artemisia vulgaris* to *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Journal of Stored Products Research*, ***42***, 339-347.

[13] Bhatt LR, Lim JA, Chai KY, Kang JI, Oh HK, Baek SH. (**2006**) Antioxidative and antimicrobial activities of essential oil from *Artemisia vulgaris*. *Natural Product Sciences*, ***12***, 226-231.

[14] Kováts N, Gölöncsér F, Ács A, Refaey M. (**2010**) Quantification of the antibacterial properties of *Artemisia absinthium*, *A. vulgaris*, *Chrysanthemum leucanthemum* and *Achillea millefolium* using the *Vibrio fischeri* bacterial biassay. *Acta Botanica Hungarica*, ***52***, 137-144.

[15] Dob T, Benabdelkader T. (**2006**) Chemical composition of the essential oil of *Artemisia herba-alba* grown in Algeria. *Journal of Essential Oil Research*, ***18***, 685-690.

[16] Ouachikh O, Bouyanzer A, Bouklah M, Desjobert JM, Costa J, Hammouti B, Majidi L. (**2009**) Application of essential oil of *Artemisia herba alba* as green corrosion inhibitor for steel in 0.5 M $H_2SO_4$. *Surface Review and Letters*, ***16***, 49-54.

[17] Kostyk BC, Borden JH, Gries G. (**1993**) Photoisomerization of antiaggregation pheromone verbenone: Biological and practical implications with respect to the mountain pine beetle, *Dendroctonus poderosae* Hopkins (Coleoptera: Scolytidae). *Journal of Chemical Ecology*, ***19***, 1749-1759.

[18] Asfaw N, Storesund HJ, Skattebøl L, Aasen AJ. (**2001**) Coexistence of chrsanthenone, filifolone and (*Z*)-isogeranic acid in hydrodistillates. Artefacts! *Phytochemistry*, ***58***, 489-492.

[19] (a) Carnat AP, Lamaison JL. (**1992**) Composition of the essential oil of *Artemisia molinieri* Quézel, Barbero et R. Loisel. *Journal of Essential Oil Research*, ***4***, 635-637; (b) Masotti V, Juteau F, Bessière JM, Viano J. (**2003**) Seasonal and phenological variations of the essential oil from the narrow endemic species *Artemisia molinieri* and its biological activities. *Journal of Agricultural and Food Chemistry*, ***51***, 7115-7121.

[20] Mirjalili BF, Meybody MHH, Arkakani MM, Rustaiyan A, Ameri N, Masoudi S, Bamoniri A. (**2006**) Chemical composition of the essential oil from aerial parts, leaves, flowers and roots of *Artemisia persica* Boiss. from Iran. *Journal of Essential Oil Research*, ***18***, 544-547.

[21] (a) Monzote Fidalgo L. (**2007**) Essential oil from *Chenopodium ambrosioides* as a promising antileishmanial agent. *Natural Product Communications*, ***2***, 1257-1262; (b) Monzote L, Nance MR, Garcia M, Scull R, Setzer WN. (**2011**) Comparative chemical, cytotoxicity and antileishmanial properties of essential oils from *Chenopodium ambrosioides*. *Natural Product Communications*, ***6***, 281-286.

[22] Cavalli JF, Tomi F, Bernardini AF, Casanova J. (**2004**) Combined analysis of the essential oil of *Chenopodium ambrosioides* by GC, GC-MS and $^{13}$C-NMR spectroscopy: Quantitative determination of ascaridole, a heat-sensitive compound. *Phytochemical Analysis*, ***15***, 275-279.

[23] Weston LA, Barney JN, Di Tommaso A. (**2005**) A review of the biology and ecology of three invasive perennials in New York State: Japanese knotweed (*Polygonum cuspidatum*), mugwort (*Artemisia vulgaris*) and pale swallow-wort (*Vincetoxicum rossicum*). *Plant and Soil*, ***277***, 53-69.

[24] Yamamoto Y. (**1995**) Allelopathic potential of *Anthoxanthum odoratum* for invading *Zoysia*-grassland in Japan. *Journal of Chemical Ecology*, ***21***, 1365-1373.

[25] Abenavoli MR, Sorgonà A, Sidari M, Badiani M, Fuggi A. (**2003**) Coumarin inhibits the growth of carrot (*Daucus carota* L. cv. Saint Valery) cells in suspension culture. *Journal of Plant Physiology*, ***160***, 227-237.

[26] Abenavoli MR, Sorgonà A, Albano S, Cacco G. (**2004**) Coumarin differentially affects the morphology of different root types of maize seedlings. *Journal of Chemical Ecology*, ***30***, 1871-1883.

[27] Nishida N, Tamotsu S, Nagata N, Saito C Sakai A. (**2005**) Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: Inhbition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. *Journal of Chemical Ecology*, ***31***, 1187-1203.

[28] Yumi O, Keiko Y, Katsuichiro K. (**2011**) Allelopathic activity of camphor released from camphor tree (*Cinnamomum camphora*). *Allelopathy Journal*, ***27***, 123-232.

[29] Kennedy JE, Davé PC, Harbin LN, Setzer WN. (**2011**) Allelopathic potential of *Sassafras albidum* and *Pinus taeda* essential oils. *Allelopathy Journal*, ***27***, 111-122.

[30] (a) Yen YP, Yeh MJ, Hsiao WF. (**2007**) Synthesis and nematicidal activity of ascaridole derivatives against *Meloidogyne incognita* and *Aphelenchoides besseyi*. *Journal of Pesticide Science*, ***32***, 49-52; (b) Torres MCM, Assunção JC, Santiago GMP, Andrade-Neto M, Silveira ER,

Costa-Lotufo LV, Bezerra DP, Filho JDBM, Viana FA, Pessoa ODL. (**2008**) Larvicidal and nematicidal activities of the leaf essential oil of *Croton regelianus*. *Chemistry and Biodiversity*, ***5***, 2724-2728; (c) Monzote L, Stamberg W, Staniek K, Gille L. (**2009**) Toxic effects of carvacrol, caryophyllene oxide, and ascaridole from essential oil of *Chenopodium ambrosioides* on mitochondria. *Toxicology and Applied Pharmacology*, ***240***, 337-347; (d) Chu SS, Hu JF, Liu ZL. (**2011**) Composition of essential oil of Chinese *Chenopodium ambrosioides* and insecticidal activity against maize weevil, *Sitophilus zeamais*. *Pest Management Science*, ***67***, 714-718; (e) Bai CQ, Liu ZL, Liu QZ. (**2011**) Nematicidal constituents from the essential oil of *Chenopodium ambrosioides* aerial parts. *E.-Journal of Chemistry*, ***8***, S143-S148.

[31] Jiménez-Osornio FMVZJ, Kumamoto J, Wasser C. (**1996**) Allelopathic activity of *Chenopodium ambrosioides* L. *Biochemical Systematics and Ecology*, ***24***, 195-205.

[32] De Martino L, Mancini E, Rolim de Almeida LF, De Feo V. (**2010**) The antigerminative activity of twenty-seven monoterpenes. *Molecules*, ***15***, 6630-6637.

[33] (a) Koitabashi R, Suzuki T, Kawazu T, Sakai A, Kuroiwa H, Kuroiwa T. (**1997**) 1,8-Cineole inhibits root growth and DNA synthesis in the root apical meristem of *Brassica campestris* L. *Journal of Plant Research*, ***110***, 1-6; (b) Romagni JG, Allen SN, Dayan FE. (**2000**) Allelopathic effects of volatile cineoles on two weedy plant species. *Journal of Chemical Ecology*, ***26***, 303-313; (c) Singh HP, Batish DR, Kaur S, Arora K, Kohli RK. (**2002**) Allelopathic effect of two volatile monoterpenes against bill goat weed (*Ageratum conyzoides* L.) *Crop Protection*, ***21***, 347-350.

[34] (a) Halligan JP. (**1975**) Toxic terpenes from *Artemisia californica*. *Ecology*, ***56***, 999-1003; (b) Jassbi AR, Zamanizadehnajari S, Baldwin IT. (**2010**) Phytotoxic volatiles in the roots and shoots of *Artemisia tridentata* as detected by headspace solid-phase microextraction and gas chromatographic-mass spectrometry analysis. *Journal of Chemical Ecology*, ***36***, 1398-1407.

[35] Steinfeld JI, Francisco JS, Hase WL. (**1999**) *Chemical Kinetics and Dynamics*, 2nd Ed. Prentice Hall, Upper Saddle River, New Jersey, USA, 247-250.

[36] Koba K, Catherine G, Raynaud C, Chaumont JP, Sanda K, Laurence N. (**2009**) Chemical composition and cytotoxic activity of *Chenopodium ambrosioides* L. essential oil from Togo. *Bangladesh Journal of Scientific and Industrial Research*, ***44***, 435-440.

[37] (a) Nelson EK. (**1920**) The composition of oil of *Chenopodium* from various sources. *Journal of the American Chemical Society*, ***42***, 1204-1208; (b) Zand R, Mesrobian RB. (**1955**) Polymerization of methacrylonitrile initiated by ascaridole and dihydroascaridole. *Journal of the American Chemical Society*, **77**, 6523-6524.

[38] Toncer O, Basbag S, Karaman S, Diraz E, Basbag M. (**2010**) Chemical composition of the essential oils of some *Achillea* species growing wild in Turkey. *International Journal of Agriculture and Biology*, ***12***, 527-530.

[39] Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry*, 4th Ed. Allured Publishing, Carol Stream, Illinois.

[40] Zar JH. (**1996**) *Biostatistical Analysis*, 3rd Ed. Prentice Hall, Upper Saddle River New Jersey, 123-137.

[41] Reed LJ, Muench H. (**1938**) A simple method of estimating fifty percent end points. *American Journal of Hygiene*, ***27***, 493-497.

[42] Sahm DH, Washington JA. (**1991**) Antibacterial susceptibility tests: Dilution methods. In *Manual of Clinical Microbiology*. Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shamody HJ (Eds.). American Society for Microbiology, Washington DC, USA.

[43] Soule HD, Vazguez J, Long A, Albert S, Brennan M. (**1973**) A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the National Cancer Institute*, ***51***, 1409-1416.

[44] Setzer WN, Setzer MC, Hopper AL, Moriarity DM, Lehrman GK, Niekamp KL, Morcomb SM, Bates RB, McClure KJ, Stessman CC, Haber WA. (**1998**) The cytotoxic activity of a *Salacia* liana species from Monteverde, Costa Rica, is due to a high concentration of tingenone. *Planta Medica*, ***64***, 583.

[45] Ferrari M, Fornasiero MC, Isetta AM. (**1990**) MTT colorimetric assay for testing macrophage cytotoxic activity *in vitro*. *Journal of Immunological Methods*, ***31***, 165-172.

[46] Rohlf JF. (**2005**) *NTSYSpc, Numerical Taxonomy and Multivariate Analysis System.* Applied Biostatistics Inc., New York.

[47] *Spartan '10 for Windows* (**2010**) Wavefunction, Inc., Irvine, California.

[48] (a) Becke AD. (**1993**) Density-functional thermochemistry. III. The role of exact exchange. *Journal of Chemical Physics*, ***98***, 5648-5652; (b) Lee C, Yang W, Parr RG. (**1988**) Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density. *Physical Review B*, ***37***, 785-789.

[49] Hehre WJ, Radom L, Schleyer PvR. (**1986**) *Ab initio Molecular Orbital Theory*. Wiley, New York.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1659 - 1662

# Chemical Compositions, Antioxidant and Antimicrobial Activity of the Essential Oils of *Piper officinarum* (Piperaceae)

**Wan Mohd Nuzul Hakimi Wan Salleh[a], Farediah Ahmad[a,*], Khong Heng Yen[b] and Hasnah Mohd Sirat[a]**

[a]*Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, Skudai, 81310 Johor Bahru, Johor, Malaysia*

[b]*School of Chemistry and Environmental Studies, Faculty of Applied Sciences, Universiti Teknologi MARA Sarawak, Kota Samarahan, 94300, Sarawak, Malaysia*

*farediah@kimia.fs.utm.my*

**Received: September 29th, 2012; October 25th, 2012**

This study was designed to investigate the antioxidant and antimicrobial activities of the essential oils from *Piper officinarum* C. DC. GC and GC/MS analysis of the leaf and stem oils showed forty one components, representing 85.6% and 93.0% of the oil, respectively. The most abundant components in the leaf oil were β-caryophyllene (11.2%), α-pinene (9.3%), sabinene (7.6%), β-selinene (5.3%) and limonene (4.6%), while β-caryophyllene (10.9%), α-phellandrene (9.3%), linalool (6.9%), limonene (6.7%) and α-pinene (5.0%) were the main components of the stem oil. The antioxidant activities were determined by using complementary tests: namely β-carotene-linoleic acid, DPPH radical scavenging and total phenolic assays. The stems oil showed weak activity ($IC_{50}$ = 777.4 μg/mL) in the DPPH system, but showed moderate lipid peroxidation inhibition in the β-carotene-linoleic acid system (88.9 ± 0.35%) compared with BHT (95.5 ± 0.30%). Both oils showed weak activity against *P. aeruginosa* and *E. coli* with M IC values of 250 μg/mL.

**Keywords:** Chemical composition, *Piper officinarum*, Essential oils, Antioxidant, Antimicrobial.

*P. officinarum* C. DC is locally known as 'lada panjang' or 'kechundai' in the Malaysian region. It is a climber that grows in Malaysia, India, China, Indonesia and the Philippines. Pepper obtained from this species has been used as an adulterant for *P. longum*. In Cambodia, Laos and Vietnam, the plant is used to treat fever, jaundice, rheumatism and neuralgia. In the Philippines, the roots are chewed to promote digestion and externally to heal wounds [1].

Previous phytochemical studies on *P. officinarum* have resulted in the isolation of a number of amide and lignan compounds [2-5]. In addition, studies on the extracts of this species showed antibacterial, interceptive, antiatherosclerotic and hypolipidaemic activities [6,7]. To the best of our knowledge there is no previous report on the chemical composition of the essential oil of *P. officinarum*. Here we report the chemical composition, antioxidant and antimicrobial activities of the leaf and stem oils of *P. officinarum*, collected from Sarawak, Malaysia.

Hydrodistillation of the fresh leaves and stems of *P. officinarum* gave pale yellow oils in yields of 0.26% and 0.22%(w/w), respectively. The chemical compositions of these oils are listed in Table 1. GC and GC-MS analysis successfully detected forty-one components each, which accounted for 85.6% and 93.0%, respectively of the chromatographic components. Sesquiterpene hydrocarbons were the major components in the leaf (41.1%) and stem (43.6%) oils, with β-caryophyllene (11.2% and 10.9%) being the most substantial compound in both oils. Other major compounds were α-pinene (9.3%), sabinene (7.6%), β-selinene (5.3%) and limonene (4.6%) for the leaf oil, and α-phellandrene (9.3%), linalool (6.9%), limonene (6.7%) and α-pinene (5.0%) for the stem oil. Monoterpene hydrocarbons were present in high concentration in both the leaf (31.7%) and stem oils (30.4%). Oxygenated monoterpenes made up a minor fraction (9.2-12.4%), while oxygenated sesquiterpenes formed 3.0-3.6% of the total oil. Nine components of the stem oil were not detected in the leaf oil. These were α-terpinolene, eugenol, α-ylangene, methyl eugenol, α-cedrene, α-bergamotene, δ-selinene, (*E*)-nerolidol and α-cadinol. On the other hand, nine components of the leaf oil were not detected in the stem oil (camphene, δ-3-carene, α-terpinene, camphor, β-cubebene, γ-elemene, (*E*, *E*)-α-farnesene, germacrene B and globulol).

The DPPH free radical scavenging activity and β-carotene/linoleic acid tests were carried out on the essential oils, together with the Folin-Ciocalteu assay, which evaluated the total phenolic content of the essential oils. The results are given in Table 2. The antioxidant activity in the DPPH radical scavenging test is due to the hydrogen donating ability of the test material. The capability of substances to donate hydrogen to convert DPPH into the non-radical form of DPPH can be followed spectrophotometrically [8]. Both oils exhibited weak DPPH radical scavenging activity ($IC_{50}$ leaf oil, 622.2 μg/mL; stem oil, 777.4 μg/mL) compared with the standard antioxidant, BHT ($IC_{50}$ = 124.4 μg/mL). The low activity was attributed to the low phenolic content of the essential oils. This was supported by the results of the Folin-Ciocalteu assay on both oils. In the β-carotene/linoleic acid assay, oxidation of linoleic acid produces hydroperoxyl radicals which simultaneously attack the chromophore of β-carotene resulting in bleaching of the reaction emulsion [9]. The effectiveness of the leaf (85.9%) and stem (88.9%) oils was compared with that of BHT (125.5%), but the inhibition values observed were significantly lower than that of BHT.

The antimicrobial activity of the essential oils was evaluated against Gram-positive bacteria, Gram-negative bacteria and fungi. Their activity potentials were assessed qualitatively and quantitatively by the presence of inhibition zones, zone diameters and minimum inhibitory concentration (MIC) values. The results are presented in Table 3. The inhibition zones were in the range of 7.0-11.2 mm.

**Table 1**: Constituents identified in *P. officinarum* leaf and stem oils.

| Components | KI [b] | Percentage [a] | |
|---|---|---|---|
| | | Leaves | Stems |
| **α-Pinene** | 930 | **9.3 ± 0.02** | **5.0 ± 0.01** |
| Camphene | 945 | 1.5 ± 0.04 | - |
| **Sabinene** | 969 | **7.6 ± 0.01** | 3.3 ± 0.02 |
| Myrcene | 988 | 1.5 ± 0.11 | 1.9 ± 0.10 |
| **α-Phellandrene** | 1002 | 2.2 ± 0.06 | **9.3 ± 0.02** |
| δ-3-Carene | 1008 | 0.7 ± 0.02 | - |
| α-Terpinene | 1014 | 0.4 ± 0.04 | - |
| **Limonene** | 1024 | **4.6 ± 0.1** | **6.7 ± 0.03** |
| 1,8-Cineole | 1026 | 1.0 ± 0.07 | 1.7 ± 0.06 |
| (*Z*)-β-Ocimene | 1030 | 0.3 ± 0.05 | 0.7 ± 0.01 |
| **γ-Terpinene** | 1055 | 3.6 ± 0.01 | 2.8 ± 0.05 |
| α-Terpinolene | 1086 | - | 0.7 ± 0.01 |
| **Linalool** | 1092 | 3.4 ± 0.02 | **6.9 ± 0.09** |
| Camphor | 1142 | 0.7 ± 0.04 | - |
| Terpinen-4-ol | 1175 | 3.6 ± 0.01 | 3.5 ± 0.03 |
| α-Terpineol | 1186 | 0.5 ± 0.12 | 0.3 ± 0.06 |
| α-Cubebene | 1345 | 0.3 ± 0.08 | 1.1 ± 0.02 |
| Eugenol | 1356 | - | 1.1 ± 0.04 |
| α-Ylangene | 1372 | - | 0.8 ± 0.03 |
| α-Copaene | 1374 | 1.7 ± 0.03 | 3.4 ± 0.05 |
| β-Cubebene | 1386 | 2.7 ± 0.02 | - |
| β-Elemene | 1389 | 1.3 ± 0.10 | 1.0 ± 0.04 |
| Methyl eugenol | 1403 | - | 2.5 ± 0.01 |
| α-Gurjunene | 1405 | 2.2 ± 0.02 | 0.1 ± 0.10 |
| α-Cedrene | 1410 | - | 0.1 ± 0.12 |
| α-Bergamotene | 1412 | - | 0.2 ± 0.05 |
| **β-Caryophyllene** | 1417 | **11.2 ± 0.02** | **10.9 ± 0.03** |
| γ-Elemene | 1434 | 0.2 ± 0.05 | - |
| Aromadendrene | 1440 | 0.5 ± 0.02 | 0.4 ± 0.01 |
| α-Humulene | 1452 | 3.2 ± 0.01 | 3.1 ± 0.04 |
| Dehydroaromadendrene | 1460 | 0.5 ± 0.04 | 0.2 ± 0.02 |
| α-Amorphene | 1483 | 3.2 ± 0.03 | 3.1 ± 0.1 |
| Germacrene D | 1485 | 0.7 ± 0.01 | 2.1 ± 0.04 |
| **β-Selinene** | 1490 | **5.3 ± 0.1** | 2.4 ± 0.1 |
| δ-Selinene | 1492 | - | 0.3 ± 0.05 |
| Cadina-1,4-diene | 1495 | 0.5 ± 0.08 | 0.3 ± 0.01 |
| α-Selinene | 1498 | 2.5 ± 0.05 | 2.8 ± 0.01 |
| **Bicyclogermacrene** | 1500 | 1.2 ± 0.02 | **5.4 ± 0.02** |
| α-Muurolene | 1502 | 0.2 ± 0.02 | 0.6 ± 0.08 |
| (*E*,*E*)-α-Farnesene | 1508 | 0.7 ± 0.05 | - |
| δ-Cadinene | 1520 | 0.2 ± 0.02 | 2.5 ± 0.02 |
| *cis*-Calamenene | 1528 | 0.2 ± 0.04 | 0.2 ± 0.04 |
| α-Calacorene | 1545 | 0.1 ± 0.01 | 0.1 ± 0.11 |
| Germacrene B | 1560 | 2.5 ± 0.1 | - |
| (*E*)-Nerolidol | 1562 | - | 0.1 ± 0.04 |
| Spathulenol | 1578 | 1.3 ± 0.01 | 1.3 ± 0.01 |
| Caryophyllene oxide | 1582 | 0.4 ± 0.03 | 0.4 ± 0.02 |
| Globulol | 1592 | 1.8 ± 0.07 | - |
| Viridiflorol | 1595 | 0.1 ± 0.02 | 0.3 ± 0.03 |
| α-Cadinol | 1652 | - | 0.9 ± 0.05 |
| **Group components** | | | |
| **Phenylpropanoids** | | **-** | **3.6 ± 0.2** |
| **Monoterpene hydrocarbons** | | **31.7 ± 0.2** | **30.4 ± 0.2** |
| **Oxygenated monoterpenes** | | **9.2 ± 0.2** | **12.4 ± 0.2** |
| **Sesquiterpene hydrocarbons** | | **41.1 ± 0.2** | **43.6 ± 0.2** |
| **Oxygenated sesquiterpenes** | | **3.6 ± 0.2** | **3.0 ± 0.2** |
| **Identified components (%)** | | **85.6 ± 0.2** | **93.0 ± 0.2** |

[a] Each value is expressed as means ± SD of three injections.
[b]Retention indices on *Ultra-1* capillary column.

**Table 2**: Antioxidant activity of *P. officinarum*[a]leaf and stem oils.

| Samples | β-Carotene/linoleic acid (%) | DPPH $IC_{50}$ (μg/mL) | TPC (mg GA/g) |
|---|---|---|---|
| Leaf oil | 85.9 ± 0.4 | 622.2 ± 0.3 | 30.4 ± 0.4 |
| Stem oil | 88.9 ± 0.3 | 777.4 ± 0.2 | 44.1 ± 0.4 |
| BHT | 125.5 ± 0.3 | 124.8 ± 0.2 | ND |

[a] Data represent mean ±SD of three independent experiments; ND – not determined.

The leaf oil was found to have a weak antimicrobial activity against *B. subtlis*, *P. aeruginosa* and *E. coli*, with MIC values of 250 μg/mL. The stem oil showed similar weak activity against *P. aeruginosa* and *E. coli*, with MIC values of 250 μg/mL. Components such as α-pinene, 1,8-cineole, γ-terpinene, linalool and α-terpineol have been found to have relatively strong antimicrobial properties [10]. These components were present in both the leaf and stem oils and,therefore, may have contributed to this antimicrobial activity. Generally, essential oils that contain high proportions of oxygenated monoterpenes have stronger antifungal activities [11]. However, the leaf and stem oils showed only weak antifungal activity against *A. niger*, with a MIC value of 500 μg/mL. The weak

**Table 3**: Antimicrobial activity of *P. officinarum* leaf and stem oils[a]

| Test microbes | | Leaf oil | Stem oil | SS | NYS |
|---|---|---|---|---|---|
| *Bacillus subtilis* | DD | 10.5 ± 0.4 | 11.2 ± 0.3 | 17.6 ± 0.2 | ND |
| | MIC | 250 | 500 | 7.8 | ND |
| *Staphylococcus aureus* | DD | 8.9 ± 0.4 | 8.6 ± 0.4 | 17.8 ± 0.2 | ND |
| | MIC | 500 | 500 | 7.8 | ND |
| *Pseudomonas aeruginosa* | DD | 8.7 ± 0.3 | 8.8 ± 0.5 | 17.2 ± 0.2 | ND |
| | MIC | 250 | 250 | 7.8 | ND |
| *Pseudomonas putida* | DD | 8.6 ± 0.5 | 8.5 ± 0.4 | 17.3 ± 0.2 | ND |
| | MIC | 500 | 500 | 7.8 | ND |
| *Escherichia coli* | DD | 8.2 ± 0.5 | 8.2 ± 0.4 | 17.5 ± 0.2 | ND |
| | MIC | 250 | 250 | 7.8 | ND |
| *Candida albicans* | DD | 7.0 ± 0.4 | 8.0 ± 0.5 | ND | 15.2 ± 0.2 |
| | MIC | 1000 | 1000 | ND | 7.8 |
| *Aspergillus niger* | DD | 7.4 ± 0.4 | 8.3 ± 0.4 | ND | 15.3 ± 0.2 |
| | MIC | 500 | 500 | ND | 7.8 |

[a] Data represent mean ±SD of three independent experiments.
DD – Disc diffusion (zone of inhibition including the diameter of disc: 6 mm); MIC – Mininum inhibitory concentration (μg/mL); SS – streptomycin sulfate; NYS – nystatin; ND – not determined.

antifungal activity of both oils was due to the small amount of oxygenated monoterpenes. Previous studies have reported that the antimicrobial activity of an essential oil results from a complex interaction between different components which may produce additive, synergistic or antagonistic effect [12].

## Experimental

***Plant materials:*** A sample of *P. officinarum* C. DC was collected from Lundu, Sarawak, Malaysia, in January 2010 and identified by Mdm Mohizar Mohamad. The voucher specimen (UiTMKS3004) was deposited at the Natural Products Research & Development Centre (NPRDC), UiTM Sarawak.

***Solvents and chemicals:*** β-Carotene, linoleic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol and dimethylsulfoxide (DMSO), HPLC grade chloroform, Folin-Ciocalteu's reagent, anhydrous sodium sulfate, sodium carbonate and polyoxyethylenesorbitan monopalmitate (Tween-40) were purchased from Merck (Darmstadt, Germany).

***Extraction of essential oils:*** The fresh leaves and stems were subjected to hydrodistillation in an all glass Dean-Stark apparatus for 8 h. The oils obtained were dried over anhydrous magnesium sulfate and stored at 4-6°C.

***Gas chromatography (GC):*** GC analyses were performed on a Hewlett Packard 6890 series II A gas chromatograph equipped with an Ultra-1 column (100% polymethylsiloxanes) (25 m long, 0.33 μm thickness and 0.20 mm inner diameter). Helium was used as carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperatures were set at 250 and 280°C, respectively. Oven temperature was kept at 50°C, then gradually raised to 280°C at 5°C/min and finally held isothermally for 15 min. Diluted samples (1/100 in diethyl ether, v/v) of 1.0 μL were injected manually (split ratio 50:1). The injection was repeated 3 times and the peak area percentages were reported as means ±SD of triplicates. Calculation of peak area percentage was carried out using GC HP Chemstation software (Agilent Technologies).

***Gas chromatography-mass spectrometry (GC-MS):*** GC-MS chromatograms were recorded using a Hewlett Packard Model 5890A gas chromatography and a Hewlett Packard Model 5989A mass spectrometer. The GC was equipped with an Ultra-1 column (25 m long, 0.33 μm thickness and 0.20 mm inner diameter). Helium was used as carrier gas at a flow rate of 1 mL/min. Injector temperature was 250°C. Oven temperature was programmed from 50°C (5 min hold) at 10°C/min to 250°C and finally held

isothermally for 15 min. For GC-MS detection, an electron ionization system, with ionization energy of 70 eV was used. A scan rate of 0.5 s (cycle time: 0.2 s) was applied, covering a mass range from 50-400 amu.

***Identification of components:*** The constituents of the oil were identified by comparison of their MS with reference spectra in the computer library (Wiley), and also by comparing their retention indices with those of either authentic compounds or data in the literature [13]. The quantitative data were obtained electronically from FID area percentage without the use of correction factor.

***Antioxidant activity - DPPH radical scavenging:*** The free radical scavenging activity was measured by the DPPH method [14], with minor modifications. Each sample of stock solution (1.0 mg/mL) was diluted to a final concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL. Then, a total of 3.8 mL of 50 µM DPPH methanolic solution (1 mg/50 mL) was added to 0.2 mL of each sample solution and allowed to react at room temperature for 30 min. The absorbance of the mixtures was measured at 517 nm. A control was prepared without sample or standard and measured immediately at 0 min. The percent inhibitions (*I*%) of DPPH radical were calculated as follows:

$$I\% = [\ (A_{blank} - A_{sample}) / A_{blank}\ ]\ x\ 100$$

where $A_{blank}$ is the absorbance value of the control reaction (containing all reagents except the test compound) and $A_{sample}$ is the absorbance value of the test compounds. The sample concentration providing 50% inhibition ($IC_{50}$) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and $IC_{50}$ values were reported as means ± SD of triplicates.

***Total phenolic content (TPC):*** Total phenolic contents of the essential oils were determined as described by Slinkard and Singleton [15]. A sample of stock solution (1.0 mg/mL) was diluted in methanol to final concentrations of 1000, 800, 600, 400, and 200 µg/mL. A 0.1 mL aliquot of sample was pipetted into a test tube containing 0.9 mL of methanol, then 0.05 mL Folin-Ciocalteu's reagent was added, and the flask thoroughly shaken. After 3 min, 0.5 mL of 5% $Na_2CO_3$ solution was added and the mixture allowed to stand for 2 h with intermittent shaking. Then, 2.5 mL of methanol was added and left to stand in the dark for 1 h. The absorbance measurements were recorded at765 nm. The same procedure was repeated for the standard gallic acid solutions and a standard curve obtained with the following equation:

$$y = 0.0021x - 0.0223,\ r^2 = 0.9928$$

The concentration of total phenolic compounds in the oils was expressed as mg of gallic acid equivalent per g of sample. Tests were carried out in triplicate and the gallic acid equivalent value was reported as mean ± SD of triplicate.

***β-Carotene-linoleic acid:*** The β-carotene-linoleic acid bleaching assay described by Miraliakbari and Shahidi [16] was used, with minor modifications. A mixture of β-carotene and linoleic acid was prepared by mixing together 0.5 mg β-carotene in 1 mL chloroform (HPLC grade), 25 µL linoleic acid and 200 mg Tween 40. The chloroform was then completely evaporated under vacuum and 100 mL of oxygenated distilled water was subsequently added to the residue and mixed gently to form a clear yellowish emulsion. The essential oils and BHT (positive control) were individually dissolved in methanol (2 g/L) and 350 µL volumes of each of them were added to 2.5 mL of the above emulsion in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50ºC for 2 h, together with a negative control (blank) containing the same volume of methanol. The absorbance values were measured at 470 nm on an ultraviolet-visible (UV-vis) spectrophotometer. Antioxidant activities (percentage inhibitions, *I*%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2 h}} - A_{\text{initial }\beta\text{-carotene}})\ x\ 100$$

where $A_{\beta\text{-carotene after 2 h}}$ is the absorbance value of β-carotene after 2 h and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance value of β-carotene at the beginning of the experiment. All tests were carried out in triplicate and percentage inhibitions were reported as means ± SD of triplicates.

***Antimicrobial activity: Microbial strains:*** *Staphylococcus aureus* (ATCC29737), *Bacillus subtilis* (ATCC6633), *Pseudomonas aeruginosa* (ATCC9027), *Pseudomonas putida* (ATCC49128), *Escherichia coli* (ATCC10536), *Candida albicans* (ATCC10231), and *Aspergillus niger* (ATCC16888) were purchased from Mutiara Scientific, Cheras, Kuala Lumpur, Malaysia. The strains were grown on nutrient agar (Oxoid, Italy) for the bacteria, and potatodextrose agar (PDA) for yeasts and fungi. For the antimicrobial tests, nutrient broth (Oxoid, Italy) for bacteria, and potato dextrose broth (PDB) for yeasts and fungi were used.

***Disc diffusion:*** Antimicrobial activity of the essential oils of *P. officinarum* was determined by the agar disc diffusion method. The essential oils were dissolved in DMSO (4 mg/mL). Antimicrobial tests were carried out by the disc diffusion method [17] using 400 µL of suspension containing $10^8$ CFU/mL of bacteria and $10^6$ CFU/mL of fungi, spread on the nutrient agar (NA) and potato dextrose agar (PDA) mediums, respectively. The disc (6 mm diameter) impregnated with 10 µL of the essential oils and DMSO (negative control) was placed on the inoculated agar, which was incubated for either 24 h at 37°C (bacteria) or 48 h at 30ºC (fungi). Streptomycin sulfate (10 µg/mL) and nystatin (100 IU) were used as the positive controls for bacteria and fungi, respectively. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. All tests and analyses were carried out in triplicate.

***Minimum inhibitory concentration (MIC):***The MIC was determined by the broth micro dilution method using 96-well microplates [18]. The inoculate of the microbial strains was prepared from 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. Essential oil (1 mg) was dissolved in DMSO (1 mL) to obtain 1000 µg/mL stock solution. A number of wells were reserved in each plate for positive and negative controls. Sterile broth (100 µL) was added to the well from row B to H. The stock solutions of samples (100 µL) were added to the wells in rows A and B. Then, the mixture of samples and sterile broth (100 µL) in row B was transferred to each well in order to obtain a twofold serial dilution of the stock samples (concentration of 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL). The inoculum (100 µL) was added to each well. The final volume in each well was 200 µL. Streptomycin sulfate for bacteria and nystatin for fungi were used as positive controls. Plates were incubated at 37°C for 24 h. Microbial growth was indicated by the presence of turbidity and a pellet at the bottom of the well.

***Statistical analysis****:* Data obtained from essential oil analysis, antioxidant and antimicrobial activity are expressed as mean values. Statistical analyses were carried out by employing one way ANOVA ($p$>0.05). A statistical package (SPSS version 11.0) was used for the data analysis.

**Acknowledgments** - The authors thank the Ministry of Science, Technology and Innovation Malaysia for financial support under vote QJ130000.7126.02H30 and the Faculty of Science, Universiti Teknologi Malaysia for research facilities.

## References

[1] Wiart C. (**2006**) *Medicinal Plants of Asia and the Pacific*.Taylor & Francis Group, Boca Raton, London, New York.pp 26-27.
[2] Gupta OP, Atal CK, Gaind KN. (**1972**) Occurrence of methyl piperate in *Piper officinarum*. *International Journal of Cancer*, ***10***, 874.
[3] (a) Gupta OP, Atal CK, Dhar KL. (**1976**) Structure of new amide from *Piper officinarum*. *Phytochemistry*, ***15***, 425; (b) Gupta OP, Gupta SC, Atal CK, Dhar KL.(**1976**) Structure of a new amide, filfiline isolated from *Piper officinarum*. *Indian Journal of Chemistry*, ***14B***, 912.
[4] Gupta OP, Gupta SC, Atal CK, Dhar KL. (**1977**) A new amide from *Piper officinarum*. *Phytochemistry*, ***16***, 1436-1437.
[5] Mrutyunjaya RR, Venkateswarlu Y. (**2011**) Isolation of three compounds from the roots of *Piper officinarum*. *Research Journal of Chemistry and Environment*, ***15***, 705-708.
[6] Sharma I, Gusain D, Dixit VP, Purohit A. (**1998**) *Piper officinarum*: A potent antiatherosclerotic and hypolipidaemic agent. *Indian Journal of Pharmaceutical Sciences*, ***60***, 391-393.
[7] Rao RM, Saranapu N, Ramanujan VB, Sastry CPS, Krishnaiah P, Srihari S. (**2011**) Antibacterial action of crude solvent extracts of *Piper officinarum*. *Asian Journal of Chemistry*, ***23***, 4024-4026.
[8] Soares JR, Dinis TCP, Cunha AP, Almeida LM. (**1997**) Antioxidant activity of some extracts of *Thymus zygis*. *Free Radical Research*, ***26***, 469-478.
[9] Frankel EN. (**1998**) *Lipid Oxidation*. The Oily Press, Dundee. pp 23-41.
[10] *Bakkali F,* Averbeck S, Averbeck D, Idaomar M. (**2008**) Biological effects *of* essential oils - A review. *Food Chemical Toxicology*, ***46***, 446-475.
[11] Kordali S, Kotan R, Cakir A. (**2007**) Screening of antifungal activities of 21 oxygenated monoterpenes *in vitro* as plant disease control agents. *Allelopathy Journal*, ***19***, 373-391.
[12] Xianfei X, Xiaoqiang C, Shunying Z, Guolin Z. (**2007**) Chemical composition and antimicrobial activity of essential oils of *Chaenomeles speciosa* from China. *Food Chemistry*, ***100***, 1312-1315.
[13] Adams RP. (**2001**) *Identification of essential oil by gas chromatography-quadrupole/mass spectroscopy*. Allured Publishing Corporation, Carol, Stream IL, USA.
[14] Loo AY, Jain K, Darah I. (**2008**) Antioxidant activity of compounds isolated from the pyroligneus acid, *Rhizopora apiculata. Food Chemistry*, ***107***, 1151-1160.
[15] Slinkard K, Singleton VL. (**1977**) Total phenol analyses: Automation and comparison with manual methods. *American Journal of Enology Viticulture*, ***28***, 49-55.
[16] Miraliakbari H, Shahidi F. (**2008**) Antioxidant activity of minor components of tree nut oils. *Food Chemistry*, ***111***, 421-427.
[17] Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. (**1999**) *Manual of Clinical Microbiology*, 7$^{th}$ed. ASM, Washington, DC, USA.
[18] Gulluce M, Sokmen M, Sahin M, Sokmen A, Adiguzel A, Ozer H. (**2004**) Biological activities of the essential oil and methanolic extract of *Micromeria fruticosa* (L.) Druce ssp. *serpyllifolia* (Bieb) PH Davis plants from the eastern Anatolia region of Turkey. *Journal of Science Food Agriculture*, ***84***, 735-741.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1663 - 1666

# Chemical Composition, Antimicrobial and Antioxidant Properties of the Volatile Oil and Methanol Extract of *Xenophyllum poposum*

**Ana M. González[a], María I. Tracanna[a], Sara M. Amani[a], Carola Schuff[b], Miguel J. Poch[a], Horacio Bach[c] and César A. N. Catalán[b,*]**

[a]*Cátedra de Farmacognosia*, *Facultad de Bioquímica*, *Química y Farmacia*, *Universidad Nacional de Tucumán*, *Ayacucho 491*, *4000, S. M. de Tucumán*, *Argentina*

[b]*INQUINOA-CONICET*, *Instituto de Química Orgánica*, *Facultad de Bioquímica Química y Farmacia*, *Universidad Nacional de Tucumán*, *Ayacucho 491*, *(T4000INI) S. M. de Tucumán*, *Argentina*

[c]*Department of Medicine*, *Division of Infectious Diseases*, *University of British Columbia*, *410-2660 Oak Street*, *Vancouver*, *Canada*

*ccatalan@fbqf.unt.edu.ar*

**Received: September 7th, 2012; Accepted: October 14th, 2012**

The essential oil and methanol extract of northwestern Argentina medicinal plant *Xenophyllum poposum*, collected in Catamarca province, were investigated. GC and GC-MS analyses of the essential oil identified 56 compounds accounting for 92.9%. The main components of the oil were δ-cadinene (16.5%), 6-hydroxytremetone (14.7%), *epi*-α-cadinol (12.0%), α-cadinol (8.8%), γ-cadinene (7.5%), 1-*epi*-cubenol (4.2%) and α-muurolene (3.0%). The essential oil exhibited antibacterial activities against five pathogenic strains as well as antifungal activities against two pathogenic fungi. The methanol extract showed antibacterial activity against two strains of *Staphylococcus aureus* and two pathogenic fungal strains. The main components isolated from the methanol extract were the antifungal 4-hydroxy-3-(isopenten-2-yl)-acetophenone, 6-hydroxytremetone, and tremetone. 6-Hydroxytremetone showed activity against all the fungal strains and one of the *S. aureus* strains assayed. Antioxidant and radical-scavenging properties of the methanol extract and essential oil were determined using the 2,2'-diphenyl-1-picrylhydrazyl assay and β-carotene bleaching (BCB) test. The methanol extract and the essential oil showed, respectively, moderate and weak antioxidant activity when compared to butylated hydroxytoluene.

**Keywords:** *Xenophyllum poposum*, *Werneria poposa*, Asteraceae, Essential oil, New chemotype, Methanol extract, Prenylated *p*-hydroxyacetophenone, 6-Hydroxytremetone, Antimicrobial activity.

*Xenophyllum poposum* (Philippi) V.A. Funk (syn. *Werneria poposa* Philippi) is a perennial rhizomatous plant belonging to the Asteraceae family and one of 21 species of the Andean genus *Xenophyllum* that has recently been extracted from the genus *Werneria s.l.* [1]. *X. poposum* is a fetid sub-shrub known under the common names "poposa", "pupusa" or "fosfosa" that grows in the high mountains of Northern Argentina, Northern Chile, Bolivia and Southern Peru at 4600-5300 m above sea level [2]. Infusions of its aerial parts are used in folk medicine for the treatment of hypertension, altitude sickness and digestive disorders, such as indigestion, intestinal inflammation, intestinal colics, and diarrhoea [3]. Also, it is used for abdominal pain, rheumatism, and as a food condiment for pneumonia convalescents [3,4]. This herb together with the "chachacoma" (*Senecio nutans* Sch. Bip.) and the "copa-copa" (*Artemisia copa* Phil.) are the most common Asteraceae in the Northwestern Argentina folk pharmacopeia [4]. *Xenophyllum poposum* is frequently confused with *X. incisum* and, in folk medicine, the uses for both species are essentially the same [5].

Previous investigations on *X. poposum* (under the old *Werneria poposa* label) reported the presence of (-)kaur-16-en-19-al from petrol extract, 4-hydroxy-3-(isopenten-2-yl)-acetophenone (**1**) and 4-hydroxy-3-(3′-hydroxyisopentyl)-acetophenone from the methylene chloride extract [6a], and the coumarins aesculetin, fraxetin, isoscopoletin, and dihydroisoscopoletin along with the flavonoid isorhamnetein from the methanol extract (ME) [6b]. Recently, the phytotoxic activities of the benzofuran (2*R*)-6-hydroxytremetone (**2**) isolated from this plant have been reported [7].

Following our investigations on herbs used in Andean traditional medicine [8], we report here the chemical composition, antimicrobial and antioxidant activities of both the essential oil and the methanol extract of *Xenophyllum poposum.*

GC and GC/MS analyses of the essential oil led to the identification of 56 compounds accounting for 92.9% of the oil. Table 1 shows the identified constituents, their percentages, retention indices and method of identification. The essential oil was dominated by sesquiterpenoids (72.2%) with almost equal amounts of sesquiterpene hydrocarbons (36.0%) and oxygenated sesquiterpenes (36.2%). The amount of monoterpenoids was very low representing barely 3.6% of the oil. Interestingly, the essential oil contains significant amounts of 6-hydroxytremetone (**2**) (14.7%) and tremetone (1.7%). Other major components of the volatile oil were: δ-cadinene (16.5%), **2** (14.7%), *epi*-α-cadinol (T-cadinol) (12.0%), α-cadinol (8.8%), γ-cadinene (7.5%), 1-*epi*-cubenol (4.2%) and α-muurolene (3.0%) and an unidentified sesquiterpene alcohol $C_{15}H_{26}O$ (5.8%) (Table 1).

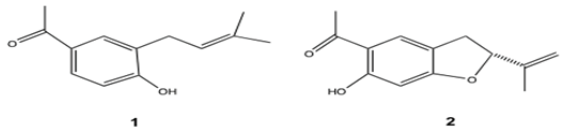
A previous investigation on *X. poposum* collected at an undisclosed location of the Argentine Puna [9] yielded a qualitative very different volatile oil, dominated by monoterpenes (76.4%), with β-pinene (21.8%), α-pinene (5.5%), terpinen-4-ol (5.3%) and α-terpinene (5.2%), as major components. The total amount of sesquiterpenoids was low (6.2%) and neither tremetone analogs nor other benzofuran derivatives were detected [9]. The dissimilar chemical composition exhibited by the essential oil of our collection strongly suggests that we are dealing with a different chemotype, a

**Table 1**: Chemical composition of *Xenophyllum poposum* essential oil.

| Compound | % | $RI_{Exp}$ | $RI_{Lit}$ [13] | Identification |
|---|---|---|---|---|
| α-Pinene | < 0.1 | 933 | 932 | a,b,c |
| β-Pinene | < 0.1 | 976 | 974 | a,b,c |
| α-Phellandrene | < 0.1 | 1004 | 1002 | a,b |
| α-Terpinene | < 0.1 | 1016 | 1014 | a,b,c |
| *p*-Cymene | < 0.1 | 1023 | 1020 | a,b,c |
| Limonene | < 0.1 | 1027 | 1024 | a,b,c |
| β-Phellandrene | < 0.1 | 1028 | 1025 | a,b |
| γ-Terpinene | < 0.1 | 1056 | 1054 | a,b |
| Undecane | < 0.1 | 1100 | 1100 | a,b,c |
| *endo*-Fenchol | 0.2 | 1114 | 1114 | a,b |
| *cis-para*-Menth-2-en-1-ol | 0.1 | 1120 | 1118 | a,b |
| 1-Terpineol | 0.1 | 1131 | 1130 | a,b,c |
| Camphene hydrate | 0.1 | 1145 | 1145 | a,b |
| Isoborneol | 0.1 | 1157 | 1155 | a,b,c |
| Borneol | 0.6 | 1167 | 1165 | a,b,c |
| Terpinen-4-ol | 0.5 | 1174 | 1174 | a,b,c |
| *p*-Cymen-8-ol | 0.1 | 1180 | 1179 | a,b |
| α-Terpineol | 1.2 | 1188 | 1186 | a,b,c |
| Piperitone | 0.2 | 1252 | 1249 | a,b,c |
| α-Cubebene | 0.1 | 1349 | 1345 | a,b |
| α-Copaene | 1.1 | 1377 | 1374 | a,b |
| β-Caryophyllene | 0.1 | 1420 | 1417 | a,b,c |
| β-Copaene | 0.1 | 1428 | 1430 | a,b |
| Aromadendrene | 0.1 | 1441 | 1439 | a,b |
| α-Humulene | 0.3 | 1454 | 1452 | a,b,c |
| *allo*-Aromadendrene | 1.0 | 1459 | 1458 | a,b,c |
| *cis*-Muurola-4(14),5-diene | 0.1 | 1467 | 1465 | a,b |
| γ-Muurolene | 0.9 | 1477 | 1478 | a,b |
| α-Amorphene | 1.1 | 1482 | 1483 | a,b |
| *trans*-Muurola-4(14),5-diene | 0.1 | 1493 | 1493 | a,b |
| *cis*-Cadina-1,4-diene | 0.5 | 1496 | 1495 | a,b |
| α-Muurolene | 3.0 | 1499 | 1500 | a,b |
| γ-Cadinene | 7.5 | 1511 | 1513 | a,b,c,d |
| δ-Cadinene | 16.5 | 1523 | 1522 | a,b,c,d |
| *trans*-Cadina-1,4-diene | 0.8 | 1533 | 1533 | a,b |
| α-Cadinene | 1.2 | 1536 | 1537 | a,b,c |
| α-Calacorene | 1.1 | 1545 | 1544 | a,b |
| Spathulenol | 0.4 | 1578 | 1577 | a,b,c |
| Viridiflorol | 0.1 | 1591 | 1592 | a,b |
| β-Oplopenone | 0.4 | 1606 | 1607 | a,b |
| 1,10-*di-epi*-Cubenol, | 1.1 | 1616 | 1618 | a,b |
| Dehydrotremetone | 0.1 | 1622 | - | b,c |
| 1-*epi*-Cubenol | 4.2 | 1626 | 1627 | a,b |
| Sesquiterpene alcohol $C_{15}H_{26}O$* | 5.8 | 1634 | - | a,b |
| *epi*-α-Cadinol (T-cadinol) | 12.0 | 1638 | 1638 | a,b,c,d |
| *epi*-α-Muurolol | 1.7 | 1641 | 1640 | a,b,c |
| α-Muurolol | 1.7 | 1645 | 1644 | a,b,c |
| α-Cadinol | 8.8 | 1652 | 1652 | a,b,c,d |
| Cadalene | 0.4 | 1672 | 1675 | a,b |
| Tremetone | 1.7 | 1727 | - | b,c,d |
| 6-Hydroxytremetone | 14.7 | 1854 | - | b,c,d |
| Nonadecane | 0.2 | 1900 | 1900 | a,b |
| Eicosane | 0.1 | 2000 | 2000 | a,b |
| Heneicosane | 0.2 | 2100 | 2100 | a,b |
| Docosane | < 0.1 | 2200 | 2200 | a,b |
| Tricosane | 0.1 | 2300 | 2300 | a,b |
| Monoterpene hydrocarbons | 0.4 | | | |
| Oxygenated monoterpenes | 3.2 | | | |
| Sesquiterpene hydrocarbons | 36.0 | | | |
| Oxygenated sesquiterpene | 36.2 | | | |
| *p*-hydroxyacetophenone derivatives | 16.5 | | | |
| Others | 0.6 | | | |
| **TOTAL IDENTIFIED** | **92.9** | | | |

[a] Retention index (RI) on an HP-5 capillary column; [b] Mass spectrum; [c] Co-injection with an authentic sample; [d] $^1$H NMR; * EIMS *m/z* (rel. abund. %): 222 [$M^+$] (3); 207 (7); 204 (23); 189 (24); 161 (55); 147 (8); 133 (20); 121 (28); 109 (100); 93 (44); 81 (28); 69 (23); 55 (26); 43 (32); 41 (40).

trait frequently found in aromatic plants. It is worth to note that later collections of *X. poposum* performed by us in December 2005, March 2009 and March 2011 at the same location (Cerro Pabellón, Catamarca province) yielded volatile oils with chemical compositions essentially identical to the March 2003 collection. The stability of the chemical profile during several years of plants gathered at the same place strongly supports that our collection reperesents a true chemotype. The essential oil composition of the samples collected in 2005, 2009 and 2011 is available on request to the corresponding author.




**1** **2**

Besides, GC-MS analysis of another sample of *X. poposum* purchased at an herbal store in Tucumán city (see *Plant Material*) showed that it belonged to the same chemotype of our Cerro Pabellón collection as its essential oil was dominated by sesquiterpenoids (72.8%), with δ-cadinene (18.8%), *epi*-α-cadinol (10.0%), γ-cadinene (9.1%), α-cadinol (5.7%), α-muurolene (3.3%) and 1-*epi*-cubenol (1.8%) as main components, which were accompanied by a significant amount of **2** (11.8%).

Most of the relevant components of the essential oil from *X. poposum* are bioactive compounds. Thus, **2** (14.7% in the essential oil) displays potent anticancer activity against HL-60 human leukaemia and HeLa cell lines [10a], plant growth inhibitory [10b], anti HIV-1 [10c] and allergenic [10d] activities. In turn, *epi*-α-cadinol (T-cadinol) (12.0%) induces dendritic cells from human monocytes and drive Th1 polarization [11a]. It has also been shown that T-cadinol inhibits induced intestinal hypersecretion in mice and electrically induced contractions of the isolated guinea pig ileum [11b], and possesses calcium antagonist properties [12a]. T-cadinol (12%) and α-cadinol (8.8%) were also reported to suppress the nitric oxide production induced by lipopolisaccharides and possess significant anti-inflammatory activity [12b]. α-Cadinol also exhibits strong antimite activity against *Dermatophagoides pteronyssinus* and *D. farinae* [12c].

The methanol extract (ME) of *X. poposum* exhibited a moderated scavenging effect in comparison to butylated hydroxytoluene (BHT) ($IC_{50}$ = 160 ppm for ME; 17 ppm for BHT), while the esential oil and compounds **1** and **2** were inactive (Figure 1). With the β-carotene bleaching method, both the ME and the essential oil showed a moderate antioxidant activity (35,9% and 27,1% resp.) in comparison to BHT (Figure 2).

The antibacterial screening of essential oil (Table 2) showed antibacterial activities against *P. aeruginosa* and both *E. coli* and *S. aureus* strains assayed. In addition, it showed antibacterial activity against the clinical strains *B. cepacia* and *H. alvei*, but at concentrations of 1.1 mg/mL. Furthermore, a strong antifungal activity against filamentous fungi was observed with minimal inhibitory concentrations (MICs) varying between 0.025-0.05 mg/mL. The ME was only active against both *S. aureus* strains assayed, but also showed similar antifungal activities as the essential oil against filamentous fungi. Interestingly, **2** exhibited weak antibacterial and antifungal activities, but a significant antifungal activity against *A. fumigatus*.

In conclusion, *X. poposum* contains significant amounts of several powerful bioactive compounds. The known inhibitory effects of T-cadinol on intestinal hypersecretion and ileum contractions [11b] as well as its calcium antagonist properties [12a] are consistent with the use of *X. poposum* in folk medicine to treat digestive disorders such as indigestion, intestinal inflammation, intestinal colics, abdominal pain and hypertension [3,4]. In addition, because of the co-occurrence of so many bioactive compounds, it should be expected that the consumption of this herb has powerful effects that deserve to be investigated in depth. To the best of our knowledge, this is the first study reporting the antioxidant and antimicrobial activities of *X. poposum* and the existence of a well-defined essential oil chemotype.

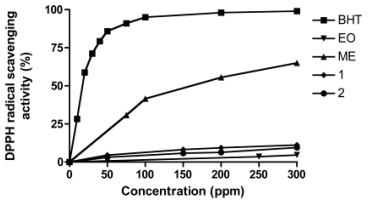



**Figure 1**: Scavenging effect of essential oil (EO) and methanol extract (ME) of *X. poposum*, compounds **1** and **2** and BHT. Free radical scavenging activity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay. BHT was used as a control. Shown is the mean ± SD of three independent experiments.

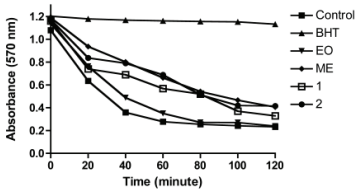



**Figure 2**: Antioxidant activity of essential oil (EO) and methanol extract (ME) of *X. poposum*, compounds **1** and **2**, and BHT at 1000 ppm in β-carotene-linoleate model system. Shown is the mean ± SD of three independent experiments.

**Table 2**: Minimal Inhibitory Concentrations (mg/mL) of essential oil and ME from *X. poposum*.

| Organism | Essential Oil | ME | **2** |
|---|---|---|---|
| Bacteria | | | |
| *Acinetobacter baumannii* | 1.0 | R | 1.0 |
| *Burkholderia cepacia** | 1.17 | NA | NA |
| *Enterobacter gergoviae** | R | NA | NA |
| *Escherichia coli* ATCC 35218 | 0.74 | NA | NA |
| *Escherichia coli* ATCC 25922 | 0.95 | R | 1.0 |
| *Hafnia alvei** | 1.11 | NA | NA |
| *Pseudomonas aeruginosa* | 0.71 | NA | NA |
| *Salmonella typhimurium* | R | R | 1.0 |
| *Staphylococcus aureus* | 0.5 | 0.25 | R |
| *Staphylococcus aureus* MRSA | 0.25 | 0.25 | 0.75 |
| Fungi | | | |
| *Aspergillus fumigatus* | 0.025 | 0.25 | 0.025 |
| *Candida albicans* | R | R | 0.75 |
| *Cryptococcus neoformans* | R | R | 1.0 |
| *Trichophyton rubrum* | 0.05 | 0.05 | 0.75 |

R= resistant. *Clinical isolate. NA= Not assayed.

## Experimental

***Plant Material:*** Aerial parts of wild growing *X. poposum* (Philippi) V.A. Funk were collected at Cerro Pabellón at 4600 m above sea level, Andalgalá Department, province of Catamarca, Argentina, in March 2003 during the flowering period. A voucher specimen has been deposited in the herbarium of Miguel Lillo Institute (LIL 29301), Tucumán, Argentina. Later collections carried out in December 2005, March 2009 and March 2011 at the same place yielded essential oils (GC-MS analysis) practically identical to the 2003 collection. Also, a commercial sample of aerial parts of *X. poposum* purchased in February 2006 at a herbal store in Tucumán city and supposedly gathered in the Calchaquí mountains, Tafí del Valle Department, Tucumán province, belonging to the same phytogeographical region of our collections in Catamarca province, was hydrodistilled to yield an essential oil with a chemical composition (see below) resembling the collection at Cerro Pabellón (Table 1).

***Methanol extract preparation and essential oil preparation:*** Air-dried aerial parts of *X. poposum* (100 g) were macerated with MeOH for 4 days. After filtering, the solvent was evaporated at reduced pressure to yield 13.2 g of ME, which was used for the antioxidant and antimicrobial assays. The essential oil was obtained by hydrodistillation of aerial parts (200 g) for 3 h in a Clevenger-type apparatus. A yield of 1.3 mL 0.65% (v/w) was obtained. Qualitative and quantitative analyses of the essential oil were carried out using a Hewlett-Packard 5890 series II GC with flame ionization detector (FID), equipped with a capillary HP-5 column (5% phenyl methyl silicone, 30 m x 0.32 mm; 0.25 μm film thickness) with nitrogen as carrier gas (1.1 mL/min). The oven was programmed for 75ºC (4 min), 75º-180º (2ºC/min) and 220º-280º (10ºC/min). Injection volume was 0.1 μL, split mode. Injector and detector temperatures were maintained at 250ºC and 270ºC, respectively. The relative amounts of individual components are based on the peak areas obtained with an integrator HP 3395 without FID response factor correction. Retention indices (RI) were obtained by co-injection of a series of standard *n*-hydrocarbons $C_8$-$C_{18}$ and the oil sample using the oven temperature program suggested by Adams [13] (60ºC to 246ºC at 3 ºC/min). The GC-MS analysis was carried out with a 5973 Hewlett Packard selective mass detector (quadrupole), source 70 eV, coupled to a HP 6890 GC fitted with a HP-5MS column (5% phenylmethyl siloxane, 30 m x 0.25 mm; film thickness 0.25 μm) with helium as carrier gas (1.0 mL/min; constant flow). Injection port was maintained at 250ºC, GC-MS interphase at 275ºC, ion source 230ºC, and MS Quad at 150ºC. The oven was programmed as above. The injection volume was 0.1 μL (split 1:80). The identification of the individual components was based on: (a) Computer matching with commercial mass spectra libraries (NBS75K, NIST, WILEY) and comparison with mass spectra available in our files, (b) Comparison of GC retention indexes (RIs) on an HP-5 column [13]. RIs were obtained by co-injection of a series of *n*-hydrocarbons $C_8$-$C_{18}$ with the oil sample. For RI measurements, an oven temperature program of 60°C to 246°C at 3°C/min was used [13]; (c) Co-injection with authentic samples (whenever available), (d) Tremetone and **2** were also isolated from the ME by column chromatography on Sigel 230-400 Mesh using hexane-EtOAc mixtures of increasing polarity as eluting solvent and characterized by NMR spectroscopy.

***Isolation of 4-hydroxy-3-(isopenten-2-yl)-acetophenone (1), tremetone and 6-hydroxytremetone (2):*** A portion of ME (2.1 g) was subjected to column chromatography over Si gel (105 g; Merck 70-230 mesh) using *n*-hexane with increasing amounts of EtOAc (0-40%) to yield 67 fractions, which were monitored and reunited on the basis of their TLC profiles and analyzed by GC-MS. Frs. 17-21 were reunited (96 mg) and re-chromatographed over Si gel (Merck 230-400 mesh) using *n*-hexane-EtOAc mixtures of increasing polarity to give 77 mg of **2**. Frs. 28-30 (14 mg) after preparative TLC (hexane-EtOAc 4:1; two developments) gave 8 mg of tremetone. Frs. 39-52 (915 mg) gave a solid residue, which on re-crystallization from *n*-heptane-ethyl acetate afforded 672 mg of crystalline 4-hydroxy-3-(isopenten-2-yl)-acetophenone (**1**), mp 96ºC. The structure of compounds **1** and **2** was confirmed by NMR spectroscopy. Compound **1** possesses significant antifungal properties [14] and was by far the main component in the ME of *X. poposum*.

***Antioxidant activity:*** The antioxidant activities of the essential oil and ME of *X. poposum* as well as compounds **1** and **2**, were measured in terms of hydrogen donating or radical scavenging capability using the stable radical DPPH as reagent. The method described by Wei and T. Shibamoto [15] with slight modifications was used. The assay was carried out in triplicate. DPPH inhibition (I%) by the samples was calculated according to the formula: I%= $(A_{blank}-A_{sample}/A_{blank})$ x 100, where $A_{blank}$ is the absorbance of the control reaction (containing all reagents except the test compound), and $A_{sample}$ is the absorbance of the blank plus test compound. Extract concentration providing 50% inhibition ($IC_{50}$) was calculated from the graph by plotting inhibition percentage against extract concentration. The antioxidant activity was determined

according to the β-carotene bleaching method [16]. Absorbance was measured at 20 min intervals until the color of β-carotene disappeared in the control reaction (t=120 min). Relative antioxidant activities (RAA%) of the extract and oil were calculated from the equation: RAA% = ($A_{Sample}/A_{BHT}$) x 100, where $A_{BHT}$ is the absorbance of the positive control BHT, and $A_{Sample}$ is the absorbance of the extract and oil. Experiments were performed in triplicate. Antioxidant capacities of the samples were compared with those of BHT and the control.

***Antimicrobial activity:*** Antibacterial assays were performed using the agar diffusion method against *Acinetobacter baumannii* (ATCC BAA-747), *Escherichia coli* (ATCC 35218 and 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Staphylococcus aureus* (ATCC 25923) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (ATCC 700698). Multi-resistant clinical isolates of *Burkholderia cepacia, Enterobacter gergoviae,* and *Hafnia alvei* provided by Dr. Cristina Estrella, Hospital Centro de Salud, San Miguel de Tucumán, Argentina, were also assayed. Antifungal activities were performed using the yeast-like *Candida albicans* (provided by Vancouver General Hospital, BC, Canada) and *Cryptococcus neoformans* var. *grubii* (kindly provided by Dr. Karen Bartlet, University of British Columbia, BC, Canada), while *Aspergillus fumigatus* (ATCC 1022) and *Trichophyton rubrum* (ATCC 18758) represented filamentous fungi. Bacteria were grown in Brain Heart Infusion broth fungi. (Laboratories Britania, Argentina) at 37ºC for 18 h, and resuspended in sterile physiological saline with reference to the value 0.5 of the McFarland scale (1.5 x $10^8$ colony forming unit /mL). Briefly, plates were prepared with a base layer of Müeller–Hinton agar (10 mL) and wells (6 mm of diameter) were made on the surface of the media. Different concentrations (50 µL) of essential oil and ME were placed in the wells. 25 µL of gentamicin and amphotericin B (0.05 mg/mL) were used as positive control for bacteria and fungi, respectively. Fungal strains were grown in Sabouraud broth (B&D), and the antifungal activity against filamentous fungi was assessed from spores obtained as reported Spores were harvested by rubbing the top of sporulated colonies in 2 mL Sabouraud broth containing 10% glycerol. Spores were aliquoted and kept at -20ºC. For yeast-like fungi, the same protocol used for bacterial strains was used, but using Sabouraud broth. The microorganisms were incubated at 37°C aerobically and after 24 h of incubation, the zones of inhibition were measured. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the well. The growth inhibition diameter was an average of four measurements, taken in 90 degrees apart. MIC values were determined by conventional agar plate dilution method [17]. All tests were performed in triplicate.

***Statistic Analysis:*** A *t*-student test was used for statistical analysis. A $P$ value <0.05 was considered significant.

**Acknowledgements** - This work was financially supported by Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT) and Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina (CONICET). The authors warmly thank Dr. Viviana Vildoza for providing the standard strains of microorganisms.

## References

[1] Funk VA. (**1997**) *Xenophyllum*, a new Andean genus extracted from *Werneria* s.l. (Compositae: Senecioneae). *Novon*, *7*, 235-241.
[2] Zuloaga FO, Morrone O. (**1999**) Catálogo de las plantas vasculares de la República Argentina. Missouri Botanical Garden Press, Missouri, USA.
[3] Alonso J, Desmarchelier C. (**2006**) Plantas Medicinales Autóctonas de la Argentina. Ediciones Fitociencia, Buenos Aires, Argentina.
[4] Giberti GC. (**1983**) Herbal folk medicine in northwestern Argentina: Compositae. *Journal of Ethnopharmacology*, *7*, 321-341.
[5] de Marchese MJA, de Heluani CS, Catalán CAN, Griffin CA, Vaughn Jr. JB, Herz W. (**2007**) Incisol, an alcohol with a novel sesquiterpene skeleton from *Xenophyllum incisum*. *Biochemical Systematics and Ecology*, ***35***, 169-175.
[6] (a) Ponce MA, Gros GE. (**1991**) Prenylated aromatic ketones from *Werneria poposa*. *The Journal of the Argentine Chemical Society*, ***79***, 197-200; (b) Ponce MA, Gros GE. (**1995**) A flavonoid and coumarins from *Werneria poposa. Ibid.* ***83***, 93-95.
[7] Romano E, Raschi AB, Gonzalez AM, Jaime G, Fortuna MA, Hernandez LR, Bach H, Benavente AM. (**2011**) Phytotoxic activities of (2*R*)-6-hydroxytremetone. *Plant Physiology and Biochemistry*, ***49***, 671-675.
[8] Lizarraga E, Castro F, Fernández F, de Lampasona MP, Catalán CAN. (**2012**) Antioxidant, hemolytic and cytotoxic activities of *Senecio* species used in traditional medicine of northwestern Argentina. *Natural Product Communications*, ***7***, 607-608.
[9] Abella L, Cortella AR, Velasco-Negueruela A, Perez-Alonso MJ. (**2000**) Ethnobotany, volatile oils and secretion tissues of *Werneria poposa* from Argentina. *Pharmaceutical Biology*, ***38***, 197-203.
[10] (a) Liu Q, Shen L, Wang T, Chen J, Qi W, Gao K. (**2010**) Novel modified furanoeremophilane-type sesquiterpenes and benzofuran derivatives from *Ligularia veitchiana*. *Food Chemistry*, ***122***, 55-59; (b) Cespedes CL, Uchoa A, Salazar JR, Perich F, Pardo F. (**2002**) Plant growth inhibitory activity of *p*-hydroxyacetophenones and tremetones from Chilean endemic *Baccharis* species and some analogous: A comparative study. *Journal of Agricultural and Food Chemistry,* ***50***, 2283–2292; (c) Piacente S, Aquino R, de Tommasi N, Pizza C, de Ugaz OL, Orellana HC, Mahamood N. (**1994**) Constituents of *Werneria ciliolata* and their in *vitro* anti-HIV activity. *Phytochemistry*, ***36***, 991–996; (d) Hausen BM, Helmke B. (**1995**) Butenylbithiophene, α-terthienyl and hydroxytremetone as contact allergens in cultivars of marigold (Tagetes sp.). *Contact Dermatitis*, ***33***, 33–37.
[11] (a) Takei M, Umeyama A, Arihara S. (**2006**) T-cadinol and calamenene induce dendritic cells from human monocytes and drive Th1 polarization. *European Journal of Pharmacology*, ***537***, 190-199; (b) Claeson P, Andersson R, Samuelsson G. (**1991**) T-cadinol: a pharmacologically active constituent of scented myrrh: introductory pharmacological characterization and high field $^1$H- and $^{13}$C-NMR data. *Planta Medica*, ***57***, 352-356.
[12] (a) Claeson P, Zygmunt P, Hogestatt ED. (**1991**) Calcium antagonistic properties of the sesquiterpene T-cadinol: a comparison with nimodipine in the isolated rat aorta. *Pharmacology and Toxicology*, ***69***, 173-177; (b) Tung YT, Huang CC, Ho ST, Kuo YH, Lin CC, Lin CT, Wu JH. (**2011**) Bioactive phytochemicals of leaf essential oils of *Cinnamomum osmophloeum* prevent lipopolysaccharide/D-galactosamine (LPS/D-GalN)-induced acute hepatitis in mice. *Journal of Agriculture and Food Chemistry*, ***59***, 8117-8123; (c) Chang ST, Chen PF, Wang SY, Wu HH. (**2001**) Antimite activity of essential oils and their constituents from *Taiwania cryptomerioides*. *Journal of Medical Entomology*, ***38***, 455-457.
[13] Adams R. (**2007**) Identification of essential oil components by Gas Chromatography/ Quadrupole Mass Spectroscopy, 4th Edition. Allured Publishing Corporation, Carol Stream, Illinois, USA.
[14] Tomás-Barberán F, Iniesta-Sanmartin E, Tomás-Lorente F, Rumbero A. (**1990**) Antimicrobial phenolic compounds from three Spanish *Helichrysum* species. *Phytochemistry*, ***29***, 1093-1095.
[15] Wei A, Shibamoto T. (**2007**) Antioxidant activities and volatile constituents of various essential oils. *Journal of Agriculture and Food Chemistry*, ***55***, 1737-1742.
[16] Jayaprakasha GK, Singh RP, Sakariah KK. (**2001**) Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. *Food Chemistry*, ***73***, 285-290.
[17] Basualdo J, Coto C, De Torres R. (**1996**) Microbiología Biomédica. Buenos Aires, Argentina: Atlante.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1667 - 1670

# Phytochemical and Micromorphological Traits of Endemic *Micromeria pseudocroatica* (Lamiaceae)

**Dario Kremer[1], Ivna Dragojević Müller[2], Edith Stabentheiner[3], Dubravka Vitali[1], Martina Kopričanec[2], Mirko Ruščić[4], Ivan Kosalec[1], Nada Bezić[4] and Valerija Dunkić[4*]**

[1]*Faculty of Pharmacy and Biochemistry*, *University of Zagreb*, *A. Kovačića 1*, *10000 Zagreb*, *Croatia*

[2]*Department of Ecology and Water Protection*, *Water Supply and Drainage*, *Patačićkina 1b*, *10000 Zagreb*, *Croatia*

[3]*Institute of Plant Sciences*, *Karl-Franzens University*, *Schubertstrasse 51*, *A-8010 Graz*, *Austria*

[4]*Department of Biology*, *Faculty of Natural Science*, *University of Split*, *Teslina 12*, *10000 Split*, *Croatia*

*dunkic@pmfst.hr*

**Received: September 26th, 2012; Accepted: October 31st, 2012**

*Micromeria pseudocroatica* Šilić is an endemic species distributed in southern Croatia. Chemical composition of the essential oil (analysed by GC and GC-MS), and the content of macroelements and trace elements analysed by ICP-AES was conducted. Additionally, a study on the types and distribution of trichomes was done by scanning electron microscopy. The essential oil was characterized by a high concentration of oxygenated monoterpenes, whose major compounds are borneol (22.7% and 24.8%) and camphor (16.1% and 13.9%). The content of Ca was highest (13202.69 mg/kg and 13223.83 mg/kg) among the investigated macroelements, while Fe was the most represented element (100.68 and 326.48 mg/kg) among the micronutrients. The content of potentially toxic elements that is Se, Cu, and Hg was below the limit of quantification. Non-glandular trichomes, peltate trichomes, and two types of capitate trichomes (type 1: one basal epidermal cell, one head cell with subcuticular space; type 2: one basal epidermal cell, two stalk cells, and one head cell with subcuticular space) were observed on leaves, bracteoles, calyx, corolla and stem.

**Keywords:** *Micromeria pseudocroatica*, Borneol, Macroelements, Trace elements, Trichomes.

The family Lamiaceae is one of the largest groups among dicotyledons, comprising more than 240 genera. Many species which belong to this family are highly aromatic due to the presence of external glandular structures that produce essential oil [1]. One of the less investigated genera in this family is *Micromeria* Benth. The essential oil composition of the *Micromeria* species is variable. The major constituents of the oil of *M. croatica* (Pers.) Schott, an endemic Illyric-Balcanic species were caryophyllene oxide and β-caryophyllene [2]. Pulegone and piperitone oxide were the main compounds in the essential oils of *M. albanica* (Griseb. ex K. Maly) Šilić and *M. thymifolia* (Scop.) Fritsch.. Spathulenol was the dominant compound in *M. parviflora* (Vis.) Rchb. [3]. The major constituents of the oil of *M. libanotica* Boiss., a species endemic to Lebanon, were isomenthone (44.5%), pulegone (13.5%) and isopulegone (6.5%) [4].

Glandular trichomes occurring in the Lamiaceae are the site of essential oil biosynthesis, secretion and accumulation and their structure has been studied by many authors [1, 5-8]. Glandular trichomes of *Micromeria fruticosa* (L.) Druce and *M. croatica* were investigated by Werker et al. [5] and Kremer et al. [2], respectively.

Although the biological activity of *Micromeria* species could be attributed to some constituents of the essential oil [9-11], the presence of other biologically active compounds such as microelements could have a synergistic effect with the essential oils. Only few literature data about the content of macroelements and trace elements in *Micromeria* species are available [2,12].

The genus *Micromeria* includes 70–90 herbs, sub-shrubs and shrubs distributed throughout the temperate belt [13-15]. Twenty one *Micromeria* species have been described for Europe [13] and nine (*M. croatica*, *M. dalmatica* Benth., *M. fruticulosa* (Bertol.) Šilić, *M. graeca* (L.) Benth., *M. juliana* (L.) Benth., *M. kerneri* Murb., *M. microphylla* (Dum.-Urv.) Benth., *M. pseudocroatica* Šilić, and *M. thymifolia* (Scop.) Fritsch.) species for Croatia [16]. *M. pseudocroatica* is an endemic species distributed in the Mediterranean part of Croatia (Pelješac Peninsula, Korčula Islands) [17,18]. It is a perennial plant with several to numerous stems up to 30 cm long, and single, pink-purple flowers [17]. *M. pseudocroatica* grows in the crevices of calcareous rocks at altitudes up to about 500 m.

The aim of the presented study is to obtain additional knowledge on the genus *Micromeria* in general, and particularly on *M. pseudocroatica*, since no data on essential oil, macroelements and trace elements, and micromorphological traits are available for *M. pseudocroatica*.

Aerial parts of wild-growing *M. pseudocroatica* were analyzed on two different localities, Pijavičino (Pi) and Prapatno (Pr) on Pelješac Peninsula (Croatia). Fifty-two components representing 86.4% of the total oil were identified in the oil from locality Pi and forty-seven components representing 90.4% of the total oil were characterized in oil from Pr. Total yield of the oil was 0.3% and 0.2% for oils from Pi and Pr, respectively. The identified components listed in order of their elution from the VF-5MS column are given in Table 1 together with their percentages of the total mass fraction of the oil. Identified components are classified on the basis of their chemical structures in seven classes.

Both samples contained higher concentration of oxygenated monoterpenes (47.3% in Pi and 43.7% in Pr) with borneol (22.7% in Pi and 24.8% in Pr) and camphor (16.1% in Pi and 13.9% in Pr) as the major components of the both oil. Similarly to our results borneol was identified as one of major compounds in the oil

**Table 1**: Phytochemical composition (%) of essential oils of *M. pseudocroatica* Šilić from the localities Pijavičino (Pi) and Prapatno (Pr).

| Component | RI | *M. pseudocroatica* (Pi) | *M. pseudocroatica* (Pr) | Identification |
|---|---|---|---|---|
| ***Monoterpene hydrocarbons*** | | **1.9** | **3.2** | |
| Camphene | 962 | 0.5 | 1.9 | RI, MS |
| *β*-Pinene | 982 | 0.2 | 0.2 | RI, MS, S |
| *p*-Cymene | 1021 | 0.2 | 0.1 | RI, MS |
| Limonene | 1032 | 0.1 | - | RI, MS, S |
| γ-Terpinene | 1057 | 0.1 | 0.1 | RI, MS |
| Terpinolene | 1089 | 0.8 | 0.9 | RI, MS |
| ***Oxygenated monoterpenes*** | | **47.3** | **43.7** | |
| *β*-Thujone | 1121 | - | 0.3 | RI, MS |
| *trans*-Pinocarveol | 1147 | 0.1 | 0.3 | RI, MS |
| Camphor | 1151 | 16.1 | 13.9 | RI, MS, S |
| Borneol | 1176 | 22.7 | 24.8 | RI, MS |
| Cymene-8-ol | 1179 | 2.1 | 0.9 | RI, MS |
| Terpinen-4-ol | 1184 | 0.6 | 0.2 | RI, MS |
| Myrtenol | 1197 | 0.6 | 0.2 | RI, MS |
| Verbenone | 1204 | 0.6 | 0.4 | RI, MS |
| Fenchyl acetate | 1218 | 0.1 | 0.3 | RI, MS |
| Thymol methyl ether | 1230 | 0.2 | 0.1 | RI, MS |
| Pulegone | 1234 | 0.5 | - | RI, MS |
| Carvacrol methyl ether | 1241 | 0.1 | 0.2 | RI, MS |
| Piperitone | 1248 | 0.3 | 0.4 | RI, MS |
| Bornyl acetate | 1285 | 0.4 | 0.2 | RI, MS |
| α-Terpenyl acetate | 1349 | 0.1 | 0.2 | RI, MS |
| Piperitone oxide | 1364 | 2.8 | 1.3 | RI, MS |
| ***Sesquiterpene hydrocarbons*** | | **23.9** | **32.8** | |
| α-Cubebene | 1345 | 1.5 | 3.5 | RI, MS |
| α-Copaene | 1377 | 0.6 | 0.3 | RI, MS |
| β-Bourbonene | 1383 | 0.3 | 0.3 | RI, MS |
| α-Gurjunene | 1407 | tr | 0.5 | RI, MS |
| β-Caryophyllene | 1424 | 11.9 | 17.8 | RI, MS, S |
| β -Copaene | 1429 | 0.2 | 0.3 | RI, MS |
| (*Z*)-β-Farnesene | 1454 | 0.2 | 0.2 | RI, MS |
| α-Humulene | 1456 | 0.6 | 0.1 | RI, MS |
| *allo*-Aromadendrene | 1465 | 0.2 | 0.3 | RI, MS |
| β-Chamigrene | 1477 | 0.1 | 0.2 | RI, MS |
| Germacrene D | 1481 | 2.1 | 2.1 | RI, MS |
| β-Bisabolene | 1494 | 0.2 | 0.2 | RI, MS |
| Bicyclogermacrene | 1500 | 0.1 | 0.3 | RI, MS |
| δ-Cadinene | 1517 | 5.9 | 6.7 | RI, MS |
| ***Oxygenated sesquiterpenes*** | | **10.7** | **9.2** | |
| Spathulenol | 1577 | 0.1 | 0.3 | RI, MS |
| Caryophyllene oxide | 1581 | 9.3 | 7.4 | RI, MS, S |
| γ-Eudesmol | 1632 | 0.2 | 0.3 | RI, MS |
| α-Cadinol | 1655 | 0.1 | - | RI, MS |
| α-Bisabolol | 1688 | 0.8 | 0.3 | RI, MS |
| Bisabolol oxide | 1748 | 0.2 | 0.9 | RI, MS |
| ***Phenolic compounds*** | | **1.0** | **0.2** | |
| Thymol | 1290 | 0.3 | - | RI, MS, S |
| Carvacrol | 1298 | 0.4 | - | RI, MS, S |
| Eugenol | 1370 | 0.3 | 0.2 | RI, MS, S |
| ***Carbonylic compounds*** | | **0.5** | **0.5** | |
| 3-Octanol acetate | 1125 | 0.3 | 0.2 | RI, MS |
| β-Ionone | 1487 | 0.2 | 0.3 | RI, MS |
| ***Hydrocarbons*** | | **1.1** | **0.8** | |
| Docosane | 2200 | 0.1 | 0.2 | RI, MS, S |
| Tricosane | 2300 | 0.3 | 0.2 | RI, MS, S |
| Tetracosane | 2400 | 0.2 | 0.1 | RI, MS, S |
| Pentacosane | 2500 | 0.1 | 0.2 | RI, MS, S |
| Hexacosane | 2600 | 0.1 | - | RI, MS, S |
| Heptacosane | 2700 | 0.3 | 0.1 | RI, MS, S |
| ***Total identified (%)*** | | **86.4** | **90.4** | |
| ***Yield (%)*** | | **0.3** | **0.2** | |

RI = VF-5MS, identification by comparison with literature [25] and/or homemade library; MS = identification by NIST02 and Wiley 7 spectral databases; S = identification confirmed with reference compound; tr-traces (mean value below 0.1%); - = not identified.

of *M. carminea* P.H. Davis, an endemic species of Turkey with percentage of 26.02%, and in the oil of *M. cristata* (Hampe) Griseb. subsp. *phrygia* P. H. Davis collected from three different localities (27–39%) [19,20]. Other main components of oil in *M. cristata* subsp. *phrygia* were camphor (9–15%) and caryophyllene oxide (4–6%) [20].

The oil from Pr contained higher concentration of sesquiterpene hydrocarbons (32.8%) than the oil from Pi (23.9%), with *β*-caryophyllene (11.9% in Pi and 17.8% in Pr) as the dominant component among this class. Caryophyllene oxide was identified as major compound of the both oil in oxygenated sesquiterpenes class with percentages of 9.3% in Pi and 7.4% in Pr. Caryophyllene-oxide was one of major compound in the oil of *M. juliana* from Croatia [9,21], *M. graeca* in Greece [22], *M. croatica* from Serbia [3] and in the oil of *M. croatica* from Croatia [2].

Groups of monoterpene hydrocarbons, phenolic compound, carbonylic compounds and hydrocarbons represented 4.5% (oil from Pi) and 4.7% (oil from Pr) of the total oil.

The contents of macroelements (K, Na, Ca, Mg) and trace elements (Al, Fe, Sr, Zn, B, Mn, Ba, Ni, Pb, V, Cr, Cd, Co, Se, Cu, Hg) in *M. pseudocroatica* from localities Pijavičino (Pi) and Prapratno (Pr) are shown in Table 2. Among the investigated macroelements the content of Ca was highest (13223.83 mg/kg and 13202.69 mg/kg from locality Pi and Pr, respectively) while the content of Na was lowest (109.54 mg/kg and 243.11 mg/kg from locality Pi and Pr, respectively). Among the micronutrients (B, Fe, Cu, Mn, Zn), the most represented element was Fe (100.68 mg/kg and 326.48 mg/kg from locality Pi and Pr, respectively). Among other trace elements, the most abundant was Al (102.49 mg/kg and 232.29 g/kg from locality Pi and Pr, respectively). The content of several toxic and potentially toxic minerals (Se, Cu, Hg) was below detection limits.

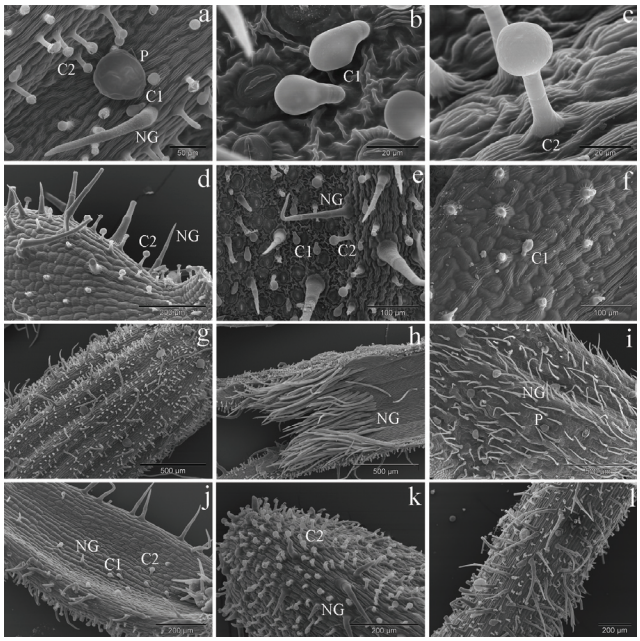
According to Kremer et al. [2] *M. croatica* had the very high potassium content (8275.00–10075.00 mg/kg), which suggests that the accumulation of this macroelement in *M. croatica* is possible affected by genetic particularity. The content of other macroelements in *M. croatica* were significantly lower than in samples of *M. pseudocroatica*. The content of Al (48.13–56.13 mg/kg) and Fe (68.63–78.00 mg/kg) in *M. croatica* was also significantly lower than in *M. pseudocroatica*. Contents of other trace elements in *M. croatica* were not significantly different [2]. Shallari et al. [12] found a significantly higher content of Zn (118 mg/kg), Pb (13 mg/kg), Cr (10 mg/kg), Cd (2 mg/kg), Co (2 mg/kg), and Ni (52 mg/kg) in *M. graeca* shoots collected at a mining site Rubik (northern Albania). These differences could be explained by high influence of environmental conditions.

**Table 2**: Content of macroelemens and traceelements in herba of *M. pseudocroatica*.

| Element | Pijavičino | | Prapratno | |
|---|---|---|---|---|
| | Contents* | RSD | Contents* | RSD (%) |
| | Macroelements (mg/kg) | | | |
| Na | 109.54 ± 0.00 | 0.00 | 243.11 ± 0.03 | 0.01 |
| K | 1922.21 ± 0.02 | 0.00 | 2034.38 ± 0.13 | 0.01 |
| Ca | 13223.83 ± 0.46 | 0.00 | 13202.69 ± 0.23 | 0.00 |
| Mg | 2244.54 ± 0.25 | 0.01 | 2636.84 ± 0.25 | 0.01 |
| | Trace elements (mg/kg) | | | |
| Al | 102.49 ± 9.42 | 9.19 | 232.29 ± 42.06 | 18.11 |
| Fe | 100.68 ± 1.27 | 1.27 | 326.48 ± 6.17 | 1.89 |
| Sr | 7.88 ± 0.54 | 6.86 | 121.99 ± 3.78 | 3.1 |
| Zn | 23.04 ± 0.05 | 0.23 | 22.55 ± 0.37 | 1.65 |
| B | 14.41 ± 0.29 | 2.01 | 12.52 ± 1.27 | 10.15 |
| Mn | 10.08 ±0.02 | 0.02 | 12.67 ± 0.17 | 1.31 |
| Ba | 4.92 ± 0.31 | 6.21 | 5.52 ± 0.81 | 14.61 |
| Ni | 0.82 ± 0.38 | 45.95 | 0.85 ± 0.11 | 13.02 |
| Pb | 0.57 ± 0.08 | 13.98 | 1.64 ± 0.22 | 13.12 |
| V | 0.29 ± 0.02 | 6.87 | 0.96 ± 0.15 | 15.49 |
| Cr | 0.20 ± 0.01 | 4.34 | 0.58 ± 0.08 | 14.32 |
| Cd | 0.09 ± 0.03 | 34.13 | 0.14 ± 0.04 | 30.09 |
| Co | 0.05 ± 0.01 | 25.22 | 0.13 ± 0.02 | 17.27 |
| Se | BLQ | – | BLQ | – |
| Cu | BLQ | – | BLQ | – |
| Hg | BLQ | – | BLQ | – |

*values are means ± SD (n = 2); RSD, (relative standard deviation; BLQ, below limit of quantitation

**Figure 1**: SEM micrographs with different types of trichomes on *M. pseudocroatica*.




Non-glandular trichomes (NG), peltate trichome (P), Type 1 capitate trichomes (C1) and Type 2 capitate trichomes (C2) on outer side of calyx (a); C1 (b) and C2 (c) trichome on the lower leaf side; distribution of the trichomes on the upper (d) and lower (e) leaf surface; C1 trichome on the upper leaf surface (f); outer side of calyx with NG, P, C1, and C2 trichomes (g); inner side of calyx with long NG trichomes (h); NG, and P trichomes on the outer side of corolla (i); NG, C1, and C2 trichomes on the adaxial (j) and abaxial (k) side of bracteole; stem with NG and P trichomes (l).

**Table 3**: Occurrence and frequency of trichomes on aerial parts of *M. pseudocroatica* from the localities Pijavičino (Pi) and Prapatno (Pr).

| | Type | Leaf | | Bracteole | | Calyx | | Corolla | | Stem |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Ad | Ab | Ad | Ab | Ou | In | Ou | In | |
| Pi | attenuate* | + | + | ± | + | + | ± | + | ±/+ | + |
| | peltate | – | +/++ | – | – | + | – | ±/+ | – | + |
| | Cap C1 | ± | +/++ | ± | + | + | ± | – | – | + |
| | cap C2 | ± | +/++ | ± | ++ | ++ | – | – | – | + |
| Pr | attenuate | + | + | ± | + | +/++ | + | +/++ | ±/+ | +/++ |
| | peltate | – | ++ | – | – | + | – | + | – | + |
| | cap C1 | ± | +/++ | ± | + | + | ± | – | – | + |
| | cap C2 | –/± | ++ | ± | +/++ | ++ | –/± | – | – | +/++ |

cap – capitates; Ad – adaxial; Ab – abaxial; Ou – outer; In - inter
trichomes: – missing, ± rare, + present, ++ abundant; *attenuate, non-glandular hairs

Both non-glandular and glandular trichomes could be observed on investigated parts (leaves, bracteoles, calyxes, corollas, and stems) of *M. pseudocroatica* (Figure 1). The occurrence and frequency of trichomes on the adaxial and abaxial side of leaves and bracteoles, outer and inner side of the calyx and corolla, and stem is shown in Table 3.

Non-glandular trichomes (Figure 1a, e) were bi-cellular to multicellular, unbranched, uniseriate and folded at different levels. The length of these trichomes varied distinctly from very short hairs on the upper leaf surface (Figure 1f) to very long hairs on the edge of inner calyx side (Figure 1h). Parts of the surface of these trichomes revealed a warty appearance due to the occurrence of cuticular micropapillae (Figure 1a). According to Payne's [23] plant hair terminology, they could be noted as attenuate hairs. These hairs were identical to non-glandular trichomes found by Kremer et al. [2] in *M. croatica*.

The glandular trichomes could further be divided into peltate and capitate types. Peltate trichomes consisted of a basal cell, a short unicellular stalk, and a multicellular head with a large subcuticular space (Figure 1a and i). They occurred on the abaxial leaf side, on the outer side of the calyx and corolla, and on the stem. Their presence in Lamiaceae is well documented [1, 5-8, 24]. Two types of capitate trichomes could be observed in *M. pseudocroatica*. Type one capitate trichome (C1) is composed of one basal epidermal cell and one elliptically formed head cell. C1 trichome was not upright but could be described as clinging to the surface (Figure 1b). This hair type was found on both on the adaxial as well as on the abaxial side of leaves and bracteoles, on the outer and inner side of calyx, and on the stem (Table 3). According to Kremer et al. [2] this type of trichomes was common in *Micromeria croatica* and it was found on the abaxial leaf surface, on stem, and on calyx. These hairs were also noticed in micrographs of *Thymus capitatus* (L.) Hoffmanns. (syn. *Coridotymus capitatus* (L.) Rchb. f.), *Majorana syriaca* (L.) Rafin., and *Satureja thymbra* L. presented by Werker et al. [5]. But, the micrographs presented by Werker et al. [5] did not show this hair type in *Micromeria fruticosa* (L.) Druce.

Type two capitate trichome (C2) is composed of one basal epidermal cell, two stalk cells and a single celled head with a subcuticular space (Figure 1c). C2 trichome was upright and quite short. It was found on both adaxial and abaxial side of leaves and bracteoles, on outer and inner side of calyx, and on stem (Table 3). This hair type was reported by Kremer et al. [2] for *M. croatica*, where it was observed on adaxial and abaxial leaf surface, on calyx, and on stem.

Comparison with literature data showed significant difference in chemical traits between in here presented *M. pseudocroatica* and closely related *M. croatica*, while micromorphological traits (trichome types) were the same. Presented results give worthy additional knowledge about chemical and micromorphological traits on the genus *Micromeria*.

## Experimental

***Herbal material and extraction:*** Randomly selected samples of wild growing plants *M. pseudocroatica* Šilić were collected during the blooming period in July of 2011 on *locus classicus* near settlement Pijavičino (GPS coordinates: 42°57'01.5" N; 17°21'52.2" E; 443 m a.s.l.'), and on locality Prapratno (GPS coordinates: 42°49'28.1" N; 17°40'23.7" E; 159 m a.s.l.'), both on Pelješac Peninsula, Croatia.

Dried aerial parts (100 g) were subjected to hydrodistillation for 3 h in Clevenger type apparatus and also used for determination of macroelements and trace elements.

***Gas chromatography and mass spectrometry (GC, GC/MS):*** Gas chromatography (GC) analyses were performed on gas chromatograph (model 3900; Varian Inc., Lake Forest, CA, USA) equipped with flame ionization detector, mass spectrometer (model 2100T; Varian Inc.) and capillary column VF-5MS (30 m × 0.25 mm i.d., coating thickness 0.25 μm; Varian Inc.). The chromatographic conditions were as follows: helium was carrier gas at 1 mL·min$^{-1}$, injector temperature was 250 ºC, and FID detector temperature was 300 ºC. VF-5MS column temperature was programmed at 60 ºC isothermal for 3 min, and then increased to 246 ºC at a rate of 3 ºC·min$^{-1}$ and held isothermal for 25 min. The injected volume was 1 μL and the split ratio was 1:20. The mass spectrometry (MS) conditions were: ionization voltage 70 eV; ion source temperature 200 ºC; mass scan range: 40–350 mass units. The analyses were carried out in duplicate. The individual peaks were identified by comparison of their retention indices (relative to C8-C25 n-alkanes for VF-5MS) to those of authentic samples and literature [25], as well as by comparing their mass spectra with the Wiley 7 MS library (Wiley, New York, NY, USA) and NIST02 (Gaithersburg, MD, USA) mass spectral database.

***Inductively coupled plasma atomic emission spectroscopy (ICP-AES)*:** For determination of macroelements (K, Na, Ca, Mg) and trace elements (Al, Fe, Sr, Zn, B, Mn, Ba, Ni, Pb, V, Cr, Cd, Co, Se, Cu, Hg), duplicates of 2 g ± 0.0005 of homogenized herbal material were wet digested with concentrated $HNO_3$ (5 mL) and 30% $H_2O_2$ (2 mL) in the microwave digestion unit (MLS-1200 MEGA Microwave Digestion Systems, Milestone, Bergamo, Italy). The digestion conditions were: 1 min at 250 W (smooth oxidation of organic matter), 1 min at 0 W (proceeding of reaction without addition of energy to avoid run-away temperatures and overpressures), 5 min at 250 W (termination of the soft oxidation of the organic compounds), 5 min at 400 W and 5 min at 600 W (final termination of oxidation processes by applying higher power). After cooling, the digested samples were diluted to 50 mL using deionised water and obtained solutions were used for further investigation.

The content of mercury was determined by atomic absorption spectrometry (AAS) on a Perkin-Elmer 4100 Zeeman ZL/FIMS 400 atomic absorption spectrometer (Perkin Elmer, Ueberlingen, Germany) using flow injection/hydride technique [26], while the content of other investigated elements was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES) on Trace Scan Thermo (Thermo Jarrel Ash Corporation, Franklin, USA) using the standard technique [27].

***Micromorphological traits:*** For SEM-investigation samples (stem, leaf, bracteoles, calyx) were transferred from 70% ethanol to 70% acetone, then dehydrated (70%, 90% and 100% acetone) and subjected to critical point drying using $CO_2$ as the drying medium (CPD030; Baltec). After that, samples were sputter coated with gold (Sputter Coater, AGAR) and examined under the scanning electron microscope XL30 ESEM (FEI) with 20 kV acceleration voltages in high vacuum mode. Common terminology was used in the description of micromorphology [23].

**Acknowledgments** - This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (projects no. 006–0000000–3178, and 177–1191192–0830). We thank Mrs. Gabrijela Čepo for check English style and grammar.

## References

[1] Giuliani C, Maleci Bini L. (**2008**) Insight into the structure and chemistry of glandular trichomes of Labiatae, with emphasis on subfamily Lamioideae. *Plant Systematics and Evolution*, ***276***, 199–208.
[2] Kremer D, Stabentheiner E, Dunkić V, Dragojević Müller I, Vujić L, Kosalec I, Ballian D, Bogunić F, Bezić N. (**2012**) Micromorphological and chemotaxonomical traits of *Micromeria croatica* (Pers.) Schott.. *Chemistry & Biodiversity*, ***9***, 755-768.
[3] Slavkovska V, Couladis M, Bojović S, Tzakou O, Pavlović M, Lakušić B, Jančić R. (**2005**) Essential oil and its systematic significance in species of *Micromeria* Bentham from Serbia & Montenegro. *Plant Systematics and Evolution*, ***255***, 1–15.
[4] Chalchat JC, Diab Y, Auezova L, Chebib H. (**2005**) Chemical composition of the essential oil of *Micromeria libanotica* Boiss., an endemic species of Lebanon. Journal *of* Essential Oil *Research*, ***17***, 449–450.
[5] Werker E, Ravid U, Putievsky E. (**1985**) Structure of glandular hairs and identification of the main components of their secreted material in some species of the *Labiatae*. *Israel Journal of Botany*, ***34***, 31–45.
[6] Werker E. (**2000**) Trichome diversity and development. In: *Advances in botanical research incorporating advances in plant pathology*, *Plant trichomes.* Vol. ***31***, Hallahand DL, Gray J, Callow JA (Ed). Academic Press, London UK. 1–35.
[7] Corsi G, Bottega S. (**1999**) Glandular hairs of *Salvia officinalis*: New data on morphology, localization and histochemistry in relation to function. *Annals of Botany*, ***84***, 657–664.
[8] Fahn A. (**2000**) Structure and function of secretory cells. In: *Advances in botanical research incorporating advances in plant pathology*, *Plant trichomes.* Vol. ***31***, Hallahand DL, Gray J, Callow JA (Ed). Academic Press, London UK. 37–75.
[9] Stojanović G, Palić I, Jursić-Janković J. (**2006**) Composition and antmicrobial activity of the essential oil of *Micromeria cristata* and *Micromeria juliana*. *Flavour and Fragrance Journal*, ***21***, 77–79.
[10] Öztürk M, Kolak U, Topçu G, Öksüz S, Choudhary I. (**2011**) Antioxidant and anticholinesterase active constituents from *Micromeria cilicica* by radical-scavenging activity-guided fractionation. *Food Chemistry*, ***126***, 31–38.
[11] Öztürk M, Kolak U, Duru ME, Harmandar M. (**2009**) GC-MS analysis of the antioxidant active fractions of *Micromeria juliana* with anticholinesterase activity. *Natural Product Communications*, ***4***, 1271-1276.
[12] Shallari S, Schwartz C, Hasko A, Morel JL. (**1998**) Heavy metals in soils and plants of serpentine and industrial sites of Albania. *Science of the Total Environment*, ***209***, 133–142.
[13] Chater AO, Guinea E. (**1972**) *Micromeria* Benth. In: *Flora Europaea*. Vol. ***3.*** Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters S, Webb DA (Ed). Cambridge University Press, Cambridge. 167–170.
[14] Wielgorskaya T. (**1995**) *Dictionary of generic names of seed plants.* Columbia University, New York. 369.
[15] Erhardt W, Götz E, Bödeker N, Seybold S. (**2002**) Zander – Handwörterbuch der Pflanzennamen. ***17***. *Auflage*, *Eugen Ulmer GmbH & Co*., Stuttgart. 576.
[16] Lovašen-Eberhardt Ž. (**2000**) *Micromeria* Benth. – bresina. In: Index florae Croaticae. Nikolić T. (Ed). *Natura Croatica*, ***3***, 19–20.
[17] Šilić Č. (**1979**) Monography of genuses *Satureja* L., *Calamintha* Miller, *Micromeria* Bentham, *Acinos* Miller and *Clinopodium* L. in flora of Yugoslavia [In Bosnian]. Zemaljski muzej BiH, Sarajevo. 237–248.
[18] Trinajstić I. (**1985**) Flora of the Korčula Islands. *Acta Botanica Croatica*, ***44***, 107–130.
[19] Baser KHC, Kirimer N, Özek T, Tümen G. (**1995**) Essential Oil of *Micromeria carminea* Davis PH. Journal *of* Essential Oil *Research*, ***7***, 457–458.
[20] Tabanca N, Kirimer N, Demirci B, Demirci F, Başer KH. (**2001**) Composition and antimicrobial activity of the essential oils of *Micromeria cristata* subsp. *phrygia* and the enantiomeric distribution of borneol. *Journal of Agriculture and Food Chemistry*, ***49***, 4300–4303.
[21] Mastelić J, Jerković I, Kuštrak D. (**2005**) Aromatic compounds of *Micromeria juliana* (L) Bentham ex Reicheneb. from Croatia. Journal *of* Essential Oil *Research*, ***17***, 516–518.
[22] Tzakou O, Couladis M. (**2001**) The essential oil of *Micromeria graeca* (L.) Bentham et Reichenb. growing in Greece. *Flavour and Fragrance Journal*, ***16***, 107–109.
[23] Payne WW. (**1978**) A glossary of plant hair terminology. *Brittonia*, ***30***, 239–255.
[24] Bini Maleci L, Servettaz O. (**1991**) Morphology and distribution of trichomes in Italian species of *Teucrium* sect. *Chamaedrys* (Labiatae) – a taxonomical evaluation. *Plant Systematics and Evolution*, ***174***, 83–91.
[25] Adams RP. (**2007**) *Identification of essential oil components by gas chromatography/ mass spectroscopy.* 4th. ed., Allured Publishing Corporation. Carol Stream IL, USA.
[26] Perkin Elmer. (**1994**) Flow Injection Mercury/Hydride Analyses Recommended Analytical Conditions and General Information. Bodenseewerk Perkin-Elmer GmbH, Überlingen, DE.
[27] Jarrel Ash Corporation. (**1995**) 'Trace Scan, Atom Scan 16/25 Plasma Spectrometer User's Guide', Franklin, Massachusetts.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1671 - 1672

# Composition and Variability of the Essential Oil of *Salvia nemorosa* (Lamiaceae) from the Vienna Area of Austria

**Remigius Chizzola**

*Institute of Applied Botany and Pharmacognosy*, *University of Veterinary Medicine Vienna*, *A – 1210 Vienna*, *Veterinaerplatz 1*, *Austria*

*remigius.chizzola@vetmeduni.ac.at*

**Received: September 28th, 2012; Accepted: October 17th, 2012**

The volatiles present in the aerial parts of *Salvia nemorosa* L. (Lamiaceae), grave sage, were analysed by GC/MS and GC in plants growing at different sites in the outskirts of Vienna, Austria. The flower oils contained mainly sabinene (37-44%), germacrene D (9-14%), β-caryophyllene (8-12%) and caryophyllene oxide (2.6-4.4%). Leaf samples had β-caryophyllene (14-41%), germacrene D (14-38%) and caryophyllene oxide (5-20%) as main compounds, while stem oils were characterized by an high hexadecanoic acid percentage besides germacrene D and β-caryophyllene.

**Keywords:** *Salvia nemorosa*, Lamiaceae, Sabinene, β-Caryophyllene, Germacrene D.

*Salvia nemorosa* L., grave sage, (Lamiaceae), a perennial with sessile leaves, purple bracts and lilac flowers in whorls, originally occurred from Eastern and Southeastern Europe eastwards to Central Asia. The natural distribution in Europe is the subcontinental dry parts of the Pannonian, Bohemian and Hercynian floral region [1a]. In the area of Vienna, Austria, the plant reaches the western border of its range [1b]. The plant bears glandular and covering trichomes [1c], and essential oils from the aerial parts rich in sesquiterpenoids [2]. As the mentioned references did not differentiate between the plant organs, the present study reports the composition of the essential oil from stem, leaves and flowers of plants collected at five different sites in the urban area of Vienna (Table 1).

The oil content of all plant parts was below 0.05%, but they had distinct oil patterns, as shown in Table 2. The oil from the flowers was dominated by sabinene (37-44%), followed by germacrene D (9-14%), β-caryophyllene (8-12%) and caryophyllene oxide (2.6-4.4%). α-Thujene (5-8%) and γ-terpinene (2-8%) were further prominent monterpenes, while β-pinene, α-terpinene, limonene and *p*-cymene were minor compounds (0.8-3.4% each). The leaf oils were rich in the sesquiterpenes β-caryophyllene (14-41%), germacrene D (14-38%) and caryophyllene oxide (5-20%). Leaf oils contained also some sabinene (up to 6.6%), other monoterpenes less than 1% each and further sesquiterpenes less than 2% each, including farnesene, α-humulene, γ-muurolene and δ-cadinene. Hexadecanoic acid (56-60%) was the main compound in the stem volatile fractions. The already mentioned sesquiterpenes β-caryophyllene and germacrene D were also major compounds in the stems. The monoterpenes sabinene and γ-terpinene were present in three out of the four stem oil samples. However, based on the composition of the oil samples, one cannot differentiate between plants of the different collecting sites.

The plants of all five locations represent the same chemotype and had similarities with plants from Serbia with caryophyllene oxide (13.4%), β-caryophyllene (10.1%) and germacrene D (5.7%) as main components. These plants had also sabinene (4.7%) and terpinen-4-ol (3.7%) as prominent monoterpenes in the oil [2a]. However, other *S. nemorosa* plants from the same country were characterized by an essential oil dominated by caryophyllene oxide (23.1%), followed by spathulenol (11.2%), and with appreciable proportions of γ-terpinene (8.6%), *E*-anethol (7.8%) and humulene epoxide II (5.2%), but devoid of sabinene [2b]. A further type of *S. nemorosa* essential oil was reported from Iran with β-caryophyllene (41.6%) and germacrene B (21.3%) as main compounds, and further sesquiterpenes such as caryophyllene oxide, spathulenol, *cis*-β-farnesene and germacrene D (each 5-7%) [2c].

## Experimental

***Plant material:*** The plants were collected during July and August 2008 at five locations in the northern and eastern part of Vienna, Austria (Table 1). Most plants were fully flowering. In each case, at least 15 plants were taken together and separated into leaf, stem and flower samples. The plant material was dried in a room in ambient air. The "Exkursionsflora für Österreich, Liechtenstein und Südtirol" [1b] was used to identify the plants. Voucher specimens were deposited in the Herbarium of the University of Vienna (WU-Generale, http:\\herbarium.univie.ac.at).

***Hydrodistillation:*** Leaf and stem samples were subjected to hydro-distillation in a Clevenger-type apparatus for 3 h using about 15 g of the plant parts and 200 mL of double distilled water. One mL *n*-hexane was added to the distillation apparatus to collect the oil.

***Microdistillation:*** The distillation of the flower samples was carried out using the automatic microdistillation unit MicroDistiller from Eppendorf (Hamburg, Germany), which allows the simultaneous distillation of 6 samples. About 0.2 to 0.3 g of finely crushed dried plant material and 10 mL distilled water were filled into the sample vial. The collecting vial containing 1 mL water, 0.5 g NaCl and 300 µL *n*-hexane was connected with a capillary to the sample vial. The heating program applied to the sample vial was 15 min at 108°C and then 45 min at 112°C. The collecting vial was kept at -2°C, where the volatiles were trapped in 0.3 mL *n*-hexane. All essential oils were stored at -18°C until GC and GC/MS analysis.

***Gas chromatography:*** The distilled oils and microdistillates were analyzed with an Agilent Technologies 6890 N GC equipped with a FID. The separation was conducted on a DB-5 narrow bore column 10 m x 0.10 mm i.d. with 0.17 µm film thickness. The

**Table 1**: Location of the sampling sites in the urban area of Vienna (Austria).

| | Location | Biotop | Coordinates | | Alt. |
|---|---|---|---|---|---|
| A | Danube border | Dry riverbank | N 48° 11´ 29´´ | E 16° 27´ 28´´ | 166 m |
| B | Donauinsel | Dry meadow | N 48° 13´ 24´´ | E 16° 24´ 53.6´´ | 168 m |
| C | Wien 21, Thayagasse | Field border | N 48° 16´ 42´´ | E 16° 26´ 19´´ | 160 m |
| D | Stammersdorf, Alte Schanzen | Meadow | N 48° 18´ 49´´ | E 16° 24´ 52.1´´ | 220 m |
| E | Bisamberg, Elisabethhöhe | Meadow | N 48° 19´ 15.5´´ | E 16° 21´ 43.4´´ | 360 m |

**Table 2**: Composition of the essential oils from *Salvia nemorosa* samples collected in the Vienna Region (Austria).

| Compound | Site | A | B | C | D | A | B | C | D | E | A | C | D | E |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RI | Stem | Stem | Stem | Stem | Leaf | Leaf | Leaf | Leaf | Leaf | Flower | Flower | Flower | Flower |
| α-Thujene | 928 | | | 0.5 | 0.7 | 0.2 | 0.4 | 1.0 | | | 4.8 | 7.6 | 5.9 | 5.3 |
| α-Pinene | 936 | | | 0.1 | | | | 0.2 | | | 0.4 | 0.8 | 0.7 | 0.3 |
| Sabinene | 973 | 5.2 | 7.9 | 3.0 | 3.9 | 2.0 | 2.2 | 6.6 | | 2.3 | 43.0 | 43.1 | 36.6 | 44.1 |
| 1-Octen-3-ol | 978 | | | | | | 0.3 | | | | | | | |
| β-Pinene | 978 | | | 0.6 | 1.0 | 0.3 | | 0.5 | | 0.2 | 1.0 | 0.8 | 1.9 | 1.5 |
| Myrcene | 989 | | | 0.3 | | 0.1 | 0.1 | 0.4 | | 0.4 | 1.2 | 1.8 | 1.6 | 1.3 |
| α-Phellandrene | 1000 | | | | | | | | | | | 0.1 | | |
| α-Terpinene | 1015 | | | | | | 0.2 | 0.3 | | | 0.8 | 2.0 | 1.2 | 1.0 |
| *p*-Cymene | 1028 | | | 1.3 | 0.8 | 0.1 | 0.2 | 1.8 | | 0.2 | 0.3 | 3.4 | 2.9 | 0.6 |
| Limonene | 1028 | | | 0.8 | | | 0.2 | 0.6 | | 0.3 | 0.9 | 1.6 | 1.5 | 1.1 |
| 1.8-Cineole | 1030 | | | | | | | | | | 0.1 | | | |
| γ-Terpinene | 1058 | | 3.3 | 2.0 | 2.1 | 0.2 | 0.9 | 2.5 | | 0.3 | 1.7 | 8.4 | 5.5 | 1.9 |
| *cis*-Sabinene hydrate | 1066 | | | | | | | | | | 0.5 | 0.7 | 0.6 | 0.6 |
| α-Terpinolene | 1086 | | | | | | 0.2 | 0.2 | | | 0.3 | 0.5 | | 0.4 |
| *trans*-Sabinene hydrate | 1098 | | | | | 0.3 | 0.1 | | | | 0.6 | 0.5 | 0.2 | 0.5 |
| Nonanal | 1109 | | | | | | | | | | | 0.0 | 1.4 | 1.6 |
| Terpinen-4-ol | 1175 | | 5.4 | 0.5 | | 0.1 | 1.1 | 0.4 | | | 1.7 | 1.6 | | |
| α-Terpineol | 1187 | | | | | | | | | | 0.1 | 0.1 | | |
| α-Cubebene | 1349 | | | | | 0.1 | 0.2 | | | 0.3 | | 0.1 | | |
| α-Ylangene | 1370 | | | | | 0.1 | 0.2 | | | | | | | |
| α-Copaene | 1375 | | | 0.4 | | 0.4 | 0.9 | 0.6 | 0.8 | 1.0 | 0.2 | 0.3 | 0.2 | 0.5 |
| *E*-β-Damascenone | 1380 | | | | | | 0.3 | | | | | | | |
| β-Bourbonene | 1383 | | 6.1 | 0.8 | 1.3 | 0.9 | 1.6 | 0.8 | 1.2 | 0.9 | 1.1 | 0.4 | 1.4 | 0.6 |
| β-Cubebene | 1388 | | | | | 0.3 | 0.5 | 0.2 | 0.4 | 0.6 | 0.1 | 0.2 | | 0.3 |
| β-Elemene | 1390 | | | | | 0.3 | | | | 0.3 | 0.1 | 0.2 | 0.2 | 0.1 |
| β-Caryophyllene | 1422 | 12.1 | 25.6 | 9.4 | 8.9 | 34.0 | 30.4 | 14.4 | 41.0 | 25.5 | 11.8 | 6.9 | 10.5 | 8.6 |
| β-Copaene | 1428 | | | 0.4 | | 0.4 | 0.9 | 0.5 | 0.8 | 0.5 | 0.1 | 0.3 | 0.2 | 0.4 |
| Geranylacetone | 1449 | | | | | | | | | 0.3 | | | | |
| Farnesene | 1453 | | | 0.4 | | 1.9 | | 0.8 | | 1.3 | 0.3 | 0.4 | 0.2 | 0.5 |
| α-Humulene | 1455 | | | 0.2 | 0.8 | 1.0 | 1.7 | 0.5 | 2.3 | 1.4 | 2.0 | 0.4 | 1.0 | 0.7 |
| γ-Muurolene | 1476 | | | 0.6 | | 0.9 | 2.4 | 1.0 | 2.0 | 1.3 | 0.2 | 0.4 | 0.2 | 0.7 |
| Germacrene D | 1481 | 7.1 | 8.0 | 6.6 | 4.7 | 14.0 | 20.1 | 15.4 | 28.3 | 38.3 | 12.9 | 8.7 | 13.0 | 13.4 |
| β-Selinene | 1487 | | | | | 0.3 | | 0.6 | 0.8 | 0.6 | | 0.1 | | 0.1 |
| γ-Amorphene | 1492 | | | | | | 1.0 | | 1.0 | 1.1 | 0.1 | | 1.1 | 0.6 |
| Bicyclogermacrene | 1494 | | | | | 0.6 | 0.7 | 0.2 | | | 0.5 | 0.1 | | |
| α-Muurolene | 1497 | | | | | 0.2 | 0.5 | 0.3 | 0.4 | 0.3 | 0.1 | 0.2 | | 0.1 |
| Germacrene A | 1503 | | | 0.4 | 0.7 | 0.5 | | 0.8 | 1.4 | 0.7 | 0.2 | | 0.2 | 0.3 |
| γ-Cadinene | 1514 | | | | 0.7 | 0.5 | 1.3 | | 1.1 | 1.0 | 0.1 | 0.4 | 0.2 | 0.5 |
| δ-Cadinene | 1525 | | | 1.1 | | 1.1 | 2.9 | 1.7 | 2.2 | 0.3 | 0.5 | 0.7 | 0.7 | 1.0 |
| Spathulenol | 1577 | | 4.6 | | 1.5 | 0.2 | 0.2 | | 0.4 | 1.7 | 0.3 | 0.1 | 1.5 | 0.6 |
| Caryophyllene oxide | 1586 | 7.2 | 25.2 | 2.7 | 2.9 | 13.2 | 19.5 | 5.3 | 7.6 | 5.4 | 4.4 | 2.1 | 2.6 | 3.2 |
| Salvial-4(14)-en-1-one | 1593 | | 13.9 | 0.4 | | 0.6 | 2.0 | 0.7 | 0.6 | 0.7 | 0.2 | 0.3 | | 0.5 |
| Hexahydro-farnesyl-acetone | 1844 | 0.8 | | 2.2 | 2.3 | 1.0 | | 2.1 | 0.9 | 0.9 | 0.2 | | 0.2 | |
| Hexadecanoic acid | 1971 | 59.8 | | 56.3 | 58.1 | 9.6 | | 24.4 | 2.2 | 4.5 | 1.8 | | 1.5 | |

analytical conditions were: carrier gas He, initial flow 0.5 mL/min (42 cm/sec), constant pressure 45.78 psi; injector temperature 250°C, split ratio 40:1, temperature program: 1 min at 60°C, with 8°C/min up to 85°C, then with 12°C/min to 280°C and held for 5 min at 280°C. The injector temperature was set at 250°C, and the injection volume was 1 μL. The FID was operated at 260°C with an air flow of 350 mL/min and a hydrogen flow of 35 mL/min. The percentage compositions of the essential oil constituents were calculated from the FID response, without any correction.

***Gas chromatography/mass spectrometry:*** To identify the volatile components, the samples were also analysed by GC/MS using a HP 6890 GC coupled to a HP 5972 quadrupole mass selective detector. Separation was accomplished with a HP5-MS fused silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm). The analytical conditions were: carrier gas He 1.3 mL/min constant flow; injector temperature 250°C, split ratio 40:1, temperature program: 2 min at 40°C, with 3°C/min up to 180°C, then with 4°C/min to 280°C and held for 4 min at 280°C. The injection volume was 1 μL. The transfer line to the MS was set at 280°C. The total ion current (*m/z* 40 to 350) was recorded and the individual compounds were identified according to their MS and their retention indices [3].

**Acknowledgments** - Thanks to Mrs Hanneliese Michitsch for her technical assistance. The research was supported by "Hochschuljubiläumsstiftung der Stadt Wien", Project H-02328/2007.

## References

[1] (a) Meusel H, Jäger E, Rauschert S, Weinert E. (**1978**) *Vergleichende Chorologie der Zentraleuropäischen Flora.* VEB Gustav Fischer Verlag, Jena, Vol II, pp. 381-382; (b) Fischer MA, Adler W, Oswald K. (**2005**) *Exkursionsflora für Österreich, Liechtenstein und Südtirol*, 2nd ed, Land Oberösterreich, Biologiezentrum der OÖ Landesmuseen, Linz, Austria, 765-790; (c) Then M, Lemberkovics E, Marczal G. (**2003**) Study of plant anatomical characteristics and essential oil composition of Hungarian *Salvia* species. *Acta Horticulturae*, ***597***, 143-148.

[2] (a) Chalchat JC, Petrovic SD, Maksimovic ZA, Gorunovic MS. (**2004**) Composition of essential oils of some wild *Salvia* species growing in Serbia. *Journal of Essential Oil Research*, ***16***, 476-478; (b) Malenčić D, Couladis M, Mimica-Dukić N, Popović M, Boža P. (**2004**) Essential oils of three *Salvia* species from the Pannonian part of Serbia. *Flavour and Fragrance Journal*, ***19***, 225-228; (c) Mirza M, Sefidkon F. (**1999**) Essential oil composition of two *Salvia* species from Iran, *Salvia nemorosa* L. and *Salvia reuterana* Boiss. *Flavour and Fragrance Journal*, ***14***, 230-232.

[3] (a) Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry*, 4th ed. Allured Pub. Corp., Carol Stream, IL; (b) McLafferty FW. (**1989**) *Registry of Mass Spectral Data*, 5th ed., New York, John Wiley & Sons.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1673 - 1675

# Essential oil Composition of *Ficus benjamina* (Moraceae) and *Irvingia barteri* (Irvingiaceae)

**Isiaka A. Ogunwande[a, *], Razaq Jimoh[a], Adedoyin A. Ajetunmobi[a], Nudewhenu O. Avoseh[a] and Guido Flamini[b]**

[a]*Natural Product Unit*, *Department of Chemistry*, *Faculty of Science*, *Lagos State University*, *P.M.B. 0001*, *Ojo*, *Lagos*, *Nigeria*

[b]*Dipartimento di Scienze Farmaceutiche*, *sede Chimica Bioorganica e Biofarmacia*, *Universita di Pisa*, *Via Bonanno 33*, *56126 Pisa*, *Italy*

*isiaka.ogunwande@lasu.edu.ng*

**Received: November 14th, 2011; Accepted: October 10th, 2012**

Essential oils obtained by hydrodistillation of leaves of two Nigerian species were analyzed for their constituents by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The leaf oil of *Ficus benjamina* L. (Moraceae), collected during the day, contained high contents of α-pinene (13.9%), abietadiene (9.7%), *cis*-α-bisabolene (8.2%) and germacrene-D-4-ol (8.4%), while the night sample was dominated by germacrene-D-4-ol (31.5%), 1,10-di-*epi*-cubenol (8.8%) and hexahydrofarnesylacetone (8.3%). This could be a possible indication of differences in emissions of volatiles by *F. benjamina* during the day and night. The main compounds of *Irvingia barteri* Hook. f. (Irvingiaceae) were β-caryophyllene (17.0%), *(E)*-α-ionone (10.0%), geranial (7.6%), *(E)*-β-ionone (6.6%) and β-gurjunene (5.1%).

**Keywords:** *Ficus benjamina*, *Irvingia barteri*, Essential oil, α-Pinene, Germacrene-D-4-ol, β-Caryophyllene.

In continuation of extensive research on the different volatile emissions from *Ficus* species [1-3] and other plant from Nigeria, we report herein the volatile compounds of *F. benjamina* and *Irvingia barteri.* *F. benjamina* L. is one of hundreds of species of figs, dozens of which are very common in cultivation. Figs are members of the family Moraceae, a huge family of plants with over 1000 species. Most Moraceae are tropical plants, and *F. benjamina* is no exception, being native to the tropics of south Asia and northern Australia. However, this is probably the most common of all the *Ficus* species. The plant is credited with many biologically active compounds and uses [4,5]. α-Copanene, cyclosativene and β-ocimene were the major compounds of its volatile oil [6]. The chemical composition of essential oils of some *Ficus* species growing in Nigeria have been reported [1-3,7-10]. Phytol and 6,10,14-trimethyl-2-pentadecanone have been described as chemical markers of Nigerian grown *Ficus* oils [2,3,8-10].

Wild mango, *Irvingia barteri* O'Rorke Bail (syn *Irvingia gabonensis*), family Irvingiaceae, is native to some Central and West African countries. The fruit is succulent and available between July and October. Due to the pleasant aroma, the roasted seeds are commonly used in Africa as flavoring in traditional dishes. *I. barteri* is known in the Nigerian languages by various names such as 'biri'or 'goron'(Hausa), 'oro' (Yoruba) and 'obono' (Igbo). The fruits have been reported to be rich in vitamin C and are widely consumed as a desert fruit or snack throughout Western and Central Africa with various nutritional and dietary aspects [11,12]. Extracts of the plant have been reported to possess antimicrobial activities and to be used as a painkiller for toothache. Some antimicrobial agents such as 3-friedelanone, betulinic acid, oleanolic acid, 3,3′,4′-tri-*O*-methylellagic acid, 3,4-di-*O*-methyl-ellagic acid and hardwickiic acid have been isolated from the plant [13]. Literature information is scanty about the volatile constituents except for the key odorant compounds of the seeds and fruits [14-16].

The plant samples yielded low contents of essential oils: 0.1% (v/w; *F. benjamina*; colorless) and 0.23% (v/w; *I. barteri*; light yellow), calculated on a dry weight basis. Forty-seven and thirty-eight components respectively were identified from the day and night samples of *F. benjamina*. The main compounds of the day sample were α-pinene (13.9%), abietadiene (9.7%), germacrene-D-4-ol (8.4%) and *cis*-α-bisabolene (8.2%). Isobornyl acetate (5.0%) and abietatriene (4.9%) were also present in sizeable quantity. However, germacrene-D-4-ol (31.5%) was the most singly abundant compound of the night sample. Other significant constituents were 1,10-*di-epi*-cubenol (8.8%), hexahydrofarnesyl-acetone (8.3%), *E*-geranyl acetone (6.2%), cubenol (5.5%) and 1, 8-cineole (4.2%). Significant differences were observed in the major classes of compounds of the day and night samples of *F. benjamina* (Table 1). Oxygenated sesquiterpenoids were the dominant class of compounds in the night sample. On the other hand, the day sample contained equal proportions of monoterpene hydrocarbons and oxygenated monoterpenes. However, diterpenoids and sulfur compounds, which were present in the day sample, could not be identified in the night sample. The volatiles emitted by plants depend on factors such as climatic conditions, morphology of the plant, and time of collection. The present result is an indication of the possible difference in emissions of volatiles by *F. benjamina* during the day and night. This is the first report of different volatile emissions of *F. benjamina*. Previous investigation into the volatiles of this plant [6] identified significant amounts of α-copaene, cyclosativene and β-ocimene. These compounds are conspicuously absent in the present study. Moreover, compounds such as phytol, 6, 10, 14-trimethyl-2-pentadecanone, and acorenone B, which are characteristics of other *Ficus* species from Nigeria [1-3, 8], were not detected in the present investigation (Table 2).

The thirty-six essential oil components of *I. barteri* were dominated by oxygenated monoterpene (34.0%) and sesquiterpene hydrocarbons (41.5%) (Table 3). The main compounds identified in

**Table 1**: Components of *F. benjamina* essential oil.

| Constituents | LRI [a] | Percent % (day) | Percent % (night) |
|---|---|---|---|
| *(E)*-2-Hexenal | 856 | - | 0.9 |
| α-Pinene | 939 | 13.9 | 1.2 |
| Camphene | 953 | 1.1 | 0.4 |
| Thuja-2,4(10)-diene | 957 | 0.8 | - |
| Benzaldehyde | 961 | 0.1 | 0.4 |
| Sabinene | 976 | 3.7 | - |
| β-Pinene | 980 | - | 0.6 |
| 6-Methyl-5-hepten-2-one | 985 | - | 0.5 |
| Myrcene | 991 | 0.8 | 0.6 |
| α-Phellandrene | 1005 | 0.7 | - |
| δ-3-Carene | 1011 | tr | - |
| α-Terpinene | 1018 | 0.1 | - |
| *p*-Cymene | 1027 | 1.2 | tr |
| Limonene | 1031 | 2.0 | 0.4 |
| 1,8-Cineole | 1034 | 2.1 | 4.2 |
| Phenylacetaldehyde | 1045 | - | 0.5 |
| γ-terpinene | 1062 | 0.1 | - |
| *cis*-Linalool oxide (furanoid) | 1076 | 0.3 | - |
| Dehydro-ρ-cymene | 1088 | 2.0 | - |
| Linalool | 1099 | 0.8 | 1.6 |
| Nonanal | 1103 | tr | 1.3 |
| Dipropyl disulfide | 1107 | 1.2 | - |
| α-Campholenal | 1127 | 1.4 | - |
| *trans*-Pinocarveol | 1140 | 2.5 | - |
| Camphor | 1145 | 1.2 | 1.2 |
| Isoborneol | 1156 | - | 1.4 |
| Pinocarvone | 1164 | 1.2 | - |
| ρ-Mentha-1,5-dien-8-ol | 1166 | 2.1 | - |
| 4-Terpineol | 1179 | 2.5 | - |
| ρ-Cymen-8-ol | 1185 | 0.2 | - |
| α-Terpineol | 1190 | - | 0.4 |
| Myrtenal | 1194 | 2.4 | - |
| Safranal | 1200 | tr | 0.9 |
| Verbenone | 1207 | 2.2 | - |
| *trans*-Carveol | 1218 | 0.2 | - |
| β-Cyclocitral | 1219 | 0.5 | 0.8 |
| Methyl carvacrol | 1244 | 0.2 | - |
| Carvone | 1245 | tr | - |
| 3,7-Dimethyl-2,6-octadienal | 1268 | tr | - |
| Isobornyl acetate | 1286 | 5.0 | 1.0 |
| Carvacrol | 1299 | - | 0.6 |
| Undecanal | 1306 | - | 0.5 |
| Dipropyl trisulfide | 1328 | 1.5 | - |
| α-Copaene | 1376 | - | tr |
| *(E)*-β-Damascone | 1409 | - | 0.5 |
| β-Caryophyllene | 1418 | 0.9 | 2.3 |
| *(E)*-α-Ionone | 1428 | 0.2 | 1.3 |
| *(E)*-Geranyl acetone | 1454 | 1.2 | 6.3 |
| *(E)*-β-Farnesene | 1458 | 0.8 | - |
| 9-*epi*-*(E)*-Caryophyllene | 1467 | - | 0.2 |
| *(E)*-β-Ionone | 1485 | - | 1.5 |
| β-Bisabolene | 1509 | - | 1.6 |
| δ-Cadinene | 1524 | 0.2 | tr |
| *cis*-α-Bisabolene | 1555 | 8.2 | - |
| *trans*-Nerolidol | 1565 | - | 0.5 |
| Germacrene D-4-ol | 1575 | 8.4 | 31.5 |
| Caryophyllene oxide | 1581 | - | 2.6 |
| *n*-Hexadecane | 1600 | - | 0.3 |
| 1,10-di-*epi*-Cubenol | 1614 | 0.3 | 8.8 |
| Cubenol | 1638 | - | 5.5 |
| Pentadecanal | 1717 | - | 1.2 |
| Hexahydrofarnesylacetone | 1845 | 1.2 | 8.3 |
| Abietatriene | 2054 | 4.9 | - |
| Abietadiene | 2080 | 9.7 | - |
| **Total** | | **90.0** | **91.0** |
| **Monoterpene hydrocarbons** | | **26.4** | **3.2** |
| **Oxygenated monoterpenes** | | **26.2** | **21.2** |
| **Sesquiterpene hydrocarbons** | | **10.1** | **4.6** |
| **Oxygenated sesquiterpenes** | | **9.9** | **57.2** |
| **Diterpenes** | | **14.6** | **-** |
| **Sulfur compounds** | | **2.7** | **-** |
| **Others** | | **0.1** | **5.6** |

[a] Retention indices on HP-5MS capillary column
tr, trace amount < 0.1%
-, not identified

**Table 2**: Major compounds of *Ficus* species from Nigeria.

| Species | Major compounds | Ref |
|---|---|---|
| *F. exasperata* | 1, 8-cineole (13.8%), (*E*)-phytol (13.7%), ρ-cymene (11.4%) | 7 |
| *F. exasperata* | α-terpineol (33.7%), α-pinene (10.8%), sabinene (5.6%) | 9 |
| *F. mucosa* | β-caryophyllene (37.0%), ethyl octanate (13.9%), methyl octanate (8.6%), caryophyllene oxide (6.0%) | 1 |
| *F. thonningii* | 6, 10, 14-trimethyl-2-pentadecanone (18.8%), phytol (14.7%), acorenone B (7.6%), β-gurjunene (6.3%) | 2 |
| *F. lutea* | acorenone (20.7%), phytol (16.2%), demethoxyageratochromene (6.0%), 6, 10, 14-trimethyl-2-pentadecanone (5.1%), zingiberene (5.2%) | 2 |
| *F. polita* | phytol (23.3%), 6, 10, 14-trimethyl-2-pentadecanone (15.0%), (*E*)-6, 10-dimethyl-5,9-undecadien-2-one (7.3%), drimenol (5.8%) | 2 |
| *F. ovata* | (*E*)-phytol (24.5%), hexadecanoic acid (10.0%), caryophyllene oxide (7.6%), 6, 10, 14-trimethyl-2-pentadecanone (6.1%) | 8 |
| *F. elasticoidies* | (*E*)-phytol (20.9%), 6, 10, 14-trimethyl-2-pentadecanone (8.7%), aβ-caryophyllene (6.8%) | 8 |
| *F. natalensis* subsp. *leprieurii* | (*E*)-phytol (37.6%), 6, 10, 14-trimethyl-2-pentadecanone (24.9%) | 8 |
| *F. elastica* | 6, 10, 14-trimethyl-2-pentadecanone (25.9%), geranyl acetone (9.9%), heneicosene (8.4%), 1, 8-cineole (8.2%) | 3 |
| *F. capensis* | α-pinene (9.3-36.7%), β-pinene (4.5-14.9%), α-cadinol (10.6%), *n*-hexanedecanoic acid (15.5-33.3%) | 10 |

**Table 3**: Essential oil composition of *I. barteri*.

| Constituents | LRI [a] | Percent % |
|---|---|---|
| Sabinene | 976 | 0.7 |
| 6-Methyl-5-hepten-2-one | 985 | tr |
| 2-Pentyl furan | 991 | 0.7 |
| *o*-Cymene | 1022 | tr |
| Limonene | 1031 | 2.6 |
| 1,8-Cineole | 1034 | tr |
| Dehydro-*p*-cymene | 1088 | tr |
| Nonanal | 1103 | 1.4 |
| 2,4-Dimethyl benzaldehyde | 1180 | tr |
| Naphthalene | 1182 | tr |
| Safranal | 1197 | tr |
| β-Cyclocitral | 1219 | 2.1 |
| Neral | 1240 | 2.9 |
| *p*-Menth-4-en-3-one | 1251 | 1.0 |
| β-Homocyclocitral | 1254 | 1.3 |
| Geranial | 1272 | 7.6 |
| 1,2,3,4-Tetrahydro-1,5,7-trimethyl naphthalene | 1310 | tr |
| 1,2-Dihydro-1,1,6-trimethyl naphthalene | 1354 | 2.1 |
| β-Caryophyllene | 1418 | 17.0 |
| *(E)*-α-Ionone | 1428 | 10.0 |
| β-Gurjunene | 1432 | 5.1 |
| *trans*- α-Bergamotene | 1439 | 1.4 |
| *(E)*-Geranyl acetone | 1453 | 2.5 |
| α-Humulene | 1455 | 1.6 |
| γ-Muurolene | 1477 | 2.9 |
| *(E)*-β-Ionone | 1488 | 6.6 |
| Valencene | 1492 | 1.9 |
| α-Muurolene | 1499 | 3.7 |
| Pentadecane | 1500 | 1.5 |
| *trans*-γ-Cadinene | 1513 | 2.8 |
| δ-Cadinene | 1524 | 3.6 |
| *trans*-Nerolidol | 1565 | 1.5 |
| Caryophyllene oxide | 1581 | 2.9 |
| Selin-11-en-4-α-ol | 1652 | tr |
| Hexahydrofarnesylacetone | 1845 | 1.7 |
| Abietatriene | 2054 | 0.9 |
| **Total** | | **90.0%** |
| **Monoterpene hydrocarbons** | | **3.3** |
| **Oxygenated monoterpenes** | | **34.0** |
| **Sesquiterpene hydrocarbons** | | **41.5** |
| **Oxygenated sesquiterpenes** | | **6.1** |
| **Diterpenes** | | **0.9** |
| **Others** | | **4.2** |

[a] Retention indices on HP-5MS capillary column; tr, trace amount < 0.1%

the oil were β-caryophyllene (17.0%), (*E*)-α-ionone (10.0%), geranial (7.6%), (*E*)-β-ionone (6.6%) and β-gurjunene (5.1%). Previous studies of the volatile contents have been concentrated on fruit samples. There were remarkable differences between this oil composition and previous studies [14-16]. The key aroma compounds in the Nigerian grown species were methional, 2-acetyl-1-pyroline, butan-2,3-dione, pentan-2,3-dione, 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine [14]. Samples from Cameroon [15,16] were found to comprise mainly of ethyl-2-methylbutyrate (11.9-14.2%), methyl-2-methylbutyrate (28.6-31.6%), zingiberene (12.4-15.7%), nerolidol (5.1-8.5%), terpinen-4-ol (8.4-7.9%) and α-terpineol (7.9-8.7%). Except for *trans*-nerolidol, the present oil lacked several of the compounds mentioned above.

## Experimental

***Plant materials:*** Fresh leaves of *Ficus benjamina* were collected from Lagos State University, Ojo, Nigeria, in June 2010. The day samples were collected at about noon on sunny days; the night samples were harvested at about 11.30 pm during darkness. The leaves of *Irvingia barteri* were harvested from Ijede Area, Ikorodu, Lagos, Nigeria, in January 2011. The plants were authenticated by Curators at the Herbarium of the Botany Department, University of Lagos, Nigeria. Voucher specimens LUH 3223 (*F. benjamina*) and LUH 3323 (*I. barteri*) were deposited at the Herbarium for future reference. All samples were air-dried under laboratory shade prior to extraction of the oils.

***Extraction of essential oils*:** Air-dried leaves (300 g) were subjected to separate hydrodistillation in an all-glass Clevenger type apparatus for 4 h in accordance with British Pharmacopoeia [17].

***Chemical analysis:*** GC analysis was accomplished with a HP-5890 series II instrument equipped with HP-wax and HP-5 capillary columns (both 30 m x 0.25 mm, 0.25 μm film thickness) with the following temperature program; $60^0$C for 10 min, rising from $5^o$C/min to $220^o$C. Both injector and detector temperatures were maintained at $250^o$C; carrier gas, nitrogen (2 mL/min); detector, FID; ratio, 1:30. The volume injected was 0.5 μL. The relative proportions of the oil constituents were percentages obtained (% area) by FID peak-area normalization, without the use of response factor.

Gas chromatography-electron ionization mass spectrometry (GC-EIMS) analysis was performed with a Varian CP-3800 gas chromatograph equipped with a HP-5 capillary column (30 m x 0.25 mm; film thickness 0.25 μm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions; injector and transfer line temperature were $220^o$C and $240^o$C, respectively. Oven temperature programmed from $60^o$C- $244^o$C at $3^o$C/min; carrier gas was helium at a flow rate of 1 mL/min; injection of 0.2 μL (10% *n*-hexane solution); split ratio 1:30. MS were recorded at 70 eV. The acquisition mass range was 30-300 m/z at a scan rate of 1 scan/s.

.

***Compound identification:*** Identification of the constituents was based on comparison of the retention times with those of authentic samples, and by comparison of their linear indices with a series of *n*-alkanes. Further identification was also made possible by the use of a MS homemade library built up from pure substances and components of known oils and MS literature data [18, 19].

## References


[1] Ogunwande IA, Saroglou V, Skaltsa E, Ogunbinu AO, Kubmawara D. (**2009**) Constituents of some essential oil-bearing plants from Nigeria. *Journal of Essential Oil Research*, ***21***, 61-66.

[2] Ogunwande IA, Sonibare MA, Thang TD, Dung NX, Soladoye MO, Morohunfolu OO. (**2008**) Comparative analysis of the oils of three *Ficus s*pecies from Nigeria. *Journal of Essential Oil Research*, ***20***, 387-389.

[3] Sonibare MA, Sonibare OO, Akharame OE, Soladoye MO. (**2009**) Chemical composition of essential oils of *Ficus elasticiodes* De Wild., *Ficus ovata* Vahl and *Ficus natalensis* subsp. *leprieurii* (Miq.) C.C. Berg from Nigeria. *Journal of Essential Oil Bearing Plants*, ***12***, 282-286.

[4] Parveen M, Ghalib RM, Mehdi SH, Ul Hasan Mattu R, Ali M. (**2009**) A novel antimicrobial triterpenic acid from the leaves of *Ficus benjamina* var. *comosa*. *Journal of Saudi Chemical Society*, ***13***, 287-290.

[5] Simo CCF, Kouam SF, Poumale HMP, Simo IK, Ngadjui BT, Green IR, Krohn K. (**2008**) Benjaminamide: A new ceramide and other compounds from the twigs of *Ficus benjamina*. *Biochemical Systematics and Ecology*, ***36***, 238-243.

[6] Grison-Pige L, Edwards AA, Hossaert-McKey H. (**1999**) Interspecies variation in floral fragrances emitted by tropical *Ficus* species. *Phytochemistry*, ***52***, 1293-1299.

[7] Sonibare MA, Ogunwande IA, Walker TM, Setzer WN, Soladoye MO, Essien E. (**2006**) Volatile constituents of *Ficus exasperata* Vahl leaves. *Natural Product Communications*, ***1***, 763-765.

[8] Ogunwande IA, Flamini G, Adefuye AE, Lawal NO, Moradeyo S, Avoseh NO. (**2011**) Chemical compositions of *Casuarina equisetifolia* L., *Eucalyptus toreliana* L. and *Ficus elastica* Roxb. ex Hornem. *South Africa Journal of Botany*, ***77***, 645-649.

[9] Oladosu IA, Zubair F, Olawore NO, Shaiq M. (**2009**) Anticandidal activity of volatile compounds from the root bark of *Ficus exasperata* Vahl-Holl (Moraceae). *Journal of Essential Oil-Bearing Plant*, ***12***, 562-568.

[10] Aboaba SA, Oladosu IA, Ekundayo O. (**2010**) Chemical composition, acetyl cholinesterase inhibitory properties and brine shrimp toxicity of essential oils from *Ficus capensis* Thunb from Nigeria. *International Journal of Biological and Chemical Sciences*, ***4***, 1491-1500.

[11] Aina JO. (**1990**) Physico-chemical changes in African mango (*Irvingia gabonensis*) during normal storage ripening. *Food Chemistry*, ***36***, 205-212.

[12] Giami SY, Okonkwo VI, Akusu MO. (**1994**) Chemical composition and functional properties of raw, heat-treated and partially proteolysed wild mango (*Irvingia gabonensis*) seed flour. *Food Chemistry*, ***40***, 237-243.

[13] Fadare DA, Ajaiyeoba EO. (**2008**) Phytochemical and antimicrobial activities of the wild mango-*Irvingia gabonensis* extracts and fractions. *African Journal of Medical Science*, ***37***, 119-24.

[14] Tairu OO, Hofmann T, Schieberle P. (**2000**) Studies on the key odorants formed by roasting of wild mango seeds (*Irvingia gabonensis*). *Journal of Agriculture and Food Chemistry*, ***48***, 2391-2394.

[15] Jirovetz L, Buchbeur G, Ngassoum MB. (**1999**) Analysis of the headspace aroma impact compounds of *Spondia cythera* (Cajarana) and *Irvingia gabonensis* (African bush mango) from Cameroon using GC, GC-MS and olfactometry. *American Laboratory*, ***9***, 18-19.

[16] Jirovetz L, Buchbeur G, Ngassoum MB. (**1999**) Aroma compounds of fruit essential oil of *Irvingia gabonensis* (African bush mango or wild mango). *Nutrition*, ***23***, 9-11.

[17] British Pharmacopoeia (**1990**) HM Stationary Office vol. II, London.

[18] Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography/ Mass Spectrometry, 4th* Ed., Carol Stream. IL: Allured Publishing.

[19] Joulain D, Koenig WA. (**1998**) *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*. E. B. Verlag, Hamburg, Germany.

NPC Natural Product Communications

2012
Vol. 7
No. 12
1677 - 1688

# Cytotoxic Agents of the Crinane Series of Amaryllidaceae Alkaloids

**Jerald J. Nair[a], Jaume Bastida[b], Francesc Viladomat[b] and Johannes van Staden[a*]**

[a]*Research Centre for Plant Growth and Development*, *School of Life Sciences*, *University of KwaZulu-Natal Pietermaritzburg*, *Private Bag X01*, *Scottsville 3209*, *South Africa*

[b]*Departament de Productes Naturals*, *Facultat de Farmacia*, *Universitat de Barcelona*, *08028*, *Barcelona*, *Spain*

*rcpgd@ukzn.ac.za*

**Received: September 14th, 2012; Accepted: October 10th, 2012**

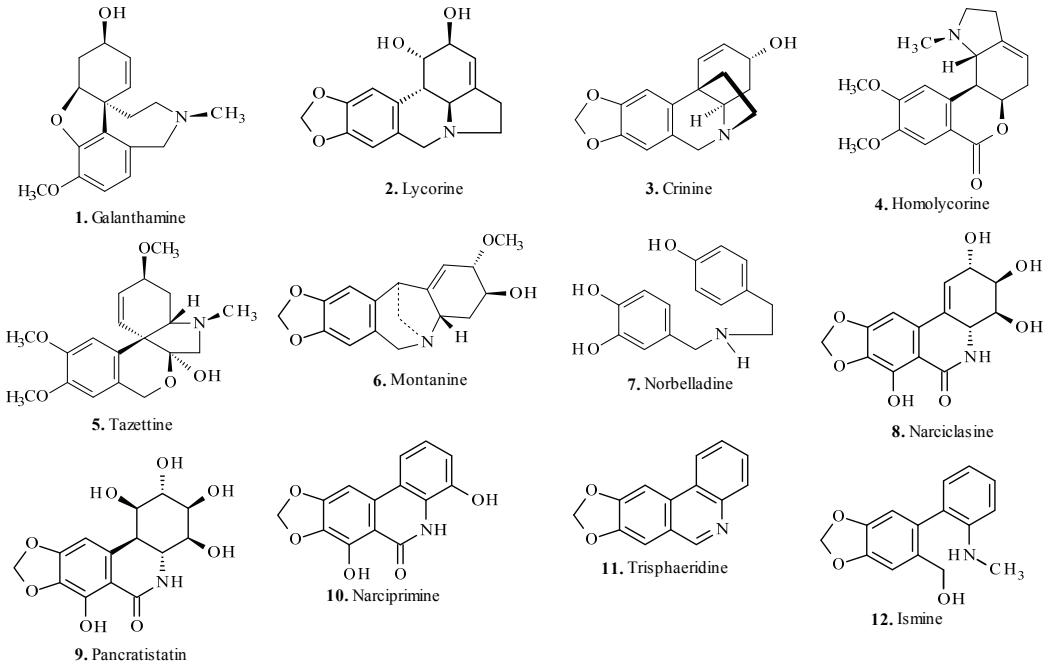
In the alkaloid galanthamine, the plant family Amaryllidaceae has endowed the pharmaceutical community with a potent and selective inhibitor of the enzyme acetylcholinestersae (AChE), of prominence in the chemotherapeutic approach towards motor neuron diseases. Following on the commercial success of this prescription drug in the treatment of Alzheimer's disease, it is anticipated that other drug candidates will in future emerge from the family. In this regard, the phenanthridones, exemplified by narciclasine and pancratistatin, of the lycorine series of Amaryllidaceae alkaloids have shown much promise as remarkably potent and selective anticancer agents, with a drug target of the series destined for the clinical market within the next decade. Given these interesting biological properties and their natural abundance, plants of the Amaryllidaceae have provided a diverse and accessible platform for phytochemical-based drug discovery. The crinane series of Amaryllidaceae alkaloids are also enriched with a significant array of biological properties. As a consequence of their close structural similarity to the anticancer agents of the lycorine series, the cytotoxic potential of crinane alkaloids has been realized through structure-activity relationship (SAR) studies involving targets of both semi-synthetic and natural origin, which has identified several members as leads with promising antiproliferative profiles. As the first of its kind, this review seeks to collate such information from the past few decades in advancing the crinane group as a viable platform for anticancer drug discovery.

**Keywords:** Alkaloids, Amaryllidaceae, Anticancer, Crinane, Cytotoxic.

Amongst the diverse array of biological properties known for the plant family Amaryllidaceae [1], two themes are recurrent: acetylcholinesterase (AChE) inhibition and cytotoxicity. Of significance in the progression of neurodegeneration associated with motor neuron diseases, the former of these has gained much prominence in both the secular and scientific media after commercialization of the Alzheimer's drug galanthamine **1** [2], derived chiefly from the Amaryllidaceous species *Galanthus nivalis* (snowdrop) and *Narcissus pseudonarcissus* (daffodil). More recently, the chemotherapeutic potential of the family has been extended to include members of the lycorine **2** series due to their potent anticancer properties [3]. Galanthamine **1**, lycorine **2** and crinine **3** are representative of the three major structural-types for these alkaloids (Scheme 1), while homolycorine **4**, tazettine **5** and montanine **6** make up the minor series of compounds discernible within the Amaryllidaceae [1c]. Other less-conspicuous members include degraded, oxidized and truncated variants such as trisphaeridine **11** and ismine **12** [1c]. Biogenetically, all of these compounds are related as a consequence of their common amino acid-derived precursor norbelladine **7** [1c].

As a distinct niche within the lycorine series, the phenanthridones exemplified by narciclasine **8** and pancratistatin **9** (Scheme 1) are known for their potent and cell line-specific anticancer activities, and are presently at various stages of development, with a clinical candidate earmarked for commercialization within the next decade [4]. Mechanistically, these phenanthridones are known to initiate cell death via the apoptotic pathway, as indicated by early activation of caspase-3 followed by flipping of phosphatidyl serine, selectively in mitochondria of cancer cells with minimal effect on normal cells [5]. Given these promising biological properties, accompanied by their synthetically-challenging molecular structures, as well as their limited availability from natural sources, these alkaloids have served to fuel sustained efforts in order to deliver a potent, selective and readily bioavailable drug target with facile synthetic access [6a-c]. Furthermore, a stronger case could be made for development of these compounds into commercially viable entities by the low interaction of narciclasine and pancratistatin with the cytochrome P450 3A4 isoenzyme, which accounts for the majority of drugs metabolized in humans [6d-f]. In addition, structure-activity relationship (SAR) studies invoking these potential drug targets have collectively facilitated the elucidation of elements of the apoptosis-inducing pharmacophore innate to these molecules [7].

Crinane alkaloids of the Amaryllidaceae, such as crinine **3** (Scheme 1), are a large and expanding group with a host of biological properties [1,8]. Structurally, they comprise the basic phenanthridine nucleus with varying degrees of oxygenation in ring-A, but usually with a methylenedioxy moiety straddling C-8 and C-9 [1c,d]. The presence of the *N*- to C-10b ethano-bridge is a diagnostic feature of their makeup, and may be either α- or β-orientated leading to two stereodefined subgroups: α- and β-crinanes (Schemes 2 and 3) [1c,d]. As a consequence, the C-10b spiro-junction may adopt one of two possible absolute configurations depending on the orientation of the ethano-bridge [1c,d]. In addition, oxygen-related substituents are usually found at various positions in ring-C and at C-11 of the bridge, while a C-1/C-2 double bond is common for many analogues of both subgroups [1c,d]. Given their close structural proximity to the potent anticancer agents of the lycorine group and their biogenesis from a common precursor, crinane compounds have recently emerged as interesting targets for cytotoxicity-based studies [1a,b]. As such, low micromolar activities in several instances were uncovered for representatives of the group, which has subsequently garnered significant interest in these alkaloids as potential leads in the chemotherapeutic approach towards cancer malignancy [1a,b].



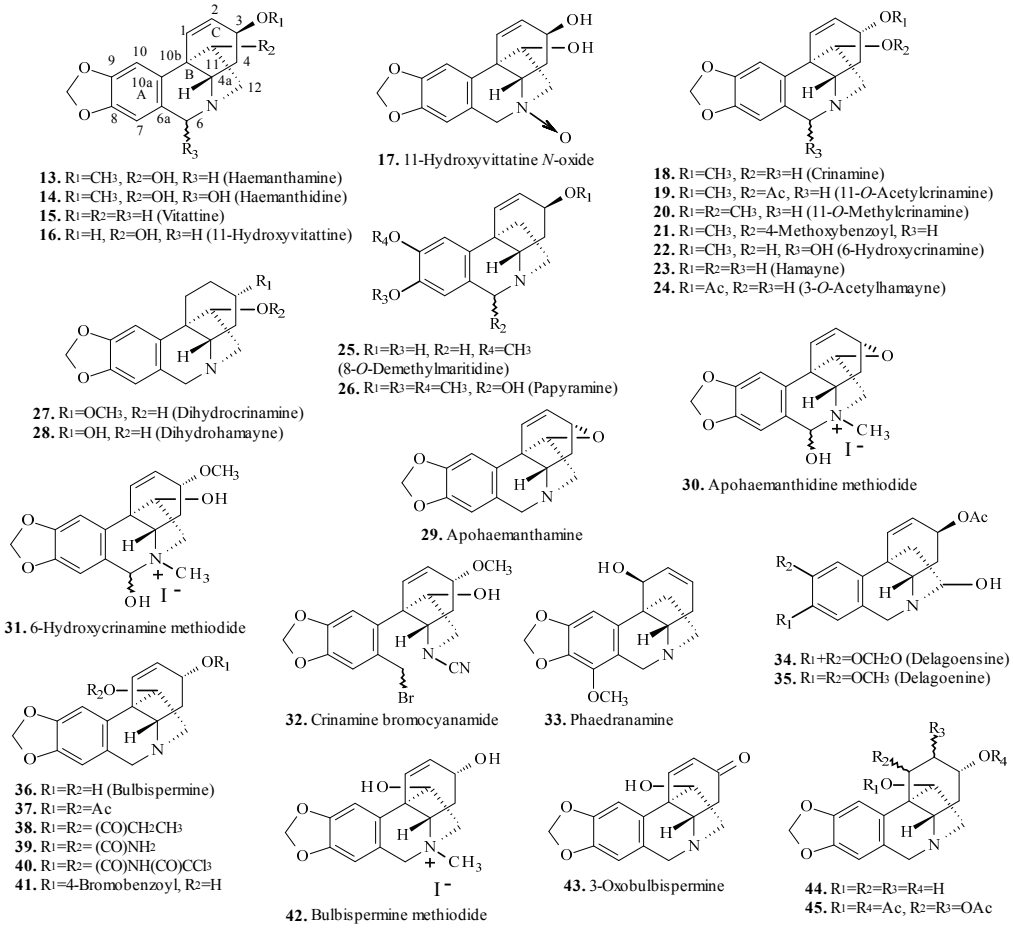

**Scheme 1**: Diverse alkaloid structures of the Amaryllidaceae, including major group representatives **1-3** as well as the common biosynthetic precursor norbelladine **7**.

While several reviews have dedicated attention to the cytotoxic properties of the lycorine series of compounds [3e,h,4d,e,6], especially the phenanthridones of the series, a comprehensive overview with the crinane series members as the focal point is absent from the literature. To this extent, we here present a detailed chronological account of such agents within the crinane series of Amaryllidaceae alkaloids, as well as their allied cytotoxic activities, taking into consideration the contributions we have made in this area over the past few years together with those of others. Core areas that will be covered include: 1) *in vitro* and *in vivo*–based studies of crinane alkaloids; 2) SAR studies utilizing both natural and synthetically-derived targets; 3) elements of the anticancer pharmacophore unraveled through these studies; and 4) mechanistic insights to the mode of operation of these cytotoxic agents.

Based on prior observations for narciclasine **8** [9a], the seminal work of Jimenez et al. (1976) [9b] sought clarity on the antiproliferative effects of a library of structurally diverse Amaryllidaceae alkaloids in cervical adenocarcinoma (HeLa) cells. Of the four crinane compounds screened (Tables 1 and 2), the α-crinane haemanthamine **13** was the most active (MIC 4 μM), while the β-crinanes crinine **3**, buphanidrine **46** and ambelline **47** exhibited no inhibitory effect on cell growth at concentrations up to 0.4 mM [9b]. It was suggested by these authors that the underlying mechanism of action of haemanthamine involved inhibition of protein synthesis by blocking the peptide bond formation step on the peptidyl transferase centre of the 60S ribosomal subunit. This dichotomy in antiproliferative activity between α- and β-crinanes is a theme commonly encountered in the literature, and will be elaborated on in several instances of this survey.

Not long after these findings, Furusawa and coworkers [10] examined various structures of the Amaryllidaceae, including several of the crinane series, for cytotoxic effects in Rauscher virus-carrying NIH/3T3 cells. During the investigation, haemanthamine **13**, crinamine **18** and 6-hydroxycrinamine **22** all exhibited minimum toxic dose (MTD) levels of 0.2 μg/mL, while crinamine bromocyanamide **32** was the next active at 5 μg/mL, and 6-hydroxycrinamine methiodide **31** was identified as the pick of these crinanes with MTD of 0.05 μg/mL. The highly strained ring system in apohaemanthidine methiodide **30** was seen to be detrimental to cytotoxicity as the compound had a MTD of 100 μg/mL, while 6-hydroxybuphanidrine methiodide **58** also exhibited the same MTD value. Again, the dichotomous behavior of α-and β-crinanes are apparent from the activity of **58** compared with the other compounds screened. Amongst the early findings on antineoplastic agents from the Amaryllidaceae, bulbs of *Amaryllis belladonna* were shown by Pettit et al. (1984) to contain two crinane compounds; ambelline **47** and undulatine **62** [11]. Of these, only ambelline was active in the murine P-388 lymphocytic leukemia assay ($ED_{50}$ 1.6 μg/mL), indicating that the compound may be more amenable towards animal models of study as it was previously shown to be inactive in human adenocarcinoma (HeLa) cells [9b].

Also in 1984, Ghosal et al. [12] isolated 1,2-epoxyambelline **61** for the first time from bulbs of *Crinum latifolium*, and subsequent cytotoxicity studies showed that at 5 μg/mL it produced moderate activation of mouse spleen lymphocytes (MSL). Furthermore, the closely-related analogue ambelline **47** in a 1:1 mixture with **61** (at 5 μg/mL) had a marked synergistic effect, producing pronounced activation of the lymphocytic cells with a stimulation index of 3.12, comparable to that of concanavalin A (stimulation index 3.23) [12]. As an extension to the work on *Crinum latifolium*, Ghosal and Singh (1986) then isolated a further two novel alkaloids crinafoline **69** and crinafolidine **71** from mature fruits of the plant [13]. Structurally, crinafoline is the 6α-hydroxy epimer corresponding to ambelline **47**, notably with the *endo* disposition for the 11-hydroxy group, while crinafolidine is the first identified natural truncated member of the crinane series, in this case with a *seco*-B-ring construction. *In vitro* studies on both compounds showed significant reductions in the viability of sarcoma 180 ascites (68.12% and 43.5% for crinafoline **69** and crinafolidine **71**, respectively) [13]. Interestingly, the B-*seco* analogue **71** was shown to be less active than the corresponding fully-cyclized crinafoline **69**, highlighting



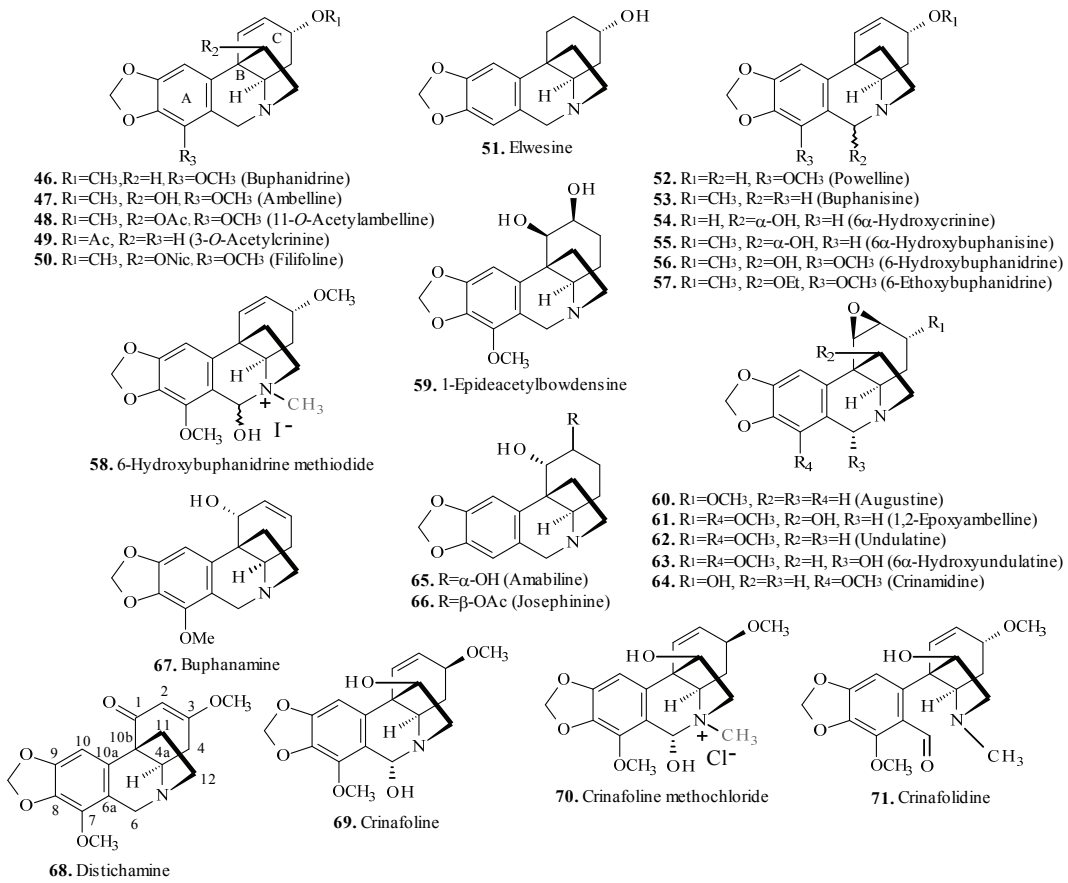

**Scheme 2**: Cytotoxic α-crinane alkaloids of the Amaryllidaceae.

the requirement of an intact phenanthridine nucleus for activity. Furthermore, quaternization of the nitrogen atom was seen to potentiate activity, as the semisynthetically-derived methochloride **70** was twice active as crinafoline **69**, with 32.31% viability shown for the sarcoma cells [13]. This work is also significant in that it represents the first demonstration of *in vivo* effects of crinane alkaloids. In this regard, compounds **69-71** produced marked reductions in growth of sarcoma 180 ascites tumors in mice with cell count values of 10.04 x $10^6$, 5.08 x $10^6$ and 6.14 x $10^6$ shown for the three compounds respectively, compared to untreated control animals (15.88 x $10^6$) [13]. *In vivo*, quaternization of the nitrogen atom was also seen to be ameliorative towards activity (**69** versus **70**), and **70** was identified as the choice of this set of compounds.

A few years later in 1991, Abd El Hafiz et al. [14] examined the constituents of *Crinum augustum* and *Crinum bulbispermum* for activity against human leukemic Molt 4 cells. Of the three crinanes (powelline **52**, 6α-hydroxycrinine **54** and 6α-hydroxybuphanisine **55**) tested, only **55** was moderately active, causing a steady decline (up to ~20%) in the viability of leukemia cells over the three-day treatment period at a dosage of 71 μg/mL [14] (Table 2). Since **54** and **55** are differentiated by their C-3 substituents, it may be construed that a small hydrogen bond acceptor (such as the methoxyl in **55**) as opposed to a donor-acceptor (hydroxyl) in this vicinity is essential for activity against Molt 4 cells. Substituent placement and geometry, as will become increasingly apparent during the course of this survey, have commonly been shown to have a distinctive influence on the cytotoxic abilities of crinane alkaloids.

The work of Likhitwitayawuid et al. (1993) stands out for the multi-cell line approach adopted by the authors in assessing the cytotoxic activities of alkaloid principles of *Crinum amabile* [15]. In the screen against ten cancers, including both human and animal cells, augustine **60** and crinamine **18** were prominent in most cases, with the best response ($ED_{50}$ 0.6 μg/mL) observed in human oral epidermoid carcinoma KB cells and viblastine-resistant KB cells for the two compounds, respectively (Tables 1 and 2) [15]. Augustine **60** was noticeably selective against KB cells as opposed to vinblastine-resistant KB cells ($ED_{50}$ > 20 μg/mL). Buphanisine **53** and amabiline **65** were conspicuous in that they exhibited $ED_{50}$ values > 20 μg/mL in most of the cells tested [15]. The fact that α- and β-crinane representatives were here indistinguishable based on cytotoxic outcomes suggests that there may be other more subtle features, as will be encountered later in the manuscript, attending the crinane alkaloid anticancer pharmacophore.

Soon after this, during the course of the phytochemical investigation of *Hymenocallis expansa*, Antoun et al. (1993) identified haemanthidine **14** as one of the cytotoxic constituents in the screen against eleven cancer cells [16]. Although the compound exhibited non-selective behavior, of note were the activities against prostate LNCaP ($ED_{50}$ 0.7 μg/mL) and P-388 murine lymphocytic leukemia ($ED_{50}$ 0.4 μg/mL) cells. Two years later in 1995, Lin et al. examined *Hymenocallis littoralis* and uncovered *inter alia* the presence of the crinane alkaloids crinine **3**, haemanthamine **13** and 8-*O*-demethylmaritidine **25** [17]. Of these, only haemanthamine was screened for cytotoxic activity and found to be indiscriminate in a panel comprising eleven cell lines with $ED_{50}$ values ranging




**Scheme 3**: Array of cytotoxic β-crinane members of the Amaryllidaceae.

from 0.3 to 1.3 μg/mL, but was markedly potent against HT-1080 human fibrosarcoma cells. The same year, we entered the cytotoxic evaluation arena via a study of 25 diverse alkaloid structures of the Amaryllidaceae against two human tumoral cell lines (Molt4 and HepG2) and one murine non-tumoral cell line (LMTK) [18]. Interestingly, α-crinanes in the library could be distinguished from their β-congeners based on activities against the above tumors [18]. Buphanidrine **46** and ambelline **47** exhibited low inhibitory interaction ($ED_{50}$ > 50 μg/mL) with both Molt4 and HepG2 cells [18], as also previously seen in adenocarcinoma HeLa cells [9b], while the α-crinanes, on the other hand, were markedly more responsive towards the tumor cells. As such, haemanthamine **13** and crinamine **18** both had $ED_{50}$ values of 0.5 μg/mL in LMTK cells, while papyramine **26** exhibited an $ED_{50}$ of 1.5 μg/mL in the same cell line [18].

Although no specific literature reference to cytotoxic properties of crinane compounds was found between 1996 and 1997, the period 1998 to 2000 saw three pieces of work from our groups pertaining to such activities of alkaloids of the South African Amaryllidaceae [19-21]. Firstly, while lycorine **2** was significantly active ($ED_{50}$ = 1.8 μg/mL), crinine **3** and ambelline **47** from bulbs of *Brunsvigia littoralis*, as well as their respective synthesized acetyl derivatives **49** and **48** were notably inactive ($ED_{50}$ > 100 μg/mL) against BL-6 mouse melanoma cells [19]. We next showed that 6-hydroxy-crinamine **22** from *Crinum delagoense* was active in the same cell line, in contrast to its other α-crinane co-constituents hamayne **23**, delagoensine **34** and delagoenine **35** [20]. The 12-hydroxy crinane analogues **34** and **35** are structurally unique as they have never been identified in any other species of Amaryllidaceae. Furthermore, these findings were significant in that they allowed for the first time a direct comparison of the cytotoxicities of C-6 and C-12 substituted crinanes, by which the former were seen to be preeminent. Finally, around this time *Brunsvigia radulosa* was examined for its phytochemical composition and cytotoxic ability against BL-6 cells [21]. Of the three crinanes assayed, the α-crinanes crinamine **18** ($ED_{50}$ = 1.8 μg/mL) and hamayne **23** ($ED_{50}$ = 9.4 μg/mL) were markedly more active than the β-crinane 1-epideacetylbowdensine **59** ($ED_{50}$ > 100 μg/mL). Furthermore, apohaemanthamine **29**, synthesized during the course of the study from crinamine **18** under strongly acidic conditions, exhibited low inhibitory interaction ($ED_{50}$ > 100 μg/mL) [21], which again could be explained in terms of ring strain generated through C-3 to C-11 etherification in **29**, as also seen for apohaemanthidine methiodide **30** and its low activity against NIH/3T3 cells [10].

A total of nine alkaloids were reported by Abou-Donia et al. (2002) from *Pancratium sickenbergeri* [22]. Of these, only haemanthidine **14** and 11-hydroxyvittatine **16** were assayed against the NCI 60-cell line library and shown to be inactive. The finding for haemanthidine **14** was surprising given the prior observations of Antoun et al. (1993) [16]. This anomaly was further highlighted by the investigation of Hohmann et al. [23] in the same year on alkaloid isolates of *Sprekelia formosissima* and *Hymenocallis* x *festalis*, during which haemanthamine **13** and haemanthidine **14** were noted for their pronounced antiproliferative properties in L5178 mouse lymphoma cells ($IC_{50}$s of 0.27 and 0.41 μg/mL for the two alkaloids, respectively). Equally impressive was the potency of both compounds in the multidrug resistant form of L5178 mouse

**Table 1**: Activities of α-crinane alkaloid representatives of the Amaryllidaceae in miscellaneous cancer cell line screens.

| Compound | No. | Cell line (Cytotoxicity index) [a] | | | | | | | | | | | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L6 | BL6 | KB | A549 | OE21 | Hs683 | U373 | SKMEL | B16F10 | Jurkat | HeLa | Vero | LOVO | 6T-CEM | HL-60 | HT29 | H460 | RXF393 | 5123tc | 3T3 | |
| Haemanthamine | **13** | 7.41 [b] | | 0.7 [b] | 4.5 | 6.8 | 7.0 | 3.5 [b] | 8.5 | 6.8 | 22 [d] | 4 | 32 [c] | | | active | | | | 15 | 0.2 [b] | 3e,f,9b,10,17,26,31, 33,35,36,38 |
| Haemanthidine | **14** | | | 3.6 [b] | 4.0 | 3.7 | 4.3 | 3.8 | 4.2 | 3.1 | | nd | | | | active | | | | | | 16,31,35,36 |
| Vittatine | **15** | | | | | | | | | | 70 [d] | | | | | | 21.91 | 15.88 | 29.57 | | | 27,29 |
| 11-Hydroxyvittatine | **16** | | | | | | | | | | 4 [d] | 72 [c] | 64 [c] | | | | | | | | | 3e,f |
| | **17** | >90 [b] | | >50 [b] | | | | | | | | | | | | | | | | | | 33 |
| Crinamine | **18** | | 1.8 [b] | 1.0 [b] | 15.9 [b] | | | 0.9 [b] | | | | | | 4.30 [b] | 2.82 [b] | 1.70 [b] | | | | 12.5 | 0.2 [b] | 10,15,21,26,28 |
| 11-Acetylcrinamine | **19** | | | | | | | | | | | | | | | | | | | na | | 26 |
| 11-Methylcrinamine | **20** | | | | 9.15 [b] | | | | | | | | | 81.0 [b] | 14.4 [b] | 6.16 [b] | | | | | | 28 |
| | **21** | | | | | | | | | | | | | | | | | | | na | | 26 |
| 6-Hydroxycrinamine | **22** | | active | | | | | | | | | | | | | | | | | | 0.2 [b] | 10,20 |
| Hamayne | **23** | | 9.4 [b] | | | | | | | | na | | | | | | | | | | | 21,29 |
| 3-*O*-Acetylhamayne | **24** | | | | 15.8 [b] | | | | | | | | | 5.49 [b] | 3.45 [b] | 4.27 [b] | | | | | | 28 |
| | **25** | >90 [b] | | >50 [b] | | | | | | | 4 [d] | 82 [c] | 82 [c] | | | | | | | | | 3e,f,33 |
| Dihydrocrinamine | **27** | | | | | | | | | | na | | | | | | | | | | | 29 |
| Dihydrohamayne | **28** | | | | | | | | | | active | | | | | | | | | | | 29 |
| Apohaemanthamine | **29** | | >100 [b] | | | | | | | | | | | | | | | | | | | 21 |
| | **30** | | | | | | | | | | | | | | | | | | | | 100 [b] | 10 |
| | **31** | | | | | | | | | | | | | | | | | | | | 0.05 [b] | 10 |
| | **32** | | | | | | | | | | | | | | | | | | | | 5 [b] | 10 |
| Phaedranamine | **33** | na | | | | | | | | | | | | | | | | | | | | 32 |
| Delagoensine | **34** | | na | | | | | | | | | | | | | | | | | | | 20 |
| Delagoenine | **35** | | na | | | | | | | | | | | | | | | | | | | 20 |
| Bulbispermine | **36** | | | | | | 11 | 38 | | | | 8 | | | | | | | | | | 36 |
| | **37** | | | | | | 63 | nd | | | | 90 | | | | | | | | | | 36 |
| | **38** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | **39** | | | | | | 50 | >100 | | | | 46 | | | | | | | | | | 36 |
| | **40** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | **41** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | **42** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| 3-Oxobulbispermine | **43** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | **44** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | **45** | | | | | | >100 | nd | | | | >100 | | | | | | | | | | 36 |
| | | BCA-1 | HT-1080 | LUC-1 | MEL-2 | COL-1 | KB-V1 | P-388 | A-431 | LNCaP | ZR-75-1 | L5178 | L5178mdr | Molt4 | LMTK | HepG2 | COL-2 | BC1 | HT | Lu1 | HSC-2 | |
| Haemanthamine | **13** | 0.7 [b] | 0.3 [b] | 3.6 [b] | | | 1.3 [b] | 5.0 [b] | 1.3 [b] | 0.6 [b] | 0.5 [b] | 0.27 [b] | 0.30 [b] | 1.2 [b] | 0.5 [b] | >50 [b] | 0.6 [b] | | | | active | 17,18,23,35 |
| Haemanthidine | **14** | | | | 1.9 [b] | | 8.4 [b] | 0.4 [b] | 3.0 [b] | 0.7 [b] | 2.6 [b] | 0.41 [b] | 0.40 [b] | | | | 6.0 [b] | >20 [b] | 1.6 [b] | 2.1 [b] | active | 16,23,35 |
| Crinamine | **18** | 1.4 [b] | 1.3 [b] | 1.4 [b] | 5.0 [b] | 1.0 [b] | 0.6 [b] | 0.7 [b] | 6.9 [b] | 1.5 [b] | 0.8 [b] | | | 0.5 [b] | 0.5 [b] | 10 [b] | | | | | | 15,18 |
| Papyramine | **26** | | | | | | | | | | | | | 15.8 [b] | 1.5 [b] | 17 [b] | | | | | | 18 |
| | | T98G | U87 | CEM | K562 | MCF-7 | G-361 | | | | | | SH-SY5Y | | | | | | | | | |
| Haemanthamine | **13** | 8 | 6 | 2.1 | 3.4 | 8.1 | 3.7 | | | | | | | | | | | | | | | 36,38 |
| Haemanthidine | **14** | 14 | 6 | | | | | | | | | | | | | | | | | | | 36 |
| 6-Hydroxycrinamine | **22** | | | | | | | | | | | | 54.5 | | | | | | | | | 37 |
| Bulbispermine | **36** | 9 | 9 | | | | | | | | | | | | | | | | | | | 36 |
| | **37** | 98 | 74 | | | | | | | | | | | | | | | | | | | 36 |
| | **38** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| | **39** | 91 | 15 | | | | | | | | | | | | | | | | | | | 36 |
| | **40** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| | **41** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| | **42** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| 3-Oxobulbispermine | **43** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| | **44** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |
| | **45** | >100 | >100 | | | | | | | | | | | | | | | | | | | 36 |

[a] Cytoxicity index values expressed as $ED_{50}$, $GI_{50}$, $IC_{50}$, MIC or $MTD_{50}$ in micromolar (μM) units unless otherwise stated. [b] Values are in μg/mL. [c] Indicates % cell viability. [d] Indicates % apoptosis. For compounds indicated as "active", no cytotoxicity indices were presented in the original work (na=not active, nd=not detected).

lymphoma cells, exhibiting $IC_{50}$ values of 0.3 and 0.40 μg/mL, respectively [23] (Table 1). Around this time, findings by Phan et al. (2003) revealed that while lycorine **2** was strongly active on HepG2, RD and FL cells, no such activity was detectable for the β-crinanes crinine **3** and crinamidine **64** isolated from the Vietnamese species *Crinum asiaticum* and *Crinum latifolium* [24]. Crinamidine is the

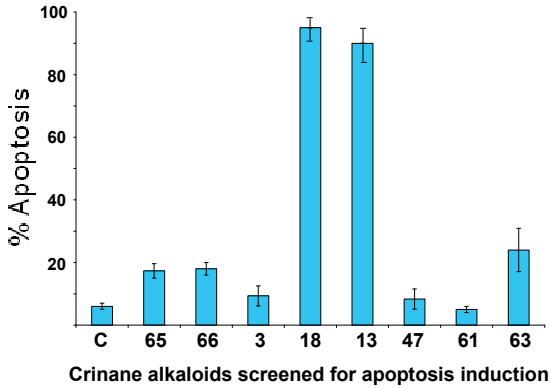



**Figure 1**: Apoptotic indices determined in rat hepatoma (5123tc) cells showing the efficacy of crinamine **18** and haemanthamine **13** at 25 □M after a 48 h treatment (after McNulty et al., 2007).[26]

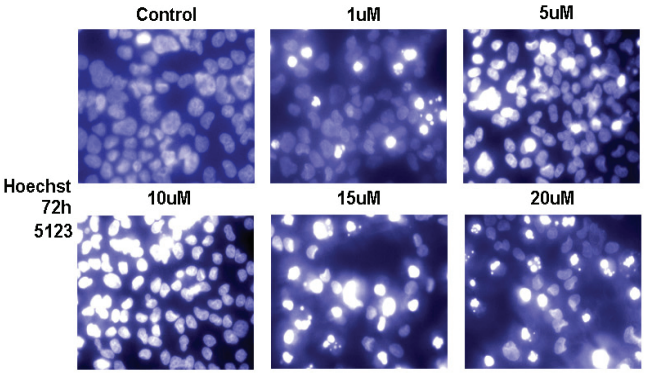



**Figure 2**: Hoechst staining of 5123tc cells 72 h after treatment with various concentrations of crinamine **18**. Apoptotic nuclei appear brighter and more fragmented than healthy nuclei (seen in control cells) (after McNulty et al., 2007).[26]

3-hydroxy analogue corresponding to undulatine **62**, previously shown to be inactive in the P-388 cell line [11]. Apart from this, during the phytochemical investigation of *Nerine filifolia*, a species of the Amaryllidaceae endemic to eastern and southeastern regions of South Africa, we identified several crinanes of the β-series, including 6α-hydroxybuphanidrine, 6α-methoxybuphanidrine, ambelline, 11-*O*-acetylambelline and filifoline [25]. Of these, only filifoline **50** was screened against rat myoblast (L6) cells and shown to be inactive [25].

The year 2007 is notable in the Amaryllidaceae alkaloid cytotoxicity calendar as it saw the first demonstration of apoptotic effects for crinane-based alkaloid structures [26]. In this regard, we examined a mini-library comprising natural and semi-synthetic derivatives of both series of crinane compounds for apoptosis-inducing effects in rat hepatoma (5123tc) cells [26]. As shown in Figure 1, the α-crinanes haemanthamine **13** and crinamine **18** were prominent amongst the other test substrates, with apoptotic indices of 90% and 95%, respectively, after a 48 h treatment at 25 μM [26]. Apoptosis induction was indicated by Hoechst staining as shown in Figure 2, by which apoptotic nuclei appeared brighter and more fragmented than healthy nuclei, in this case upon treatment with crinamine **18**. Furthermore, respective $ED_{50}$ values were established at 15 μM and 12.5 μM for haemanthamine and crinamine (Figure 3) [26]. Of the other compounds screened, only the activity of 6α-hydroxyundulatine **63** was noteworthy (25% apoptotic at the same concentration and treatment period) [26]. These results were even more impressive considering the selectivities of haemanthamine **13** and crinamine **18**, both of which were seen to exhibit negligible apoptotic effects in normal human embryonic kidney (293t) cells (Figure 4).

Apart from uncovering these novel effects in hepatoma cells, this work was also significant in shedding light on the underlying structural elements of the pharmacophore resident within these crinanes [26]. These were seen to include: 1) α-orientation for the 5,10b-ethano bridge, which renders the B,C-ring junction conformationally similar to the phenanthridone drug target pancratistatin **9**. As such, the α-crinanes crinamine and haemanthamine were clearly far superior to the other β-crinanes screened; 2) the stereochemistry at C-3 may not be significant given the relative configurations of the methoxyl group in crinamine and haemanthamine; and 3) requirement of a free hydroxyl at the C-11 position, the function of which may involve hydrogen bond donor-acceptor interactions, as acyl-derivatives (acetate and benzoate) were notably inactive [26].

However, as will be discussed later, the apoptosis-inducing pharmacophore is shown to incorporate other essential structural ingredients. The only work in 2008 on cytotoxicity studies of crinanes was that of Silva et al. [27] in which vittatine **15** from *Hippeastrum vittatum* was shown to be active against HT29, H460 and RXF393 cells with $IC_{50}$ values of 21.91, 15.88 and 29.57 μg/mL in the three cells, respectively. Interestingly, the dichloromethane and butanol extracts of the plant were markedly more active than neat vittatine **15** in all three cell lines. For example, the best $IC_{50}$ value for the butanol extract was ascertained in RXF393 cells (2.93 μg/mL), while that for the dichloromethane extract was 0.62 μg/mL in H460 cells.

The year 2009 saw a flurry of activity in the cytotoxicity field pertaining to crinane alkaloids. The first two related to major works involving a diverse Amaryllidaceae alkaloid library screen against HeLa, Vero and Jurkat cells [3e,f]. Of the crinane alkaloids evaluated (Tables 1 and 2), including 11-hydroxyvittatine **16**, 8-*O*-demethylmaritidine **25**, ambelline **47**, buphanamine **67** and buphanisine **53**, only haemanthamine **13** stood out for significantly reducing the viability of HeLa and Vero cells to 21% and 32%, respectively, over a 24 h period at a treatment dose of 25 μM [3e,f]. Furthermore, amongst these crinanes, only haemanthamine **13** was capable of apoptosis induction in Jurkat cells (22% over the same period and dosage) [3e,f]. Another important aspect of these investigations was the discovery that buphanamine **67** was the most potent of the twenty-nine compound library in the collagen type 1 invasion assay [3e,f]. At 25 μM it produced only ~2% invasion of collagen type 1 by HeLa cells [3e,f], and given its low cytotoxicity, even at relatively high dosages, it may play a significant future chemoprotective role in combination with the phenanthridone anticancer drug targets narciclasine **8** and pancratistatin **9**.

The work of Sun et al. (2009) on the chemical constituents of *Crinum asiaticum* from Yunnan province of China revealed *inter alia* the presence of the α-crinanes crinamine **18**, 11-*O*-methylcrinamine **20** and 3-*O*-acetylhamayne **24** [28]. Subsequent cytotoxicity studies in four cell lines (A549, LOVO, 6T-CEM and HL-60) showed, as expected, crinamine to be superior to the other functionalized analogues [28]. For comparison purposes, in the HL-60 cell line $IC_{50}$ values determined were 1.70, 6.16 and 4.27 μg/mL for the three compounds, respectively [28]. As mentioned above, and again seen here for this crinamine subset, the direct influence of substituents on the cytotoxic ability of crinane alkaloids is quite pronounced, and should thus guide SAR studies aimed at identifying a possible clinical candidate. Finally, the year closed out with further apoptotic studies by our research group on selected

**Table 2**: Activities of β-crinane alkaloid representatives of the Amaryllidaceae in miscellaneous cancer cell line screens.

| Compound | No. | Cell line (Cytotoxicity index) [a] | | | | | | | | | | | | | | | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L6 | BL6 | SKW-3 | HL-60 | HL-60/Dox | MDA-MB-231 | A549 | OE21 | Hs683 | U373 | SKMEL | B16F10 | Jurkat | HeLa | Vero | 5123tc | Molt4 | LMTK | HepG2 | FI | RD | |
| Crinine | **3** | | >100 [b] | 16.95 | 20.86 | 14.04 | 68.11 | | | | | | | | na | | 9 [d] | | | na | na | na | 9b,19,24,26,34 |
| Buphanidrine | **46** | | | | | | | | | | | | | | na | | | >50 [b] | >50 [b] | >50 [b] | | | 9b,18,38 |
| Ambelline | **47** | | >100 [b] | >100 | >100 | >100 | >100 | >10 | 86 | >10 | >10 | >10 | >10 | 4 [d] | 79 [c] | 89 [c] | 8 [d] | >50 [b] | >50 [b] | >50 [b] | | | 3e,f,18,19,26,31,34 |
| 11-Acetylambelline | **48** | | >100 [b] | | | | | | | | | | | | | | | | | | | | 19 |
| 3-*O*-Acetylcrinine | **49** | | >100 [b] | | | | | | | | | | | | | | | | | | | | 19 |
| Filifoline | **50** | na | | | | | | | | | | | | | | | | | | | | | 25 |
| Elwesine | **51** | | | >100 | >100 | >100 | >100 | | | | | | | | | | | | | | | | 34 |
| Powelline | **52** | | | | | | | | | | | | | | | | | na | | | | | 14 |
| Buphanisine | **53** | | | | | | | >10 | 97 | >10 | >10 | >10 | >10 | 5 [d] | 99 [c] | 79 [c] | | | | | | | 3e,f,15,31,36 |
| 6α-Hydroxycrinine | **54** | | | | | | | | | | | | | | | | | na | | | | | 14 |
| | **55** | | | | | | | | | | | | | | | | | active | | | | | 14 |
| | **56** | | | 45.03 | 95.95 | 76.69 | >100 | | | | | | | | | | | | | | | | 34 |
| | **57** | | | 59.43 | >100 | 95.22 | >100 | | | | | | | | | | | | | | | | 34 |
| | **59** | | >100 [b] | | | | | | | | | | | | | | | | | | | | 21 |
| Augustine | **60** | | | | | | | | | | 0.6 [b] | | | | | | | | | | | | 15 |
| 1,2-Epoxyambelline | **61** | | | | | | | | | | | | | | | | 5 [d] | | | | | | 26 |
| Hydroxyundulatine | **63** | | | | | | | | | | | | | | | | 25 [d] | | | | | | 26 |
| Crinamidine | **64** | | | | | | | | | | | | | | | | | | | na | na | na | 24 |
| Amabiline | **65** | | | | | | | | | | >20 [b] | | | | | | 17 [d] | | | | | | 15,26 |
| Josephinine | **66** | | | | | | | | | | | | | | | | 18 [d] | | | | | | 26 |
| Buphanamine | **67** | | | | | | | >10 | >10 | >10 | >10 | >10 | >10 | 3 [d] | 96 [c] | 90 [c] | | | | | | | 3e,f,31,36 |
| Distichamine | **68** | | | | | | | | | | | | | | 2.2 | | | | | | | | 38 |
| | | BCA-1 | HT-1080 | LUC-1 | MEL-2 | COL-1 | KB-V1 | KB | P-388 | A-431 | LNCaP | ZR-75-1 | MSL | CEM | K562 | MCF-7 | G-361 | 3T3 | S-180 | T98G | U87 | | |
| Buphanidrine | **46** | | | | | | | | | | | | | >50 | >50 | >50 | >50 | | | | | | 38 |
| Ambelline | **47** | | | | | | | | 1.6 | | | | active | | | | | | | >100 | >100 | | 11,12,36 |
| Buphanisine | **53** | >20 [b] | >20 [b] | >20 [b] | >20 [b] | >20 [b] | 19.8 [b] | >20 [b] | >5 [b] | >20 [b] | >20 [b] | >20 [b] | | | | | | | | >100 | >100 | | 15,36 |
| | **58** | | | | | | | | | | | | | | | | | 100 | | | | | 10 |
| Augustine | **60** | 2.8 [b] | 1.2 [b] | 3.7 [b] | 3.2 [b] | 2.4 [b] | >20 [b] | 0.6 [b] | 0.6 [b] | 4.9 [b] | 1.7 [b] | 1.8 [b] | | | | | | | | | | | 15 |
| 1,2-Epoxyambelline | **61** | | | | | | | | | | | | active | | | | | | | | | | 12 |
| Undulatine | **62** | | | | | | | | na | | | | | | | | | | | | | | 11 |
| Amabiline | **65** | >20 [b] | >20 [b] | >20 [b] | >20 [b] | >20 [b] | >20 [b] | >20 [b] | >5 [b] | >20 [b] | >20 [b] | >20 [b] | | | | | | | | | | | 15 |
| Buphanamine | **67** | | | | | | | | | | | | | | | | | | | >100 | >100 | | 36 |
| Distichamine | **68** | | | | | | | | | | | | | 4.5 | 4.1 | 2.3 | 14.7 | | | | | | 38 |
| Crinafoline | **69** | | | | | | | | | | | | | | | | | | 68.12 [c] | | | | 13 |
| | **70** | | | | | | | | | | | | | | | | | | 32.31 [c] | | | | 13 |
| Crinafolidine | **71** | | | | | | | | | | | | | | | | | | 43.5 [c] | | | | 13 |

[a] Cytoxicity index values expressed as $ED_{50}$, $GI_{50}$, $IC_{50}$, MIC or $MTD_{50}$ in micromolar (μM) units unless otherwise stated. [b] Values are in μg/mL. [c] Indicates % cell viability. [d] Indicates % apoptosis. For compounds indicated as "active", no cytotoxicity indices were presented in the original work (na=not active, nd=not detected). Cell line abbreviations are as follows: A-431 (human epidermoid carcinoma), A549 (human lung carcinoma), BC1 (human breast cancer), BCA-1 (human breast cancer), B16F10 (mouse melanoma), BL6 (mouse melanoma), CEM (human lymphoblastic leukemia), COL-1 (human colon cancer), COL-2 (human colon cancer), FI (human cervical adenocarcinoma), G-361 (human melanoma), H460 (human lung carcinoma), HeLa (human cervical adenocarcinoma), HepG2 (human hepatoma), HL-60 (human promyelocytic leukemia), HL-60/Dox (human leukemia/doxorubicin resistant), Hs683 (human neuronal glioma), HSC-2 (human squamous carcinoma), HT (human sarcoma), HT29 (human colon adenocarcinoma), HT-1080 (human fibrosarcoma), Jurkat (human lymphoblast), K562 (human myelogenous leukemia), KB (human oral epidermoid carcinoma), KB-V1 (vinblastine-resistant KB), L6 (rat myoblast), L5178 (mouse lymphoma), L5178mdr (multidrug resistant L5178), LMTK (murine fibroblast), LNCaP (hormone-dependent human prostatic cancer), LOVO (human colon adenocarcinoma), Lu1 (human lung cancer), LUC-1 (human lung cancer), MCF-7 (human breast cancer), MDA-MB-231 (human adenocarcinoma), MEL-2 (human melanoma), Molt4 (human T lymphoma), MSL (mouse spleen lymphocyte), OE21 (oesophageal squamous carcinoma), P-388 (murine lymphoid neoplasm), RD (human rhabdomyosarcoma), RXF393 (human renal carcinoma), S-180 (mouse ascites tumor), SH-SY5Y (human neurobalstoma), SKMEL (human skin melanoma), SKW-3 (human T-cell leukemia), 3T3 (mouse embryonic fibroblast), 5123tc (rat hepatoma), 6T-CEM (human T-lymphoblastic leukemia), T98G (human glioblastoma), U87 (human glioblastoma), U373 (human glioblastoma astrocytoma), Vero (monkey kidney epithelial), ZR-75-1 (hormone-dependent breast cancer).

crinane alkaloids [29], based on the initial discovery for α-crinanes of the series [26]. This work is notable for demonstrating the apoptosis-inducing ability of the α-crinane vittatine **15** in Jurkat cells and for highlighting further structural features attending the crinane alkaloid pharmacophore. In this case, vittatine induced apoptosis in Jurkat cells in a dose dependent manner, notably with ~70% of cells exhibiting characteristic apoptotic morphology, as indicated by Hoechst staining, Annexin-V binding and caspase-3 activation, after 24 h with a single 20 μM dose [29]. The other pharmacophoric elements uncovered in the process include: 1) the modulatory effect of the C-1/C-2 double bond as shown by the markedly lower activities for dihydro-analogues of crinamine **18** and hamayne **23**; and 2) the presence of a small substituent at C-11 (H or OH), the function of which does not appear to involve hydrogen bonding but is subject to steric constraints alone [29].

About 90% of cancer patients die from tumor metastases [30]. Metastatic cancer cells are intrinsically resistant to apoptosis and therefore unresponsive to a large majority of anticancer drugs, most of which function through apoptosis induction [30]. Recent findings by Van Goietsenoven et al. (2010) are thus significant in

focusing on the cytotoxic abilities of diverse Amaryllidaceae alkaloids in apoptosis-resistant cancer cells [31]. Of the five crinane alkaloids evaluated, ambelline **47**, buphanamine **67** and buphanisine **53** exhibited low activities against both apoptosis-resistant (A549, OE21, U373 and SKMEL) and apoptosis-sensitive (Hs683 and B16F10) cells [31]. On the other hand, haemanthamine **13** and haemanthidine **14** were markedly active in both cell-forms ($IC_{50}$s 3.1- 8.5 μM) with the best activity seen for the latter in B16F10 cells ($IC_{50}$ = 3.1 μM) [31]. Also in 2010, phaedranamine **33**, the enantiomer of buphanamine, was isolated for the first time from the South American Amaryllidaceae species *Phaedranassa dubia* and shown to be inactive towards L6 cells ($IC_{50}$ > 300 μM) [32].

In 2011, two further Amaryllidaceous species (*Galanthus trojanus* and *Crinum zeylanicum*) were examined by our group for cytotoxic effects in various cell lines [33,34]. Firstly, 11-hydroxyvittatine-*N*-oxide **17** and 8-*O*-demethylmaritidine **25** from *Galanthus trojanus* were both weakly active in L6 cells ($IC_{50}$s > 90 μg/mL) and KB cells ($IC_{50}$s > 50 μg/mL) in contrast to the co-constituent haemanthamine which exhibited $IC_{50}$s of 7.41 and 0.97 μg/mL in the two cell lines, respectively [33]. The result for 8-*O*-demethylmaritidine is in accordance with previous findings by Lin et al. (1995), who showed that the compound was unresponsive in a multi-cell line screen [17], and thus emphasizes the significance of the A-ring attached 1,3-dioxolane moiety to the crinane anticancer pharmacophore.

Secondly, bulbs of *Crinum zeylanicum* were shown to contain *inter alia* the crinane alkaloids crinine **3**, ambelline **47**, elwesine **51**, 6-hydroxybuphanidrine **56** and 6-ethoxybuphanidrine **57**, all of the β-subgroup [34] (Table 2). In the four cell lines screened, only crinine was notably active with $IC_{50}$s of 16.95, 20.86, 14.01 and 68.11 μM in SKW-3, HL-60, HI-60/Dox and MDA-MB-231 cells, respectively [34]. The role of the double bond to the integrity of the crinane pharmacophore could be gauged directly by comparison of these responses to those of elwesine **51**, the dihydro-analogue of crinine, which exhibited $IC_{50}$s > 100 μM across the four cells [34]. Further mechanistic tests on the active compound crinine via oligonucleosomal DNA fragmentation revealed the apoptotic cell death mode as responsible for its cytotoxicity [34]. Apart from this, Jitsuno et al. (2011) showed that haemanthamine **13** and haemanthidine **14** from *Lycoris albiflora* were active against HL-60 and HSC-2 cells (Table 1) [35].

The year 2012 has already seen three detailed studies of the cytotoxic properties of crinane alkaloids [36-38]. Firstly, Luchetti et al. (2012) uncovered the cytotoxic ability of bulbispermine **36**, the 11-*endo* analogue corresponding to hamayne **23**, in various cells including apopotosis-resistant and apoptosis-sensitive forms [36]. For example, in the apoptosis-resistant line T98G the compound had a $GI_{50}$ of 9 μM, matched closely by its activity in apoptosis-sensitive HeLa cells ($GI_{50}$ of 8 μM) [36]. Further results showed as expected, the prominence of haemanthamine **13** and haemanthidine **14**, as well as the low activity of buphanamine **67**, buphanisine **53** and ambelline **47** in these cells [36]. SAR studies revealed that the semi-synthetic transformation products (**37**-**45**) of bulbispermine, with the exception of **39** ($GI_{50}$ 15 μM in U87 cells), were weakly active ($GI_{50}$s > 100 μM) in most test screens. Secondly, work by Adewusi et al. (2012) on the South African amaryllid *Boophone disticha* uncovered 6-hydroxycrinamine **22** as the cytotoxic agent in neuroblastoma (SH-SY5Y) cells with $IC_{50}$s of 54.5 and 61.7 μM, as determined by the MTT and neutral red assays, respectively [37]. Finally, we

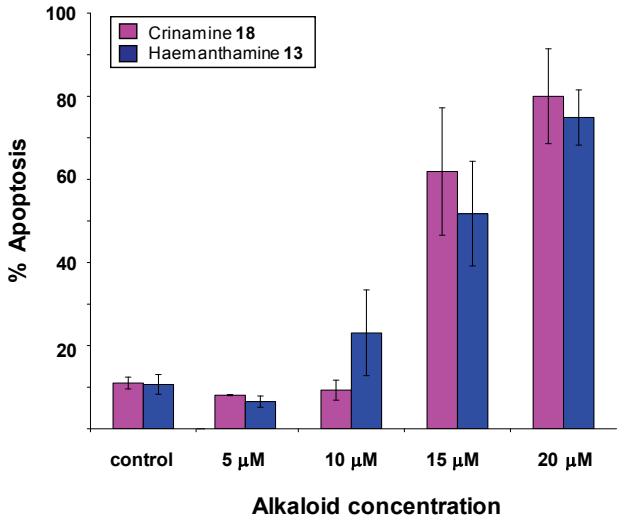



**Figure 3**: Apoptosis responses in 5123tc cells treated over 72 h with various concentrations of crinamine and haemanthamine. Effective dose ($ED_{50}$) was calculated as the concentration inducing apoptosis in 50% of cells (after McNulty et al., 2007).[26]

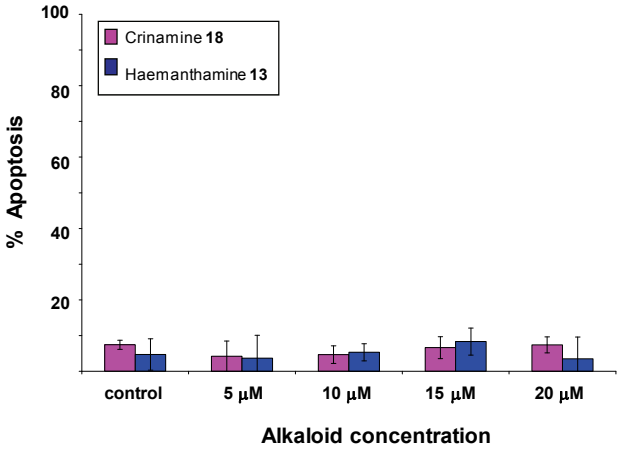



**Figure 4**: Normal human embryonic kidney (293t) cells are unaffected by treatment with various doses of crinamine and haemanthamine after 72 h (after McNulty et al., 2007).[26]

very recently revealed the antiproliferative properties of the rare Amaryllidaceae crinane constituent distichamine **68** in a mini-panel of cancer cells comprising acute lymphoblastic leukemia (CEM), chronic myelogenous leukemia (K562), breast adenocarcinoma (MCF7), malignant melanoma (G-361) and cervical adenocarcinoma (HeLa), in addition to the normal human fibroblast (BJ) cell line [38]. By means of the Calcein AM assay, distichamine **68** was seen to be active against all cancer cell lines ($IC_{50}$s 2.2-14.7 μM), with the adherent cell line HeLa shown to be the most sensitive ($IC_{50}$ 2.2 μM) to this alkaloid [38].

Mechanistic studies then invoked flow cytometry to quantify the distribution of CEM cells across the different phases of the cell cycle, and to determine the sub-$G_1$ fraction as a measure of the proportion of apoptotic cells [38]. The analysis showed that treatment with distichamine **68** increased the proportion of $G_2$/M phase cells in a dose-dependent manner, with concomitant reductions in the proportion of $G_0/G_1$ and S cells (Figure 5) [38]. In addition, the proportion of cells with sub-$G_1$ amounts of DNA (apoptotic cells) increased (up to 23.7 %) following a 24 h

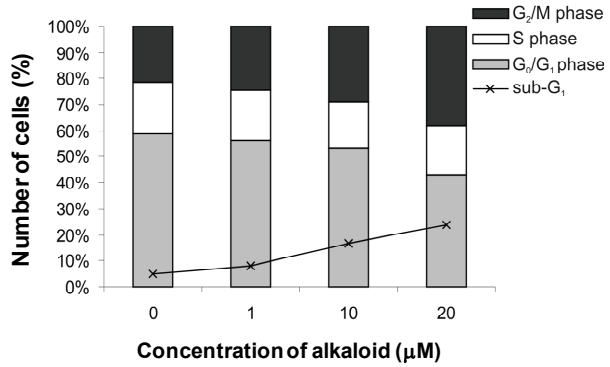



**Figure 5**: Histograms obtained by flow cytometric analysis showing the distributions of CEM cells across the $G_0/G_1$, S and $G_2/M$ phases of the cell cycle after 24 h treatment with distichamine **68**, and the sub-$G_1$ fraction of cells (relative to untreated controls). The bars show the percentage of cells in the corresponding phase (after Nair et al., 2012).[38]

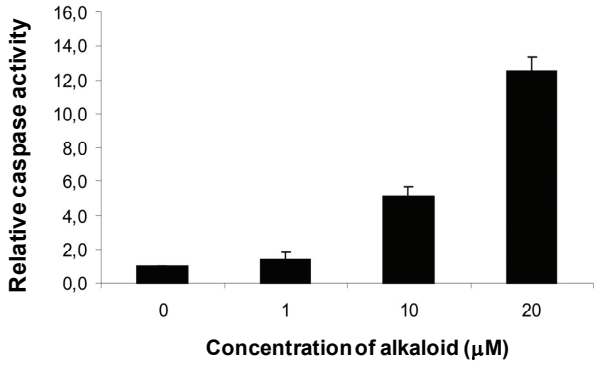



**Figure 6**: Increases in the activities of caspase-3 and 7 in CEM cells treated with distichamine **68** for 24 h, relative to untreated controls. The data shown represent averages from at least three independent experiments performed in triplicate (after Nair et al., 2012).[38]

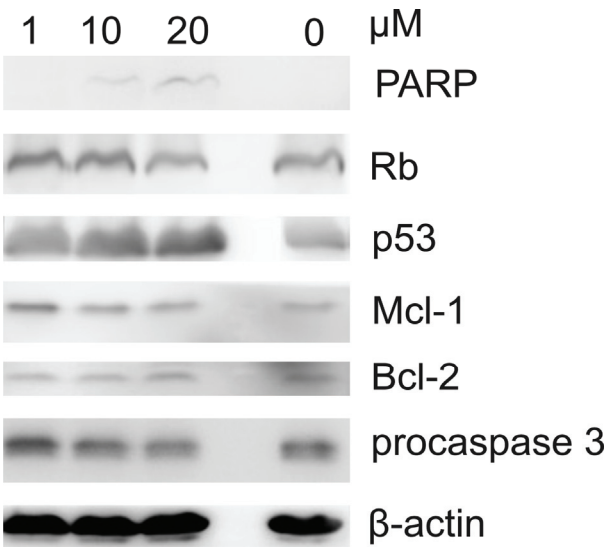



**Figure 7**: Western blot analysis of apoptosis-related proteins (PARP fragment 89 kDa, Rb, p53, Mcl-1, Bcl-2 and procaspase 3) in leukemia (CEM) cells treated with distichamine **68** compared to their expression in untreated control cells. The expression of β-actin was monitored as a protein loading control (after Nair et al., 2012).[38]

treatment with **68**, relative to that observed in untreated cells, indicating that the compound was clearly capable of cell cycle disturbance and apoptosis induction in CEM cells [38].

Furthermore, distichamine induced a 12.5-fold increase in the activity of caspase-3/7 after 24 h at the highest concentration (20 μM) compared to untreated controls (Figure 6). In addition, Western blot analysis was used to detect changes in expression of apoptosis-related proteins in CEM leukemia cells after treatment with distichamine. As shown in Figure 7, treatment with **68** (at 10 and 20 μM) induced cleavage of PARP (89 kDa fragment) after 24 h which also corresponded with decreased levels of procaspase-3. As described above, increased caspase-3 activity was observed over the same treatment period and dosage (Figure 6). Expression of the tumor suppressor protein p53 was discernible in the CEM cell line control, and **68** caused its enhanced expression after 24 h, notably at 10 and 20 μM (Figure 7). No change in the expression of the antiapoptotic protein Bcl-2 was observed, but at 20 μM, a decreased level of the antiapoptotic protein Mcl-1 was detected, indicating the onset of apoptosis. Mcl-1 is necessary for cell viability, and its decreased expression has been suggested as the cause of cell death in CEM cells [39a]. It also plays a crucial role in regulating the apoptosis of T-cells [39b].

Western blot analysis here demonstrated the dose-dependent decrease of procaspase-3 and cleavage of PARP after 24 h treatment with distichamine **68** in CEM leukemia cells (Figure 7), indicating that the compound induces caspase-3 activated apoptosis [38]. Given the unique C-ring structural formulation for distichamine, involving shift of the double bond to C-2/C-3 with concomitant vinylization of the C-3 methoxy group and oxidation at C-1, these findings point at further attendant structural features of the crinane apoptosis-inducing pharmacophore. It now appears that a $sp^2$–hybridised C-3 centre is tolerated at the active site, since most other cytotoxic crinane alkaloids possess a C-1/C-2 double bond.

Furthermore, oxygenation at C-1, as is characteristic of the anticancer phenanthridone pancratistatin **9**, was here shown to be amenable towards activity. Interestingly, in the case of distichamine, a keto-group is positioned at C-1, as a consequence of which three contiguous $sp^2$–centres are formed in ring-C, similar to that seen for the active phenanthridone narciprimine **10** [38].

In summary, a more comprehensive view has emerged of crinane alkaloids of the Amaryllidaceae as potent and selective cytotoxic agents with significant potential for preclinical development. This promising status was drawn out of multi-cell line approaches invoking targets of synthetic as well as natural origin. As such, both *in vitro* and *in vivo* models of study support the ameliorative effects of crinane compounds as anticancer agents. This survey has accounted for 54 cancer cell lines (Tables 1 and 2), including human and animal forms, against which the cytotoxic abilities of a total of 71 crinane alkaloids have been measured. As also shown in Tables 1 and 2, of the α-series, not surprisingly, haemanthamine **13**, haemanthidine **14** and crinamine **18** are the most commonly targeted agents in such studies. Within the β-series, ambelline **47** and buphanisine **53** appear routinely across most of the cell lines screened. In terms of potency, synthetic 6-hydroxycrinamine methiodide **31** is the most active crinane identified to date with a MTD of 0.05 μg/mL in NIH/3T3 cells. In animal models of study, the natural α-crinanes, haemanthamine **13**, crinamine **18** and 6-hydroxycrinamine **22** were the most active each with MTD of 0.2 μg/mL in NIH/3T3 cells. In human cancers, haemanthamine was again the most

potent compound with an $ED_{50}$ of 0.3 μg/mL in HT-1080 fibrosarcoma cells. Furthermore, selective cytotoxic abilities have been demonstrated for crinamine and haemanthamine, both of which, for example, targeted rat hepatoma (5123tc) cells as opposed to normal human embryonic kidney (293t) cells. Of the β-crinanes evaluated, augustine **60** was the most active with an $ED_{50}$ of 0.6 μg/mL in both KB (human oral epidermoid carcinoma) and U373 (human glioblastoma astrocytoma) cells. Given the close structural proximity of these alkaloids to the potent phenanthridone anticancer agents of the family, such as narciclasine and pancratistatin, as well as their common biogenetic origin, it is possible that a common, though still elusive, biological target may be operable for these agents.

Stucture-activity relationship studies, again involving natural as well as synthetically-derived targets, have provided useful insights to the structural details of the anticancer pharmacophore attending these molecules. Features that have come to light from such studies include: 1) an intact phenanthridine nucleus is essential since truncated analogues were markedly less active, as seen for crinafoline **69** against crinafolidine **71**; 2) presence of the A-ring methylenedioxyphenyl moiety since structures incorporating other substituents (as in 8-*O*-demethylmaritidine **25**) were seen to be less active; 3) B-ring modification effects such as quaternization of the nitrogen atom were shown to be deleterious; 4) small substituents with hydrogen bond donor/acceptor capability at C-11 (β to the nitrogen atom) with either *exo*- or *endo*-disposition were ameliorative (as in crinamine **18** and bulbispermine **36**, respectively), but less so for substitutions α to the nitrogen atom at C-6 (6-hydroxycrinamine **22**) or C-12 (delagoensine **34**); 5) stereochemistry at C-3 may not be significant given the relative configurations of the methoxyl group in crinamine **18** and haemanthamine **13**, as well as its planar geometry in distichamine **68**; 6) additional oxygenation of ring-A, reminiscent of the potent anticancer phenanthridones narcicalsine **8** and pancratistatin **9**, is viewed as beneficial to the pharmacophore, as shown for distichamine **68**; and 7) although active compounds were found across both α- and β-members of the series, the geometry of the 5,10b-ethano bridge appears to play a significant role given the consistent potency of the former series across most of the cell lines screened. In addition, mechanistic studies have recently uncovered the apoptotic mode of death as responsible for the cytotoxicity of several crinane compounds. These distinctive features should prove useful in guiding the development of a clinical drug target. As such, efforts to further refine the active pharmacophore associated with these molecules are an ongoing concern in our laboratories.

**Acknowledgment** - Financial support of this work by the University of KwaZulu-Natal is gratefully acknowledged.

## References

[1] (a) Bastida J, Lavilla R, Viladomat F. (**2006**) Chemical and biological aspects of *Narcissus* alkaloids. In *The Alkaloids.* Cordell GA (Ed). Elsevier, Amsterdam, Netherlands, 87-179; (b) Bastida J, Berkov S, Torras L, Pigni NB, De Andrade JP, Martinez V, Codina C, Viladomat F. (**2011**) Chemical and biological aspects of Amaryllidaceae alkaloids. In *Recent Advances in Pharmaceutical Sciences.* Muñoz-Torrero D (Ed). Transworld Research Network, Trivandrum, India, 65-100; (c) Jin Z. (**2011**) Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports*, ***28***, 1126-1142; (d) Viladomat F, Bastida J, Codina C, Nair JJ, Campbell WE. (**1997**) Alkaloids of the South African Amaryllidaceae. In *Recent Research Developments in Phytochemistry*. Vol. ***1***, Pandali SG (Ed). Research Signpost Publishers, Trivandrum, India. 131- 171.

[2] (a) Harvey AL. (**1995**) The pharmacology of galanthamine and its analogues. *Pharmacology & Therapeutics*, ***68***, 113-128; (b) Houghton PJ, Ren Y, Howes M-J. (**2006**) Acetylcholinesterase inhibitors from plants and fungi. *Natural Product Reports*, ***23***, 181-199; (c) Williams P, Sorribas A, Howes M-J. (**2011**) Natural drugs as a source of Alzheimer's drug leads. *Natural Product Reports*, ***28***, 48-77; (d) Lopez S, Bastida J, Viladomat F, Codina C. (**2002**) Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. *Life Sciences*, ***71***, 2521-2529; (e) Elgorashi EE, Stafford GI, Van Staden J. (**2004**) Acetylcholinesterase enzyme inhibitory effects of Amaryllidaceae alkaloids. *Planta Medica*, ***70***, 260-262; (f) McNulty J, Nair JJ, Little JRL, Brennan JD, Bastida J. (**2010**) Structure-activity relationship studies on acetylcholinesterase inhibition in the lycorine series of Amaryllidaceae alkaloids. *Bioorganic and Medicinal Chemistry Letters*, ***20***, 5290-5294; (g) Nair JJ, Van Staden J. (**2012**) Acetylcholinesterase inhibition within the lycorine series of Amaryllidaceae alkaloids. *Natural Product Communications*, *7*, 959-962.

[3] (a) De Leo P, Dalessandro G, De Santis A, Arrigoni O. (**1973**) Inhibitory effect of lycorine on cell division and cell elongation. *Plant and Cell Physiology*, ***14***, 481-486; (b) Liu J, Hu W, He L, Ye M, Li Y. (**2004**) Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. *FEBS letters*, ***578***, 245-250; (c) Liu J, Li Y, Tang LJ, Zhang GP, Hu WX. (**2007**) Treatment of lycorine on SCID mice model with human APL cells. *Biomedicine & Pharmacotherapy*, ***61***, 229-234; (d) Li S, Chen C, Zhang H, Guo H, Wang H, Wang L, Zhang X, Hua S, Yu J, Xiao P, Li Y, Liu J, Tang L, Shi Y, Ren W, Hu W. (**2007**) Apoptosis induced by lycorine in KM3 cells is associated with the G0/G1 cell cycle arrest. *Oncology Reports*, ***17***, 377-384; (e) Evidente A, Kornienko A. (**2009**) Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochemistry Reviews*, ***8***, 449-459; (f) Evidente A, Kireev AS, Jenkins AR, Romero AE, Steelant WFA, Van Slambrouck S, Kornienko A. (**2009**) Biological evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives: discovery of novel leads for anticancer drug design. *Planta Medica*, ***75***, 501-507; (g) McNulty J, Nair JJ, Bastida J, Pandey S, Griffin C. (**2009**) Structure-activity studies on the lycorine pharmacophore: a potent inducer of apoptosis in human leukemia cells. *Phytochemistry*, ***70***, 913-919; (h) Lamoral-Theys D, Decaestecker C, Mathieu V, Dubois J, Kornienko A, Kiss R, Evidente A, Pottier L. (**2010**) Lycorine and its derivatives for anticancer drug design. *Mini-Reviews in Medicinal Chemistry*, ***10***, 41-50; (i) Evdokimov NM, Lamoral-Theys D, Mathieu V, Andolfi A, Frolova LV, Pelly SC, Van Otterlo WAL, Magedov IV, Kiss R, Evidente A, Kornienko A. (**2011**) In search of a cytostatic agent derived from the alkaloid lycorine: synthesis and growth inhibitory properties of lycorine derivatives. *Bioorganic & Medicinal Chemistry*, ***19***, 7252-7261.

[4] (a) Pettit GR, Gaddamidi V, Cragg GM, Herald DL, Sagawa Y. (**1984**) Isolation and structure of pancratistatin. *Journal of the Chemical Society Chemical Communications*, ***24***, 1693-1694; (b) Pettit GR, Gaddamidi V, Herald DL, Singh SB, Cragg GM, Schmidt JM, Boettner FE, Williams M, Sagawa Y. (**1986**) Antineoplastic agents. 120. *Pancratium littorale*. *Journal of Natural Products*, ***49***, 995-1002; (c) Pettit GR, Pettit GR III, Backhaus RA, Boyd MR, Meerow AW. (**1993**) Cell growth inhibitory isocarbostyrils from *Hymenocallis*. *Journal of Natural Products*, ***56***, 1682-1687; (d) Kornienko A, Evidente A. (**2008**) Chemistry, biology and medicinal potential of narciclasine and its congeners. *Chemical Reviews*, ***108***, 1982-2014; (e) Ingrassia L, Lefranc F, Mathieu V, Darro F, Kiss R. (**2008**) Amaryllidaceae isocarbostyril alkaloids and their derivatives as promising antitumor agents. *Translational Oncology*, ***1***, 1-13; (f) Ingrassia L, Lefranc F, Dewelle J, Pottier L, Mathieu V, Spiegl-Kreinecker S, Sauvage S, El Yazidi M, Dehoux M, Berger W, Van Quaquebeke E, Kiss R. (**2009**) Structure-activity relationship analysis of novel derivatives of narciclasine (an Amaryllidaceae isocarbostyril derivative) as potential anticancer agents. *Journal of Medicinal Chemistry*, ***52***, 1100-1114; (g) Van Goietsenoven G, Hutton J, Becker J-P, Lallemand B, Robert F, Lefranc F, Pirker C, Vandenbussche G, Van Antwerpen P, Evidente A, Berger W, Prévost M, Pelletier J, Kiss R, Kinzy TG, Kornienko A, Mathieu V. (**2010**) Targeting of eEF1A with Amaryllidaceae isocarbostyrils as a strategy to combat melanomas. *The FASEB Journal*, ***24***, 4575-4584.

[5] (a) Kekre N, Griffin C, McNulty J, Pandey S. (**2005**) Pancratistatin causes early activation of caspase-3 and the flipping of phosphatidyl serine followed by rapid apoptosis specifically in human lymphoma cells. *Cancer Chemotherapy & Pharmacology*, ***56***, 29-38; (b) McLachlan A, Kekre N,

McNulty J, Pandey S. (**2005**) Pancratistatin: A natural anticancer compound that targets mitochondria specifically in cancer cells to induce apoptosis. *Apoptosis*, ***10***, 619-630.

[6] (a) Hudlicky T. (**1996**) Design constraints in practical syntheses of complex molecules: current status, case studies with carbohydrates and alkaloids, and future perspectives. *Chemical Reviews*, ***96***, 3-30; (b) Rinner U, Hudlicky T. (**2005**) Synthesis of Amaryllidaceae constituents-an update. *Synlett*, ***3***, 365-387; (c) Manpadi M, Kornienko A. (**2008**) Total synthesis of pancratistatin. A review. *Organic Preparations and Procedures International*, ***40***, 107-161; (d) McNulty J, Nair JJ, Singh M, Crankshaw DJ, Holloway AC, Bastida J. (**2009**) Selective cytochrome P450 3A4 inhibitory activity of Amaryllidaceae alkaloids. *Bioorganic and Medicinal Chemistry Letters*, ***19***, 3233-3237; (e) McNulty J, Nair JJ, Singh M, Crankshaw DJ, Holloway AC, Bastida J. (**2010**) Cytochrome P450 3A4 inhibitory activity studies within the lycorine series of alkaloids. *Natural Product Communications*, ***5***, 1195-1200; (f) McNulty J, Thorat A, Vurgun N, Nair JJ, Makaji E, Crankshaw DJ, Holloway AC, Pandey S. (**2011**) Human cytochrome P450 liability studies of *trans*-dihydronarciclasine: a readily available, potent and selective cancer cell growth inhibitor. *Journal of Natural Products*, ***74***, 106-108.

[7] (a) McNulty J, Mo R. (**1998**) Diastereoselective intramolecular nitroaldol entry to lycoricidine alkaloids. *Journal of the Chemical Society Chemical Communications*, 933-934; (b) McNulty J, Mao J, Gibe R, Mo R, Wolf S, Pettit GR, Herald DL, Boyd MR. (**2001**) Studies directed towards the refinement of the pancratistatin cytotoxic pharmacophore. *Bioorganic & Medicinal Chemistry Letters*, ***11***, 169-172; (c) Pettit GR, Melody N, Herald DL. (**2001**) Antineoplastic agents. 450. Synthesis of (+)-pancratistatin from (+)-narciclasine as relay. *Journal of Organic Chemistry*, ***66***, 2583-2587; (d) Hudlicky T, Rinner U, Gonzales D, Akgun H, Schilling S, Siengalewicz P, Martinot TA, Pettit GR. (**2002**) Total synthesis and biological evaluation of *Amaryllidaceae* alkaloids: narciclasine, *ent*-7-deoxypancratistatin, regioisomer of 7-deoxypancratistatin, 10b-*epi*-deoxypancratistatin, and truncated derivatives. *Journal of Organic Chemistry*, ***67***, 8726-8743; (e) Rinner U, Hillebrenner HL, Adams DR, Hudlicky T, Pettit GR. (**2004**) Synthesis and biological activity of some structural modifications of pancratistatin. *Bioorganic & Medicinal Chemistry Letters*, ***14***, 2911-2915; (f) Rinner U, Hudlicky T, Gordon H, Pettit GR. (**2004**) A β-carboline-1-one mimic of the anticancer Amaryllidaceae constituent pancratistatin: synthesis and biological evaluation. *Angewandte Chemie International Edition in English.*, ***43***, 5342-5346; (g) McNulty J, Larichev V, Pandey S. (**2005**) A synthesis of 3-deoxydihydrolycoricidine: refinement of a structurally minimum pancratistatin pharmacophore. *Bioorganic & Medicinal Chemistry Letters*, ***15***, 5315-5318; (h) Pettit GR, Eastham SA, Melody N, Orr B, Herald DL, McGregor J, Knight JC, Doubek DL, Pettit GR III, Garner LC, Bell JA. (**2006**) Isolation and structural modification of 7-deoxynarciclasine and 7-deoxy-*trans*-dihydronarciclasine. *Journal of Natural Products*, ***69***, 7-13; (i) McNulty J, Nair JJ, Griffin C, Pandey S. (**2008**) Synthesis and biological evaluation of fully functionalized *seco*-pancratistatin analogues. *Journal of Natural Products,* ***71***, 357-363.

[8] (a) Ghosal S, Saini KS, Razdan S. (**1985**) *Crinum* alkaloids: their chemistry and biology. *Phytochemistry*, ***24***, 2141-2156; (b) Fennell CW, Van Staden J. (**2001**) *Crinum* species in traditional and modern medicine. *Journal of Ethnopharmacology*, ***78***, 15-26; (c) Tram NTM, Titorenkova TV, Bankova VS, Handjieva NV, Popov SS. (**2002**) *Crinum* L. (Amaryllidaceae). *Fitoterapia*, ***73***, 183-208.

[9] (a) Ceriotti G. (**1967**) Narciclasine: an antimitotic substance from *Narcissus* bulbs. *Nature*, ***213***, 595-596; (b) Jimenez A, Santos A, Alonso G, Vazquez D. (**1976**) Inhibitors of protein synthesis in eukaryotic cells. Comparative effects of some Amaryllidaceae alkaloids. *Biochimica et Biophysica Acta*, ***425***, 342-348.

[10] Furusawa E, Irie H, Combs D, Wildman WC. (**1980**) Therapeutic activity of pretazettine on Rauscher leukemia: comparison with the related Amaryllidaceae alkaloids. *Experimental Chemotherapy*, ***26***, 36-45.

[11] Pettit GR, Gaddamidi V, Goswami A, Cragg GM. (**1984**) Antineoplastic agents. 99. *Amaryllis belladonna*. *Journal of Natural Products*, ***47***, 796-801.

[12] Ghosal S, Saini KS, Arora VK. (**1984**) 1,2-Epoxyambelline, an immunostimulant alkaloid from *Crinum latifolium*. *Journal of Chemical Research (S)*, 232-233.

[13] Ghosal S. (**1986**) Chemical constituents of Amaryllidaceae. Part 24. Crinafoline and crinafolidine, two antitumor alkaloids from *Crinum latifolium.* *Journal of Chemical Research (S)*, 312-313.

[14] Abd El Hafiz MA, Ramadan MA, Jung ML, Beck JP, Anton R. (**1991**) Cytotoxic activity of Amaryllidaceae alkaloids from *Crinum augustum* and *Crinum bulbispermum*. *Planta Medica*, ***57***, 437-439.

[15] Likhitwitayawuid K, Angerhofer CK, Chai H, Pezzuto JM, Cordell GA, Ruangrungsi N. (**1993**) Cytotoxic and antimalarial alkaloids from the bulbs of *Crinum amabile*. *Journal of Natural Products*, ***56***, 1331-1338.

[16] Antoun MD, Mendoza NT, Rios YR, Proctor, GR, Wickramaratne DBM, Pezzuto JM, Kinghorn AD. (**1993**) Cytotoxicity of *Hymenocallis expansa* alkaloids. *Journal of Natural Products*, ***56***, 1423-1425.

[17] Lin L-Z, Hu S-F, Chai H-B, Pengsuparp T, Pezzuto JM, Cordell GA, Ruangrungsi N. (**1995**) Lycorine alkaloids from *Hymenocallis littoralis*. *Phytochemistry*, ***40***, 1295-1298.

[18] Weniger B, Italiano L, Beck JP, Bastida J, Bergoñon S, Codina C, Lobstein A, Anton R. (**1995**) Cytotoxic activity of Amaryllidaceae alkaloids. *Planta Medica*, ***61***, 77-79.

[19] Campbell WE, Nair JJ, Gammon DW, Bastida J, Codina C, Viladomat F, Smith PJ, Albrecht CF. (**1998**) Cytotoxic and antimalarial alkaloids from *Brunsvigia littoralis*. *Planta Medica*, ***64***, 91-93.

[20] Nair JJ, Campbell WE, Gammon DW, Albrecht CF, Viladomat F, Codina C, Bastida J. (**1998**) Alkaloids from *Crinum delagoense*. *Phytochemistry*, ***49***, 2539-2543.

[21] Campbell WE, Nair JJ, Gammon DW, Codina C, Bastida J, Viladomat F, Smith PJ, Albrecht CF. (**2000**) Bioactive alkaloids from *Brunsvigia radulosa*. *Phytochemistry*, ***53***, 587-591.

[22] Abou-Donia AH, Amer ME, Darwish FA, Kassem FF, Mammoda HM, Abdel-Kader MS, Zhou B-N, Kingston DGI. (**2002**) Two new alkaloids of the crinane series from *Pancratium sickenbergeri*. *Planta Medica*, ***68***, 379-381.

[23] Hohmann J, Forgo P, Molnár J, Wolfard K, Molnár A, Thalhammer T, Mathé I, Sharples D. (**2002**) Antiproliferative Amaryllidaceae alkaloids isolated from the bulbs of *Sprekelia formosissima* and *Hymenocallis x festalis*. *Planta Medica*, ***68***, 454-457.

[24] Phan TS, Tran BD, Bach D, Phan MG, Nguyen TM, Hoang TH, Le MH. (**2003**) Investigation of antimicrobial and cytotoxic properties of alkaloids from some species of *Crinum* in Vietnam. *Tap Chi Duoc Hoc*, ***4***, 18-21.

[25] Nair JJ, Campbell WE, Brun R, Viladomat F, Codina C, Bastida J. (**2005**) Alkaloids from *Nerine filifolia*. *Phytochemistry*, ***66***, 373-382.

[26] (a) McNulty J, Nair JJ, Codina C, Bastida J, Pandey S, Gerasimoff J, Griffin C. (**2007**) Selective apoptosis-inducing activity of crinum-type Amaryllidaceae alkaloids. *Phytochemistry*, ***68***, 1068-1074; (b) Griffin C, Sharda N, Sood, D, Nair JJ, McNulty J, Pandey S. (**2007**) Selective cytotoxicity of pancratistatin-related natural Amaryllidaceae alkaloids: evaluation of the activity of two new compounds. *Cancer Cell International*, *7*, 10-16.

[27] Silva AFS, De Andrade JP, Machado KRB, Rocha AB, Apel MA, Sobral MEG, Henriques AT, Zuanazzi JAS. (**2008**) Screening for cytotoxic activity of extracts and isolated alkaloids from bulbs of *Hippeastrum vittatum*. *Phytomedicine*, ***15***, 882-885.

[28] Sun Q, Shen Y-H, Tian J-M, Tang J, Su J, Liu R-H, Li H-L, Xu X-K, Zhang W-D. (**2009**) Chemical constituents of *Crinum asiaticum* L. var. *sinicum* Baker and their cytotoxic activities. *Chemistry & Biodiversity*, ***6***, 1751-1757.

[29] McNulty J, Nair JJ, Bastida J, Pandey S, Griffin C. (**2009**) Structure activity studies on the crinane alkaloid apoptosis-inducing pharmacophore. *Natural Product Communications*, ***4***, 483-488.

[30] Simpson CD, Anyiwe K, Schimmer AD. (**2008**) Anoikis resistance and tumor metastasis. *Cancer Letters*, ***272***, 177-185.
[31] Van Goietsenoven G, Andolfi A, Lallemand B, Cimmino A, Lamoral-Theys D, Gras T, Abou-Donia A, Dubois J, Lefranc F, Mathieu V, Kornienko A, Kiss R, Evidente A. (**2010**) Amaryllidaceae alkaloids belonging to different structural subgroups display activity against apoptosis-resistant cancer cells. *Journal of Natural Products*, ***73***, 1223-1227.
[32] Osorio EJ, Berkov S, Brun R, Codina C, Viladomat F, Cabezas F, Bastida J. (**2010**) *In vitro* antiprotozoal activity of alkaloids from *Phaedranassa dubia* (Amaryllidaceae). *Phytochemistry Letters*, ***3***, 161-163.
[33] Kaya GI, Sarıkaya B, Onur MA, Somer NU, Viladomat F, Codina C, Bastida B, Lauinger IL, Kaiser M, Tasdemir D. (**2011**) Antiprotozoal alkaloids from *Galanthus trojanus*. *Phytochemistry Letters*, ***4***, 301-305.
[34] Berkov S, Romani S, Herrera M, Viladomat F, Codina C, Momekov G, Ionkova I, Bastida J. (**2011**) Antiproliferative alkaloids from *Crinum zeylanicum*. *Phytotherapy Research*, ***25***, 1686-1692.
[35] Jitsuno M, Yokosuka A, Hashimoto K, Amano O, Sakagami H, Mimaki Y. (**2011**) Chemical constituents of *Lycoris albiflora* and their cytotoxic activities. *Natural Product Communications*, ***6***, 187-192.
[36] Luchetti G, Johnston R, Mathieu V, Lefranc F, Hayden K, Andolfi A, Lamoral-Theys D, Reisenauer MR, Champion C, Pelly SC, van Otterlo WAL, Magedov IV, Kiss R, Evidente A, Rogelj S, Kornienko A. (**2012**) Bulbispermine: a crinine-type Amaryllidaceae alkaloid exhibiting cytostatic activity toward apoptosis-resistant glioma cells. *ChemMedChem*, ***7***, 815-822.
[37] Adewusi EA, Fouche G, Steenkamp V. (**2012**) Cytotoxicity and acetylcholinesterase inhibitory activity of an isolated crinine alkaloid from *Boophone disticha* (Amaryllidaceae). *Journal of Ethnopharmacology*, ***143***, 572-578.
[38] Nair JJ, Rárová L, Strnad M, Bastida J, Van Staden J. (**2012**) Apoptosis-inducing effects of distichamine and narciprimine, rare alkaloids of the plant family Amaryllidaceae. *Bioorganic and Medicinal Chemistry Letters, **22***, 6195-6199.
[39] (a) Opferman JT, Letai A, Beard C, Sorcinelli MD, Ong CC, Korsmeyer SJ. (**2003**) Development and maintenance of B and T lymphocytes require antiapoptotic MCL-1. *Nature*, ***426***, 671-676; (b) Inuzuka H, Shaik S, Onoyama I, Gao D, Tseng A, Maser RS, Zhai B, Wan L, Gutierrez A, Lau AW, Xiao Y, Christie AL, Aster J, Settleman J, Gygi SP, Kung AL, Look T, Nakayama KI, DePinho RA, Wei W. (**2011**) $SCF^{FBW7}$ regulates cellular apoptosis by targeting MCL1 for ubiquitylation and destruction. *Nature*, ***471***, 104-109.

# Natural Product Communications

# 2012 Volume 7

**Natural Product Communications 7 (1-12) 1-1690 (2012)**

**ISSN 1934-578X (print)**
**ISSN 1555-9475 (online)**

# NPC Natural Product Communications



**EDITOR-IN-CHIEF**

**DR. PAWAN K AGRAWAL**
*Natural Product Inc.*
*7963, Anderson Park Lane,*
*Westerville, Ohio 43081, USA*
*agrawal@naturalproduct.us*

**HONORARY EDITOR**

**PROFESSOR GERALD BLUNDEN**
*The School of Pharmacy & Biomedical Sciences,*
*University of Portsmouth,*
*Portsmouth, PO1 2DT U.K.*
*axuf64@dsl.pipex.com*

**EDITORS**

**PROFESSOR ALEJANDRO F. BARRERO**
*Department of Organic Chemistry,*
*University of Granada,*
*Campus de Fuente Nueva, s/n, 18071, Granada, Spain*
*afbarre@ugr.es*

**PROFESSOR ALESSANDRA BRACA**
*Dipartimento di Chimica Bioorganicae Biofarmacia,*
*Universita di Pisa,*
*via Bonanno 33, 56126 Pisa, Italy*
*braca@farm.unipi.it*

**PROFESSOR DEAN GUO**
*State Key Laboratory of Natural and Biomimetic Drugs,*
*School of Pharmaceutical Sciences,*
*Peking University,*
*Beijing 100083, China*
*gda5958@163.com*

**PROFESSOR YOSHIHIRO MIMAKI**
*School of Pharmacy,*
*Tokyo University of Pharmacy and Life Sciences,*
*Horinouchi 1432-1, Hachioji, Tokyo 192-0392, Japan*
*mimakiy@ps.toyaku.ac.jp*

**PROFESSOR STEPHEN G. PYNE**
*Department of Chemistry*
*University of Wollongong*
*Wollongong, New South Wales, 2522, Australia*
*spyne@uow.edu.au*

**PROFESSOR MANFRED G. REINECKE**
*Department of Chemistry,*
*Texas Christian University,*
*Forts Worth, TX 76129, USA*
*m.reinecke@tcu.edu*

**PROFESSOR WILLIAM N. SETZER**
*Department of Chemistry*
*The University of Alabama in Huntsville*
*Huntsville, AL 35809, USA*
*wsetzer@chemistry.uah.edu*

**PROFESSOR YASUHIRO TEZUKA**
*Institute of Natural Medicine*
*Institute of Natural Medicine, University of Toyama,*
*2630-Sugitani, Toyama 930-0194, Japan*
*tezuka@inm.u-toyama.ac.jp*

**PROFESSOR DAVID E. THURSTON**
*Department of Pharmaceutical and Biological Chemistry,*
*The School of Pharmacy,*
*University of London, 29-39 Brunswick Square,*
*London WC1N 1AX, UK*
*david.thurston@pharmacy.ac.uk*

**ADVISORY BOARD**

Prof. Berhanu M. Abegaz
*Gaborone, Botswana*

Prof. Viqar Uddin Ahmad
*Karachi, Pakistan*

Prof. Øyvind M. Andersen
*Bergen, Norway*

Prof. Giovanni Appendino
*Novara, Italy*

Prof. Yoshinori Asakawa
*Tokushima, Japan*

Prof. Lee Banting
*Portsmouth, U.K.*

Prof. Julie Banerji
*Kolkata, India*

Prof. Anna R. Bilia
*Florence, Italy*

Prof. Maurizio Bruno
*Palermo, Italy*

Prof. César A. N. Catalán
*Tucumán, Argentina*

Prof. Josep Coll
*Barcelona, Spain*

Prof. Geoffrey Cordell
*Chicago, IL, USA*

Prof. Ana Cristina Figueiredo
*Lisbon, Portugal*

Prof. Cristina Gracia-Viguera
*Murcia, Spain*

Prof. Duvvuru Gunasekar
*Tirupati, India*

Prof. Kurt Hostettmann
*Lausanne, Switzerland*

Prof. Martin A. Iglesias Arteaga
*Mexico, D. F, Mexico*

Prof. Leopold Jirovetz
*Vienna, Austria*

Prof. Karsten Krohn
*Paderborn, Germany*

Prof. Hartmut Laatsch
*Gottingen, Germany*

Prof. Marie Lacaille-Dubois
*Dijon, France*

Prof. Shoei-Sheng Lee
*Taipei, Taiwan*

Prof. Francisco Macias
*Cadiz, Spain*

Prof. Imre Mathe
*Szeged, Hungary*

Prof. Joseph Michael
*Johannesburg, South Africa*

Prof. Ermino Murano
*Trieste, Italy*

Prof. M. Soledade C. Pedras
*Saskatoon, Canada*

Prof. Luc Pieters
*Antwerp, Belgium*

Prof. Peter Proksch
*Düsseldorf, Germany*

Prof. Phila Raharivelomanana
*Tahiti, French Polynesia*

Prof. Luca Rastrelli
*Fisciano, Italy*

Prof. Monique Simmonds
*Richmond, UK*

Prof. John L. Sorensen
*Manitoba, Canada*

Prof. Valentin Stonik
*Vladivostok, Russia*

Prof. Winston F. Tinto
*Barbados, West Indies*

Prof. Sylvia Urban
*Melbourne, Australia*

Prof. Karen Valant-Vetschera
*Vienna, Austria*


## INFORMATION FOR AUTHORS

Full details of how to submit a manuscript for publication in Natural Product Communications are given in Information for Authors on our Web site http://www.naturalproduct.us.


Authors may reproduce/republish portions of their published contribution without seeking permission from NPC, provided that any such republication is accompanied by an acknowledgment (original citation)-Reproduced by permission of Natural Product Communications. Any unauthorized reproduction, transmission or storage may result in either civil or criminal liability.

The publication of each of the articles contained herein is protected by copyright. Except as allowed under national "fair use" laws, copying is not permitted by any means or for any purpose, such as for distribution to any third party (whether by sale, loan, gift, or otherwise); as agent (express or implied) of any third party; for purposes of advertising or promotion; or to create collective or derivative works. Such permission requests, or other inquiries, should be addressed to the Natural Product Inc. (NPI). A photocopy license is available from the NPI for institutional subscribers that need to make multiple copies of single articles for internal study or research purposes.



**To Subscribe**: Natural Product Communications is a journal published monthly. 2012 subscription price: US$1,995 (Print, ISSN# 1934-578X); US$1,995 (Web edition, ISSN# 1555-9475); US$2,495 (Print + single site online); US$595 (Personal online). Orders should be addressed to Subscription Department, Natural Product Communications, Natural Product Inc., 7963 Anderson Park Lane, Westerville, Ohio 43081, USA. Subscriptions are renewed on an annual basis. Claims for nonreceipt of issues will be honored if made within three months of publication of the issue. All issues are dispatched by airmail throughout the world, excluding the USA and Canada.

# Natural Product Communications

Contents of Volume **7**
**2012**

## *Number 1*


**New Iridoid from Aerial Parts of *Mussaenda roxburghii***
Utpal Chandra De, Ranjit Ghosh, Sanjib Chowdhury and Biswanath Dinda **1**

**A New Megastigmane Glycoside, Phoenixoside A, from *Phoenix dactylifera***
Sumbul Azmat, Aqib Zahoor, Rehana Ifzal, Viqar Uddin Ahmad and Faryal Vali Mohammed **3**

**New 3,4-*Seco-ent*-kaurene Dimers from *Croton micans***
Elsa Mateu, Katiuska Chavez, Ricarda Riina, Reinaldo S. Compagnone, Franco Delle Monache and Alírica I. Suárez **5**

**Diacarperoxide S, New Norterpene Cyclic Peroxide from the Sponge *Diacarnus megaspinorhabdosa***
Sabrin R. M. Ibrahim **9**

**Constituents of Kenyan *Gardenia volkensii***
Esther W. Kinuthia, Moses K. Langat, Elizabeth M. Mwangi and Peter K Cheplogoi **13**

**Oral Administration of *Cimicifuga racemosa* Extract Attenuates Immobilization Stress-Induced Reactions**
Isao Nadaoka, Kazuki Watanabe, Masaaki Yasue, Manabu Sami, Yasushi Kitagawa and Yoshihiro Mimaki **15**

**Complete NMR Assignments of Tubulosine**
Venkata Siva Satyanarayana Kantamreddi and Colin W. Wright **19**

**Application of Mixture Analysis to Crude Materials from Natural Resources (III)[1]: NMR Spectral Studies to Analyze Chalcones from *Angelica keiskei***
Eriko Fukuda, Masaki Baba, Yoshihiro Uesawa, Osamu Kamo, Kazunori Arifuku, Koji Tsubono and Yoshihito Okada **21**

**A Validated Chromatographic Method for the Determination of Flavonoids in *Copaifera langsdorffii* by HPLC**
João Paulo B. de Sousa, Ana Paula S. Brancalion, Milton G. Júnior and Jairo K. Bastos **25**

**Luteolin Induces Mitochondria-dependent Apoptosis in Human Lung Adenocarcinoma Cell**
Qing Chen, Shengming Liu, Jinghong Chen, Qianqian Zhang, Shijie Lin, Zhiming Chen and Jianwei Jiang **29**

**Diuretic Activity of *Lophophytum leandri***
Antonio Bracci, Anibal G. Amat, Francesco Maione, Carla Cicala, Nicola Mascolo and Vincenzo De Feo **33**

**Evaluation of the Hypocholesterolemic Effect and Phytochemical Screening of the Hydroethanolic Extract of *Crataegus aronia* from Jordan**
Entisar K. Al-Hallaq, Fatma U. Afifi and Shtaywy S. Abdalla **35**

**Secondary Metabolites, Cytotoxic Response by Neutral Red Retention and Protective Effect Against $H_2O_2$ Induced Cytotoxicity of *Sedum caespitosum***
Didem Şöhretoğlu and Suna Sabuncuoğlu **39**

**Effect of *Hibiscus sabdariffa* and its Anthocyanins on some Reproductive Aspects in Rats**
Badreldin H. Ali, Intisar Al-Lawati, Sumyia Beegam, Amal Ziada, Suhail Al salam, Abderrahim Nemmar and Gerald Blunden **41**

**New Galloyl Derivative from Winged Sumac (*Rhus copallinum*) Fruit**
Hang Ma, Tao Yuan, Antonio González-Sarrías, Liya Li, Maxwell E. Edmonds and Navindra P. Seeram **45**

**Phytochemical Analysis and Antioxidant Capacity of BM-21, a Bioactive Extract Rich in Polyphenolic Metabolites from the Sea Grass *Thalassia testudinum***
Erik L. Regalado, Roberto Menendez, Olga Valdés, Ruth A. Morales, Abilio Laguna, Olivier P. Thomas, Yasnay Hernandez, Clara Nogueiras and Anake Kijjoa **47**

**Herbicidal Activity of Curvulinic Acid Isolated from *Nimbya alternantherae***
Jun Li, Yonghao Ye, Xiaoyang Wang and Liyao Dong **51**

**Xanthones with Antiproliferative Effects on Prostate Cancer Cells from the Stem Bark of *Garcinia xanthochymus***
Feng Ji, Zhanlin Li, Gaofeng Liu, Shengli Niu, Nan Zhao, Xiaoqiu Liu and Huiming Hua **53**

**Aromatic Hydroxyl Group Plays a Critical Role in Antibacterial Activity of the Curcumin Analogues**
Mi Kyoung Kim, Jun Cheol Park and Youhoon Chong **57**

**Analysis of Danshen and Twelve Related *Salvia* Species**
Luyang Lu, Yuan Liu, Zhifeng Zhang and Hao Zhang **59**

**Meliloester, a New Melilotic Ester from *Melilotus alba***
Rasheeda Khatoon, Nikhat Saba, Aqib Zahoor, Shazia Summer and Viqar Uddin Ahmad **61**

**A New Long-Chain Unsaturated Ester and Other Constituents of *Hypericum tomentosum***
Ouassila Touafek, Zahia Kabouche, Joël Boustie and Christian Bruneau **63**

**Microbial Conversion of Tomato by a Plant Pathogenic Bacterium *Pectobacterium atrosepticum*: a Plant-Microbial Approach to Control Pathogenic *Candida* Species**
Vivek K. Bajpai, Sun Chul Kang, Soon-Gu Lee and Kwang-Hyun Baek **65**

**Antibacterial and Antiparasitic Effects of *Bothropoides lutzi* venom**
Ramon R.P.P.B. de Menezes, Alba F. C. Torres, Thiala S. J. da Silva, Daniel F. de Sousa, Danya B. Lima, Diva B. Norjosa, Nádia A. P. Nogueira, Maria F. Oliveira, Márcia R. de Oliveira, Helena S. A. Monteiro and Alice M. C. Martins **71**

**Ternary Liquid-Liquid Equilibria Measurement for Epoxidized Soybean Oil + Acetic Acid + Water**
Shuang-Fei Cai, Li-Sheng Wang, Guo-Qing Yan, Yi Li, Yun-Xia Feng and Rong-Gang Linghu **75**

**Chemical Constituents of the Essential Oil from Aerial Parts and Fruit of *Anisosciadium orientale***
Vahid Rowshan, Ahmad Hatami, Atefeh Bahmanzadegan and Mahnaz Yazdani **79**

**GC-MS Analysis of *Ziziphora clinopodioides* Essential Oil from North Xinjiang, China**
Xiaoying Zhou, Qian Yu, Haiyan Gong and Shuge Tian **81**

**Analysis of the Essential Oil of *Teucrium polium* ssp. *capitatum* from the Balkan Peninsula**
Violeta Mitić, Olga Jovanović, Vesna Stankov-Jovanović, Bojan Zlatkovic and Gordana Stojanovic **83**

**Composition of the Essential Oil of *Pogostemon travancoricus* var. *travancoricus***
Ramar Murugan and Gopal Rao Mallavarapu **87**

**Liquid $CO_2$ Extraction of *Jasminum grandiflorum* and Comparison with Conventional Processes**
Om Prakash, Deeptanjali Sahoo and Prasant Kumar Rout **89**

**Chemical Composition of Volatile Oils from the Pericarps of Indian Sandalwood (*Santalum album*) by Different Extraction Methods**
Xin Hua Zhang, Jaime A. Teixeira da Silva, Yong Xia Jia, Jie Tang Zhao and Guo Hua Ma **93**

**Fast Quality Assessment of German Chamomile (*Matricaria chamomilla* L.) by Headspace Solid-Phase Microextraction: Influence of Flower Development Stage**
Mohammad Rafieiolhossaini, An Adams, Hamid Sodaeizadeh, Patrick Van Damme and Norbert De Kimpe **97**

**Floral Scent Composition of *Plumeria tuberculata* Analyzed by HS-SPME**
Disnelys Báez, Jorge A. Pino and Diego Morales **101**

**Identification and Quantification of the Antimicrobial Components of a Citrus Essential Oil Vapor**
Carol A. Phillips, Konstantinos Gkatzionis, Katie Laird, Jodie Score, Avinash Kant and Mark D. Fielder **103**

**Composition, Antioxidant and Antimicrobial Activities of the Leaf Essential Oil of *Machilus japonica* from Taiwan**
Chen-Lung Ho and Yu-Chang Su **109**

**Chemical Composition, Antimicrobial, Antiradical and Anticholinesterase activity of the Essential Oil of *Pulicaria stephanocarpa* from Soqotra**
Nasser A. Awadh Ali, Rebecca A. Crouch, Mohamed A. Al-Fatimi, Norbert Arnold, Axel Teichert, William N. Setzer and Ludger Wessjohann **113**

**Composition of the Essential Oils and Antibacterial Activities of *Hymenocrater yazdianus*, *Stachys obtusicrena* and *Nepeta asterotricha* Three Labiatae Herbs Growing Wild in Iran**
Shiva Masoudi, Abdolhossein Rustaiyan, Razieh Mohebat and Mohammad Hossein Mosslemin **117**

**Antibacterial Activities of Essential Oils Extracted from Leaves of *Murraya koenigii* by Solvent-Free Microwave Extraction and Hydro-Distillation**
Naciye Erkan, Zhou Tao, H. P. Vasantha Rupasinghe, Burcu Uysal and Birsen S. Oksal **121**

**Chemical Composition, Antifungal and Herbicidal Effects of Essential oil Isolated from *Chersodoma argentina* (Asteraceae)**
Rosana Alarcón, Soledad Ocampos, Adriana Pacciaroni and Virginia Sosa **125**

**Essential Oil Composition and Acaricidal Activity of *Schinus terebinthifolius* from Atlantic Forest of Pernambuco, Brazil against *Tetranychus urticae***
Aline Fonseca do Nascimento, Claudio Augusto Gomes da Camara, Marcílio Martins de Moraes and Clécio Souza Ramos **129**

**Evaluation of the Anti-*Leishmania major* Activity of *Satureja bakhtiarica* Essential Oil *in vitro***
Ghasem Mohammadpour, Eisa Tahmasbpour Marzony and Mahin Farahmand **133**

***Spartium junceum* Aromatic Water: Chemical Composition and Antitumor activity**
Teresa Cerchiara, Serafina V. Straface, Giuseppe Chidichimo, Emilia L. Belsito, Angelo Liguori, Barbara Luppi, Federica Bigucci and Vittorio Zecchi **137**

## ***Additions/Coreections***

**The Chemical Composition of the Essential Oils of *Euphorbia caracasana* and *E. cotinifolia* (Euphorbiaceae) from Venezuela**
Janne Rojas, Shirley Baldovino, Marietta Vizcaya, Luis B. Rojas and Antonio Morales
*Natural Product Communications*, ***4*** (4), 571 (2009) **141**

## *Number 2*

**Antiviral Furanosesquiterpenes from *Commiphora erythraea***
Elio Cenci, Federica Messina, Elisabetta Rossi, Francesco Epifano and Maria Carla Marcotullio **143**

**Sesquiterpenes from the Inner Bark of the Silver Birch and the Paper Birch**
Roderick J. Weston and Gerald J. Smith **145**

**Two New Ballonigrin-type Diterpenoids from the Roots of *Ballota limbata***
Umar Farooq, Afsar Khan, Ather Farooq Khan, Saleha Suleman Khan, Rizwana Sarwar, Viqar Uddin Ahmad and Amir Waseem **149**

**A Novel Anticancer Diterpenoid from *Jatropha gossypifolia***
Abiodun Falodun, Udo Kragl, Serge-Mitherand Tengho Touem, Alexander Villinger, Thomas Fahrenwaldt and Peter Langer **151**

**Antihepatitis B Virus Constituents of *Solanum erianthum***
Shen-Chieh Chou, Tsurng-Juhn Huang, En-Hung Lin, Chun-Huei Huang and Chang-Hung Chou **153**

**Physical Properties and Molecular Conformations of Indole Alkaloids and Model Protein Interactions – Theoretical *vs.* Experimental Study**
Bojidarka Ivanova and Michael Spiteller **157**

**Antiparasitic and Anticancer Constituents of the Endophytic Fungus *Aspergillus* sp. strain F1544**
Sergio Martínez-Luis, Lilia Cherigo, Elizabeth Arnold, Carmenza Spadafora, William H. Gerwick and Luis Cubilla-Rios **165**

**Chemical and Bioactivity Evaluation of the Bark of *Neonauclea purpurea***
Netiya Karaket, Kanyaratt Supaibulwatana, Supatsara Ounsuk, Valérie Bultel-Poncé, Van Cuong Pham and Bernard Bodo **169**

**Antifeedants from Chinese Medicinal Herb, *Erythrina variegata* var. *orientalis*, Against Maize Weevil *Sitophilus zeamais***
Zhi Long Liu, Sha Sha Chu, Guo Hua Jiang and Shao Liang Liu **171**

**Kaempferol Tri- and Tetraglycosides from the Flowers of *Clematis* Cultivar**
Keisuke Sakaguchi, Junichi Kitajima and Tsukasa Iwashina **173**

**Flowers from *Kalanchoe pinnata* are a Rich Source of T Cell-Suppressive Flavonoids**
Marcela A. S. Coutinho, Michelle F. Muzitano, Elaine A. Cruz, Maria C. Bergonzi, Carlos R. Kaiser, Luzineide W. Tinoco, Anna R. Bilia, Franco F. Vincieri, Bartira Rossi-Bergmann and Sônia S. Costa **175**

**Novel Flavanes from *Livistona halongensis***
Tran Van Loc, Pham Duc Thang, Nguyen The Anh, Pham Thi Ninh, Trinh Thi Thuy and Tran Van Sung **179**

**Chemical Profiling of Constituents of *Smilacis glabrae* Using Ultra-high Pressure Liquid Chromatography Coupled with LTQ Orbitrap Mass Spectrometry**
Xiong Li, Yu Feng Zhang, Liu Yang, Yi Feng, Yuan Hui Deng, Yi Ming Liu and Xing Zeng **181**

**Phenolic Constituents from the Heartwood of *Artocapus altilis* and their Tyrosinase Inhibitory Activity**
Mai Ha Khoa Nguyen, Hai Xuan Nguyen, Mai Thanh Thi Nguyen and Nhan Trung Nguyen **185**

**Chemical Constituents of two Sages with Free Radical Scavenging Activity**
Omur L. Demirezer, Perihan Gurbuz, Ayşe Kuruuzum-Uz, Zuhal Guvenalp Cavit Kazaz and Ali A. Donmez **187**

**Synthesis of Uliginosins A and B**
George A. Kraus and Feng Liu **191**

**Eucleanal: A New Napthalene Derivative from *Euclea divinorum***
Margaret Mwihaki Ng'ang'a, Hidayat Hussain, Sumesh Chhabra, Caroline Langat-Thoruwa, Karsten Krohn, Javid Hussain, Ahmed Al-Harrasi and Ivan Robert Green **193**

**A Double-blind, Randomized, Clinical Trial on the Antileishmanial Activity of a *Morinda citrifolia* (Noni) Stem Extract and its Major Constituents**
Fouzia A. Sattar, Fayaz Ahmed, Nadeem Ahmed, Samina A. Sattar, Muhammad A. K. Malghani and Muhammad I. Choudhary **195**

**Biotransformation of Gambogenic Acid by *Chaetomium globosum* CICC 2445**
Si-Jia Tao, Yang Wang, Xing Zhang, Shu-Hong Guan and De-An Guo **197**

**Between Species Diversity of *Hypericum perforatum* and *H. maculatum* by the Content of Bioactive Compounds**
Edita Bagdonaite, Valdimaras Janulis, Liudas Ivanauskas and Juozas Labokas **199**

**Total Phenolic Content and Antioxidant Activity of *Salvia* spp. Exudates**
Laura Giamperi, Anahi Bucchini, Angela Bisio, Emanuela Giacomelli, Giovanni Romussi and Donata Ricci **201**

**An Efficient Microwave Assisted Extraction of Phenolic Compounds and Antioxidant Potential of *Ginkgo biloba***
Pushpinder Kaur, Abha Chaudhary, Bikram Singh and Gopichand **203**

**Synthesis and Anti-tumor Activity Evaluation of Rhein-Aloe Emodin Hybrid Molecule**
Ye-fei Yuan, Xiang-yu Hu, Ying He and Jia-gang Deng **207**

**A New Peptide Isolated from a Marine Derived *Streptomyces bacillaris***
Youcai Hu and John B. MacMillan **211**

**Biological Activity and Tissue Specific Accumulation of Fluorescently Labeled Methyl Jasmonate**
Naoki Kitaoka, Yuzou Sano, Seizo Fujikawa, Kensuke Nabeta and Hideyuki Matsuura **215**

**A New Resin Glycoside from *Ipomoea maxima***
Kazune Koryudzu, Midori A. Arai, Firoj Ahmed, Samir K. Sadhu and Masami Ishibashi **219**

**Hypoglycemic Constituents of *Gynura divaricata* subsp. *formosana***
Shen-Chieh Chou, Lee-Ming Chuang and Shoei-Sheng Lee **221**

**Constituents of South Indian Vetiver Oils**
Gopal Rao Mallavarapu, Kodakandla V. Syamasundar, S. Ramesh and Bhaskaruni R. Rajeswara Rao **223**

**Composition of Seed Essential Oils of *Rhododendron tomentosum***
Asta Judzentiene, Rita Butkiene, Jurga Budiene, Félix Tomi and Joseph Casanova **227**

**The Leaf Essential Oils of Five Vietnamese *Desmos* Species (Annonaceae)**
Do Ngoc Dai, Tran Minh Hoi, Tran Dinh Thang and Isiaka A. Ogunwande **231**

**Essential Oil Composition of *Cachrys cristata* – a rare and Endangered Species in the Flora of Serbia**
Jelena S. Matejić, Ana M. Džamić, Mihailo S. Ristić, Vladimir N. Ranđelović and Petar D. Marin **235**

**Volatiles from *Magnolia grandiflora* Flowers: Comparative Analysis by Simultaneous Distillation-Extraction and Solid Phase Microextraction**
Disnelys Báez, Jorge A. Pino and Diego Morales **237**

**Essential Oil Composition, Cytotoxic and Antibacterial Activities of Five *Etlingera* Species from Borneo**
Charles Santhanaraju Vairappan, Thilahgavani Nagappan and Kishneth Palaniveloo **239**

**Essential Oil Composition and Antibacterial Activity of *Monticalia greenmaniana* (Asteraceae)**
José Cárdenas, Janne Rojas, Luís Rojas-Fermin, María Lucena and Alexis Buitrago **243**

**Bioactivity of the Essential Oil of an Argentine Collection of *Acanthospermum hispidum* (Asteraceae)**
Mariana Alva, Susana Popich, Susana Borkosky, Elena Cartagena and Alicia Bardón **245**

**Antibacterial Activity and Comparison of the Volatile Constituents Obtained by Several Extraction Methods from the Flowers, Stems and Leaves of *Astrodaucus orientalis***
Marzieh Torabbeigi, Parviz Abroomand Azar, Anousheh Sharifan and Zahra Aghaei Meibodi **249**

***In vitro* Inhibitory Activity of Essential Oil Vapors against *Ascosphaera apis***
Pavel Kloucek, Jakub Smid, Jaroslav Flesar, Jaroslav Havlik, Dalibor Titera, Vojtech Rada, Ondrej Drabek and Ladislav Kokoska **253**

**Chemical Composition and Biological Activity of Essential Oil from *Pulicaria undulata* from Yemen**
Nasser A. Awadh Ali, Farukh S. Sharopov, Mehdi Alhaj, Gabrielle M. Hill, Andrea Porzel, Norbert Arnold, William N. Setzer, Jürgen Schmidt and Ludger Wessjohann **257**

**Composition, Antioxidant, Antimicrobial and Anti-wood-decay Fungal Activities of the Twig Essential Oil of *Taiwania cryptomerioides* from Taiwan**
Chen-Lung Ho, Su- Sing Yang, Tsong-Min Chang and Yu-Chang Su **261**

**Essential Oil from the Leaves of *Annona vepretorum*: Chemical Composition and Bioactivity**
Emmanoel Vilaça Costa, Lívia Macedo Dutra, Paulo Cesar de Lima Nogueira, Valéria Regina de Souza Moraes, Marcos José Salvador, Luis Henrique Gonzaga Ribeiro and Fernanda Ramos Gadelha **265**

**Chemical Composition and Insecticidal Activity of the Essential Oil of *Artemisia eriopoda* Against Maize Weevil, *Sitophilus zeamais***
Guo Hua Jiang, Quan Ru Liu, Sha Sha Chu and Zhi Long Liu **267**

**The Chemical Composition of the Essential Oils of *Cirsium palustre* and *C. rivulare* and their Antiproliferative Effect**
Jolanta Nazaruk, Ewa Karna and Danuta Kalemba **269**

## *Review/Account*

**Phytotherapy of Alcoholism**
Michał Tomczyk, Marijana Zovko-Končić and Lech Chrostek **273**

## *Number 3*

**Efficient Preparation of Incensole and Incensole Acetate, and Quantification of These Bioactive Diterpenes in *Boswellia papyrifera* by a RP-DAD-HPLC Method**
Michael Paul and Johann Jauch **283**

**A Comparison Investigation on the Solubilization of Betulin and Betulinic Acid in Cyclodextrin Derivatives**
Hai Ming Wang, Codruta M. Şoica and Gerhard Wenz **289**

**Two New Antimicrobial Metabolites from the Endophytic Fungus, *Seimatosporium* sp.**
Hidayat Hussain, Karsten Krohn, Barbara Schulz, Siegfried Draeger, Mamona Nazir and Muhammad Saleem **293**

**Simulation of Intramolecular Hydrogen Bond Dynamics in Manzamine A as a Sensitive Test for Charge Distribution Quality**
Dmitry A. Shulga, Dmitry I. Osolodkin, Vladimir A. Palyulin and Nikolay S. Zefirov **295**

**A Quinoline Based bis-Urea Receptor for Anions: A Selective Receptor for Hydrogen Sulfate**
Tiffany H. Russ, Avijit Pramanik, Maryam E. Khansari, Bryan M. Wong and Md. Alamgir Hossain **301**

**DNA Binding Studies of Vinca Alkaloids: Experimental and Computational Evidence**
Prateek Pandya, Surendra P. Gupta, Kumud Pandav, Ritu Barthwal, B. Jayaram and Surat Kumar **305**

**Natural Flavonoids Interact with Dinitrobenzene System in Aprotic Media: An Electrochemical Probing**
Nasima Arshad, Naveed K. Janjua, Athar Y. Khan, Azra Yaqub, Torsten Burkholz and Claus Jacob **311**

**Live Cell Imaging of a Fluorescent Gentamicin Conjugate**
Jorge O. Escobedo, Yu-Hsuan Chu, Qi Wang, Peter S. Steyger and Robert M. Strongin **317**

**Molecular Recognition of Carbohydrates: Evaluation of the Binding Properties of Pyrazole-based receptors and their Comparison with Imidazole- and Indole-based Systems**
Claudia Sonnenberg, André Hartmann and Monika Mazik **321**

**Transformations of Griseofulvin in Strong Acidic Conditions – Crystal Structures of 2'-Demethylgriseofulvin and Dimerized Griseofulvin**
Barbara Leśniewska, Said Jebors, Anthony W. Coleman and Kinga Suwińska **327**

**Rapid Acyl Migration Between Pyrogallyl 1,2- and 1,3-Dipivaloates**
Yaming Shan, Jimmy (Jingyue) Liu, Nigam P. Rath and George W. Gokel **333**

**Toward Engineering Intra-Receptor Interactions Into Bis(crown ethers)**
Martin R. Krause and Stefan Kubik **337**

**Monitoring Stepwise Proteolytic Degradation of Peptides by Supramolecular Domino Tandem Assays and Mass Spectrometry for Trypsin and Leucine Aminopeptidase**
Garima Ghale, Nikolai Kuhnert and Werner M. Nau **343**

**Intracellular Localization of PNA in Human Cells upon its Introduction by Electroporation**
Eri Noguchi, Narumi Shigi and Makoto Komiyama **349**

**Fliposomes: pH-controlled Release from Liposomes Containing New *trans*-2-Morpholinocyclohexanol-based Amphiphiles that Perform a Conformational Flip and Trigger an Instant Cargo Release upon Acidification**
Yu Zheng, Xin Liu, Nataliya M. Samoshina, Vyacheslav A. Chertkov, Andreas H. Franz, Xin Guo and Vyacheslav V. Samoshin **353**

**Competition of Ester, Amide, Ether, Carbonate, Alcohol and Epoxide Ligands in the Dirhodium Experiment (Chiral Discrimination by NMR Spectroscopy)**
Jens T. Mattiza, Vera Meyer, Gülsüm Özüduru, Tanja Heine, Joerg Fohrer and Helmut Duddeck **359**

**Synthesis of Acetylene Bridged Germanium Phthalocyanines**
Felicitas Plenzig, Alexey Lyubimtsev and Michael Hanack **363**

**Host-Guest Chemistry of Alkaloids**
Anatoly K. Yatsimirsky **369**

**Proanthocyanidins: Oligomeric Structures with Unique Bio-chemical Properties and Great Therapeutic Promise**
Zhanjie Xu, Peng Du, Peter Meiser and Claus Jacob **381**

**Aurones: Interesting Natural and Synthetic Compounds with Emerging Biological Potential**
Clemens Zwergel, François Gaascht, Sergio Valente, Marc Diederich, Denyse Bagrel and Gilbert Kirsch **389**

**Sulfur and Sulfur Compounds in Plant Defence**
Ifeanyi D. Nwachukwu, Alan J. Slusarenko and Martin C. H. Gruhlke **395**

**The Effect of Diallyl Polysulfanes on Cellular Signaling Cascades**
Mathias Montenarh and Nathaniel. E. B. Saidu **401**

**Interactions of Calix[*n*]arenes with Nucleic Acids**
Miao Li, Max Sena Peters and Thomas Schrader **409**

## *Number 4*

**Determination of the Absolute Stereochemistry of Limonene and α-Santalol by Raman Optical Activity Spectroscopy**
Akira Sakamoto, Nao Ohya, Toshio Hasegawa, Hiroaki Izumi, Nakako Tokita and Yoshiaki Hamada **419**

**Four New Eremophilane-Type Alcohols from *Cremanthodium helianthus* Collected in China**
Yoshinori Saito, Mayu Ichihara, Yasuko Okamoto, Xun Gong, Chiaki Kuroda and Motoo Tori **423**

**Culcitiolides A-D, Four New Eremophilane-Type Sesquiterpene Derivatives from *Senecio culcitioides***
Hiroshi Nozaki, Ken-ichiro Hayashi, Mikiko Kawai, Taichi Mitsui, Masahiro Kido, Hiroyuki Tani, Daisuke Takaoka, Hidemitsu Uno, Susumu Ohira, Atsuto Kuboki and Nobuyasu Matsuura **427**

**Complex Diversity in *Ligularia kanaitzensis***
Anna Shimizu, Yurika Suzuki, Atsushi Torihata, Ryo Hanai, Yoshinori Saito, Motoo Tori, Xun Gong, and Chiaki Kuroda **431**

**Chemical Constituents from *Farfugium japonicum* var. *formosanum***
Sung-Fei Hsieh, Tain-Jye Hsieh, Mohamed El-Shazly, Ying-Chi Du, Chin-Chung Wu, Tsong-Long Hwang, Yang-Chang Wu and Fang-Rong Chang **435**

**Transannular Cyclization of (4*S*,5*S*)-Germacrone-4,5-epoxide into Guaiane and Secoguaiane-type Sesquiterpenes**
Masanori Kuroyanagi, Osamu Shirota, Setsuko Sekita and Takahisa Nakane **441**

**Four New Guaianolides and Acetylenic Alcohol from *Saussurea katochaete* Collected in China**
Yoshinori Saito, Yuko Iwamoto, Yasuko Okamoto, Xun Gong, Chiaki Kuroda and Motoo Tori **447**

**Four New Bisabolane-type Sesquiterpenes from *Ligularia lankongensis***
Hiroshi Hirota, Yurie Horiguchi, Satoru Kawaii, Chiaki Kuroda, Ryo Hanai and Xun Gong **451**

**Enantioselective Synthesis of the Bisabolane Sesquiterpene (+)-1-Hydroxy-1,3,5-bisabolatrien-10-one and Revision of its Absolute Configuration**
Stefano Serra **455**

**Stereoselective Synthesis of Bicyclo[3.1.1]heptane Derivatives via Intramolecular Photocycloaddition Reaction**
Kiyoshi Honda, Mari Konishi, Manabu Kawai, Akihisa Yamada, Yasuhiko Takahashi, Yujiro Hoshino and Seiichi Inoue **459**

**Acaricidal and Repellent Activity of Terpenoids from Seaweeds Collected in Pernambuco, Brazil**
Flávia Souza Born, Éverson Miguel Bianco and Claudio Augusto Gomes da Camara **463**

**Glucosylated Sesquiterpene Alcohols from *Fraxinus griffithii***
Rene Angelo Macahig, Liva Harinantenaina, Sachiko Sugimoto, Katsuyoshi Matsunami, Hideaki Otsuka, Yoshio Takeda and Takakazu Shinzato **467**

**Chemical Constituents of the Aerial Parts of *Scutellaria lateriflora* and their α-Glucosidase Inhibitory Activities**
Minpei Kuroda, Katsura Iwabuchi and Yoshihiro Mimaki **471**

**Phorbol Esters with Wnt Signal-Augmenting Effects Isolated from *Excoecaria indica***
Tatsuhiro Yamaguchi, Kazufumi Toume, Midori A. Arai, Firoj Ahmed, Samir K. Sadhu and Masami Ishibashi **475**

**Triangulene C, a New Cubitane-based Diterpenoid from the Soft Coral *Sinularia triangula***
Huey-Jen Su, Nai-Lun Lee, Mei-Chin Lu and Jui-Hsin Su **479**

**Cladieunicellin H, a New Hemiketal Eunicellin-based Diterpenoid from the Octocoral *Cladiella* sp.**
Yung-Husan Chen, Chia-Ying Tai, Tsong-Long Hwang and Ping-Jyun Sung **481**

**A New Diterpene from the Leaves of *Andrographis paniculata* Nees**
Parvataneni Radhika, Yejella Rajendra Prasad and Kattupalli Sowjanya **485**

**New Meroterpenoids from the Marine Sponge *Aka coralliphaga***
Larisa K. Shubina, Anatoly I. Kalinovsky, Tatyana N. Makarieva, Sergey N. Fedorov, Sergey A. Dyshlovoy, Pavel S. Dmitrenok, Irina I. Kapustina, Ernesto Mollo, Natalia K. Utkina, Vladimir B. Krasokhin, Vladimir A. Denisenko and Valentin A. Stonik **487**

**New Triterpenoid Saponins from Leaves of *Panax japonicus* (3). Saponins of the Specimens Collected in Miyazaki Prefecture**
Kouichi Yoshizaki and Shoji Yahara **491**

**Oropheayunnol, an Unusual 22,23-epoxy Apotirucallane Triterpenoid from *Orophea yunnanensis***
Wen-Jian Lan, Jun Wang, Yan-Qiong Guo and Sheng Yin **495**

**New Metabolites from the Marine-derived Fungus *Aspergillus fumigatus***
Shamil Sh. Afiyatullov, Olesya I. Zhuravleva, Alexandr S. Antonov, Anatoly I. Kalinovsky, Michail V. Pivkin, Ekaterina S. Menchinskaya and Dmitry L. Aminin **497**

**A New Unusual $\Delta^{11(12)}$-Oleane Triterpene and Anti-complementary Triterpenes from *Prunella vulgaris* Spikes**
Dongsheng Du, Zhihong Cheng and Daofeng Chen **501**

**Anilides and Toluidides of 3β-Acetyloleanolic Acid**
Barbara Bednarczyk – Cwynar **507**

**Putralone, a Novel 10α-Hydroxy-25-nor D:A friedo-oleanane Triterpenoid from *Putranjiva roxburghii***
Tulika Mukherjee, Tapas Sarkar, Piyali Paul, Ajit K. Chakraborty, Parasuraman Jaisankar and Siba Brata Mukhopadhyay **511**

**Ardisikivuoside, a New Triterpenoid Saponin from *Ardisia kivuensis* (Myrsinaceae)**
Blanche L. Ndontsa, Alembert Tchinda, Rémy B. Teponno, James S. Mpetga, Michel Frédérich and Pierre Tane **515**

**Triterpene Glycosides from the Sea Cucumber *Eupentacta fraudatrix*. Structure and Biological Action of Cucumariosides $A_1$, $A_3$, $A_4$, $A_5$, $A_6$, $A_{12}$ and $A_{15}$, Seven New Minor Non-sulfated Tetraosides and Unprecedented 25-Keto, 27-Norholostane Aglycone**
Alexandra S. Silchenko, Anatoly I. Kalinovsky, Sergey A. Avilov, Pelageya V. Andryjaschenko, Pavel S. Dmitrenok, Ekaterina A. Martyyas and Vladimir I. Kalinin **517**

**A New Geranylated Aromatic Compound from the Mushroom *Hericium erinaceum***
Yasunori Yaoita, Shiori Yonezawa, Masao Kikuchi and Koichi Machida **527**

**Formation of Tetrahydrocurcumin by Reduction of Curcumin with Cultured Plant Cells of *Marchantia polymorpha***
Kei Shimoda, Naoji Kubota, Hirotaka Hirano, Masahiro Matsumoto, Hatsuyuki Hamada and Hiroki Hamada **529**

**Regioselective Formation of Silybin-23-β-D-glucoside by Glucosylation of Silybin with Cultured Plant Cells of *Eucalyptus perriniana***
Kei Shimoda, Hiroya Imai, Tadakatsu Mandai and Hiroki Hamada **531**

## *Review/Account*

**Terpenoids and Related Compounds from Plants of the Family Compositae (Asteraceae)**
Yasunori Yaoita, Masao Kikuchi and Koichi Machida **533**

**Diversity of Furanoeremophilanes in Major *Ligularia* Species in the Hengduan Mountains**
Chiaki Kuroda, Ryo Hanai, Hajime Nagano, Motoo Tori and Xun Gong **539**

# *Number 5*

## *Gerald Blunden Award (2011)*

**On-line (HPLC-NMR) and Off-line Phytochemical Profiling of the Australian Plant, *Lasiopetalum macrophyllum***
Michael Timmers and Sylvia Urban **551**

## *Original Paper*

**Iridoid and Phenolic Glycosides from the Roots of *Prismatomeris connata***
Shixiu Feng, Jijiang Bai, Shengxiang Qiu (Samuel), Yong Li and Tao Chen **561**

**Effect of some *ent*-Kaurenes on the Viability of Human Peripheral Blood Mononuclear Cells**
Yndra Cordero, Grecia M. Corao, José A. Cova and Alfredo Usubillaga **563**

**Steroidal Glycosides from *Veronica chamaedrys* L. Part I. The Structures of Chamaedrosides C, $C_1$, $C_2$, E, $E_1$ and $E_2$**
Alexandra Marchenko, Pavel Kintya, Bozena Wyrzykiewicz and Elena Gorincioi **565**

**Identification of the Plant Origin of the Botanical Biomarkers of Mediterranean type Propolis**
Milena Popova, Boryana Trusheva, Simone Cutajar, Daniela Antonova, David Mifsud, Claude Farrugia and Vassya Bankova **569**

**Alkaloids from Some Amaryllidaceae Species and Their Cholinesterase Activity**
Lucie Cahlíková, Nina Benešová, Kateřina Macáková, Radim Kučera, Václav Hrstka, Jiří Klimeš, Luděk Jahodář and Lubomír Opletal **571**

**Phytochemical and Biological Activity Studies of the Bhutanese Medicinal Plant *Corydalis crispa***
Phurpa Wangchuk, Paul A. Keller, Stephen G. Pyne, Thanapat Sastraruji, Malai Taweechotipatr, Roonglawan Rattanajak, Aunchalee Tonsomboon and Sumalee Kamchonwongpaisan **575**

**On the Biosynthetic Pathway of Papaverine *via* (*S*)-Reticuline – Theoretical vs. Experimental Study**
Bojidarka Ivanova and Michael Spiteller **581**

**Diversification of Exudate Flavonoid Profiles in Further *Primula spp*.**
Tshering Doma Bhutia and Karin M. Valant-Vetschera **587**

**Three New Biflavonoids from Chinese Dragon's Blood, *Dracaena cochinchinensis***
Jing Guan and Shun-Xing Guo **591**

**Secondary Metabolites from Polar Fractions of *Piper umbellatum***
Turibio Kuiate Tabopda, Anne-Claire Mitaine-Offer, Tomofumi Miyamoto, Chiaki Tanaka, Bonaventure Tchaleu Ngadjui and Marie-Aleth Lacaille-Dubois **595**

**A New Antimycobacterial Furanolignan from *Leucophyllum frutescens***
Blanca Alanís-Garza, Ricardo Salazar-Aranda, Rosalba Ramírez-Durón, Elvira Garza-González and Noemí Waksman de Torres **597**

**Water-soluble Constituents of the Heartwood of *Streblus asper***
Jun Li, Mao-Tong Tang, Qiang Wu, Hong Chen, Xiao-Tao Niu, Xin-Lan Guan, Jian Li, Sheng-Ping Deng, Xiao-Jian Su and Rui-Yun Yang **599**

**Lichen Depsides and Depsidones Reduce Symptoms of Diseases Caused by Tobacco Mosaic Virus (TMV) in Tobacco Leaves**
Ingrid Ramírez, Soledad Araya, Marisa Piovano, Marcela Carvajal, Alvaro Cuadros-Inostroza, Luis Espinoza, Juan Antonio Garbarino and Hugo Peña-Cortés **603**

**Antioxidant, Hemolytic and Cytotoxic Activities of *Senecio* Species used in Traditional Medicine of Northwestern Argentina**
Emilio Lizarraga, Felipe Castro, Francisco Fernández, Marina P. de Lampasona and César A. N. Catalán **607**

**Isolation of Antitrypanosomal Compounds from *Vitis repens*, a Medicinal Plant of Myanmar**
Khine Swe Nyunt, Ahmed Elkhateeb, Yusuke Tosa, Kensuke Nabata, Ken Katakura and Hideyuki Matsuura **609**

**Evaluation of Analgesic Activities of Tremetone Derivatives Isolated from the Chilean Altiplano Medicine *Parastrephia lepidophylla***
Julio Benites, Eunices Gutierrez, José López, Mauricio Rojas, Leonel Rojo, Maria do Céu Costa, Maria Pilar Vinardell and Pedro Buc Calderon **611**

**Ajuganane: A New Phenolic Compound from *Ajuga bracteosa***
Javid Hussain, Naeema Begum, Hidayat Hussain, Farman Ullah Khan, Najeeb Ur Rehman, Ahmed Al-Harrasi and Liaqat Ali **615**

**Base-mediated Transformations of 3,5-Dibromoverongiaquinol from the Sponge *Aplysina* sp. to Cavernicolins-1, -2 and a Subereatensin Analogue**
Elena A. Santalova **617**

**The Origin of Virgin Argan Oil's High Oxidative Stability Unraveled**
Saïd Gharby, Hicham Harhar, Dominique Guillaume, Aziza Haddad and Zoubida Charrouf **621**

**Chemical Composition of Essential Oils from a Multiple Shoot Culture of *Telekia speciosa* and Different Plant Organs**
Anna Wajs-Bonikowska, Anna Stojakowska and Danuta Kalemba **625**

**Evaluation of Volatile Constituents of *Cochlospermum angolense***
Michele Leonardi, Silvia Giovanelli, Pier Luigi Cioni, Guido Flamini and Luisa Pistelli **629**

**GC-MS Analysis of Aroma of *Medemia argun* (Mama-n-Khanen or Mama-n-Xanin), an Ancient Egyptian Fruit Palm**
Arafa I. Hamed, Michele Leonardi, Anna Stochmal, Wieslaw Oleszek and Luisa Pistelli **633**

**Composition of the Essential Oil of Wild Growing *Artemisia vulgaris* from Erie, Pennsylvania**
Jack D. Williams, Ayman M. Saleh and Dom N. Acharya **637**

**Chemical Composition of the Essential Oils from the Flower, Leaf and Stem of *Lonicera japonica***
Nenad Vukovic, Miroslava Kacaniova, Lukas Hleba and Slobodan Sukdolak **641**

***Jasminum sambac* Flower Absolutes from India and China – Geographic Variations**
Norbert A. Braun and Sherina Sim **645**

***In vitro* Bioactivity of Essential Oils and Methanol Extracts of *Salvia reuterana* from Iran**
Javad Safaei Ghomi, Reihaneh Masoomi, Fereshteh Jookar Kashi and Hossein Batooli **651**

**Investigation of the Volatile Constituents of Different *Gynura* Species from Two Chinese Origins by SPME/GC-MS**
Jian Chen, An Adams, Sven Mangelinckx, Bing-ru Ren, Wei-lin Li, Zheng-tao Wang and Norbert De Kimpe **655**

**Volatiles from *Michelia champaca* Flower: Comparative analysis by Simultaneous Distillation-Extraction and Solid Phase Microextraction**
Disnelys Báez, Diego Morales and Jorge A. Pino **659**

**Chemical Composition and Antibacterial Activity of the Essential Oil of *Espeletia nana***
Alexis Peña, Luis Rojas, Rosa Aparicio, Libia Alarcón, José Gregorio Baptista, Judith Velasco, Juan Carmona and Alfredo Usubillaga **661**

**The Composition and Antimicrobial Activities of *Cyperus conglomeratus*, *Desmos chinensis* var. *lawii* and *Cyathocalyx zeylanicus* Essential Oils**
Abdulkhader Hisham, Koranappallil B. Rameshkumar, Neelam Sherwani, Salim Al-Saidi and Salma Al-Kindy **663**

**Composition, Antimicrobial and Free-radical Scavenging Activities of the Essential Oil of *Plectranthus marrubatus***
Kaleab Asres, Solomon Tadesse, Avijit Mazumder and Franz Bucar **667**

**Constituents and Antimicrobial Activity of the Essential Oils from Flower, Leaf and Stem of *Helichrysum armenium***
Khodam-Ali Oji and Ali Shafaghat **671**

## ***Review/Account***

**Plant Essential Oils and Mastitis Disease: Their Potential InhibitoryEffects on Pro-inflammatory Cytokine Production in Response toBacteria Related Inflammation**
Ibrahim Taga, Christopher Q. Lan and Illimar Altosaar **675**

## *Number 6*

**Distribution of Drimane Sesquiterpenoids and Tocopherols in Liverworts, Ferns and Higher Plants: Polygonaceae, Canellaceae and Winteraceae Species**
Yoshinori Asakawa, Agnieszka Ludwiczuk, Liva Harinantenaina, Masao Toyota, Mayumi Nishiki, Alicia Bardon and Kaeko Nii **685**

**A Novel Isopimarane Diterpenoid with Acetylcholinesterase Inhibitory Activity from *Nepeta sorgerae*, an Endemic Species to the Nemrut Mountain**
Anıl Yılmaz, Pınar Çağlar, Tuncay Dirmenci, Nezhun Gören and Gülaçtı Topçu **693**

**Synthesis of Taxane ABC Tricyclic Skeleton from Lycoctonine**
Xiao-Xia Liang, Pei Tang, Qiao-Hong Chen and Feng-Peng Wang **697**

**Two Antiproliferative Saponins of *Tarenna grevei* from the Madagascar Dry Forest**
Liva Harinantenaina, Peggy J. Brodie, Martin W. Callmander, L. Jérémie Razafitsalama, Vincent E. Rasamison, Etienne Rakotobe and David G. I. Kingston **705**

**Revisit to 25*R*/25*S* Stereochemical Analysis of Spirostane-type Steroidal Sapogenins and Steroidal Saponins via $^1$H NMR Chemical Shift Data**
Pawan K. Agrawal, Torsten Burkholz and Claus Jacob **709**

**Structure-Cardiac Activity Relationship of $C_{19}$-Diterpenoid Alkaloids**
Xi-Xian Jian, Pei Tang, Xiu-Xiu Liu, Ruo-Bing Chao, Qiao-Hong Chen, Xue-Ke She, Dong-Lin Chen and Feng-Peng Wang **713**

**Unusual Reactions of a 7,17-*seco*-type $C_{19}$-Diterpenoid Alkaloid Derived from Deltaline**
Ling Wang, Qiao-Hong Chen, and Feng-Peng Wang **721**

**Cytotoxic and Anti-HIV Phenanthroindolizidine Alkaloids from *Cryptocarya chinensis***
Tian-Shung Wu, Chung-Ren Su and Kuo-Hsiung Lee **725**

**New Indole Alkaloid from *Peschiera affinis* (Apocynaceae)**
Allana Kellen L. Santos, Luciana L. Machado, Ayla Marcia C. Bizerra, Francisco José Q. Monte, Gilvandete M. P. Santiago, Raimundo Braz-Filho and Telma L. G. Lemos **729**

**Indole Alkaloids from *Vinca major* and *V. minor* Growing in Turkey**
Fatemeh Bahadori, Gülaçtı Topçu, Mehmet Boğa, Ayla Türkekul, Ufuk Kolak and Murat Kartal **731**

**Determination of the Absolute Configuration of 19-OH-(-)-eburnamonine Using a Combination of Residual Dipolar Couplings, DFT Chemical Shift Predictions, and Chiroptics**
Pablo Trigo-Mouriño, Roxana Sifuentes, Armando Navarro-Vázquez, Chakicherla Gayathri, Helena Maruenda and Roberto R. Gil **735**

**Unusual Nitrogenous Derivatives from *Alstonia***
Shin-Jowl Tan, G. Subramaniam, Noel F. Thomas and Toh-Seok Kam **739**

**Photoactivated [3+2] Addition of 6,7-*seco*-angustilobine B to Fullerene [$C_{60}$]**
Allan Patrick G. Macabeo, Dietmar Gehle, Karsten Krohn, Scott G. Franzblau and Alicia M. Aguinaldo **743**

**Flavone *C*-glycosides from *Anthurium andraeanum***
Benjamin R. Clark, Jon Y. Suzuki, Barbara J. Bliss and Robert P. Borris **747**

**LC-PDA-ESI/MS Identification of the Phenolic Components of Three Compositae Spices: Chamomile, Tarragon, and Mexican Arnica**
Long-Ze Lin and James M. Harnly **749**

**Cinnamoylphenethyl Amides from *Polygonum hyrcanicum* Possess Anti-Trypanosomal Activity**
Fahimeh Moradi-Afrapoli, Nargues Yassa, Stefanie Zimmermann, Soodabeh Saeidnia, Abbas Hadjiakhoondi, Samad N. Ebrahimi and Matthias Hamburger **753**

**Comparative Study of the *in vitro* Bioactivities of *Bupleurum rigidum* and *B. fruticescens***
Jose M. Prieto, Makanjuola O. Ogunsina, Andrea Novak, Amit Joshi, Judit Kokai, Ines Da Costa Rocha and Manuel Pardo de Santayana **757**

**Influence of Nutrient Medium Composition on *in vitro* Growth, Polyphenolic Content and Antioxidant Activity of *Alchemilla mollis***
Marina Stanilova, Rossen Gorgorov, Antoaneta Trendafilova, Milena Nikolova and Antonina Vitkova **761**

**Saracoside: A New Lignan Glycoside from *Saraca indica*, a Potential Inhibitor of DNA Topoisomerase IB**
Tulika Mukherjee, Sayan Chowdhury, Ashish Kumar, Hemanta K Majumder, Parasuraman Jaisanka and Sibabrata Mukhopadhyay **767**

**Secondary Metabolite Production by the Basidiomycete, *Lentinus strigellus,* under Different Culture Conditions**
Bartholomeu A. Barros-Filho, Maria C. F. de Oliveira, Jair Mafezoli, Francisco G. Barbosa and Edson Rodrigues-Filho **771**

**Phenylethanoid Glycosides isolated from *Paraboea paniculata***
Tan Hor Yue, Anthony Ho Siong Hock, Lim Chan Kiang and Lim Yang Mooi **775**

**Villarinol, a new Alkenoyloxyalkenol Derivative from the Endemic Philippine Rubiaceae species *Villaria odorata***
Allan Patrick G. Macabeo, Jalil A. Avila, Grecebio Jonathan D. Alejandro, Scott G. Franzblau, Simeon F. Kouam, Hidayat Hussain and Karsten Krohn **779**

**Isolation and Characterization of New Ceramides from Aerial Parts of *Lepidaploa cotoneaster***
Edlene O. dos Santos, Marilena Meira, Ademir E. do Vale, Jorge M. David, Luciano P. de Queiróz and Juceni P. David **781**

***cis*-Aconitic Anhydride Ethyl Ester and Phenolic Compounds from the Seeds of *Alisma orientale***
Ming Zhao, Jing-ying Chen, Li-jia Xu, Tanja Goedecke, Xiao-qi Zhang, Jin-ao Duan and Chun-tao Che **785**

**Suppression of Nitric Oxide Synthase by Thienodolin in Lipopolysaccharide-stimulated RAW 264.7 Murine Macrophage Cells**
Eun-Jung Park, John M. Pezzuto, Kyoung Hwa Jang, Sang-Jip Nam, Sergio A. Bucarey and William Fenical **789**

## ***Review/Account***

**Structural Characterization and Biological Effects of Constituents of the Seeds of *Alpinia katsumadai* (Alpina Katsumadai Seed)**
Joo-Won Nam and Eun-Kyoung Seo **795**

**Inventory, Constituents and Conservation of Biologically Important Sumatran Plants**
Dayar Arbain **799**

**Herbal Medicine in Healthcare-An Overview**
Mohammed Mosihuzzaman **807**

## *Number 7*

**Chemical Constituents of *Blumea balsamifera* of Indonesia and Their Protein Tyrosine Phosphatase 1B Inhibitory Activity**
Azis Saifudin, Ken Tanaka, Shigetoshi Kadota and Yasuhiro Tezuka **815**

**A New Sesquiterpene from an Endophytic *Aspergillus versicolor* Strain**
Xiang-Hong Liu, Feng-Ping Miao, Xiao-Dong Li, Xiu-Li Yin and Nai-Yun Ji **819**

**Skin Permeation of Cacalol, Cacalone and 6-*epi*-Cacalone Sesquiterpenes from a Nanoemulsion**
María Luisa Garduño-Ramírez, Beatriz Clares, Valeri Domínguez-Villegas, Concepción Peraire, María Adolfina Ruiz, María Luisa García and Ana C. Calpena **821**

**Compounds with Antiproliferative Activity on Five Human Cancer Cell Lines from South Korean *Carpesium triste***
Hyung-In Moon **825**

**Biogenetic-type Synthesis of 2-Hydroxy-4,4,7-trimethyl-1(4*H*)-naphthalenone, a Modified Apocarotenoid from *Ipomoea pes-caprae***
Kamalesh P. Pai Fondekar, Shashikumar K. Paknikar, Savia Torres and Shrivallabh P. Kamat **827**

**Ixoroid: A New Triterpenoid from the Flowers of *Ixora coccinea***
Muhammad Ali Versiani, Ambreen Ikram, Salman Khalid, Shaheen Faizi and Iftikhar Ahmed Tahiri **831**

**Distinguishing Between *R*- and *S*-Antcin C and Their Cytotoxicity**
Ting-Yu Lin, Shih-Chang Chien, Yueh-Hsiung Kuo and Sheng-Yang Wang **835**

**Chemical Investigation of Saponins from Twelve Annual *Medicago* Species and their Bioassay with the Brine Shrimp *Artemia salina***
Aldo Tava and Luciano Pecetti **837**

**Inhibition of cPLA$_2$ and sPLA$_2$ Activities in Primary Cultures of Rat Cortical Neurons by *Centella asiatica* Water Extract**
Patrícia P. Defillipo, André H. Raposo, Alessandra G. Fedoce, Aline S. Ferreira, Hudson C. Polonini, Wagner F. Gattaz and Nádia R. B. Raposo **841**

**Triterpene Glycosides from the Sea Cucumber *Eupentacta fraudatrix*. Structure and Cytotoxic Action of Cucumariosides A$_2$, A$_7$, A$_9$, A$_{10}$, A$_{11}$, A$_{13}$ and A$_{14}$, Seven New Minor Non-Sulfated Tetraosides and an Aglycone with an Uncommon 18-Hydroxy Group**
Alexandra S. Silchenko, Anatoly I. Kalinovsky, Sergey A. Avilov, Pelageya V. Andryjaschenko, Pavel S. Dmitrenok, Ekaterina A. Martyyas and Vladimir I. Kalinin **845**

**Two New Asterosaponins from the Far Eastern Starfish *Lethasterias fusca***
Natalia V. Ivanchina, Anatoly I. Kalinovsky, Alla A. Kicha, Timofey V. Malyarenko, Pavel S. Dmitrenok, Svetlana P. Ermakova and Valentin A. Stonik **853**

**Corylucinine, a new Alkaloid from *Corydalis cava* (Fumariaceae), and its Cholinesterase Activity**
Zdeněk Novák, Jakub Chlebek, Lubomír Opletal, Pavel Jiroš, Kateřina Macáková, Jiří Kuneš and Lucie Cahlíková **859**

**Improved Method for Isolation of Lycopsamine from Roots of Comfrey (*Symphytum officinale*)**
Damjan Janeš, Boštjan Kalamar and Samo Kreft **861**

**Trigonelline and other Betaines in Species of Laminariales**
Gerald Blunden, Michael D. Guiry, Louis D. Druehl, Kazuhiro Kogame and Hiroshi Kawai **863**

**Anticomplement and Antimicrobial Activities of Flavonoids from *Entada phaseoloides***
Ke Li, Shihua Xing, Mengyue Wang, Ying Peng, Yuqiong Dong and Xiaobo Li **867**

**Antioxidant Compounds from Algerian *Convolvulus tricolor* (Convolvulaceae) Seed Husks**
Nassira Kacem, Anne-Emmanuelle Hay, Andrew Marston, Amar Zellagui, Salah Rhouati and Kurt Hostettmann **873**

**Quality Control and Analytical Test Method for *Taxus baccata* Tincture Preparation**
Pamela Vignolini, Beatrice Gehrmann, Matthias Friedrich Melzig, Leonardo Borsacchi, Arianna Scardigli and Annalisa Romani **875**

**Chalcones in Bioactive Argentine Propolis Collected in Arid Environments**
Eliana Solórzano, Nancy Vera, Soledad Cuello, Roxana Ordoñez, Catiana Zampini, Luis Maldonado, Enrique Bedascarrasbure and María I. Isla **879**

**Inhibitory Effect of Hexahydrocurcumin on Human Platelet Aggregation**
Huei-Ping Dong, Rei-Cheng Yang, I-Chun Chunag, Li-Ju Huang, Hsing-Tan Li, Hsin-Liang Chen and Chung-Yi Chen **883**

**Biotransformation of Salvianolic acid B by *Fusarium oxysporum* f. sp. *Cucumerinum* and Its Two Degradation Routes**
Shidong Kan, Huimin Lin, Ji'an Li, Lei Shao and Daijie Chen **885**

**Phytopathogenic Fungal Inhibitors from Celery Seeds**
Tao Liu, Fu-Guang Liu, Hui-Qin Xie and Qing Mu **889**

**Synthesis and Antimicrobial Activities of Some Sulphur Containing Chromene Derivatives**
Tuba Şerbetçi, Seher Birteksöz, Soizic Prado, Sylvie Michel and François Tillequin **891**

**Effect of Polyamines on Shoot Multiplication and Furanocoumarin Production in *Ruta graveolens* Cultures**
Renuka Diwan and Nutan Malpathak **895**

**Antihyperglycemic agents from *Ammannia multiflora***
Harish C. Upadhyay, Natasha Jaiswal, Akhilesh K. Tamrakar, Arvind K. Srivastava, Namita Gupta and Santosh K. Srivastava **899**

**Free Radical Scavenging Activities of Naturally Occurring and Synthetic Analogues of Sea Urchin Naphthazarin Pigments**
Natalia K. Utkina and Natalia D. Pokhilo **901**

***Drynariae Rhizoma* Increases Immune Response in Mice**
Hyo-Jin An, Gil-Goo Lee and Kyung-Tae Lee **905**

**Antioxidant, Antimicrobial and Wound Healing Activities of *Boesenbergia rotunda***
Rungrat Jitvaropas, Suphaket Saenthaweesuk, Nuntiya Somparn, Amornnat Thuppia, Seewaboon Sireeratawong and Waranyoo Phoolcharoen **909**

**Revisit to (*Z*)-Civetone Synthesis**
Hisahiro Hagiwara, Teppei Adachi, Tomomi Nakamura, Takashi Hoshi and Toshio Suzuki **913**

**Search for Bioactive Compounds from *Cantharellus cibarius***
Włodzimierz Maria Daniewski, Witold Danikiewicz, W. Marek Gołębiewski, Mirosław Gucma, Agnieszka Łysik, Jacek Grodner and Elżbieta Przybysz **917**

**Fatty Acid Composition of *Juniperus* Species (*Juniperus* Section) Native to Turkey**
Ayşegül Güvenç, Nurgün Küçükboyacı and Ahmet Ceyhan Gören **919**

**c-AMP Dependent Protein Kinase A Inhibitory Activity of Six Algal Extracts from South Eastern Australia and Their Fatty Acid Composition**
Ana Zivanovic and Danielle Skropeta **923**

**Quantitative and Physical Evaluation of Patchouli Essential Oils Obtained from Different Sources of *Pogostemon cablin***
Norma Hussin, Luigi Mondello, Rosaria Costa, Paola Dugo, Nik Idris Nik Yusoff, Mohd Ambar Yarmo, Ahmad Ab.Wahab and Mamot Said **927**

**Essential Oil Composition of *Prasium majus* from Croatia**
Igor Jerković, Marko Šuste, Željan Maleš and Kroata Hazler Pilepić **931**

**Composition and Antipathogenic Activities of the Twig Essential Oil of *Chamaecyparis formosensis* from Taiwan**
Chen-Lung Ho, Kuo-Feng Hua, Kuan-Ping Hsu, Eugene I-Chen Wang and Yu-Chang Su **933**

***In vitro* Antimicrobial Properties and Chemical Composition of *Santolina chamaecyparissus* Essential Oil from Algeria**
Samah Djeddi, Khadidja Djebile, Ghania Hadjbourega, Zoubida Achour, Catherine Argyropoulou and Helen Skaltsa **937**

**Chemical Composition and *in vitro* Antimicrobial Activity of the Essential Oil of the Flowers of *Tridax procumbens***
Rajesh K. Joshi and Vijaylaxmi Badakar **941**

**Chemical Composition and Antimicrobial Activity of Essential Oil of *Heracleum rigens***
Nataraj Jagannath, Hanumanthaiah Ramakrishnaiah, Venkatarangaiah Krishna and Prameela Javarayi Gowda **943**

**Chemical Composition and *in vitro* Evaluation of Antimicrobial and Anti-acetylcholinesterase Properties of the Flower Oil of *Ferula lutea***
Mansour Znati, Aymen Jabrane, Hafedh Hajlaoui, Fethia Harzallah-Skhiri Jalloul Bouajila, Joseph Casanova and Hichem Ben Jannet **947**

**Determination of Antioxidant Properties of 26 Chilean Honeys and a Mathematical Association Study with their Volatile Profile**
Elizabeth Sánchez, Marisa Piovano, Erika Valdés, Manuel E. Young, Cristian A. Acevedo and Mauricio Osorio **951**

**Chemical Constituents and Antioxidant and Biological Activities of the Essential Oil from Leaves of *Solanum spirale***
Sukanya Keawsa-ard, Boonsom Liawruangrath, Saisunee Liawruangrath, Aphiwat Teerawutgulrag and Stephen G. Pyne **955**

## *Review/Account*

**Acetylcholinesterase Inhibition within the Lycorine Series of Amaryllidaceae Alkaloids**
Jerald J. Nair and Johannes van Staden **959**

**Alkaloids Produced by Endophytic Fungi: A Review**
Yanyan Zhang, Ting Han, Qianliang Ming, Lingshang Wu, Khalid Rahman and Luping Qin **963**

## *Number 8*

**Terpenoids from *Cichorium intybus***
Chang-Xin Zhou, Li Zou, Zong-Zheng Zhao, Hong Zhu, Qiao-Jun He, Bo Yang and Li-She Gan **971**

**(6*R*,9*S*)-6′′-(4′′-Hydroxybenzoyl)-Roseoside, a New Megastigmane Derivative from *Ouratea polyantha* and its Effect on Hepatic Glucose-6-phosphatase**
Jairo Bermúdez, María Rodríguez, Masahisa Hasegawa, Freddy González-Mujica, Sandra Duque and Yoichiro Ito **973**

**The Cytotoxic Activity of Diterpenoids from *Isodon* species**
Ayumi Ohsaki, Masaaki Ozawa, Kanki Komiyama, Akio Kishida and Takahiko Isobe **977**

**Anti-proliferative Activity and Apoptotic Potential of Britannin, a Sesquiterpene Lactone from *Inula aucheriana***
Maryam Hamzeloo Moghadam, Homa Hajimehdipoor, Soodabeh Saeidnia, Azadeh Atoofi, Roxana Shahrestani, Roger W. Read and Mahmoud Mosaddegh **979**

**Betulin as an Antitumor Agent Tested *in vitro* on A431, HeLa and MCF7, and as an Angiogenic Inhibitor *in vivo* in the CAM Assay**
Cristina Adriana Dehelean, Stefana Feflea, Judit Molnár, Istvan Zupko and Codruta Soica **981**

**Constituents of Twig Bark of Pear Cultivars (*Pyrus* species)**
Hideyuki Tomosaka, Hideaki Tamimoto, Yuki Tsukagoshi, Yasutsugu Suzuki, Hisako Ooka and Michiya Ota **987**

**Bio-assay Guided Isolation of α-Glucosidase Inhibitory Constituents from *Eclipta alba***
Deepak Kumar, Raghuvir H. Gaonkar, Rina Ghosh and Bikas C. Pal **989**

**Chromolithic Method Development, Validation and System Suitability Analysis of Ultra-Sound Assisted Extraction of Glycyrrhizic Acid and Glycyrrhetinic Acid from *Glycyrrhiza glabra***
Suphla Gupta, Rajni Sharma, Pankaj Pandotra, Sundeep Jaglan and Ajai Prakash Gupta **991**

**Furostanol Saponin and Diphenylpentendiol from the Roots of *Asparagus racemosus***
Upendra Sharma, Neeraj Kumar and Bikram Singh **995**

**Quinolizidine Alkaloids from *Sophora velutina* subsp. *zimbabweensis* (Fabaceae: Sophoreae)**
Erick Korir, Joyce J. Kiplimo, Neil R. Crouch, Nivan Moodley and Neil. A. Koorbanally **999**

**Flavonoids from the Japanese Monotypic Genus, *Nipponanthemum***
Ayumi Uehara and Tsukasa Iwashina **1005**

**The Inhibitory Effects of Representative Chalcones Contained in *Angelica keiskei* on Melanin Biosynthesis in B16 Melanoma Cells**
Enos Tangke Arung, Shoko Furuta, Kazuhiro Sugamoto, Kuniyoshi Shimizu, Hiroya Ishikawa, Yoh-ichi Matsushita and Ryuichiro Kondo **1007**

**Flavonoid Constituents of *Pistacia integerrima***
Zia Ullah, Rashad Mehmood, Muhammad Imran, Abdul Malik and Rehana A. Afzal **1011**

**Antioxidant and Anti-cholinesterase Activity of *Globularia meridionalis* Extracts and Isolated Constituents**
Rosa Tundis, Marco Bonesi, Federica Menichini, Monica R. Loizzo, Filomena Conforti, Giancarlo Statti, Filippo M. Pirisi and Francesco Menichini **1015**

**Antioxidant Activities and Total Phenolic and Flavonoid Contents in Three Indigenous Medicinal Vegetables of North-east India**
Jyotirekha G. Handique, Manas Pratim Boruah and Dipika Kalita **1021**

***In Vitro* Antiviral Activity of *Heterophyllaea pustulata* Extracts**
Brenda S. Konigheim, Mauricio Beranek, Laura R. Comini, Javier J. Aguilar, Juliana Marioni, José L. Cabrera, Marta S. Contigiani and Susana C. Núñez Montoya **1025**

**Re-investigation of the Anthraquinone Pool of *Rhamnus* spp.: Madagascin from the Fruits of *Rhamnus cathartica* and *R. intermedia***
Francesco Epifano, Salvatore Genovese, Dario Kremer, Marco Randic, Giuseppe Carlucci and Marcello Locatelli **1029**

**Two New Phloroglucinol Derivatives and Five Photosensitizing Pheophorbides from *Syzygium polyanthum* Leaves (Salam)**
Lee Wei Har, Khozirah Shaari, Lee Hong Boon, Fadzly A. Kamarulzaman and Intan S. Ismail **1033**

**Production of *Curcuminoids* in Different *in vitro* Organs of *Curcuma longa***
Laura Pistelli, Alessandra Bertoli, Federica Gelli, Laura Bedini, Barbara Ruffoni and Luisa Pistelli **1037**

**Two New Alkylresorcinols from *Homalomena wendlandii* and Their Cytotoxic Activity**
Luis A. Sánchez, Dionisio Olmedo, José Luis López-Pérez, Todd D. Williams and Mahabir P. Gupta **1043**

**Amides and an Alkaloid from *Portulaca oleracea***
Tetsuo Kokubun, Geoffrey C. Kite, Nigel C. Veitch and Monique S. J. Simmonds **1047**

**Synthesis and Biological Evaluation of Combretastatin A-4 and Three Combretastatin-Based Hybrids**
Miguel A. González, David Pérez-Guaita, Lee S. Agudelo-Goméz, Verónica Tangarife-Castaño, Bibiana Zapata and Liliana Betancur-Galvis **1051**

**Two New *p*-Terphenyl Derivatives from the Marine Fungal Strain *Aspergillus* sp. AF119**
Shao-Song Liu, Bao-Bing Zhao, Chun-Hua Lu, Jing-Jing Huang and Yue-Mao Shen **1057**

**Quantitative Analysis of Fructo-oligosaccharides in *Gynura divaricata* subsp. *formosana* by High Performance Anion Exchange Chromatography−Pulsed Amperometric Detection**
Shen-Chieh Chou and Shoei-Sheng Lee **1063**

**Polyketide Metabolites from the Endophytic Fungus *Microdiplodia* sp. KS 75-1**
Yoshihito Shiono, Taiji Hatakeyama, Tetsuya Murayama and Takuya Koseki **1065**

**Muscone Exerts Neuroprotection in an Experimental Model of Stroke via Inhibition of the Fas Pathway**
Gang Wei, Dong-Feng Chen, Xiao-Ping Lai, Dong-Hui Liu, Ru-Dong Deng, Jian-Hong Zhou, Sai-Xia Zhang, Yi-Wei Li, Hui Li and Qiong-Dan Zhang **1069**

**Cytokinin and Auxin Effect on the Terpenoid Profile of the Essential Oil and Morphological Characteristics of Shoot Cultures of *Artemisia alba***
Kalina Danova, Milka Todorova, Antoaneta Trendafilova and Ljuba Evstatieva **1075**

**Variation in the Volatile Constituents of Different Plant Parts of *Ligusticopsis wallichiana* from Western Himalaya, India**
Rajendra C. Padalia, Ram S. Verma, Amit Chauhan, Chandan S. Chanotiya and Anju Yadav **1077**

**Determination of Chemical Constituents of Leaf and Stem Essential Oils of *Artemisia monosperma* from Central Saudi Arabia**
Merajuddin Khan, Ahmad A. Mousa, Kodakandla V. Syamasundar and Hamad Z. Alkhathlan **1079**

**Chemical Composition of the Essential Oils of *Centaurea formanekii* and *C. orphanidea* ssp. *thessala*, Growing Wild in Greece**
Mariem Ben Jemia, Carmen Formisano, Svetlana Bancheva, Maurizio Bruno and Felice Senatore **1083**

**Phytochemical Profiles of Volatile Constituents from *Centaurea ragusina* Leaves and Flowers and their Antimicrobial Effects**
Olivera Politeo, Mirjana Skocibusic, Ivana Carev, Franko Burcul, Igor Jerkovic, Mladenka Sarolic and Mladen Milos **1087**

**Chemical Composition and Antimicrobial Activity of Essential Oil of Different Parts of *Seseli rigidum***
Mirjana Marčetić, Dragana Božić, Marina Milenković, Branislava Lakušić and Nada Kovačević **1091**

**Chemical Composition, Olfactory Analysis and Antibacterial Activity of *Thymus vulgaris* Chemotypes Geraniol, 4-Thujanol/Terpinen-4-ol, Thymol and Linalool Cultivated in Southern France**
Erich Schmidt, Jürgen Wanner, Martina Höferl, Leopold Jirovetz, Gerhard Buchbauer, Velizar Gochev, Tania Girova, Albena Stoyanova and Margit Geissler **1095**

**Chemical Composition, Antimicrobial, Antioxidant and Cytotoxic Activity of Essential Oils of *Plectranthus cylindraceus* and *Meriandra benghalensis* from Yemen**
Nasser A. Awadh Ali, Martina Wurster, Annika Denkert, Norbert Arnold, Iman Fadail, Gamal Al-Didamony, Ulrike Lindequist, LudgerWessjohann and William N. Setzer **1099**

**Electrophysiological Responses of the *Naupactus bipes* Beetle to Essential Oils from Piperaceae Species**
Clécio S. Ramos, Marisi G. Soares, Adalberto M. da Silva, Luciane G. Batista-Pereira, Arlene G. Corrêa and Massuo J. Kato **1103**

**Inhibition of Essential Bacterial Peptidyl-tRNA Hydrolase Activity by Tropical Plant Extracts**
Hana McFeeters, Morgan J. Gilbert, Rachel M. Thompson, William N. Setzer, Luis R. Cruz-Vera, and Robert L. McFeeters **1107**

## ***Review/Account***

**Analytical Profiling of Bioactive Constituents from Herbal Products, using Metabolomics - A Review**
Nanjappan Satheeshkumar, Narayanan Nisha, Nirmal Sonali, Jayabalan Nirmal, Gaurav K. Jain, and Vudataneni Spandana **1111**

## *Number 9*

**Catalytic and Molecular Properties of Rabbit Liver Carboxylesterase Acting on 1,8-Cineole Derivatives**
María del H. Loandos, Ana C. Muro, Margarita B. Villecco, Marcelo F. Masman, Paul G.M. Luiten, Sebastian A. Andujar, Fernando D. Suvire and Ricardo D. Enriz **1117**

**Antiparasitic and Anticancer Carvotacetone Derivatives of *Sphaeranthus bullatus***
Francis Machumi, Abiy Yenesew, Jacob O. Midiwo, Matthias Heydenreich, Erich Kleinpeter, Babu L. Tekwani, Shabana I. Khan, Larry A. Walker and Ilias Muhammad **1123**

**Naturally Occurring Limonene to Cinnamyl-type γ-Butyrolactone Substituted Aldol Condensation Derivatives as Antioxidant Compounds**
Pushpinder Kaur, Pralay Das, Abha Chaudhary and Bikram Singh **1127**

**Sesquiterpenes from *Onopordum illyricum* and their Antifeedant Activity**
Sergio Rosselli, Antonella Maria Maggio, Marisa Canzoneri, Monique S. J. Simmonds and Maurizio Bruno **1131**

**Three New Lactarane Sesquiterpenoids from the Mushroom *Russula sanguinea***
Yasunori Yaoita, Moe Hirao, Masao Kikuchi and Koichi Machida **1133**

**Royleanumin, a New Phytotoxic *neo*-Clarodane Diterpenoid from *Teucrium royleanum***
Shabir Ahmad, Muhammad Arfan, Naheed Riaz, Riaz Ullah, Ziarat Shah and Azhar Ul-Haq Ali Shah **1137**

**Bioactive-guided Identification of Labdane Diterpenoids from Aerial Parts of *Aristeguietia glutinosa* as anti-*Trypanosoma cruzi* agents**
Javier Varela, María L. Lavaggi, Mauricio Cabrera, Alejandra Rodríguez, Patricio Miño, Ximena Chiriboga, Hugo Cerecetto and Mercedes González **1139**

**Characterization of Yew Tree (*Taxus*) Varieties by Fingerprint and Principal Component Analyses**
Kalle Truus, Merike Vaher, Maria Borissova, Marju Robal, Tuuli Levandi, Rando Tuvikene, Peeter Toomik and Mihkel Kaljurand **1143**

**Identification of Triterpenes from Milkweed (*Asclepias syriaca*)**
Erzsébet Háznagy-Radnai, Edit Wéber, Szilvia Czigle and Imre Máthé **1147**

**Evaluation of the Anti-angiogenic Activity of Saponins from *Maesa lanceolata* by Different Assays**
Kenn Foubert, Annelies Breynaert, Mart Theunis, Rita Van Den Bossche, Guido R. Y De Meyer, André Van Daele, Ahmad Faizal, Alain Goossens, Danny Geelen, Edward M. Conway, Arnold Vlietinck, Luc Pieters and Sandra Apers **1149**

**Conversion of Protopanaxadiol Type Saponins to Ginsenoside Rg3 by Lemon**
Cheng-Peng Sun, Wei-Ping Gao, Bao-Zhong Zhao and Le-Qin Cheng **1155**

**Triterpene Glycosides from the Sea Cucumber *Eupentacta fraudatrix*. Structure and Biological Activity of Cucumariosides $B_1$ and $B_2$,Two New Minor Non-Sulfated Unprecedented Triosides**
Alexandra S. Silchenko, Anatoly I. Kalinovsky, Sergey A. Avilov, Pelageya V. Andryjaschenko, Pavel S. Dmitrenok, Ekaterina A. Martyyas and Vladimir I. Kalinin **1157**

**Osteoclastogenesis Inhibitory Effect of Ergosterol Peroxide Isolated from *Pleurotus eryngii***
Satoru Yokoyama, Tran Hai Bang, Kuniyoshi Shimizu and Ryuichiro Kondo **1163**

**A Novel Sterol Sulfate and New Oligosaccharide Polyester from the Aerial Parts of *Polygala sibirica***
Yue Lin Song, Si Xiang Zhou, He Lin Wei, Yong Jiang and Peng Fei Tu **1165**

**Angustinine – A New Benzopyridoquinolizine Alkaloid from *Alangium lamarckii***
Mumu Chakraborty and Sibabrata Mukhopadhyay **1169**

**Antifungal Activity of Hydrochloride Salts of Tylophorinidine and Tylophorinine**
Mini Dhiman, Rajashri R. Parab, Sreedharannair L. Manju, Dattatraya C. Desai and Girish B. Mahajan **1171**

**Evaluation of Antibacterial and Anti-biofilm Activities of Cinchona Alkaloid Derivatives against *Staphylococcus aureus***
Malena E. Skogman, Janni Kujala, Igor Busygin, Reko Leino, Pia M. Vuorela and Adyary Fallarero **1173**

**Simultaneous Determination of Alkaloids and Flavonoids from Aerial Parts of *Passiflora* Species and Dietary Supplements using UPLC-UV-MS and HPTLC**
Bharathi Avula, Yan-Hong Wang, Chidananda Swamy Rumalla, Troy J. Smillie and Ikhlas A. Khan **1177**

**Chemical Fingerprinting by RP-RRLC-DAD and Principal Component Analysis of *Ziziphora clinopodioides* from Different Locations**
Shuge Tian, Qian Yu, Lude Xin, Zhaohui Sunny Zhou and Halmuart·Upur **1181**

**Simultaneous Determination of Four Major Constituents of Semen Vaccariae Using HPLC-DAD**
Haijiang Zhang, Wei Yao, Yunyun Chen, Peipei He, Yao Chen, Peipei Chen, Jia Han and Xiaoyu Li **1185**

**Biological Activity of Isoflavonoids from *Azorella madreporica***
Luisa Quesada, Carlos Areche, Luis Astudillo, Margarita Gutiérrez, Beatriz Sepúlveda and Aurelio San-Martín **1187**

**Analysis of Homoisoflavonoids in *Caesalpinia digyna* by HPLC-ESI-MS, HPLC and Method Validation**
Somendu K. Roy, Amit Srivastava and Sanjay M. Jachak **1189**

**Oligonol-induced Degradation of Perilipin 1 is Regulated through Lysosomal Degradation Machinery**
Junetsu Ogasawara, Kentaro Kitadate, Hiroshi Nishioka, Hajime Fujii, Takuya Sakurai, Takako Kizaki, Tetsuya Izawa, Hitoshi Ishida and Hideki Ohno **1193**

**Plant Growth Inhibitor from the Malaysian Medicinal Plant *Goniothalamus andersonii* and Related Species**
Tomoko Takemura, Tsunashi Kamo, Raihan Ismil, Baki Bakar, Naoya Wasano, Syuntaro Hiradate and Yoshiharu Fujii **1197**

**Furanone Derivatives from Terrestrial *Streptomyces* spp.**
Muhammad Arfan, Khaled A. Shaaban, Anja Schüffler and Hartmut Laatsch **1199**

**Cytotoxicity Analysis of Active Components in Bitter Melon (*Momordica charantia*) Seed Extracts Using Human Embryonic Kidney and Colon Tumor Cells**
Elizabeth S. Chipps, Renuka Jayini, Shoko Ando, April D. Protzman, M. Zubayed Muhi, M. Abdul Mottaleb, Ahmed Malkawi and M. Rafiq Islam **1203**

**Phytochemical and Biological Studies on Some Egyptian Seaweeds**
Khaled N. M. Elsayed, Mohamed M. Radwan, Sherif H. M. Hassan, Mohamed S. Abdelhameed, Ibraheem B. Ibraheem, and Samir A. Ross **1209**

**Electrochemical Characterization of the Marine Antioxidant Gadusol**
Ernesto Maximiliano Arbeloa, Cristina Luján Ramírez, Raúl Ariel Procaccini and María Sandra Churio **1211**

**Insecticidal Properties of Annonaceous Acetogenins and Their Analogues. Interaction with Lipid Membranes**
Lilian Di Toto Blessing, Juan Ramos, Sonia Diaz, Aída Ben Altabef, Alicia Bardón, Margarita Brovetto, Gustavo Seoane and Adriana Neske **1215**

**Activity of Alkanediol Alkanoates against Pathogenic Plant Fungi *Rhizoctonia solani* and *Sclerotium rolfsii***
Paraj Shukla, Suresh Walia, Vivek Ahluwalia, Balraj S. Parmar and Muraleedharan G. Nair **1219**

**A Somaclonal Variant of Rose-Scented Geranium (*Pelargonium* spp.) with Moderately High Content of Isomenthone in its Essential Oil**
Swaroop S Kulkarni, Nagawara S Ravindra, Kalavagunta V N S Srinivas and Raghavendra N Kulkarni **1223**

**Comparative Study of the Chemical Composition of Essential Oils of Five *Tagetes* Species Collected in Venezuela**
Kaylin Armas, Janne Roja, Luis Rojas and Antonio Morales **1225**

**Chemical Composition and Antiphytoviral Activity of Essential Oil of *Micromeria graeca***
Elma Vuko, Valerija Dunkić, Nada Bezić, Mirko Ruščić and Dario Kremer **1227**

**Chemical Composition and Biological Activity of the Essential Oil from Jamaican *Cleome serrata***
Megil J. McNeil, Roy B. R. Porter and Lawrence A. D. Williams **1231**

**Chemical Compositions and Biological Activities of *Amomum subulatum* Essential Oils from Nepal**
Prabodh Satyal, Noura S. Dosoky, Brittany L. Kincer and William N. Setzer **1233**

**Chemical Composition and Antimicrobial Activity of *Salvia x jamensis* Essential Oil**
Daniele Fraternale, Guido Flamini, Angela Bisio, Maria Cristina Albertini and Donata Ricci **1237**

**Antimicrobial and Antioxidant Activities of *Stachys lavandulifolia* subsp. *lavandulifolia* Essential Oil and its Infusion**
Gökalp İşcan, Betül Demirci, Fatih Demirci, Fatih Göger, Neşe Kırımer, Yavuz B. Köse and Kemal Hüsnü Can Başer **1241**

**Composition, Anticancer, and Antimicrobial Activities *in vitro* of the Heartwood Essential Oil of *Cunninghamia lanceolata* var. *konishii* from Taiwan**
Yu-Chang Su, Kuan-Ping Hsu, Eugene I-Chen Wang and Chen-Lung Ho **1245**

***Review/Account***

**Secondary Metabolites of *Eichhornia crassipes* (Waterhyacinth): A Review (1949 to 2011)**
Pottail Lalitha, Shubashini.K.Sripathi and Ponnusamy Jayanthi **1249**

## *Number 10*

**HPLC Analysis and Cytotoxic Activity of *Vernonia cinerea***
Mom Khay, Phirom Toeng, Valérie Mahiou-Leddet, Fathi Mabrouki, Kim Sothea, Evelyne Ollivier, Riad Elias and Sok-Siya Bun **1259**

**Antiplasmodial Activity of the Ethnobotanical Plant *Cassia fistula***
Mary H. Grace, Carmen Lategan, Rocky Graziose, Peter J. Smith, Ilya Raskin and Mary Ann Lila **1263**

**Trichilone, a New $C_{21}$ Steroid from *Trichilia connaroides***
Qiang Zhang, Yu Zhang, Hong-Ping He, Ying-Tong Di and Xiao-Jiang Hao **1267**

**Three Novel Immunosuppressive Steroidal Glycosides from the Stems of *Stephanotis mucronata***
Xiao-yu Li, Shu-ling Zong, Feng-yang Chen, Shi-fang Xu and Yi-ping Ye **1269**

**Optimization of Carbon Source for Hairy Root Growth and Withaferin A and Withanone Production in *Withania somnifera***
Ganeshan Sivanandhan, Manoharan Rajesh, Muthukrishnan Arun, Murugaraj Jeyaraj, Gnanajothi Kapil Dev, Markandan Manickavasagam, Natesan Selvaraj and Andy Ganapathi **1271**

**Quantitative Analysis of Substituted *N,N*-Dimethyl-tryptamines in the Presence of Natural Type XII Alkaloids**
Bojidarka Ivanova and Michael Spiteller **1273**

**HPLC Analysis of Stemokerrine and Oxystemokerrine in the Thai Medicinal Plant *Stemona kerrii***
Sumet Kongkiatpaiboon, Vichien Keeratinijakal and Wandee Gritsanapan **1277**

**Identification of Pavinane Alkaloids in the Genera *Argemone* and *Eschscholzia* by GC-MS**
Lucie Cahlíková, Radim Kučera, Anna Hošťálková, Jiří Klimeš and Lubomír Opletal **1279**

**Effect of Piperine on the Pharmacokinetics and Pharmacodynamics of Glimepiride in Normal and Streptozotocin - induced Diabetic Rats**
Ciddi Veeresham, Samala Sujatha and Thatipamula Sandya Rani **1283**

***In Silico* Prediction of the Cosmetic Whitening Effects of Naturally Occurring Lead Compounds**
Pedro Fong and Henry H. Y. Tong **1287**

**Profiling Flavonoid Cytotoxicity in Human Breast Cancer Cell Lines: Determination of Structure-Function Relationships**
Sina Yadegarynia, Anh Pham, Alex Ng, Duong Nguyen, Tetiana Lialiutska, Anthony Bortolazzo, Valentin Sivryuk, Martina Bremer and J. Brandon White **1295**

**Two New Flavonoids from the Seeds of *Derris scandens***
Rachakunta Munikishore, Aluru Rammohan, Adivireddy Padmaja, Duvvuru Gunasekar, Alexandre Deville and Bernard Bodo **1305**

**Dihydroflavonol and Flavonol Derivatives from *Macaranga recurvata***
Mulyadi Tanjung, Euis H. Hakim, Elfahmi, Jalifah Latip and Yana M. Syah **1309**

**Flavonoids Bearing an *O*-arabinofuranosyl-(1→3)-rhamnoside Moiety from *Cladocolea micrantha*: Inhibitory Effect on Human Melanoma Cells**
Anderson C. Guimarães, Alvicler Magalhães, Marcos J. Nakamura, Antonio C. Siani, Christina Barja-Fidalgo and André L. F. Sampaio **1311**

**Effect of Seasonality on Chemical Composition and Antibacterial and Anticandida Activities of Argentine Propolis. Design of a Topical Formulation**
María Inés Isla, Yanina Dantur, Ana Salas, Carolina Danert, Catiana Zampini, Myriam Arias, Roxana Ordóñez, Luis Maldonado, Enrique Bedascarrasbure and María Inés Nieva Moreno **1315**

**Larvicidal Activity of Isoflavonoids from *Muellera frutescens* Extracts against *Aedes aegypti***
Charlotte Nirma, Alice M S. Rodrigues, Charlie Basset, Lionel Chevolot, Romain Girod, Christian Moretti, Didier Stien, Isabelle Dusfour and Véronique Eparvier **1319**

**Phenolic Compounds in Five *Epilobium* Species Collected from Estonia**
Indrek Remmel, Lauri Vares, Lauri Toom, Vallo Matto and Ain Raal **1323**

**A New Prenylated Acetophenone from the Root Bark of *Derris indica***
Naushad Edayadulla and Penugonda Ramesh **1325**

**Phytotoxic Furanocoumarins from the Shoots of *Semenovia transiliensis***
Shobha Sondhia, Stephen O. Duke, Solomon Green III, Nadezhda G. Gemejiyeva, Leonid K. Mamonov and Charles L Cantrell **1327**

**Chemoenzymatic Synthesis of Two New Halogenated Coumarin Glycosides as Potential Antifungal Agents**
Liangbin Zhou, Ling Liu, Tian Tian, Bailin Xue and Rongmin Yu **1331**

**Two New Naphthoquinone Derivatives from the Stem Bark of *Callicarpa maingayi***
Sumayah Mohammed Asiri, Khozirah Shaari, Faridah Abas, Nabil Ali Al-Mekhlafi and Nordin H. Lajis **1333**

**Novel Sorbicillin Analogues from the Marine Fungus *Trichoderma* sp. Associated with the Seastar *Acanthaster planci***
Wen-Jian Lan, Yang Zhao, Zhong-Liang Xie, Li-Zhen Liang, Wei-Yan Shao, Long-Ping Zhu, De-Po Yang, Xiao-Feng Zhu and Hou-Jin Li **1337**

**Cytotoxic Bioactivity of some Phenylpropanoic Acid Derivatives**
Guillermo F. Reta, Carlos E. Tonn, Carla Ríos-Luci, Leticia G. León, Eduardo Pérez-Roth, José M. Padrón, and Osvaldo J. Donadel **1341**

**Anti-*Helicobacter pylori* Activities of Natural Isopentenyloxycinnamyl Derivatives from *Boronia pinnata***
Cécile Ribeiro da Silva, Valérie Michel, Salvatore Genovese, Marie-Christine Prévost, Francesco Epifano and Eliette Touati **1347**

**Thymofolinoates A and B, New Cinnamic Acid Derivatives from *Euphorbia thymifolia***
Riaz Hussain, Bakhat Ali, Muhammad Imran and Abdul Malik **1351**

**Efficient Counter-current Chromatographic Isolation and Structural Identification of Two New Cinnamic Acids from *Echinacea purpurea***
Ying Lu, JiaYin Li, MiLu Li, Xia Hu, Jun Tan and Zhong Hua Liu **1353**

**Norlignans from *Asparagus cochinchinensis***
Xing-Nuo Li, Chu Chu, Dong-Ping Cheng, Sheng-Qiang Tong and Ji-Zhong Yan **1357**

**Novel Application of Mahua (*Madhuca* sp.) Flowers for Augmented Protease Production from *Aeromonas* sp. S1**
Amrik Bhattacharya, Vandana Saini and Anshu Gupta **1359**

**Chemical Composition of the Fatty Oils of the Seeds of *Cleome viscosa* Accessions**
Rashmi Kumari, Gopal Rao Mallavarapu, Vinod Kumar Jain and Sushil Kumar **1363**

**Essential Oil and other Constituents from *Magnolia ovata* Fruit**
Letícia F. L. Barros, Carlos Augusto Ehrenfried, Dilamara Riva, Andersson Barison, Renato de Mello-Silva and Maria Elida A. Stefanello **1365**

**Chemical Composition of the Essential Oil of *Zingiber zerumbet* var. *darcyi***
Virendra S. Rana, Mercedes Verdeguer and Maria A. Blazquez **1369**

**Compositions of the Volatile Oils of *Citrus macroptera* and *C. maxima***
Virendra S. Rana and Maria A. Blazquez **1371**

**Chemical Compositions of Essential Oils from *Xyloselinum vietnamense* and *X. selinum leonidii***
Tran Huy Thai, Nguyen Sinh Khang, Nguyen Thi Hien, Tran Minh Hoi and Nguyen Tien Dat **1373**

**Chemotaxonomical Markers in Essential Oil of *Murraya koenigii***
Thilahgavani Nagappan, Perumal Ramasamy and Charles Santhanaraju Vairappan **1375**

**Essential Oil Composition and Antibacterial Activity of *Anthemis mixta* and *A. tomentosa* (Asteraceae)**
Carmen Formisano, Daniela Rigano, Felice Senatore, Francesco Maria Raimondo, Antonella Maggio and Maurizio Bruno **1379**

**Antifungal and Antibacterial Activity of the Essential oil of *Chamaecyparis lawsoniana* from Spain**
Jesús Palá-Paúl, Jaime Usano-Alemany, Elena Granda and Ana-Cristina Soria **1383**

**Chemical Composition and Biological Activities of Essential Oil from *Hyptis crenata* Growing in the Brazilian Cerrado**
Ivana Maria Póvoa Violante, Walmir Silva Garcez, Carolina da Silva Barbosa and Fernanda Rodrigues Garcez **1387**

**Larvicidal Activity against *Aedes aegypti* of Essential Oils from Northeast Brazil**
Patrícia L. Lavor, Gilvandete M. P. Santiago, Roberto W. da Silva Gois, Leôncio M. de Sousa, Gabrieli da P. Bezerra, Nirla R. Romero, Ângela M. C. Arriaga, Telma L. G. Lemos, Péricles B. Alves and Paulo C. S. Gomes **1391**

**Composition and Antipseudomonal Effect of Essential Oils Isolated from Different Lavender Species**
Anna Végh, Tímea Bencsik, Péter Molnár, Andrea Böszörményi, Éva Lemberkovics, Krisztina Kovács, Béla Kocsis and Györgyi Horváth **1393**

**Essential Oil of *Croton argyrophylloides*: Toxicological Aspects and Vasorelaxant Activity in Rats**
Aldair de França-Neto, Ana Carolina Cardoso-Teixeira, Thiago Coutinho Medeiros, Maria do Socorro Quinto-Farias, Celia Maria de Souza Sampaio, Andrelina Noronha Coelho-de-Souza, Saad Lahlou and José Henrique Leal-Cardoso **1397**

**Antimicrobial Activity of Blended Essential Oil Preparation**
Sarin Tadtong, Supatcha Suppawat, Anchalee Tintawee, Phanida Saramas, Suchada Jareonvong and Tapanee Hongratanaworakit **1401**

## *Number 11*

**Conformational Analysis of Antistaphylococcal Sesquiterpene Lactones from *Inula helenium* Essential Oil**
Polina D. Blagojević and Niko S. Radulović **1407**

**Ophiobolin O and 6-Epi-ophiobolin O, Two New Cytotoxic Sesterterpenes from the Marine Derived Fungus *Aspergillus* sp.**
Dahai Zhang, Seketsu Fukuzawa, Masayuki Satake, Xianguo Li, Takafumi Kuranaga, Ai Niitsu, Kentaro Yoshizawa and Kazuo Tachibana **1411**

**Anti-Human Coronavirus (anti-HCoV) Triterpenoids from the Leaves of *Euphorbia neriifolia***
Fang-Rong Chang, Chiao-Ting Yen, Mohamed EI-Shazly, Wen-Hsun Lin, Ming-Hong Yen, Kuei-Hsiang Lin and Yang-Chang Wu **1415**

**30-Normedicagenic Acid Glycosides from *Chenopodium foliosum***
Paraskev T. Nedialkov, Zlatina Kokanova-Nedialkova, Daniel Bücherl, Georgi Momekov, Jörg Heilmann, and Stefan Nikolov **1419**

**A New Monodesmosidic Triterpenoid Saponin from the Leaves of *Pometia pinnata***
Faryal Vali Mohammad, Mushtaq Noorwala, Viqar Uddin Ahmad, Aqib Zahoor and Nordin HJ. Lajis **1423**

**Antiproliferative Oleanane Saponins from *Dizygotheca elegantissima***
Antonio Vassallo, Mariasabina Pesca, Letizia Ambrosio, Nicola Malafronte, Nadia Dekdouk Melle, Fabrizio Dal Piaz and Lorella Severino **1427**

**New Holostan-type Triterpene Glycosides from the Sea Cucumber *Apostichopus japonicus***
Zenglei Wang, Wei Gong, Guoquan Sun, Hua Tang, Baoshu Liu, Ling Li, Yanghua Yi and Wen Zhang **1431**

**Freshwater Clam Extract Inhibits Inflammatory Responses in LPS-activated Macrophages by Reducing the Activation of Mitogen-activated Protein Kinases and NF-κB**
Kuo-Feng Hua, Guan-Ming Chen, Chen-Lung Ho, Ming-Chung Chen, Yi-Lin Sophia Chen, Wei-Jung Chen, Jeng-Fang Huang, Yuan-Shing Perng and Chai-Ching Lin **1435**

**A New Monoterpenoid Oxindole Alkaloid from *Hamelia patens* Micropropagated Plantlets**
David Paniagua-Vega, Carlos M. Cerda-García-Rojas, Teresa Ponce-Noyola and Ana C. Ramos-Valdivia **1441**

**Preparation of *O*-Methyl Substituted 2-Oxofuro- and 2-Oxopyrrolidinoindolines by Reductive Lactonization of Oxindol-3-ylacetic Acids**
Martha S. Morales-Ríos, Ernesto Rivera-Becerril, Perla Y. López-Camacho, Nadia A. Pérez-Rojas and Oscar R. Suárez-Castillo **1445**

**Classification Using NMR-based Metabolomics of *Sophora flavescens* Grown in Japan and China**
Ryuichiro Suzuki, Yuriko Ikeda, Akari Yamamoto, Toyoe Saima, Tatsuya Fujita, Tatsuo Fukuda, Eriko Fukuda, Masaki Baba, Yoshihito Okada and Yoshiaki Shirataki **1453**

**Treatment of Licorice Seeds with Colchicine: Changes in Seedling DNA Levels and Anthocyanin and Glycyrrhizic Acid Contents of Derived Callus Cultures**
Françoise Bernard, Nahid Moghbel and Sahar Hassannejad **1457**

**Effects of Anhydroicaritin and 2″-Hydroxy-3″-en-anhydroicaritin on the Proliferation and Differentiation of MC3T3–E1 Osteoblasts**
Yanping Li, Xuehui Zhang, Hui Peng, Rongtao Li and Xuliang Deng **1461**

**Chemical and Biological Investigation of Some Secondary Metabolites in *Atriplex halimus* Growing in Egypt**
Amal Kabbash and Nagwa Shoeib **1465**

**Flavonoids in Selected *Primula* spp.: Bridging Micromorphologywith Chemodiversity**
Tshering Doma Bhutia, Karin M. Valant-Vetschera, Wolfram Adlassnig and Lothar Brecker **1469**

**Neohesperidin Induces Cellular Apoptosis in Human Breast Adenocarcinoma MDA-MB-231 Cells via Activating the Bcl-2/Bax-mediated Signaling Pathway**
Fei Xu, Jia Zang, Daozhen Chen, Ting Zhang, Huiying Zhan, Mudan Lu and Hongxiang Zhuge **1475**

**Rotenoid Derivatives from *Derris trifoliata* with Nitric Oxide Production Inhibitory Activity**
Chihiro Ito, Tomiyasu Murata, Hugh T.-W. Tan, Norio Kaneda, Hiroshi Furukawa and Masataka Itoigawa **1479**

**Bioactive Compounds and Antioxidative, Antileukemic and Anti-MMPs Activity of *Eleutherococcus* Species Cultivated in Poland**
Daniel Załuski, Helena D. Smolarz and Urszula Gawlik-Dziki **1483**

**Chemical Profile, Radical Scavenging and Cytotoxic Activity of Yellow Gentian Leaves (*Genitaneae luteae folium*) Grown in Northern Regions of Montenegro**
Jasmina Balijagić, Teodora Janković, Gordana Zdunić, Jelena Bošković, Katarina Šavikin, Dejan Gođevac, Tatjana Stanojković, Miodrag Jovančević and Nebojša Menković **1487**

***Thymus mastichina*: Chemical Constituents and their Anti-cancer Activity**
Joana Gordo, Patrícia Máximo, Eurico Cabrita, Ana Lourenço, Abel Oliva, Joana Almeida, Mariana Filipe, Pedro Cruz, Rita Barcia, Miguel Santos and Helder Cruz **1491**

**Bioactive Lipids Metabolites in *Amanita virosa***
Francesca Cateni, Marina Zacchigna, Bojan Doljak, Marko Anderluh, Giuseppe Procida and Andrej Piltaver **1495**

**Glycoglycerolipids from *Stellera chamaejasme***
Liping Liu, Junqi Zhang, Xiaoyang Wang and Hongbing Wang **1499**

**Effect of 1,5-Anhydro-D-Fructose on the Inhibition of Adipogenesis in 3T3-L1 Adipocytes**
Akiko Kojima-Yuasa, Yohei Deguchi, Yotaro Konishi and Isao Matsui-Yuasa **1501**

**Preparation of a Novel Glucuronomannan from *Auricularia auricala* and its Immunological Activity**
Zonghong Li, Dan Jiang, Hongtao Bi, Dazheng Liu, Sungju Jang and Yifa Zhou **1507**

**Microwave-Assisted Isolation of Essential Oils from *Nepeta crispa* and *N. racemosa* and Comparisons with the Conventional Method**
Ameneh Porgham Daryasari, Mojtaba Soleimani, Azam Ghorbani, Homayon Kheiri and Mohammad Porgham Daryasari **1511**

**Composition of the Essential Oils from *Peucedanum cervaria* and *P. alsaticum* Growing Wild in the Urban Area of Vienna (Austria)**
Remigius Chizzola **1515**

**Volatile Oil Constituents of Different Parts of *Artemisia chamaemelifolia* and the Composition and Antibacterial Activity of the Aerial Parts of *A. turcomanica* from Iran**
Shiva Masoudi, Abdolhossein Rustaiyan and Maryam Vahedi **1519**

**Volatile Composition and Biological Activity of Key Lime *Citrus aurantifolia* Essential Oil**
Federica Spadaro, Rosaria Costa, Clara Circosta and Francesco Occhiuto **1523**

**Chemical Composition, Antioxidant Properties and Antimicrobial Activity of the Essential Oil of *Murraya paniculata* Leaves from the Mountains of Central Cuba**
Elisa Jorge Rodríguez, Guillermo Ramis-Ramos, Yvan Vander Heyden, Ernesto F. Simó-Alfonso, María Jesús Lerma-García, Yanelis Saucedo-Hernández, Urbano Monteagudo, Yeni Morales, Beatriz Holgado and José Manuel Herrero-Martínez **1527**

**Chemical Composition and Anti-proliferative Properties of *Bursera graveolens* Essential Oil**
Lianet Monzote, Gabrielle M. Hill, Armando Cuellar, Ramón Scull and William N. Setzer **1531**

**Caryophyllene-Rich Essential Oil of *Didymocarpus tomentosa*: Chemical Composition and Cytotoxic Activity**
Prameela Javarayi Gowda, Hanumanthaiah Ramakrishnaiah, Venkatarangaiah Krishna, Sivakrishna Narra and Nataraj Jagannath **1535**

**Effects of Inhaled Lavender Essential Oil on Stress-Loaded Animals: Changes in Anxiety-Related Behavior and Expression Levels of Selected mRNAs and Proteins**
Mizuho Takahashi, Akiharu Yoshino, Ayako Yamanaka, Chihiro Asanuma, Tadaaki Satou, Shinichiro Hayashi, Yoshinori Masuo, Kiyomi Sadamoto and Kazuo Koike **1539**

## *Review/Account*

***Trichoderma* Secondary Metabolites that Affect Plant Metabolism**
Francesco Vinale, Krishnapillai Sivasithamparam, Emilio L. Ghisalberti, Michelina Ruocco, Sheridan Woo and Matteo Lorito **1545**

**An Inside to the Better Understanding of the Ethnobotanical Route to Drug Discovery- the Need of the Hour**
Vivekananda Mandal, Velmani Gopal and Subhash C. Mandal **1551**

## *Number 12*

**A Comparative Study of the Antioxidant/Prooxidant Effects of Carvacrol and Thymol at Various Concentrations on Membrane and DNA of Parental and Drug Resistant H1299 Cells**
Aysun Ozkan and Ayse Erdogan **1557**

**New Secoiridoid Glycosides from the Buds of *Lonicera macranthoides***
Jiang Liu, Jing Zhang, Feng Wang and Xingfu Chen **1561**

**Chemical Constituents from the Aerial Parts of *Gynura bicolor***
Jian Chen, Sven Mangelinckx, An Adams, Wei-lin Li, Zheng-tao Wang and Norbert De Kimpe **1563**

**Chemical Constituents of *Ligularia nelumbifolia* and *L. subspicata* Hybrid Collected in Shangrila County, Yunnan Province of China**
Ryo Hanai, Hiroka Yamada, Yurika Suzuki, Hajime Nagano, Takayuki Kawahara, Jiao-Jun Yu, Xun Gong and Chiaki Kuroda **1565**

**A Practical, Enantiospecific Synthesis of (*S*)-*trans*-γ-Monocyclofarnesol**
Stefano Serra **1569**

**ACAT Inhibitory Activity of Exudates from *Calocedrus macrolepis* var. *formosana***
Yu-Hsin Hsieh, Kuan-Jung Chen, Shih-Chang Chien, Wen-Ling Cheng, Jun-Hong Xiao and Sheng-Yang Wang **1573**

**Cucurbitane-Type Triterpenoids from the Fruit Pulp of *Momordica charantia***
**Yun-Wen Liao, Chiy-Rong Chen, Yueh-Hsiung Kuo, Jue-Liang Hsu, Wen-Ling Shih, Hsueh-Ling Cheng, Tzou-Chi Huang and Chi-I Chang** **1575**

**Two Novel Phenethylamine Alkaloids from *Streptomyces* sp. YIM10049**
Xueqiong Yang, Guangwei He, Lixing Zhao, Yabin Yang, Yun Liu, Lihua Xu and Zhongtao Ding **1579**

**Alkaloids from *Cinnamomum philippinense***
Hsing-Tan Li, Wei-Jen Li, Hui-Ming Wu and Chung-Yi Chen **1581**

**Conversional Synthesis of Heteratisine**
Ling Wang, Qi-Feng Chen and Feng-Peng Wang **1583**

**Flavonoids and Triterpenes from the leaves of *Artocarpus fulvicortex***
Shajarahtunnur Jamil, Muhammad Taher, Hasnah M. Sirat and Nur Azlin Othman **1587**

**Dihydrospinochalcone-A and Epi-flemistrictin-B, Natural Isocordoin Derivatives from the Root Extract of *Lonchocarpus xuul***
Fabiola Escalante-Erosa, Brenda González-Morales, Ramiro F. Quijano-Quiñones, Gumersindo Miron-López and Luis M. Peña-Rodríguez **1589**

**Phenolic Compounds, Including Novel *C*-glycosylflavone, from the Flowers of the Tall Bearded Iris Cultivar 'Victoria Falls'**
Takayuki Mizuno, Tsutomu Yabuya, Nobuhiro Sasaki and Tsukasa Iwashina **1591**

**Two New Biisoflavonoids from the Roots of *Daphne oleoides***
Shazia Yasmeen, Muhammad Aijaz Anwar, Sadia Ferheen, Nighat Afza, Abdul Malik and Lubna Iqbal **1595**

**Biflavonoids from the Unripe Fruits of *Clusia paralicola* and their Antioxidant Activity**
Rafaela Ferreira Oliveira, Celso Amorim Camara, Maria de Fátima Agra and Tania Maria Sarmento Silva **1597**

**Ochnaflavone and Ochnaflavone 7-*O*-methyl Ether two Antibacterial Biflavonoids from *Ochna pretoriensis* (Ochnaceae)**
Tshepiso J. Makhafola, Babatunde B. Samuel, Esameldin E. Elgorashi and Jacobus N. Eloff **1601**

**Factors Affecting the Separation and Bioactivity of Red Clover (*Trifolium pratense*) Extracts Assayed against *Clostridium sticklandii*, a Ruminal Hyper Ammonia-producing Bacterium**
Isabelle A. Kagan and Michael D. Flythe **1605**

**Exploitation of the Antioxidant Potential of *Geranium macrorrhizum* (Geraniaceae): Hepatoprotective and Antimicrobial Activities**
Niko S. Radulović, Milan B. Stojković, Snežana S. Mitić, Pavle J. Randjelović, Ivan R. Ilić, Nikola M. Stojanović and Zorica Z. Stojanović-Radić **1609**

**Differential Expression of Benzophenone Synthase and Chalcone Synthase in *Hypericum sampsonii***
Lili Huang, Hong Wang, Hechun Ye, Zhigao Du, Yansheng Zhang, Ludger Beerhues and Benye Liu **1615**

**Efficient Preparation of Hangekobokuto (Banxia-Houpo-Tang) Decoction by Adding Perilla Herb before Decoction is Finished**
Megumi Sumino, Yuko Saito, Fumio Ikegami, Yoshiro Hirasaki and Takao Namiki **1619**

**Anti-neutrophilic Inflammatory Secondary Metabolites from the Traditional Chinese Medicine, Tiankuizi**
Chia-Lin Lee, Tsong-Long Hwang, Chieh-Yu Peng, Chao-Jung Chen, Yuan-Shiun Chang and Yang-Chang Wu **1623**

**DART MS Based Chemical Profiling for Therapeutic Potential of *Piper betle* Landraces**
Vikas Bajpai, Renu Pandey, Mahendra Pal Singh Negi, Nikhil Kumar and Brijesh Kumar **1627**

**Total Phenolic Content and Antioxidative Properties of Commercial Tinctures Obtained from Some Lamiaceae Plants**
Adam Kowalczyk, Izabela Biskup[a] and Izabela Fecka **1631**

**Synthesis, Antifungal Activity and Structure-Activity Relationships of Vanillin oxime-*N-O*-alkanoates**
Vivek Ahluwalia, Nandini Garg, Birendra Kumar, Suresh Walia and Om P. Sati **1635**

**Cinnamic Acid Derivatives Acting Against *Aspergillus* Fungi. *Taq* Polymerase I a Potential Molecular Target**
Lucas J. Gutierrez, Maria L. Mascotti, Marcela Kurina-Sanz, Carlos R. Pungitore, Ricardo D. Enriz and Fernando A. Giannini **1639**

**Insecticidal Effects of Acetogenins from *Rollinia occidentalis* Seed Extract**
Diego Tolosa, Olga Álvarez Colom, Alicia Bardón and Adriana Neske **1645**

**Conservation and Multiplication of Encapsulated Micro Shoots of *Rauvolfia vomitoria* - an Endangered Medicinal Tree: ISSR and RAPD based Evaluation of Genetic Fidelity of Converted Plantlets**
Shakti Mehrotra, Liaq Ur Rahman, Jahnvi Mishra and Arun K. Kukreja **1647**

**Bioactivities of Volatile Components from Nepalese *Artemisia* Species**
Prabodh Satyal, Prajwal Paudel, Ananad Kafle, Suraj K. Pokharel, Bimala Lamichhane, Noura S. Dosoky, Debra M. Moriarity and William N. Setzer **1651**

**Chemical Compositions, Antioxidant and Antimicrobial Activity of the Essential Oils of *Piper officinarum* (Piperaceae)**
Wan Mohd Nuzul Hakimi Wan Salleh, Farediah Ahmad, Khong Heng Yen and Hasnah Mohd Sirat **1659**

**Chemical Composition, Antimicrobial and Antioxidant Properties of the Volatile Oil and Methanol Extract of *Xenophyllum poposum***
Ana M. González, María I. Tracanna, Sara M. Amani, Carola Schuff, Miguel J. Poch, Horacio Bach and César A. N. Catalán **1663**

**Phytochemical and Micromorphological Traits of Endemic *Micromeria pseudocroatica* (Lamiaceae)**
Dario Kremer, Ivna Dragojević Müller, Edith Stabentheiner, Dubravka Vitali, Martina Kopričanec, Mirko Ruščić, Ivan Kosalec, Nada Bezić and Valerija Dunkić **1667**

**Composition and Variability of the Essential Oil of *Salvia nemorosa* (Lamiaceae) from the Vienna Area of Austria**
Remigius Chizzola **1671**

**Essential oil Composition of *Ficus benjamina* (Moraceae) and *Irvingia barteri* (Irvingiaceae)**
Isiaka A. Ogunwande, Razaq Jimoh, Adedoyin A. Ajetunmobi, Nudewhenu O. Avoseh and Guido Flamini **1673**

## ***Review/Account***

**Cytotoxic Agents of the Crinane Series of Amaryllidaceae Alkaloids**
Jerald J. Nair, Jaume Bastida, Francesc Viladomat and Johannes van Staden **1677**

# Natural Product Communications

Volume **7** (1-12)
**2012**
Author Index


Abas, F....1333
Abdalla, SS....35
Abdelhameed, MS....1209
Acevedo, CA....951
Acharya, DN....637
Achour, Z....937
Adachi, T....913
Adams, A....97,655,1563
Adlassnig, W....1469
Afifi, FU....35
Afiyatullov, SS....497
Afza, N....1595
Afzal, RA....1011
Agra,MF....1597
Agrawal, PK....709
Agudelo-Goméz, LS....1051
Aguilar, JJ....1025
Aguinaldo, AM....743
Ahluwalia, V....1219,1635
Ahmad, F....1659
Ahmad, S....1137
Ahmad, VU....3,61,149,1423
Ahmed, F....195,219,475
Ahmed, N....195
Ajetunmobi, AA....1673
Al salam, S....41
Alanís-Garza, B....597
Alarcón, L....661
Alarcón, R....125
Albertini, MC....1237
Al-Didamony, G....1099
Alejandro, GJD....779
Al-Fatimi, MA....113
Alhaj, M....257
Al-Hallaq, EK....35
Al-Harrasi, A....193,615
Ali, B....1351
Ali, BH....41
Ali, L....615
Ali, NAA....113
Alkhathlan, HZ....1079
Al-Kindy, S....663
Al-Lawati, I....41
Almeida, J....1491
Al-Mekhlafi, NA....1333
Al-Saidi, S....663
Altabef, AB....1215
Altosaar, I....675
Alva, M....245
Alves, PB....1391
Amani, SM....1663
Amat, AG....33
Ambrosio, L....1427
Aminin, DL....497
An, HJ....905
Anderluh, M....1495
Ando, S....1203
Andryjaschenko, PV..517,845,1157
Andujar, SA....1117
Anh, NT....179
Antonov, AS....497
Antonova, D....569
Anwar, MA....1595
Aparicio, R....661
Apers, S....1149
Arai, MA....219,475
Araya, S....603
Arbain, D....799
Arbeloa, EM....1211
Areche, C....1187
Arfan, M....1137,1199
Argyropoulou, C....937
Arias, M....1315
Arifuku, K....21
Armas, K....1225
Arnold, E....165
Arnold, N....113,257,1099
Arriaga, AMC....1391
Arshad, N....311
Arun, M....1271
Arung, ET....1007
Asakawa, Y....685
Asanuma, C....1539
Asiri, SM....1333
Asres, K....667
Astudillo, L....1187
Atoofi, A....979
Avila, JA....779
Avilov, SA....517,845,1157
Avoseh, NO....1673
Avula, B....1177
Awadh Ali, NA....257,1099
Azar, PA....249
Azmat, S....3

Baba, M....21,1453
Bach, H....1663
Badakar, V....941
Baek, KH....65
Báez, D....101,237,659
Bagdonaite, E....199
Bagrel, D....389
Bahadori, F....731
Bahmanzadegan, A....79
Bai, J....561
Bajpai, V....1627
Bajpai, VK....65
Bakar, B....1197
Balijagić, J....1487
Bancheva, S....1083
Bang, TH....1163
Bankova, V....569
Baptista, JG....661
Barbosa, CS....1387
Barbosa, FG....771
Barcia, R....1491
Bardón, A....245,685,1215,1645
Barison, A....1365
Barja-Fidalgo, C....1311
Barros, LFL....1365
Barros-Filho, BA....771
Barthwal, R....305
Başer, KHC....1241
Basset, C....1319
Bastida, J....1677
Bastos, JK....25
Batista-Pereira, LG....1103
Batooli, H....651
Bedascarrasbure, E....879,1315
Bedini, L....1037
Bednarczyk – Cwynar, B....507
Beegam, S....41
Beerhues, L....1615
Begum, N....615
Belsito, EL....137
Bencsik, T....1393
Benešová, N....571
Benites, J....611
Beranek, M....1025
Bergonzi, MC....175
Bermúdez J, ....973
Bernard, F....1457
Bertoli, A....1037
Betancur-Galvis, L....1051
Bezerra, GP....1391
Bezić, N....1227,1667
Bhattacharya, A....1359
Bhutia, TD....587,1469
Bi, H....1507
Bianco, EM....463
Bigucci, F....137
Bilia, AR....175
Birteksöz, S....891
Bisio, A....201,1237
Biskup, I....1631
Bizerra, AM....729
Blagojević, PD....1407
Blazquez, MA....1369,1371
Blessing, LDT....1215
Bliss, BJ....747
Blunden, G....41,863
Bodo, B....169,1305
Boğa, M....731
Bonesi, M....1015
Boon, LH....1033
Borissova, M....1143
Borkosky, S....245
Born, FS....463
Borris, RP....747
Borsacchi, L....875
Bortolazzo, A....1295
Boruah, MP....1021
Bošković, I....1487
Bossche, RVD....1149
Böszörményi, A....1393
Bouajila, J....947
Boustie, J....63
Božić, D....1091
Bracci, A....33
Brancalion, APS....25
Braun, NA....645
Braz-Filho, R....729
Brecker, L....1469
Bremer, M....1295
Breynaert, A....1149
Brodie, PJ....705
Brovetto, M....1215
Bruneau, C....63
Bruno, M....1083,1131,1379
Bucar, F....667
Bucarey, SA....789
Bucchini, A....201
Buchbauer, G....1095
Bücherl, D....1419
Budiene, J....227
Buitrago, A....243
Bultel-Poncé, V....169
Bun, SS....1259
Burcul, F....1087
Burkholz, T....311,709
Busygin, I....1173
Butkiene, R....227

Cabrera, JL....1025
Cabrera, M....1139
Cabrita, E....1491
Çağlar, P....693
Cahlíková, L....1279
Cahlíková, L....571
Cahlíková, L....859
Cai, SF....75
Calderon, PB....611
Callmander, MW....705
Calpena, AC....821
Camara, CA....1597
Camara, CAG....463
Cantrell, CL....1327
Canzoneri, M....1131
Cárdenas, J....243
Cardoso-Teixeira, AC....1397
Carev, I....1087
Carlucci, G....1029
Carmona, J....661
Cartagena, E....245
Carvajal, M....603
Casanova, J....227,947
Castro, F....607
Catalán, CAN....607,1663
Cateni, F....1495
Cenci, E....143
Cerchiara, T....137
Cerda-García-Rojas, CM....1441
Cerecetto, H....1139
Chakraborty, AK....511
Chakraborty, M....1169
Chang, CI....1575
Chang, FR....435,1415
Chang, TM....261
Chang, YS....1623
Chanotiya, CS....1077
Chao, RB....713
Charrouf, Z....621
Chaudhary, A....203,1127
Chauhan, A....1077
Chavez, K....5
Che, CT....785
Chen, CJ....1623
Chen, CR....1575
Chen, CY....883,1581
Chen, D....501,885
Chen, DF....1069
Chen, DL....713
Chen, FY....1269
Chen, GM....1435
Chen, H....599
Chen, HL....883
Chen, J....29,655,1563
Chen, JY....785
Chen, KJ....1573
Chen, MC....1435

Chen, P....1185
Chen, Q....29
Chen, QF....1583
Chen, QH....697,713,721
Chen, T....561
Chen, WJ....1435
Chen, X....1561
Chen, Y....1185
Chen, YH....481
Chen, YLS....1435
Chen, Z....29
Chen, D....1475
Cheng, DP....1357
Cheng, HL....1575
Cheng, LQ....1155
Cheng, WL....1573
Cheng, Z....501
Cheplogoi, PK....13
Cherigo, L....165
Chertkov, VA....353
Chevolot, L....1319
Chhabra, S....193
Chidichimo, G....137
Chien, SC....835,1573
Chihiro Ito, C....1479
Chipps, ES....1203
Chiriboga, X....1139
Chizzola, R....1515,1671
Chlebek, J....859
Chong, Y....57
Chou, CH....153
Chou, SC....153,221,1063
Choudhary, MI....195
Chowdhury, S....1,767
Chrostek, L....273
Chu, C....1357
Chu, SS....171,267
Chu, YH....317
Chuang, LM....221
Chunag, IC....883
Churio, MS....1211
Cicala, C....33
Cioni, PL....629
Circosta, C....1523
Clares, B....821
Clark, BR....747
Coelho-de-Souza, AN....1397
Coleman, AW....327
Colom, OÁ....1645
Comini, LR....1025
Compagnone, RS....5
Conforti, F....1015
Contigiani, MS....1025
Conway, EM....1149
Corao, GM....563
Cordero, Y....563
Corrêa, AG....1103
Costa, EV....265
Costa, MDC....611
Costa, R....927,1523
Costa, SS....175
Coutinho, MAS....175
Cova, JA....563
Crouch, NR....999
Crouch, RA....113
Cruz, EA....175
Cruz, H....1491
Cruz, P....1491
Cruz-Vera, LR....1107
Cuadros-Inostroza, A....603
Cubilla-Rios, L....165
Cuellar, A....1531
Cuello, S....879
Cutajar, S....569
Czigle, S....1147

da Camara, CAG....129
da Silva, AM....1103
da Silva, CR....1347
da Silva, JAT....93
da Silva, TSJ....71
Daele, AV....1149
Dai, DN....231
Damme, PV....97
Danert, C....1315
Daniewski, WM....917
Danikiewicz, W....917
Danova, K....1075
Dantur, Y....1315
Daryasari, AP....1511
Daryasari, MP....1511
Das, P....1127
Dat, NT....1373
David, JM....781
David, JP....781
de França-Neto, A....1397
De Kimpe, N....1563
De Kimpe, N....655
de Lampasona, MP....607
de Mello-Silva, R....1365
de Menezes, RRPB....71
de Moraes, MM....129
de Oliveira, MCF....771
de Oliveira, MR....71
de Sousa, DF....71
de Sousa, JPB....25
de Torres NW....597
De, UC....1
Defillipo, PP....841
Deguchi, Y....1501
Dehelean, CA....981
Demirci, B....1241
Demirci, F....1241
Demirezer, OL....187
Deng, JUG....207
Deng, RD....1069
Deng, SP....599
Deng, X....1461
Deng, YH....181
Denisenko, VA....487
Denkert, A....1099
Desai, DC....1171
Dev, GK....1271
Deville, A....1305
Dhiman, M....1171
Di, YT....1267
Diaz, S....1215
Diederich, M....389
Dinda, B....1
Ding, Z....1579
Dirmenci, T....693
Diwan, R....895
Djebile, K....937
Djeddi, S....937
Dmitrenok, PS....487,517
Dmitrenok, PS....845,853,1157
do Nascimento, AF....129
Doljak, B....1495
Domínguez-Villegas, V....821
Donadel, OJ....1341
Dong, HP....883
Dong, L....51
Dong, Y....867
Donmez, AA....187
dos Santos, EO....781
Dosoky, NS....1233
Drabek, O....253
Draeger, S....293
Druehl, LD....863
Du, D....501
Du, P....381
Du, YC....435
Du, Z....1615
Duan, JA....785
Duddeck, H....359
Dugo, P....927
Duke, SO....1327
Dunkić, V....1227,1667
Duque, S....973
Dusfour, I....1319
Dutra, LM....265
Dyshlovoy, SA....487
Džamić, AM....235

Ebrahimi, SN....753
Edayadulla, N....1325
Edmonds, ME....45
Ehrenfried, CA....1365
EI-Shazly, M....1415
Elfahmi....1309
Elgorashi, EE....1601
Elias, R....1259
Elkhateeb, A....609
Eloff, JN....1601
Elsayed, KNM....1209
El-Shazly, M....435
Enriz, RD....1117,1639
Eparvier, V....1319
Epifano, F....143,1029,1347
Erdogan, A....1557
Erkan, N....121
Ermakova, SP....853
Escalante-Erosa, F....1589
Escobedo, JO....317
Espinoza, L....603
Evstatieva, L....1075

Fadail, I....1099
Fahrenwaldt, T....151
Faizal, A....1149
Faizi, S....831
Fallarero, A....1173
Falodun, A....151
Farahmand, M....133
Farooq, U....149
Farrugia, C....569
Fecka, I....1631
Fedoce, AG....841
Fedorov, SN....487
Feflea, S....981
Feng, S....561
Feng, Y....181
Feng, YX....75
Fenical, W....789
Feo, VD....33
Ferheen, S....1595
Fernández, F....607
Ferreira, AS....841
Fielder, MD....103
Filipe, M....1491
Flamini, G....629,1237,1673
Flesar, J....253
Flythe, MD....1605
Fohrer, J....359
Fondekar, KPP....827
Fong, P....1287
Formisano, C....1083
Formisano, C....1379
Foubert, K....1149
Franz, AH....353
Franzblau, SG....743,779
Fraternale, D....1237
Frédérich, M....515
Fujii, H....1193
Fujii, Y....1197
Fujikawa, S....215
Fujita, T....1453
Fukuda, E....21,1453
Fukuda, T....1453
Fukuzawa, S....1411
Furukawa, H....1479
Furuta, S....1007

Gaascht, F....389
Gadelha, FR....265
Gan, LS....971
Ganapathi, A....1271
Gao, WP....1155
Gaonkar, RH....989
Garbarino, JA....603
Garcez, FR....1387
Garcez, WS....1387
García, ML....821
Garduño-Ramírez, ML....821
Garg, N....1635
Garza-González, E....597
Gattaz, WF....841
Gawlik-Dziki, U....1483
Gayathri, C....735
Geelen, D....1149
Gehle, D....743
Gehrmann, B....875
Geissler, M....1095
Gelli, F....1037
Gemejiyeva, NG....1327
Genovese, S....1029,1347
Gerwick, WH....165
Ghale, G....343
Gharby, S....621
Ghisalberti, EL....1545
Ghomi, JS....651
Ghorbani, A....1511
Ghosh, R....1,989
Giacomelli, E....201
Giamperi, L....201
Giannini, FA....1639
Gil, RG....735
Gilbert, MJ....1107
Giovanelli, S....629
Girod, R....1319
Girova, T....1095
Gkatzionis, K....103
Gochev, V....1095
Gođevac, D....1487
Goedecke, T....785
Göger, F....1241
Gois, RWS....1391
Gokel, GW....333
Gołębiewski, WM....917
Gomes, PCS....1391
Gong, H....81
Gong, W....1431
Gong, X....423,431,447,451,539,
Gong, X....1565
González, AM....1663
González, M....1139
González, MA....1051
González-Morales, B....1589
González-Mujica, F....973
González-Sarrías, A....45
Goossens, A....1149
Gopal, V....1551
Gopichand....203
Gordo, J....1491
Gören, AC....919
Gören, N....693
Gorgorov, R....761
Gorincioi, E....565
Gowda, PJ....1535
Gowda, PJ....943
Grace, MH....1263
Granda, E....1383
Graziose, R....1263
Green III, S....1327
Green, IR....193
Gritsanapan, W....1277
Grodner, J....917
Gruhlke, MCH....395
Guan, J....591
Guan, SH....197
Guan, XL....599
Gucma, M....917
Guillaume, D....621
Guimarães, AC....1311
Guiry, MD....863
Gunasekar, D....1305

Guo, DA....197
Guo, SX....591
Guo, X....353
Guo, YQ....495
Gupta, A....1359
Gupta, AP....991
Gupta, MP....1043
Gupta, N....899
Gupta, S....991
Gupta, SP....305
Gurbuz, P....187
Gutierrez, E....611
Gutierrez, LJ....1639
Gutiérrez, M....1187
Güvenç, A....919

Haddad, A....621
Hadjbourega, G....937
Hadjiakhoondi, A....753
Hagiwara, H....913
Hajimehdipoor, H....979
Hajlaoui, H....947
Hakim, EH....1309
Hamada, H....529,531
Hamada, Y....419
Hamburger, M....753
Hamed, AI....633
Han, J....1185
Han, T....963
Hanack, M....363
Hanai, R....431,451,539,1565
Handique, JG....1021
Hao, XJ....1267
Har, LW....1033
Harhar, H....621
Harinantenaina, L....467,685,705
Harnly, JM....749
Hartmann, A....321
Harzallah-Skhiri, F....947
Hasegawa, M....973
Hasegawa, T....419
Hassan, SHM....1209
Hassannejad, S....1457
Hatakeyama, T....1065
Hatami, A....79
Havlik, J....253
Hay, AE....873
Hayashi, K....427
Hayashi, S....1539
Háznagy-Radnai, E....1147
He, G....1575
He, HP....1267
He, P....1185
He, QJ....971
He, Y....207
Heilmann, J....1419
Heine, T....359
Hernandez, Y....47
Herrero-Martínez, JM....1527
Heyden, YV....1527
Heydenreich, M....1123
Hien, NT....1373
Hill, GM....257,1531
Hiradate, S....1197
Hirano, H....529
Hirao, M....1133
Hirasaki, Y....1619
Hirota, H....451
Hisham, A....663
Hleba, L....641
Ho, CL....109,261,933, 1245,1435
Hock, AHS....775
Höferl, M....1095
Hoi, TM....231,1373
Holgado, B....1527
Honda, K....459
Hongratanaworakit, T....1401
Horiguchi, Y....451
Horváth, G....1393
Hoshi, T....913
Hoshino, Y....459
Hossain, MA....301
Hošťálková, A....1279
Hostettmann, K....873
Hrstka, V....571
Hsieh, SF....435
Hsieh, TJ....435
Hsieh, YH....1573
Hsu, JL....1575
Hsu, KP....933,1245
Hu, X....1353
Hu, XY....207
Hu, Y....211
Hua, H....53
Hua, KF....933,1435
Huang, CH....153
Huang, JF....1435
Huang, JJ....1057
Huang, L....1615
Huang, LJ....883
Huang, TC....1575
Huang, TJ....153
Hussain, H....193,293,615,779
Hussain, J....193,615
Hussain, R....1351
Hussin, N....927
Hwang, TL....435,481,1623

Ibraheem, IB....1209
Ibrahim, SRM....9
Ichihara, M....423
Ifzal, R....3
Ikeda, Y....1453
Ikegami, F....1619
Ikram, A....831
Ilić, IR....1609
Imai, H....531
Imran, M....1011,1351
Inoue, S....459
Iqbal, L....1595
İşcan, G....1241
Ishibashi, M....219,475
Ishida, H....1193
Ishikawa, H....1007
Isla, MI....879,1315
Islam, MR....1203
Ismail, IS....1033
Ismil, R....1197
Isobe, T....977
Ito, Y....973
Itoigawa, M....1479
Ivanauskas, L....199
Ivanchina, NV....853
Ivanova, B....157,581,1273
Iwabuchi, K....471
Iwamoto, Y....447
Iwashina, T....173,1005,1591
Izawa, T....1193
Izumi, H....419

Jabrane, A....947
Jachak, SM....1189
Jacob, C....311,381,709
Jagannath, N....943,1535
Jaglan, S....991
Jahodář, L....571
Jain, GK....1111
Jain, VK....1363
Jaisanka, P....767
Jaisankar, P....511
Jaiswal, N....899
Jamil, S....1587
Janeš, D....861
Jang, KH....789
Jang, S....1507
Janjua, NK....311
Janković, T....1487
Jannet, HB....947
Janulis, V....199
Jareonvong, S....1401
Jauch, J....283
Jayanthi, P....1249
Jayaram, B....305
Jayini, R....1203
Jebors, S....327
Jemia, MB....1083
Jerković, I....931,1087
Jeyaraj, M....1271
Ji, F....53
Ji, NY....819
Jia, YX....93
Jian, XX....713
Jiang, D....1507
Jiang, GH....171,267
Jiang, J....29
Jiang, Y....1165
Jimoh, R....1673
Jiroš, P....859
Jirovetz, L....1095
Jitvaropas, R....909
Joshi, A....757
Joshi, RK....941
Jovančević, M....1487
Jovanović, O....83
Judzentiene, A....227
Júnior, MG....25

Kabbash, A....1465
Kabouche, Z....63
Kacaniova, M....641
Kacem, N....873
Kadota, S....815
Kagan, IA....1605
Kaiser, CR....175
Kalamar, B....861
Kalemba, D....269,625
Kalinin, VI....517,845,1157
Kalinovsky, AI....487,497,517
Kalinovsky, AI....845,853,1157
Kalita, D....1021
Kaljurand, M....1143
Kam, TS....739
Kamarulzaman, FA....1033
Kamat, SP....827
Kamchonwongpaisan, S....575
Kamo, O....21
Kamo, T....1197
Kan, S....885
Kaneda, N....1479
Kang, SC....65
Kant, A....103
Kantamreddi, KSS....19
Kapustina, II....487
Karaket, N....169
Karna, E....269
Kartal, M....731
Kashi, FJ....651
Katakura, K....609
Kato, MJ....1103
Kaur, P....203,1127
Kawahara, T....1565
Kawai, H....863
Kawai, M....427,459
Kawaii, S....451
Kazaz, ZGC....187
Keawsa-ard, S....955
Keeratinijakal, V....1277
Keller, PA....575
Khalid, S....831
Khan, A....149
Khan, AF....149
Khan, AY....311
Khan, FU....615
Khan, IA....1177
Khan, M....1079
Khan, SI....1123
Khan, SS....149
Khang, NS....1373
Khansari, ME....301
Khatoon, R....61
Khay, M....1259
Kheiri, H....1511
Kiang, LC....775
Kicha, AA....853
Kido, M....427
Kijjoa, A....47
Kikuchi, M....527,533,1133
Kim, MK....57
Kimpe, ND....97
Kincer, BL....1233
Kingston, DGI....705
Kintya, P....565
Kinuthia, EW....13
Kiplimo, JJ....999
Kırımer, N....1241
Kirsch, G....389
Kishida, A....977
Kitadate, K....1193
Kitagawa, Y....15
Kitajima, J....173
Kitaoka, N....215
Kite, GC....1047
Kizaki, T....1193
Kleinpeter, E....1123
Klimeš, J....571,1279
Kloucek, P....253
Kocsis, B....1393
Kogame, K....863
Koike, K....1539
Kojima-Yuasa, A....1501
Kokai, J....757
Kokanova-Nedialkova, Z....1419
Kokoska, L....253
Kokubun, T....1047
Kolak, U....731
Komiyama, K....977
Komiyama, M....349
Kondo, R....1007,1163
Kongkiatpaiboon, S....1277
Konigheim, BS....1025
Konishi, M....459
Konishi, Y....1501
Koorbanally, NA....999
Kopričanec, M....1667
Korir, E....999
Koryudzu, K....219
Kosalec, I....1667
Köse, YB....1241
Koseki, T....1065
Kouam, SF....779
Kovačević, N....1091
Kovács, K....1393
Kowalczyk, A....1631
Kragl, U....151
Krasokhin, VB....487
Kraus, GA....191
Krause, MR....337
Kreft, S....861
Kremer, D....1029,1227,1667
Krishna, V....943
Krohn, K....193,293,743,779
Kubik, S....337
Kuboki, A....427
Kubota, N....529
Kučera, R....571,1279
Küçükboyacı, N....919
Kuhnert, N....343
Kujala, J....1173
Kukreja, AK....1647
Kulkarni, RN....1223
Kulkarni, SS....1223
Kumar, A....767
Kumar, B....1627,1635
Kumar, D....989
Kumar, N....995,1627
Kumar, S....305,1363

Kumari, R ....1363
Kuneš, J....859
Kuo, YH....835,1575
Kuranaga, T ....1411
Kurina-Sanz, M ....1639
Kuroda, C..... 423,431,447,451,539
Kuroda, C....1565
Kuroda, M....471
Kuroyanagi, M....441
Kuruuzum-Uz, A ....187

Laatsch, H....1199
Labokas, J ....199
Lacaille-Dubois, MA....595
Laguna, A ....47
Lahlou, S....1397
Lai, XP....1069
Laird, K....103
Lajis, NH....1333
Lajis, NHJ....1423
Lakušić, B....1091
Lalitha, P....1249
Lan, CQ....675
Lan, WJ....495,1337
Langat, MK....13
Langat-Thoruwa, C....193
Langer, P....151
Lategan, C....1263
Latip, J ....1309
Lavaggi, ML ....1139
Lavor, PL....1391
Leal-Cardoso, JH....1397
Lee, CL ....1623
Lee, GG....905
Lee, KH....725
Lee, KT ....905
Lee, NL ....479
Lee, SG ....65
Lee, SS....221,1063
Leino, R ....1173
Lemberkovics, E....1393
Lemos, TLG....1391
Lemos, TLG....729
León, LG....1341
Leonardi, M ....629
Leonardi, M ....633
Lerma-García, MJ....1527
Leśniewska, B....327
Levandi, T....1143
Li, H....1069
Li, HJ ....1337
Li, HT ....883,1581
Li, J ....51,599,885
Li, JY ....1353
Li, K....867
Li, L ....45,1431
Li, ML....1353
Li, R ....1461
Li, W ....655,1563
Li, WJ ....1581
Li, X....181,867,1185,1411
Li, XD ....819
Li, XN....1357
Li, XY ....1269
Li, Y....75,561,1461
Li, YW ....1069
Li, Z ....53,1507
Lialiutska, T....1295
Liang, LZ....1337
Liang, XX ....697
Liao, YW ....1575
Liawruangrath, B ....955
Liawruangrath, S ....955
Liguori, A ....137
Lila, MA ....1263
Lima, DB ....71
Lin, CC ....1435
Lin, EH ....153
Lin, H....885
Lin, KH ....1415
Lin, LZ ....749
Lin, S....29
Lin, TY....835
Lin, WH ....1415
Lindequist, U....1099
Linghu, RG....75
Liu, B....1431,1615
Liu, D ....1507
Liu, DH ....1069
Liu, F....191
Liu, FG....889
Liu, G ....53
Liu, J....333,1331,1499,1561
Liu, QR....267
Liu, S....29
Liu, SL ....171
Liu, SS....1057
Liu, T....889
Liu, X ....53,353
Liu, XH ....819
Liu, XX ....713
Liu, Y ....59,1579
Liu, YM....181
Liu, ZH....1353
Liu, ZL ....171,267
Lizarraga, E....607
Loandos, MH ....1117
Loc, TV ....179
Locatelli, M....1029
Loizzo, MR ....1015
López, J ....611
López-Camacho, PY ....1445
López-Pérez, JL ....1043
Lorito, M ....1545
Lourenço, A ....1491
Lu, CH....1057
Lu, L....59
Lu, M....1475
Lu, MC ....479
Lu, Y ....1353
Lucena, M ....243
Ludwiczuk, A....685
Luiten, PGM....1117
Luppi, B ....137
Łysik, A....917
Lyubimtsev, A....363

Ma, GH....93
Ma, H....45
Mabrouki, F....1259
Macabeo, APG....743,779
Macahig, RA ....467
Macáková, K....571,859
Machado, LL....729
Machida, K....527,533,11333
Machumi, F ....1123
MacMillan, JB....211
Mafezoli, J....771
Magalhães, A ....1311
Maggio, A ....1379
Maggio, AM....1131
Mahajan, GB ....1171
Mahiou-Leddet, V....1259
Maione, F ....33
Majumder, HK ....767
Makarieva, TN ....487
Makhafola, TJ ....1601
Malafronte, N....1427
Maldonado, L ....1315
Maldonado, L ....879
Maleš, Z ....931
Malghani, MAK....195
Malik, A ....1011,1351,1595
Malkawi, A....1203
Mallavarapu, GR....87,223,1363
Malpathak, N....895
Malyarenko, TV....853
Mamonov, LK....1327
Mandai, T ....531
Mandal, SC ....1551
Mandal, V ....1551
Mangelinckx, S....655,1563
Manickavasagam, M ....1271
Manju, SL....1171
Marčetić, M ....1091
Marchenko, A....565
Marcotullio, MC....143
Marin, PD ....235
Marioni, J....1025
Marston, A....873
Martínez-Luis, S....165
Martins, AMC ....71
Martyyas, EA....517,845,1157
Maruenda, H....735
Marzony, ET....133
Mascolo, N ....33
Mascotti, ML ....1639
Masman, MF ....1117
Masoomi, R ....651
Masoudi, S....117,1519
Masuo, Y ....1539
Matejić, JS ....235
Mateu, E ....5
Máthé, I....1147
Matsui-Yuasa, I ....1501
Matsumoto, M ....529
Matsunami, K ....467
Matsushita, Y....1007
Matsuura, H ....215,609
Matsuura, N ....427
Mattiza, JT....359
Matto, V....1323
Máximo, P ....1491
Mazik, M ....321
Mazumder,.A....667
McFeeters, H ....1107
McFeeters, RL ....1107
McNeil, MJ....1231
Medeiros, TC....1397
Mehmood, R....1011
Mehrotra, S....1647
Meibodi, ZA ....249
Meira, M....781
Meiser, P....381
Melle, ND....1427
Melzig, MF....875
Menchinskaya, ES....497
Menendez, R....47
Menichini, F ....1015
Menković, N....1487
Messina, F ....143
Meyer, GRY ....1149
Meyer, V....359
Miao Li, M ....409
Miao, FP ....819
Michel, S....891
Michel, V....1347
Midiwo, JO....1123
Mifsud, D....569
Milenković, M....1091
Milos, M....1087
Mimaki, Y ....15,471
Ming, Q....963
Miño, P ....1139
Miron-López, G....1589
Mishra, J ....1647
Mitaine-Offer, AC....595
Mitić, SS....1609
Mitić, V....83
Mitsui, T ....427
Miyamoto, T....595
Mizuno, T ....1591
Moghadam, MH ....979
Moghbel, N....1457
Mohammad, FV....1423
Mohammadpour, G ....133
Mohammed, FV....3
Mohebat, R....117
Mollo, E ....487
Molnár, J ....981
Molnár, P ....1393
Momekov, G ....1419
Monache, FD ....5
Mondello, L ....927
Monte, FJQ ....729
Monteagudo, U ....1527
Monteiro, HSA ....71
Montenarh, M ....401
Monzote, L....1531
Moodley, N ....999
Mooi, LY ....775
Moon, HI....825
Moradi-Afrapoli, F ....753
Moraes, VRS....265
Morales, A ....1225
Morales, D ....101,237,659,
Morales, RA....47
Morales, Y ....1527
Morales-Ríos, MS....1445
Moreno, MIN....1315
Moretti, C....1319
Mosaddegh, M ....979
Mosihuzzaman, M ....807
Mosslemin, MH ....117
Mottaleb, MA....1203
Mousa, AA....1079
Mpetga, JS ....515
Mu, Q....889
Muhammad, I....1123
Muhi, MZ....1203
Mukherjee, T....511
Mukherjee, T....767
Mukhopadhyay, S....767,1169
Mukhopadhyay, SB ....511
Müller, ID ....1667
Munikishore, R ....1305
Murata, T ....1479
Murayama, T....1065
Muro, AC ....1117
Murugan, R ....87
Muzitano, MF ....175
Mwangi, EM ....13

Nabata, K ....609
Nabeta, K ....215
Nadaoka, I....15
Nagano, H....539,1565
Nagappan, T....239,1375
Nair, JJ ....959,1677
Nair, MG....1219
Nakamura, MJ....1311
Nakamura, T ....913
Nakane, T....441
Nam, JW ....795
Nam, SJ....789
Namiki, T....1619
Narra, VKS ....1535
Nau, WM ....343
Navarro-Vázquez, A ....735
Nazaruk, J ....269
Nazir, M....293
Ndontsa, BL....515
Nedialkov, PT....1419
Negi, MPS....1627
Nemmar, A....41
Neske, A....1215,1645
Ng, A....1295
Ng'ang'a, MM ....193
Ngadjui, BT ....595
Nguyen, D....1295
Nguyen, HX....185
Nguyen, MHK ....185
Nguyen, MTT ....185
Nguyen, NT ....185
Nii, K....685
Niitsu, A....1411

Nikolov, S....1419
Nikolova, M....761
Ninh, PT....179
Nirma, C....1319
Nirmal, J....1111
Nisha, N....1111
Nishiki, M....685
Nishioka, H....1193
Niu, S....53
Niu, XT....599
Noguchi, E....349
Nogueira, NAP....71
Nogueira, PCL....265
Nogueiras, C....47
Noorwala, M....1423
Norjosa, DB....71
Novak, A....757
Novák, Z....859
Nozaki, H....427
Núñez Montoya, SC....1025
Nwachukwu, ID....395
Nyunt, KS....609

Ocampos, S....125
Occhiuto, F....1523
Ogasawara, J....1193
Ogunsina, MO....757
Ogunwande, IA....231,1673
Ohira, S....427
Ohno, H....1193
Ohsaki, A....977
Ohya, N....419
Oji, KA....671
Okada, Y....1453
Okada, Y....21
Okamoto, Y....423,447
Oksal, BS....121
Oleszek, W....633
Oliva, A....1491
Oliveira, MF....71
Oliveira, RF....1597
Ollivier, E....1259
Olmedo, D....1043
Ooka, H....987
Opletal, L....571,859,1279
Ordoñez, R....879,1315
Osolodkin, DI....295
Osorio, M....951
Ota, M....987
Othman, NA....1587
Otsuka, H....467
Ounsuk, S....169
Ozawa, M....977
Ozkan, A....1557
Özüduru, G....359

Pacciaroni, A....125
Padalia, RC....1077
Padmaja, A....1305
Padrón, LM....1341
Paknikar, SK....827
Pal, BC....989
Palaniveloo, K....239
Palá-Paúl, J....1383
Palyulin, VA....295
Pandav K....305
Pandey, R....1627
Pandotra, P....991
Pandya, P....305
Paniagua-Vega, D....1441
Parab, RR....1171
Park, EJ....789
Park, JC....57
Parmar, BS....1219
Paul, M....283
Paul, P....511
Pecetti, L....837
Peña, A....661
Peña-Cortés, H....603
Peña-Rodríguez, LM....1589
Peng, CY....1623
Peng, H....1461
Peng, Y....867
Peraire, C....821
Pérez-Guaita, D....1051
Pérez-Rojas, NA....1445
Pérez-Roth, E....1341
Perng, YS....1435
Pesca, M....1427
Peters, MS....409
Pezzuto, JM....789
Pham, A....1295
Pham, VC....169
Phillips, CA....103
Phoolcharoen, W....909
Piaz, FD....1427
Pieters, L....1149
Pilepić, KH....931
Piltaver, A....1495
Pino, JA....101,237,659
Piovano, M....603,951
Pirisi, FM....1015
Pistelli, L....629,633,1037
Pivkin, MV....497
Plenzig, F....363
Poch, MJ....1663
Pokhilo, ND....901
Politeo, O....1087
Polonini, HC....841
Ponce-Noyola, T....1441
Popich, S....245
Popova, M....569
Porter, RBR....1231
Porzel, A....257
Prado, S....891
Prakash, O....89
Pramanik, A....301
Prasad, YR....485
Prévost, MC....1347
Prieto, JM....757
Procaccini, RA....1211
Procida, G....1495
Protzman, AD....1203
Przybysz, E....917
Pungitore, CR....1639
Pyne, SG....575,955

Qin, L....963
Qiu (Samuel), S....561
Queiróz, LP....781
Quesada, L....1187
Quijano-Quiñones, RF....1589
Quinto-Farias, MS....1397

Raal, A....1323
Rada, V....253
Radhika, P....485
Radulović, NS....1407
Radulović, NS....1609
Radwan, MM....1209
Rafieiolhossaini, M....97
Rahman, K....963
Rahman, LU....1647
Raimondo, FM....1379
Rajesh, M....1271
Rajeswara Rao, BR....223
Rakotobe, E....705
Ramakrishnaiah, H....943,1535
Ramasamy, P....1375
Ramesh, P....1325
Ramesh, S....223
Rameshkumar, KB....663
Ramírez, CL....1211
Ramírez, I....603
Ramírez-Durón, R....597
Ramis-Ramos, G....1527
Rammohan, A....1305
Ramos, CS....129,1103
Ramos, J....1215
Ramos-Valdivia, AC....1441
Rana, VS....1369,1371
Ranđelović, VN....235
Randic, M....1029
Randjelović, PJ....1609
Rani, TS....1283
Raposo, AH....841
Raposo, NRB....841
Rasamison, VE....705
Raskin, I....1263
Rath, NP....333
Rattanajak, R....575
Ravindra, NS....1223
Razafitsalama, LJ....705
Read, RW....979
Regalado, EL....47
Remmel, I....1323
Ren, B....655
Reta, GF....1341
Rhouati, S....873
Riaz, N....1137
Ribeiro, LHG....265
Ricci, D....201,1237
Rigano, D....1379
Riina, R....5
Ríos-Luci, C....1341
Ristić, MS....235
Riva, D....1365
Rivera-Becerril, E....1445
Robal, M....1143
Rocha, IDC....757
Rodrigues, AMS....1319
Rodrigues-Filho, E....771
Rodríguez, A....1139
Rodríguez, EJ....1527
Rodríguez, M....973
Rojas, J....243,1225
Rojas, L....661,1225
Rojas, M....611
Rojas-Fermin, L....243
Rojo, L....611
Romani, A....875
Romero, NR....1391
Romussi, G....201
Ross, SA....1209
Rosselli, S....1131
Rossi, E....143
Rossi-Bergmann, B....175
Rout, PK....89
Rowshan, V....79
Roy, SK....1189
Ruffoni, B....1037
Ruiz, MA....821
Rumalla, CS....1177
Ruocco, M....1545
Rupasinghe, HPV....121
Ruščić, M....1227,1667
Russ, TH....301
Rustaiyan, A....117,1519

Saba, N....61
Sabuncuoğlu, S....39
Sadamoto, K....1539
Sadhu, SK....219
Sadhu, SK....475
Saeidnia, S....753
Saeidnia, S....979
Saenthaweesuk, S....909
Sahoo, D....89
Said, M....927
Saidu, NEB....401
Saifudin, A....815
Saima, T....1453
Saini, V....1359
Saito, Y....423,431,447,1619
Sakaguchi, K....173
Sakamoto, A....419
Sakurai, T....1193
Salas, A....1315
Salazar-Aranda, R....597
Saleh, AM....637
Salleh, WMNHW....1659
Salvador, MJ....265
Sami, M....15
Samoshin, VV....353
Samoshina, NM....353
Sampaio, ALF....1311
Sampaio, CMS....1397
Samuel, BB....1601
Sánchez, E....951
Sánchez, LA....1043
San-Martín, A....1187
Sano, Y....215
Santalova, EA....617
Santayana, MP....757
Santiago, GMP....1387
Santiago,GMP....729
Santos, AKL....729
Santos, M....1491
Saramas, P....1401
Sarkar, T....511
Sarolic, M....1087
Sarwar, R....149
Sasaki, N....1591
Sastraruji, T....575
Satake, M....1411
Satheeshkumar, N....1111
Sati, OP....1635
Satou, T....1539
Sattar, FA....195
Sattar, SA....195
Satyal, P....1233
Saucedo-Hernández, Y....1527
Šavikin, K....1487
Scardigli, A....875
Schmidt, E....1095
Schmidt, J....257
Schrader, T....409
Schuff, C....1663
Schüffler, A....1199
Schulz, B....293
Score, J....103
Scull, R....1531
Seeram, NP....45
Sekita, S....441
Selvaraj, N....1271
Senatore, F....1083,1379
Seo, EK....795
Seoane, G....1215
Sepúlveda, B....1187
Şerbetçi, T....891
Serra, S....455,1569
Setzer, WN....113,257, 1099,1107
Setzer, WN....,1233, 1531,1651
Severino, L....1427
Shaaban, KA....1199
Shaari, K....1033,1333
Shafaghat, A....671
Shah, AUA....1137
Shah, Z....1137
Shahrestani, R....979
Shan, Y....333
Shao, L....885
Shao, WY....1337
Sharifan, A....249
Sharma, R....991
Sharma, U....995
Sharopov, FS....257
She, XK....713
Shen, YM....1057
Sherwani, N....663
Shigi, N....349
Shih, WL....1575
Shimizu, A....431
Shimizu, K....1007,1163
Shimoda, K....529,531
Shinzato, T....467

Shiono, Y ..... 1065
Shirataki, Y ..... 1453
Shirota, O ..... 441
Shoeib, N ..... 1465
Shubina, LK ..... 487
Shukla, P ..... 1219
Shulga, DA ..... 295
Siani, AC ..... 1311
Sifuentes, R ..... 735
Silchenko, AS ..... 517,845,1157
Silva, TMS ..... 1597
Sim, S ..... 645
Simmonds, MSJ ..... 1047,1131
Simó-Alfonso, EF ..... 1527
Singh, B ..... 203,995,1127
Sirat, HM ..... 1587,1659
Sireeratawong, S ..... 909
Sivanandhan, G ..... 1271
Sivasithamparam, K ..... 1545
Sivryuk, V ..... 1295
Skaltsa, H ..... 937
Skocibusic, M ..... 1087
Skogman, ME ..... 1173
Skropeta, D ..... 923
Slusarenko, AJ ..... 395
Smid, J ..... 253
Smillie, TJ ..... 1177
Smith, GJ ..... 145
Smith, PJ ..... 1263
Smolarz, HD ..... 1483
Soares, MG ..... 1103
Sodaeizadeh, H ..... 97
Şöhretoğlu, D ..... 39
Soica, C ..... 981
Şoica, CM ..... 289
Soleimani, M ..... 1511
Solórzano, E ..... 879
Somparn, N ..... 909
Sonali, N ..... 1111
Sondhia, S ..... 1327
Song, YL ..... 1165
Sonnenberg, C ..... 321
Soria, AC ..... 1383
Sosa, V ..... 125
Sothea, K ..... 1259
Sousa, LM ..... 1391
Sowjanya, K ..... 485
Spadafora, C ..... 165
Spadaro, F ..... 1523
Spandana, V ..... 1111
Spiteller, M ..... 157,581,1273
Srinivas, KVNS ..... 1223
Sripathi, SK ..... 1249
Srivastava, A ..... 1189
Srivastava, AK ..... 899
Srivastava, SK ..... 899
Stabentheiner, E ..... 1667
Stanilova, M ..... 761
Stankov-Jovanović, V ..... 83
Stanojković, T ..... 1487
Statti, G ..... 1015
Stefanello, MEA ..... 1365
Steyger, PS ..... 317
Stien, D ..... 1319
Stochmal, A ..... 633
Stojakowska, A ..... 625
Stojanovic, G ..... 83
Stojanović, NM ..... 1609
Stojanović-Radić, ZZ ..... 1609
Stojković, NB ..... 1609
Stonik, VA ..... 487,853
Stoyanova, A ..... 1095
Straface, SV ..... 137
Strongin, RM ..... 317
Su, CR ..... 725
Su, HJ ..... 479
Su, JH ..... 479
Su, XJ ..... 599
Su, YC ..... 109,261,933,1245
Suárez, AI ..... 5
Suárez-Castillo, OR ..... 1445
Subramaniam, G ..... 739
Sugamoto, K ..... 1007
Sugimoto, S ..... 467
Sujatha, S ..... 1283
Sukdolak, S ..... 641
Sumino, M ..... 1619
Summer, S ..... 61
Sun, CP ..... 1155
Sun, G ..... 1431
Sung, PJ ..... 481
Sung, TV ..... 179
Supaibulwatana, K ..... 169
Suppawat, S ..... 1401
Šuste, M ..... 931
Suvire, FD ..... 1117
Suwińska, K ..... 327
Suzuki, Y ..... 1565
Suzuki, JY ..... 747
Suzuki, R ..... 1453
Suzuki, T ..... 913
Suzuki, Y ..... 431,987
Syah, YM ..... 1309
Syamasundar, KV ..... 1079,1309

Tabopda, TK ..... 595
Tachibana, K ..... 1411
Tadesse, S ..... 667
Tadtong, S ..... 1401
Taga, I ..... 675
Taher, M ..... 1587
Tahiri, IA ..... 831
Tai, CY ..... 481
Takahashi, M ..... 1539
Takahashi, Y ..... 459
Takaoka, D ..... 427
Takeda, Y ..... 467
Takemura, T ..... 1197
Tamimoto, H ..... 987
Tamrakar, AK ..... 899
Tan, SJ ..... 739
Tan, HTW ..... 1479
Tan, J ..... 1353
Tanaka, C ..... 595
Tanaka, K ..... 815
Tane, P ..... 515
Tang, H ..... 1431
Tang, MT ..... 599
Tang, P ..... 697,713
Tangarife-Castaño, V ..... 1051
Tani, H ..... 427
Tanjung, M ..... 1309
Tao, SJ ..... 197
Tao, Z ..... 121
Tava, A ..... 837
Taweechotipatr, M ..... 575
Tchinda, A ..... 515
Teerawutgulrag, A ..... 955
Teichert, A ..... 113
Tekwani, BL ..... 1123
Teponno, RB ..... 515
Tezuka, Y ..... 815
Thai, TH ..... 1373
Thang, PD ..... 179
Thang, TD ..... 231
Theunis, M ..... 1149
Thomas, NF ..... 739
Thomas, OP ..... 47
Thompson, RM ..... 1107
Thuppia, A ..... 909
Thuy, TT ..... 179
Tian, S ..... 81,1181,1331
Tillequin, F ..... 891
Timmers, M ..... 551
Tinoco, LW ..... 175
Tintawee, A ..... 1401
Titera, D ..... 253
Todorova, M ..... 1075
Toeng, P ..... 1259
Tokita, N ..... 419
Tolosa, D ..... 1645
Tomczyk, M ..... 273
Tomi, F ..... 227
Tomosaka, H ..... 987
Tong, HHY ..... 1287
Tong, SQ ..... 1357
Tonn, CE ..... 1341
Tonsomboon, A ..... 575
Toom, L ..... 1323
Toomik, P ..... 1143
Topçu, G ..... 693,731
Torabbeigi, M ..... 249
Tori, M ..... 423,431,447,539
Torihata, A ..... 431
Torres, AFC ..... 71
Torres, S ..... 827
Tosa, Y ..... 609
Touafek, O ..... 63
Touati, E ..... 1347
Touem, ST ..... 151
Toume, K ..... 475
Toyota, M ..... 685
Tracanna, MI ..... 1663
Trendafilova, A ..... 761,1075
Trigo-Mouriño, P ..... 735
Trusheva, B ..... 569
Truus, K ..... 1143
Tsubono, K ..... 21
Tsukagoshi, Y ..... 987
Tu, PF ..... 1165
Tundis, R ..... 1015
Türkekul, A ..... 731
Tuvikene, R ..... 1143

Uehara, A ..... 1005
Uesawa, Y ..... 21
Ullah, R ..... 1137
Ullah, Z ..... 1011
Uno, H ..... 427
Upadhyay, HC ..... 899
Upur, H ..... 1181
Ur Rehman, N ..... 615
Urban, S ..... 551
Usano-Alemany, J ..... 1383
Usubillaga, A ..... 563,661
Utkina, NK ..... 487
Utkina, NKI ..... 901
Uysal, B ..... 121

Vahedi, M ..... 1519
Vaher, M ..... 1143
Vairappan, CS ..... 239,1375
Valant-Vetschera, KM ..... 587,1469
Valdés, E ..... 951
Valdés, O ..... 47
Vale, AE ..... 781
Valente, S ..... 389
van Staden, J ..... 959,1677
Varela, J ..... 1139
Vares, L ..... 1323
Vassallo, A ..... 1427
Veeresham, C ..... 1283
Végh, A ..... 1393
Veitch, NC ..... 1047
Velasco, J ..... 661
Vera, N ..... 879
Verdegue, M ..... 1369
Verma, RS ..... 1077
Versiani, MA ..... 831
Vignolini, P ..... 875
Viladomat, F ..... 1677
Villecco, MB ..... 1117
Villinger, A ..... 151
Vinale, F ..... 1545
Vinardell, MP ..... 611
Vincieri, FF ..... 175
Violante, IMP ..... 1387
Vitali, D ..... 1667
Vitkova, A ..... 761
Vlietinck, A ..... 1149
Vuko, E ..... 1227
Vukovic, N ..... 641
Vuorela, PM ..... 1173

Wahab, AA ..... 927
Wajs-Bonikowska, A ..... 625
Walia, S ..... 1219
Walia, S ..... 1635
Walker, LA ..... 1123
Wang, EIC ..... 1245
Wang, F ..... 1561
Wang, FIC ..... 933
Wang, FP ..... 697,713,721,1583
Wang, H ..... 1499,1615
Wang, HM ..... 289
Wang, J ..... 495
Wang, L ..... 721,1583
Wang, LS ..... 75
Wang, M ..... 867
Wang, Q ..... 317
Wang, SY ..... 835,1573
Wang, X ..... 51,1499
Wang, Y ..... 197
Wang, YH ..... 1179
Wang, Z ..... 655,1431,1563
Wangchuk, P ..... 575
Wanner, J ..... 1095
Wasano, N ..... 1197
Waseem, A ..... 149
Watanabe, K ..... 15
Wéber, E ..... 1147
Wei, G ..... 1069
Wei, HL ..... 1165
Wenz, G ..... 289
Wessjohann, L ..... 113,257,1099
Weston, RJ ..... 145
White, JB ..... 1295
Williams, JD ..... 637
Williams, LAD ..... 1231
Williams, TD ..... 1043
Wong, BM ..... 301
Woo, S ..... 1545
Wright, CW ..... 19
Wu, CC ..... 435
Wu, HM ..... 1581
Wu, L ..... 963
Wu, Q ..... 599
Wu, TS ..... 725
Wu, YC ..... 435,1415,1623
Wurster, M ..... 1099
Wyrzykiewicz, B ..... 565

Xiao, JH ..... 1573
Xie, HQ ..... 889
Xie, ZL ..... 1337
Xin, L ..... 1181
Xing, S ..... 867
Xu, F ..... 1475
Xu, L ..... 1579
Xu, LJ ..... 785
Xu, SF ..... 1269
Xu, Z ..... 381
Xue, B ..... 1331

Yabuya, T ..... 1591
Yadav, A ..... 1077
Yadegarynia, S ..... 1295
Yahara, S ..... 491
Yamada, A ..... 459
Yamada, H ..... 1565
Yamaguchi, T ..... 475
Yamamoto, A ..... 1453
Yamanaka, A ..... 1539
Yan, GQ ..... 75
Yan, JZ ..... 1357
Yang, B ..... 971

Yang, DP....1337
Yang, L....181
Yang, RC....883
Yang RY....599
Yang, SS....261
Yang, X....1575
Yang, Y....1575
Yao, W....1185
Yaoita, Y....527,533,1133
Yaqub, A....311
Yarmo, MA....927
Yasmeen, S....1595
Yassa, N....753
Yasue, M....15
Yatsimirsky, AK....369
Yazdani, M....79
Ye, H....1615
Ye, Y....51
Ye, YP....1269
Yen, CT....1415
Yen, KH....1659
Yen, MH....1415
Yenesew, A....1123
Yi, Y....1431
Yılmaz, A....693
Yin, S....495
Yin, XL....819
Yokoyama, S....1163
Yonezawa, S....527
Yoshino, A....1539
Yoshizaki, K....491
Yoshizawa, K....1411
Young, ME....951
Yu, J....1565
Yu, Q....81,1181
Yu, R....1331
Yuan, T....45
Yuan, YF....207
Yue, TH....775
Yusoff, NIK....927

Zacchigna, M....1495
Zahoor, A....3,61,1423
Załuski, D....1483
Zampini, C....879,1315
Zang, J....1475
Zapata, B....1051
Zdunić, G....1487
Zecchi, V....137
Zefirov, NS....295
Zellagui, A....873
Zeng, X....181
Zhan, H....1475
Zhang, D....1411
Zhang, H....59
Zhang, J....1499,1561
Zhang, Q....29,1267
Zhang, QD....1069
Zhang, SX....1069
Zhang, T....1475
Zhang, W....1431
Zhang, X....197,1461
Zhang, XH....93
Zhang, XQ....785
Zhang, Y....963,1267,1615
Zhang, YF....181
Zhang, Z....59
Zhao, BB....1057
Zhao, JT....93
Zhao, L....1575
Zhao, M....785
Zhao, N....53
Zhao, Y....1337
Zhao, ZZ....971
Zheng, Y....353
Zhou, CX....971
Zhou, JH....1069
Zhou, L....1331
Zhou, X....81
Zhou, Y....1507
Zhu, H....971
Zhu, LP....1337
Zhu, XF....1337
Zhuge, H....1475
Zhuravleva, OI....497
Ziada, A....41
Zimmermann, S....753
Zivanovic, A....923
Zlatkovic, B....83
Znati, M....947
Zong, SL....1269
Zou, L....971
Zovko-Končić, M....273
Zupko, I....981
Zwergel, C....389

# Natural Product Communications

Volume **7** (1-12)
**2012**
Keywords Index


A2780 705
A549 29
Abiodone 151
Absolute 89
Absolute configuration 419,835
ABTS 901,1211,1631
Acanthaceae 485
*Acanthospermum hispidum* 245
Acaricidal activity 129,463
Acetic acid 75
Acetophenone 1325
Acetylcholinesterase 571,859,959
Acetylene bridged oligomers 363
Acetylenic 1077
Acetylenic alcohol 447
Acid-triggered leakage 353
Aconitine-type alkaloid 713
Actein 15
Acteoside 775
Acute toxicity 1397
Acyl migration 333
Acyl phloroglucinol 191
Acyl-CoA 1573
Acylglycerols 917
Adulteration 807
*Aedes aegypti* 1319,1391
Aerial parts 1165
*Aeromonas* sp. S1 1359
Agathisflavone 973
*Ajuga bracteosa* 615
Ajuganane 615
*Aka coralliphaga* 487
Alangiaceae 1169
Alangium alkaloids 19
*Alangium lamarckii* 19,1169
Alantolactone 1407
Albican-11 819
Alcoholism 273
Alcohols, Ethers 359
Aldol condensation 1127
Algae 923
*Alisma orientale* 785
Alkaloid 959,1169,1579
Alkaloids 369,739,799,963, 1177,1249,1365,1677
Alkanediol dialkanoates 1219
Alkanes 1363
Alkenoyloxy alkenol 779
Alkylresorcinol 1043
Allelochemicals 1327
Allelopathy 1137,1233,1651
Allicin 395
Aloe-emodin 207
*Alpinia katsumadai* 795
*Alstonia* 739
Alzheimer´s disease 571,859,959
*Amanita virosa* 1495
Amaryllidaceae 959,1677
Amazonian mistletoe 1311
Amide alkaloids 595
Amides 359
Aminobutyric acid betaine 863
Aminoglycosides 317
Ammaniol 899
*Ammannia multiflora* 899
AMP-activated protein kinase 1501
*Ampelopsis japonica* 1287
Anacardiaceae 1011
Analgesic activity 611
Analysis of hallucinogens 1273
*Andrographis paniculata* 485
*Angelica keiskei* 21,1007
Angiogenesis 1149
Angustinine 1169
Anhydro-D-fructose 1501
Anhydroicaritin 1461
Anilides 507
Anion coordination 301
Anion selectivity 301
*Anisosciadium orientale* 79
*Annona vepretorum* 265
Annonaceous acetogenins 1215
Annual *Medicago* spp 837
*Anthemis mixta* 1379
*Anthemis tomentosa* 1379
Anthocyanins 41,1457
Anthraquinone extracts 1025
Anthraquinones 1029
*Anthurium* 747
Antiangiogenic effect 981
Antibacterial 597,891,1107
Antibacterial activity 57,121,239, 243,245,249,661,955,1095,1187, 1315,1383, 1387,1393,1519,1601
Anti-bacterial compounds 1347
Antibacterial effect 71
Antibiotic 1107
Anticancer 1123,1557,1677
Anticancer activity 1245
Anticholinesterase activity 575, 693,731,947,1015
Antcin C 835
Anticomplement 867
Anti-complementary activity 501
Antifeedant 171
Antifeedant activity 245,1131
Antifungal 891,1171
Antifungal activity 125,1051,1219
Antifungal activity 1315,1383,1387,1431,1635
Anti-HBV599
Anti-hepatitis B virus 153
Antiherpetic activity 1025
Antihypercholesterolemic agents 35
Antihyperglycemic 899
Anti-inflammatory 435,575,675,757,
Antileishmanial 1123,1465
Antimalarial 1209
Anti-melanoma activity 1311
Antimicrobial 103,113, 867,909,947, 1099,1171,1173,1209,1231,
Antimicrobial 1233,1241,1401,1651, 1659
Antimicrobial activity 109,261,293, 381,485,663,667,937,941,943, 1065, 1087,1091,1237,1245,1379,1527, 1609,1663
Antimicrobial effect 671
Antimicrobial 9
Anti-multidrug resistance 1465
Antimycotic effect 65
Anti-neutrophilic inflammatory activity 1623
Antinutritional effects 381
Antioxidant 47,203,607,757,909, 1021,1099, 1127,1241,1373,1483, 1557,1659
Antioxidant activity 109,201,261, 611,693,731,875,951,955, 1015, 1527, 1597,1631
Antioxidants 381
Antiparasite effect 71
Antipathogenic activity 933
Antiplasmodial 575,1123,1263,
Antiproliferative 705
Anti-proliferative activity 269
Antiradical activity 875
Anti-*Trypanosoma cruzi* activity 1139
Antitrypanosomal activities 609
Antitumor 1341
Antitumor activity 137
Antiviral 1025
Antiviral activity 143,603,1051
Anti-wood-decay fungal activity 261
*Antrodia cinnamomea* 835
Anxiolytic-like effect 1539
Apiaceae 79,235,1077,1373,1515
*Apium graveolens* 889
*Aplysina* 617
Apocarotenoid 827
Apocynaceae 101,729
Apoptosis 29,401,1203,1295
*Apostichopus japonicas* 1431
Apotirucallane 495
Aqueous extract 41
*Arabidopsis thaliana* root inhibition assay 215
Arabinopyranosyl dimethyl-secologanol 1561
Aracaceae 3
Araceae 1043
Araliaceae 1427
*Ardisia kivuensis* 515
Ardisikivuoside 515
Argentine propolis 879
Argentinean propolis 1315
Arid environments 879
*Aristeguietia glutinosa* 1139
Aroma compounds 633
Aromatic water 137
*Artemia salina* 837
*Artemisia alba* 1075
*Artemisia chamaemelifolia* 1519
*Artemisia dubia* 1651
*Artemisia eriopoda* 267
*Artemisia indica* 1651
*Artemisia monosperma* 1079
*Artemisia turcomanica* 1519
*Artemisia vulgaris* 637,1391,1651
Arteriosclerosis 1573
*Artocapus altilis* 185
*Artocarpus fulvicortex* 1587
Ascaridol 227
Ascaridole 1651
*Asclepiadaceae* 1269
*Asclepias syriaca* 1147
*Asparagus cochinchinensis* 1357
*Asparagus racemosus* 995
*Aspergillus* 1639
*Aspergillus fumigatus* 497
*Aspergillus* sp 1057,1411
*Aspergillus* sp. strain F1544 165
*Aspergillus versicolor* 819
Asteraceae 243,423,431,435,447, 451,533,611,625,671,941,1005, 1079, 1083,1123,1131,1225,1379, 1407,1663
Asterosaponins 853
*Astrodaucus orientalis* 249
Atomic charges 295
Atorvastatin 35
*atpB-rbcL* 1565
*atpB-rbcL* 451
*Atriplex halimus* L 1465
*Auricularia auricala* 1507
Aurones 389
Autofluorescence 1469
*Azorella madreporica* 1187

B lymphocyte proliferations 1507
B16 melanoma cells 1007
Ballonigrin lactone A and B149
*Ballota limbata*149
Basidiomycete 771
Basidiomycetes 1495
Bax-mediated signaling pathway 1475
Bayogenin 837
Bearded iris 1591
Benzenoids 89
Benzoic acid hexyl ester 651
Benzophenone synthase 1615
Benzylidenebenzofuran-3(2H)-ones 389
Bergapten 1327
Betaines 863
*Betula papyrifera* 145
*Betula pendula* 145
Betulin 289,981
Betulinic Acid 289
Bhutanese traditional medicine 575
Bicyclogermacrene 651
Bicyclogermacrene 87
Bicyclo[3.1.1]heptane ring system 459
Biflavonoid glucosides 1597
Bi-hydroxyorobol 1595
Biisoflavonoids 1595
Binding sites 359
Bioactivity 879
Bioassay-guided isolation 1327
Biocontrol 1545
Biofilms 1173
Bioflavonoids 591
Biological activities 963
Biological activity 265,389,795
Biomarkers 1539
Biossay 837
Biosynthetic pathway 581

Biotherapy 675
Biotransformation 197,885
Bis(crown ethers) 337
Bisabolane 451
Bisabolane sesquiterpenes 455
Bisabolol 117
Bitter Melon 1203
Black cardamom 1233
Blended essential oil 1401
*Blumea balsamifera* 815
BM-21 47
*Boesenbergia rotunda* 909
Bone regenerative medicine 1461
Borneo 239
Borneol 671,1667
Bornyl acetate 943
*Boswellia papyrifera* 283
*Bothropoides lutzi* 71
Bridged cyclobutanes 459
Brine shrimp lethality 1233
Britannin 979
Bud absolute 645
*Bupleurum* 757
*Bursera graveolens* 1531
Butyrolactone 1127
Butyrylcholinesterase 571,859

$C_{19}$ –Diterpenoid Alkaloids 697,721, 713,1583
Cacalol 821
Cacalone 821
*Cachrys cristata* 235
Cadambine 169
Cadinene 671
Cadinol 261,629,671,933
*Caesalpinia digyna* 1189
Caffeoylquinic acids 607,749,873
Calceoralarioside E 775
Calixarenes 409
*Callicarpa maingayi* 1333
Callicarpaquinone A 1333
Callicarpaquinone B 1333
*Calocedrus macrolepis* Kurz var. *formosana* 1573
Camalexin 395
Cambodian biodiversity 1259
Camphor 937,1651
Cancer 401
Cancer prevention 789
*Candida* species 65
*Canisctrocarpus cervicornis* 463
Cantharellaceae 917
*Cantharellus cibarius* 917
Capparidaceae 1363
Caprifoliaceae 1561
*Caprifoliaceae* 641
*Capsella bursa-pastoris* 51
Carbohydrate receptors 321
Carbon source 1271
Carbonates 359
Cardioactive evaluation 713
Carotene 827
*Carpesium triste* 825
Carvacrol 109,1557
Carvotacetones 1123
Carvotanacetone 257
Caryophyllene 117,629,1375,1535, 1671,1673
Caryophyllene oxide 117,1083
Caryophyllene 8*R*,9*R*-oxide 815
Caspases 401
*Cassia fistula* 1263
Catalytic reaction 1411
Catechin derivatives 181
Cavernicolin 617
$CCl_4$-induced liver damage 1609
CD 735
Cedrol 1245
Celery seed 889
Cell apoptosis 1475
cell cycle arrest 1295
Cell division 349
Cell growth inhibition 53
Cell morphology 137
Cell proliferation 401
*Centaurea formanekii* 1083
*Centaurea orphanidea* ssp. *Thessala* 1083
*Centaurea ragusina* L 1087
*Centella asiatica* 841
Ceramides 781
Cerebral ischemia 1069
*C*-glycosylflavones 1591
*Chaetomium globosum* CICC 2445 197
Chalcone 1589
Chalcone synthase 1615
Chalcones 879,1007
Chalkbrood disease 253
*Chamaecyparis formosensis* 933
*Chamaecyparis lawsoniana* 1383
Chamomile 97,749
Chanterelle 917
ChemGPS-NP 1173
Chemical composition 103,947, 1531,1659
Chemical constituents 795,955
Chemistry 799
Chemodiversity 587
Chemoenzymatic synthesis 1331
Chemotaxonomy 1375
Chemotype 1095
*Chenopodium foliosum* 1419
*Chersodoma argentina* 125
Chikusetsusaponin 491
Chiral differentiation 359
Chiral dirhodium complexes 359
Chiral resolution 369
Chlorocoumarin-glucopyranoside 1331
Cholesterol acyltransferase 1573
Chorioallantoic membrane 981
Chromenes 891
Chromolith-100,
*Cichorium intybus* 971
*Cimicifuga racemosa* 15
Cimiracemoside F 15
Cinchona alkaloid 1173
Cineole 117,1117,1233,1651
Cinnamamides1047
Cinnamic acid derivatives 1351
Cinnamic acids 1353
*Cinnamomum philippinense* 1581
*Cinnamosma* 685
Cinnamoyl phenylethylamides 1047
Cinnamoylphenethyl amide 753
circular dichroism 591
*Cirsium palustre* 269
*Cirsium rivulare* 269
*cis*-Aconitic anhydride ethyl ester 785
Citronellol 1223
Citronelol 101
*Citrus aurantifolia* 1523
*Citrus macroptera* 1371
*Citrus maxima* 1371
Civetone 913
*Cladiella* 481
Cladieunicellin H 481
*Cladocolea micrantha* 1311
Clary sage oil 1401
*Clematis* 173
*Cleome serrate* 1231
*Cleome viscosa* 1363
Cleomeaceae 1231
Clonogenic assay 853
Clusiaceae 63
Clusiaceae, *Clusia paralicola* 1597
Cochlospermaceae 629
*Cochlospermum angolense* 629
*Codium fragile* 819
Colon cancer 1491
Combretastatin A-4 1051
Comfrey 861
*Commiphora erythraea* (Burseraceae) 143
Compositae 221,533,781,1063,1519
Computer-aided drug discovery 1287
Concrete 89
Conformational Analysis 735,1407
Conformational switch 353
Conservation 799
Constituent 533
Contact toxicity 267
Controlled release 353
Conversion 1155
Convolvulaceae 219
*Convolvulus tricolor* 873
*Copaifera langsdorffii* 25
*Corbicula fluminea* 1435
*Corydalis cava* 859
*Corydalis crispa* 575
Corylucinine,
Countercurrent chromatography 973
Crassulaceae 175
*Crataegus aronia* 35
*Cremanthodium helianthus* 423
Crinane 1677
*Croton argyrophylloides* 1397
*Croton micans* 5
*Cryptocarya chinensis* 725
Crystal structure 327
Cubenol 937
Cubitane 479
Cucumariosides 517,845,1157
Cucumber mosaic virus 1227
Cucurbitaceae 1575
Cucurbitane 1575
Cucurbituril 343
Culcitiolide A-D 427
Cultured plant cells 529
Cultured plant cells 531
*Cunninghamia lanceolata* var. *konishii* 1245
Cupressaceae 261,919,933
*Cupressus sempervirens* 569
Curcumin 529,1037
Curcumin analogue 57
Curry leaf 121
Curvulinic acid 51
*Cyathocalyx zeylanicus* Champ 663
Cyclic voltammetry 311,1211
[3+2] Cycloaddition 743
Cyclodextrin Derivatives 289
*Cymbopogon flexuosus* 1391
CYP2C9 1283
*Cyperus conglomeratus* Rott 663
Cytokines 675
Cytotoxic 9,239,891,1099,1677
Cytotoxic activity 1051
Cytotoxic activity 607,825,955, 1057,1333,1499,1535
Cytotoxicities 1337
Cytotoxicity 39,45,495,835,853,971, 977,979, 1025,1043,1203,1233, 1259,1263,1411,1419,1487,1531, 1651

Dammarane type saponin 491
Danshen 59
*Daphne oleoides* 1595
DART-MS 1627
Date palm 3
Degradation 1193
Deltaline 1583
Demethoxycurcumin, *bis*-Demethoxycurcumin 1037
Demethylgriseofulvin 327
Density functional theory 1651
Deoxyactein 15
Depsides 603
Depsidones 603
Dereplication 1033
dereplication 551
*Derris indica* 1325
*Derris scandens* 1305
*Derris trifoliate* 1479
*Desmos chienensis*231
*Desmos chinensis* var. *lawii* 663
*Desmos cochinchinensis*231
*Desmos dumosus*231
*Desmos penducolosus* var. *tonkinensis*231
*Desmos penduculosus*231
Deuteration experiment 721
DFT 735
Diabetes 815
Diabetes mellitus 989,1563
*Diacarnus megaspinorhabdosa* 9
Diacarperoxide S 9
Diallyl polysulfanes 401
Dibenzothiophenes 891
Dibromoverongiaquinol 617
Dichlorophthalocyanine 363
Diclofenac acid 821
*Didymocarpus tomentosa* 1535
Dietary Supplements 1177
Diethylene glycol diesters 1219
Differentiation 1461
Dihydrocadambine 169
Dihydrocurcumin 529
Dihydroenmein 977
Dihydroflavonol 1309
Dihydroisocordoin 1589
Dihydrospinochalcone A 1589
Dihydroxygambogenic acid 197
Dihydroxylupanine 999
Dihydroxyolic-11-ene-28-oic acid 501
Dihydroxyphenethyl-apiofuranosyl-glucopyranoside 775
Diiminoisoindoline 363
Dimers 5
Dimethoxybenzoquinone 169
Dimethoxycinnamoyl-embinin 747
Dimethoxysesamin 597
Dinitrobenzene 311
Diosphenol 827
Diphenylpentendiol 995
Diplophyllin 1407
Direct micelles 1211
Disaccharides 595
Disc Diffusion assay 1087
Diterpene glucoside 471
Diterpenoid 475
Diterpenoid 479
Diterpenoids149
Diuretic activity 33
Diversity 451
*Dizygotheca elegantissima* 1427
DNA 409,1557
DNA binding constants 305
DNA double-strand break 1057
DNA interaction 305
DNA topoisomerase IB 767
Docking 1117
Documentation 807
DOSY 21
DPPH 113,901,1021,1127,1631
DPPH free radical scavenging assay 775
DPPH radical scavenging 1465
DPPH radical-scavenging activity 487

*Dracaena cochinchinensis* 591
Drimanes 685
Drug discovery 1111,1551
Drug-DNA binding 305
Drupamine 1639
*Drynariae Rhizoma* 905
DSC 1215
D-Valine 211

Ebenaceae 193
*Echinacea purpurea* 1353
Eclalbasaponins 989
*Eclipta alba* 989
Efficacy 807
*Eichhornia crassipes* 1249
Elastase 481
Electroantennography 1103
Electroporation 349
Elemanes 1131
Elemene 237,659,1083
Elemental sulfur 395
*Eleutherococcus* 1483
Eleutherosides 1483
Enantioselective synthesis 455
Encapsulation 1647
Endophytic fungi 293,963
Enlargement of B ring 697
*Entada phaseoloides* 867
Enthalpy-entropy compensation 337
*ent*-Labdane diterpenes 485
Enzyme assay-guided fractionation 471
Enzyme assays,
Enzyme inhibition 381
Enzyme inhibitors 923
Enzyme-mediated resolution 1569
Enzymes 343
Epi-flemistrictin B 1589
Epi-ophiobolin O 1411
Epoxides 359
Epoxidized soybean oil 75
Eremophilane 423
Eremophilane 427
Ergosterol peroxide 1163
Erysopine 171
Erysovine 171
*Erythrina variegate* 171
*Eschscholzia pulchella* 1279
*Espeletia nana* 661
Essential enzyme inhibition 1107
Essential oil 1083,1091,1095,1103,
Essential oil 79,81,87,89,109,113,
117,125,133, 239,245,257,261,269,
625,629,637, 661,667, 931,933,937,
943,955,1225,1227,1231,1237,1241,
1245,1365,1369,1371,1373,1375,
1379,1383,1387,1397,1511,1515,
1527,1531,1535,1663,1673
Essential oil composition 83, 129
223,227,235,243,249,265,651,663,
671,941,1519
Essential oil profile 1075
Essential oil, Bark 145
Essential oils 103,253,641,675,1077,
1099,1391,1393,1659
Esters 359
Ethnobotanical 1551
Ethnomedicine 1551
Ethyl secologanoside 1561
Ethylene glycol diesters 1219
*Etlingera* 239
*Eucalyptus perriniana* 531
*Euclea divinorum* 193
Eucleanal 193
Eudesmanes 1131
Eudesmol 261
Eunicellin 481
*Eupentacta fraudatrix* 517,845,1157
*Euphorbia neriifolia* 1415
*Euphorbia thymifolia* 1351
Euphorbiaceae 5,475,1309,1351,
1415
Expression analysis 1539
Extract 151
Extraction 203
Exudate 1469
Exudate Flavonoids 587
Exudates 201

Fabaceae 61,999,1305
Falcarinol 941,1091,1077
Farnesene 97
*Farfugium japonicum* 435
Farina 587
Fas death pathway 1069
Fatty acids 919,923
Fatty oil composition 1363
Ferns 685
Ferruginol 261,1573
Ferruloyl-embinin 747
*Ferula lutea* 947
*Ficus benjamina* 1673
Fingerprint 1181
Fingerprint analysis 1143
Fingerprinting 807
Flavanol 1309
Flavanones 1491
Flavone 153,747
Flavone glycosides 595,1491
Glycoside 747
Flavones 1005
Flavonoid 1469
Flavonoid glycosides 551,1011,1185
Flavonoids 33,179,187,199,311,749,
761,867, 1015,1177,1249,1305,
1311,1323,1487,1587
Flavonol glycosides173,1465
Flavonols 25,1005
Fliposomes 353
Floral scent 101,237
Flower 659
Flower absolute 645
Flower development stage 97
Flower essential oil 947
Flowers 175,1087
Fluorescence 305,343
Fluorescence microscopy 1173
Fluorescence staining 981
Fluorescent-labeled compound 215
Folk medicine 675
Forced swimming test 905
Fractional crystallization 1569
Fragmentation pathway 1279
Fragrance ingredient 913
Frankincense resins 283
*Fraxinus griffithii* 467
Free radical scavenging activity 187,667
French Guiana 1319
Freshwater clam 1435
Friedelane-type 987
Fructo-oligosaccharides 221,1063
Fruit 1575
Fruit oil 1515
FTIR 1215
Fullerene[$C_{60}$], Photoaddition 743
Fumigant 267
Fumiquinazolines 497
Fumoquinone 165
Furanocoumarin production 895
Furanocoumarins 1327
Furanodienone 143
Furanoeremophilanes 431,539,1565
Furanones 1199
Furospirostane 565
Furostane 565
Spirostane 565

*Fusarium oxysporum* f. sp. *Cucumerinum* 885

Gadusolate 1211
Galenical preparations 1631
Gallic acid 1051
Gambogenic acid 197
*Garcinia xanthochymus* 53
*Gardenia volkensii* 13
Gastroprotective activity 1187
GC (FID, RI, MS) 227
GC 81,641,931
GC/FID 1535
GC/GC-MS analysis 93
GC/MS 89,101,237,571,641,659,
931,941,947,1237,1241,1527,1535
GC/MS analysis 79
GC-EIMS analysis 987
GC-FID 89,1523
GC-FID response factor 927
GC-MS 81,113,257,629,633,637,
655, 889,919,1087,1099,1279,1523
GC-MS analyses 937
GC-MS, Patchouli 927
Gene expression 1615
Genetic fidelity 1647
Gentamicin 317
*Gentiana lutea* leaves 1487
Geographic variations 645
Geranial 101,237,1223
*Geranium macrorrhizum* 1609
Geranylated aromatic compound 527
Germacrane 441,1131
Germacrene B 87
Germacrene D 87,117,235,237,243,
629,651,1671
Germacrene-D-4-ol 1673
Germacrone-4,5-epoxide 441
Germination 51
Germplasm conservation 1647
*Ginkgo biloba* 1287
*Ginkgo biloba* 203,1287
Ginsenoside Rg3 1155
Glandular Trichome 1469
Glimepiride 1283
*Globularia meridionalis* 1015
Glucose uptake 899
Glucose-6-phosphatase 973
Glucosidase 471,989
Glucosinolates 395
Glucosylation 531
Glucuronomannan 1507
Glycerol 2-phosphate dehydrogenase (GPDH) 1501
Glycinebetaine 863
Glycoglycerolipid 1499
Glycolipid 1263
Glycosides 1427,1563
Glycyrrhetinic acid 991
*Glycyrrhiza glabra* 991,1457
Glycyrrhizic acid 99,1457
Goniothalamin 1197
*Goniothalamus* 1197
Griseofulvin dimerization 327
Growth inhibition 41
Growth phase dependent addition 895
Guaiane 441
Guaianolides 447
Guest 369
Guttiferae 53
*Gynura bicolor* 655,1563
*Gynura divaricata* subsp. *Formosana* 221,1063
*Gynura divaricate* 655
*Gynura medica* 655

HAB 1605
Hairy roots 1271
*Hamelia patens* 1441
Hangekobokuto 1619
HCA 1181
Head space solid-phase microextraction 249
Hederagenin 837,1423
HeLa cell 349
*Helichrysum armenium* 671
*Helicobacter pylori* infection 1347
Hengduan Mountains 539
HEp-2 cells 143
HER2 1295
*Heracleum rigens* 943
Herbal medicine 807
Herbicidal activity 51,125
Hericiaceae 527
*Hericium erinaceum* 527
Heteratisine 1583
*Heterophyllaea pustulata* 1025
Hexadecanoic acid 1083
Hexahydrocurcumin 883
Hexose transport assay 221
*Hibiscus sabdariffa* 41
High-speed counter-current chromatography 1353
Holistic approach 807
*Homalomena wendlandii* 1043
Homoisoflavonoids 1189
Honey 951
Horner-Wadsworth-Emmons reaction 1569
Host 369
Host-guest complex 301
Hot-taste 685
HPAEC-PAD 1063
HPLC 25,59,199,283,1021,1185,
1189
HPLC analysis 1259,1277
HPLC/DAD/MS 875
HPLC-ESI/MS 873
HPLC-ESI-MS 1189
HPLC-NMR 551
HS-SPME 101,629,633
Human coronaviruses (HCoVs) 1415
Human mononuclear cells 563
Human platelets 883
Human tumor cell lines 825
Humulene 1375
HUVEC 1149
Hybrid 1565
Hydrocharitaceae 47
Hydro-distillation 121,249,637,1523
Hydrogel 1315
Hydroxycurcumin 57
Hydroxy-3″-en-anhydroicaritin 1461
Hydroxy-trifluoromethylcoumarin-glucoside 1331
Hydroxybenzaldehyde 779
Hydroxybenzoyl-roseoside 973
Hydroxyderricin 21
Hydroxyisovaleric acid 211
Hydroxy-olean-12-en-28-oic acid 13
Hydroxy-olivacine 729
Hydroxypolyneuridine 731
hydroxytremetone 1663
*Hymenocrater yazdianus* 117
*Hypericum maculatum* 199
*Hypericum perforatum* 199
*Hypericum sampsonii* 1615
*Hypericum tomentosum* L 63
*Hyptis crenata* 1387

Ichthyotoxic 1033
Immobilization stress 15
Immunohistochemical staining 1539
Immunosuppressive activities 1269
Immunosuppressive effect 175
*In vitro* cultures 1441

Incensole 283
Incensole acetate 283
Indian sandalwood 93
Indole 659
Indole alkaloids 157,735
Inducible nitric oxide synthase 789
Induction of plant resistance 1545
Inflammation 675,789,1435
Infusion 1241
Inhibitors 1495
Insecticidal 1233
Insecticides 1319
Intellectual property rights 807
Interleukin-2 1419
Intracellular localization 349
Intramolecular Hydrogen bond 295
Intra-receptor interactions 337
*Inula aucheriana* 979
*Inula helenium* L 1407
Ionones 827
*Ipomoea maxima* 219
*Ipomoea pes-caprae* 827
Iridaceae 1591
Iridoid 1,13
Iridoid glycosides 561
Irregular monoterpenoids 1075
*Irvingia barteri* 1673
Isoalantolactone 1407
Isoalantolactone 625
*iso*-Ascaridol 227
Isocordoin 1589
*Isodon* 977
Isoflavones 1187
Isoflavonoids 1319,1605
Isolation 861,917
Isolithospermic acid 885
Isomenthone 1223
Isopimarane diterpene 693
Isoquinoline alkaloids 575,581
Isorhamnetin-3-*O*-glycosides 873
Isothermal titration calorimetry 337
isovalencenol 223
Isovallesiachotamine 731
ISSR 1647
ITS 1565
ITS 431,451,539,1565
*Ixora coccinea* 831
Ixoroid 831

Jasmine oil 1401
*Jasminum grandiflorum* L. 89
*Jasminum sambac* 645
*Jatropha gossypifolia* 151
*Juniperus* 919
*Juniperus chinensis* 455
*Juniperus communis* var. *Communis* 919
*Juniperus communis* var. *Saxatilis* 919
*Juniperus drupacea 919*
*Juniperus formosana* 455
*Juniperus oxycedrus* subsp. *Macrocarpa* 919
*Juniperus oxycedrus* subsp. *Oxycedrus* 919
Jurkat 45 1483

Kaempferol glycoside 173
*Kalanchoe pinnata* 175
Kamebanin 977
Kampo medicine 1619
Kaurene derivatives 563
Khusimol 223

Labdane diterpenoids 1139
Labiatae 615
Lactarane sesquiterpenoid 1133
Lady's mantle 761
Lamiaceae 83,87,117,149,187,471,667, 1137,1387,1671
Laminariales 863
Landraces 1627
Larvicidal activity 1391
Lathyrane diterpenes 151
Lauraceae 109,1581
*Laurencia dendroidea* 463
*Lavandula officinalis* 1539
Lavender oil 1401
Lavender species 1393
LC-DAD-ESI-MS 1037
LC-MS/MS 181
LC-PDA-ESI/MS 749
Lead compound discovery 1107
Leaf 491
Leaves 1079,1087
Leguminosae 1325,1479,1589
*Leishmania amazonensis* 1531
*Leishmania donovani* 165
*Leishmania major* 133
Leishmanicidal activity 195
Lemon 1155
*Lentinus strigellus* 771
*Lepidaploa cotoneaster* 781
*Lethasterias fusca* 853
*Leucophyllum frutescens* 597
Lichen compounds,
Light petroleum extract 889
Lignan glycosides 767
Lignans 597,1043,1357
Lignoids 1365
*Ligularia* 539
*Ligularia kanaitzensis* 431
*Ligularia lankongensis* 451
*Ligularia nelumbifolia* 1565
*Ligularia subspicata* 1565
*Ligusticopsis wallichiana* 1077
Liliaceae 1357
Lime Mexicana 1523
Limonene 79,419,671,943,1079,1127,1371,1383
α-Santalol 419
*Lindera aggregate* 1287
Linear retention index (LRI) 927
Linoleic acid 1363
Lipids 1495
Liquid $CO_2$ extract 89
Liquid-liquid equilibria 75
*Listeria innocua* 121
Liverworts 685
*Livistona halongensis* 179
L-Lactic acid 211
*Lonchocarpus xuul* 1589
Long-chain unsaturated esters 63
*Lonicera japonica* 641
*Lonicera macranthoides* 1561
*Lophophytum leandri*,
Loranthaceae 1311
Low-density lipoproteins 35
LPS 1435
Lupane-type 987
Lupenyl acetate 1147
Lutein 1263
Luteolin 29
L-Valine 211
Lycoctonine 697
Lycoctonine type 721
Lycoctonine-type alkaloid 713
Lycopsamine 861
Lycorine 959
Lymphocyte proliferation 175
Lysosome 1193

*Macaranga recurvate* 1309
Macarecurvatins A and B 1309
*Machilus japonica* 109
Macroalgae 1209
Macrocycles 343
Macroelements 1667
Macrophages 789,1507
*Macrophomina phaseolina* 1635
Madagascin 1029
*Maesa lanceolata* 1149
*Magnolia grandiflora* 237
*Magnolia ovate* 1365
Magnoliaceae 237,1365
Mahua flower 1359
Mangiferin 1591
Manzamine A 295
MAPK 1435
*Marchantia polymorpha* 529
Marfey's method 211
Marine fungus 1337
Marine products 1211
Marine sponge 487
Marine-derived fungus 497
Mass spectrometry 157,343,581,637,1295
Mastitis 675
MCF-7 165
MDA-MB-231 cells 1475
*Medemia argun* 633
Media optimization 1359
Medicagenic acid 837
Medicinal plants 799,1323
Megastigmane glycoside 3
Megastigmane sesquiterpene 785
Megastigmanes 973
Melanin inhibition 1007
Meliloester 61
Melilotic ester 61
*Melilotus alba* 61
*Melissa officinalis* 1631
Membrane 1557
*Mentha piperita* 1631
*Meriandra* 1099
Metabolomics 1111,1453
Metal chelation 381
Metalloproteinases 1483
Methanol extract 1609,1663
Method validation 1189
Methoxytremetone 611
Methyl benzoate 659
Methyl jasmonate 215
Methyl octanoate 633
Methylene linker 305
Mexican arnica 749
*Michelia champaca* 659
Microbial conversion 65
Microdilution assay 1087
*Microdiplodia* sp 1065
*Micromeria graeca* 1227
*Micromeria pseudocroatica* 1667
Microrhizomes 1037
Microwave 203
Microwave assisted HS-SPME 249
Microwave extraction, 121
Microwave hydrodistillation 1511
Minor fatty acids 1363
Mitochondria 29
Mitochondrial Toxicity 1295
Mixture analysis 21
Molecular dynamics 295,1639
Molecular Modeling 1117
Molecular recognition 321,337,409
Molluscicidal activity 245
*Momordica charantia* 1203,1575
Monensin 1199
Monoterpenes231
Monotypic genus 1005
*Monticalia greenmaniana* 243
Moraceae 599,1587
*Morinda citrifolia* 195
Morindicone 195
Morinthone 195
Morphological characteristics 1075
Mosher's reagents 211
Mouse model 1347
Mouse peritoneal macrophage 905
MTT assay 775,979,981
*Muellera frutescens* 1319
Multiple shoots 625
*Murraya koenigii* (L.) Spreng 1375
*Murraya koenigii* L 121
*Murraya Paniculata* 1527
Muscone 1069
Mushrooms 527,1133,1495
Musk xylene 1083
*Mussaenda roxburghii* 1
Muurolol 933
*Mycobacterium tuberculosis* 597
Myrcene 661
Myristicin 79
Myrrhone 143
Myrsinaceae 515

Nanoemulsion 821
Naphthazarins 901
NAPRALERT 1551
*Narcissus poeticus* var *recurvus* 571
Natural antibacterial 675
Natural antioxidants 311
Natural products 191,293,551,735,1111
*Naupactus bipes* 1103
Nebularine 9
Nematocidal 1233
Neohesperidin 1475
*Neonauclea purpurea* 169
*Nepeta asterotricha* 117
*Nepeta crispa* 1511
*Nepeta racemosa* 1511
*Nepeta sorgerae* 693
*Nerine filamentosa* 571
*Nerine filifolia* 571
Neuronal apoptosis 1069
Neurons 841
Neutral red assay 39
New chemotype 1663
NF-κB 1435
*Nicotiana tabacum* 603
*Nimbya alternantherae* 51
*Nipponanthemum* 1005
Nitric oxide 789,905,1479,1507
Nitrogenous compounds 739
Nivefuranone 1065
*N*-methylenehydroxycytisine 999
NMR 3,19,173,193,227,289,359, 487,511,591,615,709,729,767,831, 873,1169,1423,1427,1453
NMR analysis 565
NMR data 1147
NMR spectroscopy 1441
Nonadyne 1077
Nootkatone 1371
Nordammarane 497
Nor-friedo-oleanane 511
Norisoprenoids 1563
Norlignan glycoside 1357
Normedicagenic acid 1419
Norsesterpene 9
Novel target 1107
NTFP 1359
Nuclear factor κB 427
Nutraceuticals 47
Nutritional alterations 1645
Nystose 221

Obesity 815
*Ochna pretoriensis* 1601
Ochnaflavone 1601
Ochnaflavone 7-*O*- methyl ether 1601

Octaalkoxylphthalocyaninatogermanium dichlorides 363
Octaalkylphthalocyaninatogermaniu m dichlorides 363
Octanoic acid 633
Ocimene 237
Odor evaluation 645
Oleaceae 467,645
Oleanolic acid 507
Olefin metathesis 913
Oleic acid 913,1363
Oligonol 1193
Oligosaccharide polyester 1165
Olivacine 729
Olivacine-2*N*-oxide 729
*O*-Methylneocaryachine 1279
*Onopordum Illyricum* 1131
Ophiobolin O 1411
Opioid receptors 1209
Optical spectroscopy 157,581
Optimized tube dilution method 1393
Organic semisynthesis 283
*Orophea yunnanensis* 495
Osteoclastogenesis inhibitory effect 1163
Ototoxic drugs 317
*Ouratea polyantha* 973
Oxidative stability 621
Oxime 1203
Oxime esters 1635
Oxindole alkaloids 1441
Oxofuroindolines 1445
Oxopyrrolidinoindolines 1445
Oxygenated oleanane saponin 705
Oxyprenylated derivatives 1347
Oxyprenylated secondary metabolites 1029
Oxystemokerrine 1277

P-388 cells 1309
p53 1295
Palmitic acid 1363
*Panax japonicas* 491
Papaveraceae 1279
*Paraboea paniculata* (Gesneriaceae) 775
Parainfluenza type 3 virus143
*Parastrephia lepidophylla* 611
*Passiflora incarnate* 1177
*Passiflora* species 1177
Pavinane alkaloids 1279
Pavine alkaloids 725
PC-3 cell line 53
PCA 1181,1453
*p*-Coumaric acid 185
*p*-Cymene 667
Pear 987
*Pectobacterium atrosepticum* 65
*Pelargonium* 1223
Peptide 211
Peptidyl-tRNA Hydrolase 1107
Pericarp 93
Perilipin 1 1193
Perilla Herb 1619
Perillaldehyde 1619
Peripheral blood 563
Permeation 821
Peroxisome proliferator-activated receptor (PPAR)γ 1501
*Peschiera affinis* 729
Petigrain oil 1401
*Peucedanum alsaticum* 1515
*Peucedanum cervaria* 1515
Pharmacodynamics 1283
Pharmacokinetics 1283
Phellandrene 1515
Phenalenones 1249
Phenanthroindolizidine 1171
Phenanthroindolizidine alkaloid 725
Phenethylamine 1579
Phenolic 185,1605
Phenolic acids 187
Phenolic compounds 47,785,1143
Phenolic glycosides 561
Phenolics 45,471
Phenylethanoid glycosides 775
Phenylethanoids 1015
Phenylglycine methyl ester 835
Phenylpropanoic acids 1341
Phenylpropanoid glycosides 181
Pheophorbides 1033
Phloroglucinol derivatives 1033
*Phoenix dactylifera* 3
Phoenixoside A 3
Phospholipase $A_2$ 841
Phospholipid 621
Photocycloaddition 459
Photocytotoxic 1033
pH-Sensitive liposomes 353
Physical properties 157
Phytochemical analysis 47,1111
Phytochemical screening 1107
Phytochemicals 1249
Phytochemistry 757,1469
Phytol 1231,1263
Phytopathogenic fungus 889
Phytotherapy 273
Phytotoxin 1327
Pinacol rearrangement 697,721
Pinene 117,237,243,661,671,943, 1079,1515,1673
*Piper betle* 1627
*Piper officinarum* 1659
*Piper tuberculatum* 1391
*Piper umbellatum* 595
Piperaceae 595,1103
Piperine 1283
*Pistacia integerrima* 1011
Pistacide A 1011
Pistacide B 1011
Pivalic acid 333
Plant 1551
Plant defence 395
Plant extracts 273
Plant growth inhibitory 1197
Plant growth promotion 1545
Plant growth regulators 761,1075
Plant hormone 215
Plant metabolites 1645
Plant origin 569
Plant secondary metabolites 1347
Plasmid relaxation 767
*Plasmodium falciparum* 165
Platelet aggregation 883
*Plectranthus* 1099
*Plectranthus marrubatus* 667
*Pleurotus eryngii* 1163
*Plumeria tuberculata* 101
*p*-Menthanone 81
PNA 349
*Pogostemon* 87
*Pogostemon cablin* 927
Pogostone 87
Polyamines 895
*Polygala sibirica* 1165
Polygalasibiricose I 1165
Polygalasterol A 1165
Polygonaceae 753
*Polygonum* 685
*Polygonum hyrcanicum* 753
*Polygonum multiflorum* 1331
Polyketide 1065
Polyphagous lepidopteran 1645
Polyphenol mixture 1193
Polyphenols 875,1323,1483
Polyploidy 1457
Poly-unsaturated fatty acid 153
*Pometia pinnata* 1423
*Pongamia glabra* 1325
POPC 1215
*Portulaca oleracea* 1047
Portulacaceae 1047
Portulacaldehyde 1047
*Prasium majus* L 931
Pregnane 1267
Prenylated *p*-hydroxyacetophenone 1663
Preparative chromatography 1147
Preservation 621
Primary culture 841
*Primula* 1469
*Primula* 587
*Dionysia* 587
Principal component analysis 1143
Prismaconnatoside 561
*Prismatomeris connate* 561
Proanthocyanidins 381
Proaporphine alkaloids 725
Processing 1185
Profiling 1627
Projection to latent structures 951
Proliferation 1461
Propolis 569
Propylene glycol diesters 1219
Protease 1359
Proteases 343
Protecting group 333
Protein kinase A 923
Protein tyrosine phosphatase 1B (PTP1B) 815
Protopanaxadiol-type saponins 1155
*Prunella vulgaris* 501
*Pseudomonas aeruginosa* 1393
*Pseudowintera* 685
Pseurotins 165
Pulegone 81
*Pulicaria* 113,257
Purine-pyrimidine specificity 305
*Putranjiva roxburghii* 511
Pyridine 1581
Pyrogallol 333,1051
Pyrogallol[4]arene 333
Pyrrolizidine alkaloids 861
*Pyrus communis* 987
*Pyrus serotina* 987

Quantitative analysis 927
Quantitative PCR 1615
Quantum chemistry 157,581
Quercetin glycosides 175
Quercetin-glucopyranoside 33

Rabbit liver carboxylesterase 1117
Radical scavenging 311,609,1609
Radical scavenging activity 901
Raman optical activity (ROA) 419
Ranunculaceae 1623
RAPD analysis 1647
Rat aorta ring assay 1149
Rat reproductive system 41
*Rauvolfia vomitoria* 1647
RAW 264.7 1479
RDC 735
3T3-L1 cells 1501
Reactive oxygen species 401,1501
Rearrangement 1445
Recognition 369
Reduction 529
Relationship 963
Resin glycoside 219
*Rhamnus* 1029
Rhein 207
Rhein-aloe emodin hybrid molecule 207
*Rhipicephalus (Boophilus) microplus* 1387
*Rhizoctonia solanii* 1219,1635
*Rhododendron tomentosum* 227
*Rhus copallinum* 45
Roasting 621
Root 991
ROS 1057
Rosmarinic acid 1619
ROSY 21
Rotamers 1591
Rotenoid derivatives 1479
Royleanumin 1137
RP-HPLC-UV/VIS 1029
RT-PCR 1539
Rubiaceae 1,561,705,779,831
*Russula sanguinea* 1133
Russulaceae 1133
*Ruta graveolens* 895
Rutaceae 1371,1523
Rutaceae 1523

sabinene 1515
Sabinene 1515,1671
Safety 807
*Salvia* 187
*Salvia nemorosa* 1671
S*alvia officinalis* 1631
*Salvia przewalskii* 59
*Salvia reuterana* 651
*Salvia* species 59,201
*Salvia x jamensis* 1237
Salvianolic acid B 885
Sampsoniones 1615
Santalaceae 93
*Santalum album* 93
*Santolina chamaecyparissus* L 937
Sapindaceae 1423
Sapogenins 837
Saponins 837,1149
SAR 899
Saracoside 767
*Satureja bakhtiarica* 133
*Saussurea katochaete* 447
*Schinus terebinthifolius* 129
*Sclerotium rolfsii* 1219
*Sclerotium rolfsii* 1635
Screening 253
*Scrophulariaceae* 565
*Scutellaria lateriflora* 471
SDE 237
SDSE 97
Sea cucumbers 517,845,1157,1431
Sea urchins 901
Seasonality 1315
Seaweeds 1209
*Seco*-angustilobine B 743
*seco-ent*-Kaurene 5
Secoguaiane 441
Secoiridoid glycosides 1561
Secoiridoids 1487
Secondary metabolites 771,1545
*Sedum caespitosum* 39
Seed oil 227,621,919
Seeds 13,785,1305
*Seimatosporium* sp 293
Selectivity index 1025
Semen Vaccariae 1185
*Semenovia transiliensis* 1327
*Semiaquilegia adoxoides* 1623
*Senecio culcitioides* 427
*Senecio nutans* 607
*Senecio spegazzinii* 607
*Senecio viridis*
Separation 1353
*Seseli rigidum* 1091
Sesquiterpene 441,451,819
Sesquiterpene alcohol 467
Sesquiterpene hydroquinones 487
Sesquiterpene lactone 1259
Sesquiterpenes 231,423,435,447,

815,1365,1535,1569
Sesquiterpenoids 145,431,539,971, 1565
Sesterterpene 1411
Shanzhiol 1
Shatavarol 995
Shatavaroside C 995
Shoot cultures 1075
Shoot multiplication 895
Shoot oil 227
Siderophores 1545
Silybin 531
Silybin glucoside 531
*Sinularia triangula* 479
*Sitophilus zeamais* 171,267
Sitosterol 779
Small molecules 1069
*Smilacis glabrae* 181
Solanaceae 65,153
*Solanum erianthum* 153
*Solanum spirale* 955
Solubilization 289
Solvent effect 333
Somaclonal variant 1223
*Sophora flavescens* 1453
*Sophora velutina* subsp. *Zimbabweensis* 999
Soqotra 113
Sorbicillin analogues 1337
*Spartium junceum* L 137
Spasmolytic activity 1523
*Sphaeranthus bullatus* 1123
Spiro-indole diketopiperazines 497
Spirostanes 709
SPME 97,237,655
SPME-GCMS 145
Sponge 617
Square-wave voltammetry 1211
SRB 825
*Stachys lavandulifolia* 1241
*Stachys obtusicrena* 117
Standardization 807
*Staphylococcus aureus* 1173
Starfish 853
Stearic acid 1363
*Stellera chamaejasme* 1499
Stem 1079
Stem extract 195
Stemokerrine 1277
*Stemona kerrii* 1277
Stemonaceae 1277
*Stephanotis mucronata* 1269
Stereochemistry 709,1441
Stereoselective synthesis 1569
*Sternbergia lutea* 571
Steroidal alkaloid glycoside 153
Steroidal glycosides 565,1269
Steroidal sapogenins 709
Steroidal saponin 995
Steroidal saponins 709
Sterol sulfate 1165
Sterols 1209
Storage 621
Streblusligнanol-glucopyranoside 599
*Streblus asper* Lour. 599
*Streptomyces* 211
*Streptomyces* sp 1199,1579
Stress 1539
Structural Elucidation 735
Structure 533
Structure activity relationships 1635
Structure elucidation 193,591
Structure identification 1353
Structure-activity relationships 57,901,1341,1415
Subereatensin 617
Suberosin 235
Submerged fermentation 1359
Substituted tryptamines 1273
Sulfates 487
Sumatra 799
Superoxide anion 481,1021
Supramolecular chemistry 321,343
*Symphytum officinale* L 861
Synergistic interactions 245
Synthesis 191,389,1445
*Syzygium polyanthum* 1033

*Tagetes* 1225
*Taiwania cryptomerioides* 261
Tannins 761,1323
Tanshinones 59
*Taq* polymerase I 1639
*Tarenna* 705
Tarragon 749
tau-cadinol 633
Taxoids 697,1143
*Taxus* varieties 1143
Tea tree oil 675
*Telekia speciosa* 625
Terpenes 973,1563
Terpenoid 533,831
Terphyl acid 1057
Terphyl diacid 1057
Terpinene 117,667
Terpinen-4-ol 117
Terpinolene 79,1079
Terrefuranone 1065
Trichloromethyl-dihydropalmatine 859
Tetrahydrocurcumin 529
Tetrahydroxy-methoxyflavane 179
Tetranor-labd-7-en-l7 1139
*Tetranychus urticae* 463
*Teucrium polium* L. ssp. *Capitatum* 83
*Teucrium royleanum* 1137
*Thalassia* 47
Thienodolin 789
Thin-layer chromatography 1605
Thrombin 1495
Thujone 1651
Thyme 253
Thymofolinoates A and B 1351
Thymol 109,667,1557
Thymol derivatives 625
*Thymus mastichina* L 1491
*Thymus vulgaris* 1095
Tiankuizi 1623
Tissue culture 1457
TLC 875,1147
Tobacco mosaic virus 603
Tocopherols 685
Toluidides 507
Tomato (*Solanum esculentum*) 65
Topical ointment 195
Total cholesterol 35
Total flavonoid 1021
Total phenolic 1021
Total phenolic content 201,203,951,1631
Total phenolics 607
Toxicity 837,1215,1645
Trace elements 1667
Traditional plant uses 33
*trans*-Anethole 1225
Transannular cyclization 441
Transfection 409
Transgenic hairy roots 1331
*trans*-Ocimenone 1225
Tremetone 611
*Trichilia connaroides* 1267
Trichilone 1267
*Trichoderma* 1545
*Trichoderma* sp 1337
Trichomes 1667
Trichorabdal B 977
*Tridax procumbens* L 941
*Trifolium pretense* 1605
Trigonelline 863
Trimethyl-1(4*H*)-naphthalenone 827
Triterpene glycosides 517,845,1157,1431
Triterpene saponin 1419
Triterpenes 187, 501,507,987,1147,1427,1491,1587
Triterpenic amides 507
Triterpenoid 13,495,497,511,1575
Triterpenoid saponin 491,515,1423
Triterpenoids 693,1415
Tropical plant extracts 1107
*brucei rhodesiense* 753
*Trypanosoma cruzi* 165
*Trypanosoma evansi* 609
Tubulosine 19
Tumor cell 1531
Tumor necrosis factor alpha 905
Turmeric, *In vitro* shoots 1037
Twig bark 987
Two spotted spider mite 129
*Tylophora indica* 1171
Tylophorinidine 1171
Tylophorinine 1171
Type XII indole alkaloids 1273
Tyramine 889
Tyrosinase 1287
Tyrosinase inhibitory activity 185

Uliginosin 191
Umbelliferae 841
Unsaturated compounds 1127
UPLC-UV 1177
Urea receptor 301
UV-Vis titrations 301

*Vaccaria hispanica* 1185
Validated chromatographic method 25
Vallesiachotamine 731
Vanillin 1635
Vascular smooth muscles 1397
Vasorelaxant effect 1397
Velutinine 999
*Vernonia cinerea* 1259
*Vernonia cotoneaster* 781
Verongida 617
*Veronica chamaedrys* L. 565
vetiselinenol 223
*Vetiveria zizanioides*223
Vetivones 223
*Villaria odorata* 779
*Vinca minor* 731
Vincristine-resistant P388 977
*Vitis repens* 609
VLC 1147
Volatile 655
volatile constituents 629
Volatile oil 93
Volatile organic compounds 951
Volatile screening 97
Volatiles 659
Vulgarole 637

Water 75
Waterhyacinth 1249
*Werneria poposa* 1663
Winged Sumac 45
Withaferin A 1271
*Withania somnifera* 1271
Withanone 1271
Wnt signal 475
Wound healing 909

X-ray crystallography 511
Xanthine/xanthine oxidase 1021
Xanthoangelol 21
Xanthone 53
Xanthotoxin 1327
*Xenophyllum poposum* 1663
*Xyloselinum leonidii* 1373
*Xyloselinum vietnamense* 1373

Ylang ylang oil 1401

Zebrafish assay 1149
Zerumbone 1369
Zingiberene 637
*Zingiber officinale* 883
*Zingiber zerumbet* var. *darcyi* 1369
Zingiberaceae 795,1369
*Ziziphora clinopodioide* 81,1181

# Natural Product Communications

## Manuscripts in Press
## Volume 7, Number 12 (2012)



**Effect of Argan and Olive Oil Consumption on the Hormonal Profile of Androgens Among Healthy Adult Moroccan Men**
Abdelfettah Derouiche, Ali Jafri, Issam Driouch, Mohammed El Khasmi, Ahmed Adlouni, Nada Benajiba, Youssef Bamou, Rachid Saile and Mohammed Benouhoud

**The Social and Environmental Context of Argan Oil Production**
Yann le Polain de Waroux

**Triterpenoids from *Argania spinosa*: 20 Years of Research**
Imane Chafchaouni-Moussaoui, Zoubida Charrouf and Dom Guillaume

**Modeling of the Distribution of Heavy Metals and Trace Elements in Argan Forest Soil and Parts of Argan Tree**
Faez A. E. Mohammed, Rahma Bchitou, Mohamed Boulmane, Ahmed Bouhaouss and Dominique Guillaume

**Argan Oil-contained Antioxidants for Human Mitochondria**
Luis C. López, Carmen Cabrera-Vique, Carmen Venegas, Laura García-Corzo, Marta Luna-Sánchez, Darío Acuña-Castroviejo and Germaine Escames

**Volatile Compound Formation During Argan Kernel Roasting**
Hanae El Monfalouti, Zoubida Charrouf, Manuela Giordano, Dominique Guillaume, Badreddine Kartah, Hicham Harhar, Saïd Gharby, Clément Denhez, and Giuseppe Zeppa

**Climatic Conditions and Herbivory Effects on Morphological Plasticity of *Argania spinosa***
Fatima Ain-Lhout, María Zunzunegui, Mari Cruz Díaz Barradas, Juan Jáuregui, Tarik Tagma and Said Boutaleb

**Chemical Changes in Extra Virgin Argan Oil after Thermal Treatment**
Saïd Gharby, Hicham Harhar, Badr Eddine Kartah, Dom Guillaume and Zoubida Charrouf

**Some Secrets of *Argania spinosa* Water Economy in a Semiarid Climate**
Mari Cruz Díaz Barradas, María Zunzunegui, Mari Paz Esquivias, Said Boutaleb, Javier Valera-Burgos, Tarek Tagma and Fátima Ain-Lhout

**Can Fruit-form be a Marker for Argan Oil Production?**
Saïd Gharby, Hicham Harhar, Badr Eddine Kartah, Hanae El Monfalouti, Clément Denhez, Miloudi Hilali, Dom Guillaume and Zoubida Charrouf[a]

**Argan Oil and Postmenopausal Moroccan Women: Impact on the Vitamin E Profile**
Hanae El Monfalouti, Zoubida Charrouf, Asma El Hamdouchi, Hanane Labraimi, Imane Chafchaouni-Moussaoui, Badreddine Kartah, Khalid El Kari, Yahya Bensouda, Abdelfettah Derouich, Sylvie Dodin, Clément Denhez, Dom Guillaume and Hassan Agnaou

**Germination Success and Seedling Development of *Argania spinosa* under Different Climatic Conditions and Browsing Intensity**
María Zunzunegui, Juan Jáuregui, Fatima Ain-Lhout, Said Boutaled, Leonor Álvarez-Cansino andMariPaz Esquivias

**Quality Parameters for Cold Pressed Edible Argan Oils**
Bertrand Matthäus

**Headspace, Solid-phase Micro-extraction, Gas Chromatographic-Mass Spectrometric Analysis of Terpenoids in the Latex of *Euphorbia* Species**
Asmita V. Patel, Stephen Sumner, H. Leslie Thompson, Gerald Blunden, David Wright, Liu Jun-feng[3] and Zan Jun-feng

**Fusaodavinvin, a Novel Metabolite Containing Sulfur from the Endophytic Fungus *Fusarium sp*. (CTGU-ZL-34)**
Liang Zhang, Yanhui Liu, Zhangshuang Deng, Zhiyong Guo, Jianfeng Chen, Xuan Tu and Kun Zou

**Anti-metastatic Activities of Bibenzyls from *Dendrobium pulchellum***
Pithi Chanvorachote, Akkarawut Kowitdamrong, Thidarat Ruanghirun, Boonchoo Sritularak, Chutichot Mungmee and Kittisak Likhitwitayawuid

**Antifeedant Activity of Spin-Labeled Derivatives of Deoxypodophyllotoxin against *Brontispa longissima* Larvae**
Gang Feng, Jing Zhang, Liu Yang, Ying-Qian Liu, Zhi-Wei Zhang, Xuan Tian, Qi-An Jin and Zheng-Qiang Peng

**Trichodermaerin, a New Diterpenoid Lactone from the Marine Fungus *Trichoderma erinaceum* Associated with the Sea Star *Acanthaster planci***
Zhong-Liang Xie, Hou-Jin Li, Lai-You Wang, Wan-Ling Liang, Wei Liu and Wen-Jian Lan

**Two New Amides from *Streptomyces michiganensis***
Jinghua Xue, Liangxiong Xu, Zi-Hua Jiang and Xiaoyi Wei

**Chemical Composition and Antibacterial Activity of Essential Oils from *Myrcia alagoensis* (Myrtaceae)**
Aline do N. Silva, Ana Paula T. Uetanabaro and Angélica M. Lucchese

**Microbial Transformations of Isophorone *by Alternaria alternata* and *Neurospora crassa***
Ismail Kiran, Özge Özşen, Turgay Çelik, Semra İlhan, Bükay Yenice Gürsu and Fatih Demirci

**Chemical Composition and Biological Activities of the Essential Oil from *Calamintha nepeta* Plants from the Wild in Southern Italy**
Emilia Mancini, Laura De Martino, Hanna Malova and Vincenzo De Feo

**Chemical Compositions and Antimicrobial and Antioxidant Activities of the Essential Oils from *Magnolia grandiflora*, *Chrysactinia mexicana*, and *Schinus molle* Found in Northeast Mexico**
Laura Guerra-Boone, Rocío Álvarez-Román, Ricardo Salazar-Aranda, Anabel Torres-Cirio, Verónica Mayela Rivas-Galindo, Noemí Waksman de Torres, Gloria María González González, and Luis Alejandro Pérez-López

**Challenges of Curcumin Bioavailability: Novel Aerosol Remedies**
Parasuraman Aiya Subramani and Venkata R. Narala

**Pancreatic Lipase Inhibitory Activity of Cassiamin A, a Bianthraquinone from *Cassia siamea***
Dilip Kumar, Aniket Karmase, Sneha Jagtap, Ruchi Shekhar and Kamlesh K. Bhutani

**Summation Solute Hydrogen Bonding Acidity Values for Hydroxyl Substituted Flavones Determined by NMR Spectroscopy**
William L. Whaley, Ekua M. Okoso-amaa, Cody L. Womack, Anna Vladimirova, Laura B. Rodgers, Margaret J. Risher and Michael H. Abraham

**Inhibition of *In Vitro* Leukotriene $B_4$ Biosynthesis in Human Neutrophil Granulocytes and Docking Studies of Natural Quinones**
Premysl Landa, Zsofia Kutil, Veronika Temml, Jan Malik, Ladislav Kokoska, Ute Widowitz, Marie Pribylova, Marcela Dvorakova, Petr Marsik, Daniela Schuster, Rudolf Bauer and Tomas Vanek

**Norcucurbitane Triterpenoids from the Fruits of *Momordica charantia* var. *abbreviata***
Yun-Wen Liao, Chiy-Rong Chen, Jue-Liang Hsu, Yun-Sheng Lin, Hsueh-Ling Cheng, Wen-Ling Shih, Yueh-Hsiung Kuo and Chi-I Chang

**Phenolic Content and DPPH Radical Scavenging Activity of the Flowers and Leaves of *Trifolium repens***
Agnieszka Kicel and Maria Wolbiś

**Effect of Nine Plant Volatiles in the Field on the Sex Pheromones of *Leguminivora glycinivorella***
Daihua Hu, Juntao Feng, Zhihui Wang, Hua Wu and Xing Zhang

**Molecular Analysis of *Vitex* Species Using Candidate DNA Barcoding and PCR-RFLP of the *mat*K Gene for Authentication of *Vitex glabrata***
Waranyoo Phoolcharoen and Suchada Sukrong

**Evaluation of the Quality of Chinese and Vietnamese Cassia Using LC-MS and Multivariate Analysis**
Ken Tanaka, Feng Li, Yasuhiro Tezuka, Shiro Watanabe, Nobuo Kawahara and Hiroaki Kida

**Conservation and Multiplication of Encapsulated Micro Shoots of *Rauvolfia vomitoria* - an Endangered Medicinal Tree: ISSR and RAPD based Evaluation of Genetic Fidelity of Converted Plantlets**
Shakti Mehrotra, Liaq Ur Rahman, Jahnvi Mishra and Arun K. Kukreja **1647**

**Bioactivities of Volatile Components from Nepalese *Artemisia* Species**
Prabodh Satyal, Prajwal Paudel, Ananad Kafle, Suraj K. Pokharel, Bimala Lamichhane, Noura S. Dosoky, Debra M. Moriarity and William N. Setzer **1651**

**Chemical Compositions, Antioxidant and Antimicrobial Activity of the Essential Oils of *Piper officinarum* (Piperaceae)**
Wan Mohd Nuzul Hakimi Wan Salleh, Farediah Ahmad, Khong Heng Yen and Hasnah Mohd Sirat **1659**

**Chemical Composition, Antimicrobial and Antioxidant Properties of the Volatile Oil and Methanol Extract of *Xenophyllum poposum***
Ana M. González, María I. Tracanna, Sara M. Amani, Carola Schuff, Miguel J. Poch, Horacio Bach and César A. N. Catalán **1663**

**Phytochemical and Micromorphological Traits of Endemic *Micromeria pseudocroatica* (Lamiaceae)**
Dario Kremer, Ivna Dragojević Müller, Edith Stabentheiner, Dubravka Vitali, Martina Kopričanec, Mirko Ruščić, Ivan Kosalec, Nada Bezić and Valerija Dunkić **1667**

**Composition and Variability of the Essential Oil of *Salvia nemorosa* (Lamiaceae) from the Vienna Area of Austria**
Remigius Chizzola **1671**

**Essential oil Composition of *Ficus benjamina* (Moraceae) and *Irvingia barteri* (Irvingiaceae)**
Isiaka A. Ogunwande, Razaq Jimoh, Adedoyin A. Ajetunmobi, Nudewhenu O. Avoseh and Guido Flamini **1673**

## ***Review/Account***

**Cytotoxic Agents of the Crinane Series of Amaryllidaceae Alkaloids**
Jerald J. Nair, Jaume Bastida, Francesc Viladomat and Johannes van Staden **1677**

# Natural Product Communications
# 2012
Volume 7, Number 12

## Contents

***Original Paper*** ***Page***

**A Comparative Study of the Antioxidant/Prooxidant Effects of Carvacrol and Thymol at Various Concentrations on Membrane and DNA of Parental and Drug Resistant H1299 Cells**
Aysun Ozkan and Ayse Erdogan **1557**

**New Secoiridoid Glycosides from the Buds of *Lonicera macranthoides***
Jiang Liu, Jing Zhang, Feng Wang and Xingfu Chen **1561**

**Chemical Constituents from the Aerial Parts of *Gynura bicolor***
Jian Chen, Sven Mangelinckx, An Adams, Wei-lin Li, Zheng-tao Wang and Norbert De Kimpe **1563**

**Chemical Constituents of *Ligularia nelumbifolia* and *L. subspicata* Hybrid Collected in Shangrila County, Yunnan Province of China**
Ryo Hanai, Hiroka Yamada, Yurika Suzuki, Hajime Nagano, Takayuki Kawahara, Jiao-Jun Yu, Xun Gong and Chiaki Kuroda **1565**

**A Practical, Enantiospecific Synthesis of (*S*)-*trans*-γ-Monocyclofarnesol**
Stefano Serra **1569**

**ACAT Inhibitory Activity of Exudates from *Calocedrus macrolepis* var. *formosana***
Yu-Hsin Hsieh, Kuan-Jung Chen, Shih-Chang Chien, Wen-Ling Cheng, Jun-Hong Xiao and Sheng-Yang Wang **1573**

**Cucurbitane-Type Triterpenoids from the Fruit Pulp of *Momordica charantia***
Yun-Wen Liao, Chiy-Rong Chen, Yueh-Hsiung Kuo, Jue-Liang Hsu, Wen-Ling Shih, Hsueh-Ling Cheng, Tzou-Chi Huang and Chi-I Chang **1575**

**Two Novel Phenethylamine Alkaloids from *Streptomyces* sp. YIM10049**
Xueqiong Yang, Guangwei He, Lixing Zhao, Yabin Yang, Yun Liu, Lihua Xu and Zhongtao Ding **1579**

**Alkaloids from *Cinnamomum philippinense***
Hsing-Tan Li, Wei-Jen Li, Hui-Ming Wu and Chung-Yi Chen **1581**

**Conversional Synthesis of Heteratisine**
Ling Wang, Qi-Feng Chen and Feng-Peng Wang **1583**

**Flavonoids and Triterpenes from the leaves of *Artocarpus fulvicortex***
Shajarahtunnur Jamil, Muhammad Taher, Hasnah M. Sirat and Nur Azlin Othman **1587**

**Dihydrospinochalcone-A and Epi-flemistrictin-B, Natural Isocordoin Derivatives from the Root Extract of *Lonchocarpus xuul***
Fabiola Escalante-Erosa, Brenda González-Morales, Ramiro F. Quijano-Quiñones, Gumersindo Miron-López and Luis M. Peña-Rodríguez **1589**

**Phenolic Compounds, Including Novel *C*-glycosylflavone, from the Flowers of the Tall Bearded Iris Cultivar 'Victoria Falls'**
Takayuki Mizuno, Tsutomu Yabuya, Nobuhiro Sasaki and Tsukasa Iwashina **1591**

**Two New Biisoflavonoids from the Roots of *Daphne oleoides***
Shazia Yasmeen, Muhammad Aijaz Anwar, Sadia Ferheen, Nighat Afza, Abdul Malik and Lubna Iqbal **1595**

**Biflavonoids from the Unripe Fruits of *Clusia paralicola* and their Antioxidant Activity**
Rafaela Ferreira Oliveira, Celso Amorim Camara, Maria de Fátima Agra and Tania Maria Sarmento Silva **1597**

**Ochnaflavone and Ochnaflavone 7-*O*-methyl Ether two Antibacterial Biflavonoids from *Ochna pretoriensis* (Ochnaceae)**
Tshepiso J. Makhafola, Babatunde B. Samuel, Esameldin E. Elgorashi and Jacobus N. Eloff **1601**

**Factors Affecting the Separation and Bioactivity of Red Clover (*Trifolium pratense*) Extracts Assayed against *Clostridium sticklandii*, a Ruminal Hyper Ammonia-producing Bacterium**
Isabelle A. Kagan and Michael D. Flythe **1605**

**Exploitation of the Antioxidant Potential of *Geranium macrorrhizum* (Geraniaceae): Hepatoprotective and Antimicrobial Activities**
Niko S. Radulović, Milan B. Stojković, Snežana S. Mitić, Pavle J. Randjelović, Ivan R. Ilić, Nikola M. Stojanović and Zorica Z. Stojanović-Radić **1609**

**Differential Expression of Benzophenone Synthase and Chalcone Synthase in *Hypericum sampsonii***
Lili Huang, Hong Wang, Hechun Ye, Zhigao Du, Yansheng Zhang, Ludger Beerhues and Benye Liu **1615**

**Efficient Preparation of Hangekobokuto (Banxia-Houpo-Tang) Decoction by Adding Perilla Herb before Decoction is Finished**
Megumi Sumino, Yuko Saito, Fumio Ikegami, Yoshiro Hirasaki and Takao Namiki **1619**

**Anti-neutrophilic Inflammatory Secondary Metabolites from the Traditional Chinese Medicine, Tiankuizi**
Chia-Lin Lee, Tsong-Long Hwang, Chieh-Yu Peng, Chao-Jung Chen, Yuan-Shiun Chang and Yang-Chang Wu **1623**

**DART MS Based Chemical Profiling for Therapeutic Potential of *Piper betle* Landraces**
Vikas Bajpai, Renu Pandey, Mahendra Pal Singh Negi, Nikhil Kumar and Brijesh Kumar **1627**

**Total Phenolic Content and Antioxidative Properties of Commercial Tinctures Obtained from Some Lamiaceae Plants**
Adam Kowalczyk, Izabela Biskup and Izabela Fecka **1631**

**Synthesis, Antifungal Activity and Structure-Activity Relationships of Vanillin oxime-*N*-*O*-alkanoates**
Vivek Ahluwalia, Nandini Garg, Birendra Kumar, Suresh Walia and Om P. Sati **1635**

**Cinnamic Acid Derivatives Acting Against *Aspergillus* Fungi. *Taq* Polymerase I a Potential Molecular Target**
Lucas J. Gutierrez, Maria L. Mascotti, Marcela Kurina-Sanz, Carlos R. Pungitore, Ricardo D. Enriz and Fernando A. Giannini **1639**

**Insecticidal Effects of Acetogenins from *Rollinia occidentalis* Seed Extract**
Diego Tolosa, Olga Álvarez Colom, Alicia Bardón and Adriana Neske **1645**

*Continued inside backcover*