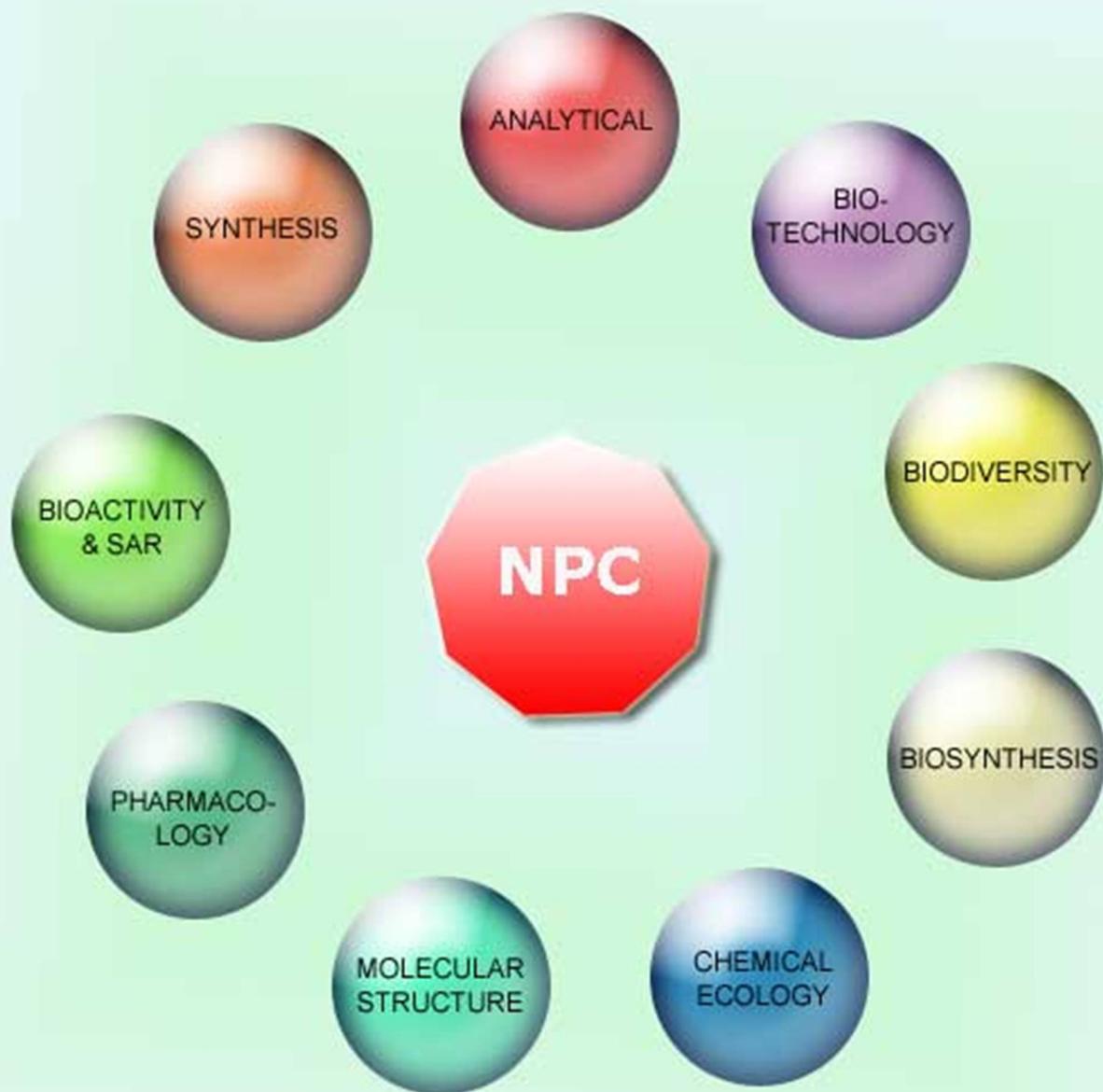


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Natural Product Communications

2010

Volume 5, Number 12

Contents

<u>Original Paper</u>	<u>Page</u>
Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence Damião P. de Sousa, Franklin F. F. Nóbrega, Camila C. M. P. Santos and Reinaldo N. de Almeida	1847
Kinetic Analysis of Genipin Degradation in Aqueous Solution Paul Slusarewicz, Keng Zhu and Tom Hedman	1853
Microbial Transformation of Marine Halogenated Sesquiterpenes Aurelio San Martin, Juana Roviroso, Alvaro Carrasco, Silvia Orejarena, Jorge Soto-Delgado, Renato Contreras and M. Cristina Chamy	1859
Two New Guaianolides from <i>Amberboa ramosa</i> Muhammad Ibrahim, Rehan Khan and Abdul Malik	1865
Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from <i>Canella winterana</i> Mary H. Grace, Carmen Lategan, Flaubert Mbeunkui, Rocky Graziose, Peter J. Smith, Ilya Raskin and Mary Ann Lila	1869
Three New 18-Oxygenated <i>ent</i>-Kaurane Diterpenoids from <i>Isodon leucophyllus</i> Hai Bo Zhang, Jian Xin Pu, Yong Zhao, Fei He, Wei Zhao, Li Guang Lou, Wei Lie Xiao and Han Dong Sun	1873
Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber <i>Cucumaria okhotensis</i>: Stimulation of Activity of Mouse Peritoneal Macrophages Dmitry L. Aminin, Alexandra S. Silchenko, Sergey A. Avilov, Vadim G. Stepanov and Vladimir I. Kalinin	1877
Three New Aaptamines from the Marine Sponge <i>Aaptos</i> sp. and Their Proapoptotic Properties Larisa K. Shubina, Tatyana N. Makarieva, Sergey A. Dyshlovoy, Sergey N. Fedorov, Pavel S. Dmitrenok and Valentin A. Stonik	1881
Isolation and Characterization of Crotosparsamide, a New Cyclic Nonapeptide from <i>Croton sparsiflorus</i> Rashad Mehmood and Abdul Malik	1885
Two New Lavandulyl Flavonoids from <i>Sophora flavescens</i> Dan Liu, Xiulan Xin, Dong-hai Su, Junying Liu, Qing Wei, Bo Li and Jian Cui	1889
Biotransformation of Naringenin to Eriodictyol by <i>Saccharomyces cerevisiae</i> Functionally Expressing Flavonoid 3' Hydroxylase Ilef Limem-Ben Amor, Alain Hehn, Emmanuel Guedon, Kamel Ghedira, Jean-Marc Engasser, Leila Chekir-Ghedrira and Mohamed Ghoul	1893
Two New 3-C-Carboxylated Flavones from the Rhizomes of <i>Caragana conferta</i> Rehan Khan, Abdul Malik, Shazia Yasmeen and Nighat Afza	1899
Kaempferol Glycosides in the Flowers of Carnation and their Contribution to the Creamy White Flower Color Tsukasa Iwashina, Masa-atsu Yamaguchi, Masayoshi Nakayama, Takashi Onozaki, Hiroyuki Yoshida, Shuji Kawanobu, Hiroshi Ono and Masachika Okamura	1903
Factors Influencing Glabridin Stability Mingzhang Ao, Yue Shi, Yongming Cui, Wentao Guo, Jing Wang and Longjiang Yu	1907
Effect of Different Strains of <i>Agrobacterium rhizogenes</i> and Nature of Explants on <i>Plumbago indica</i> Hairy Root Culture with Special Emphasis on Root Biomass and Plumbagin Production Moumita Gangopadhyay, Saikat Dewanjee, Somnath Bhattacharyya and Sabita Bhattacharya	1913
Fujianmycin C, A Bioactive Angucyclinone from a Marine Derived <i>Streptomyces</i> sp. B6219 Muna Ali Abdalla, Elisabeth Helmke and Hartmut Laatsch	1917
Dioscorealide B from the Traditional Thai Medicine Hua-Khao-Yen Induces Apoptosis in MCF-7 Human Breast Cancer Cells via Modulation of Bax, Bak and Bcl-2 Protein Expression Jiraporn Saekoo, Potchanapond Graidist, Wilairat Leeanansaksiri, Chavaboon Dechsukum and Arunporn Itharat	1921

Continued Overleaf

- Inhibition of Protein Tyrosine Phosphatase 1 β by Hispidin Derivatives Isolated from the Fruiting Body of *Phellinus linteus***
Yeon Sil Lee, Il-Jun Kang, Moo Ho Won, Jae-Yong Lee, Jin Kyu Kim and Soon Sung Lim 1927
- A New Azafluorenone from the Roots of *Polyalthia cerasoides* and its Biological Activity**
Kanchana Pumsalid, Haruthai Thaisuchat, Chatchanok Loetchutinat, Narong Nuntasaeen, Puttanan Meepowpan and Wilart Pompimon 1931
- Evaluation of Antiviral Activities of Curcumin Derivatives against HSV-1 in Vero Cell Line**
Keivan Zandi, Elissa Ramedani, Khosro Mohammadi, Saeed Tajbakhsh, Iman Deilami, Zahra Rastian, Moradali Fouladvand, Forough Yousefi and Fatemeh Farshadpour 1935
- Hyloglyceride and Hylodiglyceride: Two New Glyceride Derivatives from *Hylodendron gabunensis***
Awazi Tengu Nyongha, Hidayat Hussain, Etienne Dongo, Ishtiaq Ahmed and Karsten Krohn 1939
- Chemical Composition and Bioactivities of the Marine Alga *Isochrysis galbana* from Taiwan**
Chi-Cheng Yu, Hsiao-Wei Chen, Mao-Jing Chen, Yu-Ching Chang, Shih-Chang Chien, Yueh-Hsiung Kuo, Feng-Ling Yang, Shih-Hsiung Wu, Jie Chen, Hsiao-Hui Yu and Louis Kuop-Ping Chao 1941
- An Efficient Protocol for High-frequency Direct Multiple Shoot Regeneration from Internodes of Peppermint (*Mentha x piperita*)**
Sanjog T. Thul and Arun K. Kukreja 1945
- Essential Oil Yield and Chemical Composition Changes During Leaf Ontogeny of Palmarosa (*Cymbopogon martinii* var. *motia*)**
Bhaskaruni R. Rajeswara Rao, Dharmendra K. Rajput, Rajendra P. Patel and Somasi Purnanand 1947
- Essential Oil Composition of Four Endemic *Ferulago* Species Growing in Turkey**
Ceyda Sibel Kılıç, Ayşe Mine Gençler Özkan, Betül Demirci, Maksut Coşkun and Kemal Hüsnü Can Başer 1951
- Essential Oils of *Daucus carota* subsp. *carota* of Tunisia Obtained by Supercritical Carbon Dioxide Extraction**
Hanan Marzouki, Abdelhamid Khaldi, Danilo Falconieri, Alessandra Piras, Bruno Marongiu, Paola Molicotti and Stefania Zanetti 1955
- Oil Constituents of *Artemisia nilagirica* var. *septentrionalis* Growing at Different Altitudes**
Flora Haider, Narendra Kumar, Ali Arif Naqvi and Guru Das Bagchi 1959
- Volatile Oil Composition of *Pogostemon heyneanus* and Comparison of its Composition with Patchouli Oil**
Ramar Murugan, Gopal Rao Mallavarapu, Kyathsandra Venkataramaiah Padmashree, Ramachandra Raghavendra Rao and Christus Livingstone 1961
- Chemical Composition of Volatile Oils of *Aquilaria malaccensis* (Thymelaeaceae) from Malaysia**
Saiful Nizam Tajuddin and Mashitah M. Yusoff 1965
- Chemical Composition and Phytotoxic Effects of Essential Oils from Four *Teucrium* Species**
Laura De Martino, Carmen Formisano, Emilia Mancini, Vincenzo De Feo, Franco Piozzi, Daniela Rigano and Felice Senatore 1969
- Chemical Constituents and Larvicidal Activity of *Hymenaea courbaril* Fruit Peel**
José Cláudio D. Aguiar, Gilvandete M. P. Santiago, Patrícia L. Lavor, Helenicy N. H. Veras, Yana S. Ferreira, Michele A. A. Lima, Ângela M. C. Arriaga, Telma L. G. Lemos, Jefferson Q. Lima, Hugo C. R. de Jesus, Pérciles B. Alves and Raimundo Braz-Filho 1977
- Caryophyllene Oxide-rich Essential Oils of Lithuanian *Artemisia campestris* ssp. *campestris* and Their Toxicity**
Asta Judzentiene, Jurga Budiene, Rita Butkiene, Eugenija Kupcinskiene, Isabelle Laffont-Schwob and Véronique Masotti 1981
- Comparison of Antibacterial Activity of Natural and Hydroformylated Essential Oil of *Thymus capitatus* Growing Wild in North Sardinia with Commercial *Thymus* Essential Oils**
Marianna Usai, Marzia Foddai, Barbara Sechi, Claudia Juliano and Mauro Marchetti 1985
- Composition and Chemical Variability of the Leaf Oil from Corsican *Juniperus thurifera* Integrated Analysis by GC(RI), GC-MS and ¹³C NMR**
Josephine Ottavioli, Joseph Casanova and Ange Bighelli 1991

**Combined Analysis by GC (RI), GC-MS and ¹³C NMR of the Supercritical Fluid Extract of
Abies alba Twigs**
Emilie Duquesnoy, Bruno Marongiu, Vincent Castola, Alessandra Piras, Silvia Porcedda and Joseph Casanova **1995**

Review/Account

Eugenol: A Natural Compound with Versatile Pharmacological Actions
Kannissery Pramod, Shahid H. Ansari and Javed Ali **1999**

Manuscripts in Press **2007**

Cummulative Index

Contents *i-xvii*
Author Index *1-7*
Keywords Index *i-viii*

LIST OF AUTHORS

Abdalla, MA.....	1917	Ferreira, YS.....	1977	Lima, MAA.....	1977	Rigano, D.....	1969
Afza, N.....	1899	Filho, RB.....	1977	Liu, D.....	1889	Rovirosa, J.....	1859
Aguiar, JCD.....	1977	Foddai, M.....	1985	Liu, J.....	1889	Saekoo, J.....	1921
Ahmed, I.....	1939	Formisano, C.....	1969	Livingstone, C.....	1961	Santiago, GMP.....	1977
Ali, J.....	1999	Fouladvand, M.....	1935	Loetchuinat, C.....	1931	Santos, CMP.....	1847
Alves, PB.....	1977	Gangopadhyay, M.....	1913	Lou, LG.....	1873	Schwob, IL.....	1981
Aminin, DL.....	1877	Ghedira, K.....	1893	Makarieva, TN.....	1881	Sechi, B.....	1985
Amor, ILB.....	1893	Ghedrira, LC.....	1893	Malik, A.....	1865,1885,1899	Senatore, F.....	1969
Ansari, SH.....	1999	Ghoul, M.....	1893	Mallavarapu, GR.....	1961	Shi, Y.....	1907
Ao, M.....	1907	Grace, MH.....	1869	Mancini, E.....	1969	Shubina, LK.....	1881
Arriaga, AMC.....	1977	Graidist, P.....	1921	Marchetti, M.....	1985	Silchenko, AS.....	1877
Avilov, SA.....	1877	Graziose, R.....	1869	Marongiu, B.....	1955,1995	Slusarewicz, P.....	1853
Bagchi, GD.....	1959	Guedon, E.....	1893	Martin, AS.....	1859	Smith, PJ.....	1869
Başer, KHC.....	1951	Guo, W.....	1907	Martino, LD.....	1969	Soto-Delgado, J.....	1859
Bhattacharya, S.....	1913	Haider, F.....	1959	Marzouki, H.....	1955	Stepanov, VG.....	1877
Bhattacharyya, S.....	1913	He, F.....	1873	Masotti, V.....	1981	Stonik, VA.....	1881
Bighelli, A.....	1991	Hedman, T.....	1853	Mbeunkui, F.....	1869	Su, DH.....	1889
Budiene, J.....	1981	Hehn, A.....	1893	Meepowpan, P.....	1931	Sun, HD.....	1873
Butkiene, R.....	1981	Helmke, E.....	1917	Mehmood, R.....	1885	Tajbakhsh, S.....	1935
Carrasco, A.....	1859	Hussain, H.....	1939	Mohammadi, K.....	1935	Tajuddin, SZ.....	1965
Casanova, J.....	1991,1995	Ibrahim, M.....	1865	Molicotti, P.....	1955	Thaisuchat, H.....	1931
Castola, V.....	1995	Itharat, A.....	1921	Murugan, R.....	1961	Thul, ST.....	1945
Chamy, MC.....	1859	Iwashina, T.....	1903	Nakayama, M.....	1903	Usai, M.....	1985
Chang, YC.....	1941	Judzentiene, A.....	1981	Naqvi, AA.....	1959	Veras, HNH.....	1977
Chao, LKP.....	1941	Juliano, C.....	1985	Nóbrega, FFF.....	1847	Wang, J.....	1907
Chen, HW.....	1941	Kalinin, VI.....	1877	Nuntasaeen, N.....	1931	Wei, Q.....	1889
Chen, J.....	1941	Kang, IJ.....	1927	Nyongha, AT.....	1939	Won, MH.....	1927
Chen, MJ.....	1941	Kawanobu, S.....	1903	Okamura, M.....	1903	Wu, SH.....	1941
Chien, SC.....	1941	Khalidi, A.....	1955	Ono, H.....	1903	Xiao, WL.....	1873
Contreras, R.....	1859	Khan, R.....	1865,1899	Onozaki, T.....	1903	Xin, X.....	1889
Coşkun, M.....	1951	Kılıç, CS.....	1951	Orejarena, S.....	1859	Yamaguchi, M.....	1903
Cui, J.....	1889	Kim, JK.....	1927	Ottaviooli, J.....	1991	Yang, FL.....	1941
Cui, Y.....	1907	Krohn, K.....	1939	Özkan, AMG.....	1951	Yasmeen, S.....	1899
de Almeida, RN.....	1847	Kukreja, AK.....	1945	Padmashree, KV.....	1961	Yoshida, H.....	1903
de Jesus, HCR.....	1977	Kumar, N.....	1959	Patel, RP.....	1947	Yousefi, F.....	1935
de Sousa, DP.....	1847	Kuo, YH.....	1941	Piozzi, F.....	1969	Yu, CC.....	1941
Dechsukum, C.....	1921	Kupcinskiene, E.....	1981	Piras, A.....	1955,1995	Yu, HH.....	1941
Deilami, I.....	1935	Laatsch, H.....	1917	Pompimon, W.....	1931	Yu, L.....	1907
Demirci, B.....	1951	Lategan, C.....	1869	Porcedda, S.....	1995	Yusoff, MM.....	1965
Dewanjee, S.....	1913	Lavor, PL.....	1977	Pramod, K.....	1999	Zandi, K.....	1935
Dmitrenok, PS.....	1881	Lee, JY.....	1927	Pu, JX.....	1873	Zanetti, S.....	1955
Dongo, E.....	1939	Lee, YS.....	1927	Pumsalid, K.....	1931	Zhang, HB.....	1873
Duquesnoy, E.....	1995	Leeanansaksiri, W.....	1921	Purnanand, S.....	1947	Zhao, W.....	1873
Dyshlovoy, SA.....	1881	Lemos, TLG.....	1977	Rajeswara Rao, BR.....	1947	Zhao, Y.....	1873
Engasser, JM.....	1893	Li, B.....	1889	Rajput, DK.....	1947	Zhu, K.....	1853
Falconieri, D.....	1955	Lila, MA.....	1869	Ramedani, E.....	1935		
Farshadpour, F.....	1935	Lim, SS.....	1927	Rao, RR.....	1961		
Fedorov, SN.....	1881	Lima, JQ.....	1977	Raskin, I.....	1869		
Feo, VD.....	1969			Rastian, Z.....	1935		

Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence

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The anticonvulsant activity of the racemate and enantiomers of linalool have been evaluated. Pretreatment of the mice with (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool increased the latency of convulsions significantly in the PTZ model. Only *rac*-linalool had an effect at the dose of 200 mg/kg. The enantiomers and their racemic mixture were effective in inhibiting the convulsant effect of PTZ at the dose of 300 mg/kg. The linalools presented pharmacological activity close to that of diazepam. In the PIC seizure model, (*R*)-(-)-linalool and *rac*-linalool presented activity at the dose of 200 mg/kg, but the *rac*-linalool was more potent than (*R*)-(-)-linalool; (*S*)-(+)-linalool had no effect at this dose. On the other hand, at the dose of 300 mg/kg this enantiomer was effective, but less potent than (*R*)-(-)-linalool and *rac*-linalool. In the MES model, linalools decreased the convulsion time of the mice in the doses of 200 and 300 mg/kg. *rac*-Linalool presented maximum effect at 300 mg/kg. Surprisingly, it increased significantly the convulsion time at a dose of 100 mg/kg. Using the parameter of tonic hind convulsions, only (*R*)-(-)-linalool produced protection from tonic extension at the dose of 200 mg/kg. When the (+)- and (-)-enantiomers, and *rac*-linalool were administered at the dose of 300 mg/kg they were also effective in preventing tonic convulsions induced by transcorneal electroshock in the animals. The (+)- and (-)-forms were equipotent and the *rac*-linalool was more effective than phenytoin. We have demonstrated that the two enantiomers have similar qualitative anticonvulsant activity, but show different potencies.

Keywords: epileptic seizure, anticonvulsant activity, terpenes, essential oils, structure-activity relationship.

In folk medicine as well as in phytotherapy, essential oils have been utilized as therapeutic agents to treat many diseases. They are used, for example, as sedatives, relaxants, and anticonvulsants [1,2]. Many essential oils possess a great variety of pharmacological activities, such as anxiolytic [3], anticonvulsant [2], and antinociceptive [4]. Evidence for the effects of their components on animal behavior has been supplied. Compounds such as linalool [5], α -terpineol [6], and citronellol [7] have anticonvulsant activity, while (+)-limonene [8] and (+)-pulegone [9] show analgesic properties, and isopulegol present an anxiolytic-like effect [10]. The monoterpene derivatives also have been shown to have several effects on the central nervous system (CNS), including antinociceptive [11], sedative [12], and antidepressant [13] activity.

A significant number of herbal medications and dietary supplements are used for treating patients with neurological and psychiatric complaints. Some of these products may be anticonvulsant and thus of possible

benefit to patients with epilepsy. There are many studies that report the antiseizure activity of a nonallopathic preparation with animal models of epilepsy. Several of these medications are prepared with plants that contain linalool [14].

Linalool (3,7-dimethyl-1,6-octadien-3-ol), one of the most common terpene alcohols (found in rosewood, linaloe, and lavender), exists in two enantiomeric forms [1,14]. (*R*)-(-)-linalool (licareol) occurs in many essential oils, such as orange blossom flower and lavender flower and has a fine fresh floral odor-character. (*S*)-(+)-Linalool (coriandrol) occurs in coriander fruits and has a more herbal, floral odor-note [15-17]. Linalool is a fragrance ingredient used in many fragrance products as well as in non-cosmetic materials, such as household cleaners and detergents [18].

Pharmacological assessment of chiral compounds in an early research phase can lead to the selection of a single isomer for development. This selection process can

maximize the potential for specific activity and minimize the potential for side-effects. For various reasons, however, many racemates have been developed and for these compounds the pharmacological picture is considerably more complex than that of the single enantiomer. Therefore, in the present paper, we studied the anticonvulsant properties of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool in three animal models of convulsion exploring differences between the enantiomers and racemic mixture with respect to their pharmacological effects.

Because the linalool enantiomers are common in many plant species, and are used in cosmetic, non-cosmetic, and pharmaceutical preparations, as well as in the food industry [19-21], it is interesting and important to know the effects and the enantioselectivity of the convulsion receptors in relation to these monoterpenes. Chiral recognition by receptors and enzymes is well demonstrated in biochemical, pharmaceutical, and chemosensory research. We report in this comparative study the findings of the central effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on the behavioral parameters of these anticonvulsant drugs in mice.

We first investigated the anticonvulsant activity involved in the PTZ-induced seizures model. The administration of PTZ caused clonic convulsions in mice. As shown in Figure 1, pretreatment of the mice with (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool increased the latency of convulsions significantly. Only *rac*-linalool ($p < 0.05$) had an effect at the dose of 200 mg/kg [$F(3.27) = 3.747$; $P = 0.02$]. This indicates that the largest potency of the *rac*-linalool is determined by the synergistic action of its constituents. When the enantiomers and their racemic mixture were administered at the dose of 300 mg/kg they were also effective in inhibiting the convulsant effect of PTZ [$F(3.23) = 14.55$; $P < 0.0001$]. The linalools presented pharmacological activity close to that of diazepam ($p < 0.001$), a standard anticonvulsant drug. PTZ is the prototype agent in the class of systemic convulsants, and is used as a screening test for anticonvulsants. Generally, compounds with anticonvulsant activity in petit mal epilepsy are effective in the PTZ-induced seizure model [22-24]. So (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool may be useful in petit mal epilepsy.

The anticonvulsant activity was also evaluated in the PIC seizure model. (*R*)-(-)-Linalool and *rac*-linalool presented activity at the dose of 200 mg/kg (Figure 2), but the *rac*-linalool ($p < 0.001$) was more potent than (*R*)-(-)-linalool ($p < 0.05$). (*S*)-(+)-Linalool had no effect at this dose. On the other hand, at the dose of 300 mg/kg [$F(3.28) = 27.23$; $P < 0.0001$], this enantiomer was effective ($p < 0.01$) but less potent than (*R*)-(-)-linalool

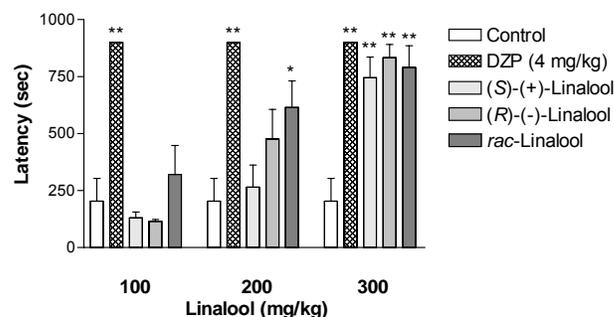


Figure 1: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on PTZ-induced seizure in mice. Values are the latency of convulsions. Values are presented as mean \pm S.E.M. for 8 mice; * $p < 0.05$, ** $p < 0.01$, when compared with vehicle (control), one-way ANOVA, followed by Tukey's test.

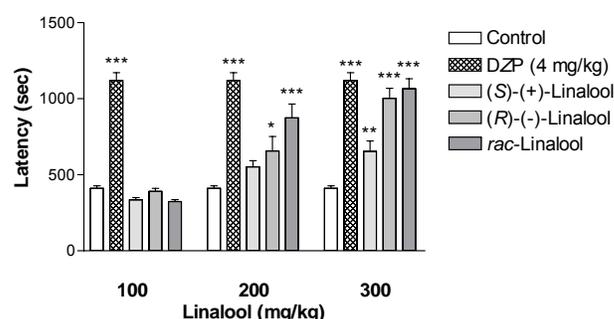


Figure 2: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on PIC-induced seizure in mice. Values are the latency of convulsions. Values are presented as mean \pm S.E.M. for 8 mice; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, when compared with vehicle (control), one-way ANOVA, followed by Tukey's test.

($p < 0.001$) and *rac*-linalool ($p < 0.001$). Again, a synergistic action was observed. The present result also shows that the chiral center at carbon 3 in the linalool molecule is important in the interaction with the receptor. The molecule with the *R*-configuration hydroxy group at carbon 3 is clearly more bioactive than the molecule with the *S* configuration. PIC seizure is a popular systemic convulsant model and is known to be a GABA antagonist exerting its effect by binding to the picrotoxin binding site, which is closely related to the chloride ionophore in the GABA_A receptor complex. Classical anticonvulsants such as carbamazepine, phenytoin and DZP have a protective effect against PIC-induced seizures [25].

In another model, MES seizure, (*S*)-(+)-linalool ($p < 0.001$), (*R*)-(-)-linalool ($p < 0.001$), and *rac*-linalool ($p < 0.001$) decreased the convulsion time of the mice in the doses of 200 [$F(3.28) = 21.40$; $P < 0.0001$] and 300 mg/kg; *rac*-Linalool presented maximum effect at 300 mg/kg. Surprisingly, it also increased significantly the convulsion time at the dose of 100 mg/kg [$F(3,28) = 100.5$; $P < 0.0001$] (Figure 3). Using the parameter of tonic hind convulsions, only (*R*)-(-)-linalool produced protection from tonic extension of about 62.5% ($p < 0.05$) at the dose of 200 mg/kg.

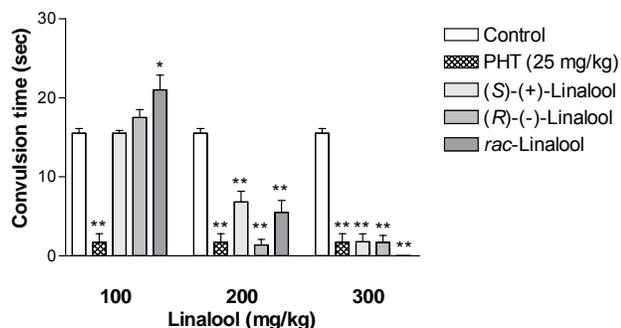


Figure 3: Effect of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on the convulsion time induced by electroconvulsive shock. The bars indicate mean \pm S.E.M. ($n = 8$). Statistically significant differences * $p < 0.05$, ** $p < 0.001$ with respect to control according to one-way ANOVA, followed by Tukey's test.

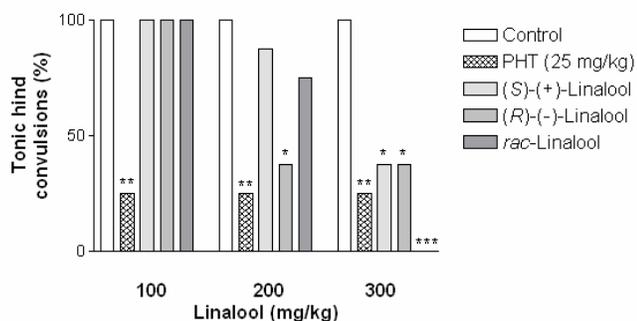


Figure 4: Effects of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool on tonic convulsions induced by electroconvulsive shock. The bars indicate percentage values. Statistically significant differences at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Fisher's exact test).

When the (+)- and (-)-enantiomers, and *rac*-linalool were administered at the dose of 300 mg/kg they were also effective in preventing tonic convulsions induced by transcorneal electroshock in the animals, producing 62.5% ($p < 0.05$), 62.5% ($p < 0.05$), and 100% ($p < 0.001$) protection, respectively. The (+)- and (-)-forms were equipotent and the *rac*-linalool was more effective than phenytoin [75.0% ($p < 0.01$)], a standard anticonvulsant drug (Figure 4). Again the influence of the stereogenic center at carbon 3 and the synergistic action was shown. The MES method is arguably the best-studied and most useful animal model of seizures. Drugs able to inhibit MES seizures in mice are considered to be candidate therapies for primary and secondarily generalized tonic-clonic epilepsies [22].

Chirality has been used to see whether more specific agents can be found for subsets of GABA-receptors. A higher affinity for these receptors results in a better pharmacological profile of the drug. The PTZ and PIC seizure models showed the involvement of the GABA-receptor in the anticonvulsant activity of (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool [26]. It has been reported that linalool also acts on glutamatergic transmission. This monoterpene significantly reduces potassium-stimulated glutamate release, as well as glutamate uptake, but not

interfering with basal glutamate release [27]. It also inhibited and delayed the behavioral expression of PTZ-kindling, but did not modify the PTZ-kindling-induced increase in L-[3H]glutamate binding [28]. Therefore, linalool acts through different mechanisms of action to inhibit the action of various convulsant agents.

The pharmacological differences between linalool enantiomers have been observed on physiological parameters of stress [29] and sedative effect in humans [17]. The chiral influence of other optically active monoterpenes on behavior experimental models also was demonstrated [9,30]. Interestingly, the combined results showed that (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool inhibit not only the action of pentylenetetrazol and picrotoxin (chemical-convulsions), but also protect the mice against MES-induced seizures. This feature may be of interest when one thinks of a good candidate for a drug designed to cause neuroprotection in response to various pro-convulsive agents. The MES and PTZ procedures are of predictive relevance regarding the clinical spectrum of activity of the investigated compounds [31].

In conclusion, we have found that (*S*)-(+)-, (*R*)-(-)- and *rac*-linalool may be effective in blocking generalized tonic-clonic partial and generalized clonic seizures. Our data also demonstrate that the two enantiomers have similar qualitative anticonvulsant activity, but differ in their potencies. The administration of a single enantiomer appears to offer no advantage over the racemic mixture.

Experimental

Chemicals: Pentylenetetrazol (PTZ), phenytoin (PHT), picrotoxin (PIC), Tween 80 and diazepam (DZP) were purchased from Sigma Chemical Co. (USA). (*R*)-(-)-Linalool [α]_D²³ = -16.2° (*c* 4.2, CHCl₃) and *rac*-linalool were purchased from Sigma Aldrich Chemical Co. (USA) and Dierberger (Brazil), respectively. (*S*)-(+)-Linalool (1.23 g) was isolated by column chromatography on silica gel (*n*-hexane/ethyl acetate, 9 : 1 v/v) from *Coriandrum sativum* L. oil (3.15 g) [17]. (*S*)-(+)-Linalool [α]_D²³ = +13.6° (*c* 2.7, CHCl₃) was identified on the basis of chromatographic (TLC) behavior, optical rotation, and spectroscopic data (¹H and ¹³C NMR and IR). These assignments agreed with literature values [32] and data of (*R*)-(-)-linalool. All drugs were injected intraperitoneally (ip).

Animals: Adult, male, 3 month old, albino Swiss mice weighing 24–30 g, were used throughout this study. The animals were randomly housed in appropriate cages at 21±1°C on a 12 h light cycle with free access to food (Purina – Brazil) and tap water. This study used 360

animals that were placed in groups, each of eight animals. All animals were acclimatized before the experiments and the behavioral observations were conducted between 08:00 and 13:00 h. Experimental protocols and procedures were approved by the Laboratório de Tecnologia Farmacêutica Animal Care and Use Committee (CEPA/LTF-UFPB #1205/06).

Statistical analysis: The behavioral data obtained were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's Test. The incidence of clonic or tonic convulsions were evaluated by Fisher's exact test. Differences were considered to be statistically significant when $p < 0.05$.

Pentylenetetrazol (PTZ)-induced convulsions: Mice were divided into 5 groups ($n = 8$). The control and positive control groups received 5% Tween 80 and diazepam (4 mg/kg), respectively. The remaining groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool at doses of 100, 200 and 300 mg/kg. Thirty min after drug administration, the mice were treated with PTZ (ip) at a dose of 60 mg/kg and observed for at least 15 min to detect the occurrence of the first episode of forelimb clonus [33].

Picrotoxin (PIC)-induced convulsion: Animals were divided into 5 groups ($n = 8$). The first group served as a control and received saline with one drop of Tween 80, while the second group was given diazepam (4 mg/kg, ip). The remaining groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool (100,

200 and 300 mg/kg, ip). After 30 min of administration, the mice were challenged with picrotoxin at a dose of 8 mg/kg (ip). Immediately after the picrotoxin injection, mice were individually placed in plastic boxes and observed for the onset of clonic seizures [34].

Maximal electroshock (MES)-induced convulsion: The maximal electroshock (MES) protocol to produce convulsions characterized by tonic hindlimb extension was used [33]. Electroconvulsive shock (130V, 150 Hz, for 0.5 s) was delivered through auricular electrodes (ECT UNIT 7801 – Ugo Basile). Mice were divided into 5 groups ($n = 8$). The first group served as a control and received saline with one drop of Tween 80, while the second group was treated with phenytoin (25 mg/kg, ip) and the other groups received an injection of (*S*)-(+)-linalool, (*R*)-(-)-linalool or *rac*-linalool (100, 200 and 300 mg/kg, ip). After 30 min all groups received the electroconvulsive shock. The convulsion time (s) and percentage of animals showing tonic convulsions, characterized by the presence of tonic hind limb extension, was carefully observed. The animals that did not exhibit tonic hindlimb extension were considered protected [35]. Phenytoin and saline were used as positive and negative controls, respectively.

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Kinetic Analysis of Genipin Degradation in Aqueous Solution

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Degradation of genipin (GP), a low toxicity natural protein crosslinking agent, in aqueous solution was monitored by HPLC at various pH levels. Degradation of GP was consistent with a mechanism consisting of a first order reaction with a reversible first step. Formation of the intermediate was slowest at more neutral pHs while formation of the irreversible product was correlated to increasing alkalinity. Degradation at all pHs was enhanced by the presence of phosphate ions. Degradation of GP most likely proceeds via the reversible opening of the dihydropyran ring by water followed by irreversible polymerization of the intermediate. Degraded solutions containing no detectable GP or intermediate, however, are still capable of crosslinking proteins.

Keywords: Genipin, crosslinking, degradation, kinetics.

Genipin (GP), whose structure is depicted in Figure 1, is the aglycone of geniposide [1,2], an iridoid glycoside that is a major component of the fruit of the gardenia plant, *Gardenia jasminoides* Ellis. GP is a potent and yet relatively non-toxic [3,4] crosslinker of proteins [5] and has elicited a great deal of interest in areas as diverse as tissue engineering [4,6], biomechanics [7,8], forensics [9] and leather tanning [10]. In addition, GP may be a promising therapeutic for the treatment of type-2 diabetes [11]. Although a complete chemical synthesis of GP has been reported [12], the process is not commercially viable and GP is still produced from plant-derived geniposide by enzymatic deglycosylation [13].

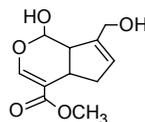


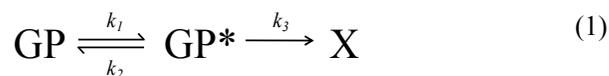
Figure 1: Structure of Genipin.

In order for GP to be utilized in both pharmacological and non-pharmacological applications, some information on the stability of GP in aqueous solution would be valuable as a basis for the design of both products with acceptable shelf lives and crosslinking reaction schemes. Although it has been reported that GP is particularly labile under both acidic [1] and alkaline conditions [14,15], its degradation kinetics have never

been studied in detail. This paper reports on the stability of GP in aqueous solution.

When GP was incubated at 24°C and pH 9 it degraded rapidly (Figure 2), but did not appear to obey the first order rate law since the correlation coefficient obtained from an exponential fit (Figure 2, broken line) was only 0.9952. The first order equation approximated the data obtained at later time points but deviated substantially in the earlier samplings.

A number of possible reaction mechanisms might better describe this data, including that of a sequential reaction with a reversible first step as depicted in Equation 1.



In this reaction GP is converted into a hypothetical intermediate (GP*) which can either reform GP or be degraded to a second product (X) in an irreversible step. The kinetics of such a reaction are governed by three kinetic constants; k_1 for the conversion of GP to GP*, k_2 for the reversion of GP* to GP and k_3 for the breakdown of GP* to X.

This reaction is described mathematically by equations 2-6, and thus the concentration of the three molecules in this reaction scheme can be determined using equations

2, 3 and 4 respectively [16], which express the concentration of each molecule at time t with respect to the starting GP concentration ($[GP]_0$) and the three rate constants.

$$[GP]_t = [GP]_0 \left(\left[\frac{\gamma_1 - k_2 - k_3}{\gamma_1 - \gamma_2} \right] e^{-\gamma_1 t} + \left[\frac{k_2 + k_3 - \gamma_2}{\gamma_1 - \gamma_2} \right] e^{-\gamma_2 t} \right) \quad (2)$$

$$[GP^*]_t = \frac{k_1 [GP]_0}{\gamma_1 - \gamma_2} (e^{-\gamma_2 t} - e^{-\gamma_1 t}) \quad (3)$$

$$[X]_t = k_1 k_3 [GP]_0 \left(\left[\frac{-1}{\gamma_2 (\gamma_1 - \gamma_2)} \right] e^{-\gamma_2 t} + \left[\frac{1}{\gamma_1 (\gamma_1 - \gamma_2)} \right] e^{-\gamma_1 t} + \frac{1}{\gamma_1 \gamma_2} \right) \quad (4)$$

Where:

$$\gamma_1^2 - (k_1 + k_2 + k_3)\gamma_1 + k_1 k_3 = 0 \quad (5)$$

$$\gamma_2 = \frac{k_1 k_3}{\gamma_1} \quad (6)$$

To determine rate constant values, data was fitted iteratively to Equation 2 using Octave software. This produced an accurate fit ($R^2=0.99994$) with values of 0.52, 1.24 and 0.99 hr^{-1} for k_1 , k_2 and k_3 respectively (Figure 2, solid line), suggesting that Equation 2 is a more accurate description of GP degradation than simple first-order decay. A wavelength scan of the degraded GP sample did not reveal the presence of new light-absorbing species in the 200-900 nm range and so subsequent experiments were limited to measuring the disappearance of GP and not the appearance of GP^* or X.

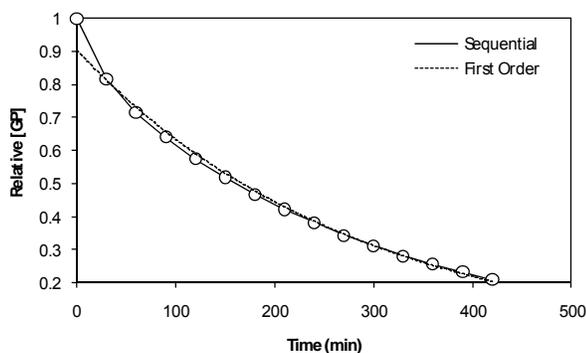


Figure 2: Degradation of GP at pH 9. GP was incubated at 24°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted exponentially (First Order) or to Equation 2 (Sequential).

GP degradation was strongly influenced by pH and was more rapid under alkaline conditions (Figure 3). Interestingly, however, while stability increased with increasing acidity from pH 9 to 6, stability at pH 4 and 5 appeared to be comparable to that at pH 7. These experiments were repeated several times at each pH and the average value of each rate constant and the half-life ($t_{1/2}$) of GP under each condition were determined (Table 1). These data suggested that this effect was due to a relative increase in the k_1 rate constant at pH 4 and 5 relative to pH 6 and that the apparent decrease in stability observed under the more acidic conditions was due to a more rapid establishment of the GP/GP^*

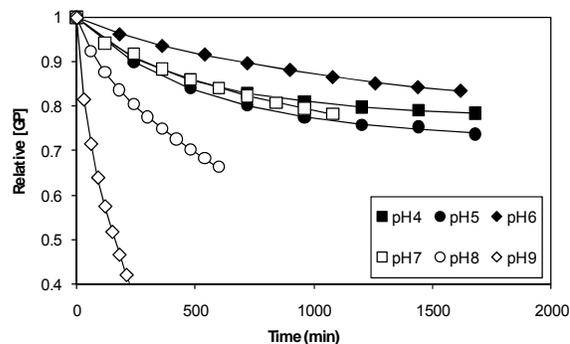


Figure 3: Degradation of GP at various pHs. GP solutions at various pHs were incubated at 24°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted using Equation 2.

Table 1: Summary of experimentally determined rate constants (in hour^{-1}) and calculated half-lives (in hours).

pH	n	k_1	k_2	k_3	$t_{1/2}$	$t_{1/2}^*$
4	4	0.033	0.126	0.005	443.2	650.7
5	4	0.039	0.113	0.006	293.1	494.4
6	3	0.017	0.116	0.046	139.8	162.7
7	4	0.029	0.138	0.056	77.9	97.1
8	3	0.105	0.468	0.273	16.9	20.8
9	3	0.491	1.406	1.196	2.9	3.8

equilibrium and not to irreversible degradation. In fact, the k_3 constant decreased substantially at pH 4 and 5, suggesting that irreversible degradation might indeed be slower under the more acidic conditions.

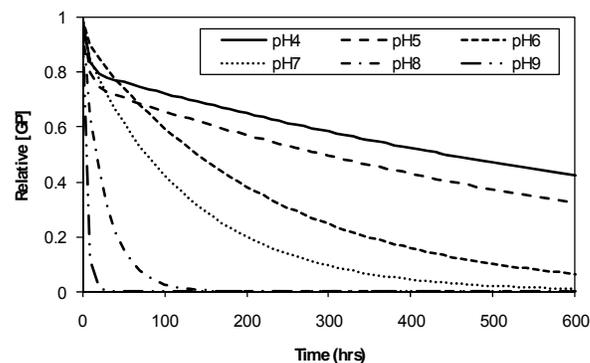


Figure 4: Calculated long-term GP degradation curves. Using experimentally determined rate constants (Table 1), the degradation of GP at various pHs was modeled and plotted using Equation 2.

The experimentally determined values for the three constants were used to plot theoretical curves for the degradation of GP at the various pHs (Figure 4). This indeed showed that at longer time scales GP was more stable at pH 4 and 5, due to the reduction in the magnitude of k_3 . This also demonstrates that the concentration of GP at any given time point does not truly reflect the stability of GP since GP^* can reform GP instead of being permanently converted to X. It is therefore the sum of $[GP]$ and $[GP^*]$ that determines the amount of irreversible degradation that has occurred.

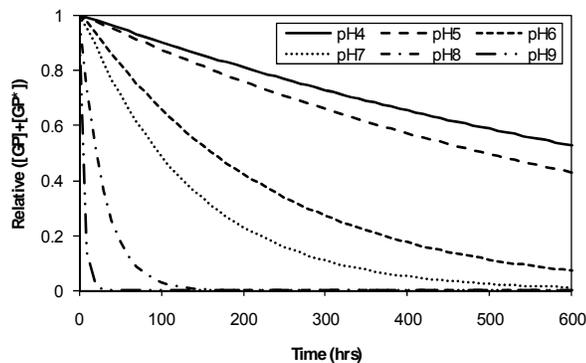


Figure 5: Calculated long-term permanent GP degradation curves. Since GP* is not a permanent breakdown product of GP, the actual breakdown was calculated by determining the loss of both GP and GP* over time using experimentally determined rate constants (Table 1) and Equations 2 and 3.

The concentration of GP + GP* was calculated at various time points to obtain curves (Figure 5) and half-lives (Table 1) that describe the true irreversible degradation of GP and demonstrate a clear and direct correlation between pH and GP stability from pH 4 to 9.

It was previously shown that GP crosslinking is enhanced by the presence of phosphate ions [17]. It was therefore investigated whether they could also affect stability. GP was incubated in the presence or absence of phosphate at pH 7, 8 or 9 and its degradation monitored. In its presence degradation was still consistent with the mechanism of Equation 1 ($R^2 > 0.999$ in all cases), but was more rapid in all cases. In general, phosphate increased the magnitude of each rate constant by approximately 1.5 to 3-fold, resulting in decreases in half-lives of 45-70% (Table 2). There was no apparent correlation between the changes in the rate constants or half-lives and pH.

Table 2: Summary of experimentally determined rate constants (hour^{-1}) and calculated half lives (in hours) in the presence of phosphate ions.

pH	k_1	k_2	k_3	$t_{1/2}$	$t_{1/2}^*$
7	0.10	0.38	0.12	26.3	35.6
8	0.24	0.83	0.34	9.2	12.4
9	0.91	2.03	2.50	1.3	1.7

As expected, GP stability increased when a sample was frozen and stored at -20°C (Figure 6). The degradation was still consistent with the mechanism of Equation 1, although the fit was not as close as in previous experiments ($R^2 = 0.998$). This was probably due to experimental errors from the use of an external calibration standard to quantify GP concentration at each time point since this experiment was conducted over a period of 4 months and not in a single run. The values for k_1 , k_2 and k_3 determined from this experiment were 0.667, 1.110, and $4.849 \times 10^{-3} \text{ hr}^{-1}$ respectively under these conditions. When compared to the average

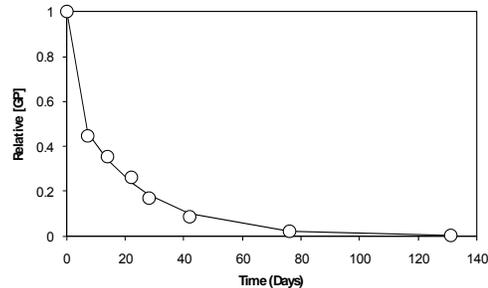


Figure 6: Degradation of GP at -20°C . GP was incubated at -20°C and pH 9 and analyzed for GP content at regular intervals by HPLC. Data were fitted to Equation 2.

values obtained from identical samples stored at 24°C (Table 2), the k_1 and k_2 rate constants decreased by only 0.36- and 0.82-fold respectively at -20°C . The k_3 constant, however, appeared to be substantially more sensitive to temperature and was reduced 515-fold. These decreases in rate constant increased the apparent half-life of GP under these conditions from 1.3 hours for an equivalent sample stored at 24°C to 124 hours, a 96-fold increase. The actual GP half life (i.e. GP+GP*), however, increased 221-fold from 1.7 hours at 24°C to 382 hours.

GP reaction with amines is thought to occur via a nucleophilic attack by the free N-electron pair on the olefinic carbon of the dihydropyran ring [18,19], resulting in ring opening and reclosure to incorporate the amine nitrogen. Crosslinking then proceeds via polymerization. It has also been suggested that since GP contains a cyclic hemi-acetal in its dihydropyran ring it may be susceptible to reversible attack and opening by water [20] to form aldehydes (Figure 7). Breaking of the dihydropyran ring in alkaline solution has been confirmed experimentally by Mi and colleagues, who also demonstrated that ring-opened GP is capable of polymerization by aldol condensation [14]. However, while Mi *et al* reported that ring opening and subsequent polymerization occurs only at very high pH levels (i.e. 13.6), our data suggests otherwise. Closer inspection of their data, in fact, shows that the absorbance at 240nm of GP solutions prepared at different pHs follows the order $7.4 > 5 > 1.2 > 9$, i.e. similar to the “apparent” GP stability observed here. Possibly the degradation of GP at lower pH was previously overlooked due to the extremely high degradation/polymerization rate observed at pH 13.6.

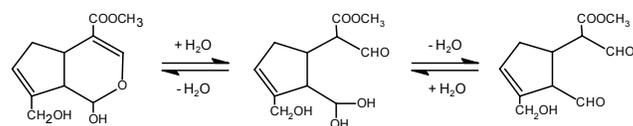


Figure 7: The proposed reversible ring-opening reactions of genipin in aqueous solution.

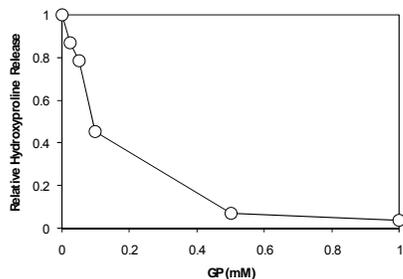


Figure 8: Protein crosslinking by GP. Samples of bovine annulus tissue were incubated with various concentrations of GP and then subjected to proteolysis. The extent of proteolysis, and hence crosslinking, was monitored by measuring the release of hydroxyproline-containing peptides from the insoluble tissue matrix.

While this paper [14] does not report the incubation times used before spectral analysis, the authors do state: “Because polymerization of genipin at strong base (pH 13.6) was quick (the color of aqueous genipin changed from transparent to brownish immediately), its intermediate reaction could only be examined at a lower temperature.” It is therefore possible that the intermediates and products of degradation were not observed previously because their formation rate at lower pHs was too slow to allow their detection upon subsequent analysis. In fact, given sufficient time, the “browning” of GP solutions was observed at all pHs reported here, including at pH4 at -20°C after 6 months (data not shown). The data therefore, suggest that GP degradation by reversible ring opening and subsequent polymerization is a phenomenon that occurs at all pH levels, albeit with dramatically different kinetics.

While the chemical identities of both GP* and X have not been determined, in light of these previous studies, it seems likely that GP* represents the open-ring structure of GP while X represents the first irreversible product of the polymerization process. It should be noted that our data are also consistent with a mechanism where GP is converted to GP* via multiple reversible steps through one or more additional intermediates. In such an instance, our k_1 and k_2 rate constants would describe a simplification of a more complex equilibrium, but would nevertheless be valid.

Interestingly, the GP sample that had been stored at -20°C for 131 days was still capable of crosslinking proteins even though it contained no detectable GP (Figure 6). The resistance of insoluble protein matrices to enzymatic proteolysis increases with the extent of crosslinking [21], due to the increased number of scission events required to release soluble peptide fragments and a decrease in the steric accessibility of the protease to its substrate. Such a protease protection assay was used to indirectly measure the ability of GP to crosslink the insoluble collagenous tissue of the annulus fibrosis of bovine spinal discs.

Treatment of tissue with a fresh solution of 0.25 mM GP resulted in a 93% decrease in the proteolysis of the tissue. The 131-day sample originally containing a 10 mM solution of GP was diluted 40-fold to produce the equivalent of a 0.25 mM solution. While this sample contained no GP by HPLC it was still capable of decreasing proteolysis by 67%.

Since the concentration of GP in the 131-day sample was below the detection limits of our assay, the levels of both GP and GP* were calculated using Equations 2 and 3 and the experimentally determined values for the three rate constants. The GP content of the sample was $21\ \mu\text{M}$ while the GP* concentration was $12\ \mu\text{M}$, giving a total of $33\ \mu\text{M}$. Since the sample was diluted 40-fold prior to use, the final concentration of GP and the reversible intermediate in the crosslinking reaction was therefore less than $1\ \mu\text{M}$. The crosslinking activity of low concentrations of fresh GP was then determined under the same conditions (Figure 8) and, at the lowest concentration tested ($25\ \mu\text{M}$), GP was only able to retard proteolysis by 13% compared to the 67% by the aged, $<1\ \mu\text{M}$ sample. This suggests that X and/or further downstream products are still capable of crosslinking proteins leading to the speculation that X corresponds to polymers of ring-opened genipin reported previously [14], and crosslinking is due to the reaction of aldehydes present in the polymer termini with the protein.

Experimental

Genipin was purchased from Challenge Bioproducts Co., Ltd. (Taiwan). All other reagents were purchased from Sigma.

HPLC Analysis: Analysis of GP was an adaptation of previous methods [3,22,23] and conducted using a Hewlett Packard Series 1050 HPLC and an Agilent Extend C-18 column fitted with a guard cartridge. The column was equilibrated in 40% methanol/0.1% perchloric acid and run isocratically in the same buffer.

For short-term kinetic studies, $50\ \mu\text{L}$ of a solution of freshly prepared 1 mM GP in buffer was injected onto the column. The sample was incubated at 24°C and further $50\ \mu\text{L}$ aliquots injected and analyzed at regular intervals thereafter. The magnitude of the intervals was determined by the rate of GP degradation under the given conditions. The samples were run for 10 minutes at 1 mL/min and absorbance was monitored at 240nm. GP eluted at approximate 6.7 minutes. Peak integration was performed using Chemstation version A.09 software at default settings. Data were normalized by dividing by the peak area of the $t=0$ sample.

Buffers were selected to contain no amines, which could react with GP and thus complicate results. All buffers used in the pH studies were 100 mM sodium acetate (pH 4), sodium cacodylate (pH 5), MES (pH 6), MOPS (pH 7), and EPPS (pH 8 and 9). In experiments containing phosphates, the appropriate buffers were supplemented with 100 mM sodium triphosphate.

A long term -20°C stability experiment was conducted with a 10 mM solution of GP in 100 mM EPPS/100 mM sodium triphosphate, pH 9. A sample of the solution was diluted 10-fold with water and analyzed as described above while the remainder was divided into aliquots and frozen at -20°C. An aliquot was thawed, diluted and analyzed at regular intervals over 4 months. In addition, before each GP analysis, a 50 µL aliquot of freshly prepared 0.5 mM uracil in water was also analyzed using the GP method as a calibration standard. The peak area of each GP peak was normalized by dividing by the area of the uracil peak produced on the

same day of analysis and then by the normalized peak area of the t=0 sample.

Half-lives were calculated iteratively using an algorithm programmed into custom Visual Basic script to determine the time at which the concentration of either GP or GP+GP* equaled 0.5.

Tissue Crosslinking: Crosslinking and analysis of bovine annulus tissue by GP was conducted as described previously [24]. Crosslinking was conducted on 20-25 mg of homogenized bovine annulus fibrosis per duplicate sample at 37°C for 1 hour with shaking in the presence of 0.5 mL of crosslinking solution. Crosslinking solutions consisted of either various concentrations of fresh GP in 100 mM EPPS/100 mM sodium triphosphate pH 9 or a 1/40 dilution into the same buffer of degraded 10 mM GP.

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Microbial Transformation of Marine Halogenated Sesquiterpenes

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The sesquiterpene pacifenol is one of the main constituents of the red alga *Laurencia claviformis*. Earlier work on the semisynthetic derivatives of pacifenol afforded a series of halogenated sesquiterpenes. The aim of the present work was to obtain new hydroxylated derivatives of halogenated sesquiterpenes by means of microbial transformation using *Aspergillus niger*, *Gibberella fujikuroi* and *Mucor plumbeus*. The best results were obtained with *M. plumbeus*. The microbiological transformation by *M. plumbeus* of pacifenol, and two semisynthetic derivatives, is described. The structures of the new compounds obtained were determined by spectroscopic means.

Keywords: *Laurencia claviformis*, *Mucor plumbeus*, biotransformation, pacifenol, pacifidiene.

Biotransformation is an important tool in the structural modification of organic compounds, especially natural products, due to its significant regio and stereoselectivities [1-3].

Filamentous fungi have frequently been used to catalyze selective hydroxylation reactions that are usually difficult to achieve by chemical means [2]. This study looks at the microbial hydroxylation of pacifenol and its derivatives **2** and **3**, using the fungal microorganisms *Aspergillus niger*, *Gibberella fujikuroi* and *Mucor plumbeus*. From screening experiments, *M. plumbeus* was selected as the best yielding microorganism and incubations with this (6 days, 27°C, 0.5 g/L) afforded, after extraction, compounds **4** – **9**.

Pacifenol (**1**), from the red alga *Laurencia claviformis* [4], when treated with *p*-toluenesulfonic acid, yielded pacifidiene (**2**). However, when pacifenol was treated with sodium hydride, compound **3** was obtained [5].

A previous study described the microbiological transformation of pacifenol by the fungus *Penicillium brevicompactum* [6]. Continuing these studies, we report here results obtained from incubations with *M. plumbeus* of pacifenol and two semi-synthetic pacifenol derivatives (**2** and **3**).

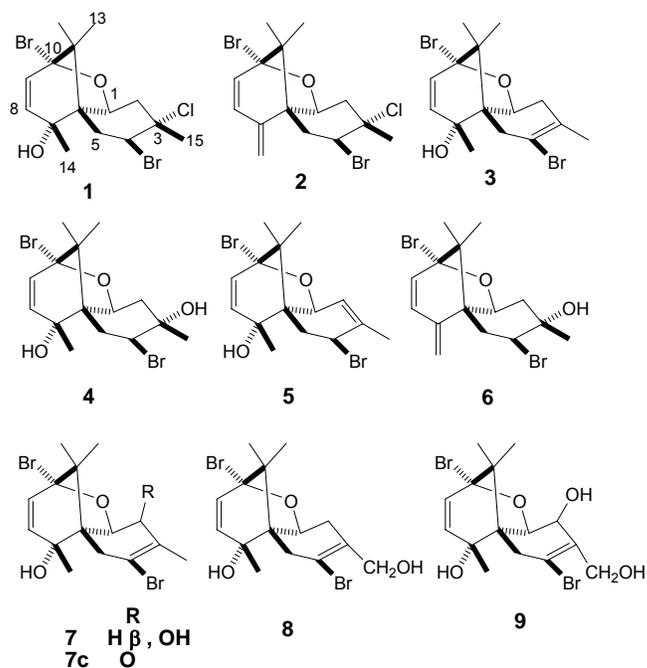


Figure 1: Compounds obtained from the biotransformation from pacifenol **1** and the semisynthetic derivatives **2** and **3**.

The fermentations were carried out for a period of 6 days. The combined broth and mycelium were extracted with ethyl acetate, and then separated into neutral and acid fractions.

In the biotransformation of pacifenol with *M. plumbeus* two metabolites that were not present in a control fermentation were detected by TLC. The ethyl acetate extract of the medium was purified by chromatography to yield the biotransformation products **4** and **5**.

Metabolite **4**, obtained in good yield, was identified by comparing the physical and spectroscopic data with those of the product obtained in the biotransformation of pacifenol with *P. brevicompactum* [6].

Comparison of the ^1H NMR spectra of compound **5** and the substrate **1** showed the disappearance of a doublet due to H-2 in compound **1** and the appearance of a broad doublet at δ_{H} 5.81, corresponding to the presence of a tri-substituted double bond in compound **5**; also, the signal of the methyl (Me-12) geminal to a chlorine atom at δ_{H} 1.72 in **1** was shifted downfield to δ_{H} 1.85 in **5**.

The position of the tri-substituted double bond was inferred from the following correlations observed in the 2D experiments and COSY and TOCSY. The HMBC interaction of the H-1 resonance at δ_{H} 4.76 (C-1, δ_{C} 75.0) with the olefinic carbon at δ_{C} 136.3, and also the correlation between Me-12 at δ_{H} 1.85 with the olefinic carbons at δ_{C} 136.3 and 126.7 ppm, and with the methine carbon bearing the bromine atom at δ_{C} 67.9, indicate the presence of the C₂-C₃ double bond. Furthermore, a COSY experiment revealed correlation between a broad doublet at δ_{H} 5.81 and a methyl at 1.85 ppm. A TOCSY spectrum, when the signal at δ_{H} 5.81 is irradiated, showed correlations with the signal at δ_{H} 4.76 and with the methyl at 1.85 ppm. Consequently, structure **5** was assigned to this compound.

The biotransformation of pacifidiene (**2**) with *M. plumbeus* gave the metabolites **1** and **6**. The molecular formula, C₁₅H₂₀Br₂O₂, of compound **6** was established based on ^{13}C NMR, DEPT and MS [*m/z*: 392]. This formula suggests that oxygen was introduced in **2** in place of the chlorine atom. Comparison of the ^1H NMR spectra of **2** and **6** showed only minor differences in the chemical shift corresponding to the geminal methyl to the heteroatom (chlorine in **2**, δ_{H} 1.73 and oxygen in **6**, δ_{H} 1.26).

As in the compound obtained from the biotransformation of pacifenol with *P. brevicompactum*, the hydroxyl group at C-3 is axial (exo face of the molecule) [6]. So, compound **6** is shown to be 3-hydroxydechloropacifidiene.

From the microbiological transformation of compound **3**, two products, **7** and **8**, were obtained, the structures

of which were established unambiguously by NMR spectroscopy. Another compound (**9**) was obtained, but only in small quantities such that only a ^1H NMR spectrum was recorded.

The ^1H NMR spectrum of compound **7** exhibited a downfield CH signal at δ_{H} 4.23 (brs, H-2). This observation suggested the introduction of an OH group at one of the CH₂ groups, i.e. C-2 or C-5. The assignments of all the protons were accomplished by interpretation of the HMQC spectrum. The position for the newly introduced OH group at C-2 was inferred on the basis of HMBC couplings of the protons resonating at δ_{H} 1.93 (Me-15) and 4.40 (H-C-1) with the newly hydroxylated methine C-atom at δ_{C} 75.3 (H-C(2)). The orientation of the OH group at C-2 was inferred from the NOESY correlations of H β -C(2) with H β -C(5), Me-15 and with Me-13; H α -C(1) has correlation only with H α -C(5). Also, there are correlations between the methyl at δ 0.98 (Me-13) with Me-15 (δ 1.93) and H β -C(5); between Me-12 with H α -C(5) and Me-14 (δ 1.34). Other correlations observed are between Me-14 with H β -C(5) and H α -C(5); between H-9 with Me-12 and between H-8 with Me-12 and Me-14; H α -C(5) with Me-14 and H α -C(1); and H β -C(5) with Me-13, H β -C(2) and Me-14. The stereochemistry of pacifenol was assigned by X-ray-diffraction, and as compound **3** is a synthetic derivative of pacifenol, the stereochemistry of this compound is assumed to be the same as that of pacifenol. Furthermore, the coupling constant between H-1 and H-2 ($J_{1,2}=1.9\text{Hz}$) indicates that the H-2 must be β and, therefore, the hydroxyl group must be pseudo-equatorial at the exoface of the molecule, which is appropriate for this hydroxylation.

In order to confirm the spectroscopic data, a DFT/GIAO approach has been used to calculate the ^1H and ^{13}C chemical shifts. This methodology has been used for NMR assignments in several natural products [7-9]. The combined approach of extensive spectroscopic analysis and quantum mechanical methods has been used for the reassignment of structures [10], and can be very helpful to either confirm or discard both rigid and flexible molecular structures [11,12]. Recently Bassarello *et al.* [13] have used this methodology to derive the stereo structures of unknown compounds by comparing the experimental NMR spectroscopic data with the corresponding calculated spectra for all the possible stereo isomers. In addition, quantum mechanical calculations of proton-proton and proton-carbon *J* coupling constants have been proposed as useful tools to assign the relative configurations of chiral organic compounds. This approach provides results that were in good agreement with the experimental data [14,15]. When heavy atoms

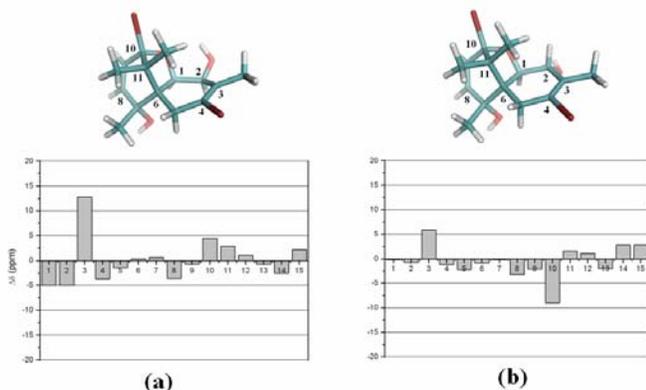


Figure 2: Deviation from calculated and observed ^{13}C NMR chemical shift for both configurations in C2 for configurations (a) and (b) of compound **7**.

are present in the structure, some spin-orbit (SO) coupling may be operative [16]. This effect has been discussed by Braddock *et al.* [10]. These authors report that the use of the extended basis set do not produce a great change in the chemical shift. They recommended that an average correction of ca. -3 ppm for bromine attached to a sp^3 carbon [17] was adequate to empirically reproduce the experimental chemical shift.

Proton-proton $J_{\text{H,H}}$ coupling constants were calculated at the mPW1PW91/6-31G(d,p) level of theory for compound **7** and optimized at the mPW1PW91/6-31G(d) level. The resultant structure for compound **7** is shown in Figure 2. The differences between theoretical and experimental values $\Delta\delta = |\delta_{\text{exp}} - \delta_{\text{calc}}|$ show a measure of the dispersion between the theoretical and experimental chemical shift values for compound **7b**. The coupling constants for protons H-1 and H-2 were calculated for both configurations. The values for $^3J_{\text{H,H}}$ for the protons at C-2 and C-1 are 8.21 Hz for isomer (a) and 1.92 Hz for isomer (b), respectively.

In summary, our results show that GIAO/DFT calculations on the optimized structure at the mPW1PW91/6-31G(d,p) level of theory provide excellent results that are in agreement with experimental values for ^{13}C chemical shifts and in fair agreement with experimental proton-proton $^3J_{\text{H,H}}$ coupling constants. The theoretical results confirm and support the experimentally derived assignments of compound **7**.

Compound **7** was treated with Jones' reagent to obtain compound **7c**, the ^1H NMR spectrum of which was very similar to that of compound **7**, with the disappearance of the H-atom geminal to the OH group at δ 4.23, and the appearance in the ^{13}C NMR spectrum of a signal due to an oxo group at δ_{C} 191.5. The assignments of all the H-C atoms were accomplished by interpretation of the

HMQC spectrum. The position of the carbonyl group was inferred on the basis of HMBC couplings, i.e. the H-C-1 proton at δ_{H} 4.75 correlates with C-6 (δ 54.1), C-7 (δ 75.0) and C-2 (δ 191.5). Me-15 (δ 2.00) correlates with C-3 (δ 135.4), with C-4 (δ 146.5) and with C-2 (δ 191.5)

The least polar compound isolated (**8**) had a molecular formula $\text{C}_{15}\text{H}_{20}\text{O}_3\text{Br}_2$, and possessed one more oxygen atom than substrate **3**. Its ^1H NMR spectrum was very similar to that of **3** except that the signal of a methyl group (Me-15) at δ_{H} 2.01 had been replaced by that of a hydroxymethylene group, two signals, forming an AB system, at δ_{H} 4.32 and 4.27 ($J = 16$ Hz). Thus, structure **8** was assigned to this compound.

The most polar substance isolated in the incubation of **3** was the triol **9**. The two novel alcoholic groups introduced in the molecule were located at C-2 and C-15, in accordance with the ^1H NMR spectrum. Thus the resonance of H-2 was similar to that of compound **7** and the resonance for the hydroxymethylene in C-15 was similar to that of compound **8**. The quantity available was insufficient to obtain a ^{13}C NMR spectrum.

Experimental

General experimental procedures: IR spectra were obtained using a Perkin-Elmer Spectrum BX FT-IR spectrometer. ^1H and ^{13}C NMR spectra were recorded in CDCl_3 at 400.13 and 100 MHz, respectively, with a Bruker AMX2-400 spectrometer. Chemical shifts are given in δ (ppm). Mass spectra were taken at 70 eV (probe) with a Micromass Autospec spectrometer. Semipreparative HPLC was carried out with a Beckman System Gold 125P. Dry column chromatography was performed on Merck 0.02-0.063 mm silica gel.

Computational details: Geometry optimization was performed using the mPW1PW91[18] exchange-correlation function, together with the standard 6-31G(d) basis set [19]. The ^1H and ^{13}C NMR chemical shifts were calculated using the GIAO (Gauge Invariant Atomic Orbitals) method [20] at this level of theory. Relative chemical shift and coupling constants were estimated by using the corresponding TMS shielding calculated at the same level of theory. Spin-spin coupling calculations were performed taking into account the contributions of the following interactions: Fermi contact (FC), paramagnetic spin-orbit (PSO), diamagnetic spin-orbit (DSO), and spin-dipole (SD). All calculations were carried out with the Gaussian 03 suite of programs [21].

Substrate **1** was isolated from *Laurencia claviformis*, an alga that grows on the coast of Easter Island. Substrate

2 was obtained by treating compound **1** with *p*-toluene sulfonic acid and compound **3** was obtained after the reaction of pacifenol with NaH in THF [5].

Incubation and isolation procedures: *Mucor plumbeus* was grown in shaking culture at 25°C for 2 days in 65-75 conical flasks (250 mL), each containing sterile medium (50 mL) [6]. The substrate was dissolved in EtOH (13-15 mL) and distributed equally between the flasks, and the incubation was allowed to continue for 6 days. The broth was separated from the mycelium by filtration, and both were extracted with EtOAc. The extracts were combined and chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient. Some mixtures were resolved by HPLC on an Ultrasphere silica gel 5 µm column (1× 25 cm), eluting with mixtures of isocratic *n*-hexane-EtOAc at 3 mL/min.

Biotransformation of pacifenol (1): The incubation of **1** (300 mg) afforded, from the neutral fraction, starting material **1** (45 mg), **4** (100 mg), and **5** (27 mg).

Compound 5

¹H NMR (δ, 400 MHz): 6.09 (1H, d, *J* = 9.9 Hz, H-9), 5.81 (1H, brd, *J* = 2.3 Hz, H-2), 5.48 (1H, d, *J* = 9.9 Hz, H-8), 4.76 (1H, d, *J* = 2.3 Hz, H-1), 4.21 (1H, dd, *J* = 5.0, 4.7 Hz, H-4), 2.07 (1H, dd, *J* = 4.7, 15.3 Hz, H-5), 2.03 (1H, dd, *J* = 5.0, 15.3 Hz, H-5'), 1.85 (3H, brs, Me-15), 1.44 (3H, s, Me-14), 1.11 (3H, s, Me-12), 1.09 (3H, s, Me-13).

¹³C NMR (δ, 100 MHz, CDCl₃): 75.0 (CH, C-1), 136.3 (CH, C-2), 126.7 (C, C-3), 67.9 (CH, C-4), 29.5 (CH₂, C-5), 55.4 (C, C-6), 77.2 (C, C-7), 134.0 (CH, C-8), 132.5 (CH, C-9), 100.3 (C, C-10), 51.9 (C, C-11), 24.7 (CH₃, C-12), 21.9 (CH₃, C-13), 25.1 (CH₃, C-14), 20.7 (CH₃, C-15).

EIMS (70 eV) *m/z* (% rel.int): 392 [M - H₂O]⁺(20), 313 [M - H₂O - ⁷⁹Br]⁺(100), 312 [M - H₂O - Br]⁺(20), 311 [M - H₂O - ⁸¹Br]⁺(90), 295(10).

Biotransformation of pacifidiene (2): The incubation of **2** (300 mg) afforded, from the neutral fraction, starting material **2** (60 mg), **6** (86 mg), and **1** (27 mg).

Compound 6

¹H NMR (δ, 400 MHz): 5.87 (1H, d, *J* = 9.9 Hz, H-9), 5.58 (1H, d, *J* = 9.9 Hz, H-8), 5.1 and 5.0 (1H each, brs, H-14 and H-14'), 4.48 (1H, d, *J* = 6.1 Hz, H-4), 4.07 (1H, dd, *J* = 4.7, 14.2 Hz, H-1), 2.66 (1H, dd, *J* = 4.7, 14.0 Hz, H-2), 2.20 (1H, dd, *J* = 6.1, 11.8 Hz, H-5a), 1.72 (1H, d, *J* = 11.8 Hz, H-5e), 1.26 (3H, brs, Me-15), 1.15 (3H, s, Me-12), 1.03 (3H, s, Me-13)

¹³C NMR (δ, 100 MHz, CDCl₃): 77.9 (CH, C-1), 43.1 (CH₂, C-2), 75.2 (C, C-3), 61.5 (CH, C-4), 32.8 (CH₂,

C-5), 53.8 (C, C-6), 147.0 (C, C-7), 133.0 (CH, C-8), 130.1(CH, C-9), 100.3 (C, C-10), 50.5 (C, C-11), 23.3 (CH₃, C-12), 20.1 (CH₃, C-13), 114.7 (CH₂, C-14), 29.7 (CH₃, C-15).

MS (EI, 70 eV) *m/z* (% rel.int): 392 [M - H₂O]⁺ (20), 313 [M - H₂O - ⁷⁹Br]⁺ (100), 312 [M - H₂O - Br]⁺ (20), 311 [M - H₂O - ⁸¹Br]⁺ (90), 295 (10).

Biotransformation of compound 3: The incubation of **3** (260 mg) afforded, from the neutral fraction, starting material **3** (50 mg), **7** (120 mg), **8** (35 mg) and **9** (1 mg).

Compound 7

¹H NMR (δ, 400 MHz): 6.06 (1H, d, *J* = 9.8 Hz, H-9), 5.42 (1H, d, *J* = 9.8 Hz, H-8), 4.40 (1H, d, *J* = 1.9 Hz, H-1), 4.23 (1H, brs, H-2), 2.93 (1H, d, *J* = 18 Hz, H-5), 2.48 (1H, d, *J* = 18 Hz, H-5'), 1.93 (3H, s, Me-15), 1.34 (3H, s, Me-14), 1.08 (3H, s, Me-12), 0.98 (3H, s, Me-13).

¹³C NMR (δ, 100 MHz, CDCl₃): 83.1 (CH, C-1), 75.3 (CH, C-2), 121.7 (C, C-3), 132.5 (C, C-4), 35.3 (CH₂, C-5), 54.0 (C, C-6), 75.1 (C, C-7), 133.8 (CH, C-8), 132.8(CH, C-9), 99.4 (C, C-10), 52.4 (C, C-11), 21.3 (CH₃, C-12), 25.1 (CH₃, C-13), 24.3 (CH₃, C-14), 20.1 (CH₃, C-15).

MS (EI, 70 eV): *m/z* (% rel.int.) = 409.96 [M⁺ + 2] (0.45), 407.96 [M]⁺ (1.2), 405.96 [M - 2]⁺ (0.7), 391.95 [M + 2-H₂O]⁺ (2.1), 389.97 [M -H₂O]⁺ (3.0), 387.97 [M⁺ - 2-H₂O]⁺ (1.7), 329.06 [M-⁷⁹Br]⁺ (18.7), 327.06 [M-⁸¹Br]⁺ (18.9), 311.04 [390-⁷⁹Br] (28.6), 309.05 [390-⁸¹Br] (26.5), 283.05 (18.5), 281.05 (12.9), 265.04 (4.7), 229.13 (11.3), 219.14 (11.2), 212.99 (10.1), 211.01 (12.0), 201.13 (19.8), 201.01 (56.0), 200.0 (26.3), 199.01 (61.7), 198.0 (24.7), 196.99 (9.1), 187.11 (13.7), 186.1 (15.0), 184.12 (19.6), 173.13 (13.9), 149.09 (11.7), 149.02 (10.7), 147.07 (14.8), 143.08 (10.5), 141.06 (9.4), 135.08 (17.7), 134.96 (10.3), 120.08 (100), 119.07 (46.6), 105.06 (29.1) 91.05 (65.2), 85.02 (45.1).

Compound 7c: To a solution of compound **7** (30 mg, 0.074 mmol) in acetone (3 mL) cooled to 0°C was added Jones' reagent (2.67 g CrO₃, 2.3 mL H₂SO₄, dilute to 10 mL with water), dropwise, until disappearance of the starting material, as monitored by TLC. The reaction was quenched by the addition of iso-propanol, followed by saturated NaHCO₃. The solution was extracted 3 times with EtOAc and the combined organic layers were washed with water, dried over MgSO₄, and filtered. The solvent was removed *in vacuo* and the residue was chromatographed on silica gel using as eluent a light petroleum-EtOAc gradient obtaining **7c**.

¹H NMR (δ, 400 MHz): 6.17 (1H, d, *J* = 9.8 Hz, H-9), 5.40 (1H, d, *J* = 9.8 Hz, H-8), 4.75 (1H, s, H-1), 3.33 (1H, ddd, *J* = 2.5, 5.1, 19.6 Hz, H-5), 2.87 (1H,

dd, $J = 1.7, 19.6$ Hz, H-5'), 2.00 (3H, s, Me-15), 1.45 (3H, s, Me-14), 1.14 (3H, s, Me-12), 0.98 (3H, s, Me-13),

^{13}C NMR (δ , 100 MHz, CDCl_3): 77.7 (CH, C-1), 191.5 (C, C-2), 135.4 (C, C-3), 146.5 (C, C-4), 36.7 (CH_2 , C-5), 54.1 (C, C-6), 75.0 (C, C-7), 134.3 (CH, C-8), 132.8 (CH, C-9), 98.2 (C, C-10), 53.0 (C, C-11), 20.7 (CH_3 , C-12), 24.7 (CH_3 , C-13), 24.9 (CH_3 , C-14), 15.7 (CH_3 , C-15).

Compound 8

^1H NMR (δ , 400 MHz): 6.11 (1H, d, $J = 9.8$ Hz, H-9), 5.44 (1H, d, $J = 9.8$ Hz, H-8), 4.69 (1H, dd, $J = 4.0, 8.0$ Hz, H-1), 4.32 (1H, d, $J = 16$ Hz, H-15), 4.27 (1H, d, $J = 16$ Hz, H-15'), 2.97 (1H, d, $J = 18.0$ Hz, H-5), 2.95 (2H, dd, $J = 8.0, 10.0$ Hz, H-2), 2.65 (1H, d, $J = 18.0$ Hz, H-5'), 2.63 (2H, dd, $J = 4.0, 10.0$ Hz, H-2'), 1.44 (3H, s, Me-14), 1.16 (3H, s, Me-12), 1.15 (3H, s, Me-13).

^{13}C NMR (δ , 100 MHz, CDCl_3): 74.7 (CH, C-1), 35.1 (C, C-2), 119.1 (C, C-3), 132.8 (C, C-4), 36.7 (CH_2 , C-5), 53.7 (C, C-6), 76.3 (C, C-7), 133.7 (CH, C-8), 133.2 (CH, C-9), 100.2 (C, C-10), 52.8 (C, C-11), 22.3 (CH_3 , C-12), 24.8 (CH_3 , C-13), 25.1 (CH_3 , C-14), 65.8 (CH_2 , C-15).

MS (EI, 70 eV): m/z (% rel.int.) = 391.95 [$\text{M} + 2\text{-H}_2\text{O}$] $^+$ (8.9), 389.95 [$\text{M} - \text{H}_2\text{O}$] $^+$ (17.7), 387.95 [$\text{M}^+ - 2\text{-H}_2\text{O}$] $^+$ (9.2), 329.05 [$\text{M} - ^{79}\text{Br}$] $^+$ (2.2), 327.06 [$\text{M} - ^{81}\text{Br}$] $^+$ (2.9), 311.04 [$^{390-79}\text{Br}$] (41.4), 309.05 [$^{390-81}\text{Br}$] (41.0), 229.03 (16.5), 227.00 (15.6), 214.99 (14.3), 212.99 (19.7), 211.00 (10.0), 201.00 (15.8), 200.00 (53.3), 199.00 (20.9), 198.00 (50.9), 196.99 (10.2), 190.95 (19.8), 188.95 (19.3), 184.12 (22.1), 183.11 (15.5), 171.11 (14.2), 169.10 (16.9), 149.01 (55.1), 147.07 (9.9), 143.08 (13.5), 141.06 (10.4), 135.07 (14.3), 134.06 (22.1), 133.09 (35.4), 132.08 (19.8), 121.09 (19.7), 120.08 (26.1), 119.07 (51.0), 115.05 (23.1), 91.05 (88.3), 57.07 (100).

Compound 9

^1H NMR (δ , 400 MHz): 6.02 (1H, d, $J = 9.8$ Hz, H-9), 5.41 (1H, d, $J = 9.8$ Hz, H-8), 4.66 (1H, brs, H-1), 4.53 (1H, d, $J = 12.7$ Hz, H-15), 4.47 (1H, brs, H-2), 4.35 (1H, d, $J = 12.7$ Hz, H-15'), 2.97 (1H, brd, $J = 18.2$ Hz, H-5), 2.60 (1H, d, $J = 18.2$ Hz, H-5'), 1.39 (3H, s, Me-14), 1.08 (3H, s, Me-12), 1.02 (3H, s, Me-13).

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Two New Guaianolides from *Amberboa ramosa*

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Two new sesquiterpene lactones named amberbins A (**1**) and B (**2**), have been isolated from the ethyl acetate soluble fraction of *Amberboa ramosa*, together with jaseocidine (**3**), crysoeriol (**4**) and 3 β ,8 α -dihydroxy-11 α -methyl-1 α H,5 α H,6 β H, 7 α H,11 β H-guai-10(14), 4(15)-dien-6,12-olide (**5**). The structures of the isolated compounds have been elucidated on the basis of their spectroscopic data.

Keywords: *Amberboa ramosa*, guaianolides, amberbins A and B.

The genus *Amberboa* belongs to the family Compositae and comprises six species. *A. ramosa* is an annual herbaceous plant, mainly found in Pakistan and India, with tonic, aperient, febrifuge, deobstruent, cytotoxic and antibacterial properties [1]. The butyrylcholinesterase inhibitory activity of the chloroform soluble fraction has also been reported [2]. A literature survey revealed that triterpenoids, flavonoids, steroids and sesquiterpene lactones have previously been reported from this species [1,3]. The chemotaxonomic and ethnopharmacological significance of the genus *Amberboa* prompted us to reinvestigate the constituents of *A. ramosa*. As a result, we herein report the isolation and structural elucidation of two new sesquiterpene lactones named as amberbins A (**1**) and B (**2**), respectively. In addition, the previously reported compounds, jaseocidine (**3**) [2], crysoeriol (**4**) [4] and 3 β ,8 α -dihydroxy-11 α -methyl-1 α H,5 α H,6 β H, 7 α H,11 β H-guai-10(14), 4(15)-dien-6,12-olide (**5**) [2] have been isolated and characterized.

Amberbin A (**1**) was isolated as white crystals. The HREI-MS exhibited an [M⁺] peak at m/z 308.3681 corresponding to the molecular formula C₁₇H₂₄O₅ (calcd. for C₁₇H₂₄O₅, 308.3693), which indicated six degrees of unsaturation. Further prominent peaks at m/z 290, 265, 250 and 247 represented the losses of [M-H₂O]⁺, [M-COCH₃]⁺, [M-COCH₃-CH₃]⁺ and [M-COCH₃-H₂O]⁺, respectively. The IR spectrum showed characteristic absorption of hydroxyl (3447 cm⁻¹), γ -lactone (1755 cm⁻¹), ester (1735 cm⁻¹) and C=C (1656 cm⁻¹). The UV absorption maxima at 197 and

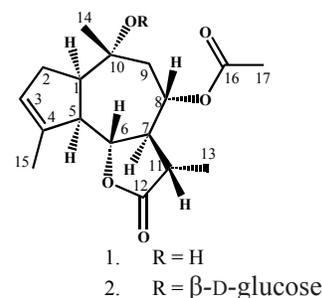


Figure 1: Structures of amberbins A (**1**) and B (**2**).

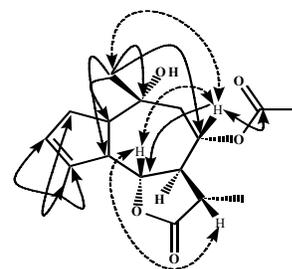
205 nm were typical of a γ -lactone [5]. Further spectral data showed close agreement with a guaianolide-type sesquiterpene [3,6-7].

The ¹H NMR spectrum showed signal for a trisubstituted double bond at δ_H 5.08. It also showed signals for oxymethine protons at δ_H 5.08 (ddd, J = 8.3, 6.0, 5.5 Hz) and 4.33 (dd, J = 10.0, 9.0 Hz). The latter was assigned to the proton geminal to the lactone oxygen atom. The larger coupling constant allowed us to assign β and axial orientation, which is characteristic of all guaianolides of the genera *Amberboa* and *Ixeris* [8]. The spectrum showed ¹H-¹H correlations with the vicinal protons at δ_H 2.78 (dd, J = 9.0, 8.5 Hz) and δ_H 2.59 (ddd, J = 10.0, 9.5, 8.5 Hz), which could subsequently be assigned to H-5 and H-7, respectively. The larger coupling constants suggested a *trans*-diaxial disposition among H-5, H-6 (β) and H-7, providing conclusive evidence for an α orientation of both H-5 and H-7 [8].

Table 1: ^1H (400 MHz) and ^{13}C NMR (100 MHz) spectroscopic data for amberbins A (**1**) and B (**2**) (CD_3OD , δ in ppm).

1			2		
Positions	δ_{C}	δ_{H} multiplicity (J in Hz)	Positions	δ_{C}	δ_{H} multiplicity (J in Hz)
1	55.5	2.71 ddd (9.0, 8.5, 8.0)	1	53.0	2.81 ddd (9.0, 8.5, 8.0)
2	34.0	2.26 dd (8.0, 7.0) 2.37 dd (9.0, 5.0)	2	34.0	2.36 dd (8.0, 7.0) 2.22 dd (9.0, 5.0)
3	127.1	5.50 m	3	126.8	5.46 m
4	143.8	-	4	144.0	-
5	52.7	2.78 dd (9.0, 8.5)	5	55.0	2.95 dd (9.0, 8.5)
6	82.1	4.33 dd (10.0, 9.0)	6	82.3	4.20 dd (10.0, 9.0)
7	54.4	2.59 ddd (10.0, 9.5, 8.3)	7	52.3	2.87 ddd (10.0, 9.5, 8.3)
8	75.5	5.08 ddd (8.3, 6.0, 5.5)	8	75.4	5.08 ddd (8.3, 6.0, 5.5)
9	44.2	1.77 dd (12.3, 5.5) 2.34 dd (12.3, 6.0)	9	42.8	1.87 dd (12.3, 5.5) 2.39 dd (12.3, 6.0)
10	73.4	-	10	81.1	-
11	42.64	2.34 dq (9.5, 5.8)	11	42.1	2.52 dq (9.5, 5.8)
12	172.1	-	12	172.1	-
13	15.8	1.23 d (6.9)	13	15.7	1.23 d (6.9)
14	29.5	1.16 s	14	26.1	1.26 s
15	21.2	2.06 s	15	17.7	1.8 s
16	180.8	-	16	181.0	-
17	17.4	1.83 s	17	21.3	2.06 s
			1'	98.4	4.54 d (7.5)
			2'	78.2	3.38 m
			3'	71.8	3.28 m
			4'	75.5	3.17 m
			5'	77.7	3.23 m
			6'	62.9	3.82 dd (2.1, 12.0) 3.6 dd (6.0, 12.0)

The coupling pattern of the proton signals for H-1 and H-9 supported the guaianolide structure [9]. The entire sequence of protons attached to the guaianolide skeleton was established by ^1H - ^1H COSY and spin decoupling experiments. Irradiation of the H-5 proton at δ_{H} 2.78 simplified the double doublet of H-6 at δ_{H} 4.33 into a doublet and the doublet of double doublets of H-1 into a double doublet. Irradiation of H-7 at δ_{H} 2.59 simplified the doublet of quartets at δ_{H} 2.34 into a quartet. Irradiation of H-11 at δ_{H} 2.34 simplified the doublet of the methyl group at δ_{H} 1.23 into a singlet, confirming the presence of a methyl group at C-11. Irradiation of H-3 at δ_{H} 5.50 turned the double doublets at δ_{H} 2.26 (H-2 α) and 2.37 (H-2 β) into doublets. The signal at δ_{H} 2.06 could be assigned to methyl protons at C-4 and further confirmed by 2J and 3J correlations with C-4 (δ_{C} 143.8), C-3 (δ_{C} 127.1) and C-5 (δ_{C} 52.7). The *O*-acetyl group was assigned to C-8 on the basis of the HMBC spectrum, in which H-8 at δ 5.08 showed an interaction with C-16 (δ_{C} 180.8). The hydroxyl group could be assigned to C-10 based on its downfield shift compared with the unsubstituted guaianolides and supported by HMBC, in which the methyl protons at C-14 (δ_{C} 1.16) showed 2J and 3J correlations with C-10 (δ_{C} 73.4), C-9 (δ_{C} 44.2) and C-1 (δ_{C} 55.5).

Figure-2. Important HMBC (—) and NOESY (---) correlations of amberbins A (**1**) and B (**2**).

The ^{13}C NMR and DEPT spectra showed 17 signals comprising four methyl, two methylene, seven methine and four quaternary carbons. The low frequency region showed four signals at δ_{C} 180.8, 172.1, 143.8 and 127.1, which could be assigned to *O*-acetyl, lactone ester and trisubstituted olefinic carbons. One oxygenated quaternary and two oxygenated methine carbons resonated at δ_{C} 73.4, 82.1 and 75.5, respectively. The position of substituents could be confirmed by HMQC, HMBC and COSY experiments.

The relative stereochemistry at various chiral centers of amberbin A was assigned through NOESY experiments [10-11] [Figure-2], which revealed *trans*/*anti*/*cis*-fusion of the α -methyl- γ -lactone moiety, the seven membered ring at C-7 and the five membered ring at C-5 and C-1. Interaction was shown between H-1 at δ_{H} 2.71, H-5 at δ_{H} 2.78 and H-7 at δ_{H} 2.59, confirming a 1,5-*cis*-fused guaianolide. H-1 also showed interaction with H-2 α . The α -orientation of the acetate group at C-8 could also be deduced through NOESY interactions between H-8 at δ_{H} 5.08, H-6 at δ_{H} 4.33 and H-11 at δ_{H} 2.34. The methyl protons attached to C-10 showed correlation with H-8 at δ_{H} 4.33 revealing its β -orientation. On the basis of this evidence, the structure of amberbin A (**1**) could be assigned as 8 α -acetoxy-10 α -hydroxy-1 α H,5 α H,6 β H,7 α H,11 β H,11 α -methylguaia-3-enolide (Figure 1).

Amberin B (**2**) was isolated as white crystals. The HRESI-MS exhibited a pseudomolecular $[\text{M}-\text{H}]^-$ peak at m/z 469.2068 corresponding to the molecular formula $\text{C}_{23}\text{H}_{33}\text{O}_{10}$ (calcd. for $\text{C}_{23}\text{H}_{33}\text{O}_{10}$; 469.2074). The EIMS showed a $[\text{M}-162]^+$ peak at m/z 308, followed by further fragments at m/z 265 $[\text{M}-162-\text{COCH}_3]^+$ and 250 $[\text{M}-162-\text{COCH}_3-\text{Me}]^+$. The IR and UV spectra were similar to those of **1**. The ^{13}C NMR and DEPT spectra were also similar to those of **1**, except for the additional signals of a hexose moiety at δ_{C} 98.4 (C-1'), 78.2 (C-2'), 71.8 (C-3'), 75.5 (C-4'), 77.7 (C-5') & 62.9 (C-6'), as well as the downfield shift of C-10 by 7.7 ppm revealing its presence at C-10. The ^1H NMR spectrum was also similar to that of **1** except for the presence of additional signals due to a hexose moiety, including the anomeric proton at δ_{H} 4.54 (d, $J = 7.5$ Hz), the

oxymethine protons between δ_{H} 3.17-3.38 and oxymethylene protons at δ_{H} 3.82 (dd, $J = 2.1, 12.0$ Hz) and 3.60 (dd, $J = 6.0, 12.0$ Hz). The position of the hexose moiety at C-10 could further be confirmed by HMBC experiments showing 3J correlation of the anomeric proton at δ_{H} 4.54 with C-10 (δ_{C} 81.1). The larger coupling constant of the anomeric proton allowed us to assign a β -configuration to the sugar moiety. Acid hydrolysis provided D-glucose, which could be identified through the sign of its optical rotation and retention time of its TMS ether by GC. The NOESY correlations were exactly the same as those of **1**. Thus, the structure of amberbin B could be assigned as 8 α -acetoxy-10 α -O-(β -D-glucopyranosyl)-1 α H,5 α H,6 β H,7 α H,11 β H,11 α -methylguaia-3-enolide (**2**).

Experimental

General: Melting points were determined on a Buchi melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-360 polarimeter. The IR spectra were recorded on a JASCO 302-A spectrophotometer with KBr discs. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer. EIMS and HREI-MS spectra were recorded on JEOL JMS-HX-110 and Varian MAT-311-A mass spectrometers. The HRESI-MS was recorded on a Jeol JMS 600H instrument. The NMR spectra were recorded on a Bruker Avance DRX 400 NMR spectrometer. Chemical shifts are given on the δ scale and referenced to TMS at 0 ppm for proton and carbon. Coupling constants (J) are in Hertz. Silica gel 230–400 mesh (E. Merck, Darmstadt, Germany) was used for column chromatography.

Plant material: The whole plant of *Amberboa ramosa* Jafri (Compositae) was collected in June 2002, from Karachi (Pakistan) and identified by Dr Surraiya Khatoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (no. KU 312 b) has been deposited.

Extraction and isolation: The shade dried plant material (22 kg) was extracted with methanol at room temperature 3 times. The solvent was evaporated under reduced pressure and the residue (217 g) was suspended in H₂O, then partitioned with *n*-hexane, CHCl₃, EtOAc and *n*-BuOH.

Column chromatography of the EtOAc soluble fraction (90 g) over silica gel and elution with *n*-hexane/EtOAc in increasing order of polarity afforded 4 major fractions A [*n*-hexane/EtOAc (8:2)], B [*n*-hexane/EtOAc (6.5:3.5)], C [*n*-hexane/EtOAc (5.5:4.5)] and D [*n*-hexane/EtOAc (4:6)], respectively.

Fraction B was chromatographed over silica gel, eluting with mixtures of *n*-hexane/EtOAc in increasing order of polarity to afford sub-fractions B_A [*n*-hexane/EtOAc (7:3)], B_B [*n*-hexane/EtOAc (6:4)] and B_C [*n*-hexane/EtOAc (6.5:3.5)]. Fraction B_A on further chromatography over silica gel and elution with *n*-hexane/EtOAc (6.5:3.5) yielded compound **3** (9 mg). Chromatography of fraction B_C and elution with *n*-hexane/EtOAc (6:4) furnished compound **4** (11.5 mg).

Fraction C was chromatographed over silica gel and eluted with mixtures of *n*-hexane/EtOAc to provide two sub-fractions C_A [*n*-hexane/EtOAc (4.5:5.5)] and C_B [*n*-hexane/EtOAc (4:6)]. Fraction C_B on further chromatography over silica gel and elution with *n*-hexane/EtOAc (7:3) afforded compound **5** (10 mg).

Fraction D was chromatographed over silica gel and eluted with mixtures of EtOAc/MeOH in increasing order of polarity to obtain compound **1** [EtOAc/MeOH (9:1)] (11 mg) and compound **2** [EtOAc/MeOH (7:3)] (13 mg), respectively.

Amberbin A [8 α -acetoxy-10 α -hydroxy-1 α H,5 α H,6 β H,7 α H,11 β H,11 α -methylguaia-3-enolide] (**1**)

White crystals.

MP: 130-132°C.

$[\alpha]_{\text{D}}^{20}$: +33 (c 0.02, CHCl₃).

IR (KBr) ν_{max} : 3447, 1775, 1735, 1656 cm⁻¹.

UV (MeOH) λ_{max} : 197 and 205 nm.

¹H NMR: Table 1.

¹³C NMR: Table 1.

HMBC: Figure 2.

EIMS (70 ev) m/z (rel. int. %) 308 [M]⁺ (12).

HREI-MS m/z [M]⁺ calcd. for C₁₇H₂₄O₅; 308.3693; found 308.3698.

Amberbin B [8 α -acetoxy-10 α -O-(β -D-glucopyranosyl)-1 α H,5 α H,6 β H,7 α H,11 β H,11 α -methylguaia-3-enolide] (**2**)

White crystals.

MP: 137-139°C.

$[\alpha]_{\text{D}}^{20}$: +51.1 (c 0.02, CHCl₃).

IR (KBr) ν_{max} : 1740, 3600, 1779 and 1649 cm⁻¹.

¹H NMR: Table 1.

¹³C NMR: Table 1.

HRESI-MS: m/z [M-H]⁻ calcd. for C₂₃H₃₃O₁₀; 469.2074; found 469.2068.

Acid hydrolysis of amberbin B (2): Amberbin B (**2**) (2 mg) in MeOH (3 mL) containing 1 N HCl (3 mL) was refluxed for 4 h, concentrated under reduced pressure, diluted with water (7 mL) and extracted with ethyl acetate. The residue recovered from the organic phase was a mixture of aglycone products, which could

not be worked up due to paucity of material. The aqueous phase was concentrated and D-glucose was identified by its optical rotation $[\alpha]_D^{20} = +50.6$ and by comparing retention times of its TMS ether (α -anomer

3.7 min, β -anomer 5.1 min) with a standard sample by gas chromatography (GC). Preparation of the TMS ether and its subsequent GC was carried out according to the reported protocol [12].

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Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from *Canella winterana*

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The hexane extract from the leaves of *Canella winterana* exhibited strong activity against the chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10) *in vitro* (IC₅₀ 2.53 µg/mL). Bioassay guided fractionation of this extract has led to the isolation of 5 drimane-type sesquiterpenoids: 9-epideoxymuzigadial, 9-deoxymuzigadial, muzigadial, 3-β-acetoxypolygodial and the newly isolated hemiacetal, named muzigodiol, with IC₅₀-values of 1.01, 2.19, 0.31, 2.77 and 7.43 µg/mL, respectively. The first four compounds were tested for their cytotoxicity using Chinese Hamster Ovarian (CHO) cells, where they showed IC₅₀-values of 1.82, 33.69, 1.18, and 58.31 µg/mL, respectively. A structure-activity relationship is discussed.

Keywords: *Canella winterana*, antimalarial, antiplasmodial, cytotoxicity, drimane sesquiterpenes.

Malaria is one of the most important health problems in tropical and subtropical regions of the world [1a]. One of the main factors contributing to the escalating prevalence and distribution of malaria is the emergence and spread of drug-resistant parasites highlighting a need for the discovery and development of novel, affordable antimalarial treatments.

Drug discovery based on results from *in vitro* antiplasmodial bioassays, i.e., bioassay-guided isolation, has revealed many active constituents from plant origins [1b]. *Canella* is one of five genera (*Canella*, *Cinnamosa*, *Pleodendron*, *Warburgia* and *Capsicocordendron* or *Cinnamodendron*) of the family Canellaceae. All plants belonging to this family are characterized by their high content of drimane-type sesquiterpenoid dialdehydes, which are considered chemosystematic markers of the Canellaceae [1c]. One of the species belonging to this family, *Cinnamosma fragrans* Baill, is traditionally used in the east and central parts of Madagascar for the treatment of malaria symptoms [2]

As a part of a large project seeking new antimalarial lead compounds, we investigated the antiplasmodial activity of *Canella winterana* (L.) Gaertn leaves. *C.*

winterana is a small tree that grows in the subtropical areas of the Florida Keys, and throughout the Caribbean region. The bark is used as a spice similar to true cinnamon, giving rise to the common name "wild cinnamon" [3]. The plant is known as a rich source of drimane sesquiterpenoids with unsaturated 1, 4-dialdehyde functionality. Most of these compounds have a very hot taste and are known for their antifeedant [4a], molluscicidal [4b] and antimicrobial activities [4c]. Several drimane sesquiterpene compounds have been reported for this plant [5a-5c]. *C. winterana* was originally screened by Spencer and co-workers during the World War II era [6a] in an effort to identify candidates with antimalarial activity. Their results showed that water extracts from stems showed very high quinine equivalent activity when administered orally to *Plasmodium gallinaceum* sporozoite infected chicks. However, further characterization of the active principles was not conducted.

In order to determine the antiplasmodial principles in *C. winterana* leaves, we followed an isolation scheme which, at every step, monitored and assessed the antiplasmodial activity of the resulting fractions. Dried powdered leaves were extracted sequentially with

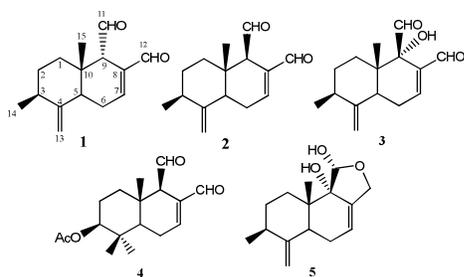


Figure 1: Structures of compounds 1-5.

n-hexane, ethyl acetate and methanol. The *n*-hexane and ethyl acetate extracts were found to be active with IC_{50} values of 2.53 and 5.30 $\mu\text{g/mL}$, respectively. HPLC and TLC of both *n*-hexane and ethyl acetate extracts indicated close similarity of their main components (data not shown). The *n*-hexane extract was subjected to further fractionation, which led to the isolation of five structurally related compounds of the drimane sesquiterpene class of compounds (1-5, Figure 1).

Compounds 1 and 2 were identified as 9-epideoxymuzigadial and its stereoisomer, 9-deoxymuzigadial, respectively. Both compounds have been previously reported for *C. winterana* [5b,5c]. Compound 3, which represented the major compound in the *n*-hexane extract (~ 8.5%), was identified as muzigadial. Muzigadial was first isolated from *Warburgia ugandensis* [4a], and then from *C. winterana* and fully identified through X-ray crystallography and given another name “canellal” [5a]. Compound 4 was identified as 3 β -acetoxypolygodial [5c]. Although compounds 1-4 have been previously reported in *C. winterana* for their antimicrobial and phytotoxic properties [5a,6b], this is the first report to assign their antiparasmodial activity. Compound 5 was purified by repeated TLC to afford white crystalline material. The ^1H and ^{13}C NMR spectra, unlike compounds 1-4, lacked the downfield signals for the aldehydic functionality. This led us to suspect cyclization to form the hemiacetal. The study of the ^1H , ^{13}C , COSY, NOSEY, HMQC and HMBC NMR spectra and with the aid of the HR MS, 5 was identified as the hemiacetal derivative of muzigadial and given the name muzigodiol (5, Figure 1). This is the first time that this compound has been isolated from either *C. winterana* or any other natural resource. The hemiacetal of muzigadial was previously prepared by fermentation of muzigadial with certain microbial cultures. In the same study, the same compound was prepared synthetically by the reaction of muzigadial with diisobutylaluminum hydride [6c].

Although the acetals are reported as natural products, they were often considered artifacts. This usually occurs by reactions of bioactive compounds when extracted with alcohols [6d]. Since we did not use methanol for

Table 1: IC_{50} -values with standard deviations of compounds 1-5 isolated from *C. winterana* and tested *in vitro* for antiparasmodial activity and cytotoxicity.

Compound	D10	CHO	SI
	IC_{50} ($\mu\text{g/mL}$) (n=3)	IC_{50} ($\mu\text{g/mL}$) (n=3)	
1	1.01 \pm 0.06	1.82 \pm 0.11	1.8
2	2.19 \pm 0.08	33.96 \pm 4.18	15.5
3	0.31 \pm 0.02	1.18 \pm 0.09	3.8
4	2.77 \pm 0.15	58.31 \pm 0.21	20.7
5	7.43 \pm 0.60	Not tested	-
Chloroquine (n=4)	18.58 \pm 0.33 ng/mL	-	-
Emetine (n=9)	-	0.08 (n=3)	-

D10: *P. falciparum* strain D10, CHO: Chinese Hamster Ovarian cell line, n = Number of replicates, SI (Selectivity index) = IC_{50} CHO/ IC_{50} D10.

extraction, and the leaves were collected fresh and directly freeze-dried, we confirm that the hemiacetal form is a natural metabolite of this plant.

Results of the *in vitro* antiparasmodial activity of the five purified sesquiterpenoids 1-5 (Table 1) showed that muzigadial (3) was the most potent sesquiterpene against the chloroquine-sensitive strain of *P. falciparum* (D10) with an IC_{50} -value of 0.31 μg . 9-Deoxymuzigadial (2) and 9-epideoxymuzigadial (1) showed antiparasmodial activity of 2.19 and 1.01 $\mu\text{g/mL}$, respectively. Comparing the structures of compounds 1-3 (Figure 1), we could see that muzigadial (3) has an additional hydroxyl group at position 9. This indicated that the hydroxyl substitution at this position increased the antiparasmodial activity of muzigadial by about 7 and 3 fold compared with its deoxy compounds 1 & 2, respectively. Muzigadial has been reported as one of the strongest antimicrobial drimane sesquiterpenes isolated from the *Warburgia* genus [4c].

Interestingly, it was found that 9-epideoxymuzigadial (1) showed higher activity than its isomer 9-deoxymuzigadial (2) (~2 fold), indicating that the α -orientation of the aldehyde group in 9-deoxy drimane sesquiterpenes is favorable for antiparasmodial activity. 3 β -Acetoxypolygodial (4) showed antiparasmodial activity with an IC_{50} of 2.77 $\mu\text{g/mL}$ which is comparable with 9-deoxymuzigadial (IC_{50} 2.19 $\mu\text{g/mL}$). Compound 4 has the same configuration of the aldehydic groups as compound 2, but they differ in ring A methyl group arrangement, and the β -acetoxyl substitution at C-3 (see Figure 1), which indicated that the substitution and arrangement of positions 3 and 4 in ring A did not affect the antiparasmodial activity. This finding supports the reported literature for the phytotoxic activity of sesquiterpenes from *C. winterana* [6b].

Muzigodiol (5), the hemiacetal cyclic form of muzigadial was the least active antiparasmodial compound with an IC_{50} of 7.43 $\mu\text{g/mL}$. The diminished activity compared with muzigadial indicated the importance of the 1, 4 dialdehydic groups for the

antiplasmodial activity. This finding was different from the published results for the phytotoxic activity of sesquiterpenes from *C. winterana*, where they found that compounds with masked aldehydic groups still retained the tested activity [6b].

The isolated sesquiterpenes **1-5** were also tested for cytotoxicity to test their selective antiplasmodial activity reflected by their selectivity index (IC_{50} CHO/ IC_{50} D10) (see Table 1). Muzigadial showed a high cytotoxicity (IC_{50} 1.18 μ g/mL, SI 3.8), indicating non-specific activity. Interestingly, the 9-deoxymuzigadial (**2**) was much less toxic than its stereoisomer 9-epideoxymuzigadial (**1**) (IC_{50} 33.96 and 1.82 μ g/mL, SI 15.5 and 1.8, respectively). 3- β -Acetoxypolygodial (**4**) was the least toxic compound to the CHO cells (IC_{50} 58.31 μ g/mL, SI 20.7), indicating that the 3 β -acetoxy substitution with 4 dimethyl arrangement has a significant effect in reducing the toxicity of this dialdehyde sesquiterpene.

The stereochemistry and the presence of a substituent may modulate the reactivity and bioactivity of the unsaturated dialdehydes considerably. It was reported that polygodial, a drimane sesquiterpene analogous to 9-deoxymuzigadial with 4 dimethyl groups, is known to react with primary amines under biomimetic conditions to form pyrroles, a reaction that has been proposed to be responsible for the bioactivity of polygodial. It was found that its 9-epimer was less bioactive, and did not undergo the same reaction because the formation of a pyrrole is less favored due to the larger distance between the aldehyde carbons [7a].

We conclude that the presence of these very reactive compounds is responsible for the biological activities of this plant and related plants containing the same type of compounds. The major component identified in this plant was muzigadial (**3**). Although muzigadial showed very strong *in vitro* antiplasmodial activity, its potential for development as an antimalarial drug is limited due to its inherent cytotoxicity and lack of selectivity. This is often the case with antimalarial compounds identified from plants [1b]. 9-Deoxymuzigadial (**2**) and 3 β -acetoxypolygodial (**4**) showed less antiplasmodial activity than muzigadial, but they were less toxic to the CHO cells. The three compounds (**2-4**) represent hits that could potentially be subjected to more detailed analysis. These compounds could also be used as scaffolds to generate leads with enhanced antiplasmodial activity, reduced cytotoxicity and improved bioavailability using different medicinal chemistry approaches to the dialdehyde reactive sites of the molecule.

Experimental

Plant material: Leaves from *C. winterana* were collected from Coral Gables, Fairchild Tropical Botanical Garden, Florida in October 2008 and identified by Dr Lena Struwe (Rutgers University). A voucher specimen (RG # 6) was deposited in the Chrysler Herbarium (CHRB) at Rutgers University, New Brunswick, NJ. The leaves were freeze-dried and stored at room temperature until extraction.

Extraction and bioassay-guided fractionation: Dried powdered leaves (200 g) were successively extracted with *n*-hexane (3 X 1L), ethyl acetate (3 x 1L) and methanol (3 x 1L) at room temperature. The solvents were evaporated to afford 13.4 g, 4.75 g and 17.5 g, respectively. The *n*-hexane extract (11.5 g) was subjected to flash chromatography using silica gel G 60 (Merck, 70-230 mesh, 2.5 x 30 cm). Gradient elution was performed using *n*-hexane with increasing amounts of ethyl acetate from 10 to 100%. Twelve fractions, 500 mL each, were collected and bioassayed for their antiplasmodial activity. Active fractions were subjected to further fractionation using flash chromatography and elution with varying solvent gradients of *n*-hexane-ethyl acetate. Separation was monitored by TLC (benzene-ethyl acetate 8:2, visualization with vanillin/H₂SO₄), as well as HPLC-UV. Fractionation was guided by antiplasmodial testing to concentrate on isolating and purifying only compounds which showed activity. Fraction 2 (5.13 g), eluted with 20% ethyl acetate in *n*-hexane, was further fractionated to afford 9-epideoxymuzigadial (**1**, 120 mg) [5c] and 9-deoxymuzigadial (**2**, 200 mg) [5b]. Fraction 3 (0.5 g), eluted with 30% ethyl acetate in *n*-hexane was further fractionated using CC and crystallization (diethyl ether) to afford another quantity of 9-deoxymuzigadial (**2**, 150 mg), muzigadial (**3**, 90 mg) [5c] and 3 β -acetoxypolygodial (**4**, 150 mg) [5c] in pure form. Fraction 4 (2.4 g) was subjected to crystallization from *n*-hexane-diethyl ether. Crystals were washed with ice cold diethyl ether to afford a larger quantity of muzigadial (**3**, 890 mg). Fraction 5 (0.58 g) was fractionated using CC and purified by preparative TLC to afford additional quantity of **4**, (50 mg), and muzigodiol (**5**, 22 mg) [6b].

In vitro antiplasmodial activity: A chloroquine-sensitive strain (D10) of *Plasmodium falciparum* was continuously cultured according to a modified method [7b], and parasite lactate dehydrogenase (pLDH) activity was used to measure parasite viability [8a]. The *in vitro* assays were performed as previously described [8b]. Chloroquine diphosphate (Sigma) served as the positive control and was made up in double distilled water and serially diluted in medium to the required concentrations. Crude extracts, fractions and purified compounds were prepared to 2 mg/mL stock solution in

10% DMSO. Stock solutions were stored at -20°C. Further dilutions were prepared on the day of the experiment. A full dose-response was performed for all compounds to determine the concentration inhibiting 50% of the parasite growth (IC₅₀-value). Test samples were tested at a starting concentration of 100 µg/mL, which was then serially diluted 2-fold in complete medium to give 10 concentrations, with the lowest one being 0.2 µg/mL. The same dilution technique was used for all samples. CQ was tested at a starting concentration of 1000 ng/mL. Muzigadial was tested at a starting concentration of 10 µg/mL. The highest concentration of solvent to which the parasites were exposed had no measurable effect on the parasite viability. Test samples were tested in triplicate on one occasion. The IC₅₀-values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

In vitro cytotoxicity assay: Active fractions and pure compounds were tested for *in vitro* cytotoxicity against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [8c]. The CHO cells were cultured in Dulbecos Modified Eagles Medium (DMEM): Hams F-12 medium (1:1) supplemented with

10% heat-inactivated fetal calf serum (FCS) and gentamycin (0.05 µg/mL). Sample preparation was the same as for antiplasmodial testing. The highest concentration of solvent to which the cells were exposed had no measurable effect on cell viability. Emetine dihydrochloride (Sigma) was used as the positive control in all cases. The initial test concentration of emetine was 100 µg/mL, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/mL. The same dilution technique was applied to all test samples with an initial concentration of 100 µg/mL to give 5 concentrations, with the lowest being 0.01 µg/mL. The concentration of test samples that inhibited 50% of the cells (IC₅₀ values) was obtained from dose-response curves, using a non-linear dose-response curve fitting analyses via GraphPad Prism v. 4 software.

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Three New 18-Oxygenated *ent*-Kaurane Diterpenoids from *Isodon leucophyllus*

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Three new 18-oxygenated *ent*-kaurane diterpenoids, isoleuconins A-C (**1–3**) and ten known diterpenoids were isolated from the aerial parts of *Isodon leucophyllus*. The structures were elucidated by 1D and 2D NMR spectroscopic analysis. All of the compounds were evaluated for their cytotoxicity. Rabdokunmin A (**13**) showed significant cytotoxicity against HT-29 cells, with an IC₅₀ value of 6.2 μM.

Keywords: *Isodon leucophyllus*, Labiatae, *ent*-kaurane, diterpenoid, cytotoxicity.

Isodon leucophyllus (Dunn) Kudo (Labiatae/Lamiaceae) is a small shrub mainly distributed in the western districts of Sichuan Province and the north-western regions of Yunnan Province, People's Republic of China [1]. Previous research reported the isolation of 28 diterpenoids (C-20 nonoxygenated and 7, 20-epoxy *ent*-kaurane), 6 flavones and one derivative of ionone [2a-2e]. In continuation of our research for new diterpenoids with antitumor activities, we have reinvestigated the aerial parts of *I. leucophyllus*, collected in Shangri-La County, Yunnan Province. As a result, along with 10 known diterpenoids (rabdoloxin A (**4**) [3a], isoscoparin I (**5**) [3b], 4-*epi*-henryine (**6**) [3c], rabdokunmin C (**7**) [3d], rabdokunmin E (**8**) [3d], rabdoinflexin B (**9**) [3e], excisanin A (**10**) [3f], phyllostachysin H (**11**) [3g], rabdoloxin B (**12**) [3a, and rabdokunmin A (**13**) [3d]), three new 18-oxygenated *ent*-kaurane diterpenoids [isoleuconins A-C (**1–3**)] were isolated from *I. leucophyllus*.

Isoleuconin A (**1**), exhibited a pseudomolecular ion peak (m/z 389.1947 [M+Na]⁺, calcd 389.1940), corresponding to the molecular formula C₂₀H₃₀O₆, with six degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene groups in according with the absorptions at 3373, 1697 and 898 cm⁻¹, respectively. Analyses of the ¹H, ¹³C and DEPT NMR data (Table 1) provided evidence that **1** possessed one *exo*-methylene group, one carbonyl

group, two tertiary methyls, five methylenes (including one oxygenated), seven methines (including four oxygenated), and four quaternary carbons. On the basis of the characteristic signals of three methines (δ_C 44.4, 62.1, and 53.6 assigned to C-5, 9, and 13), three quaternary carbons (δ_C 37.3, 51.4, 39.5 assigned to C-4, 8 and 10), two methyls (δ_C 17.4, 18.0 assigned to C-19 and 20), and an oxygenated methylene (δ_C 70.1, assigned to C-18), together with the *exo*-methylene group (δ_C 149.2 s, 113.6 t, elucidated as C-16 and 17), we presumed that **1** should be an *ent*-kaur-16-ene diterpenoid.

Chemical shift values of some typical carbon signals of **1** were similar to those of the known compound **4** [3a]. The main difference between them was that the conjugated carbonyl group at C-15 of **4** was reduced to a secondary hydroxyl group in **1**. HMBC correlations H-15/C-8, H-15/C-9, H-15/C-7, and H-15/C-16, and the related ¹H-¹H COSY correlations H-15/H₂-17 confirmed the above deduction. ROESY correlations H-15/H-13 α , H-15/H-7 β indicated that the hydroxyl group located at C-15 adopted a β -orientation, as shown in Figure 1. The signal for C-9 (δ_C 70.1) in **4** shifted upfield to δ_C 62.1 (C-9) in **1**, caused by the γ -steric compression effect between HO-15 and H-9 β . That could also support the view that HO-15 in **1** was β -oriented. Consequently, compound **1** was elucidated as 7 α ,12 α ,14 β ,15 β ,18-pentahydroxy-*ent*-kaur-16-en-11-one, to which the name isoleuconin A was given.

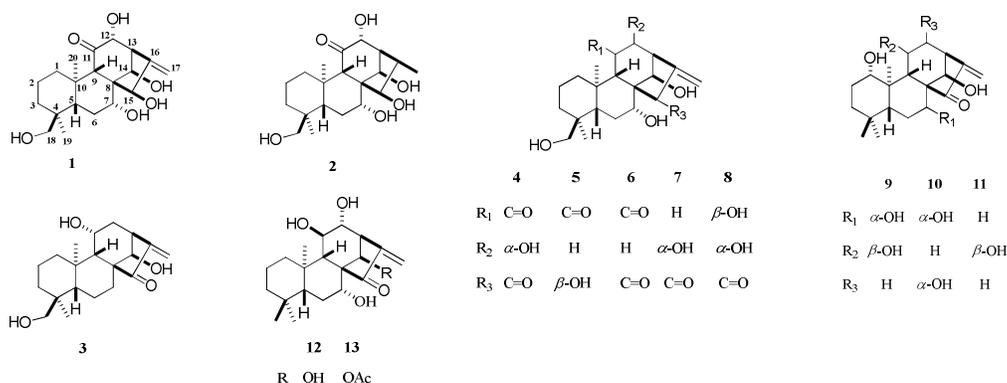


Table 1: ^1H and ^{13}C NMR spectroscopic data of isoleuconins A–C (1–3) (δ in ppm, J in Hz, recorded at 400 MHz and 100 MHz, respectively).

No.	1 ^a		2 ^b		3 ^b	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1 α	1.48 ^c (d, 13.2)	39.1 t	2.2 ^c (m)	40.9 t	1.91 (d, 13.0)	39.5 t
1 β	0.97 ^c (m)		1.59 (m)		1.07 ^c (m)	
2 α	1.40 (m)	17.3 t	1.71 (m)	18.5 t	1.70 ^c (m)	18.5 t
2 β	1.35 ^c (m)		1.50 (m)		1.50 ^c (m)	
3 α	1.02 ^c (m)	34.0 t	1.32 (d, 12.7)	35.6 t	1.39 (m)	35.7 t
3 β	1.32 ^c (m)		1.82 ^c (m)		1.81 ^c (m)	
4		37.3 s		38.3 s		38.1 s
5 β	1.17 (s, overlap)	44.4 d	1.96 (d, 12.0)	46.7 d	1.80 ^c (m)	49.0 d
6 α	1.61 (m)	28.5 t	2.22 ^c (m)	30.8 t	1.45 ^c (m)	18.5 t
6 β	1.68 (m)		2.49 (d, 9.4)		1.85 ^c (m)	
7 α		73.2 d		76.4 d	2.61 (d, 13.8)	26.7 t
7 β	3.60 (dd, 4.0, 11.2)		4.53 (m)		2.21 (d, 13.8)	
8		51.4 s		54.7 s		57.5 s
9 β	2.15 (s)	62.1 d	3.30 (s)	64.5 d	2.18 (s)	66.9 d
10		39.5 s		40.1 s		38.9 s
11 β		210.3 s		211.4 s	4.28 (s)	64.9 d
12 α		78.4 d		74.9 d	2.34 (m)	41.7 d
12 β	3.48 (d, 3.1)		4.50 (s)		2.43 (m)	
13 α	2.69 (br. d)	53.6 d	2.85 (d, 5.6)	54.6 d	3.36 (s)	46.4 d
14 α	5.07 (br. s)	71.2 d	6.10 (s)	74.3 d	4.90 (s)	73.4 d
15	4.94 (br. s)	72.9 d	5.57 (m)	73.8 d		209.2 s
16 α		149.2 s	3.35 ^c (m)	35.0 d		150.6 s
17 α	5.10 (br. s)	113.6 t	1.22 (d, 7.7)	11.3 q	5.35 (s)	112.8 t
17 β	5.24 (br. s)				6.22 (s)	
18 α	2.81 (d _{AB} , 11.3)	70.1 t	3.34 ^c (m)	71.3 t	3.32 (m)	71.4 t
18 β	3.26 (d _{AB} , 11.3)		3.66 (d, 10.3)		3.62 (m)	
19 α	0.59 (s)	17.4 q	0.85 (s)	18.2 q	0.80 (s)	18.0 q
20 α	1.13 (s)	18.0 q	1.78 (s)	19.3 q	1.08 ^c (s)	18.5 q

a: in $\text{CDCl}_3+\text{CD}_3\text{OD}$, b: in $\text{C}_5\text{D}_5\text{N}$, c: revealed by HSQC correlations.

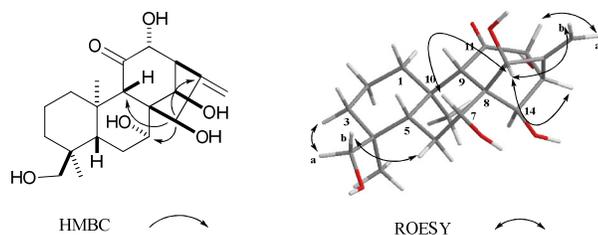


Figure 1: Key HMBC and ROESY correlations for **1**.

Isoleuconin B (**2**) was obtained as a grey amorphous powder. The HR-ESI-MS gave a pseudomolecular ion peak (m/z 391.2109 $[\text{M}+\text{Na}]^+$, calcd 391.2096), corresponding to the molecular formula $\text{C}_{20}\text{H}_{32}\text{O}_6$, with five degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl and carbonyl groups with absorptions at 3386 and 1698 cm^{-1} , respectively. The absorption for *exo*-methylene disappeared in the IR spectrum of **2**. Typical carbon signals of three methines (δ_{C} 46.7, 64.5, 54.6 assigned as C-5, 9, and 13), three quaternary carbons (δ_{C} 38.3, 54.7, 40.1 assigned as C-4, 8 and 10), two methyls (δ_{C} 18.2, 19.3 assigned as C-19 and 20), and an oxygenated methylene (δ_{C} 71.3, assigned as C-18) indicated that **2** also should be an

ent-kaurane diterpenoid. Carefully comparing the ^{13}C NMR data of compound **2** with those of **1**, we found that the *exo*-methylene of compound **1** was reduced to a methyl (δ_{C} 11.3, q, assigned as C-17) in compound **2**. HMBC correlations $\text{H}_3\text{-17}/\text{C-16}$, $\text{H}_3\text{-17}/\text{C-13}$, and $\text{H}_3\text{-17}/\text{C-15}$, and $^1\text{H-}^1\text{H}$ COSY correlations $\text{H}_3\text{-17}/\text{H-16}$, $\text{H-15}/\text{H-16}$, and $\text{H-16}/\text{H-13}$ proved the above elucidation. ROESY correlations $\text{H}_3\text{-17}/\text{H-12}\beta$ and $\text{H-16}\alpha/\text{H-13}\alpha$ revealed that the methyl located at C-16 adopted the β -orientation. Thus, the structure of compound **2** was established as $7\alpha,12\alpha,14\beta,15\beta,18$ -pentahydroxy-16 β -methyl-*ent*-kaur-11-one, to which the name isoleuconin B was assigned.

The molecular formula for isoleuconin C (**3**) was established as $\text{C}_{20}\text{H}_{30}\text{O}_4$ on the basis of HR-ESI-MS data (m/z 357.2040 $[\text{M}+\text{Na}]^+$, calcd 357.2041). The IR spectrum revealed the presence of hydroxyl, carbonyl, and *exo*-methylene from absorptions at 3371, 1713, 1648, and 928 cm^{-1} , respectively. Analyses of the ^1H -, ^{13}C - and DEPT-NMR data (Table 1) provided evidence that **3** possessed one conjugated carbonyl group (δ_{C} 209.2 assigned to C-15), one *exo*-methylene (δ_{C} 150.6,

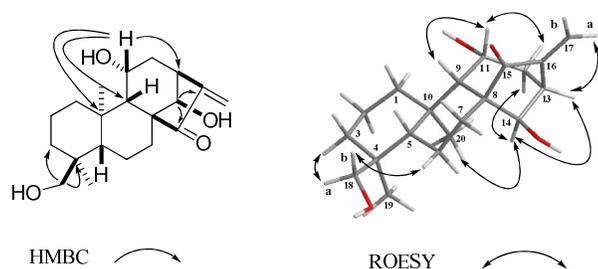


Figure 2: Key HMBC and ROESY correlations for **3**.

112.8, assigned to C-16 and 17), two oxygenated methines (δ_C 64.9, 73.4, assigned to C-11 and 14), one oxygenated methylene (δ_C 71.4 assigned to C-18) and other typical carbon signals of an *ent*-kaurane diterpenoid. HMBC correlations H₂-17/C-16, H₂-17/C-13, and H₂-17/C-15 indicated that the *exo*-methylene was located at C-16 and conjugated with the carbonyl group (C-15). HMBC correlations H-11/C-8, H-11/C-10, and H-11/C-13, and H-14/C-16, H-14/C-15 disclosed that C-11 and C-14 were substituted by two hydroxyl groups, which was confirmed by ¹H-¹H COSY correlations H-11/H-12/H-13/H-14 and H-11/HO-11, H-14/HO-14. ROESY correlations H-14/H-20, H-14/H-13 α , and H-14/H-12 α revealed that H-14 adopted an α -orientation, as shown in Figure 2. ROESY correlations H-11/H-9 β and H-11/H-12 β revealed that H-11 adopted a β -orientation. HMBC correlations H-18/C-3 and H-18/C-4 indicated that C-18 was substituted by one hydroxyl group. Like compound **1**, this substitution also could be proved by the upfield shifted carbon signals (δ_C 35.7, C-3; δ_C 49.0, C-5) caused by the γ -steric compression effect between HO-18 and H-3 β and H-5 β in **3** compared with those signals (δ_C 41.8, C-3; δ_C 53.2, C-5) of compound **12**. Therefore, the structure of compound **3** was determined as 11 α ,14 β ,18-trihydroxy-*ent*-kaur-16-en-15-one, and named as isoleuconin C.

Compounds **1–13** were evaluated for their cytotoxic activities against SK-OV-3, BEL-7402 and HT-29 cell lines, using the sulforhodamine B (SRB) method with adriamycin as the positive control [3h]. As may be seen from Table 2, compound **3** exhibited weak activities against SK-OV-3 and BEL-7402 cell lines, while compounds **1** and **2** were found to be inactive against all of these cells. Their analogue **4** exhibited weak inhibitory effects against two kinds of cell lines; more importantly, another analogue, **6**, showed significant activity against these cell lines. Carefully comparing the structure of **6** with that of **4**, we found that the α -hydroxyl group C-12 of compound **4** disappeared in compound **6**. The above results disclosed that in the diterpenoids with a structure like **1**, the α -hydroxy group located at C-12 weakened the cytotoxicity of the *ent*-kaurane diterpenoid. On the other hand, in diterpenoid with a structure like **12** and **13**, esterification with the hydroxyl group located at C-14 could

Table 2: Cytotoxicity bioassay result^a for compounds **1–13**.

Compd	SK-OV-3	BEL-7402	HT-29
1	>100	>100	>100
2	>100	>100	>100
3	38.0	40.8	82.8
4	38.9	98.9	35.7
5	>100	>100	>100
6	8.6	23.5	6.6
7	55.3	71.3	74.7
8	>100	>100	>100
9	40.0	46.7	29.7
10	>100	30.2	6.2
11	17.1	24.0	17.0
12	9.9	32.8	6.6
13	7.4	26.2	6.2
ADR	0.17	0.067	0.092

^a Results are expressed as IC₅₀ values in μ M. Cell lines: SK-OV-3 (human ovarian cancer cell line); BEL-7402 (human lung cancer cell line); HT-29 (human colon cancer cell line).

improve the cytotoxicity. The above results further proved that the cyclopentanone conjugated with an *exo*-methylene is the active center of *ent*-kauranoids. [3i]

Experimental

General: Optical rotations, Perkin-Elmer Model 241 polarimeter; UV, Shimadzu UV-2401 PC UV-VIS spectrophotometer; IR, Bio-Rad Fts-135 spectrophotometer; MS, VG Auto spec-3000 spectrometer or Finnigan MAT 90; NMR, Bruker AV-400 or DRX-500 instrument.

Plant material: Aerial parts of *Isodon leucophyllus* (Dunn) Kudo were collected and air dried in Shangri-La county of Yunnan Province in August, 2004. The identity of the plant material was verified by Prof. Xi-Wen Li, and a voucher specimen was deposited in the Herbarium of the Kunming Institute of Botany.

Extraction and isolation: Powdered aerial parts of *I. leucophyllus* (1.8 kg) were extracted with 70% aq. acetone (3 \times 6 L) at RT for 3 days each time. The extract was evaporated *in vacuo* to remove acetone, then partitioned between H₂O and EtOAc. The EtOAc extract (78 g) was decolorized with MCI gel, and then chromatographed over a silica gel column (650g, 100-200 mesh, Qingdao marine chemical factory), eluted with a gradient solvent system [CHCl₃-CH₃COCH₃ (1:0, 9:1, 8:2, 7:3, 2:1, 1:1, 0:1)] to afford fractions A–G, monitoring by TLC (volume of each collection was 1000 mL). Fraction E (2:1, 5 g) was submitted to chromatography over a RP-18 column (100 g, 40-63 μ m, Merck Company) eluted with 30% \rightarrow 100% MeOH–H₂O to give fractions E1–E7, monitoring by TLC (volume of each collection was 250 mL). Fraction E4 (590 mg) was chromatographed over a silica gel column (200-300 mesh, 20 g) eluted with a gradient solvent system of CHCl₃-CH₃OH (60:1 \rightarrow 10:1) to afford a mixture of 2 diterpenoids (volume of each collection was 50 mL), which were purified by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 9.4 \times 250 mm, 72% MeOH–H₂O, λ_{max} = 210 nm) to yield compound **1** (46 mg) and compound **2** (14 mg). Fraction E2 (105 mg) was subjected to a silica gel CC, eluting

with a gradient solvent system [CHCl₃–CH₃OH (40:1→5:1)] to afford a mixture of diterpenoids, which was purified by semi-preparative HPLC (45% MeOH–H₂O, λ_{\max} = 210 nm) to obtain compound **5** (45 mg). Fraction C (8:2, 11 g) was submitted to CC over a RP-18 column (200 g, 40–63 μ m, Merck Company, 30%→100% MeOH–H₂O) to give fractions C1–C5, monitoring by TLC (volume of each collection was 250 mL). In the sixth, seventh and eighth bottles of elution solvent belonging to fraction C1, compound **4** (3.1 g) was separated as needle crystals. The mother liquid of compound **4** was subjected to RP-18 CC (100 g, 40–63 μ m, Merck Company, 30%→100% MeOH–H₂O) to obtain compounds **6** (434 mg) and **13** (78 mg). Fraction C2 (2.3 g) was subjected to silica gel CC (200–300 mesh, 40g) eluting with a gradient solvent system of light petroleum–CH₃COCH₃ (1:0 to 0:1, volume of each collection was 50 mL). Compound **9** (509 mg) was separated out as needle crystals from the first bottle of fraction C2. Compound **7** (3.1 mg) and compound **8** (1.7 mg) were isolated by semi-preparative HPLC (40% MeOH–H₂O, 45% MeOH–H₂O, λ_{\max} = 230 nm) from the fourth and fifth bottle of elution solvent. The tenth bottle (23 mg) of the above elution was subjected to RP-18 CC (5 g, 40–63 μ m, Merck Company, 30%→100% MeOH–H₂O) to obtain a mixture mainly contained two diterpenoids. The mixture was separated by semi-preparative HPLC (24% ACN–H₂O, λ_{\max} = 230 nm) to obtain compound **10** (5 mg) and one unpurified diterpenoid, and then purified by semi-preparative HPLC (38% MeOH–H₂O, λ_{\max} = 230 nm) to obtain compound **3** (3 mg). Compound **11** (4.0 mg) was isolated from fraction F (1:1) after continued CC on RP-18, and then purified by semi-preparative HPLC (42% MeOH–H₂O, λ_{\max} = 238 nm). Compound **12** (2.4 g) separated as granular crystals from the EtOAc extract when dissolved in acetone.

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Isoleuconin A (1)

Grey tablet-like crystals
 $[\alpha]_D^{23.6}$: +52.0 (c = 12.60 mg/mL, MeOH).
 IR (KBr): 3373, 2930, 2873, 1697, 1455, 898, cm⁻¹.
 UV λ_{\max} (MeOH) nm (log ϵ): 204 (3.66).
¹H and ¹³C NMR: Table 1.
 HR-ESI MS: m/z [M+Na]⁺, calculated for C₂₀H₃₀O₆Na (calcd. 389.1940); found: 389.1947.

Isoleuconin B (2)

Grey powder
 $[\alpha]_D^{23.8}$: +12.0 (c = 5.82 mg/mL, MeOH).
 IR (KBr): 3386, 2931, 2874, 1697, 1453 cm⁻¹.
 UV λ_{\max} (MeOH) nm (log ϵ): 203 (3.23).
¹H and ¹³C NMR: Table 1.
 HR-ESI MS: m/z [M+Na]⁺, calculated for C₂₀H₃₂O₆Na (calcd. 391.2096); found: 391.2109.

Isoleuconin C (3)

White powder
 $[\alpha]_D^{18.9}$: -76.0 (c = 0.79 mg/mL, MeOH).
 IR (KBr): 3443, 3371, 2935, 2855, 1713, 1648, 928 cm⁻¹.
 UV λ_{\max} (MeOH) nm (log ϵ): 236 (3.81).
¹H and ¹³C NMR: Table 1.
 HR-ESI MS: m/z [M+Na]⁺, calculated for C₂₀H₃₀O₄Na (calcd. 357.2041); found: 357.2040.

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Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber *Cucumaria okhotensis*: Stimulation of Activity of Mouse Peritoneal Macrophages

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Six monosulfated triterpene glycosides, frondoside A₁ (**1**), okhotoside B₁ (**2**), okhotoside A₁-1 (**3**), frondoside A (**4**), okhotoside A₂-1 (**5**) and cucumarioside A₂-5 (**6**), isolated from *Cucumaria okhotensis* Levin et Stepanov, stimulate spreading and lysosomal activity of mouse macrophages and ROS-formation in the macrophages. The highest macrophage spreading and stimulation of their lysosomal activity was induced by glycosides **1**, **4** and **6**. All glycosides similarly stimulate ROS formation in macrophages, but glycoside **2** caused minimal stimulation.

Keywords: triterpene glycosides, *Cucumaria okhotensis*, macrophages, ROS formation, spreading, lysosomal activity.

Triterpene glycosides of sea cucumbers possess a wide spectrum of biological activity caused by their membranolytic action [1]. During recent decades their immunomodulatory activity in subtoxic doses has attracted attention. Monosulfated glycosides from the sea cucumbers *Cucumaria japonica* [2–5] and *C. frondosa* [6] stimulate mammalian cell immunity while their activity is effective in doses significantly less than the cytotoxic ones against immune cells [7].

Recently it was found that populations of *C. japonica* in the northern parts of the Sea of Japan and Sea of Okhotsk, including the shallow waters of the Kamchatka Peninsula and North Kuril Islands, really are a series of endemic species that differ from *C. japonica* by several features, including composition of triterpene glycosides [8,9]. Because monosulfated glycosides from *C. japonica* are used for the preparation of immunostimulants [5], it is of interest to investigate the immunostimulatory activity of the glycosides isolated recently from the newly described *C. okhotensis* Levin et Stepanov, found near the south-western shore of Kamchatka Peninsula [10].

The structures of the studied monosulfated glycosides from *C. okhotensis* are presented in Figure 1. Glycosides

1 and **4** were also isolated from *C. frondosa* [11,12], and glycoside **6** from *C. conicospermium* [13]. Because the immunomodulatory action of frondoside A (**4**) has been studied previously [6], we used it as a positive control. To study immunomodulatory activity we chose spreading and lysosomal activity of mouse macrophages, and the formation of reactive oxygen species (ROS) in them.

Macrophage adhesion onto an extracellular matrix, followed by their spreading, is necessary to follow their functional activity. These indexes reflect the initial stage of phagocytosis and macrophage ability for phagocytosis. It is accompanied by changes in macrophage geometric parameters, including cell area and perimeter. The cell may elongate and change shape because of the formation of lamellae and filopodia [14,15].

All the studied glycosides induced an increase of all these indexes on the fourth day after intraperitoneal injection. Macrophages of mice administrated with frondoside A (**4**) had larger geometrical parameters and their shape was more differentiated because of an increase in spreading, elongation, and formation of lamellae and filopodia compared with macrophages of mice from the control group.

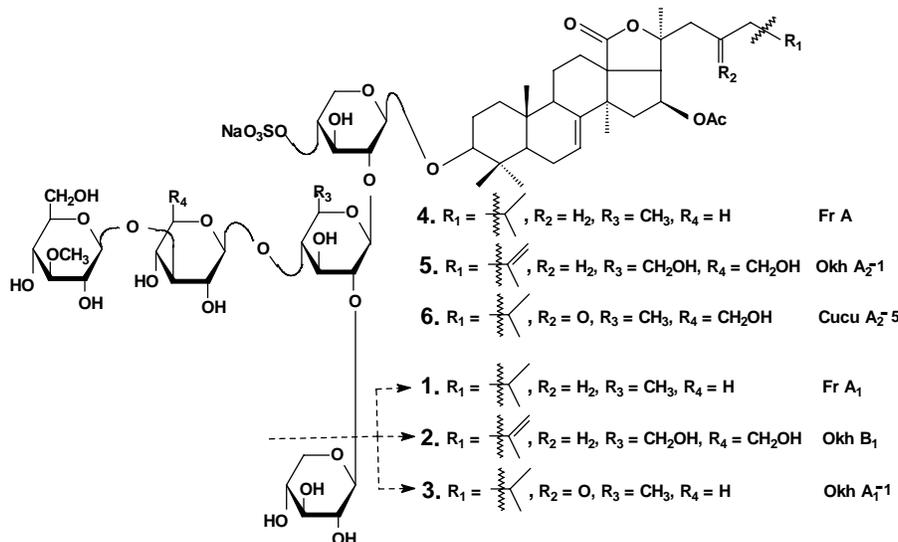


Figure 1: Chemical structures of monosulfated triterpene glycosides from *Cucumaria okhotensis*: 1 – frondoside A₁ [9,11]; 2 – okhotoside B₁ [9]; 3 – okhotoside A₁-1 [8]; 4 – frondoside A [9,12]; 5 – okhotoside A₂-1 [8]; 6 – cucumarioside A₂-5 [9,13].

Table 1: Effect of triterpene glycosides on peritoneal macrophage spreading. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 µg/mouse. On the fourth day the mice were killed by perversical dislocation followed by isolation of macrophages that were then stained with the fluorescent probe 5-carboxyfluorescein diacetate and analyzed. The data are presented as $m \pm se$ (n=100); * – $p < 0.05$.

Substance	Geometric parameters of macrophages, pixels			
	Cell area	Perimeter	Maximal chord	Shape
Control	92.163 ± 13.111	80.698 ± 9.484	11.050 ± 1.094	0.189 ± 0.016
Fr A ₁ (1)	239.315 ± 25.444*	104.849 ± 7.578*	21.284 ± 1.465*	0.245 ± 0.012*
Okh B ₁ (2)	107.989 ± 15.989	120.333 ± 8.621*	14.774 ± 0.863*	0.075 ± 0.003*
Okh A ₁ -1 (3)	154.146 ± 18.282*	142.343 ± 12.40*	19.214 ± 1.452*	0.106 ± 0.007*
Fr A (4)	250.125 ± 33.284*	128.659 ± 12.240*	22.472 ± 1.611*	0.210 ± 0.011*
Okh A ₂ -1 (5)	157.138 ± 22.937*	88.400 ± 8.956	17.493 ± 1.064*	0.285 ± 0.011*
Cucu A ₂ -5 (6)	229.608 ± 39.828*	125.411 ± 16.941*	22.592 ± 2.453*	0.206 ± 0.014

Almost all the studied glycosides induced statistically significant increases in cell area, perimeter, maximal chord and integral index of cell shape at an injection dose of 0.2 µg/mouse. Quantitative estimation of the influence of the glycosides on macrophage spreading is presented in Table 1. The most effective glycosides were frondoside A₁ (1), frondoside A (4) and cucumarioside A₂-5 (6). These substances activate spreading 2–2.5 fold greater than the control. Frondoside A was more active than frondoside A₁ and cucumarioside A₂-5.

Lysosomal activity is one of the important markers of physiological and biochemical macrophage status. Almost all the studied glycosides induced a statistically significant increase in lysosomal activity of peritoneal macrophages on the fourth day after a single intraperitoneal injection of 0.2 µg/mouse. The most effective glycosides were frondoside A₁ (1), frondoside A (4) and cucumarioside A₂-5 (6). These compounds activated lysosomal activity approximately 1.5–2.5 times greater than that of control cells (Figure 2). The most active glycoside was frondoside A, whereas the activity of glycoside 6 was similar to that of glycoside 1.

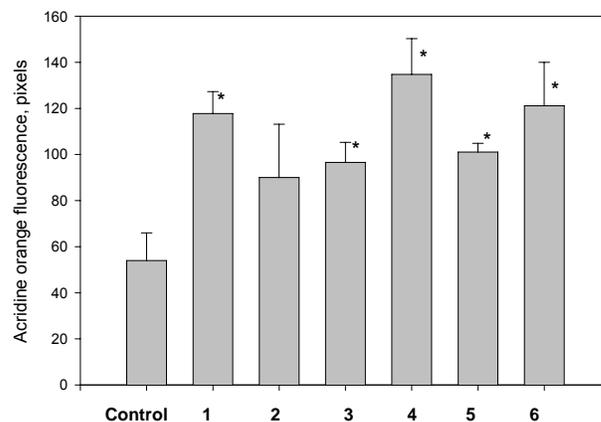


Figure 2: Influence of triterpene glycosides on lysosomal activity of mouse peritoneal macrophages. Glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose 0.2 µg/mouse. On the fourth day the mice were killed by perversical dislocation followed by isolation of macrophages. The lysosomes were stained with acridine orange and analyzed. The data are presented as $m \pm se$ (n=100); * – $p < 0.05$.

The ability to generate ROS, singlet oxygen, hydrogen peroxide and the products of their interaction with each other, and NO, the so called “oxidative burst”, is

important for the ability of macrophages to kill ingested microorganisms.

Almost all the studied glycosides induced statistically significant increases of ROS formation in peritoneal macrophages isolated from mice on the fourth day after a single intraperitoneal injection at a dose of 0.2 $\mu\text{g}/\text{mouse}$ (Figure 3). All the glycosides activated lysosomal activity 1.3–1.8 folds greater than control cells. Okhotoside B₁ (**2**) showed minimal activity.

Hence all the studied monosulfated glycosides isolated from *Cucumaria okhotensis* showed immunostimulatory activity of mouse macrophages, as indicated by an increase in macrophage spreading, their lysosomal activity and ROS-formation. This indicates that *C. okhotensis* can be used as a source for immunostimulant preparations.

The activity of the studied glycosides was changed depending on their structure, but not significantly. Glycosides **4** and **5** having a fifth terminal monosaccharide unit (xylose) were more active than their corresponding tetrasaccharide analogs **1** and **2**.

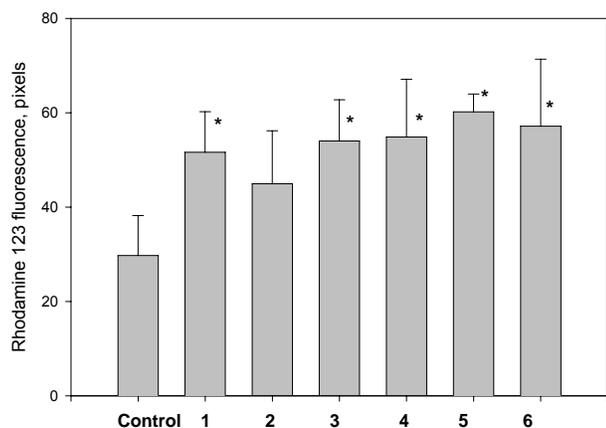


Figure 3: Influence of triterpene glycosides on the ROS-formation in peritoneal mouse macrophages. The glycosides were administrated by intraperitoneal injection of 0.5 mL at a dose of 0.2 $\mu\text{g}/\text{mouse}$. On the fourth day the mice were killed by perversal dislocation followed by isolation of macrophages. The cells were stained with dihydrorhodamine 123 probe and analyzed. The data are presented as $m \pm se$ ($n=100$); *– $p < 0.05$.

Experimental

Triterpene glycosides: The glycosides were isolated from *Cucumaria okhotensis* using common procedures of hydrophobic chromatography of the extracts on teflon powder Polichrom-1, followed by chromatography on a Si gel column and HPLC [8,9]. Structures and purity of individual isolated frondoside A₁ (**1**), okhotoside B₁ (**2**), okhotoside A₁-1 (**3**), frondoside A (**4**), okhotoside A₂-1 (**5**) and cucumarioside A₂-5 (**6**) were confirmed by ¹³C NMR

spectra [8,9]. Individual substances were dissolved in sterile distilled water at a concentration of 1 mg/mL (initial concentration). Forty μL initial glycoside solution were added to 960 μL of distilled water to produce a solution of 40 $\mu\text{g}/\text{mL}$. Ten μL of this solution was added to 990 μL of distilled water to obtain the final concentration of 0.4 $\mu\text{g}/\text{mL}$. The final concentration was used for injection into mice.

Determination of immunomodulatory activity: CBA mice (female, 20 g) were used for *in vivo* testing. The glycosides were intraperitoneally injected (0.5 mL of a glycoside solution in distilled water) at a final glycoside dose of 0.2 $\mu\text{g}/\text{mouse}$. The control mice were injected with distilled water. On the fourth day after injection the mice were killed by perversal dislocation and peritoneal macrophages were isolated by standard procedures. The estimation of immunomodulatory activity was carried out by staining and localization of lysosome in macrophages, determination of reactive oxygen species (ROS) formation in macrophages, and determination of macrophage spreading on an extracellular matrix. Molecular fluorescent probes followed by cell imaging analysis were used. Mice peritoneal liquor (250 μL) containing macrophages was placed onto a microscope cover slip and incubated for 1 h at 37°C. After macrophage adhesion, the cover slip was washed twice with phosphate buffered saline (PBS, pH 7.5). Then 250 μL of a solution of the fluorescent probe was added to the cell monolayer in drops and the cells were incubated at 37°C. To determine macrophage lysosomal activity, a solution of acridine orange (Calbiochem, 100 $\mu\text{g}/\text{mL}$ in PBS) was applied and the cells were incubated for 30 min. To estimate ROS formation, a solution of dihydrorhodamine 123 (Sigma, 100 $\mu\text{g}/\text{mL}$ in PBS containing 0.5 mM of sodium azide) was used. The cells were incubated for 10 min. To estimate macrophage spreading, a solution of 5-carboxyfluorescein diacetate (Molecular Probes, 50 $\mu\text{g}/\text{mL}$ in PBS) was applied. The cells were incubated for 60 min. The cell monolayer was washed twice with PBS. Cover slips were placed on the stage of a fluorescent scanning device composed on the base of an inverted microscope Axiovert 200 (Zeiss, Germany). A luminiscent 75 W Optosource xenon arc lamp and a DAC-controlled monochromator Optoscan (Cairn Research Ltd., UK) were used as light sources for inducing fluorescence at $\lambda_{\text{exc}} = 488 \text{ nm}$; a HQ FITC filter-block (Chroma Technology Corp., USA) and Fluor 40 \times /1.3 oil objective lens (Zeiss, Germany) were used to visualize cell fluorescence. Cell fluorescence images were recorded using a Hamamatsu Orca-ER C4742-95 digital monochrome video camera (Hamamatsu Photonics K.K., Japan) and an IBM-compatible computer having a Fireware data interface.

The intensity of fluorescence of 100 randomly chosen cell images was estimated using an AQM Advance 6 computer program (Kinetic Imaging Ltd., UK) and calculated along with average intensity of fluorescence for each cell, in pixels. Geometric cell parameters (area, perimeter, maximal chord and shape) were also estimated. Each experiment was repeated in triplicate. Average value, standard error, standard deviation and p-values were calculated and plotted on the chart using a SigmaPlot 3.02 computer program (Jandel Scientific, USA).

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Three New Aaptamines from the Marine Sponge *Aaptos* sp. and Their Proapoptotic Properties

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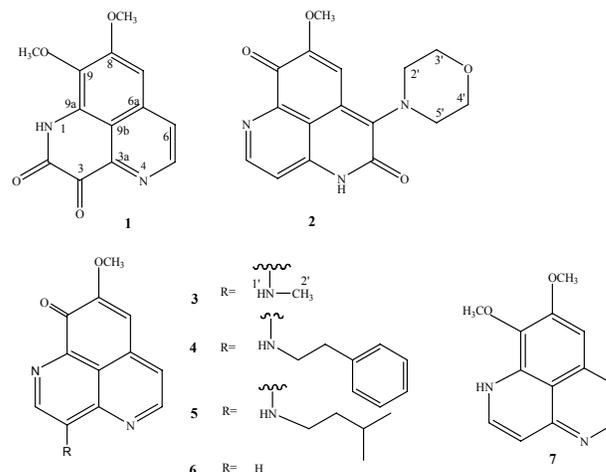
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Three new aaptamine-type alkaloids, 2,3-dihydro-2,3-dioxoaaptamine (**1**), 6-(*N*-morpholinyl)-4,5-dihydro-5-oxo-demethyl(oxy)aaptamine (**2**) and 3-(methylamino)demethyl(oxy)aaptamine (**3**), along with known aaptamines were isolated from the sponge *Aaptos* sp. Their structures were determined on the basis of detailed analysis of their 1D and 2D NMR spectroscopic and mass spectral data. The isolated compounds induced apoptosis in human leukemia THP-1 cells.

Keywords: aaptamine, benzo[*de*][1,6]-naphthyridine alkaloids, marine sponge, apoptosis, NMR.

The genus *Aaptos* comprises about 20 species of sponges that are mostly known as a rich source of benzo[*de*][1,6]-naphthyridine alkaloids (aaptamines) possessing antioxidant [1], antiviral [2], antimicrobial [3], antifungal [4], antiparasitic [5], cytotoxic [3a,3b,6], and other activities [7]. The various and interesting bioactivities of these compounds have raised a high interest in their syntheses and the preparation of derivatives [7]. During our search for anticancer metabolites from marine organisms [8-10], we recently isolated a new aaptamine derivative [10] from the Vietnamese sponge *Aaptos* sp. In this paper we report the results of a study on another collection of a sponge belonging to the same genus which has resulted in the isolation of three new benzo[*de*][1,6]-naphthyridine alkaloids, namely 2,3-dihydro-2,3-dioxoaaptamine (**1**), 6-(*N*-morpholinyl)-4,5-dihydro-5-oxo-demethyl(oxy) aaptamine (**2**) and 3-(methylamino)demethyl(oxy)aaptamine (**3**), along with the known 3-(phenethylamino)demethyl-(oxy)aaptamine (**4**) [11], 3-(isopentylamino)-demethyl(oxy)aaptamine (**5**) [11], demethyl(oxy)-aaptamine (**6**) [3a] and aaptamine (**7**) [12].

Compound **1** was obtained as a yellow amorphous solid, the molecular formula for which was established as C₁₃H₁₀N₂O₄ from the [M + Na]⁺ ion at *m/z* 281.0544 (calcd for C₁₃H₁₀N₂O₄Na, 281.0546) and [M + H]⁺ ion at *m/z* 259.0727 (calcd for C₁₃H₁₁N₂O₄, 259.0726) in the HRESIMS (positive ion mode). The ¹H NMR spectrum of **1** exhibited two doublets of coupled protons at δ 8.73 and 8.07 (each 1H, d, *J*=5.4 Hz), one isolated



singlet at δ 7.30 and singlets for two methoxyl groups at δ 4.04 and 4.09 (each 3H), which are characteristic for aaptamines [3b]. The signal of the exchangeable proton at δ 11.4 (1H, br.s) in the ¹H NMR spectrum recorded in DMSO-*d*₆, together with the IR absorption at 3375 cm⁻¹ suggested the presence of a NH group.

Careful analysis of the ¹³C NMR data, and the HSQC, HMBC and 1D NOE correlations (Table 1) allowed us to establish the modified aaptamine core and attribute proton signals to the 5, 6 and 7 positions. Among all the carbon signals of **1**, two quaternary oxygenated carbons at δ 178.8 and 158.4 did not show any HMBC correlation with protons. These signals have been assigned to the C-3 keto group and C-2 amide carbonyl by comparison of ¹³C NMR data of N-1 – C-3 fragment

Table 1: ^1H and ^{13}C NMR spectroscopic data for **1** and **2** in CD_3OD .

Position	1				2						
	$\delta_{\text{H}}^{\text{a}}$ (mult, J in Hz)	$\delta_{\text{C}}^{\text{b}}$	HMBC (H \rightarrow C)			$\delta_{\text{H}}^{\text{a}}$ (mult, J in Hz)	$\delta_{\text{C}}^{\text{b}}$	HMBC (H \rightarrow C)			
			J^{c}	J^{d}	J^{e}			J^{c}	J^{d}	J^{e}	
1	11.4 br.s ^c										
2		158.4			8.61 d (5.4)	149.1	3	3a, 9a	9b	H3	
3		178.8			7.34 d (5.4)	112.1	2	9b	9a	H2	
3a		145.7				145.1 ^d	3				
4					10.18 br.s ^c						
5	8.73 d (5.4)	146.1	6	3a, 6a	9b	H6				162.4	
6	8.07 d (5.4)	126.4	5, 6a	7, 9b	9a	H5, H7				143.9	
6a		136.9								122.5	
7	7.30 s	102.3	8	6, 9, 9b	9a	H6, 8-OMe	7.18 s	8	6, 9, 9b	9a	H2', 8-OMe
8		158.9								154.8	
9		139.4								178.9	
9a		125.8								142.6 ^d	
9b		116.9								116.5	
1'											
2'					3.70 m	54.3	3'	6, 5'		H3', H7	
3'					3.88 m	68.9	2'	4'		H7, H2'	
4'					3.88 m	68.9	5'	3'		H5'	
5'					3.70 m	54.3	4'	6, 2'		H3'	
8-OMe	4.09 s	57.0	8				8			H7	
9-OMe	4.04 s	62.2	9								

^a Measured at 700 MHz, ^b at 175 MHz. Assignments were confirmed from HSQC, HMBC and NOE data. ^c In $\text{DMSO}-d_6$, ^d Interchangeable signals.

with literature data for known bis indol alkaloids containing an unusual α -keto enamide functionality [13] and aaptanone, having a rare oxygenated 1,6-naphthyridine core [14]. This suggestion was in agreement with IR data (two carbonyl bands at 1697 and 1714 cm^{-1}) and an ESI MS/MS experiment, where MS/MS fragmentation of the $[\text{M}+\text{H}]^+$ ion gave a daughter ion at m/z 187 indicative of the loss of a $\text{C}_2\text{H}_2\text{NO}_2$ fragment. Thus the structure of **1** was determined as 2,3-dihydro-2,3-dioxoaaptamine, the first naturally occurring 2,3-disubstituted aaptamine alkaloid. Earlier, compound **1** was obtained during attempts at the synthesis of aaptamine [15].

The molecular formula of **2** was determined as $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_4$ from the HRESIMS ions for $[\text{M}+\text{H}]^+$ at m/z 314.1129 (calcd for $\text{C}_{16}\text{H}_{16}\text{N}_3\text{O}_4$, 314.1135) and $[\text{M} + \text{Na}]^+$ at m/z 336.0949 (calcd for $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_4\text{Na}$, 336.0954). In the ^1H NMR spectrum, signals observed at δ 8.61 and 7.34 (each 1H, d, $J=5.4$ Hz), along with the singlet at 7.18 (1H, s), were characteristic of the aaptamine alkaloids, suggesting the benzo[de][1,6]-naphthyridine skeleton. Signals for one methoxyl group at δ 3.97 (3H, s) in the ^1H NMR spectrum and a carbonyl group at δ 178.9 in the ^{13}C NMR spectrum were similar to the characteristic 8-OMe and C-9 carbonyl signals observed in demethyl(oxy)aaptamine [3a]. Detailed analysis of the 1D and 2D NMR spectra (Table 1) allowed us to establish a modified demethyl(oxy)-aaptamine core substituted at C-5 and C-6, and confirm the position of aromatic protons at C-2, C-3 and C-7. In addition, signals for four methylene groups at δ 3.70 (4H, m) and 3.88 (4H, m) in the ^1H NMR spectrum and δ 54.3 and 68.9 in the ^{13}C NMR spectrum resembled signals for the morpholinyl fragment in 3-(*N*-morpholinyl)-9-demethyl(oxy)aaptamine [10]. The ^1H - ^1H COSY correlations from H_2 -2' to H_2 -3' and

from H_2 -4' to H_2 -5', as well as HMBC correlations $\text{H}-2'/\text{C}-3'$, $\text{C}-5'$ and $\text{H}-3'/\text{C}-2'$, $\text{C}-4'$ confirmed the presence of a morpholinyl fragment in the structure. Furthermore, HMBC correlations from $\text{H}-2'$ and $\text{H}-5'$ to C-6 and NOE interactions between $\text{H}-2'$, $\text{H}-3'$ and $\text{H}-7$ indicated attachment of this fragment to the benzo[de][1,6]-naphthyridine part at C-6. The ^1H NMR spectrum recorded in $\text{DMSO}-d_6$ shows a signal for an exchangeable proton at δ 10.18 (1H, br.s). The quaternary carbon at δ 162.4 did not show any correlations in the HMBC spectrum and was assigned to the amide carbonyl carbon. This supposition was in agreement with the IR absorption band at 1716 cm^{-1} and literature data [14]. Thus, the structure of **2** was determined as 6-(*N*-morpholinyl)-4, 5-dihydro-5-oxodemethyl(oxy)aaptamine.

Only few substituted morpholine derivatives have so far been isolated from marine sources [16]. The first morpholinyl fragment in an aaptamine-type alkaloid was recently reported in another collection of *Aaptos* sp [10].

Alkaloid **3** was obtained as an orange amorphous solid and its molecular formula was established to be $\text{C}_{13}\text{H}_{11}\text{N}_3\text{O}_2$ from the $[\text{M} + \text{Na}]^+$ ion at m/z 264.0768 (calcd for $\text{C}_{13}\text{H}_{11}\text{N}_3\text{O}_2\text{Na}$, 264.0743) in the HRESIMS (positive ion mode). ^1H and ^{13}C NMR data of **3** (Table 2) were similar to those of **4** and **5** [11] showing the same C-3 substituted benzo[de][1,6]-naphthyridine moiety. The difference was in the NMR signals of a substituent. A methylamino group at C-3 was deduced from signals at δ 3.22 (3H, s) in the ^1H NMR (CD_3OD) and δ 29.6 in the ^{13}C NMR spectra. The 1H multiplet at δ 7.0 in the ^1H NMR spectrum recorded in CDCl_3 and its 1H-1H COSY correlation with $\text{H}-2'$, as well as the IR absorption band at 3400 cm^{-1} confirmed the presence of NH. Again, the J^3 HMBC correlation from H_3 -2' to

Table 2. ¹H and ¹³C NMR spectroscopic data for **3** in CD₃OD.

Position	$\delta_{\text{H}}^{\text{a}}$ (J in Hz)	$\delta_{\text{C}}^{\text{b}}$	HMBC (H→C)			1D NOE
			J ²	J ³	J ⁴	
2	8.30 s	130.5	3	3a, 9a	9b	
3		147.9				
3a		137.1				
5	8.83 d (4.5)	152.3	6	3a, 6a	9b	H6
6	7.70 d (4.9)	124.0	5	7, 9b	3a, 9a	H5, H7
6a		137.5				
7	6.99 s	108.6	8	6, 9, 9b	9a	H6, 8-OMe
8		158.8				
9		177.2				
9a		134.1				
9b		119.2				
1'	7.00m ^c					
N-Me	3.22 s	29.6		3	2	
8-OMe	3.97 s	56.7	8		7	H7

^a Measured at 700 MHz, ^b at 175 MHz. Assignments were confirmed by HSQC, HMBC and NOE data. ^c In CDCl₃.

C-3 confirmed the connection of the methylamino substituent to the benzo[*de*][1,6]-naphthyridine moiety at C-3. Based on these data, the structure of **3** was assigned as 3-(methylamino)-demethyl(oxy)aaptamine.

We have studied the apoptosis inducing activity of alkaloids **1-7** and isoaptamine. The substances were found to induce a dose-dependent apoptosis of human leukemia THP-1 cells. Isoaptamine was shown to be the most active inducer of apoptosis (34% of early and 62% of late apoptosis at 12.5 μM concentration), whereas alkaloids **2** and **5** were the least active (89% and 80% of total apoptosis at 320 and 400 μM concentration, respectively). By comparison of the activity of compounds **3** (40% of early and 56% of late apoptosis at 208 μM concentration) and **5** (38% of early and 42% of late apoptosis at 400 μM concentration), it was concluded that the apoptosis inducing activity of the substances studied decreased with an increase of the side chain length at position 3.

Experimental

General: NMR, Bruker Avance III-700 spectrometer; IR, Bruker Vector 22 spectrophotometer; UV, UV-1601 spectrophotometer (Shimadzu); HREIMS, AMD-604S (Intectra, Germany) mass spectrometer. Low pressure column liquid chromatography was performed using Polichrom-1 (powder Teflon, Biolar, Latvia) and silica gel KSK (50-100 μm , Sorbpolimer, Russia). HPLC was performed using a Shimadzu LC-10 AD instrument equipped with an UV-Vis detector and a Develosil ODS-UG-5A (250 \times 4.6 mm) column.

Animal material: The *Aaptos* sp. was collected by scuba during the scientific cruise of R/V "Academic Oparin" in June 2007, in Vang Fong Bay, Vietnam, at a depth of 5-10 m, and identified by Dr Krasokhin V.B. A voucher specimen (PIBOC #O34-064) is kept in the marine invertebrate collection of the Pacific Institute of Bioorganic Chemistry (Vladivostok, Russia).

Extraction and isolation: Animal material (400 g, dry weight) was cut and extracted with EtOH immediately after collection (3 x 3 L). The EtOH extract, after evaporation *in vacuo*, was redissolved in EtOH-H₂O (5:1) and extracted with *n*-hexane. The EtOH-H₂O layer after evaporation was subjected to CC on Polichrom-1 using H₂O with increasing amounts of EtOH as eluents. The fractions eluted with 10% EtOH (5 g) were further chromatographed on a silica gel column using chloroform with increasing amounts of EtOH as eluent to obtain 3 fractions: 1a (50 mg), 1b (250 mg) and 1c (330 mg), containing aaptamine-type alkaloids. Reversed-phase chromatography (Develosil ODS-UG-5, CH₃CN/H₂O, 20%→60%, 25 min, 1 mL/min, 280 nm) of fraction 1a afforded the new compounds **1** (1 mg), **2** (0.5 mg) and **3** (1 mg), together with the known 3-(phenethylamino)demethyl(oxy)aaptamine (6 mg, 0.0015% dry weight) and 3-(isopentylamino)demethyl(oxy)aaptamine (5 mg, 0.0012% dry weight). Repeated chromatography of fractions 1b and 1c using silica gel CC (CHCl₃/EtOH, 7:1) resulted in the isolation of 9-demethyl(oxy)aaptamine (70 mg, 0.018% dry weight), and aaptamine (80 mg, 0.02% dry weight).

2,3-Dihydro-2,3-dioxoaaptamine (1)

0.001 g (0.00025% dry weight), crimson amorphous solid.

IR (CHCl₃): 3375 (br), 1714, 1697, 1602, 1305 cm⁻¹.

UV/Vis λ_{max} (EtOH) nm (log ϵ): 280 (3.7), 376 (3.07), 447 (3.03).

¹H and ¹³C NMR (CD₃OD): Table 1.

HRMS-ESI: *m/z* [M+H]⁺ calcd for C₁₃H₁₁N₂O₄ 259.0726; found: 259.0727.

6-(*N*-Morpholinyl)-4,5-dihydro-5-oxo-demethyl(oxy)aaptamine (2)

0.0005 g (0.00012% dry weight), yellow amorphous solid.

IR (CHCl₃): 3400 (br), 1716, 1652, 1602 cm⁻¹.

UV/Vis λ_{max} (EtOH) nm (log ϵ): 241 (1.55), 286 (1.27), 487 (1.20).

¹H and ¹³C NMR (CD₃OD): Table 1.

HRMS-ESI: *m/z* [M+H]⁺ calcd for C₁₆H₁₆N₃O₄, *m/z* 314.1135; found: 314.1129.

3-(Methylamino)demethyl(oxy)aaptamine (3)

0.001 g (0.00025% dry weight), orange amorphous solid.

IR (CHCl₃): 3400, 1683, 1274 cm⁻¹.

UV/Vis λ_{max} (EtOH) nm (log ϵ): 246 (3.84), 280 (3.64), 411 (3.39), 497 (3.63).

¹H and ¹³C NMR (CD₃OD): Table 2.

HRMS-ESI: *m/z* [M+Na]⁺ calcd for C₁₃H₁₁N₃O₂Na, *m/z* 264.0743; found: 264.0768.

Cell culture: Human monocytic leukemia THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C and 5% CO₂ in RPMI containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

Apoptosis assay: Early and late apoptosis induced by aaptamine and its derivatives in THP-1 cells was analyzed by flow cytometry using the Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA) and PE Annexin V Apoptosis Detection Kit (BD Biosciences, San Diego, CA, USA) according to the manufacturer's protocol. Briefly, the onset of early and late apoptosis was analyzed using Phycoerythrin (PE), Annexin V and 7-amino-Actinomycin (7-AAD) double staining. THP-1 cells in a 6-well plate, 2×10⁵ cells/well, in 10% RPMI were treated with various concentrations

of the substances for 24 h. After incubation, cells were washed with PBS by centrifugation at 1000 rpm (170 rcf) for 5 min, and processed for detection of apoptosis. A total of 1×10⁵-2×10⁵ cells were resuspended in 500 µL of 1× binding buffer. Then, 5 µL of PE Annexin V and 5 µL of 7-AAD were added, and the cells were incubated at room temperature for 15 min in the dark and analyzed by flow cytometry.

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Isolation and Characterization of Crotosparsamide, a New Cyclic Nonapeptide from *Croton sparsiflorus*

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Crotosparsamide (1), a new cyclic nonapeptide, has been isolated from the *n*-butanol soluble sub-fraction of *Croton sparsiflorus* along with *p*-hydroxy methylcinnamate and kaempferol, which are reported for the first time from this species. Their structures were determined by chemical and spectral studies including ESIMS, and 1D and 2D NMR spectroscopic data.

Keywords: *Croton sparsiflorus*, Euphorbiaceae, cyclic peptide, crotosparsamide.

Cyclic peptides have been isolated from marine and micro organisms [1] as well as from higher plants [2] of the families Caryophyllaceae [3,4], Labiatae [5,6], Linaceae [7], Annonaceae [8], Amaranthaceae [9] and Euphorbiaceae [10,11]. Plant cyclopeptides [12,13] are cyclic compounds formed with the peptide bonds of 2-37 protein or non-protein amino acids, mainly L-amino acids. From higher plants, cyclolinopeptide A was the first Caryophyllaceae-type cyclopeptide isolated from the seeds of *Linum usitatissimum*. The structure was determined in 1959 [7]. The cyclopeptides are divided into two classes, five subclasses and eight types [2] on the basis of their structure and distribution in plants. Highly functionalized natural cyclic peptides are being used as therapeutic agents. Many of these bioactive cyclic peptides contain tryptophan residues such as diazonamides, complestatin and celogentins. These peptides are extensively modified through incorporation of an oxygen functionality into the amino acid residues and/or oxidative cross-linking of the indole group of the tryptophan side chain [14].

The aim of our research was to isolate and identify bioactive and structurally novel compounds from *Croton* species. The genus *Croton* is an important genus of Euphorbiaceae comprising 1300 species growing as trees, shrubs and herbs in tropical and subtropical regions of both hemispheres [15]. *C. sparsiflorus* (syn. *C. bonplandianus*) is a shrub growing in sandy clay soil in Asia and South America [16]. A literature survey revealed that a number of alkaloids have so far been reported from this plant [17,18]. The

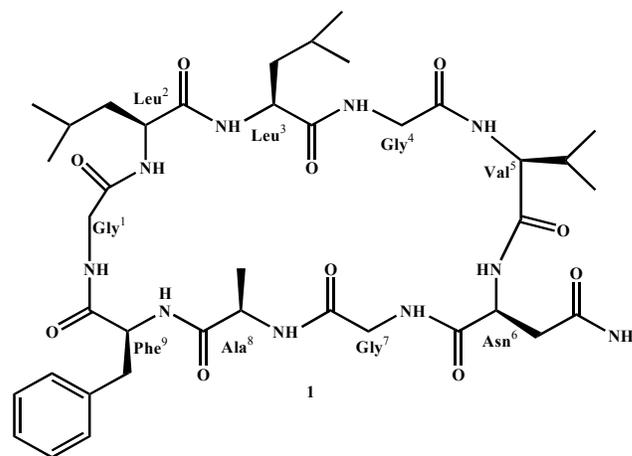


Figure 1: Structure of crotosparsamide (1).

chemotaxonomic and ethnopharmacological importance of the genus *Croton* prompted us to carry out further phytochemical studies on *C. sparsiflorus*. As a result, we herein report the isolation and structural elucidation of a new cyclic peptide named, crotosparsamide (1) (Figure 1), along with *p*-hydroxy methylcinnamate (2) and kaempferol (3), reported for the first time from this species.

The 80% ethanolic extract of the whole plant of *C. sparsiflorus* was suspended in water and extracted successively with *n*-hexane, dichloromethane, ethyl acetate, *n*-butanol, and water. Column chromatographic techniques applied to the *n*-butanol soluble fraction led to the isolation of a cyclic nonapeptide, which was named crotosparsamide (1). The ethyl acetate soluble

Table 1: ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) spectroscopic data of crotosparsamide (**1**).

Residue	δ_{C}	δ_{H} [multi, (Hz)]	^1H - ^1H COSY	^1H - ^{13}C HMBC	^1H - ^1H NOESY
Gly ¹					
C=O	172.2				
C ^{α}	44.3	4.02 ^a (d, 8.5 Hz) 3.58 ^a (d, 8.5 Hz)	3.58 4.02	174.7, 172.2 174.7, 172.2	3.58 4.02
Leu ²					
C=O	174.8				
C ^{α}	53.1	4.64 (d, 8.5 Hz)	1.77, 1.41	174.8, 172.2, 41.7	1.71, 1.41
C ^{β}	41.7	1.77 (m) 1.41 (m)	4.64, 1.71, 1.41 4.64, 1.71, 1.41	174.8, 53.1, 25.1, 24.0 174.8, 53.1, 25.1, 23.7	1.41, 1.00, 0.98 4.64, 1.77, 0.98
C ^{γ}	25.1	1.71 (h, 6.0 Hz)	1.77, 1.0, 0.98	41.7, 24.0, 23.7	4.64, 1.00, 0.98
C ^{δ}	24.0	1.00 (d, 6.0 Hz)	1.71	41.7, 25.1	1.77, 1.71, 0.98
C ^{δ'}	23.7	0.98 (d, 6.0 Hz)	1.71	41.7, 25.1, 24.0	1.77, 1.41, 1.00
Leu ³					
C=O	174.6				
C ^{α}	51.9	4.62 (d, 8.0 Hz)	1.95, 1.37	174.8, 174.6, 40.7, 25.8	1.60, 1.37
C ^{β}	40.7	1.95 (m) 1.37 (m)	4.62, 1.60, 1.37 4.62, 1.60, 1.37	174.6, 51.9, 25.8 174.6, 51.9, 25.8	1.37, 0.96 4.62, 1.95, 0.92
C ^{γ}	25.8	1.60 (h, 6.0 Hz)	1.95, 0.96, 0.92	40.7, 21.9, 21.6	4.62, 0.96, 0.92
C ^{δ}	21.6	0.92 (d, 6.0 Hz)	1.60	40.7, 25.8, 21.9	1.60, 0.92
C ^{δ'}	21.9	0.96 (d, 6.0 Hz)	1.60	40.7, 25.8	1.95, 1.60, 0.92
Gly ⁴					
C=O	170.9				
C ^{α}	43.8	3.90 (d, 8.5 Hz) 3.78 (d, 8.5 Hz)	3.78 3.90	174.6, 170.9 174.6, 170.9	3.78 3.90
Val ⁵					
C=O	175.2				
C ^{α}	63.8	3.65 (d, 7.5 Hz)	2.06	175.2, 170.9, 30.4, 19.9, 19.3	2.06, 1.06, 0.99
C ^{β}	30.4	2.06 (m)	3.65, 1.06, 0.99	175.2, 63.8, 19.9, 19.3	3.65, 1.06, 0.99
C ^{γ}	19.9	1.06 (d, 6.5 Hz)	2.06	63.8, 30.4, 19.3	3.65, 2.06
C ^{γ'}	19.3	0.99 (d, 6.5 Hz)	2.06	63.8, 30.4, 19.9	3.65, 2.06
Asn ⁶					
C=O	173.7				
C ^{α}	52.6	4.52 (t, 7.5 Hz)	3.00, 2.87	175.2, 174.4, 173.7, 36.6	3.00, 2.87
C ^{β}	36.6	3.00 (dd, 7.5, 8.5 Hz) 2.87 (dd, 7.5, 8.5 Hz)	4.52, 2.87 4.52, 3.00	175.4, 52.6 175.4, 52.6	7.51, 4.52, 2.87 7.05, 4.52, 3.00
$^{\nu}\text{C}=\text{O}$	175.4				
NH ₂		7.51 7.05	7.05, 3.00, 2.87 7.51, 3.00, 2.87	175.4, 36.6 175.4, 36.6	7.05, 3.00 7.51, 2.87
Gly ⁷					
C=O	171.6				
C ^{α}	44.1	4.03 ^a (d, 8.5 Hz) 4.59 ^a (d, 8.5 Hz)	3.59 4.03	173.7, 171.6 173.7, 171.6	3.59 4.03
Ala ⁸					
C=O	173.4				
C ^{α}	50.8	3.96 (q, 7.5 Hz)	1.31	173.4, 171.6, 16.6	1.31
C ^{β}	16.6	1.31 (d, 7.5 Hz)	3.96	173.4, 50.8	3.96
Phe ⁹					
C=O	174.7				
C ^{α}	58.4	4.29 (t, 8.0 Hz)	3.18	174.7, 173.3, 37.4, 138.1	3.18
C ^{β}	37.4	3.18 (d, 8.0 Hz)	4.29	174.7, 138.1, 130.4, 58.4	4.29
Ph: C-1	138.1				
C-2, C-6	130.4	7.30 (d, 6.0 Hz)	7.26	138.1, 129.6, 128.0, 37.4	7.26
C-3, C-5	129.6	7.26 (m)	7.30, 7.22	130.4, 128.0, 138.1	7.30, 7.22
C-4	128.0	7.22 (m)	7.26	130.4, 129.6	7.26

^a Signals partially obscured.

fraction provided *p*-hydroxy methylcinnamate (**2**), and kaempferol (**3**).

Crotosparsamide (**1**) was obtained as white crystals (MeOH), mp 309°C, $[\alpha]_{\text{D}}^{25}$ -48 (*c* 0.02, MeOH). The UV spectrum (MeOH) showed bands at 204 and 193 nm, while the IR spectrum showed absorptions at 3306 (HN), 1665 (amide), and 1606-1400 cm^{-1} (aromatic moiety). The HR-ESI-MS showed the $[\text{M}+\text{H}]^+$ peak at *m/z* 829.4521 consistent with the formula $\text{C}_{39}\text{H}_{61}\text{O}_{10}\text{N}_{10}$. The ^1H NMR spectrum showed two N-H signals at δ 7.51 and 7.05, which were coupled with each other

and each gave HMBC correlations to a methylene carbon signal at δ 36.6. This indicated the presence of an $-\text{NH}_2$ group. The ^{13}C NMR spectrum showed 10 carbonyl signals (δ 170.9-175.4) and 9 carbon resonances in the region of δ 43.8-63.8. The DEPT ^{13}C NMR spectrum revealed that six of them represent methine and three methylene carbons. All these data suggested that **1** was a peptide consisting of nine amino acids, out of which three are glycine and another asparagine. The ^1H and ^{13}C NMR spectra of **1** also showed signals indicating the presence of a mono-substituted benzene ring, suggesting the presence of a

phenylalanine moiety. The ^1H - ^1H COSY spectrum along with HMQC and HMBC spectra allowed the determination of the amino acids leucine, valine and alanine besides glycine, asparagine and phenylalanine. Since the molecular formula showed 15 double bond equivalents, it was evident that compound **1** is a cyclic nonapeptide. The sequence of amino acids was determined by the extensive use of a combination of ^1H - ^1H COSY, NOESY and HMBC. In the HMBC correlations, all the α -carbons showed $^{1,3}J$ correlations with the carbonyls of the adjacent amino acid, as shown in Table 1. The sequence of amino acids in **1** was further confirmed by the evidence of peptide fragments in the ESI-MS-MS at m/z 275 (Phe-Ala-Gly), m/z 489 (Phe-Ala-Gly-Asn-Val), m/z 545 (Phe-Ala-Gly-Asn-Val-Gly), m/z 659 (Phe-Ala-Gly-Asn-Val-Gly-Leu), and m/z 771 (Phe-Ala-Gly-Asn-Val-Gly-Leu-Leu). On the basis of the above evidence the amino acid sequence in **1** was determined as cyclo (-Gly¹-Leu²-Leu³-Gly⁴-Val⁵-Asn⁶-Gly⁷-Ala⁸-Phe⁹-) (Figure 1). The absolute configurations of the amino acids of **1** were shown to be all L, by comparison of the chromatograms from GC analysis of suitably derivatized L- and D-amino acid standards with those obtained from the acid hydrolyzate of the peptide [19]. This finding is consistent with the observation that all cyclic peptides isolated to date from the Euphorbiaceae family consist exclusively of L-amino acids [10,11].

The known compounds *p*-hydroxy methylcinnamate (**2**) and kaempferol (**3**) were also isolated from the ethyl acetate soluble fraction and identified by comparison of physical and NMR data with those in the literature [20,21].

Experimental

General experiment procedures: Column chromatography was carried out using silica gel (230-400 mesh, E. Merck, Darmstadt, Germany), Diaion HP-20 ion exchange resin (Nippon Rensui Co., Mitsubishi Chemical Corporation, Tokyo, Japan) and Sephadex LH-20 (25-100 μ , Amersham Biosciences Limited, Stockholm, Sweden). TLC was performed using precoated silica gel F₂₅₄ plates and detection was achieved at 254 and 366 nm, and by spraying with ceric sulfate in 10% H₂SO₄ and ninhydrin [2]. The UV spectra were recorded on a Hitachi UV-3200 spectrophotometer, while the IR spectra were recorded as KBr pellets on a Jasco 302-A spectrometer. Optical rotation was recorded on a Jasco P-2000 polarimeter. Mass spectra were measured in electron spray ionization mode (LRESIMS, HRESIMS and ESI-MS-MS) on QSTAR XL spectrometers and ions are given in m/z (%). Melting points were determined on a Gallenkamp apparatus and are uncorrected. The ^1H , ^{13}C

and 2D (^1H - ^1H COSY, HMQC, HMBC, NOESY) NMR spectra were recorded on a Bruker AMX-400 spectrometer in CD₃OD using standard pulse sequence. Chemical shifts are reported in ppm (δ), relative to tetramethylsilane used as an internal standard, and scalar coupling are reported in Hz. GC analyses were carried out using a Shimadzu GC-17A Gas Chromatograph.

Plant material: The whole plant of *Croton sparsiflorus* Morong (18 kg) was collected in 2005 from Karachi district, province of Sindh, and identified by Prof. Dr Surraiya Kahtoon, Plant Taxonomist, Department of Botany, University of Karachi, where a voucher specimen (No. 4309 KUH) has been deposited in the herbarium.

Extraction and isolation: The freshly collected whole plant material of *C. sparsiflorus* (18 Kg) was shade dried, cut into small pieces, and extracted with 80% ethanol (3 x 20 L, 10 days each) at room temperature. The combined ethanolic extract was evaporated under reduced pressure at rt. to yield a residue (300 g), which was suspended in water and successively extracted with *n*-hexane (50 g), CH₂Cl₂ (10 g), EtOAc (6 g), *n*-BuOH (14 g) and water (220 g). The *n*-butanol soluble fraction was subjected to CC over diaion HP-20, eluting with water, water-methanol and methanol in decreasing order of polarity. The fraction obtained with water-methanol (1:1) was re-chromatographed over Sephadex LH-20 and eluted with mixture of CHCl₃-MeOH in increasing order of polarity. The fractions eluted with 15% CHCl₃/MeOH provided a semi-pure compound, which was re-chromatographed over silica gel and eluted with CH₂Cl₂: MeOH (9.0:1.0) to afford crotosparsamide (**1**) (12 mg).

The ethyl acetate soluble fraction was subjected to CC over silica gel and divided into 8 sub-fractions by eluting with *n*-hexane, *n*-hexane-CH₂Cl₂, and CH₂Cl₂-MeOH in increasing order of polarity. The fractions eluted with *n*-hexane: CH₂Cl₂ (2.0:8.0) were re-chromatographed over silica gel and eluted with the same solvent system to afford compound **2** (10 mg). The fractions eluted with CH₂Cl₂ were triturated with dry acetone, and the residue was re-chromatographed over silica gel, eluting with CH₂Cl₂: MeOH (9.9:0.1) to afford compound **3** (20 mg) as a yellow amorphous powder.

Crotosparsamide (1)

White crystals (MeOH).

MP: 309°C.

$[\alpha]_{\text{D}}^{25}$: -48 (*c* 0.02, MeOH).

UV $\lambda_{\text{max}}^{\text{MeOH}}$ nm (log ϵ): 204 (1.9), 193 (6.0).

IR (KBr) ν_{\max} cm^{-1} : 3306, 1665, 1606, 1540, 1248.
 ^1H and ^{13}C NMR: Table 1.
 LRESIMS m/z : 829 $[\text{M}+\text{H}]^+$.
 HRESIMS m/z : 829.4521 (calcd for $\text{C}_{39}\text{H}_{61}\text{O}_{10}\text{N}_{10}$, 829.4500).

Acid hydrolysis of 1: A solution (1 mg) of **1** was heated with 6 N HCl (2 mL) at 110°C for 10 h. The solution was concentrated to dryness and the residue esterified with 4 N HCl and methanol by heating at 100°C for 20 min. After cooling, the residue was dissolved in 200 μL of trifluoroacetic anhydride/ethyl trifluoroacetate (1:1) and heated for 15 min at 115°C. The obtained solution was concentrated to dryness, the residue dissolved in toluene and subjected to gas chromatography (Chirasil-

L-Val capillary column) with H_2 as carrier gas at a flow rate maintained at 1.5 mL/min and an oven temperature increasing from 60°C to 180°C at a rate of 4°C/min. The retention times were noted of the peaks corresponding to the eluted amino acid derivatives. Similarly, derivatized D- and L-amino acid standards were subjected to GC analysis under identical conditions and the retention times obtained for the derivatives were as follows: D- and L-alanine: 4.65 and 5.40 min; D- and L-valine: 8.34 and 9.24 min; D- and L-leucine: 11.11 and 12.58 min; D- and L-asparagine: 14.51 and 14.71 min; and D- and L-phenylalanine: 21.15 and 21.75 min. Comparison of data indicated that all the amino acids of the peptide have the L-configuration.

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Two New Lavandulyl Flavonoids from *Sophora flavescens*Dan Liu¹, Xiulan Xin^{2,*}, Dong-hai Su², Junying Liu², Qing Wei², Bo Li² and Jian Cui^{3,*}¹School of Science, Dalian Nationalities University, Dalian 116600, China²Beijing Vocational College of Electronic Science and Technology, Beijing 100029, China³Center University for Nationalities, Chinese Minority Traditional Medical Center, Beijing 10081, China

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Two novel lavandulyl flavonoids, (2*S*)-7-methoxyl-4'',5''-dihydroxynorkurarinone (**1**) and (2*S*)-6''-hydroxynorkurarinone-7-*O*-β-D-galactoside (**2**), were isolated from the rhizome of *Sophora flavescens*. Their structures were elucidated by spectral methods, including 2D NMR spectroscopy. Both compounds showed cytotoxic activity against Hela cells, with **2** being more active than **1**.

Keywords: *Sophora flavescens* Ait, lavandulyl flavanone, (2*S*)-7-methoxyl-4'',5''-dihydroxy norkurarinone, (2*S*)-6''-hydroxynorkurarinone-7-*O*-β-D-galactoside, cytotoxicity.

The root of *Sophora flavescens* Ait (family Papilionaceae) is a well-known Chinese herbal medicine (Chinese name "Ku-Shen") used to treat gastric disturbance and eczema, and as an antifebrile and anthelmintic [1]. The major constituents are quinolizidine alkaloids and flavonoids [2-7]. Pharmacological research was focused on the lavandulyl flavonoids, which have significant cytotoxicity [8] and are glycosidase inhibitors [9]. This paper reports the isolation and characterization of two minor lavandulyl flavanones from the MeOH extract of *S. flavescens*.

Compound **1** was obtained as yellow powder. Its HR-ESIMS provided a molecular ion [M-H]⁻ at *m/z* 471.1957 (calcd. 471.2019), suggesting a molecular formula of C₂₆H₃₂O₈. In the ¹H NMR spectrum, four aromatic protons, and three methyl and one methoxy groups were observed. The aromatic protons at δ 6.46 (d, *J* = 2.5 Hz), 6.43 (dd, *J* = 2.5, 9.0 Hz) and 7.38 (d, *J* = 9.0 Hz) formed an ABX spin system. In addition, 5-OH was observed at δ 12.17, due to the hydrogen-bonding effect. The ¹³C NMR spectrum of **1** indicated five oxygenated aromatic carbons at δ 162.4, 162.7, 161.9, 156.9 and 156.2, and a carbonyl carbon at δ 198.0. The splitting patterns of the B ring in the ¹H NMR spectrum and the chemical shifts in the ¹³C NMR spectrum indicated that two oxygen-bearing groups were located on the B-ring of **1**. In the HMBC

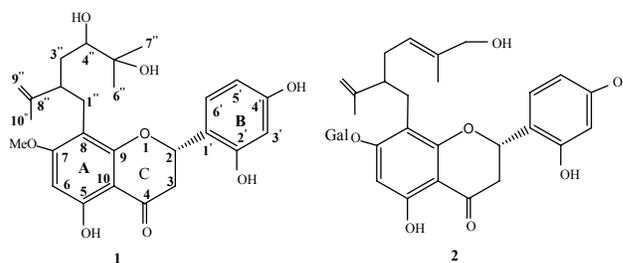


Figure 1: The structures of compounds **1** and **2**.

spectrum, H-5' (δ 6.43), correlated with C-1' (δ 117.6), C-6' (δ 128.3) and C-4' (δ 156.2), while the carbon at δ 156.9 (C-2') correlated with the aromatic protons at δ 6.46 (d, *J* = 2.5 Hz) and δ 7.38 (d, *J* = 9.0 Hz), suggesting oxygen groups at C-2' and C-4'. The methoxy group was located at C-7, due to the HMBC correlation of its protons (δ 3.10) with C-7. The H-1'' protons at δ 2.59 and δ 2.67 showed long range correlations with the carbon signals at δ 107.4 (C-8) and δ 161.9 (C-9). Furthermore, the aromatic proton at δ 6.00 correlated with C-7 (δ 162.7) and C-5 (δ 162.4), suggesting that a lavandulyl skeleton is located at C-8 of the A ring. In addition, the HMBC correlations of the carbon at δ 74.5 with the six protons at δ 1.03 (Me-6'' and 7'') and δ 3.22 (H-4''), and HMBC correlations of C-4'' (δ 77.4) with H-6''/H-7'', H-3'' and H-2'', implied that hydroxyl groups were present at C-4'' and C-5'' of the lavandulyl group. The circular dichroism (CD) spectrum of **1** showed a negative absorption at

Table 1: ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopic data of **1** and **2** (DMSO- d_6).

No.	Compound 1		Compound 2	
	H	C	H	C
2	5.64 (dd, $J = 3.0, 13.0$ Hz)	75.1	5.85 (dd, $J = 3.0, 13.0$ Hz)	75.0
3	2.76 (m) 3.04 (m)	42.4	2.82 (m) 2.92 (m)	43.2
4	---	198.0	---	197.7
5	12.17 (s)	162.4	12.2 (s)	162.7
6	6.00 (s)	95.9	6.00 (s)	96.0
7	---	162.7	---	165.3
8	---	107.4	---	107.5
9	---	161.9	---	161.9
10	---	102.9	---	102.9
1'	---	119.6	---	120.8
2'	---	156.9	---	159.0
3'	6.46 (d, $J = 2.5$ Hz)	103.3	6.77 (d, $J = 2.5$ Hz)	104.3
4'	---	156.2	---	155.9
5'	6.43 (dd, $J = 2.5, 9.0$ Hz)	109.6	6.64 (dd, $J = 2.5, 8.5$ Hz)	110.1
6'	7.38 (d, $J = 9.0$ Hz)	128.3	7.49 (d, $J = 8.5$ Hz)	127.7
1''	2.67 m 2.59 m	28.3	2.62 m 2.51 m	27.6
2''	2.89 m	44.1	2.49 m	47.4
3''	1.36 m 1.55 m	34.0	2.13 m 1.96 m	31.1
4''	3.22 m	77.4	5.24 (brt, $J = 6.0, 6.5$ Hz)	124.2
5''	---	74.5	---	136.2
6''	1.03 (s)	20.1	3.79 (brs)	68.2
7''	1.03 (s)	20.0	1.45 (s)	13.6
8''	---	148.6	---	148.7
9''	4.67 brs 4.65 brs	111.6	4.60 brs	111.1
10''	1.65 s	18.6	1.66 s	19.1
7-OMe	3.10 s	48.8	---	---
Glu-1			4.82 (d, $J = 7.0$ Hz)	102.8
2			3.45 (m)	74.3
3			3.50 (m)	77.5
4			3.41 (m)	71.0
5			---	77.4
6			3.89 (m) 3.71 (m)	62.4

286 nm and a positive absorption at 310 nm, due to the $n \rightarrow \pi^*$ transition of flavanone, indicating that the absolute configuration of C-2 was *S*. Based on the above analysis, **1** was characterized as (2*S*)-7-methoxy-4'',5''-dihydroxynorkurarinone.

Compound **2** was obtained as yellow powder. The molecular formula of **2** was determined to be $\text{C}_{31}\text{H}_{38}\text{O}_{12}$ from the high-resolution ESI-MS *pseudo*-molecular ion $[\text{M}-\text{H}]^-$ at m/z 601.2189 (calcd. 601.2285). This evidence suggested that a sugar residue may be linked

to a flavonoid moiety. Similar to compound **1**, the ^1H NMR spectrum showed aromatic proton signals at δ 6.77 (d, $J = 2.5$ Hz), 6.64 (dd, $J = 2.5, 8.5$ Hz) and 7.49 (d, $J = 8.5$ Hz) forming an ABX spin system, suggesting that two oxygen atoms were attached to C-2' and C-4' of **2**. The hydrogen-bonded 5-OH of **2** was observed at δ 12.2. In the HMBC spectrum, the proton at δ 2.62 (H-1'') correlated with the carbon signals of C-2'' (δ 47.4), C-8 (δ 107.5) and C-9 (δ 161.9), indicating that a group with a lavandulyl skeleton is located at C-8 of the A ring. The HMBC correlation of the proton at δ 3.79 with the carbon signals at δ 136.2 (C-5'') and δ 13.6 (C-7'') suggested that a hydroxyl group is present at C-6'' of the lavandulyl skeleton. The carbon signals at δ 102.8, 74.3, 77.5, 71.0, 77.4 and 62.4 indicated the presence of a hexose [6,7]. By acid hydrolysis and thin-layer chromatography using reference samples, the sugar was identified as D-galactose. In the HMBC spectrum, the proton at δ 4.82 correlated with the carbon signal at δ 165.3 (C-7), showing that the β -D-galactopyranose moiety was at C-7. In the CD spectrum, a positive absorption at 310 nm, and a negative absorption at 291 nm were observed, indicating that the absolute configuration of C-2 was also *S*. Based on the above data, compound **2** was characterized as (2*S*)-6''-hydroxynorkurarinone-7-*O*- β -D-galactopyranoside.

Compounds **1** and **2** showed cytotoxic activity against Hela cell with IC_{50} values of 51.2 and 9.5 $\mu\text{mol/L}$, respectively, which implied that the introduction of a sugar into the skeleton of a lavandulyl flavonoid increases the cytotoxicity.

Experimental

General experimental procedure: IR spectra were recorded in KBr pellets on a NEXUS-470 FTIR (Nicolet) spectrophotometer. ^1H NMR and ^{13}C NMR spectra were measured with an INOVA-500 spectrometer (500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) in DMSO- d_6 with TMS as internal standard. Optical rotations were obtained with a Perkin-Elmer 243B polarimeter. TLC analyses were performed on silica gel G. All chemicals were purchased from Beijing Chemical Factory. Silica gels were produced by Qingdao Haiyang Chemical Group Co., China.

Extraction and isolation: The dried roots of *S. flavescens* Ait were collected from Hebei Province, China. A voucher specimen was identified by Prof. X.L. Xin, and deposited at the School of Science, Dalian Nationalities University, China. The dried roots (5 Kg) were extracted by refluxing with MeOH 3 times. After removal of MeOH, water (1 L) was added to the residue and partitioned with equal volumes of light petroleum, EtOAc and *n*-BuOH, successively, three times with

each solvent. The EtOAc layer (90 g) was concentrated *in vacuo* and the residue subjected to silica gel CC, eluting with CHCl₃/CH₃OH (100:1-1:1) in a gradient manner. Eight fractions were obtained; fraction V (5.2 g) was purified over Sephadex LH-20 and by preparative HPLC (Ultimate 3000 HPLC system, YMC-ODS column (5 μ m, \varnothing 10 \times 250mm)) with CH₃OH-H₂O, 45:55 (v/v) to afford compounds **1** (6 mg) and **2** (5 mg), respectively.

Acid hydrolysis: Acid hydrolysis of compound **2** was carried out by refluxing 2 mg in 5 mL of 6% aqueous HCl for 3 h. The reaction mixture was extracted with 30 mL EtOAc to obtain the aglycone, which was separated by TLC using EtOAc-H₂O-MeOH-HOAc (13: 3: 3: 4); sugars were detected using thymol in H₂SO₄ (0.5 g thymol in 95 mL EtOH and 5 mL H₂SO₄), followed by heating at 120°C for 10 min.

(2S)-7-Methoxyl-4'', 5''-dihydroxy norkurarinone (1)

Yellow powder (MeOH).

$[\alpha]_D^{22}$: -16 (c 0.3, MeOH).

CD (MeOH): $[\theta]_{310} +8051$, $[\theta]_{286} -18056$.

IR (KBr) ν_{\max} (cm⁻¹): 3300, 2950, 1750, 1625, 1170.

¹H NMR and ¹³C NMR: Table 1.

HR-ESIMS: $m/z = 471.1957$ [M-H]⁻ (calcd. 471.2019).

(2S)-6''-Hydroxynorkurarinone-7-O- β -D-galactoside (2)

Yellow powder (MeOH).

$[\alpha]_D^{22}$: -58 (c 0.2, MeOH).

CD (MeOH): $[\theta]_{310} +5606$, $[\theta]_{291} -13218$.

IR (KBr) ν_{\max} (cm⁻¹): 3375, 2930, 1700, 1653, 1170.

¹H NMR and ¹³C NMR: Table 1.

HR-ESIMS: $m/z = 601.2189$ [M-H]⁻ (calcd. 601.2285).

Bioassay: Hela cells were maintained in RPMI-1640 medium with 10% (v/v) fetal bovine serum and cultured in 96 well microtiter plates. Appropriate dilutions of **1** and **2** were added and the cells cultured at 37°C in 5% CO₂ for 72 h. The MTT method was used to evaluate the survival rates of the cancer cells [12]. The concentration of test compound that gave 50% inhibition of cell growth was expressed as the IC₅₀ value. Results were expressed as the mean value of triplicate determinations.

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Biotransformation of Naringenin to Eriodictyol by *Saccharomyces cerevisiae* Functionally Expressing Flavonoid 3' Hydroxylase

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To increase the biological activities of flavonoids and to enhance their stability and solubility by functionalization reactions (polymerization, esterification, alkylation, glycosylation and acylation), an increase in the number of hydroxyl groups in these molecules is needed. Hydroxylation reactions may be achieved using either chemical or enzymatic methods, the latter being more highly specific than the former. In our study, the flavonoid 3' hydroxylase (F3'H) from *Gerbera hybrid*, functionally expressed in *Saccharomyces cerevisiae*, was used to hydroxylate naringenin (the first flavonoid core synthesized in plants). Furthermore, we studied factors that may affect naringenin hydroxylation by recombinant cell-like yeast growth on selective or rich media and plasmid stability.

The whole recombinant cells hydroxylated naringenin at position 3' to give eriodictyol. In a selective media, the yeast failed to grow to high cell densities (maximum 5 g/L), but the plasmid stability was nearly 90 %, and naringenin hydroxylation reached 100 %. In a rich complex media, the biomass reached 10 g/L, but the yield of naringenin hydroxylation reached only 71 %, and the plasmid stability decreased. When yeast functionally expressing F3'H from *Gerbera hybrid* was used, in a selective media, 200 mg/L of eriodictyol from naringenin was produced.

Keywords: flavonoid, F3'H from *Gerbera hybrid*, hydroxylation, plasmid stability.

Flavonoids are secondary metabolites found in plants [1], in which their roles are as diverse as their structures, contributing to plant protection from environmental aggression [2,3]. In addition, flavonoids have potential benefits to human health [4], but the major hurdles of their formulation are their low solubility and stability. In order to take advantage of their beneficial properties, functionalization reactions (polymerisation, esterification, alkylation, glycosylation and acylation) have been suggested by several authors as a promising route. However, these reactions need free hydroxyl groups in aglycons or in glycosides to react with functional groups. Through hydroxylation, the basic flavonoid backbone can serve as useful starting materials for the development of new drugs.

However, the ability to generate either regioselective or stereoselective compounds through chemical hydroxylation presents a major obstacle for the development of new pharmaceuticals. Fortunately, biological hydroxylation using either enzymes or transgenic microbes has the ability to overcome this problem. Enzymatic hydroxylation reactions have been achieved, in particular with either flavonoid hydroxylase (FH) directly extracted from plants or from recombinant *Escherichia coli* or *Saccharomyces cerevisiae* functionally (Table 1) expressing FHs [5,6,12]. In the present study, the 3' hydroxylation of naringenin was evaluated using the microsomal fraction and whole recombinant yeast expressing the F3'H from *Gerbera hybrid*. Also we investigate the effect of media

Table 1: Origin of flavonoid hydroxylation enzyme functionally expressed in recombinant cells.

Flavanone 3 hydroxylase	Flavonoid 3' hydroxylase	Flavonoid 3'5' hydroxylase
<i>Malus domestica</i> cv. M9 [6] <i>Ammi majus</i> , <i>Anethum graveolens</i> , <i>Pimpinella anisum</i> [7] <i>Petunia hybrida</i> [8-10] <i>Petroselinum crispum</i> cv. Italian Giant plants [11]	<i>Brassica napus</i> [12] <i>Callistephus chinensis</i> line '01', <i>Osteospermum</i> hybrid line '082', <i>Gerbera hybrid</i> line 'D1', <i>Hieracium pilosella</i> [5], <i>Sorghum</i> [13] <i>Torenia hybrida</i> [14], <i>Gentian</i> [15] <i>Arabidopsis thaliana</i> [16], <i>Petunia hybrida</i> [17]	<i>Callistephus chinensis</i> line '01', <i>Osteospermum hybrid</i> cv. 'Bamba', <i>Pericallis cruenta</i> cv. 'Blue Bicolor' [5] <i>Verbena temari</i> , <i>Butterfly pea</i> [18] <i>Catharanthus roseus</i> , <i>Petunia hybrida</i> [19]

Table 2: Effect of microsome extraction pressure on naringenin bioconversion yield and protein quantity.

Protein (g/L)	Manual extraction	French press extraction				
		500 bars	750 bars	1000 bars	1500 bars	2000 bars
Naringenin bioconversion yield (%)*	100	66	90	97	83	85

*: Relative to the activity of microsomes extracted manually (100%). The quantity of proteins used for the reaction of naringenin hydroxylation was standardized to 0.25 g/L

culture on naringenin bioconversion, yeast growth and plasmid stability.

Indeed, the functional expression of *Gerbera hybrid* F3'H has been previously studied by Seitz *et al.* [5]; the ORF of isolated cDNA is constituted by 1539 base pairs; the encoded protein has a specific activity of 5.63 pkat/mg. The recombinant activity has been explored using the microsomal fraction to convert naringenin to eriodictyol [5]. Generally, the preparation of the microsomal fraction is done manually. In order to facilitate the microsome extraction procedure, we have used a modified extraction method based on the use of a constant disruption system. As can be seen in Table 2, the increase of extraction pressure was accompanied with an increase of protein yield and a decrease in naringenin bioconversion yield. A pressure of 750 and 1000 bars gave nearly the same bioconversion yield as that obtained with manual extraction, but the protein content of the microsomal fraction was more important. Whereas 500, 1500 and 2000 bars gave no amelioration of naringenin bioconversion, this may be attributed to insufficient extraction pressure (500 bars) or to the deleterious effect of pressure on enzyme structure and activity (1500 and 2000 bars). Manual extraction gave the best ratio for bioconversion/ protein quantity, but the use of a constant disruption system is easier and a more comfortable tool, since it permits the characterization of the naringenin bioconversion reaction and enzyme constants.

Whole cells and purified enzymes are both used as biocatalysts, but the former presents the advantages of simplicity, convenient manipulation and low costs, while enzyme purification is tedious and expensive. Moreover, the purification of enzymes could lead to significant loss of activity and cofactors such as NADPH, usually required for enzymes like F3'H [5,12-17]. Consequently, in order to probe the whole cell activity, 1 g/L of recombinant yeast was incubated with naringenin as substrate. In the course of the incubation,

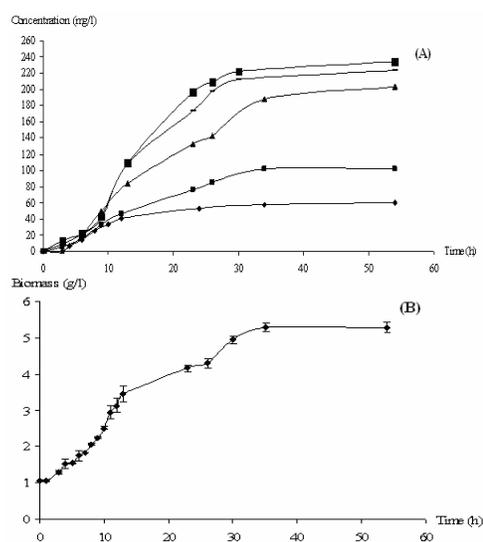


Figure 1 (A): Eriodictyol production by recombinant *S. cerevisiae* expressing the F3'H of *G. hybrid* in the presence of various concentrations of naringenin. (■): 2025 mg/L, (○): 675 mg/L, (▲): 225 mg/L, (■): 100 mg/L, (◆): 50 mg/L. **(B):** Growth curve of the recombinant *S. cerevisiae* expressing the F3'H of *G. hybrid* (means of growth curves of the recombinant yeast strain with different concentrations of naringenin (0, 50, 100, 225, 675 and 2025 mg/L) with standard deviation).

the initial naringenin concentration (100 mg/L) started to decrease and a new product appeared (data not shown). The new product was identified as eriodictyol (LC/MS). Thus, the whole recombinant yeast can be used to hydroxylate naringenin.

In order to investigate the maximum bioconversion ability of *S. cerevisiae* strain harbouring F3'H, the naringenin was added to the culture media at final concentrations of 50, 100, 225, 675 and 2025 mg/L; product formation and residual substrate concentrations against incubation time were plotted (Figure 1). We analysed the yeast growth and the naringenin biotransformation kinetics with *S. cerevisiae* expressing F3'H. In *S. cerevisiae* strain harbouring F3'H, recombinant protein production was induced by galactose in the YPL medium, as described in the experimental section. The growth of the recombinant

strain was not affected by the naringenin concentration (Figure 1). The bioconversion kinetic and growth were slow for the first 10 hours; the biomass was doubled, and 33 to 50 mg/L of naringenin was converted to eriodictyol. Within 10 to 33 hours of the exponential growth phase, the biomass was multiplied by 5 (when compared with the initial biomass) and the growth was associated with the bioconversion kinetic, both of which progress faster than during the first 10 hours after induction. Nearly 200 mg/L of eriodictyol were synthesized from 225, 675 and 2025 mg/L of naringenin. Within 33 to 54 h, the stationary growth phase was attained and only an extra 5 mg/L of eriodictyol, at a maximum, was formed (Figure 1).

It is clear that increasing naringenin concentrations enhanced the production yield and bioconversion kinetic but there is a limit of saturation (of substrate bioconversion). It is not necessary to increase naringenin concentration over 225 mg/L because the bioconversion yield decreases 3 and 9 folds when compared to a total bioconversion (for 675 and 2025 mg/L of naringenin, respectively).

The bioconversion yield is influenced by the initial naringenin concentration and also by the culture media composition. The latter dictates the growth rate of the recombinant yeast, the cell yield, the plasmid stability and naringenin bioconversion. Consequently, we tested, in parallel, the effect of culture conditions on the factors cited above.

Two stage culture modes were used: (i) a growth phase to form the initial biomass and (ii) an induction phase. We tested two culture media; a minimal medium without uracil (to select recombinant yeast harboring plasmid) and a rich medium. We combined these two media, as indicated in Table 3. As can be seen in Table 3, independently of the media used to prepare the inoculums, when the induction was done in YPL rich medium, the biomass production was nearly 10 g/L compared with 5 g/L in SC minimal medium. Despite the use of the same quantity of carbon source and the same initial biomass (adjusted to 1 g/L), biomass production was not the same, indicating that minimal

medium presents a limitation in its saline, vitamin or nitrogen composition.

Within 33 hours of induction, it was notable that SC-SC and YPL-SC two stage culture modes gave the same maximum bioconversion yield (100%), but in SC-YPL and YPL-YPL culture mode the bioconversion yield did not exceed 71% (Table 3). This indicates the importance of induction media in the conversion reaction. Indeed, SC minimal media exert a selection pressure to stabilize the plasmid in cells, and consequently, the F3H expression is maintained to a high level allowing the production of the maximum amount of eriodictyol (100 mg/L of naringenin were totally converted to eriodictyol). As can be shown in Figure 2, after 30 hours of induction, only 58 % of cells in YPL medium contain plasmid, while in SC medium, 90 % of the yeast contains plasmid. We noticed that during the exponential phase, between 6 and 24 hours, the fraction of plasmid free cells increased. This phenomenon may be due to the acceleration of cell division. However, in the stationary phase, this fraction decreased, probably because the media components become limiting and only cells containing the recombinant plasmid can survive uracil limitation.

Over-viewing our work, we have successfully produced eriodictyol, a 3'hydroxylated flavonoid from naringenin, by using a whole cell activity. Recombinant *S. cerevisiae* harboring F3'H from *Gerbera hybrid* was used as a selective single step for high-yield hydroxylation to replace a series of chemical synthetic reactions that suffers from drawbacks of simultaneous formation of diverse by-products and low isolated yield.

Contrary to the works reported in Table 1, we have used another easy method for microsomes (containing the F3'H) extraction, to test enzymatic activity. The use of a cell disruption system at 750 and 1000 bars gave nearly the same naringenin bioconversion yield as that of the classic manual extraction. Moreover, in the present study, we demonstrated that it is not necessary to extract enzyme to achieve the hydroxylation reaction. The whole recombinant *S. cerevisiae* cell expressing flavonoid hydroxylases can hydroxylate naringenin and

Table 3: Effect of culture mode on growth of the recombinant yeast strain and eriodictyol production. SC: selective medium, YPL: rich medium.

Medium used to two stage culture mode		Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)	Biomasse (g/L)	Eriodictyol (mg/L)
Inoculum media	Induction and bioconversion media	Time after induction of recombinant hydroxylase synthesis (h)									
		0	5.5	11	22	33					
SC min	SC min	1	0	1.5	17.5	2.1	47.6	4.4	90.4	5.1	100
		YPL	1	0	1.1	30.7	2.2	85.3	4.7	94	4.8
SC min	YPL	1	0	1.5	12	2.5	36.8	8	71.2	8.3	71
		YPL	1	0	1.7	20	3.7	61.1	8.9	70.4	9.9

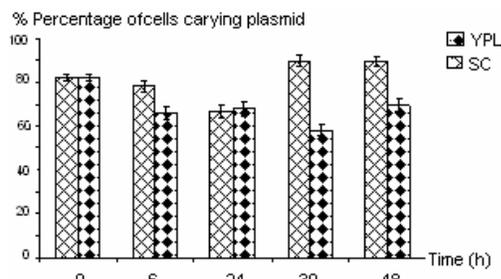


Figure 2: Effect of culture media on the percentage of plasmid containing cells. SC: selective medium, YPL: rich medium.

other flavonoids (like apigenin ;data not shown)). As substrate concentration increased, the naringenin bioconversion yield decreased and goes much slower. This may be ascribed to a substrate saturation phenomenon, low substrate solubility in aqueous medium, low substrate penetration to cells, and limitation in medium composition... Special techniques, such as two-phase biocatalysis and cell immobilization and/or permeabilization could be used to increase the concentration tolerance of this reaction [20].

Naringenin bioconversion to eriodictyol depends on the production of the recombinant F3'H enzyme. Synthesis of the cloned gene product is influenced by genetic and environmental factors, including plasmid stability, cell growth rate and medium composition. We have tested four two stage culture modes using two culture media: the SC minimal and YPL rich medium. We found that the naringenin bioconversion yield depends on the media used to induce the recombinant F3'H production, independently of the medium used to form the initial yeast biomass. SC minimal medium used as an inductor gave the best bioconversion/biomass ratio; 20 mg of eriodictyol per g of recombinant yeast (dry weight), when compared with the YPL rich complex medium. The induction in this latter case gave a ratio of 7.1 mg of eriodictyol per g of recombinant yeast. In addition, culture in the minimal medium offers the advantage of maintaining high plasmid stability. Thus, the decrease of naringenin bioconversion, in rich media, may be correlated to plasmid instability. It is notable (Figure 2) that the fraction of plasmid-carrying cells decreased during the exponential growth phase, but increased during the stationary phase. This phenomenon may be ascribed to the death rate difference between the plasmid-free and plasmid carrying cells caused by starvation in the stationary phase. Indeed, the high segregational instability of recombinant *S. cerevisiae* is primarily caused by asymmetric cell division, which can lead to the formation of 9 to 43 buds per cell [21]. In contrast to binary cell division in *E. coli*, budding results in an unequal distribution of plasmids among daughter cells, leading to an increased probability of plasmid-free cell emergence, especially in

the exponential growth phase. In theory, plasmid-free cells respond to environmental changes much quicker than plasmid carrying cells. Plasmid-free cells may grow faster than plasmid carrying cells when there is plenty of growth substrate, but they also may lyse or die faster upon depletion of growth substrate (uracil). Thus, pulse addition of growth substrate at an appropriate time interval, with a significant starvation period between two consecutive feedings during fedbatch fermentation may have positive effects on stabilizing plasmid and enhancing protein production [22]. Other various operating strategies have been proposed to address the problem of competitive plasmid instability in recombinant yeast. These include cycling growth rate changes, cycling of substrate levels, cycling dissolved oxygen tension, dilution rate cycling, and autoselection systems [23].

Our work represents the first report of using recombinant yeast whole cell activity to achieve a single flavonoid hydroxylation step. Yan *et al* [24] have reported the use of whole cells expressing multiple enzymes to produce eriodictyol from caffeic acid. Their recombinant system produced 6.5 mg/L of eriodictyol from 180 mg/L of caffeic acid. In the present work, we produced 200 mg/L of eriodictyol from 225 mg/L of naringenin. Consequently, eriodictyol formation is 24 orders of magnitude higher than that reported [24]. This work opens up alternative routes for the production of additional hydroxylated flavonoid structures from other non-hydroxylated flavonoid precursors, using recombinant *S. cerevisiae* cells and by engineering substrate specificity of the F3'H by using site directed mutagenesis, laboratory evolution or chimeragenesis and engineering improved redox partners [25]. Generally, the relatively poor stability of P450 monooxygenases, such as F3'H, still impede the technical application of these enzymes. Immobilization of enzyme or recombinant permeabilized yeast has proven useful for *in vitro* biotransformations [26]. A combination of chemical, physical and biological approaches will contribute to the production of polyhydroxylated flavonoid structures needed for functionalization reactions, like glycosylation [4,27], acylation [4], methylation [4,28], and sulfation [4,29].

Experimental

Plasmid and cell transformation: The F3'H from *Gerbera hybrid* was generously given by Pr. Christian Seitz (Technical University Munich, Germany) in yeast plasmid pYES2.1/V5-His-TOPOZ® [5]. The *Saccharomyces cerevisiae* INVSc1 strain was purchased from Invitrogen, (Paisley, UK). The yeast transformation was carried out according to the manufacture's prescription. Chemicals used in this study were purchased from Sigma Chemicals (St. Louis, (USA)).

Hydroxylation reaction by microsomes extracted from *S. cerevisiae* expressing *Gerbera hybrid* F3'H:

Microsomes were prepared as described by Pompon *et al.* [30]. Ten mL of the minimal growth medium (SC minimal composed of 20 g glucose, 1.92 g yeast synthetic drop-out media without uracil, and 6.7 g yeast nitrogen base without amino acids in 1L of distilled water) were inoculated with the recombinant yeast and grown to stationary phase. Cells were harvested by centrifugation (3000 rpm for 5 min), then used to inoculate 250 mL of rich induction medium composed of 20 g/L galactose, 10 g/L peptone and 10 g/L yeast extract (YPL) and incubated overnight at 28°C, with shaking at 200 rpm. Cells were harvested by centrifugation at 7000 g for 10 min at 4°C. The cell pellet was washed with TEK buffer (10 mM Tris-HCL (pH=7.5), 1 mM EDTA and 100 mM KCl). After centrifugation, cells were suspended in 50 mL TESB buffer (50 mM Tris-HCl (pH=7.5), 1 mM EDTA, 0.6 mM sorbitol, 1% BSA and 20 mM β -mercaptoethanol). The cell suspension was passed through either a constant cell disruption system or disrupted manually, as described [30]. Five extraction pressures were applied: 500, 750, 1000, 1500 and 2000 bars. The suspension was centrifuged at 10000 g for 15 min at 4°C. The supernatant was recovered and the microsomes precipitated with MgCl₂ (125 mM) for 30 min at 4°C. Microsomes were then recovered by centrifugation for 20 min at 15000 rpm at 4°C and suspended in 3 mL cold TEG (50 mM Tris-HCL (pH=7.5), 1 mM EDTA, 30% glycerol). Protein quantification was determined by the bicinchoninic acid method [31].

Determination of naringenin hydroxylation product by whole cells of *S. cerevisiae* expressing F3'H:

Ten mL of culture grown to stationary phase in SC minimal medium was used to inoculate 100 mL of a rich induction medium. Naringenin was added at a concentration of 100 mg/L and the mixture was incubated on a shaker at 28°C for 55 h. 750 μ L of supernatant was collected and mixed with 250 μ L of acetonitrile. Identification of the hydroxylation products was monitored using HPLC and LC/MS analysis.

Effect of substrate concentration on bioconversion kinetics with *S. cerevisiae* expressing F3'H:

Thirty mL of culture grown to stationary phase in SC minimal medium was used to inoculate 250 mL of SC minimal medium. Cells were harvested by centrifugation at 3000 rpm for 5 min before their transfer to SC medium, with different concentrations (50, 100, 225, 675 and 2025 mg/L) of the substrate. The mixture was incubated on a shaker at 28°C for 55 h. 750 μ L of the supernatant was collected and mixed with 250 μ L of acetonitrile. Quantification of the metabolites and parent material was monitored using HPLC.

Determination of the appropriate culture modes for growth of the recombinant *Saccharomyces cerevisiae* and naringenin bioconversion:

A single colony of recombinant yeast strain was placed in 30 mL of (20%) glucose supplemented SC minimal media or in YPL rich medium and incubated at 28°C till stationary phase. The culture was centrifuged for 3 min at 5000 rpm and the pellet suspended in (20%) galactose supplemented fresh minimal (SC) or complex media (YPL). This gives four types of culture, as seen in Table 3. Naringenin was added to a final concentration of 100 mg/L and its transformation was assessed for 34 h.

Analytical method: Cell density was measured by the dry cell weight method. For determining dry cell weight, 10 mL of sample was spun in a pre-weighed centrifuge tube, at 5000 rpm for 5 min. The cell pellet was washed with deionized water and dried at 100°C for 24 h. The tube was weighed along with the dried pellet and used to calculate the dry weight of the sample. Total cell concentration was determined by measuring the optical density at 600 nm (OD_{600nm}). One unit of OD_{600nm} was found to be equivalent to about 0.45 g/L dried cell weight. The percentage of plasmid-containing cells was determined by the replica-plating technique. The percentage of plasmid stability was calculated as the ratio of the number of plasmid-containing cells forming the clones in selective plates (SC min) to the total number of cells growing on non selective YPL medium (including plasmid-free and plasmid-containing cells). Cell samples were diluted to make the colony counts within the range of 30-300. All plate counts were taken from the average of at least 3 replicates.

HPLC analysis: A Thermo Finnigan (San Jose, CA, USA) HPLC apparatus equipped with an U.V. 6000 LP detector was used. For analytical scale, the mobile phase consisted of water and acetonitrile and was programmed as follows; 10% acetonitrile from 0 min to 12 min, then a linear gradient was used starting from 40% acetonitrile to 60% at 17 min, followed by 8 min of a constant eluant of 10% acetonitrile. The flow rate was 1 mL/min; detection wavelength 296 nm; column temperature 28°C.

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Two New 3-C-Carboxylated Flavones from the Rhizomes of *Caragana conferta*

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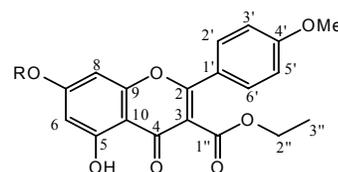
Confertins A (**1**) and B (**2**), new 3-C-carboxylated flavones, have been isolated from the ethyl acetate soluble fraction of the rhizomes of *Caragana conferta*. Their structures have been assigned on the basis of spectroscopic studies.

Keywords: *Caragana conferta*, 3-C-carboxylated flavones, confertins A and B.

The genus *Caragana* belongs to the family Fabaceae and comprises over 80 species out of which ten have so far been identified in Pakistan [1]. Some of these are used for the treatment of hypertension, irregular menstruation, and fatigue. The extracts and compounds from the genus have antitumor, antiviral, anti-inflammation, hypertensive, sedative, acetylcholinesterase inhibitory, and immunosuppressant activities [2].

C. conferta is mainly found in the Ziarat, Gilgit and Kashmir valleys of Pakistan at an altitude of 7000-12000 feet above sea level [1]. A literature survey revealed that one isoflavone and two 4-hydroxyisoflavones have so far been reported from this species [3,4]. The ethnopharmacological and chemotaxonomic importance of *Caragana* species prompted us to carry out phytochemical studies on the rhizomes of *C. conferta*. Herein we report the isolation and structural elucidation of two new 3-C-carboxylated flavones named as confertin A (**1**) and confertin B (**2**), respectively.

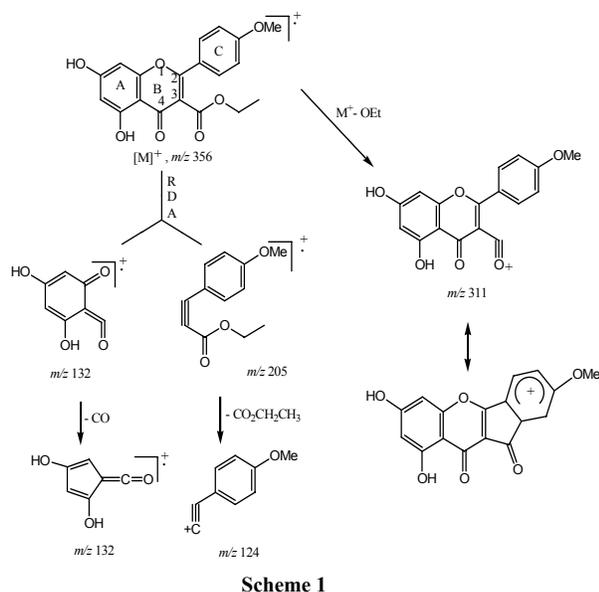
Confertin A (**1**) was obtained as light yellow needles, mp 184°C. The infrared (IR) spectrum showed absorption bands for hydroxyl (3450 cm⁻¹), conjugated ester (1700 cm⁻¹), conjugated ketone C=O (1660 cm⁻¹), aromatic (1545 cm⁻¹) and OCH₃ (1250 cm⁻¹) functionalities. The UV spectrum showed absorption bands at 265, 295 and 330 nm, which are characteristic of a C-3-substituted flavone-type skeleton [5]. Addition of AlCl₃ and NaOAc resulted in bathochromic shifts of 30 nm and 12 nm, respectively, indicating a free phenolic group at both C-7 and C-5, respectively [6]. The high resolution electron



Compound 1. R = H
Compound 2. R = COCH₃

impact mass spectrum (HREI-MS) of **1** exhibited the [M]⁺ peak at *m/z* 356.0513 (Calcd. for C₁₉H₁₆O₇: 356.0584) with twelve degrees of unsaturation.

The broad-band decoupled (BB) and DEPT ¹³C-NMR spectra of **1** showed 19 signals, comprising two methyl, six methine, one methylene and ten quaternary carbons. The signals at δ 182.5 (C-4) and 162.4 (C-1'') were due to the conjugated carbonyl and ester moieties, while the conjugated olefinic carbons resonated at δ 152.0 and 123.5, respectively. The methoxyl group gave a signal at δ 55.7, while oxygenated aromatic carbons appeared at δ 166.7, 163.3 and 161.2. In the HREI-MS the peak at *m/z* 311.2661 (C₁₇H₁₁O₆) resulted from the loss of an ethoxyl group. The retro Diels-Alder (RDA) fragments A⁺ at *m/z* 152.1041 (C₇H₄O₄) and B⁺ at *m/z* 205.2221 (C₁₂H₁₃O₃) revealed the presence of two hydroxyl groups in ring A and one methoxyl and ethyl ester groups in rings B and C. The daughter ion fragments at *m/z* 124.0941 (C₆H₄O₃) and *m/z* 132.1591 (C₉H₉O) resulted from the loss of CO and an ethoxyl carbonyl group from the fragments A⁺ and B⁺, respectively (Scheme 1) [7]; the latter ion peak provided evidence



for the presence of an ethoxy carbonyl group at C-3. Further evidence was provided by the carbon signals at δ 123.5 (C-3), δ 152.0 (C-2) and δ 182.5 (C-4), and the absence of a proton signal for H-3 in the ^1H and ^{13}C NMR spectra, confirming the presence of an ethyl ester group at C-3.

The ^1H NMR spectrum showed a chelated hydroxyl group at δ 12.40 as a singlet and meta coupled protons of ring-A at δ 6.23 (d, $J = 1.6$ Hz) and 6.34 (d, $J = 1.6$ Hz), thereby confirming the presence of hydroxyl groups at C-5 and C-7, respectively. Protons for a methoxyl group resonated at δ 3.75. Its presence at C-4' was confirmed by the protons of ring-B showing an AA' BB' pattern at δ 6.94 (d, $J = 8.8$ Hz) and 7.20 (d, $J = 8.4$ Hz). The presence of an ethoxyl group was revealed by a typical two-proton quartet at δ 4.20 and a three-proton triplet at δ 1.03. The position of the methoxyl group at C-4' was further confirmed by HMBC correlations in which the methoxyl protons at δ 3.75 showed 3J correlations with C-4' (δ 55.7). Irradiation of these protons at δ 3.75 caused strong NOE enhancement of neighboring protons at C-3 and C-5. The NMR assignments were facilitated by ^1H - ^1H COSY and HMQC experiments. The positions of the substituents were further authenticated through HMBC

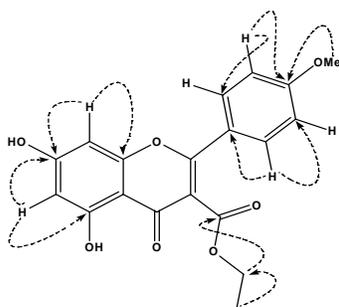


Figure- 2. Important HMBC correlations of confertin A (1)

Table 1: ^1H - and ^{13}C -NMR data (CDCl_3) of compounds **1** and **2** at 400 and 100 MHz, respectively; δ in ppm, J in Hz. Assignments were confirmed by COSY, HMQC and HMBC experiments.

Position	1		2	
	δ_{H} ($J = \text{Hz}$)	δ_{C}	δ_{H} ($J = \text{Hz}$)	δ_{C}
2	-	152.0	-	149.2
3	-	123.5	-	110.2
4	-	182.5	-	175.7
5	-	163.3	-	150.7
6	6.23 (d, $J = 1.6$)	105.0	6.48 (d, $J = 2.0$)	109.7
7	-	166.7	-	162.7
8	6.34 (d, $J = 1.6$)	94.9	6.72 (d, $J = 2.0$)	101.1
9	-	158.5	-	158.1
10	-	100.5	-	123.2
1''	-	162.4	-	161.8
2''	4.13 (q)	63.4	4.07 (q)	62.3
3''	1.03 (t)	13.8	0.92 (t)	13.4
1'	-	125.0	-	126.0
2'	6.94 (d, $J = 8.8$)	132.1	7.09 (d, $J = 8.4$)	131.0
3'	7.20 (d, $J = 8.4$)	114.5	6.87 (d, $J = 8.8$)	113.6
4'	-	161.2	-	159.6
5'	7.20 (d, $J = 8.4$)	114.5	6.87 (d, $J = 8.8$)	113.6
6'	6.94 (d, $J = 8.8$)	132.1	7.09 (d, $J = 8.4$)	131.0
4'-OCH ₃	3.75 (s)	55.7	3.75 (s)	55.2
7-CO	-	-	-	169.9
7-CH ₃	-	-	2.30 (s)	21.0

^a) Arbitrary C-atom numbering.

correlations (Figure 2). The structure of confertin A (**1**) could, therefore, be assigned as ethyl 5, 7-dihydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-3-carboxylate.

Confertin B (**2**) was also obtained as light yellow needles, mp 186°C. The IR and UV spectra were similar to those of **1**. However, a bathochromic shift of 28 nm was observed with AlCl_3 , but no such shift was observed with NaOAc , indicating the absence of a phenolic group at C-7. The HREI-MS showed an $[\text{M}]^+$ ion at m/z 398.3630 corresponding to the molecular formula $\text{C}_{21}\text{H}_{18}\text{O}_8$ (Calcd. for $\text{C}_{21}\text{H}_{18}\text{O}_8$: 398.3670).

The BB and DEPT ^{13}C NMR spectra of compound **2** showed twenty-one carbon signals comprising three methyl, six methine, one methylene and eleven quaternary carbons. The spectrum was similar to that of **1** with additional signals for an acetyl group (δ 169.9 and δ 21.0). The ^1H NMR spectrum was also similar to that of **1**, with an additional signal for an acetyl group at δ 2.30 (COCH_3). The signal for the chelated hydroxyl group at C-5 was observed at δ 12.3 as a singlet. Compound **2** is therefore, the acetylated derivative of **1**. The UV spectrum indicated acetylation of the phenolic group at C-7. The HMBC correlations were in complete agreement with the assigned structure of confertin B (**2**) as ethyl 7-(acetyloxy)-5-hydroxy-2-(4-methoxyphenyl)-4-oxo-4*H*-chromene-3-carboxylate.

This is the first report of the natural occurrence of 3-*C*-carboxylated flavones, although these type of compounds have previously been synthesized [8].

Experimental

General: Melting points were determined on a Gallenkamp apparatus and are uncorrected. ^1H - and ^{13}C -NMR spectra and two-dimensional correlation spectroscopy (COSY, NOSEY, HMQC, and HMBC) were recorded on a Bruker AV-400 spectrometer (400 MHz for ^1H - and 300 MHz for ^{13}C -NMR) in CDCl_3 with TMS as internal standard. Chemical shifts (δ) are shown in ppm relative to TMS. The UV spectra were obtained on a Hitachi UV-3200 spectrophotometer. The IR spectra were recorded on a JASCO 302-A spectrometer in CHCl_3 . Thin layer chromatography (TLC) was carried out on pre-coated silica gel 60G F₂₅₄ plates (20 x 20 cm, 0.2 mm layer thickness, E. Merck, Darmstadt, Germany) and visualized under UV light (254 nm) and also by spraying with ceric sulfate reagent. Silica gel 230-400 mesh (E. Merck, Darmstadt, Germany) was used for column chromatography. The HREI-MS were recorded on a JEOL JMS-HX-110 mass spectrometer.

Plant material: Rhizomes of *Caragana conferta* Benth were collected from Gilgit valley (Pakistan) and identified by a Senior Scientist of the National Agriculture Research Center (NARC), Islamabad Pakistan. A voucher specimen has been deposited in the herbarium of the Department of Botany, University of Karachi (voucher no 319).

Extraction and isolation: The air-dried powdered rhizomes (12 kg) were extracted with MeOH (3 x 25 L) at room temperature. The combined extract was evaporated to yield the residue (250 g), which was divided into *n*-hexane (50 g), ethyl acetate (EtOAc) (120 g) and water (80 g) soluble sub-fractions. The EtOAc fraction was subjected to column chromatography (CC) over silica gel, eluting with mixtures of *n*-hexane and EtOAc in increasing order of polarity to obtain 3 major fractions, A, B and C. Fraction A obtained from

n-hexane/EtOAc (4:6) was again chromatographed over silica gel using *n*-hexane/EtOAc (8:2) as eluent to afford two successive fractions A_A and A_B. CC of the sub fraction A_A gave the compound **1** (20 mg) through elution with *n*-hexane/EtOAc (5:5), while elution with *n*-hexane/EtOAc (6:4) provided compound **2** (11 mg). Fractions B and C could not be worked up due to paucity of material.

Confertin A (1)

Light yellow needles.

MP: 184°C

IR (KBr) ν_{max} cm^{-1} : 3450 (OH), 1700 (ester), 1660 (conjugated ketone), 1545 (aromatic C=C), and 1250 cm^{-1} (OCH₃).

UV (CHCl_3) λ_{max} (log ϵ): 330 (4.43), 295 (3.99) and 265 nm (4.73).

^1H and ^{13}C NMR: Table 1

HREI-MS: m/z 356.0584 (C₁₉H₁₆O₇), 311.2661 (C₁₇H₁₁O₆), 205.2221 (C₁₂H₁₃O₃), 152.1041 (C₇H₄O₄), 132.1591 (C₉H₉O), 124.0941 (C₆H₄O₃).

Confertin B (2)

Light yellow needles.

MP: 186°C.

IR (KBr) ν_{max} cm^{-1} : 3440, 1700-1720 (ester), 1660 (conjugated ketone), 1540 (aromatic C=C), and 1252 cm^{-1} (OCH₃).

UV λ_{max} (log ϵ): 332 (4.12), 294 (3.85) and 267 nm (4.50).

^1H and ^{13}C NMR: Table 1.

HREI-MS: m/z 398.3630 (Calcd. for C₂₁H₁₈O₈: 398.3670).

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Kaempferol Glycosides in the Flowers of Carnation and their Contribution to the Creamy White Flower Color

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Three flavonol glycosides were isolated from the flowers of carnation cultivars 'White Wink' and 'Honey Moon'. They were identified from their UV, MS, ¹H and ¹³C NMR spectra as kaempferol 3-*O*-neohesperidoside, kaempferol 3-*O*-sophoroside and kaempferol 3-*O*-glucosyl-(1→2)-[rhamnosyl-(1→6)-glucoside]. Referring to previous reports, flavonols occurring in carnation flowers are characterized as kaempferol 3-*O*-glucosides with additional sugars binding at the 2 and/or 6-positions of the glucose. The kaempferol glycoside contents of a nearly pure white flower and some creamy white flower lines were compared. Although the major glycoside was different in each line, the total kaempferol contents of the creamy white lines were from 5.9 to 20.9 times higher than the pure white line. Thus, in carnations, kaempferol glycosides surely contribute to the creamy tone of white flowers.

Keywords: carnation, creamy white flower, flavonols, kaempferol 3-*O*-glycosides.

Anthocyanin flower pigments of carnations have been reported for some pink, red, red-purple and mauve cultivars. Pelargonidin 3-*O*-glycoside was found in salmon and red cultivars, pelargonidin 3,5-di-*O*-glycoside in pink, cyanidin 3-*O*-glycoside in lavender and crimson, and cyanidin 3,5-di-*O*-glycoside in lavender and magenta ones [1,2]. In 1986, Terahara *et al.* reported that the major anthocyanins in pink and red carnations were pelargonidin and cyanidin 3-*O*-glucosides acylated with malic acid [3], and isolated pelargonidin and cyanidin 3-*O*-(6''-*O*-malylyl-β-glucopyranosides) from the red cultivar 'Scania' and the purplish-red one 'Nina' [4]. Recently, cyclic 5-3 malylyl pelargonidin and cyanidin were also identified from deeper colored cultivars [5,6]. The yellow color in the petals of carnations is due to the accumulation of a chalcone, chalcononaringein 2'-*O*-glucoside (i.e., isosalipurposide) [7-9]. Some flavonoids also have co-pigmentation effects, which influence coloration by interaction with anthocyanins. Information of flavonoid structures is important to understand the coloration mechanism in carnation flowers. Recently, one

C-glycosylflavone, isovitexin 7-*O*-(6''-malylylglucoside) and two flavonols, kaempferol 3-*O*-glucosyl-(1→2)-[rhamnosyl-(1→6)-glucoside] and kaempferol 3-*O*-(6''-malylylglucosyl)-(1→2)-[rhamnosyl-(1→6)-glucoside] were isolated from the petals of transgenic carnation cultivars 'Moondust' and 'Moon shadow' [10]. Of their flavonoids, it was proved that isovitexin 7-*O*-(6''-malylylglucoside) exhibits the strongest co-pigment effect.

In previous studies, only cyanic cultivars were investigated. As the first step of our study, acyanic cultivars were investigated seeking compounds whose occurrence in carnation flowers has not yet been reported. We selected a white flower cultivar 'White Wink' and a cream one 'Honey Moon' and detected three kaempferol 3-*O*-glycosides. In addition, the flavonoid contents of some white flower lines were compared to determine the contribution of kaempferol glycosides, including those identified here, to the creamy tone coloration of carnation flowers.

In this survey, three flavonoids (**1** - **3**) were obtained as pale yellow powders from the petals of *D. caryophyllus* 'Honey Moon' and 'White Wink'. Acid hydrolysis of these liberated kaempferol as the aglycone, and glucose and rhamnose (**1** and **3**), and glucose alone (**2**) as the sugars. In the UV spectra, the bathochromic shift of band I by addition of NaOMe, with an increase in the intensity relative to the spectrum of the MeOH solution, showed the presence of free 4'-hydroxyl and substituted 3-hydroxyl groups [11]. Moreover, though band I bathochromically shifted on the addition of AlCl₃, there was no hypsochromic shift on addition of HCl, showing the presence of a monohydroxyl group in ring B, and a free 5-hydroxyl group. The appearance of an accompanying peak on NaOMe addition, and a bathochromic shift of band II on addition of NaOAc showed the presence of a free 7-hydroxyl group. Thus, it was proved that the sugars are attached at the 3-hydroxyl position of their glycosides. Finally, flavonoids **1** and **2** were identified as kaempferol 3-*O*-neohesperidoside and kaempferol 3-*O*-sophoroside by ESIMS, and ¹H and ¹³C NMR, respectively. In flavonoid **3**, the shifts of 3-*O*-glucosyl C-2 (δ 82.9) and C-6 (δ 66.5) were recognized from the ¹³C NMR spectra. The attachment of rhamnose to the 6-position and another glucose to the 2-position of 3-*O*-glucose were determined by the presence of HMBC and/or NOESY signals. Thus, flavonoid **3** was completely identified as kaempferol 3-*O*- β -glucopyranosyl-(1 \rightarrow 2)-*O*-[α -rhamnopyranosyl-(1 \rightarrow 6)-*O*- β -glucopyranoside] (kaempferol 3-*O*-2^G-glucosylrutinoside).

The kaempferol glycoside content of a nearly pure white line 'MB3' was compared with those of five common white cultivars or lines, 'White Sim', 'Bridal White', 'Annabel', 'Milky Way' and 'BD2349-08' (Table 1). Three kaempferol glycosides, i.e. 3-*O*-neohesperidoside (**1**), 3-*O*-sophoroside (**2**) and 3-*O*-(2^G-glucosylrutinoside) (**3**) were detected in the flowers of the cultivars except for 'White Sim', which contained flavonoids **1** and **3** alone. The fourth kaempferol glycoside (**4**), which was not isolated from the flowers of 'White Wink' and 'Honey Moon', was found in all

the cultivars except for 'White Sim'. However, it could not be characterized. Although the major glycoside was different in each of the cultivars, the total kaempferol contents of the five creamy white cultivars or line were from 5.9 (BD2349-08) to 20.9 (White Sim) times higher than that of the nearly pure white line 'MB3'.

Kaempferol 3-*O*-neohesperidoside has been isolated from the leaves of *Nerisyrenia linearifolia* and *N. gracilis* (Cruciferae) [12]. Recently, kaempferol 3-*O*-sophoroside has been isolated from the purple flowers of *Gladiolus* cultivar 'Ariake' as a co-pigment, together with kaempferol 3-*O*-rutinoside and quercetin 3-*O*-rutinoside [13]. Kaempferol 3-*O*-2^G-glucosylrutinoside has been reported from *Hosta ventricola* (Liliaceae) [14]. In carnation, they have been reported from the flowers [15,16], roots and stems [17] of common cultivars. The basic structure of these glycosides was characterized as kaempferol 3-*O*-glucoside, with additional sugar moieties at the 2 and/or 6-positions of the 3-*O*-glucose. White carnation cultivars have been divided into three flower flavonoid types, i.e. flavonoid-lacking cultivars, naringenin accumulating cultivars, and kaempferol accumulating cultivars [18]. Moreover, it was shown that the flavonoid biosynthesis pathway is blocked before chalcone synthesis in flavonoid-lacking cultivars, whereas it is blocked at the flavanone-3-hydroxylase step in naringenin accumulating cultivars [19]. However, flavonoid identification had been not performed at the glycosylation level. In this experiment, it was shown that four kaempferol glycosides are involved, i.e. 3-*O*-neohesperidoside, 3-*O*-sophoroside, 3-*O*-(2^G-glucosylrutinoside) and an unknown glycoside. Although quercetin glycosides produce pale yellow colors, in the case of yellow *Clematis* species and cultivars, kaempferol glycosides do not apparently act as yellow pigments [20, 21]. In carnation, however, creamy white flowers contained over five times higher levels of kaempferol glycosides than a nearly pure white flower. The presence of such amounts of kaempferol glycosides surely contributes to the creamy tone in the white flowers of carnations.

Table 1: Quantitative HPLC analysis of the flowers of six white carnation cultivars and lines.

Cultivars/lines	Colors	1 *	2	3	4	Total
MB3	pure white	0.2 \pm 0.1	0.1 \pm 0.0	0.5 \pm 0.3	0.1 \pm 0.1	0.9 (1.0)
White Sim	white	□	10.4 \pm 0.4	8.4 \pm 0.3	□	18.8 (20.9)
Bridal White	white	2.5 \pm 0.2	3.1 \pm 0.1	2.4 \pm 0.1	4.1 \pm 0.2	12.1 (13.4)
Annabel	white	0.3 \pm 0.0	0.5 \pm 0.0	4.3 \pm 0.4	1.5 \pm 0.1	6.6 (7.3)
Milky Way	white	1.3 \pm 0.1	0.7 \pm 0.0	4.5 \pm 0.3	2.0 \pm 0.6	8.5 (9.4)
BD2349-08	white	0.9 \pm 0.1	0.5 \pm 0.0	2.5 \pm 0.1	1.4 \pm 0.1	5.3 (5.9)

Each 1 g fresh petals was extracted with 6 mL 10% HOAc in MeOH.

1 = kaempferol 3-*O*-neohesperidoside, **2** = kaempferol 3-*O*-sophoroside, **3** = kaempferol 3-*O*-(2^G-glucosylrutinoside) and **4** = unknown kaempferol glycoside.

* Calculated from the absorption coefficient of authentic quercetin 3-*O*-rutinoside (μ mol/g fresh weight).

() = Relative amounts of total flavonoids as peak area of pure white line 'MB3' is 1.00.

Experimental

General: Prep. HPLC was performed on a Wakosil II 5C18 AR Prep (I.D. 20×250 mm) with a flow-rate of 9 mL/min and by monitoring absorbance at 360 nm. The solvent system used included linear gradient elution for 25 min using 25-50% of solvent B (0.5% TFA, 10% formic acid, 40% MeCN) in solvent A (0.5% TFA, 10% formic acid). Paper chromatography was performed using solvent systems: BAW (*n*-BuOH/HOAc/H₂O = 4:1:5, upper phase), BEW (*n*-BuOH/EtOH/H₂O = 4:1:2.2), 15% HOAc and 5% HOAc for flavonol glycosides; BAW, BEW and Forestal (HOAc/HCl/H₂O = 30:3:10) for flavonol aglycones; and BBPW (*n*-BuOH/benzene/pyridine/H₂O = 5:1:3:3) and BTPW (*n*-BuOH/toluene/pyridine/H₂O = 5:1:3:3) for glycosidic sugars. UV spectral analysis and acid hydrolysis were performed according to published procedures [11]. The ESIMS were analyzed on a TSQ system (Thermo Quest, San Jose, CA, USA). 1D and 2D NMR spectra were measured on a Bruker AVANCE 800.

Plant materials: The flowers of two carnation cultivars 'White Wink' and 'Honey Moon' were used for flavonoid isolation. They were cultivated in Japan by Tobacco Inc., Co. Ltd., Oyama, Tochigi, Japan. A nearly white line 'MB3' and four white cultivars or lines, 'Bridal White', 'Annabel', 'Milky Way' and 'BD2349-08' were cultivated by Kirin Agribio Co., Ltd. The other white cultivar 'White Sim' was cultivated by the National Institute of Floricultural Science, National Agriculture and Bio-oriented Research Organization, Tsukuba, Ibaraki, Japan. Live specimens are growing in the nurseries mentioned above.

Extraction and isolation: Dry petals (each 200 g) of 'Honey Moon' and 'White wink' were extracted with 80% MeOH for 2 days at room temperature. After concentration *in vacuo*, the residue was dissolved in a small volume of 10% MeOH and applied to a Sephadex LH-20 column and eluted with 70% EtOH. The fractions containing the flavonoids were concentrated and purified by prep. HPLC. The isolated flavonoids were concentrated to dryness and freeze dried.

Kaempferol 3-*O*-neohesperidoside (1)

Although UV and ¹H NMR spectroscopic data of flavonoid **1** have been published [12], ¹³C NMR data have not been reported previously.

¹³C NMR (150 MHz, DMSO-*d*₆ + TFA-*d* = 9:1): (kaempferol) δ 156.9 (C-2), 133.3 (C-3), 177.9 (C-4), 160.3 (C-5), 98.9 (C-6), 164.5 (C-7), 94.1 (C-8), 156.7 (C-9), 104.6 (C-10), 121.5 (C-1'), 131.2 (C-2'), 115.5 (C-3'), 159.4 (C-4'), 115.5 (C-5'), 131.2 (C-6'); (3-glucose) δ 99.1 (C-1), 78.1 (C-2), 77.7 (C-3), 69.7

(C-4), 77.9 (C-5), 61.2 (C-6); (rhamnose) δ 101.1 (C-1), 71.0 (C-2), 70.6 (C-3), 72.3 (C-4), 68.5 (C-5), 17.7 (C-6).

ESIMS: *m/z* 611.1604 [M+H]⁺ (base, calcd. for C₂₇H₃₀O₁₆, 611.1607); *m/z* 633.1426 [M+Na]⁺ (base, calcd. for C₂₇H₃₀O₁₆Na, 633.1426).

Kaempferol 3-*O*-sophoroside (2)

UV, ¹H and ¹³C NMR spectroscopic data have been reported by Budzianowski [14].

Kaempferol 3-*O*-β-glucopyranosyl-(1→2)-[rhamnopyranosyl-(1→6)-glucopyranoside] (3)

PC (Rf): 0.26 (BAW), 0.36 (BEW), 0.66 (15%HOAc), 0.60 (5%HOAc); UV (365 nm) – dark purple, UV/NH₃ – dark greenish yellow.

UV λ_{max} nm (MeOH): 267, 345. +NaOMe: 281, 325, 404 (inc.). +AlCl₃: 274, 304, 353, 396. +AlCl₃/HCl: 275, 302, 346, 393. +NaOAc: 274, 307, 384. +NaOAc/H₃BO₃: 266, 352.

¹H NMR (800 MHz, DMSO-*d*₆ + TFA-*d* = 9:1): δ 7.99 (2H, dd, *J* = 2.0 and 8.9 Hz, H-2',6'), 6.90 (2H, dd, *J* = 2.1 and 8.9 Hz, H-3',5'), 6.40 (1H, d, *J* = 2.1 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.55 (1H, d, *J* = 7.1 Hz, 3-glucosyl H-1), 4.59 (1H, d, *J* = 7.9 Hz, *t*-glucosyl H-1), 4.32 (1H, d, *J* = 1.3 Hz, rhamnosyl H-1), 0.93 (3H, d, *J* = 6.2 Hz, rhamnosyl Me).

¹³C NMR (150 MHz, DMSO-*d*₆ + TFA-*d* = 9:1): (kaempferol) δ 156.9 (C-2), 133.4 (C-3), 178.0 (C-4), 161.6 (C-5), 98.8 (C-6), 164.4 (C-7), 94.2 (C-8), 157.0 (C-9), 104.6 (C-10), 121.5 (C-1'), 131.5 (C-2'), 115.7 (C-3'), 160.3 (C-4'), 115.7 (C-5'), 131.5 (C-6'); (3-glucose) δ 99.1 (C-1), 82.9 (C-2), 76.9 (C-3), 70.0 (C-4), 76.1 (C-5), 66.5 (C-6); (*t*-glucose) δ 104.5 (C-1), 74.8 (C-2), 77.0 (C-3), 70.0 (C-4), 77.5 (C-5), 61.3 (C-6); (rhamnose) δ 100.9 (C-1), 70.8 (C-2), 71.0 (C-3), 72.3 (C-4), 68.7 (C-5), 18.0 (C-6).

Analysis of flavonoid contents: White petals of 'Bridal White', 'Annabel', 'Milky Way' 'BD2349-08' and 'White Sim' were extracted with 10% HOAc in MeOH. The extracts were analyzed by HPLC using an HP1100 system with photodiode array detector (Agilent Technologies-Yokokawa Analytical Systems, Tokyo, Japan) and an Inertsil ODS-2 column (4.6 mm x 250 mm, GL science, Tokyo, Japan) combined with an Inertsil ODS-2 guard column at 40°C with a flow rate of 0.8 mL/min. Absorption spectra were monitored at 240-580 nm. A linear gradient of 10-50% of solvent B (1.5% H₃PO₄, 40% MeCN, 50% HOAc) in solvent A (1.5% H₃PO₄) was run for 40 mins. Flavonoids were identified and quantified based on absorption spectra and the absorption at 360 nm.

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Factors Influencing Glabridin Stability

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Glabridin, a polyphenolic isoflavan of *Glycyrrhiza glabra*, has shown a variety of pharmaceutical properties. We have previously studied the isolation of glabridin using macroporous resin and found that it is partially degraded, giving a dark color. To illustrate the degradation of glabridin, the present work studied the stability of glabridin under various conditions. Licorice extract containing about 20% glabridin, obtained from *G. glabra* by silica gel column chromatography, was used in the stability study. Seven different factors (temperature, illumination, humidity, pH, solvent, oxygen, and oxidant) were studied and content changes were determined through HPLC analysis. Except for oxygen, all the above factors had an effect on the stability of glabridin, with illumination being the main one. Moreover, the interactions between temperature and pH, temperature and humidity, and illumination and pH can promote the degradation of glabridin. In conclusion, we suggest that a dark, dry and airtight environment provides the optimized condition for the long-term storage of glabridin.

Keywords: Glabridin, stability, illumination, solvent.

Glabridin is a polyphenolic isoflavan found in the hydrophobic extract of *Glycyrrhiza glabra* [1]. Recent studies have shown that glabridin has a wide range of pharmacological effects, including antioxidant, antimicrobial, anti-inflammatory, and anti-gastric ulcer activities, as well as inhibitory effects on the formation of melanin and low density lipoprotein [2–5]. Due to its many applications, interest in glabridin has intensified. However, its isolation is complicated because of its low content and high decomposition rate in the preparation process [6,7]. Up to now, there are no reports referring to the stability of glabridin. Temperature, illumination, humidity, oxidant, oxygen, pH and solvent are known to be effective factors which may influence the stability of various compounds. Thus, in our study, licorice extract containing a high proportion of glabridin was first obtained from *G. glabra*. This was then used to determine the effects of the seven parameters on the stability of glabridin, from which a rational protocol for its preparation and preservation was proposed.

An extract of licorice root was treated by silica gel chromatography [6,7]. The glabridin concentration of the resulting brown product was then determined by an HPLC method [8]. Under optimized chromatographic conditions, the peak of glabridin could easily be distinguished from other co-eluting compounds.

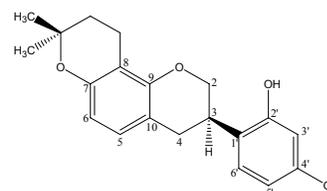


Figure 1: Structure of glabridin.

The calibration curve was obtained through the least-square method, which is widely used in linear analysis. The established linear regression equation was: $Y=1154.9X-33.101$, where Y is the peak area of glabridin and X the concentration of each injection. The correlation coefficient (R^2) was 0.9967 for glabridin. $S_{y,x}$ was 13.202, while b was 1154.9. Thus, the LOD and LOQ within the linearity of 0.9967 were 0.034 mg/mL and 0.114 mg/mL, respectively.

Intra-day and inter-day precisions were measured by repeated injections of standard glabridin on the same and three different days [8]. The relative standard deviation (RSD) values for both situations were 2.02% and 2.48%, respectively, which were considered to be acceptable. Linear analyses at four other wavelengths (210, 254, 310 and 377 nm) were also carried out to investigate the specificity of the HPLC method. The concentration of glabridin was 0.72, 0.72, 0.73, 0.75

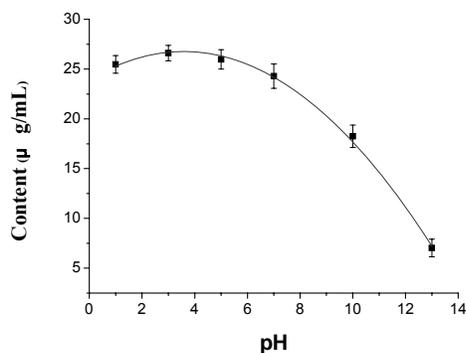


Figure 2: Degradation of glabridin at different pHs.

and 0.67 mg/mL at the above wavelengths, respectively, with a RSD value of 3.7%, which indicated that the established HPLC method could be used for the stability study of glabridin.

The effects of temperature, illumination, humidity, oxidant, oxygen, pH and solvent on the chemical stability of glabridin were assessed. The stability of glabridin at pH values of 1.0, 3.0, 5.0, 7.0, 10.0 and 13.0 were evaluated. In acid and neutral solutions the compound was mainly stable, whereas in alkaline solutions it underwent severe degradation within 24 hours. At pH 13.0, degradation was 71.0% (Figure 2). Friedman [9] reported that the susceptibility of plant phenolic compounds to pH strongly depended on their structure. Glabridin, with two hydroxyl substituents at positions 2' and 4', can exhibit a low degree of ionization. Under alkaline conditions, a neutralization reaction may be aroused leading to the degradation of glabridin and other flavones [9].

Thermal stability of glabridin (pH 8.0) was studied at 4, 25, 40, 60, 80 and 100°C. The interaction between pH and temperature was carried out as well. Glabridin exhibited great stability below 60°C at a specific pH, while it started to degrade moderately after 5 hours above 60°C (Figure 3). The results were in agreement with earlier studies on other kinds of isoflavonoid [10]. Meanwhile, the close relationship between the pH of the solvent and the stability of glabridin at high temperature

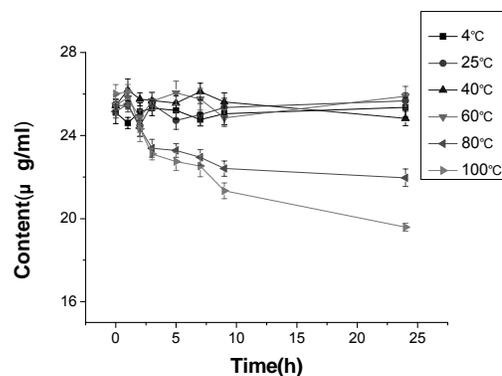


Figure 3: Degradation of glabridin at different temperatures.

is shown in Figure 4. Glabridin at pH 5 remained almost stable, whereas at pH 1, 3, 7 and 10 it degraded significantly at 100°C. Stintzing [11] found that the thermal degradation of isoflavonoids was highly dependent on their substitution pattern and solvent pH. A molecule with hydroxyl groups at positions 7 and 4' was more stable under high temperature compared with ones with other substituents at these positions. Glabridin, with a hydroxyl substituent at position 4' and one side ring on ring A, showed stability under high temperature in our study. The effect of the side ring remains to be demonstrated.

The optical stability of glabridin was examined in the dark, natural light and ultraviolet light (Figure 5). Glabridin remained almost unchanged in the dark after 24 h, while it was remarkably unstable in both natural and ultraviolet light after 8 h, the contents being reduced by 26.3% and 54.1%, respectively. It was obvious that the light degradation of glabridin was closely correlated with the exposure time to light and the type of light source. We speculated that the light degradation was promoted by the absorbed energy from photons. Since the energy of ultraviolet light was more powerful than that of natural light, glabridin degraded more in the former conditions [12]. Interaction between pH and illumination was also carried out. Glabridin in acid (pH 1) and alkaline (pH 13) solutions was more unstable than when in neutral solution (pH 7), indicating the synergistic effect between the pH of the solvent and light.

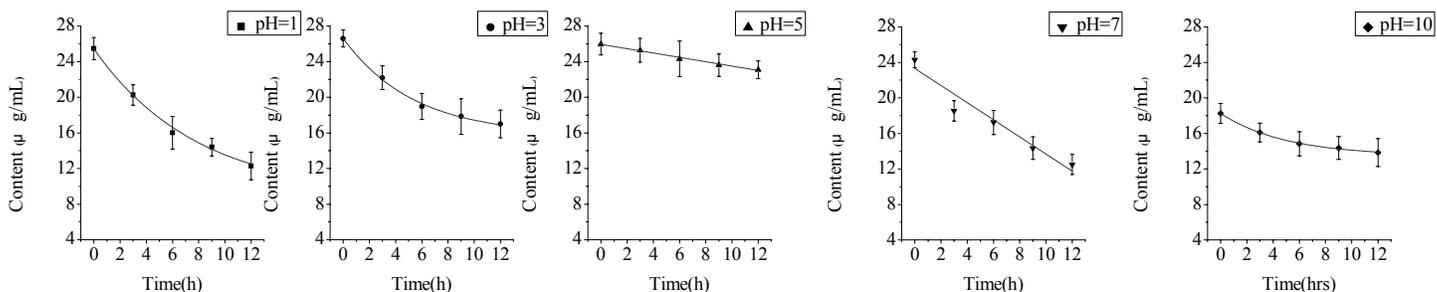


Figure 4: Degradation of glabridin at different pHs at 100°C.

Table 1: Average content of glabridin in seven solvents during 45 days.

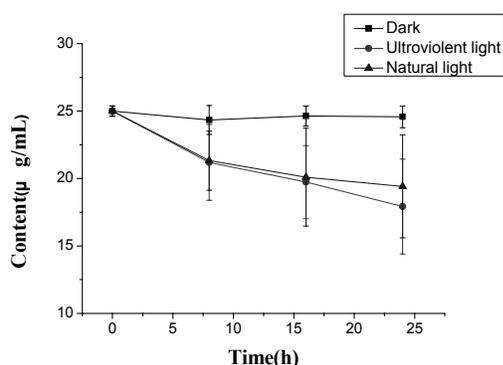
	Methanol	Ethanol	Acetone	Ethyl acetate	Methylene dichloride	Chloroform	Propylene glycol
Content ($\mu\text{g/mL}$)	30.0 \pm 0.6	29.9 \pm 1.0	31.5 \pm 3.7	31.6 \pm 3.9	8.7 \pm 1.6	8.9 \pm 0.6	30.0 \pm 0.9
RSD	2.3%	4.3%	3.2%	4.1%	26.2%	8.9%	3.6%

Table 2: Degradation rate of glabridin at different pHs under ultraviolet light.

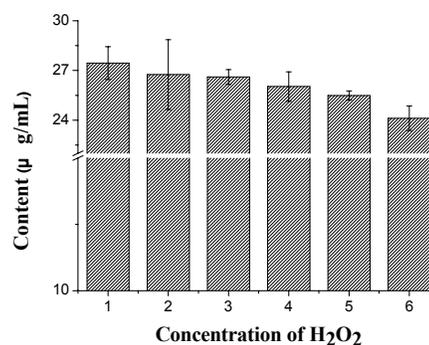
Degradation rate	pH 1	pH 7	pH 13	Stock solution
8 h	16.4%	13.1%	16.8%	13.1%
16 h	50.6%	28.0%	49.7%	19.1%

Table 3: Degradation of glabridin at different relative humidities and different temperatures.

Degradation rate	75% at 60°C	75% at 25°C	90% at 60°C	90% at 25°C
5 th day	14.2%	5.9%	13.7%	8.3%
10 th day	22.9%	12.7%	22.2%	14.9%

**Figure 5:** Degradation of glabridin under light.

Glabridin examined in seven different organic solvents demonstrated varying stability (Table 1). It was apparent that the stability was dependent on the properties of the solvents. Methanol was the most suitable for glabridin, followed by acetone under the same conditions. On the contrary, glabridin was equally unstable in methylene dichloride and chloroform. The reason why the solvent could affect the stability of phenolic compounds was because of its ionizability, relative volatility and dissolved oxygen. In the present study, the solvents were first degassed to create low-oxygen conditions. The solvents can be divided into two groups, protonic and nonprotonic [13]. An analogous dissociation to water was observed in organic solvents and was described as the pKa value. For glabridin dissolved in protonic solvents with higher pKa values, the solvation effect between solvents and glabridin was easily initiated. Nevertheless, when dissolved in nonprotonic solvents with lower pKa values, solvation energies were too negative to start a solvation effect. It was concluded that glabridin with two hydroxyl substituents on the benzene ring was more unstable in protonic solvents because of the solvation effect [13], which was inconsistent with our results. We presumed that it was the relative volatility of the solvents rather than the ionizability that caused the degradation of glabridin, which remains to be confirmed in a further study.

**Figure 6:** Degradation of glabridin with different concentrations of H₂O₂ (1: Contrast; 2: Stock solution with water; 3: 5% H₂O₂; 4: 10% H₂O₂; 5: 20% H₂O₂; 6: 30% H₂O₂).

The effect of oxygen and H₂O₂ was carried out at room temperature. Glabridin displayed different stabilities under these two conditions. As shown in Figure 6, the stability of glabridin in H₂O₂ was concentration dependent, while the content of glabridin remained unchanged after exposure to air in the dark (data not shown). Glabridin was reported to possess the ability to scavenge oxygen free radicals, which may explain its content change in H₂O₂. We supposed that the stability of glabridin was relevant to the type of free radical [14].

The degradation of glabridin was positively related to relative humidity and treatment time (Figure 7). The interaction between humidity and temperature indicated that a high temperature can promote the degradation of glabridin at the same relative humidity. In our oxidant study, glabridin in CH₃OH/H₂O (80/20) also degraded after several days. We have mentioned that the stability of glabridin was in part correlated with the ionizability of the solvent. From the results above, it seemed that water was one of the factors that had an impact on the stability of glabridin for its strong ionizing ability. Thus we suggest that glabridin should be kept in a dry environment during storage.

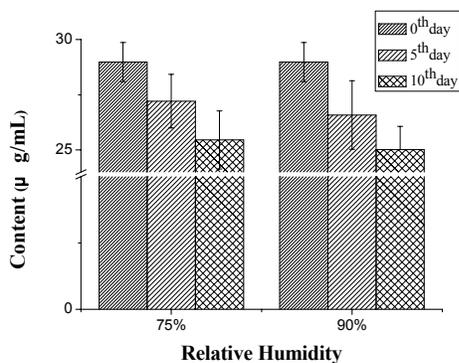


Figure 7: Degradation of glabridin at different humidities.

In conclusion, the results from this study show that the stability of glabridin in licorice extract depends on illumination, temperature, pH, solvent, humidity and oxidant, of which illumination is the main factor. Interaction effects between temperature and pH, illumination and pH, and temperature and humidity increased the degradation rate of glabridin. To minimize degradation, we suggest that pure glabridin and licorice extract should be stored in a dark, dry and airtight environment. A suitable solvent is recommended, high temperature should be avoided, and pH should be carefully controlled in the isolation process. However, it is worth noting that oxygen can promote a variety of reactions. Our studies showed that the direct impact of oxygen on the stability of glabridin was weak, but the interactions it may cause with other factors still need to be investigated. Thus the presence of oxygen should be avoided both in the preservation and isolation of glabridin to reduce its degradation.

Experimental

Apparatus: HPLC determinations were performed on Agilent 1200 Series (Agilent Technologies Inc., America) equipment, consisting of an Iso pump, UV detector and Man Injection. UV Penetration Reflection Analysis Instrument (Beijing Institute of New Technology, China) was used for photolysis treatment. Thermo analysis was performed on a 101A-1BY Infrared Radiation Electric Oven (Hangzhou LanTian Chemical Instrument Plant, China). A 320-S Acidometer (Mettler Toledo, Switzerland) was used in the adjustment of the pKa of the test solutions.

Chemicals: Glabridin (>97%) was purchased from National Vaccine & Serum Institute of China. The solvents used for extraction and chromatographic isolation were of analytical grade. Ultrapure water was prepared using the MRR-2(D) ultrapure water production system (Hangzhou Repure Ltd. China). Other solvents were of high-performance liquid chromatography (HPLC) grade.

Isolation of glabridin: Powdered roots of *Glycyrrhiza glabra* (provided by Xinjiang Production and Construction Corps) were extracted in acetone (1:5, w/v) at room temperature for 24 h. After solvent evaporation, the brown crude extract was extracted in chloroform (1:3, w/v) to obtain the hydrophobic constituents. This fraction (after evaporation) was separated on a silica gel column with CH₂Cl₂, 2% CH₂Cl₂/CH₃OH, 5% CH₂Cl₂/CH₃OH and 10% CH₂Cl₂/CH₃OH. The fractions collected were first examined through HPLC and the fraction of 5% CH₂Cl₂/CH₃OH containing glabridin was subsequently used for the stability study.

HPLC analysis: HPLC separation was performed on a Kromasil C18 reverse-phase column (5 µm particle size, 150 x 4.6 mm i.d.) with a flow rate of 1.0 mL/min and column temperature of 25°C. A mixture of methanol–water containing 0.2% acetic acid (70/30, v/v) was used as the mobile phase, which was filtered through a 0.45 µm membrane and degassed by sonication. The column effluent was monitored at 282 nm with UV detection. Prior to HPLC analysis, all the standards and extracts were filtered through a 0.45 µm syringe membrane filter.

Calibration curve: The linearity of the HPLC method was established by triplicate injections of standard glabridin in the range of 0.125–0.625 mg/mL. The linear regression curve was obtained using the least-squares method with the peak area (*Y*-axis) of glabridin as the dependent variable and the concentration (*X*-axis) of each injection as the independent variable.

Validation of the HPLC method: The precision was measured by means of intra-day and inter-day repeatability. Intra-day precision was obtained by 6 injections of standard glabridin on the same day, while inter-day precision was established by 3 repeated injections on days 1, 2 and 3 at the same concentration. The specificity of the method was evaluated through linear analysis at 5 different wavelengths (210, 254, 282, 310 and 377 nm). A linear regression curve was obtained through the least-squares method. The concentration of glabridin was subsequently determined at the above 5 wavelengths. The RSD value of concentration was used to evaluate the purity of the glabridin peak. Limit of detection (LOD) and limit of quantification (LOQ) were determined through the following equation: $LOD=3S_{y,x}/b$ and $LOQ=10S_{y,x}/b$, where $S_{y,x}$ is the standard deviation of the *Y* value distribution and *b* is the slope of regression equation.

Statistical analysis: All data were recorded as means ± standard deviation of either duplicate or triplicate measurements. Analyses of variance (ANOVA) and

significance differences between the means were performed through SPSS 7.0 for Windows. Relative standard deviation (RSD) was also calculated to evaluate the stability.

Chemical stability evaluations: In this study, seven factors involved in glabridin stability were studied. The 5% CH₂Cl₂/CH₃OH eluent was evaporated to obtain a brown solid extract, which was dissolved in methanol to a final concentration of 0.5 mg/mL (stock solution) and used in the following experiments.

The thermal stability of glabridin was studied at 4, 25, 40, 60, 80 and 100°C. Capped glass bottles with 10 mg extract were stored in an oven (fridge for 4°C) at different temperatures for 1, 2, 3, 5, 7, 9 and 24 h, respectively. The extract was then dissolved in 5 mL methanol and filtered through a 0.45 µm syringe membrane filter before HPLC analysis. Each temperature group was repeated 3 times.

For the optical stability study, an aliquot (10 mL) of the stock solution was placed in an open container and treated in the dark, natural light and ultraviolet light, respectively, for 8, 16 and 24 h [12]. The gross weight of each sample was determined at the beginning. Before HPLC analysis, the gross weight of each sample was adjusted to its initial value to avoid the influence of solvent evaporation. The analysis of each group was repeated 3 times.

H₂O₂ of varying concentration (5%, 10%, 20% and 30%) was used in the study of the oxidant effect on stability of glabridin. Sample solutions were prepared by mixing a stock solution with different concentrations of H₂O₂ (4:1, v/v) and storing in a well-capped bottle to avoid solvent evaporation. Meanwhile, water and methanol were added to the stock solution (1:4, v/v) to contrast with groups 1 and 2, respectively. All samples were kept in the dark at room temperature for 14 days. Each group was repeated 3 times.

The effect of pH on chemical stability of glabridin was studied at 6 different levels (1.0, 3.0, 5.0, 7.0, 10.0 and 13.0) at room temperature [9]. Sodium hydroxide solution, sodium chloride solution and hydrochloric acid solution were prepared and then mixed with stock solution (500 µg/mL) at a ratio of 9:1. Sample solutions were kept in the dark at room temperature for 24 h. Each group was repeated 2 times.

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The solvent effect on the stability of glabridin was carried out in 7 different organic solvents (methanol, ethanol, acetone, ethyl acetate, chloroform, methylene dichloride and propylene glycol). Licorice extract (25 mg) was dissolved in 100 mL of solvent, degassed and kept hermetically sealed at room temperature in the dark. An aliquot (20 µL) of the sample solution was periodically removed and subjected to HPLC analysis on days 0, 7, 12, 19, 25, 32, 35 and 42.

The effect of humidity was studied at relative humidities of 75% and 90%. A constant humidity environment was established in desiccators with super-saturated solutions. In our study, NaCl and KNO₃ were used to set up the constant humidity environment of 75% and 90%, respectively. Licorice extract (10 mg) was transferred into an open container and placed in the desiccators. The treated extracts were then dissolved in 5 mL methanol and subjected to HPLC analysis on days 5 and 10. Each group was repeated twice.

A control group with stock solution was kept hermetically sealed in the dark. The experimental group with stock solution was exposed to air in the dark. After 24 h, the contents of glabridin in both groups were determined. The effect of oxygen was characterized by the content change.

Moreover, the interaction effects between temperature and pH, illumination and pH, and temperature and humidity were studied. The effect of pH on thermal stability was studied at 5 different pHs (1.0, 3.0, 5.0, 7.0 and 10.0) at 100°C [9]. The content of glabridin was determined after 3, 6, 9 and 12 h. The optical stability of glabridin at different pHs (1.0, 7.0 and 13.0) was studied under UV light [12]. Stock solution (10 mL) was filled into an open container and treated under UV light for 8 and 16 h, respectively. The effect of humidity on thermal stability was carried out at relative humidities of 75% and 90% at 60°C. Licorice extract (10 mg) was transferred into an open container and placed in the desiccators. The treated extracts were then dissolved in 5 mL methanol and subjected to HPLC analysis on days 5 and 10. Each group was repeated twice.

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Effect of Different Strains of *Agrobacterium rhizogenes* and Nature of Explants on *Plumbago indica* Hairy Root Culture with Special Emphasis on Root Biomass and Plumbagin Production

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The aim of the present study was to determine the effect of three strains of *Agrobacterium rhizogenes* (ATCC 15834, A₄ and LBA 9402) and the nature of explants (leaf and stem) on hairy root induction, growth and plumbagin production in *Plumbago indica*. The first appearance of hairy roots, the transformation frequency, dry root biomass and plumbagin accumulation were found to be maximum in hairy roots induced in leaf explants infected with *A. rhizogenes* ATCC 15834 as compared with the other two bacterial strains. The hairy roots generated from stem explants infected with all three strains were not found to be productive in terms of the selected parameters. Finally, the insertion of the *rolB* gene of *A. rhizogenes* ATCC 15834 in hairy roots of *P. indica* derived from leaf explants was confirmed by PCR analysis.

Keywords: *Agrobacterium rhizogenes*, hairy root, *Plumbago indica*, plumbagin.

Plumbago indica Linn. (Family: Plumbaginaceae) is a rich source of a therapeutically active, root specific metabolite, plumbagin. This has been reported to possess filaricidal, anticancer, antimicrobial, and antifertility activity [1-4]. During the past few decades, several attempts have been made to scale up production of plumbagin *in vitro* by suspension culture [5] and cell culture [6], but low product yield coupled with instability necessitates improvement for better commercial exploitation. Thus, it is necessary to scale up the production of plumbagin without hampering the natural flora through modern biotechnological applications.

Hairy root culture offers a valuable source of root derived phytochemicals [7]. *Agrobacterium rhizogenes*, a Gram negative soil grown bacterium, is the causative agent of hairy root formation in dicotyledonous plants. Hairy root cultures are capable of unlimited growth and produce superior yields of root specific plant secondary metabolites. In the present study, it was aimed to determine the effect of three different strains

of *A. rhizogenes* (ATCC 15834, A₄ and LBA 9402) and the nature of the explants (leaf and stem) on hairy root induction, growth and plumbagin accumulation.

Three strains of *A. rhizogenes* were utilized to infect leaf and stem explants of *P. indica*. A virulent strain of *A. rhizogenes* was selected on the basis of transformation frequency, appearance of hairy roots in minimum time, root biomass and plumbagin accumulation. The transformation frequency was found to be highest when *P. indica* leaf explant was infected with *A. rhizogenese* ATCC 15834 (Figure 1). Similarly, the appearance of hairy roots was best observed (6.6 ± 0.5th day) when *P. indica* leaf explants were infected with *A. rhizogenese* ATCC 15834 (Figure 1). The highest dry root biomass and plumbagin contents were found when *P. indica* leaf explant was infected with *A. rhizogenese* ATCC 15834 (Figure 2).

A. rhizogenes ATCC 15834-induced hairy roots of *P. indica*, emerging from leaf lamina at the exponential phase, are shown in Figures 3 A-B.

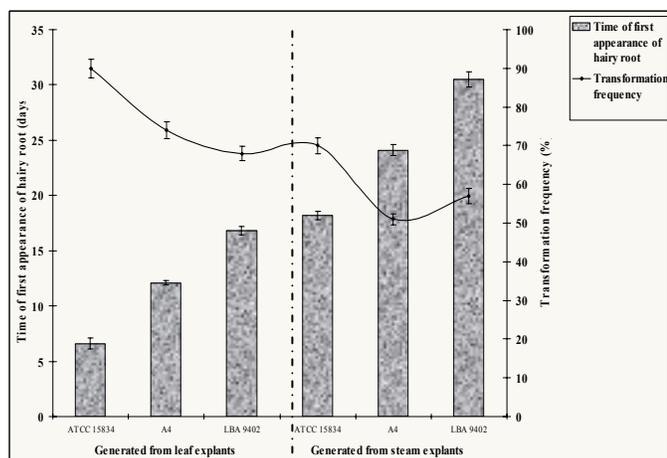


Figure 1: Effect of different strains of *A. rhizogenes* on the hairy root formation from *P. indica* hairy roots derived from leaf and stem explants.

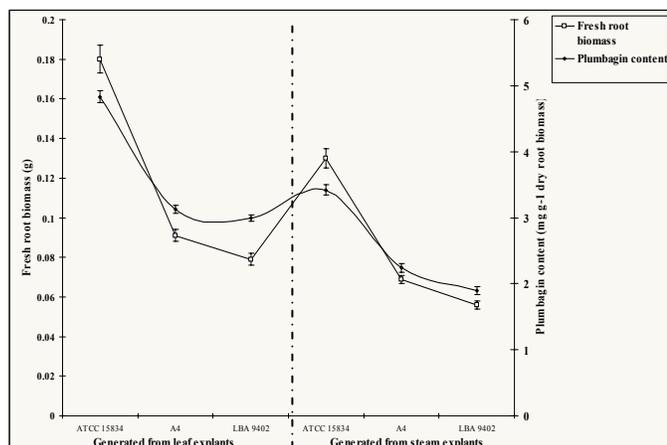


Figure 2: Effect of different strains of *A. rhizogenes* on the hairy root biomass and plumbagin production from *P. indica* hairy roots derived from leaf and stem explants.

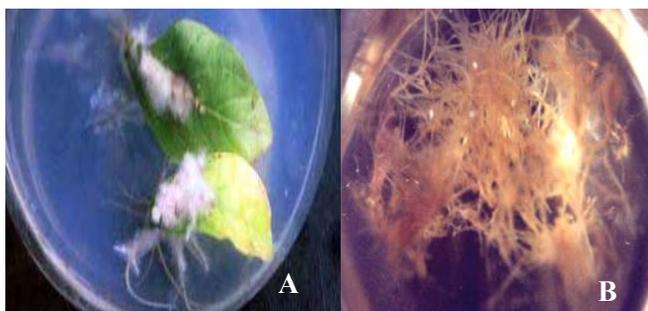


Figure 3: *A. rhizogenes* ATCC 15834 induced *P. indica* hairy roots emerging from leaf lamina (A) and at exponential phase of growth medium (B).

The best results in term of hairy root generation, root biomass and plumbagin content, were generated from leaf explants of *P. indica* infected with *A. rhizogenes* ATCC 15834. These were, therefore, subjected to polymerase chain reaction (PCR) analysis for confirmation of *rolB* insertion. The presence of *therolB* gene (*A. rhizogenes* ATCC 15834 strain) in the genomic DNA from randomly selected hairy root clones derived from leaf and stem explants was tested by PCR

amplification using *rolB* forward and reverse primers. All the selected hairy root clones showed amplification of the 780 bp fragment (*rolB*) and the DNA isolated from non-transformed roots gave no such amplification.

In the present study, the effect of different strains of *A. rhizogenes* (ATCC 15834, A₄ and LBA 9402) and the nature of the explants (leaf and stem) on hairy root induction, growth and plumbagin production in *P. indica* have been investigated. The strain specificity observed in the present study agrees with the hypothesis that the ability for infection of a given species by different *A. rhizogenes* strains is different [8,9]. It was also observed that the nature of the explants had a significant effect on hairy root culture and metabolite production. It is believed that transformation by *A. rhizogenes* is species specific and strongly depends on the bacterial strains and culture conditions [10,11]. The effectiveness of ATCC 15834 in hairy root culture and secondary metabolite production has been previously documented for other species [7,12]. Due to the better performance of ATCC 15834 than the other two strains, it will be used for subsequent experiments.

Experimental

Bacterial culture: Three different strains of *A. rhizogenes* viz. ATCC 15834 (pRi 15834), A₄ (pRi A₄) and LBA 9402 (pRi 1855) were used in the experiments. *A. rhizogenes* ATCC 15834 (pRi 15834) strain was maintained in solid *Agrobacterium* broth (AB) medium [13]. *A. rhizogenes* A₄ (pRi A₄) and LBA 9402 (pRi 1855) strains were maintained in Yeast-Mannitol Broth (YMB) medium [14].

Induction and establishment of hairy roots: Bacterial (*A. rhizogenes* strains viz. ATCC 15834, A₄ and LB 9402) infection was made artificially to the stem and leaf explants of *in vitro* grown axenic *P. indica* plants by wounding the surface tissue with the needle of a loaded syringe, showing absorbance (A₆₀₀) 0.5.

Frequency of successful transformation and time (days) taken for first appearance of hairy roots from leaf and stem explants with the 3 *A. rhizogenes* strains were recorded. Developing fresh hairy roots arising from the infected explants were cut at the tips and transferred to the MS solid medium (gelled with 0.8% agar) containing 3% sucrose supplemented with cefotaxime (cefotaxime sodium, ALKEM, India) to eliminate bacteria from the culture. Vigorously growing hairy roots free from bacterial contamination were selected from the young root tips and were transferred to 250 mL Erlenmeyer flasks containing 50 mL liquid Murashige, Skoog (MS) medium [15], pH 5.6, with 3% sucrose, on an orbital shaker at 70 rpm and maintained

under darkness at $25 \pm 2^\circ\text{C}$. Dry root biomass (g) was measured by drying a weighed amount of fresh root, obtained during the exponential phase of root culture, in an oven at 60°C for 4 h.

Quantification of plumbagin by high performance liquid chromatography: Standard plumbagin (Sigma Aldrich, USA) solution for HPLC was prepared by dissolving in HPLC methanol ($1\mu\text{g}\ \mu\text{L}^{-1}$) and kept at 4°C as a stock solution. Before use, a working solution was freshly prepared ($50\ \mu\text{g}\ \text{mL}^{-1}$). Hairy root tissue was crushed with 50% (v/v) methanol and the homogenized root material was centrifuged at 10000 g for 20 mins. The supernatant was collected and filtered. The filtered sample was transferred to a PhenomenexTM (Torrance, USA) C₁₈ column (Luna, 4 μm , 250 x 4.6 mm) using a JASCO HPLC (Tokyo, Japan) system equipped with a PU-2080 PlusTM pump and a PU-2075 PlusTM UV-VIS detector. For the mobile phase, an isocratic linear solvent system of acetonitrile and water (80: 20, v/v) with a flow rate of $1\ \text{mL}\ \text{min}^{-1}$ for 15 min was used to elute the plumbagin. The chromatogram was monitored at a wave length of 410 nm and analyzed on a MicrosoftTM Windows 2000TM platform with DataApex ClarityTM software (Prague, Czech Republic). Identification of plumbagin was performed on the basis of retention time and chromatographic behavior in comparison with an authentic standard.

Confirmation of rolB gene in hairy roots: The transformed root nature of two randomly selected hairy root clones derived from leaf explants of *P. indica* infected with *A. rhizogenes* ATCC 15834 was checked by detecting the rol B gene in their genomes by PCR analysis. For extraction of genomic DNA from hairy root clones and non-transformed *in vitro* root clones (negative control) the procedure of mini preparation of

plant genomic DNA was used [16]. Plasmid DNA from *A. rhizogenes* ATCC 15834 was isolated by the alkaline lyses method [17], which served as a positive control. The primer sequences (Bangalore Genie, India) used to amplify a 780 bp fragment of the *rolB* gene of the isolated DNA samples were: 5'-ATG GAT CCG AAA TTG CTATTC CTT CCA CGA-3'- Forward primer and 3'-TTA GGC TTC TTT CTT CAG GTT TAC TGCAGC-5' - Reverse primer.

A 25 μL volume of reaction mixture used consisted of 20 ng (2 μL) of template DNA, 100 ng (1 μL) of forward and reverse primer; 1 μL (2.5 mM) dNTPs; 0.5 μL (0.5 U) Taq DNA polymerase; 2.5 μL 10X Taq buffer, and 17 μL H₂O. PCR amplification was programmed at an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min, followed by final extension for 10 min at 72°C . Amplified products were separated by electrophoresis analyzed along with a 100 bp DNA ladder (Bangalore Genie, India) on a 1.5% agarose gel stained with ethidium bromide with 1% Tris borate EDTA (TBA) as running buffer.

Data analysis: All sets of experiments were carried out in triplicate and the data were analyzed statistically using SPSS software (10.0.5, 1999, SPSS Inc). Variability in data was expressed as the mean \pm standard errors.

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Fujianmycin C, A Bioactive Angucyclinone from a Marine Derived *Streptomyces* sp. B6219 [1]

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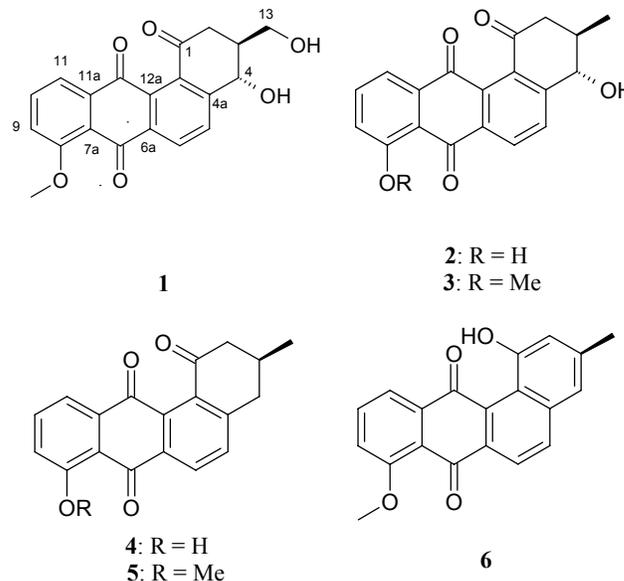
From a marine-derived streptomycete, a new bioactive angucyclinone, fujianmycin C (**1**), has been isolated along with five known, metabolites fujianmycins A (**2**) and B (**3**), ochromycinone (**4**), ochromycinone methyl ether (**5**), and tetrangulol methyl ether (**6**). The structure elucidation of fujianmycin C (**1**) was performed by detailed analysis of data such as ¹H, ¹³C, ¹H,¹H COSY, HSQC, HMBC and NOESY spectra. Fujianmycin C (**1**) exhibited antibacterial activity against *Streptomyces viridochromogenes* (Tü57).

Keywords: marine streptomycetes, angucyclinones, fujianmycin C.

Fujianmycins [2] are members of the angucyclinone family. Currently, more than 40 derivatives of the latter are known. They are the third class of quinone antibiotics after the tetracyclines and anthracyclines [3]. Most of them are found as free aglycones, and all are oxygenated at C-4. While the related linear anthracyclines usually have low pharmaceutical activities, the angucyclinones show pronounced antibacterial activity, such as inhibiting prolyl-endopeptidases of *Flavobacterium meningosepticum* non-competitively (IC₅₀ = 8.9 μM) [4], and enhancing the cytotoxicity of colchicine. Of special interest is the inhibition of mycobacteria [5] and of *Helicobacter pylori* [6,7]. Both angucyclines/angucyclinones and anthracyclines have been isolated from various streptomycete strains.

Extracts obtained from the marine derived *Streptomyces* sp. B6219 exhibited antibacterial activity against *Streptomyces viridochromogenes* (Tü57), *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli*. An unselective antitumor activity against six cancer cell lines was found with average IC₅₀ and IC₇₀ values of 17.0 and 36.0 μg/mL, respectively [8].

The strain B6219 was cultivated on M₂⁺ medium (50% seawater) and incubated on a linear shaker for 8 days. Both culture filtrate and mycelium were extracted with either XAD-16 resin or ethyl acetate, respectively.



Separation by chromatographic techniques (see experimental part) afforded fujianmycins A (**2**) and B (**3**), ochromycinone (**4**), ochromycinone methyl ether (**5**), tetrangulol methyl ether (**6**) and a new member of the angucyclinone family, fujianmycin C (**1**).

Fujianmycin C (**1**) and the other compounds were obtained as yellow needle crystals. On TLC they showed a UV absorbing band, which turned to yellow

with anisaldehyde/sulfuric acid spray reagent. Compounds **2** and **4** turned to violet with 2N NaOH indicating a *peri*-hydroxy quinone moiety, while the other compounds did not react. Due to this behavior and their similar UV/vis and NMR spectra, a group of related quinones was expected.

In the ^1H NMR spectrum of **1**, two doublets and a triplet of three adjacent aromatic protons and an AB system of two further protons in the *ortho* position in another ring appeared; the patterns were confirmed by COSY data. In the aliphatic region, a methoxy singlet at δ 3.95, two methylene groups at δ 3.82/3.75 (CH₂-13) and 2.99/2.77 (CH₂-2) and two methine signals at δ 4.75 (H-4) and δ 2.37 (H-3) were visible. The ^{13}C NMR spectrum displayed 20 signals, a ketone carbonyl, two quinone carbonyls, twelve sp^2 and five sp^3 carbon signals.

The CI mass spectrum afforded a *pseudomolecular* ion at m/z 353 $[\text{M} + \text{H}]^+$, and the HRESIMS of **1** delivered the formula C₂₀H₁₆O₆. A quinone nucleus was predicted based of the UV/vis data, the presence of carbonyl signals at δ 182.7 and 185.4 and the positive test for *peri*-hydroxy groups in the related components **2** and **4**.

The triplet of H-10 showed HMBC correlations with C-8 and 11a. The doublet at δ 7.66 (H-11) displayed a correlation with a quinone carbonyl and must be in a *peri*-position relative to the latter (C-8 or C-11). Further correlations (Figure 1) confirmed the methoxy group in the same ring, so that it must occupy the opposite *peri*-position (C-11 or C-8). Proton H-6 correlated with the second quinone carbonyl (C-7) and must be in a *peri*-position of a second ring on the same side as the methoxy group. Further HMBC and COSY correlations completed the angucyclinone skeleton, as drawn in Figure 1. Compound **1** is the new 13-hydroxyfujianmycin B and was named fujianmycin C. To date, it seems to be the second hydroxymethyl-angucyclinone after YM-181741 [9,10].

The relative configuration at C-3 and C-4 was supposed to be the same *trans* orientation as for fujianmycins A (**2**) and B (**3**). Force field calculations resulted in a *bis*-axial orientation of the hydrogens H-3 and H-4, which required a coupling constant of $J_{\text{H-3,4}} \sim 10$ Hz. This agrees well with the experimental value of 9.3 Hz for H-4. The small NOE between 3-H and 4-H seemed to indicate their *cis* orientation, but this was also observed for fujianmycin B (**3**) and was therefore neglected. As the observed optical rotation of **1** (+18°, MeOH) was nearly the same as for **2** (+17°, CHCl₃) and as their biosyntheses are certainly closely related, a (3*S*,4*S*) configuration can be assumed.

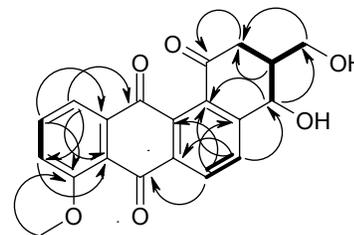


Figure 1: Selected H,H COSY (—) and HMBC (---) correlations of **1**.

Fujianmycin C (**1**) showed weak antibacterial activity against *Streptomyces viridochromogenes* Tü 57, causing inhibition zones of 14 mm at 40 μg / disk, but was not cytotoxic.

Further components were identified as fujianmycins A (**2**) [11] and B (**3**), ochromycinone (**4**) [12], ochromycinone methyl ether (**5**), and tetrangulol methyl ether (**6**) [13] by interpretation of their NMR spectroscopic data by means of AntiBase [14]. These compounds were responsible for the cytotoxicity of the crude extract and contributed also to its antibacterial properties. It is obvious that all these compounds have a close biosynthetic relationship.

Experimental

General experimental procedures: see ref. [15]. R_f values were measured on Polygram SIL G/UV₂₅₄ TLC cards from Macherey-Nagel & Co. (Düren, Germany) with CH₂Cl₂/5% MeOH.

Taxonomy of *Streptomyces* sp. B6219: Strain B6219 was derived from sediment of a mangrove forest in Galapagos and was isolated on casein peptone agar [16] containing 50% of natural seawater. The reference culture of B6219 is kept on yeast extract-malt extract agar in the Collection of Marine Actinomycetes at the Alfred-Wegener-Institute for Polar and Marine Research in Bremerhaven.

The almost complete 16S rRNA gene sequence of strain B6219 was deposited in GenBank under the accession no. HQ419059. The sequence is 99 % similar to that of the type strain *Streptomyces griseorubiginosus* (accession no. AJ781339).

The substrate mycelium is brown. Spores are light grey. Spore chains are straight to flexuous (*Rectiflexibiles*). The surface of spores is smooth to warty. Melanin pigment is produced on peptone-yeast extract-iron agar and tyrosine agar [17]. Optimum growth temperature is at about 30°C. The strain neither reproduces at 4°C nor at 45°C. The strain does not develop in media with 7% or higher seawater salinity. Starch and chitin are degraded, casein is not hydrolyzed [18]. The strain is

nitrate reductase positive. For a photograph, see supplementary material Figure S1.

Fermentation, extraction and isolation: The marine derived *Streptomyces* sp. B6219 was cultivated on M₂⁺ medium (10 g malt extract, 4 g glucose, 4 g yeast extract per L, 50% seawater) for 8 days and extracted with ethyl acetate (mycelium) and XAD-16 resin (filtrate), respectively, as described for related strains [19]. The resulting yellow brownish combined crude extract (6.43 g) was defatted using cyclohexane and chromatographed on a silica gel column using CH₂Cl₂/MeOH (gradient 0 to 10 % MeOH) to deliver 4 fractions FI-FIV. Fraction FII was purified on Sephadex LH-20 using MeOH to afford 2 sub-fractions. FIIa was subsequently purified using PTLC (CH₂Cl₂/5% MeOH) and again Sephadex LH-20 using MeOH to afford fujianmycin C (**1**, 1.90 mg, *R_f* = 0.38), while FIIb after purification on Sephadex LH-20 afforded fujianmycin A (**2**, 6.4 mg, *R_f* = 0.43) and ochromycinone (**4**, 1.41 mg, *R_f* = 0.36). Separation of FIII on silica gel (CH₂Cl₂/MeOH gradient 0 to 10 % MeOH) and Sephadex LH-20 delivered 2 sub-fractions: FIIIa afforded on PTLC (CH₂Cl₂/5% MeOH) fujianmycin B (**3**, 8.0 mg, *R_f* = 0.33; spectra see supplementary material), while FIIIb was purified on Sephadex LH-20 to deliver ochromycinone methyl ether (**5**, 1.4 mg, *R_f* = 0.40). FIV delivered, in a similar way, tetrangulol methyl ether (**6**, 1.0 mg, *R_f* = 0.30).

Fujianmycin C (**1**)

Yellow needles

R_f: 0.38 (CH₂Cl₂-MeOH, 95:5).

[α]_D²⁰: +18° (*c* 0.1, CH₃OH).

UV/VIS: λ_{max} (log ε) = (MeOH): 225 (4.41), 256 (4.46), 375 (3.87).

¹H NMR (300 MHz, CD₃OD): δ 8.33 (1H, d, *J* = 8.2 Hz, H-6), 8.04 (1H, d, *J* = 8.2 Hz, H-5), 7.79 (1H, t, *J* = 8.4, 7.6 Hz, H-10), 7.66 (1H, d, *J* = 7.6 Hz, H-11), 7.51 (1H, d, *J* = 8.5 Hz, H-9), 4.75 (1H, d, *J* = 9.3 Hz, H-4), 3.95 (3H, s, 8-OCH₃), 3.82, 3.95 (2H, ABX, *J* = 10.9, 5.5, 4.6 Hz, CH₂-13), 2.99, 2.77 (2H, ABX, *J* = 16.2, 6.3, 10.4 Hz, CH₂-2), 2.37 (1H, m, H-3).

¹³C NMR (125 MHz, CD₃OD): δ 200.0 (C_q-1), 185.4 (C_q-12), 182.7 (C_q-7), 161.5 (C_q-8), 153.6 (C_q-4a), 138.6 (C_q-11a), 137.0 (CH-10), 136.7 (C_q-12b), 135.3 (C_q-12a), 135.1 (C_q-6a), 132.3 (CH-5), 130.7 (CH-6), 121.5 (C_q-7a), 120.2 (CH-11), 119.1 (CH-9), 69.4 (CH-4), 63.3 (CH₂-13), 56.9 (8-OCH₃), 46.4 (CH-3), 41.0 (CH₂-2).

CIMS: *m/z* (%) 353 [M + H]⁺ (100).

HRESIMS: *m/z* 353 [M + H]⁺ calcd for C₂₀H₁₇O₆: 353.10196; found: 353.10205.

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Dioscorealide B from the Traditional Thai Medicine Hua-Khao-Yen Induces Apoptosis in MCF-7 Human Breast Cancer Cells via Modulation of Bax, Bak and Bcl-2 Protein Expression

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Dioscorealide B is a pharmacologically active compound from the rhizome of the Thai medicinal plant *Dioscorea membranacea*. Here, we demonstrated that *in vitro* treatment of dioscorealide B resulted in a cytotoxic effect on MCF-7 human breast cancer cells ($IC_{50} = 2.82 \mu\text{M}$). To determine whether this compound induces apoptosis in MCF-7, the Annexin V assay was performed. The data showed that the number of apoptotic cells were increased 7–12 folds over that of the control cells after treatment with various concentrations of dioscorealide B (3, 6 and 12 μM) for 24 hours. Dioscorealide B-induced apoptosis was associated with modulation of the multidomain Bcl-2 family members Bax, Bak and Bcl-2. After treatment with 3 μM dioscorealide B, acceleration of the level of proapoptotic proteins Bax and Bak were observed at 6 hours and 12 hours, respectively, while the decrease in the expression of antiapoptotic protein Bcl-2 was observed 3 hours after the treatment. These effects of dioscorealide B might result in the activation of caspase-8, -9 and -7, which lead to apoptosis in MCF-7 cells. Taken together, the results of this study provide evidence that dioscorealide B possesses an antitumor property against human breast cancer cells and thus provide the molecular basis for the further development of dioscorealide B as a novel chemotherapeutic agent for breast cancer treatment.

Keywords: Dioscorealide B, apoptosis, Bcl-2, MCF-7.

Breast cancer is the most common cancer in women worldwide, comprising 16% of all female cancers. It is estimated that 519,000 women died in 2004 due to this disease, and although it is thought to be a problem of the developed world, a majority (69%) of all breast cancer deaths occurs in developing countries [1]. The American Cancer Society estimated that 192,370 new cases of invasive breast cancer would be diagnosed in women in the U.S. and about 40,170 breast cancer deaths were expected in 2009 [2]. These statistics accentuate the immediate need for improvements in detection, diagnosis, and treatment of this disease. Breast cancer is currently controlled through surgery

and/or radiotherapy, and is frequently supported by adjuvant chemo- or hormonal therapies. However, these classical treatments are impeded by unwanted side effects and, most importantly, the development of tumor resistance. The medicinal use of naturally occurring substances or natural agents then becomes an alternative for the patients suffering from cancer [3,4].

Dioscorealide B (Figure 1) is a pharmacologically active compound from the ethanol extract of *Dioscorea membranacea* Pierre (Dioscoreaceae), a plant known in Thailand as "Hua-Khao-Yen", which is used in Thai traditional medicines as an ingredient in several

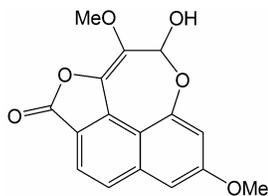


Figure 1: Chemical structure of dioscorealide B

preparations, including those used in the treatments of lymphopathy, dermatopathy, venereal diseases, leprosy, bacterial infections and cancers [5–7]. Previous study revealed that dioscorealide B, one of the isolated compounds from *D. membranacea*, has cytotoxic activity against MCF-7 human breast cancer cells and has been shown to serve as an anti-proliferative and selective cytotoxic agent. The compound selectively inhibited the proliferation of breast cancer cell line (MCF-7), without being significantly cytotoxic towards non-malignant cells (SVK) [8]. In the present study, some mechanisms related to apoptosis, which might underlie the antiproliferation activity of dioscorealide B, have been investigated.

In this study, the cytotoxic effect of dioscorealide B on MCF-7 was determined by SRB assay. Dioscorealide B showed a cytotoxic effect on MCF-7 with an IC_{50} value of $2.82 \pm 0.36 \mu\text{M}$ (Figure 2), whereas vinblastine, the positive control, had a stronger cytotoxic effect with an IC_{50} value of $1.65 \pm 0.13 \text{ nM}$. This result confirmed that dioscorealide B was able to inhibit *in vitro* growth of MCF-7.

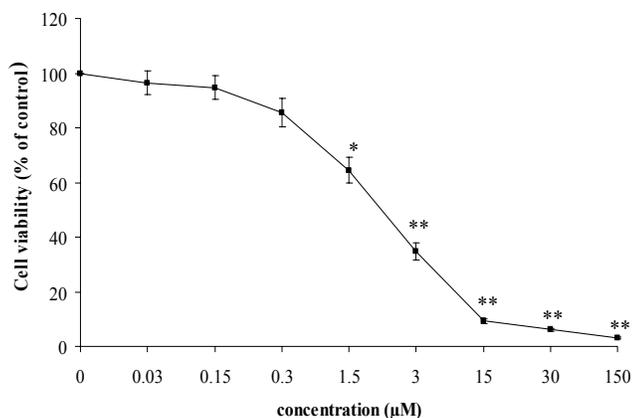


Figure 2: The antiproliferative effect of dioscorealide B on MCF-7 cells. MCF-7 cells were incubated with various concentrations of dioscorealide B. After 72 hours, cell proliferation was determined by SRB assay. Results are expressed as percent of viable cells in the studied group as compared with those in the control group. (* $p < 0.05$ and ** $p < 0.01$)

Cancer is an aberrant net accumulation of atypical cells which can arise from an excess of proliferation, an insufficiency of apoptosis, or a combination of the two [9]. Increased frequency of apoptosis could result in cell loss in tumors and promote tumor regression. In cancer

treatment, a major recent focus is on activating the apoptotic program in the cell [10], and there is evidence that many anticancer agents cause tumor cell death via the mechanisms of apoptosis [11–13]. Thus, we extended our study to examine whether the anti-tumor property against breast cancer cells is mediated through apoptosis induction after dioscorealide B treatment. MCF-7 cells were stained with Annexin V-FITC and PI, and then subsequently analyzed by flow cytometry. This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in the early apoptotic cells. MCF-7 cells were challenged with dioscorealide B at 3, 6 and 12 μM for 24 h. We found that dioscorealide B significantly induced apoptosis in a dose-dependent manner compared with control cells. The numbers of apoptotic cells in MCF-7 were increased to 7.02, 11.87 and 12.17 fold after 3, 6 and 12 μM dioscorealide B treatment, respectively (Figure 3B).

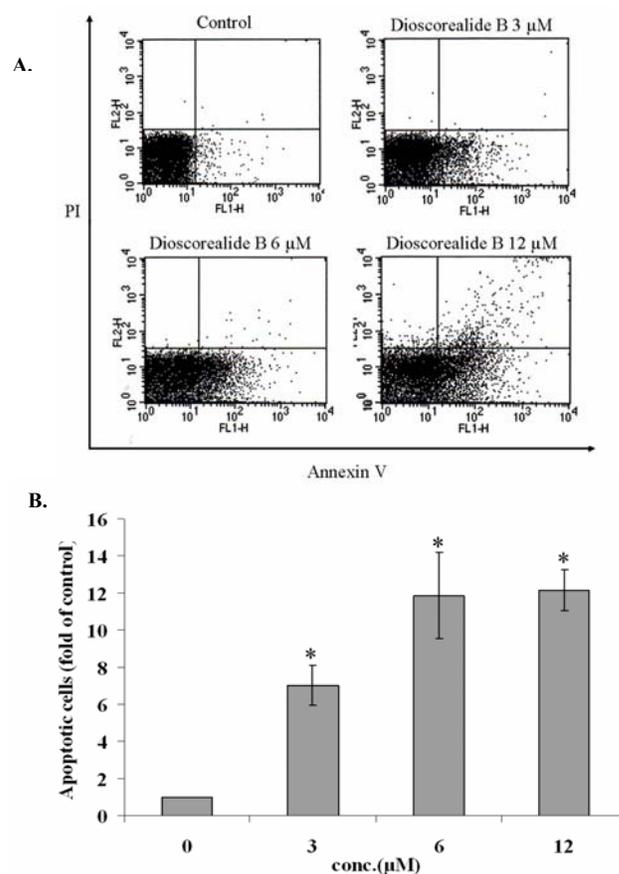


Figure 3: Induction of apoptosis in MCF-7 cells by dioscorealide B. MCF-7 cells were treated with dioscorealide B or vehicle (control) for 24 h. Apoptotic cells were detected as An+/PI- cells, while viable cells were An-/PI- cells and necrotic cells were PI+. Panel 3 A, representative dot plots after five days of dioscorealide B exposure. Panel 3 B, the fold increase of apoptotic cells compared with control was determined from three experiments (mean \pm SD). Statistical analysis on log transformed data; asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

Caspases are members of a family of cysteine proteases that play a crucial role in the apoptotic pathway [14,15]. There are two major mechanisms that initiate the caspase cascade: the extrinsic, involving caspase-8; and the intrinsic pathway, involving caspase-9 as the apical caspase, which leads to the proteolytic activation of effector caspases, including caspase-3 and -7, which cleave the cellular substrate, resulting in cell death [16,17]. The extrinsic pathway is triggered by the interaction between a death ligand, such as TNF or FasL, and its cognate receptor, TNF-R or Fas (CD95), inducing the trimerization of the receptors, which recruit adaptor proteins such as FADD and procaspase-8, leading to the activation of caspase-8 [18,19]. The intrinsic pathway is regulated by the Bcl-2 family of proteins, including proapoptotic members such as Bax and Bak, and anti-apoptotic members such as Bcl-2 and Bcl-X_L [20-22]. In response to apoptotic signals, proapoptotic Bcl-2 family members translocate to and alter the permeability of the mitochondrial membrane [23]. These proteins are thought to either form channels in the outer mitochondrial membrane or to alter the activity of existing channels, leading to changes in the mitochondrial membrane potential and cytochrome c release. Cytochrome c then interacts with Apaf-1 and procaspase-9, which in turn activates caspase-9 [24,25].

To gain insights into the mechanism of apoptosis induction in MCF-7, the effect of dioscorealide B treatment on levels of Bcl-2 family proteins by western blotting was studied and the results are shown in Figure 4. Following treatment with 3 μM of dioscorealide B, the formation of anti-apoptotic protein Bcl-2 was down-regulated after three hours, whereas the expression of proapoptotic protein Bax was upregulated after six hours. The dioscorealide B treatment also caused an increase in the protein level of Bak, as observed at twelve hours.

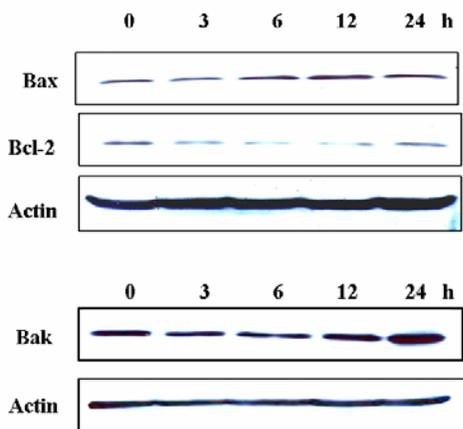


Figure 4: Immunoblotting for Bax, Bak and Bcl-2 using lysates from MCF-7 cells treated with 3 μM dioscorealide B for the indicated time periods. The blots were stripped and reprobbed with anti-actin antibody to normalize for differences in protein loading.

Next, we examined whether the caspases are involved in dioscorealide B-induced apoptosis. Immunoblot analyses of lysates obtained from MCF-7 cells treated with dioscorealide B at 3 μM were examined and results are shown in Figure 5. The cleaved form of caspase-7 increased after three hours and procaspase-7 was totally cleaved after twenty-four hours of treatment, suggesting that this compound induces the activation of caspase-7. Dioscorealide B increased the activity of caspase-7 activity in a dose- and time-dependent manner, as demonstrated in Figures 6 and 7. After incubation with 3 μM of dioscorealide B, the caspase-7 activity significantly increased to 230% at six hours. The effect of dioscorealide B on caspase-8 and caspase-9 activities is shown in Figure 8. Our findings revealed that activation of caspase-9 significantly increased after treatment of the cells with dioscorealide B, starting from three hours of exposure time, but caspase-8 activity showed a significant elevation after only one hour of treatment.

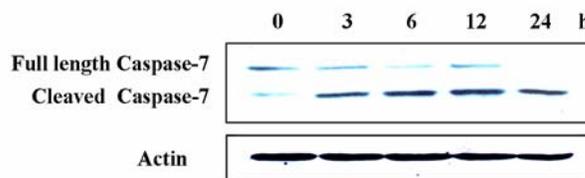


Figure 5: Western Blot analysis of apoptotic proteins in dioscorealide B treated cells. Proteins from MCF-7 cells treated with 3 μM of dioscorealide B for the indicated times were resolved on 12% SDS-PAGE and subjected to Western Blotting analysis with antibodies against caspase-7 and actin.

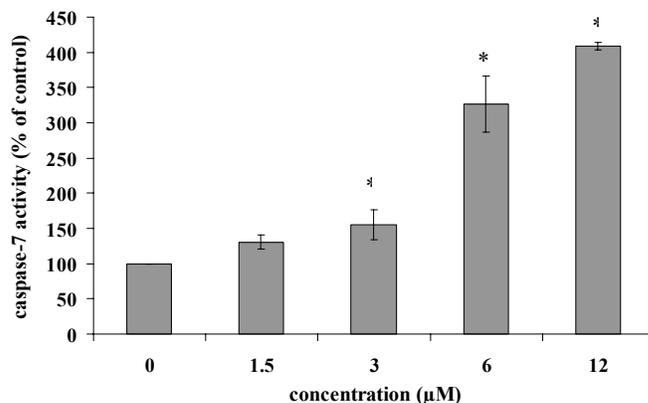


Figure 6: The effect of induction of caspase-7 activity by dioscorealide B in MCF-7 breast cancer carcinomas. MCF-7 cells were treated with 1.5, 3, 6 and 12 μM of dioscorealide B for 3 h. The data represent the mean value of 3 replications from 2 independent experiments.

In a further experiment, the effect of caspase inhibitors on dioscorealide B-induced apoptosis was studied. In MCF-7, cells were pretreated with either 50 μM of the caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK for 3 h prior to treatment with 3 μM of dioscorealide B. As shown in

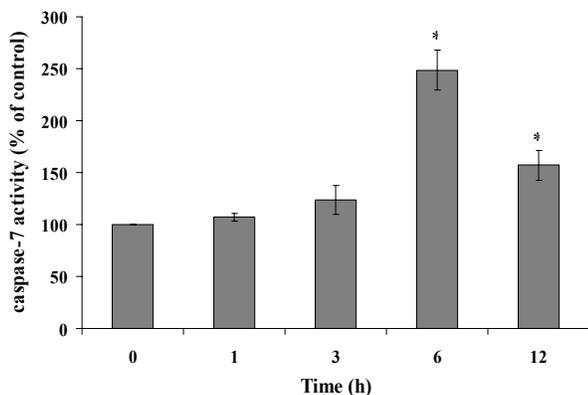


Figure 7: The activation of caspase-7 in MCF-7 by dioscorealide B. MCF-7 cells were incubated with 3 μ M of dioscorealide B for the indicated times. The data represent the mean value of 3 replications from 2 independent experiments. Asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

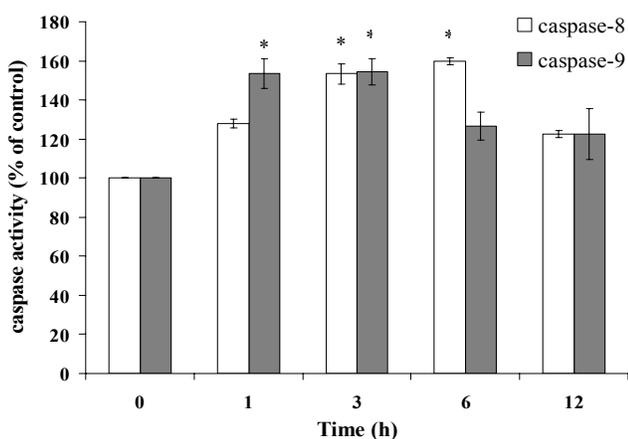


Figure 8: The activation of caspase-8 and caspase-9 in MCF-7 by dioscorealide B. MCF-7 cells were incubated with 3 μ M of dioscorealide B for the indicated times. Asterisks indicate ANOVA significance $p < 0.05$ versus corresponding control.

Figure 9, pretreatment of MCF-7 cells with either the caspase-8 inhibitor or the caspase-9 inhibitor significantly decreased the caspase-7 activity. These results suggested that this bioactive compound might be involved in both intrinsic and extrinsic apoptotic pathways. This observation further supports the fact that induction of apoptosis in MCF-7 cells by dioscorealide B is mediated through reduction of antiapoptotic protein Bcl-2 expression and induction of proapoptotic protein Bax and Bak expression, leading to the activation of caspase-9 and -7, respectively. However, the mechanism of dioscorealide B in the extrinsic pathway needs to be refined. The role of the TNF superfamily such as Fas, Fas ligand, TNF-R1 and TNF- α might be investigated in the further study. In this study, we found that dioscorealide B caused an increase in both caspase-8 and -9 activities. Bid, a BH3 domain-containing proapoptotic Bcl-2 family member, might be involved in dioscorealide B-induced apoptosis. Bid is a specific proximal substrate of caspase-8 in the Fas apoptotic

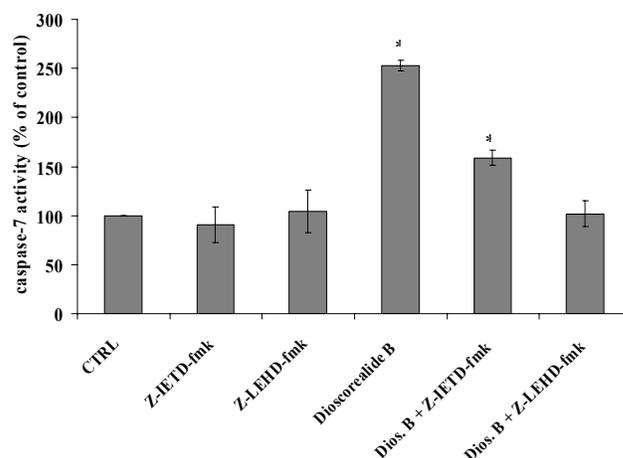


Figure 9: The effect of caspase-8 and -9 inhibitor on dioscorealide B-induced apoptosis. MCF-7 cells were pre-incubated with Z-IETD-FMK (50 μ M) for 3 h before challenge with 3 μ M dioscorealide B. After 6 h of treatment, caspase-7 activity was measured by caspase-Glo[®] assay. Each value is the mean \pm SD of 3 determinations. The asterisk indicates a significant difference between control and dioscorealide B-treated cells, as analyzed by ANOVA $p < 0.05$.

signaling pathway. While full-length BID is localized in cytosol, truncated BID (tBID) translocates to mitochondria and, therefore, transduces apoptotic signals from the cytoplasmic membrane to mitochondria. tBID induces first the clustering of mitochondria around the nuclei and release of cytochrome c independent of caspase activity, and then the loss of mitochondrial membrane potential, cell shrinkage, and nuclear condensation in a caspase-dependent fashion [26].

In conclusion, for the first time, the mechanisms of dioscorealide B against breast cancer were elucidated. The results of the present study indicated that dioscorealide B treatment decreased the expression of antiapoptotic protein Bcl-2 and increased the expression of proapoptotic proteins Bax and Bak, which leads to the activation of caspase-9 and -7, respectively. These studies thus provide the molecular basis for the further development of dioscorealide B as a novel chemotherapeutic agent for breast cancer treatment.

Experimental

Materials: Reagents were purchased from the following suppliers: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin from Gibco BRL (Gaithersburg, MD, USA); anti- β -actin antibody from Sigma-Aldrich Co. (St. Louis, MO, USA); antibodies against Bak and caspase-7 from Cell Signaling (Beverly, MA, USA); antibody against Bcl-2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-Bax antibody from US biological (Massachusetts, USA), Annexin V-FITC and propidium iodide (PI) from BD

Pharmingen (Franklin Lake, NJ, USA), Caspase-Glo[®] assay from Promega (Madison, WI), caspase-8 inhibitor (Z-IETD-FMK) and caspase-9 inhibitor (Z-LEHD-FMK) from Calbiochem (Darmstadt, Germany).

Plant materials: The rhizomes of *D. membranacea* Pierre (Dioscoreaceae) were collected from Pa-tue, Chumporn, Thailand. Authentication of plant materials was carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium voucher (SKP A062041305) is kept. Specimens are also kept in the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand.

Isolation of dioscorealide B: Dioscorealide B was isolated following the method previously described and agreed in all respects as regards reported chromatographic and spectral data [8].

Cell culture conditions: MCF-7 human breast cancer cell line was kindly provided by Dr P. Twentyman and Dr P. Rabbits of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK. Cells were cultured in monolayers in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin and maintained at 37°C in a humidified atmosphere of 5% CO₂.

SRB assay: The cytotoxicity assay was carried out using the sulforhodamine B (SRB) assay [8,27]. Briefly, 3,000 of MCF-7 cells were plated per well in 96-well culture plates kept in an incubator at 37°C. After overnight incubation, the cells were treated without or with dioscorealide B 0.03, 0.15, 0.3, 1.5, 3, 15, 30, 150 µM, with 6 replications. The cells were incubated for 72 h and then the medium was removed and washed. The survival percentage was measured colorimetrically using the SRB assay, and IC₅₀ values were calculated by means of the Prism program. Cells incubated with regular cell culture media with 0.2% DMSO were used as a negative control and vinblastine sulfate as a positive control.

Annexin V- FITC assay: Induction of apoptosis by dioscorealide B treatment was measured by an Annexin V-FITC apoptosis detection kit, following the manufacture's instructions. Briefly, MCF-7 cells (1×10⁶ cells) were seeded in 12-well plates and treated with dioscorealide B at 3, 6 and 12 µM. After 24 h, cells were collected, washed with cold PBS twice, gently

resuspended in 100 µl of staining solution (containing annexin V, fluorescein and propidium iodide in a HEPES buffer). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and propidium iodide).

Caspase-7, -8 and -9 activity assay: The Caspase-Glo[®]-3/7, -8 and -9 assays were used to measure caspase-3/7, caspase-8 and caspase-9 activity. Briefly, 10⁴ cells were cultured in 96-well plates and treated with dioscorealide B (3, 6 or 12 µM). After the incubation period, caspase-Glo[®] reagent was added to each well according to the manufacturer's instructions. Plates were mixed on a plate shaker for 30 secs and incubated at room temperature for 1 h. Luminescence was measured using a luminometer. The assay was performed in triplicate.

Western blotting: After treatment, cells were washed with ice-cold PBS and lysed with SDS lysis buffer. The protein concentration of the supernatant was determined by the Bradford method. The lysates were subjected to electrophoresis on a 12% SDS-polyacrylamide gel and then transferred to a PVDF membrane. After the membrane was blocked in Tris-buffer saline (TBST) containing 0.05% Tween 20 and 5% nonfat powdered milk, the membranes were incubated with primary antibodies specific for Bax, Bak, Bcl-2 and caspase-7. After washing 3 times with TBST for 10 min each, the membrane was incubated with horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA).

Statistics: Data were expressed as means ± SEM. Statistical comparisons of the results were made using analysis of variance (ANOVA) and a *P* value less than 0.05 was considered significant.

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Inhibition of Protein Tyrosine Phosphatase 1 β by Hispidin Derivatives Isolated from the Fruiting Body of *Phellinus linteus*

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Protein tyrosine phosphatase 1 β (PTP1 β) acts as a negative regulator of insulin signaling. Selective inhibition of PTP1 β has served as a potential drug target for the treatment of type 2 diabetes mellitus. We evaluated the inhibitory effect of *Phellinus linteus* against PTP1 β as part of our ongoing search for natural therapeutic and preventive agents for diabetes mellitus. Fractions of the *P. linteus* extract were found to exhibit significant inhibitory activities against PTP1 β . In an attempt to identify bioactive components, we isolated, from the most active ethyl acetate fraction, five hispidin derivatives (phelligradimer A, davallialactone, hypholomine B, interfungins A, and inoscavin A) and four phenolic compounds (protocatechuic acid, protocatechualdehyde, caffeic acid, and ellagic acid). The chemical structures of these compounds were elucidated from spectroscopic evidence and by comparison with published data. All the compounds strongly inhibited PTP1 β activity in an *in vitro* assay; their IC₅₀ values ranged from 9.0 \pm 0.01 to 58.2 \pm 0.3 μ M. Our results indicated that the hispidin skeleton may be an important moiety for inhibitory activity of the above compounds against PTP1 β . Thus, hispidin derivatives could be a potent new class of natural PTP1 β inhibitors.

Keywords: *Phellinus linteus*, hispidin, protein tyrosine phosphatase 1 β , diabetes.

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important signaling molecules, such as members of the MAP kinase family. Among them, protein tyrosine phosphatase 1 β (PTP1 β) plays a critical role in the signal transduction of both the insulin and leptin pathway [1,2]. This role has been demonstrated for the dephosphorylated insulin receptor and other proteins in intact cells and thus, PTP1 β can act as a negative regulator of the insulin signaling pathway [3]. Also, the deletion of the PTP1 β gene in mice has been reported to induce marked insulin sensitivity and prolonged the autophosphorylation of the insulin receptor [4]. PTP-1 β inhibitors would increase insulin sensitivity by blocking the PTP1 β -mediated

negative regulation of the insulin signaling pathway [5]. Therefore, PTP1B has emerged as a novel target for the treatment of diabetes and obesity, with numerous compounds having been developed as PTP1 β inhibitors [6]. Small molecule inhibitors of PTP-1 β , such as oxalamides, benzoic acid, and phenoxyacetic acids were reported [7]. Of them, only ertiprotafib progressed to clinical trials prior to discontinuation at phase II due to insufficient efficacy, coupled with unwanted side effects [8]. Although several types of PTP1 β inhibitors have been reported, they have low selectivity and poor pharmacokinetic properties; therefore, new types of PTP1 β inhibitors with improved pharmacological properties are still being sought.

Phellinus linteus (Berkeley & M. A. Curtis), an orange colored mushroom in the family Hymenochaetaeaceae,

Table 1: Inhibitory effects on PTP1 β of MeOH extract and its fractions isolated from the fruiting body of *P. linteus*.

Fractions	PTP1 β inhibitory activity ^a (μ g/mL)
MeOH Extract	23.4 \pm 1.9
<i>n</i> -hexane fr.	-
Methylene chloride fr.	-
Ethylacetate fr.	17.2 \pm 0.8
<i>n</i> -BuOH fr.	35.9 \pm 2.1
Water fr.	-

^a IC₅₀ values were determined by regression analyses and are expressed as mean \pm SD of 3 replicates.

has been used in oriental countries for centuries to prevent ailments such as gastrointestinal dysfunction, diarrhea, hemorrhage, inflammation, and cancer [9]. Although they produce a large and diverse variety of secondary metabolites, polysaccharides have been considered to be responsible for their biological effect. β -Glucans derived from *P. linteus* act as an effective immunomodulator, exhibit a wide range of antitumor activity and have been shown to prevent metastasis [10]. Also, polysaccharide of *P. linteus* has been reported to be effective for diabetes in non-obese diabetic mouse [11].

A recent investigation of the chemical constituents of the genera *Inonotus* and *Phellinus* resulted in isolation of hispidin derivatives with a novel carbon skeleton, such as phellifuropyranone A [12], phellinusfurans A and B [13], phellinone [14], phellinsin A [15], inoscavins A–D and methylinoscavin A–D [16,17], phelligrindins A–G [18–20] and interfungins A–C [21]. However, there have been few studies on the biological activities of these compounds. In our recent study, hispidin derivatives from *P. linteus* exerted potent antioxidant effects, as well as marked inhibitory effects on rat lens aldose reductase and advanced glycation end product formation, corresponding to high values of total phenolic content and total flavonoid content [22,23].

While screening for PTP1 β inhibitors from natural resources, the MeOH extract of the fruiting body of *P. linteus* was found to inhibit PTP1 β activity at a concentration of 16.3 μ g/mL; this led us to investigate the PTP1 β inhibitory components from this plant. In this paper, we report the isolation and structural elucidation of active principles with an inhibitory activity against PTP1 β .

To identify the active principles, we performed bioassay-guided fractionation and purification by using repeated column chromatography, which resulted in 5 hispidin derivatives and 4 phenolic compounds (Figure 1). The isolated compounds were identified by analysis of their NMR and MS data as phelligrindimer A (1), davallialactone (2), hypholomine B (3), interfungin A (4) and inoscavin A (5), as well as hispidin (6), protocatechuic acid (7), protocatechualdehyde (8), caffeic acid (9), and ellagic acid (10). In addition, these

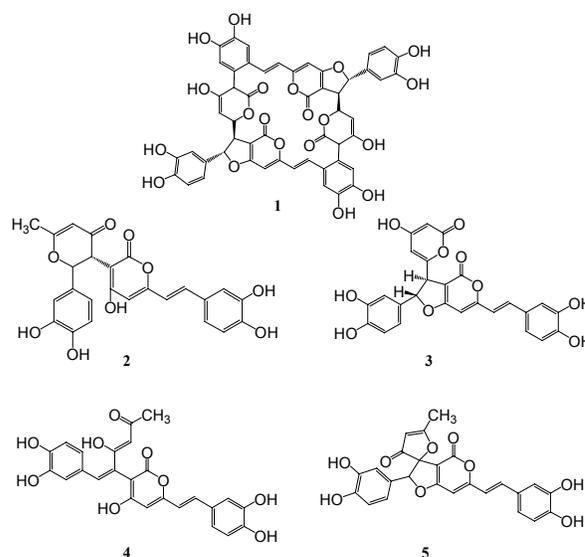


Figure 1: Chemical structures of the compounds isolated from *P. linteus*. 1: phelligrindimer A; 2: davallialactone; 3: hypholomine B; 4: interfungin A; 5: inoscavin A.

data were compared with those found in the literature [24–27]. The inhibitory activities of the isolated compounds against PTP1 β were measured using *p*-nitrophenyl phosphate (*p*NPP) as the substrate; the results are summarized in Table 2. The known PTP1 β inhibitor, ursolic acid (IC₅₀ = 3.9 \pm 0.3 μ M), was used as a positive control. The isolated compounds 1–10 dose-dependently inhibited the PTP1 β activity with IC₅₀ values ranging from 9.0 \pm 0.6 to 58.2 \pm 3.4 μ M.

The hispidin derivatives 1–6 were found to show greater inhibitory activities against PTP1 β than the phenolic compounds 7–10. Of the hispidin derivatives, compounds 1 and 3 with a hispidin moiety exhibited a greater inhibitory activity against PTP1 β than compounds 2, 4, and 5, which have a hispidolone moiety. As shown in Table 2, the inhibitory effects of the hispidin derivatives were enhanced when a hispidin dimer or tetramer was formed. Among the hispidin derivatives, compound 1, a hispidin tetramer, exhibited the highest inhibitory activity against PTP1 β with an IC₅₀ of 8.9 \pm 0.6 μ M. This result indicates that compound 1 is approximately 2- to 3-fold more effective than other hispidin derivatives. Compounds 1–6 are highly oxygenated and functionalized aromatic compounds that possess the unique basic structural unit, namely, 6-[2-(3,4-dihydroxyphenyl)ethenyl]-4-hydroxy-2H-pyran-2-one (hispidin, 6). It has been reported that compound 3, which has an unusual structure with 2,3-dihydro-4-H-furo[3,2-c]pyran-4-one, may be biosynthesized by oxidative coupling of two hispidin mediates, while compound 1 may be sequentially or simultaneously formed by oxidative coupling of four hispidin mediates and/or by oxidative coupling of two hypholomine B molecules [24].

Table 2: Inhibitory effects of compounds isolated from the fruiting body of *P. linteus* on PTP1 β .

Compounds	Concentration (μ M)	Inhibition (%)	IC ₅₀ (μ M)
Phelligradimer A (1)	10	56	8.9 \pm 0.6
	5	29	
	1	9	
Davallialactone (2)	25	51	24.2 \pm 1.0
	10	26	
	2.5	11	
Hypholomine B (3)	25	91	12.9 \pm 0.9
	10	42	
	2.5	12	
Interfungins A (4)	25	59	20.1 \pm 1.7
	10	32	
	2.5	12	
Inoscavin A (5)	50	95	27.3 \pm 2.1
	25	46	
	10	15	
Hispidin (6)	50	80	33.6 \pm 3.8
	25	32	
	10	10	
Protocatechuic acid (7)	100	88	52.9 \pm 3.6
	50	48	
	10	15	
Protocatechualdehyde (8)	100	82	58.6 \pm 3.4
	50	42	
	10	14	
Caffeic acid (9)	100	93	44.5 \pm 2.5
	50	52	
	10	25	
Ellagic acid (10)	50	88	26.2 \pm 1.9
	25	49	
	10	23	
Ursolic acid ^b	5	65	3.9 \pm 0.3
	2.5	30	
	1	10	

^aIC₅₀ values were determined by regression analyses and are expressed as mean \pm SD of 3 replicates. ^bPositive control.

Compounds **2** and **5** are known to be biosynthesized by rearrangement of compound **4**, which is biosynthesized by the condensation of hispidin and 6-(3,4-dihydroxyphenyl)-4-hydroxy-3,5-hexadiene-2-one (hispolon) [21]. This suggests that a dihydrofuro[3,2-c]pyran-4-one moiety is formed by a cyclization linkage between the A and B rings via an oxygen-bridge that might increase the inhibitory activity against PTP1 β . Phelligradins H and I have an unprecedented carbon skeleton (pyrano-[4,3-c]isochromen-4-one) as the structural element. A previous study reported inhibitory activity of these 2 derivatives from *P. igniarius* against PTP1 β [20]. Although the structure-activity relationships of the hispidin derivatives were not thoroughly investigated, the dihydrofuro[3,2-c]pyran-4-one moiety appeared to correlate with the inhibitory activity against PTP1 β . The hispolon moiety might be responsible for a loss of PTP1 β activity. Thus, the inhibitory activities of the hispidin derivatives might provide valuable information regarding the structure-activity relationship for the development of novel PTP1 β inhibitors.

Experimental

Plant materials: The fresh fruiting bodies of *P. linteus* were provided by Samsung Herb Medicine Co., Ltd.,

Chuncheon, Korea and the voucher specimen (No. RIC-021) was deposited at the Regional Innovation Center, Hallym University, Republic of Korea.

Chemicals and equipment: ¹H and ¹³C NMR spectra were recorded on a Bruker DPX 400 spectrometer. Sephadex LH 20 (GE Healthcare Bio-Science AB, Sweden) and cosmosil 75C18-OPN (Nacalai tesque, Kyoto, Japan) were used as column packing material. PTP1 β (human, recombinant) drug discovery kit was purchased from BIOMOL[®] International Inc. (USA).

Isolation and identification: The lyophilized fruiting bodies of *P. linteus* (1 kg) were extracted 3 times with MeOH for 5 h. After removal of MeOH under reduced pressure, a dark brown residue (50 g, 5%) was obtained. This was suspended in water and then partitioned sequentially with *n*-hexane (8 g, 0.8%), methylene chloride (4.9 g, 0.5%), ethylacetate (EtOAc, 22 g, 2.2%), *n*-butanol (14.1 g, 1.4%), and water (1 g, 0.1%). Sequential fractionation indicated that the EtOAc-soluble fraction showed PTP1 β inhibitory activity. Therefore, this fraction was chromatographed over Sephadex LH 20 eluting with MeOH. Among the 8 pools (Fr.1-Fr.8), combined by their HPLC profiles, Fr. 7 was found to have the highest activity. Mixed frs. 5, 7, and 8 was subjected to Lichroprep[®] RP-18 CC using a stepwise gradient of AcCN-H₂O (1:4 to 4.5:5.5) to afford compounds **1-6**. Fractions 1-3 were each purified by reversed phase HPLC using an isocratic solvent system of 15% AcCN to isolate compounds **7-9**, respectively. Compound **10** was obtained as crude crystals from Fr. 4, which was further purified by HPLC using an isocratic solvent system of 20% AcCN. The structures of **1-10** were identified by comparison of observed spectroscopic data with published values.

Assay for PTP1 β inhibitory activity: The PTP 1 β tyrosine phosphatase drug discovery kit is a colorimetric, non-radioactive assay designed to measure the phosphatase activity of purified PTP1 β [28]. The enzyme activity was measured by using IR5 phosphopeptide (Insulin Receptor B residues 1142-1153, pY-114) as a substrate. To each 96-well (final volume: 125 μ L) was added 75 μ M IR5 substrate and PTP1 β (2.5 ng/well) in a buffer containing 100 mM MES (pH 6.0), 0.3 M NaCl, 2 mM EDTA, 2 mM dithiothreitol (DTT) and 0.1% NP-40 with or without test compounds. Following incubation at 37°C for 30 min, the reaction was terminated with BIOMOL REDTM reagent. The amount of produced *p*-nitrophenol was estimated by measuring the absorbance at 620 nm. The non-enzymatic hydrolysis of IR5 substrate was corrected by measuring the increase in absorbance at 620 nm obtained in the absence of PTP1 β enzyme.

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A New Azafluorenone from the Roots of *Polyalthia cerasoides* and its Biological Activity

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Chromatographic separation of the ethyl acetate extract of roots of *Polyalthia cerasoides* has led to the isolation of the new compound, 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone. This compound exhibited potent cytotoxic activities with IC₅₀ values in the range of 2.64-3.58 $\mu\text{g}\cdot\text{mL}^{-1}$ for A549, GLC4 and GLC4/Adr cells, but was not recognized by ABC11/MRP1 protein. The compound also showed very strong inhibition of *M. tuberculosis* using a broth microdilution method, with an MIC value of 0.78 $\mu\text{g}\cdot\text{mL}^{-1}$, which was equal to that of ofloxacin, one of the four antibiotic drugs used as a positive control.

Keywords: *Polyalthia cerasoides*, Annonaceae, azafluorenone, antimycobacterial activity, cytotoxicity.

Polyalthia cerasoides (Roxb.) Bedd. (Annonaceae) is a medium-sized tree up to 5-15 meters tall, occurring mainly in Asiatic and Oceanic areas such as Burma (Myanmar), South China, India, Indochina, Thailand, and Laos (Khammouan) [1a,1b]. Previous phytochemical studies of various parts of *P. cerasoides* have resulted in the characterization of a benzofuran derivative [2a], oxoprotoberberine alkaloids [2b], dimeric aporphine alkaloids, a sesquiterpene, an isoquinoline alkaloid and triynoic acid [2c], and phytosterols [2d]. The constituents of *Polyalthia* species have shown remarkable antibacterial [2e], antifungal [3a], cytotoxic [3b], and antimalarial [2e] properties. In traditional Thai medicine, *P. cerasoides* is used for the treatment of tuberculosis. Based on already known preliminary data from bioassay-guided fractionation, we undertook further cytotoxicity studies of the crude *n*-hexane, ethyl acetate, acetone and methanol extracts. We present here the results obtained from testing the ethyl acetate extract for cytotoxic activity. From this extract, a new azafluorenone was isolated and examined for its antimycobacterial activity.

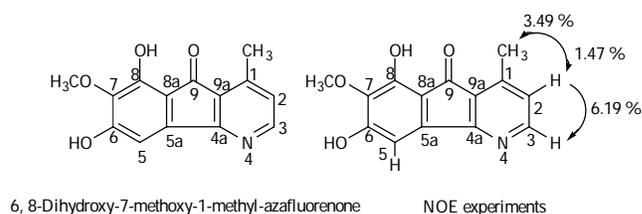


Figure 1: Chemical structure and NOE experiments.

The new azafluorenone was isolated as yellow needle crystals from ethanol (mp 200.9-202.7°C). In the ESIMS, the molecular weight was indicated by the HRAPCIMS peak at m/z 257 [M^+], in combination with the measurement of the [$M+H$]⁺ ion at m/z 258.0764 (calcd. 258.0761 for $C_{14}H_{12}NO_4$). The presence of a hydroxyl group was suggested in the mass spectrum by a fragment ion base peak at m/z 239 (100) due to the direct loss of an 18 a.m.u. water unit from the $C_{14}H_{12}NO_4^+$ ion. In addition, the fragment ions at m/z 242 (M^+-Me) and 224 (M^+-Me-H_2O) in the mass spectrum indicated the presence of an OMe group in the position next to the OH group. As a unique group of alkaloids, azafluorenones

Table 1: ^{13}C NMR and ^1H NMR spectroscopic data of the azafluorenone and ^1H - ^{13}C and ^1H - ^1H correlations exhibited in the 2D NMR spectra in CDCl_3 .

Carbon	$\delta^{13}\text{C}$ (DEPT)	$\delta^1\text{H}$ (J/Hz)	HMBC correlation	COSY correlation
1	147.41 (C)	-	H-1CH ₃ , H-3	-
2	124.16 (CH)	6.80 (1H, d, 5.53)	H-1CH ₃ , H-2, H-3	H-3, H-1CH ₃
3	150.80 (CH)	8.16 (1H, d, 5.53)	H-2	H-2
4	-	-	-	-
4a	166.35 (C)	-	H-3, H-5	-
5	104.30 (CH)	6.87 (1H, s)	-	-
5a	125.35 (C)	-	-	-
6	151.89 (C)	-	H-5	-
7	139.13 (C)	-	H-7OCH ₃ , H-5	-
8	145.92 (C)	-	-	-
8a	119.75 (C)	-	H-5	-
9	191.58 (C=O)	-	H-1CH ₃	-
9a	129.72 (C)	-	H-1CH ₃ , H-2	-
1-CH ₃	17.09 (CH ₃)	2.56 (3H, s)	-	H-2
7-OCH ₃	60.94 (OCH ₃)	4.13 (3H, s)	-	-

show a characteristic UV spectrum; the substituted onychines (1-methyl-4-azafluorenone) give two absorption bands in the UV spectrum (MeOH); band I at 207 nm (2.35) and band II at 269 nm (2.48) that exhibit $\pi \rightarrow \pi^*$ of an aromatic unit together with a double bond, and $n \rightarrow \pi^*$ from a conjugated carbonyl chromophore. In the IR spectrum, absorption bands attributable to hydroxyl (3526 cm^{-1} and 3329 cm^{-1}) and the carbonyl of an unsaturated ketone (1701 cm^{-1}) were observed. In addition, a C-O stretching vibration occurs at 1350 cm^{-1} indicating an ether moiety.

The ^1H NMR spectrum displayed only three unique signals: three aromatic [δ_{H} 6.80, d, $J = 5.53\text{ Hz}$; 6.87, s, and 8.16, d, $J = 5.53\text{ Hz}$], one methoxy resonance (δ_{H} 4.13, s) and one C-methyl signal (δ_{H} 2.56, s). All the direct proton-carbon connectivities were assigned following HMQC analysis. Using COSY, ^1H - ^1H coupling constants and HMBC correlations, a trisubstituted pyridine moiety was readily established. HMBC correlations from protons of the methyl group at δ_{H} 2.56 (1-Me) to three carbons of the pyridine unit [δ_{C} 147.41(C-1), 129.72 (C-9a), and 124.16 (C-2)] indicated its *para*-position relative to the pyridine ring system. An additional weak HMBC correlation from this methyl group to a carbon at δ_{C} 191.58 (C-9) suggested that the carbonyl functionality was attached *ortho* to the methyl moiety. Moreover, three carbon signals of the pyridine ring at δ_{C} 166.35 (C-4a), 147.41 (C-1) and 124.16(C-2) were HMBC correlated with the proton signals at δ_{H} 8.16 (H-3), 6.87 (H-5) and 8.16 (H-2). The one aromatic proton at δ_{H} 6.87 (s) exhibited HMBC correlations to three quaternary carbons [δ_{C} 151.89 (C-6), 139.13 (C-7), and 119.75 (C-8a)], three of which emerged to be connected to an oxygen heteroatom on account of their ^{13}C chemical shifts. The HMBC spectrum of this compound, which provided reciprocity from δ_{H} 4.13 (7-OMe) to the carbon at δ_{C} 139.13, clearly showed that the methoxy group was located on an aromatic ring. An additional strong HMBC correlation from proton δ_{H} 6.87 (5-H) to a carbon at δ_{C} 139.13 suggested that a methoxy group was located

at the C-7 position. Moreover, a series of NOE experiments were carried out in order to assign the methoxy group at position 7. Enhancements were observed in the pyridine ring, but not in the phenolic ring (Figure 1). Based on the above data, the compound was identified as 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone, an onychine analogue not previously reported.

The inhibition effects of *P. cerasoides* root extracts were investigated using the MTT assay. The IC_{50} value was determined against three human lung cancer cell lines. Of the four extracts, the ethyl acetate one exhibited the highest antiproliferative activity with IC_{50} values of 2.7, 4.3 and $3.4\text{ }\mu\text{g}\cdot\text{mL}^{-1}$ for A549, GLC4 and GLC4/Adr cells, respectively (Table 2). Strong growth inhibitory activity was also found for the *n*-hexane and acetone extracts with IC_{50} values in the range from 3.5 to $11.4\text{ }\mu\text{g}\cdot\text{mL}^{-1}$; the methanol extract was only weakly active. The ethyl acetate extract was, therefore, considered for subsequently purification and the new active compound, 6,8-dihydroxy-7-methoxy-1-methyl-azafluorenone, was isolated. This compound showed inhibitory activity against the three cancer cell lines equal to or higher than that of the ethyl acetate extract. The IC_{50} values of the crude extracts and the pure compound showed similar values against both GLC4 and GLC4/Adr cells. It is proposed that neither the crude extracts nor the pure compound are recognized by ABCC1/MRP1 protein.

The new compound was also examined for its efficacy against *M. tuberculosis* using a broth microdilution method. It demonstrated very strong inhibition, with a MIC value of $0.78\text{ }\mu\text{g}/\text{mL}$, equal to that of ofloxacin, one of four antibiotic drugs used as positive controls. This antimycobacterial activity is consistent with a previous report for compounds isolated from *P. cerasoides* roots [2c]. Three compounds isolated from seeds exhibited antiproliferative action against the CACO-2 cell line, and two phytosterols showed antimutagenic activity [2d]. It has been reported that some extracts of this plant have antimalarial [2c] and antioxidant potential [4a].

Table 2: Antiproliferative, anti-*Mycobacterium tuberculosis*, and H₃₇Ra effects.

<i>P. cerasoides</i> (roots)	%IC ₅₀ (µg.mL ⁻¹)						MIC (µg.mL ⁻¹) H ₃₇ Ra
	A549		GLC4		GLC4/Adr		
	Mean	SD	Mean	SD	Mean	SD	
<i>n</i> -Hexane extract	11.4	0.3	4.9	4.2	3.5	2.3	-
Ethyl acetate extract	2.7	0.8	4.3	3.4	3.4	1.6	-
Acetone extract	10.7	1.3	7.4	4.7	7.3	1.8	-
Methanol extract	14.4	0.4	45.5	16.0	28.7	5.7	-
6,8-Dihydroxy-7-methoxy-1-methyl-azafluorenone	2.6	0.2	2.9	0.7	3.6	1.0	0.78

Experimental

General experimental procedures: IR, Shimadzu 8900 FTIR spectrophotometer; Melting point, Büchi 322 micromelting point apparatus; NMR (1D and 2D), Brüker DPX 400 spectrometer; ESIMS (positive mode), Finnigan LC-Q Advantage Thermoquest spectrometer equipped with Xcalibur software; HRMS, Finnigan INCOS 50 and Brüker Daltonics (micro TOF); CC, Silica gel 60 (Merck, 70-230 mesh); TLC analysis on Si gel GF₂₅₄ precoated plates with UV detection.

Plant material: Roots of *P. cerasoides* were collected in Amnartcharoen Province, Thailand, in March 2009. A voucher specimen (BKF no. 151499) has been deposited at the Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand.

Extraction and isolation: Air-dried powdered roots (5 kg) were successively defatted with *n*-hexane, WP0455 (18 L x 3 days x 7 times) and then sequentially extracted at room temperature with ethyl acetate, WP0456 (18 L x 3 days x 7 times), acetone, WP0457 (18 L x 3 days x 6 times), and methanol, WP0458 (18 L x 3 days x 6 times), followed by filtration. The filtrates were combined and evaporated to dryness under reduced pressure to afford 14.1, 45.6, 144.0 and 359.5 g of residues, respectively. Based on the bioassay results (carried out by our collaborators from Chiang Mai University of Thailand), the ethyl acetate extract was chosen for further study. This (45.6 g) was subjected to a coarse separation by silica gel CC (400 g), eluting with various proportions of ethyl acetate-*n*-hexane, followed by an increasing amount of methanol in ethyl acetate, and finally with methanol. Fractions (1000 mL each) were collected and combined on the basis of TLC behavior. The solvents were evaporated to dryness to afford 7 fractions (F₁ – F₇). Fraction F₃ (2.27 g), eluted by 10% ethyl acetate-*n*-hexane, was obtained as a semisolid. Further separation was achieved by CC over silica gel (100 g), eluting with *n*-hexane, followed by an increasing amount of methanol in ethyl acetate, and finally with methanol. Fractions were collected and combined before the solvents were removed under reducing pressure to afford subfractions A₁ – A₂. Subfraction A₂ (0.87 g), was separated by CC over

silica gel to yield a yellow solid subfraction B₂ (0.38 g). This was recrystallized from ethanol to give purified yellow needles (0.38 g) of azafluorenone.

6, 8-Dihydroxy-7-methoxy-1-methyl-azafluorenone

MP: 200.9-202.7°C.

IR (KBr): 3329, 1701, 1350 cm⁻¹.

¹H and ¹³C NMR (CDCl₃): Table 1.

HRAPCIMS: *m/z* 257 [M⁺].

Cell lines and culture conditions: The crude extracts and the pure compound were tested on 3 cancer cell lines {non small cell lung cancer (A549), adriamycin-sensitive small cell lung cancer (GLC4), and adriamycin-resistant small cell lung cancer (GLC4/Adr) expressing ABCC1/MRP1 [4b,4c]}. All were cultivated in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 37°C incubator under 5% CO₂.

Anti-proliferation assay: The cellular viability of cell A549 was determined using the sulforhodamine B (SRB) method. The sulforhodamine B assay is used for cell density determination, based on the measurement of cellular protein content. The cellular viability of cell lines GLC4 and GLC4/Adr were determined by using the conventional MTT-colorimetric method. Cells were incubated under cell culture conditions with various concentrations of pure compound up to 62.5 µg.mL⁻¹ and crude extracts up to 250 µg.mL⁻¹ for 72 h; the cells without either crude extracts or pure compound served as a blank. At 72 h, 50 µL of MTT (2.5 mg.mL⁻¹) was added to each well. The culture plates were gently shaken and incubated for 4 h. MTT in solution was converted to blue formazan crystals by mitochondrial succinate dehydrogenase of the living cells. The formazan crystals formed within the cells were solubilized with 50 µL DMSO and shaken well. The optical density (OD) of the blue formazan chromophore was determined at 550 nm in an automated plate reader [4d]. The cytotoxic parameter was expressed as the concentration of compound in which the cellular proliferation was inhibited by 50% (%IC₅₀). The percentage of cell-growth inhibition (%IC) was calculated using the formula:

$$\%IC = \frac{(C72 - S72)}{(C72 - C0)} \times 100$$

C0 is the OD value representing the initial cell amount of non treated cells (control), C72 is the OD value representing the cell amount of the control at 72 h and S72 is the OD value representing the cell amount of the treated cells at 72 h

Anti-Myco bacterium tuberculosis, H₃₇Ra: Green fluorescent protein (GFP) expressing *Mycobacterium tuberculosis* strain H37Ra was established by Changsen *et al.* [4e]. H₃₇Ra *gfp* was cultivated on 7H10 agar containing 30 µg.mL⁻¹ kanamycin at 37°C for either 4 weeks or until growth was observed. Starter cultures were prepared by fully looping 2-3 single colonies into 7H9 broth supplemented with 0.2% v/v glycerol, 0.1 % w/v casitone, 0.05% v/v Tween 80, 10% v/v Middlebrook OADC enrichment solution (BD Biosciences) and 30 µg.mL⁻¹ kanamycin. The mixture was then incubated at 37°C in a 200 rpm shaker incubator until the optical density (OD) at 550 nm was between 0.5 and 1. For batch cultivation, the starter cultures were transferred at the rate of 1/10 volume to the 7H9 broth and incubated at 37°C in a 200 rpm shaker incubator until the OD550 nm was approximately 0.5 to 1. The cells were pelleted, washed and suspended in PBS buffer, and then sonicated 8 times for 15 secs each. The sonicated samples were then aliquoted and frozen at -80°C for up to 2 to 3 months prior to use. Titer stocks were determined by the colony forming unit (cfu) assay and the seeding density for anti-TB assay was optimized by serial dilutions. The dilution that grew at logarithmic phase on day 7 was used as an optimal bacterial seeding density. For assay in a 384-well format, the seeding was approximately

2x10⁴ to 1x10⁵ cfu/mL/well. The assay was performed in duplicate, each well containing 5 µL of test sample serially diluted in 5% dimethyl sulfoxide, followed by 45 µL of cell suspension prepared as described above. Plates were incubated at 37°C for 7 days and the fluorescence signals were measured using a SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom-reading mode at the excitation and emission wavelengths of 485 nm and 535 nm. Fluorescence signals on day zero were used as background, which was used to subtract the signals on day 7. The percentage growth inhibition was calculated from the mean of the fluorescence unit of cells treated with sample (FUT) and untreated cells (FUC), according to the following equation:

$$\% \text{ Inhibition} = [1 - (\text{FUT} / \text{FUC})] \times 100$$

The lowest drug concentration that inhibits cell growth by 90% is reported as the Minimum Inhibitory Concentration (MIC). Rifampicin, streptomycin, isoniazid and ofloxacin were used as positive controls, and 0.5% DMSO as a negative control.

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Evaluation of Antiviral Activities of Curcumin Derivatives against HSV-1 in Vero Cell Line

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Antiviral drug resistance is one of the most common problems in medicine, and, therefore, finding new antiviral agents, especially from natural resources, seems to be necessary. This study was designed to assay the antiviral activity of curcumin and its new derivatives like gallium-curcumin and Cu-curcumin on replication of HSV-1 in cell culture. The research was performed as an *in vitro* study in which the antiviral activity of different concentrations of three substances including curcumin, Gallium-curcumin and Cu-curcumin were tested on HSV-1. The cytotoxicity of the tested compounds was also evaluated on the Vero cell line.

The CC₅₀ values for curcumin, gallium-curcumin and Cu-curcumin were 484.2 µg/mL, 255.8 µg/mL and 326.6 µg/mL, respectively, and the respective IC₅₀ values 33.0 µg/mL, 13.9 µg/mL and 23.1 µg/mL. The calculated SI values were 14.6, 18.4 and 14.1, respectively. The results showed that curcumin and its new derivatives have remarkable antiviral effects on HSV-1 in cell culture.

Keywords: Herpes simplex virus type 1 (HSV-1), curcumin, gallium-curcumin, Cu-curcumin, cell culture.

Viral diseases have always been a major health problem and scientists have continually tried to find new antiviral compounds. Cold sores, one of these viral diseases, are caused by herpes simplex virus type 1 (HSV-1), which is a DNA virus of the herpesviridae family [1].

Most complications caused by HSV-1 are self-limited, but HSV-1 can establish lifelong latent infection in sensory ganglia and some factors will cause the reactivation of the virus [2]. Herpes simplex virus-1 infection is common worldwide, with 45% to 98% of the world population being infected in different populations [1,2]. Due to the high prevalence of HSV-1 infections, several antiviral drugs have been developed for the treatment of HSV-1 infections, but many of them show severe side effects and are unable to cure the infections completely. Nearly all clinically effective antiviral drugs are nucleoside analogues. However,

following a long period of their use, drug resistance has emerged [3]. Therefore, finding novel anti HSV-1 agents with low side effects is necessary. It has been suggested that natural products from plants can exhibit anti HSV-1 activities. Such natural products need to be isolated and screened for their potential to act as antiviral compounds [4]. One of these natural antiviral agents is curcumin, a major antioxidant compound and a principal constituent of the spice turmeric (*Curcuma longa*) [5], a native plant from south India and Indonesia. This spice has been used for hundreds of years for flavoring and coloring of many kinds of foods and is also used as a food preservative [6,7].

Regarding the use of turmeric in Indian and Chinese traditional medicine many scientists are interested to reveal the therapeutic and biological functions of this compound. Curcumin can affect the metabolism of cells and organisms [8]. Also, it has anti-tumor, antioxidant,

Table 1: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of curcumin. Each value represents the mean of four replicate assays

Concentration ($\mu\text{g/mL}$)	CPE Inhibition (%)
12	0
18	10
24	30
30	40
36	55
42	75
48	90
54	100

antiinflammatory, antiviral and anti-infectious properties [6,9-12]. Although various antiviral effects of curcumin have been reported, more studies are necessary to develop it as an alternative antiviral drug for HSV-1 treatment or for providing a template for the synthesis of new anti HSV-1 agents [13].

In this study, we evaluated the *in vitro* anti-HSV-1 activity of curcumin and its novel derivatives gallium-curcumin and Cu-curcumin. Based on our knowledge, there has been no study of the antiviral activity of these last two compounds.

The cytotoxicities of curcumin, gallium-curcumin and Cu-curcumin on Vero cells were determined by calculation of their CC_{50} values as 484.2, 255.8 and 326.6 $\mu\text{g/mL}$, respectively. Regarding the collected data for the antiviral activity of curcumin (Table 1), gallium-curcumin (Table 2), and Cu-curcumin (Table 3) against HSV-1 in cell culture, the IC_{50} values of these compounds, calculated using STATA software, were 33.0, 13.9 and 23.1 $\mu\text{g/mL}$, respectively. As shown in Tables 1, 2 and 3, 12 $\mu\text{g/mL}$ of curcumin, 4 $\mu\text{g/mL}$ gallium-curcumin and 8 $\mu\text{g/mL}$ of Cu-curcumin could not prevent CPE presentation, which is related to HSV-1 replication in cell culture. However, 54 $\mu\text{g/mL}$ of curcumin, 30 $\mu\text{g/mL}$ of gallium-curcumin and 42 $\mu\text{g/mL}$ of Cu-curcumin totally prevented viral CPE presentation.

Table 2: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of gallium-curcumin. Each value represents the mean of four replicate assays.

Concentration ($\mu\text{g/mL}$)	CPE Inhibition (%)
4	0
8	30
12	55
18	75
24	90
30	100

Based on our knowledge, no research has been reported up to now on the activity of the new derivatives of curcumin, Cu-curcumin and gallium-curcumin against viruses, especially HSV-1.

Antibacterial activities of curcumin and curcuminoids have been reported [14]. Curcumin has also been shown

to inhibit the growth of several types of viruses and malignant cells [15]. Curcumin can inhibit the expression of immediate early genes of HSV-1 [8], and also inhibit the activity of HIV-1 integrase, which is necessary for replication of this virus [16].

Table 3: Inhibition of HSV-1 related cytopathic effect (CPE) by using different concentrations of Cu-curcumin. Each value represents the mean of four replicate assays.

Concentration ($\mu\text{g/mL}$)	CPE Inhibition (%)
8	0
12	10
18	40
24	60
30	75
36	90
42	100

Various derivatives related to curcumin were synthesized and tested as inhibitors of the replication cycle of some viruses [17]. Synthetic modification of antiviral agents in order to improve either their antiviral activity or pharmacological properties is of interest [18]. Therefore, two new complexes, Cu-curcumin and gallium-curcumin, were chosen for the present study.

In this study, the CC_{50} values obtained showed that the cytotoxicity of curcumin is less than those of its new derivatives gallium- and copper-curcumins. Based on the differences in the CC_{50} values of gallium-curcumin and Cu-curcumin it could be concluded that the gallium compound was more cytotoxic than the copper one to Vero cells.

In most studies, DMSO showed antiviral and cytotoxic effects *in vitro* on different cell types [19]. Thus we tested the probable DMSO cytotoxic and virucidal effects in the current study. The concentration of DMSO used in our study was less than 2%, which is the lowest concentration for an antiviral effect; therefore we ignored the effect of DMSO in our study.

Based on the data obtained, it could be concluded that curcumin and its new derivatives exhibited *in vitro* anti-HSV-1 activities. Such agents could be developed either as anti HSV-1 compounds or provide a template for the synthesis of new anti HSV-1 agents. Our results can be considered as an early step in elucidating the molecular basis of the antiviral activities of curcumin. Future research is necessary to determine the possible *in vivo* anti-HSV-1 activity of curcumin and its related derivatives. Also, the mechanism(s) of action of these compounds should be revealed in future studies.

Experimental

Cell and virus: The African green monkey kidney cell line (Vero) was used as an appropriate cell line for

HSV-1 propagation. The cells were cultured using Dulbecco minimum essential medium (Gibco) containing 10% fetal bovine serum (Gibco). The antiviral activity assay was carried out on the KOS strain of HSV-1. The virus was propagated in Vero cells and the titer of propagated viral stock was fixed as TCID₅₀ mL⁻¹ by using Karber's method. After titration, viral stock was dispensed in sterile tubes, which were stored at -70°C until the date of use.

Preparation of curcumin and its derivatives: Curcumin was purchased from Sigma, and the curcumin derivatives were prepared as previously described [20]. Dimethyl sulfoxide (DMSO) was used as the solvent for curcumin and its derivatives.

Cytotoxicity assay: Cytotoxicity values of all compounds were determined by culturing Vero cells for 96 h in the presence of increasing amounts of each substance. Three wells for each concentration of each compound were used. Viable cells were determined by the trypan blue exclusion test. Results were plotted as a dose response curve and 50% cell growth inhibitory concentration (CC₅₀) was obtained by using STATA modeling software.

Antiviral activity assay: The cytopathic inhibition assay was used to determine the semi-quantitative antiviral activity of each test compound. Briefly, Vero cells were grown in a 96-well cell culture microplate (2×10³ cells/well). The cultured plates were incubated at 37°C in the presence of 5% CO₂ until the cells showed 80% confluency. Subsequently, the culture medium was removed from each well and 100 TCID₅₀ of virus suspension and different concentrations of curcumin and its derivatives from minimal to maximal non-cytotoxic concentrations were added to each well of the

cell culture microplate. For each concentration of curcumin and its derivatives 4 wells were chosen.

For the virus control, 100 TCID₅₀ with the highest amount of DMSO which did not show cytotoxicity were added to 4 wells. Also, in each microplate, 4 wells were treated with DMSO without virus as a negative control for virus. In addition, 4 wells of each row were treated with the highest level of each curcumin based compound which did not previously exhibit cytotoxicity.

The plates were incubated at 37°C in a humidified CO₂ atmosphere (5% CO₂) and were investigated every day for cytopathic effect (CPE) presentation up to 5 days post infection.

The degree of inhibition was expressed as percent yield of virus control (% virus control = CPE experimental group/CPE virus control × 100). The concentration of each compound which reduced 50% of CPE presentation with respect to virus control was estimated from graphic plots defined as 50% inhibited concentration (IC₅₀) expressed in µg per mL by using STATA modeling software. The selectivity index (SI) was measured from the ratio of CC₅₀/IC₅₀ [21,22].

Statistical analysis: The STATA statistical analysis package was used for curve plotting in order to calculate IC₅₀ and CC₅₀ values.

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Hyloglyceride and Hylodiglyceride: Two New Glyceride Derivatives from *Hylodendron gabunensis*

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Phytochemical investigation of *Hylodendron gabunensis* resulted in the isolation of two new glyceride derivatives, hyloglyceride (**1**) and hylodiglyceride (**2**). The structures of the two new compounds were determined by comprehensive analysis of their 1D and 2D NMR spectroscopic, and HREIMS data. One known compound was also isolated and identified as β -sitosterol.

Keywords: *Hylodendron gabunensis*, Fabaceae, glyceride derivatives, hyloglyceride, hylodiglyceride.

The African continent is one of the continents endowed with the richest biodiversity in the world, with an avalanche of plants used as foods, herbs and health foods, and for therapeutic purposes [1]. In the course of phytochemical studies of medicinal plants from Africa [2], we investigated *Hylodendron gabunensis* Taub. (Fabaceae). Previous studies of this species resulted in the isolation of two apiitol derivatives [3]. We now report on the structure elucidation of two new glyceride derivatives, namely, hyloglyceride (**1**) and hylodiglyceride (**2**) (Figure 1), together with the known compound β -sitosterol [4] from *H. gabunensis*. Glycerides and diglyceride derivatives have been reported from plants, endophytic fungi and marine organisms [5].

Hyloglyceride (**1**) was obtained as white powder. Its molecular formula was assigned as C₃₄H₆₈NO₄ on the basis of the ion at m/z 540.5110 [M]⁺ in the HREIMS, and ¹H and ¹³C NMR spectral analyses. The IR spectrum revealed absorption bands for an hydroxyl group at 3420 cm⁻¹ and C=O at 1733 cm⁻¹. The ¹³C (DEPT) and ¹H NMR spectra of **1** showed signals for a fatty acid moiety [δ 14.0 (q), 22.6 (t), 24.8 (t), 29.2 (t), 32.8 (t), 34.1 (t), 174.3 (s), δ 0.85 (3H, t, J = 6.4 Hz), 1.27 (br s, n x CH₂), 1.60 (2H, m), 2.33 (2H, t, J = 7.5 Hz)], two oxymethylene [δ _C 63.2, 65.1; δ _H 4.40 (1H, dd, J = 11.2, 3.5 Hz), 4.37 (1H, dd, J = 11.2, 5.5 Hz), 3.70 (1H, dd, J = 11.2, 3.5 Hz), 3.60 (1H, dd, J = 11.2, 3.5 Hz)], and one oxymethine [δ 70.2, δ 3.92 (1H, m)].

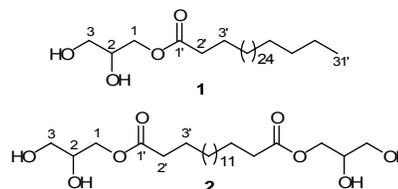


Figure 1: Structure of hyloglyceride (**1**) and hylodiglyceride (**2**) isolated from *H. gabunensis*.

These data suggested that **1** was a fatty acid-1-glyceride [5]; this was supported by the HMBC spectrum, which showed correlations of H-1 and H-3a with C-2, and H-1, H-2 with C-3, indicating the presence of an asymmetrically substituted glycerol moiety. Furthermore, correlations of H-1, H-2', and H-3' with C-1' indicated that the acyl of the fatty acid moiety was attached to C-1. According to the molecular weight obtained from the HREIMS, the fatty acid moiety should be hentriacontanoic acid. Based on the literature which reported that glyceride analogue exhibiting +ve optical rotation had an absolute configuration of 2S, [5a,6] the structure of **1** was elucidated as (2S)-1-O-hentriacontanoyl glycerol (Figure 1).

Hylodiglyceride (**2**) was found to have a molecular formula of C₂₃H₄₄O₈ from the molecular ion peak at m/z 448.3030 [M]⁺ in the HREIMS. The IR spectrum revealed absorption bands for an hydroxyl group at 3420 cm⁻¹ and C=O at 1730 cm⁻¹. The ¹H and ¹³C NMR spectra of **2** were similar to those of compound **1** (see

Experimental), with differences observed in the fatty acid. A methyl group seen in **1** (δ_C 14.0, t; δ_H 0.85 (t, $J = 6.4$ Hz) is missing in **2** and replaced by another glycerol moiety, as observed from the 1H and ^{13}C NMR spectra. The 1H and ^{13}C NMR spectra showed peaks for half of the molecule. According to the HREIMS, the di fatty acid moiety should be heptadecanedioic acid. Based on these findings, hylodiglyceride was established as **2** (Figure 1).

Experimental

General experimental procedures: Optical rotation was recorded on a Perkin–Elmer 241 MC polarimeter at the sodium D-line. IR spectra were obtained from a Nicolet-510P spectrophotometer; ν_{max} in cm^{-1} . EIMS and HREIMS were carried out using a MAT 8200 and Micromass LCT mass spectrometers. The 1H NMR spectra were recorded on a Bruker AMX-500 instrument using TMS as an internal reference.

Plant material: The bark of *H. gabunensis* was collected at Mont Elounden 11- Yaounde, in the central region of Cameroon, in December 2007, and identified by Dr Nole Tsabang, Ministry of Scientific Research. A voucher specimen (N° 9336) has been deposited at the National Herbarium, Yaounde, Cameroon

Extraction and isolation: Dried and powdered bark (12.0 kg) of *H. gabunensis* was extracted with EtOAc at room temperature for 48 h and then filtered. The filtrate was concentrated under vacuum to give 200 g of crude residue. The ethyl acetate-fraction (200 g) was then subjected to CC (silica gel, *n*-hexane, *n*-hexane-EtOAc, EtOAc and EtOAc-MeOH, in order of increasing polarity), yielding 7 fractions (F₁ to F₇). Fraction F₇ was eluted with a mixture of CH₂Cl₂, CH₂Cl₂-MeOH (order of increasing polarity) yielding hylodiglyceride (**2**, 9.0 mg). Fraction F₅ [*n*-hexane-EtOAc (8.0:2.0)] was similarly subjected to CC, yielded hyloglyceride

(**1**, 11.0 mg). Finally, fraction F₂ gave β -sitosterol (7 mg) [*n*-hexane-EtOAc (8.5:1.5)].

Hyloglyceride (1)

Colorless powder.

MP: 64–66°C.

$[\alpha]_D^{20}$: +6.2 (*c* 0.25, CH₂Cl₂).

IR ν_{max} (CH₂Cl₂): 3420, 2920, 2850, 1733, 1469, 1389, 1180 cm^{-1} .

1H NMR (500 MHz, CDCl₃): δ 0.85 (3H, t, $J = 6.4$ Hz, Me-31'), 1.27 (54H, br s, H-4' to H-30'), 1.60 (2H, m, H-3'), 2.33 (2H, t, $J = 7.5$ Hz, H-2'), 3.60 (1H, dd, $J = 11.2, 3.5$ Hz), 3.70 (1H, dd, $J = 11.2, 3.5$ Hz), 4.37 (1H, dd, $J = 11.2, 5.5$ Hz), 3.92 (1H, m), 4.40 (1H, dd, $J = 11.2, 3.5$ Hz).

^{13}C NMR (125 MHz, CDCl₃): δ 14.0 (CH₃, C-31'), 22.6 (CH₂, C-29'), 24.8 (CH₂, C-3'), 29.2 (CH₂, C-4' to C-28'), 32.8 (CH₂, C-30'), 34.1 (CH₂, C-2'), 63.3 (CH₂, C-3), 65.2 (CH₂, C-1), 70.2 (CH₂, C-2), 174.3 (C, C-1').

HREIMS: m/z 540.5110 [M]⁺ (calcd. 540.5118 for C₃₄H₆₈NO₄).

Hylodiglyceride (2)

Colorless powder.

MP: 87°C.

$[\alpha]_D^{20}$: +10.2 (*c* 0.20, CH₂Cl₂).

IR ν_{max} (CH₂Cl₂): 3420, 2920, 2850, 1730, 1469, 1389, 1180 cm^{-1} .

1H NMR (500 MHz, CDCl₃): δ 1.27 (br s, H-4' to H-8'), 1.60 (2H, m, H-3'), 2.33 (2H, t, $J = 7.5$ Hz, H-2'), 3.60 (1H, dd, $J = 11.2, 3.5$ Hz), 3.70 (1H, dd, $J = 11.2, 3.5$ Hz), 4.37 (1H, dd, $J = 11.2, 5.5$ Hz), 3.92 (1H, m), 4.40 (1H, dd, $J = 11.2, 3.5$ Hz).

^{13}C NMR (125 MHz, CDCl₃): δ 24.9 (CH₂, C-3'), 29.7 (CH₂, C-4' to C-8'), 34.2 (CH₂, C-2'), 63.3 (CH₂, C-3), 65.2 (CH₂, C-1), 70.3 (CH₂, C-2), 174.3 (C, C-1').

HREIMS: m/z 448.3030 [M]⁺ (calcd. 448.3036 for C₂₃H₄₄O₈).

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Chemical Composition and Bioactivities of the Marine Alga *Isochrysis galbana* from Taiwan

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The present study investigated the chemical composition of *Isochrysis galbana* Parke, a marine microalga which is widely used as a feedstock in aquaculture. From gas chromatography/mass spectrometric analysis the mono-sugar compositions of *I. galbana* were 2.1% fucose, 2.5% rhamnose, 2.7% arabinose, 8.5% xylose, 15.7% mannose, 32.7% galactose and 35.8% glucose. The polysaccharides of *I. galbana* were able to induce prointerleukin-1 β (pro-IL-1 β) protein expression within murine macrophages. Furthermore, five kinds of chlorophyll and one sterol were separated from the ethanolic extracts, including pheophorbide-a, ethyl pheophorbide-a, 10S-10-hydroxypheophytin-a, 10R-10-hydroxypheophytin-a, (13²-R)-pheophytin-a, and brassicasterol. In addition, the major soluble components of the ethanol/*n*-hexane extract were 9-octadecenoic acid (*E*) (38.4%), hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), and octadecanoic acid (7.2%), but only a few polyunsaturated fatty acids were found, such as 9,12,15-octadecatrienoic acid (1.9%), 9,12-octadecadienoic acid (*Z,Z*) (3.4%), and docosahexaenoic acid (0.2%). This is the first occasion that polysaccharides from *I. galbana* have been demonstrated to exert immunomodulatory properties by the induction of IL-1 within macrophages.

Keywords: *Isochrysis galbana*, extracts, chemical compositions, polysaccharides, bioactivity, pro-IL-1 β .

Marine microalgal biomasses play an important role as primary producers in the animal food chain. *Isochrysis galbana* Parke, a golden-brown flagellate marine microalga, is widely used as an aquaculture feed for young fish and in bivalve hatcheries because it is rich in polyunsaturated fatty acids (PUFA) [1]. Many studies have focused on the relationship between fatty acids and algal growth [2-4]. In addition, because of the high content of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), *I. galbana* is recognized for its beneficial effects on human health, and is considered to be a good substitute for fish oil in the human diet. The purpose of the present study was to analyze the chemical composition of *I. galbana*, including *n*-hexane/ethanol extracts; ethanol extracts; total carbon, hydrogen, oxygen, nitrogen and sulfur; and total polysaccharides.

First we analyzed the ratio of carbon, hydrogen, oxygen, nitrogen, sulfur and ash in *I. galbana* cultured in the laboratory. Based on dry weight, the values obtained were: C (56.3%), H (7.5%), O (20.3%), N (6.25%), S (1.0%) and ash (8.6%). *I. galbana* also yielded a high content of *n*-hexane/ethanol (1/1) and ethanol extractive (21.7% and 38.2%, respectively).

Sixteen fatty acids were identified. These are given in Table 1, where all compounds are listed in order of their elution from the DB-5HT column. The major fatty acid was 9-octadecenoic acid (*E*) (38.4% of total), followed by hexadecanoic acid (23.3%), tetradecanoic acid (15.7%), octadecanoic acid (7.2%), and 9-octadecenoic acid (*Z*) (3.2%). The composition of fatty acids in *I. galbana* found in this study differs from the results of

Table 1: Chemical composition of *I. galbana* ethanol/*n*-hexane extractive.

Compound ID	RT ^a	Conc. (%)	Identification ^b
Tetradecanoic acid, methyl ester	14.51	15.7	MS, KI, ST
Pentadecanoic acid, methyl ester	16.52	0.8	MS, KI, ST
11,14,17-Eicosatrienoic acid, methyl ester	17.76	0.1	MS, KI
(<i>Z</i>)-9-Hexadecenoic acid, methyl ester	18.04	2.5	MS, KI
Hexadecanoic acid, methyl ester	18.50	23.3	MS, KI, ST
Heptadecanoic acid, methyl ester	20.53	0.2	MS, KI, ST
9,12,15-Octadecatrienoic acid, methyl ester,	21.22	1.9	MS, KI, ST
9,12-Octadecadienoic acid (<i>Z,Z</i> -), methyl ester	21.54	3.4	MS, KI
9-Octadecenoic acid (<i>E</i> -), methyl ester	21.66	38.4	MS, KI, ST
9-Octadecenoic acid (<i>Z</i> -), methyl ester	21.77	3.2	MS, KI, ST
Octadecanoic acid, methyl ester	22.16	7.2	MS, KI, ST
Arachidonic acid, ethyl ester	24.30	0.09	MS, KI
Heneicosanoic acid, methyl ester	27.14	0.1	MS, KI
4,7,10,13,16,19-Docosahexaenoic acid, methyl ester, (all- <i>Z</i> -)	27.40	0.2	MS, KI
Docosanoic acid, methyl ester	28.67	2.8	MS, KI
Total saturated		50.1	
Total monoenoic		44.1	
Total PUFA		5.8	

^a Retention time on a DB-5 HT column with reference to *n*-alkanes.

^b MS, NIST and Wiley library spectra, and the literature; RI, retention index; ST, authentic standard compounds.

an earlier study, which showed higher DHA (22:6n-3) (7.91%) [5].

Also, some differences were found from the study by Lin *et al.*, who determined DHA to be a major fatty acid at every growth phase [4]. It is interesting that we found a few fatty acids, such as pentadecanoic acid, heptadecanoic acid, and heneicosanoic acid, which had not been previously found [4]. This could be a result of different growth conditions.

Few existing studies have focused on the variety of pigments from *I. galbana*. Herein we have separated and identified the pigments from 1.65 g of an ethanolic extract of *I. galbana*. At least five chlorophyll compounds (Figure 1) were found including (13²-*R*)-pheophytin-a (1.5 mg; RT = 8.03 min), pheophorbide-a (1 mg; RT = 15.82 min), 10*R*-10-hydroxypheophytin-a (3 mg; RT = 22.54 min), 10*S*-10-hydroxypheophytin-a (1 mg; RT = 23.51 min), and ethyl pheophorbide-a (4 mg; RT = 34.92 min). We also found brassicasterol (2 mg; RT = 15.10 min).

In 1981, Volkman *et al.* [6] were the first to report that 24-methyl-22-dehydrocholesterol is the dominant sterol in *I. galbana* [6]. Our experimental results were similar. Park *et al.* [7] demonstrated that autotrophically grown *I. galbana* contains three major sterols (24-oxocholesterol acetate, ergost-5-en-3-ol, and cholest-5-en-24-1, 3-(acetyloxy)-,3-ol), with 24-methylcholesta-5,22-dien-3-ol as a minor sterol [7]. They found that the total sterol content clearly decreased during dark culture, with such decreases being particularly evident in two major sterols, 24-oxocholesterol acetate and ergost-5-en-3-ol.

Our study showed that the monosaccharide composition of a polysaccharide (IP) from *I. galbana* was 2.07%

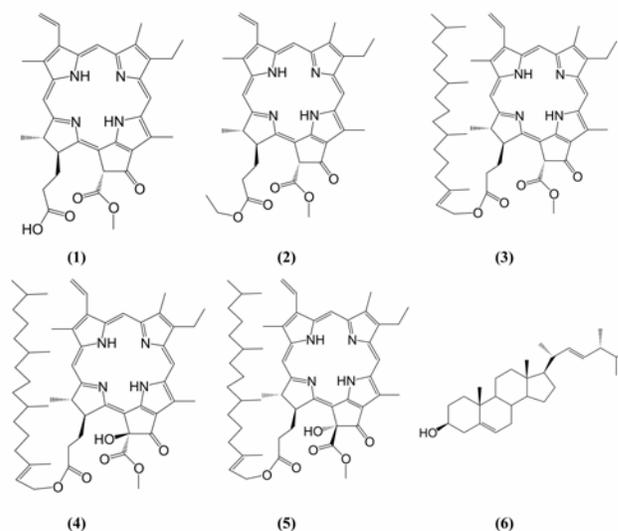


Figure 1: The chemical structure of (1) pheophorbide-a; (2) ethyl pheophorbide-a; (3) (13²-*R*)-pheophytin-a; (4) 10*R*-10-hydroxypheophytin-a; (5) 10*S*-10-hydroxypheophytin-a; (6) brassicasterol.

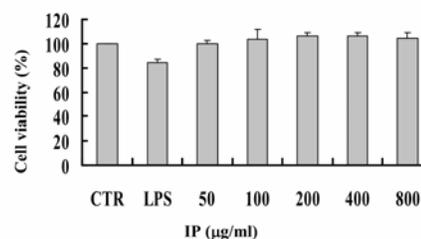


Figure 2: Effect of *Isochrysis* polysaccharide (IP) on cell viability. J774A.1 macrophages (5×10^3 /well) were treated with IP or DMSO (control) for 24 h, followed by incubation with MTT reagent. Absorbance (A550-A690) was measured by spectrophotometry. Data are expressed as mean \pm SE from three separate experiments.

fucose, 2.50% rhamnose, 2.72% arabinose, 8.49% xylose, 15.70% mannose, 32.73% galactose and 35.79% glucose. No cytotoxic effect was observed after J774A.1 cells were treated with various concentrations of IP for a period of 24 hours, as measured by MTT assay (Figure 2).

It is well known that IL-1 β is secreted mainly from activated macrophages; this could activate other immune cells and modulate immune responses. An IP-induced IL-1 β precursor, pro-IL-1 β , was detected in whole cell lysates after IP stimulation by means of Western-blotting analysis. In this time course study, the expression of pro-IL-1 β protein within IP-stimulated cells was detected at six hours post-stimulation. In addition, the expression of pro-IL-1 β increased with increasing IP concentrations in a dose-dependent manner. Such results demonstrate that IP stimulates pro-IL-1 β expression, a result that would appear to be similar to the ability of polysaccharides isolated from the algae *Rhizoclonium riparium* (Roth) Harvey and *Chlorella pyrenoidosa* Chick to stimulate pro-IL-1 β expression within murine macrophages [8,9].

Fabregas *et al.* found that endocellular extracts of *I. galbana* clearly inhibited viral hemorrhagic septicemia virus (VHSV) replication at a dose of 20 µg/mL, and that *I. galbana* contained sulfated soluble exopolysaccharides [10].

Experimental

Algal culture and collection: *I. galbana* used in this study was obtained from the Tungkang Biotechnology Research Center, Fisheries Research Institute, Republic of China. Purified *I. galbana* CCMP 1324 (0.9 L, 680 nm, OD 1.10) was inoculated and cultured in Walne's medium [9]. This included nutrient, vitamin and trace metal solutions in a 10 L PET tank containing 8.1 L seawater autoclaved at 120°C for 20 min, to which was added 9.0 mL of nutrient solution and 0.9 mL of vitamin solution. The culture medium was agitated gently by bubbling air with a flow rate of 4.7 L/min and a culture time of 7 d. Continuous illumination at an irradiance of 5900 lx was provided by fluorescent lamps. The culture medium was then centrifuged (Himac CR22-GII, Hitachi, Japan) continuously at 12000 rpm at 25°C. After lyophilization the yield of alga was 0.1 g/L (dry weight) culture medium.

Total C, H, O, N, S: Total carbon, hydrogen, oxygen, nitrogen and sulfur contents were determined by CHN elemental analysis. Freeze-dried samples (15 g each) were combusted in a 2400 CHN/O elemental analyzer (Perkin-Elmer, Waltham MA, USA) [11].

Extraction and composition of n-hexane/ethanol extracts of *I. galbana*: Ten grams of sample was extracted in a Soxhlet apparatus with *n*-hexane: ethanol (95% v/v) (50:50) for 48 h. The solution was rotary-evaporated at 65 °C to provide a hydro-ethanolic extractive (HAE) (2.175 g). The HAE (0.5 g) was trimethylsilylated with Sylon HTP (HMDS/TMCS/pyridine, 3:1:9) trimethylsilylation reagent (Supelco, Bellefonte PA, USA). The final derivatives were kept in *n*-hexane for gas chromatography–mass spectrometric (GC-MS) analysis. A Hewlett-Packard HP 6890 gas chromatograph equipped with a DB-5HT fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness; Agilent Technologies, Santa Clara CA, USA) and a FID detector were used for quantitative determination of the components. The oven temperature was programmed as follows: 100°C for 2 min, rising to 275°C at 5°C/min; injector temperature, 270°C; carrier gas, He with a flow rate of 1 mL/min; detector temperature, 250°C; split ratio 50.1:1. One µL sample was injected. Identification of the oil components was based on their retention indices and MS results. The GC analysis parameters listed above and the MS were obtained (full scan mode; scan time, 0.3 s; mass range, MHz 30–500) in the electron impact (EI) mode at 70 eV.

Extraction, purification and identification of ethanolic extracts of *I. galbana*: Dry alga (5 g) was treated with ethanol (95% v/v for 10 d, repeated 3 times) at room temperature. Then the extract was concentrated to provide the ethanolic extract (AE). AE (1.65 g) was applied to a silica gel column (Si 60) and eluted with acetone/*n*-hexane to give 43 sub-fractions. Each eluted fraction was 150 mL. The chlorophyll compounds were purified by preparative HPLC (KNAUER RI detector 2400, pump 100; KNAUER, Germany) on a Merck (Germany) Hibar Fertigsaule RT column Si 60 (25 cm length, 1 cm i.d., 5.0 µm). The separation conditions were as follows: flow rate 4 mL/min; mobile phase, acetone/*n*-hexane = 1/9. The sterol was separated by a Phenomenex Luna silica (2) column (25 cm length, 1 cm i.d., 5.0 µm) under the following conditions: flow rate 4 mL/min; mobile phase, acetone/*n*-hexane = 1/15. The structures of the compounds were confirmed by comparison of physical and spectral data (including optical rotation, EIMS, ¹H NMR) with previously reported values.

Extraction of polysaccharides from *I. galbana*: Five grams dry alga was extracted with *n*-hexane/ethanol. The extractive was ground into a fine powder, and then suspended in 100 mL distilled water. After autoclaving at 121°C for 30 min, the extract was filtered through a 0.2 µm membrane. The extract was then vacuum-concentrated at 50°C, giving a final volume of 30 mL to which 5 volumes of 95% ethanol was added slowly at 4°C. Then the mixture was centrifuged to produce a precipitate of ca. 1.910 g, dry wt. Sixty mg of the precipitate was treated further with 3 mg proteinase K for removal of the peptide part, and dialyzed against H₂O (Spectra/Por[®] membrane, molecular weight cutoff 1,000 Da), resulting in 17.5 mg polysaccharide [*I. galbana* (IP)].

Sugar composition analysis: Sugar composition was determined by GC-MS. The polysaccharide content of *I. galbana* was determined by methanolysis with 0.5 M methanolic HCl at 80°C for 16 h, and trimethylsilylation with Sylon HTP. The final trimethylsilylated (TMS) derivatives were kept in *n*-hexane for GC-MS analysis [12]. Carbohydrate analysis was done with inositol as the internal standard; integrated peak area was used to establish the relative amounts of the constituents. Compounds were identified by comparing of their mass spectrometric fragmentation patterns with those of authentic standards, and the quantity of compounds was obtained by integrating the peak area of the spectra.

Microculture tetrazolium (MTT) assay for cell viability: J774A.1 macrophages were seeded in 96-well plates at a density of 5 × 10³ cells/well. Cells were incubated with IP for 24 h. Cell viability was determined using colorimetric MTT assays.

Cell cultures: Murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC) (Rockville MD, USA). All cells were propagated in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 2 mM L-glutamine (Life Technologies, Carlsbad CA, USA), and cultured in a 37°C, 5% CO₂ incubator [8,9].

Western blotting: Whole cell lysates were separated by 12% SDS-PAGE and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was then incubated with anti-IL-1 β antibody at room temperature for 2 h. After washing 3 times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system

(DuPont NEN[®] Research Products, Boston MA, USA) according to the manufacturer's instructions [8,9].

Statistical analysis: All values are given as mean \pm SE. Data analysis involved one-way ANOVA with subsequent Scheffé test.

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An Efficient Protocol for High-frequency Direct Multiple Shoot Regeneration from Internodes of Peppermint (*Mentha x piperita*)

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A simple, repeatable and efficient protocol for direct multiple shoot regeneration from internodal explants has been defined in peppermint (*Mentha x piperita* var. Indus). *In vitro* regenerated shoots of peppermint were excised into 4 to 8 mm long internodes and cultured on Murashige and Skoog's medium supplemented with different cytokinins. In the hormonal assay, 3.0 mg L⁻¹ zeatin or 6-isopentenyl adenine independently supplemented to half strength MS medium exhibited multiple shoot regeneration, while thiadiazorn (0.1-3.0 mg L⁻¹) showed no morphogenetic effect. A maximum of 85% *in vitro* cultured explants showed multiple shoot formation with an average of 7 shoots per explant on MS medium supplemented with zeatin. Multiple shoots were initiated within three weeks of cultivation. Internodes with regenerated multiple shoots were transferred to half - strength MS medium without supplementing with any plant growth hormone for shoot elongation and rhizogenesis. Rooted plants acclimatized and grew to maturity under glasshouse conditions. The plantlets developed were phenotypically identical to the parent plant and exhibited 96 % survival.

Keywords: *Mentha x piperita*, plant growth hormones, internodes, multiple shoot regeneration, cytokinins.

Peppermint (*Mentha piperita* L.), an allopolyploid (2n=72) and a natural hybrid of *M. aquatica* x *M. spicata* is cultivated in India and subtropical regions. Its essential oil is valued commercially as an additive to food products, cosmetics and pharmaceuticals [1]. *M. piperita* var. Indus [2] produces high menthofuran (27.2%) and pulegone (15.4%) levels in its essential oil. In the present investigation, we report a simple and efficient method of direct multiple shoot regeneration and formation of complete plantlets from internodal segments of *M. piperita* var. Indus without the production of a callus phase.

Effect of cytokinins on multiple shoot induction:

Multiple shoot induction in internodal segments was observed within 3 weeks on half strength MS medium, while a comparatively low regeneration response was observed in full strength MS medium. Of the three cytokinins (Z, 2-iP and TDZ) tested (0.1-3.0 mg L⁻¹), Z at higher levels (>1.0 mg L⁻¹) and 2-iP at all levels (0.1-3.0 mg L⁻¹) tested exhibited the highest (85%) multiple shoot regeneration response (Table 1) and an average of 7 shoots/explant were obtained with Z (3.0 mg L⁻¹), while a comparatively low response (55%) was observed with 2-iP (3.0 mg L⁻¹). None of the explants showed regeneration in medium supplemented with

Table 1: Effect of cytokinins on morphogenetic response in *M. piperita*.

Cytokinin (mg l ⁻¹)	Morphogenetic response (%)		
	Z	TDZ	2-iP
0.0	—	—	—
0.1	—	—	R (25)
0.5	—	R (35)	R (50)
1.0	MS (25)	—	R (40)
2.0	MS (35)	—	MS (35)
3.0	MS (85)	—	MS (50)

MS - multiple shoots; R - rooting; — no response ; figure in parentheses denotes percentage of cultures showing morphogenetic response

TDZ at any of the levels tested. This is contrary to the earlier reports where regeneration of plantlets in the presence of TDZ has been reported [3-5]. Coconut water (25%), along with either TDZ or BA or 2-iP were evaluated for their effect on organogenesis. Amongst these cytokinins, TDZ was found to be the most effective for inducing shoot formation in peppermint (*Mentha x piperita*) leaf explants [4]. These results are in contrast to our present study in which none of the concentrations of TDZ tested induced shoot regeneration. This indicated that the TDZ levels tested alone may not be able to induce multiple shoot induction. Occasionally TDZ and 2-iP at certain low levels also showed root initiation in cultured explants, but shoot regeneration was not observed along with the rooting (Table 1). Leaf disks and petioles of *M. citrata*

Ehrh., *M. piperita* cv. Black mitcham, *M. spicata* L. and *M. gracilis* Sole ex Baker also exhibited high multiple shoot regeneration responses with 2iP [6], as has also been observed in the present study. Coconut water in the MS medium supplemented with 4.5 μM TDZ has been found to affect adventitious shoot formation in callus of *Mentha x gracilis* [3]. Excision of the growing shoots at each sub-culture passage further enhanced the elongation of dormant buds formed during the initial shoot formation.

Shoot elongation and root induction: The half strength MS medium was found to be more effective for rapid and healthy growth of explants. Shoot elongation and rhizogenesis were much more rapid and significant in half strength MS medium in contrast to MS full strength. Although Z and 2-iP stimulated multiple shoot formation, they slowed down the development and elongation of shoots. Simultaneous shoot elongation and rooting was observed in nodal explants and therefore, single shoots separated from multiple shoot clumps were sub-cultured in growth hormone free half strength MS medium. The observed effects of shoot elongation and root initiation on medium devoid of hormones support the reported work [7]. About 90-95% of the single shoots elongated and formed roots within three weeks.

Recovery of plantlets: Of the plantlets transferred to glasshouse conditions, 96% showed survival and grew to maturity. No phenotypic variation was observed among the control (sucker grown parent plants) and *in vitro* raised plants. This study thus demonstrated a simple and efficient protocol for direct multiple shoot regeneration and production of true-to-type plants that could be adapted for study of transgenic *M. piperita*.

Experimental

Establishment of aseptic cultures: Nodal segments were collected from healthy young plants from the National Gene Bank at CIMAP, Lucknow. These were surface sterilized and inoculated on half strength MS medium [8] under reported culture conditions [9].

Shoot induction and multiplication: Internodal segments (4 to 8 mm in size) from pre-cultured mother stocks were placed on half strength MS medium supplemented either with zeatin (Z), thidiazuron (TDZ) or N6-2(2-isopentyl) adenine (2-iP) at 0.1, 0.5, 1.0, 2.0 and 3.0 mg L⁻¹ levels. Full and half strength MS basal media were tested to define optimal concentration of cytokinins for multiple shoot induction. Ten replicates of each level of all the 3 cytokinins were used and the experiment was repeated twice. Multiple shoots were separated and sub-cultured on fresh cytokinin free half strength MS medium for further shoot elongation and rooting.

Acclimatization of plantlets: The rooted shoots measuring 4-6 cm in length with 5-7 leaves were taken from the culture vessel, washed gently under running tap water and kept in a culture tube containing water for 5-7 days. After this hardening phase, plants were transferred to plastic trays containing soil and vermicompost mixture (2:1) and acclimatized in a glass house under normal day length conditions.

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Essential Oil Yield and Chemical Composition Changes During Leaf Ontogeny of Palmarosa (*Cymbopogon martinii* var. *motia*)

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Changes in leaf biomass yield, essential oil yield, and chemical composition were investigated during leaf ontogeny of palmarosa {*Cymbopogon martinii* (Roxb.) Wats. var. *motia* Burk., family Poaceae}. Eleven leaves representing different developmental stages, serially numbered from the apex to the base of the plant were utilized for the study. Leaf biomass yield increased up to the eighth leaf. Essential oil recovery increased up to the third leaf; thereafter it decreased. Minimum essential oil recovery was observed in the eleventh leaf. Essential oil yield/leaf increased up to the sixth leaf. Essential oil yield and concentrations of linalool, α -terpineol, geranyl isobutyrate and geraniol were relatively higher in the essential oils of mature, older leaves. Essential oil recovery, and percentages of myrcene, β -caryophyllene, geranyl acetate, (*E,Z*) farnesol and geranyl hexanoate were higher in the essential oils of young, expanding leaves.

Keywords: palmarosa, *Cymbopogon martinii* var. *motia*, leaf ontogeny, leaf biomass yield, essential oil yield, essential oil composition, geraniol.

Palmarosa {*Cymbopogon martinii* (Roxb.) Wats. var. *motia* Burk., family Poaceae} essential oil finds extensive use in perfumery (soaps, cosmetics, rose-like perfumes), flavoring (tobacco, food products, non-alcoholic beverages), aromatherapy, and in medicine (stiff joints, lumbago, skin diseases) [1]. The major constituents of the essential oil are linalool, geranyl acetate and geraniol. Leaves and inflorescences are the chief sources of essential oil. Leaf sheath [1], seeds [2], and stalks [3] also possess essential oil. In the aromatic crops lemongrass (*Cymbopogon flexuosus*) [4], citronella (*Cymbopogon winterianus*) [5], rose-scented geranium (*Pelargonium* species) [6], sage (*Salvia officinalis*) [7], clove (*Eugenia caryophyllata*) [8], and peppermint (*Mentha x piperita*) [9] changes in essential oil yield and composition were reported during leaf ontogeny. In the present study, we describe in detail, the changes in leaf growth, biomass yield, essential oil recovery, essential oil yield, and essential oil composition during palmarosa leaf development.

Leaf area increased significantly up to the sixth leaf due to increases in leaf length and width (Table 1). Earlier formed, older leaves reached smaller sizes than the latter formed young leaves; a similar pattern was

observed in rose-scented geranium [6]. Leaf biomass yield increased significantly up to the eighth leaf due to increase in leaf size. There was a significant decrease in biomass yield of the eleventh leaf in comparison with the ninth leaf, due to its smaller size and loss of moisture. In spite of the smaller leaf sizes, the biomass yields of the ninth and tenth leaves were at par with that of the eighth leaf. Decrease in biomass yield with advancement of leaf age was reported in lemongrass [4], and citronella [5].

The essential oil recovery increased significantly up to the third leaf, declined up to the seventh leaf, but with no further significant decrease up to the tenth leaf. The eleventh leaf recorded the lowest essential oil recovery. Young, expanding leaves exhibited higher recovery due to high biosynthetic activity [5,9,10], rapid formation of oil glands, and accumulation of essential oil in them [9,10]. The relationship between oil gland density, and leaf oil recovery was demonstrated in rose-scented geranium [6], and mints [11,12]. Crop responses to the relationship between leaf age, and essential oil recovery varied. In lemongrass, and citronella, the essential oil recovery increased in young, expanding leaves, and decreased with leaf age [4,5]. In rose-scented geranium,

Table 1: Length, width, area, yield, essential oil recovery, and essential oil yield of palmarosa leaves.

Leaf number	Leaf length (cm)	Leaf width (cm)	Leaf area (cm ²)	Leaf yield (mg/leaf)	Oil recovery (mL/100 g)	Oil yield (μL/leaf)
1	8.92	0.34	3.03	29.0	1.26	0.36
2	12.75	0.59	7.52	54.0	1.44	0.78
3	16.96	0.90	15.26	88.8	1.64	1.46
4	22.41	1.16	25.99	172.2	1.47	2.53
5	28.96	1.31	37.94	267.3	1.35	3.61
6	34.50	1.24	42.78	376.8	1.28	4.82
7	34.99	1.21	42.34	450.7	1.10	4.96
8	34.69	1.04	36.08	513.0	0.99	5.08
9	33.87	0.85	28.79	501.3	0.98	4.91
10	29.65	0.74	21.94	493.8	0.91	4.49
11	21.32	0.55	11.73	468.5	0.82	3.84
LSD ^a (<i>P</i> =0.05)	2.85	0.09	4.25	26.0	0.17	0.53

^aLSD = least significant difference**Table 2:** Percentage composition of essential oils of palmarosa leaves.

Leaf number	Myrcene	Linalool	β-Caryophyllene	α-Terpineol	Geranyl acetate	Geranyl isobutyrate	Geraniol	(<i>E,Z</i>) Farnesol	Geranyl hexanoate
1	0.18	1.04	0.60	0.26	3.76 ^a	0.09	85.24 ^b	2.75 ^a	2.38 ^a
2	0.12	0.99 ^b	0.75	0.31	3.32	0.10	86.31	2.73	1.75
3	0.12	1.34	0.78 ^a	0.20 ^b	2.48	0.09	88.22	2.55	1.54
4	0.14	1.30	0.56	0.24	1.52	0.08 ^b	90.05	2.42	1.52
5	0.14	1.72	0.48	0.27	1.21	0.17	91.30	2.33	1.35
6	0.32 ^a	1.96 ^a	0.43	0.35	0.90	0.16	91.44	2.30	1.33
7	0.20	1.86	0.53	0.45	0.59	0.16	92.18	2.23	1.26
8	0.13	1.64	0.53	0.56 ^a	0.42	0.18	92.09	2.24	1.24
9	0.11 ^b	1.67	0.51	0.38	0.40	0.16	92.60	2.21	1.18
10	0.12	1.52	0.36 ^b	0.27	0.39	0.19 ^a	93.25 ^a	2.15	1.08
11	0.13	1.64	0.38	0.42	0.33 ^b	0.18	92.03	2.14 ^b	1.07 ^b
LSD ^c (<i>P</i> =0.05)	0.02	0.20	0.16	0.17	0.21	0.03	0.81	0.06	0.11

^aHighest concentrations recorded in the leaves of different ages; ^bLowest percentages observed in the leaves of different ages; ^cLSD = least significant difference.

the essential oil recovery decreased with leaf age [6], whereas in peppermint, after an initial increase in the young leaves, it remained constant with increasing leaf age [9].

Nine constituents of the essential oils, accounting for 96.3-99.5%, were identified and are listed in Table 2. All the leaves produced essential oils of good quality with high levels of geraniol, the principal component of palmarosa essential oil, for which it is internationally traded. The leaves were, however, distinct in possessing the maximum (first, third, sixth, eighth, and tenth leaves), and the minimum (first, second, third, fourth, ninth, tenth, and eleventh leaves) concentrations of individual compounds. The percentages of myrcene, β-caryophyllene, geranyl acetate, (*E,Z*) farnesol, and geranyl hexanoate were comparatively higher in young leaves, and declined with leaf age. Mature, older leaves had relatively greater amounts of linalool, α-terpineol, geranyl isobutyrate, and geraniol. The accumulation of specific essential oil constituents in leaves of different ages is a function of relative abundance and activity of the enzymes responsible for their synthesis [10,13-15]. The relationship between levels and activities of the enzymes and accumulation of specific constituents in leaves of different ages has been demonstrated in lemongrass [13]. Essential oil composition variation in leaves of different ages was reported in lemongrass [4],

citronella [5], rose-scented geranium [6], sage [7], clove [8], and mints [9,11,16]. To the best of our knowledge, this is the first report on chemical profile changes in palmarosa leaf essential oils during leaf ontogeny.

Experimental

Leaf sample collection: Palmarosa cultivar Trishna was cultivated in the research farm of the Central Institute of Medicinal and Aromatic Plants Research Center, Hyderabad, India, following standard cultivation practices. The rainy season encourages luxuriant growth and produces high biomass and essential oil yields of palmarosa [17]. The present study was conducted during the rainy season when the crop was ready for harvest. Uniformly growing, healthy palmarosa plants were selected at random. Leaves were numbered serially (from one to eleven) from the apex proceeding downwards to the base of the plant. The first leaf on the inflorescence was the youngest, and the eleventh leaf, the oldest. Two hundred leaves of each developmental stage were collected in 3 replicates (11 leaves X 200 each X 3 replications = 6600 leaves in total). The fresh weights were recorded separately for all the leaves and presented as biomass yield. A separate set of 100 leaves each was collected for leaf area estimation. Leaf lengths and leaf widths were measured. Leaf areas (length X width) were computed.

Essential oil isolation: Leaf samples, in triplicate (11 leaves X 3 replications = 33 samples) were distilled in a Clevenger-type glass apparatus for 3 h. The essential oil samples were dried over anhydrous sodium sulfate and stored in a refrigerator in sealed vials until analysis. Essential oil recovery (mL/100 g biomass) and yield (biomass yield X essential oil recovery) were calculated.

GC analysis: GC analyses were carried out using a Varian Star 3400CX GC fitted with a flame ionization detector (FID) and an electronic integrator. Separation of the compounds was achieved employing a Supelcowax-10 capillary column (30 m X 0.25 mm X 0.25 µm film thickness) coated with carbowax 20 M (polyethylene glycol). Nitrogen was the carrier gas at 1 mL/min flow rate. The column temperature program was: 80°C (2 min) to 220°C (5 min) at 7°C/min ramp rate. The injector and the detector temperatures were 200°C and 240°C, respectively. Samples (0.1 µL) were injected with a 1:50 split ratio. Retention indices were generated with a standard solution of *n*-alkanes (C₈-C₂₃). Peak areas and retention times were measured by an electronic integrator. The relative amounts of individual compounds were computed from GC peak areas without FID response factor correction.

GC/MS analysis: GC-MS analyses were performed on a Hewlett-Packard 5890 GC coupled to a HP-5970 mass selective detector (MSD) and quadrupole EI mass analyzer. A HP-1 column (coated with methyl silicone)

(25 m X 0.25 mm X 0.25 µm film thickness) was used as the stationary phase. Helium was the carrier gas at 1 mL/min flow rate. Temperature was programmed from 60° to 220°C at 5°C/min ramp rate. The injector and the GC-MS interface temperatures were maintained at 250°C and 280°C, respectively. Mass spectra were recorded over 40–400 amu range at one span/s with 70 eV ionization energy and EI mode of ionization. The ion source and the detector temperatures were maintained at 250°C and 150°C, respectively. The samples (0.1 µL) were injected with a 1:50 split ratio.

Identification of constituents: Essential oil components were identified by comparing the retention times of the GC peaks with standard compounds run under identical conditions, by comparison of retention indices with published literature [18,19], and by comparison of MS with those reported in the literature [20,21], and stored in NIST and Wiley libraries.

Statistical analysis: The data were statistically analyzed employing analysis of variance technique [22]. The significance of differences between treatment variance and error variance was tested with variance (F) ratio. Least significant difference (LSD) values at 5% probability level (P=0.05) were calculated by multiplying standard error of difference (SEd) values with tabulated t values.

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Essential Oil Composition of Four Endemic *Ferulago* Species Growing in Turkey

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The essential oils from aerial parts of *Ferulago pachyloba* (Fenzl) Boiss., *F. platycarpa* Boiss. & Bal., *F. isaurica* Peşmen, and *F. longistylis* Boiss. (Apiaceae) were obtained by hydrodistillation and analyzed by GC and GC-MS. The highest oil yield (1.50%) was obtained from *F. pachyloba* followed by *F. longistylis* (0.16%), *F. isaurica* (0.08%) and *F. platycarpa* (0.07%). Fifty-three compounds were identified in the oil of *F. pachyloba* with (*Z*)- β -ocimene (25.7%) and α -pinene (9.8%) as main constituents; sixty-seven in the oil of *F. platycarpa* with 2,3,6-trimethylbenzaldehyde (29.8%) and *cis*-chrysanthenyl acetate (24.2%) as main components; seventy-eight in the oil of *F. isaurica* with nonacosane (25.5%) and hexadecanoic acid (14.8%) as main constituents; and fifty-nine in the oil of *F. longistylis* with 2,3,6-trimethylbenzaldehyde (32.7%) and bornyl acetate (12.6%) as main components. Quantitative and qualitative differences in the oil compositions of these four species were observed.

Keywords: *Ferulago pachyloba*, *F. platycarpa*, *F. isaurica*, *F. longistylis*, Apiaceae, essential oil composition, GC analysis, GC-MS analysis.

Ferulago W. Koch. is a perennial genus of the Apiaceae family represented by nearly forty species, thirty-two of which exist in the flora of Turkey, seventeen being endemic. This suggests that the gene centre for this genus is Anatolia [1-7]. The species of this genus are known as kuzukemirdi, kuzukişnişi, kuzubaşı, çakşırotu, çağşır, asaotu and kişniş in different regions of Turkey.

Ferulago species have been used since antiquity for the treatment of intestinal worms, hemorrhoids and as a sedative, tonic and digestive. Moreover, they are used against ulcers, snake bites, spleen diseases and headache [8]. It has also been reported that gums obtained from the incision of the roots of some species are used as seasoning and as a carminative [9]. However, the plants are mainly known as aphrodisiacs and as a preferred fodder to increase animal productivity [10].

Ferulago species have been reported to contain flavonoids, quinones, coumarin esters, sesquiterpenes, coumarins, furanocoumarins and aromatic compounds [11-17]. The genus is rich in essential oil and several species have been studied; *F. angulata* (Schlecht.) Boiss., *F. asparagifolia* Boiss., *F. galbanifera* (Mill.)

W. D. J. Koch, *F. humilis* Boiss., *F. sandrasica* Pesmen et Quézel, *F. aucheri* Boiss., *F. confusa* Velen, *F. idaea* Özhatay et E. Akalın, *F. macrosciadia* Boiss. et Bal., *F. mughlae* Pesmen, *F. silaifolia* (Boiss.) Boiss., *F. bernardii* L. Tomkovich & M. Pimenov. *F. carduchorum* Boiss. et Hausskn., *F. contracta* Boiss. et Hausskn., *F. isaurica* Pesmen, *F. syriaca* Boiss., *F. nodosa* (L.) Boiss., *F. thyrsoflora* (Sm.) W. D. J. Koch, *F. phialocarpa* Rech. f. et Riedl, *F. sylvatica* (Besser) Reichb., *F. thirkeana* (Boiss.) Boiss., *F. trachycarpa* Boiss., *F. longistylis* Boiss. The major constituents of the oils of these foregoing species were β -ocimene, α -pinene, α - and β -phellandrene, limonene, myrcene and *p*-cymene [18]. The aim of this paper is to present and compare the chemical composition of the essential oils of four endemic *Ferulago* species; *F. pachyloba*, *F. platycarpa*, *F. isaurica* and *F. longistylis* growing in Turkey. Gas chromatographic (GC) and gas chromatographic-mass spectroscopic (GC-MS) analysis helped us to establish the composition and the relationship of the essential oil constituents. To the best of our knowledge, this is the first report on the chemical analysis of *F. pachyloba* and *F. platycarpa*. The identified constituents are presented in Table 2.

Table 1: Collection data for the investigated *Ferulago* species.

Code	<i>Ferulago</i> species	Specimen Herbarium Number	Collection place, and altitude	Collection date	Oil Yield [‡] (%)
A	<i>F. pachyloba</i>	GAZI [‡]	Niğde, Aladağlar, around Demirkazık, rocky slopes, 2600 m	August 2007	1.5
B	<i>F. platycarpa</i>	AEF* 23173	Nevşehir, Üçhisar, Gemil Mount, northern slopes, 1450 m	July 2004	0.07
C	<i>F. isaurica</i>	AEF 22957	Alanya – Antalya road, 6 km after Derince turn, rocky slopes, 1110 m	September 2002	0.08
D	<i>F. longistylis</i>	AEF 23795	Erzincan, Sakaltutan Pass, roadsides, 2000 m	July 2006	0.16

[‡] Herbarium of Gazi University Faculty of Arts and Sciences

* Herbarium of Ankara University Faculty of Pharmacy

[‡] Yields are given on moisture free basis

A total of fifty-three compounds representing 96.6% of the oil were identified in the essential oil of *F. pachyloba*. (*Z*)- β -ocimene, α -pinene, sabinene and δ -cadinene were the major components, amounting to 25.7%, 9.8%, 6.3% and 5.6%, respectively. The analysis of *F. platycarpa* resulted in the identification of sixty-seven volatile compounds representing 94.1% of the oil. 2,3,6-Trimethylbenzaldehyde at 29.8% was the most abundant compound in the volatile oil, followed by *cis*-chrysanthenyl acetate (24.2%), nonacosane (7.7%) and α -pinene (4.2%). Seventy-eight compounds were characterized in the oil of *F. isaurica* representing 86.3% of the oil. The major constituents were nonacosane (25.5%), hexadecanoic acid (14.8%), bornyl acetate (5.3%) and terpinen-4-ol (4.6%). Fifty-nine compounds, representing 92.5% of the oil, were identified in the oil of *F. longistylis* with 2,3,6-trimethylbenzaldehyde (32.7%), bornyl acetate (12.6%), *p*-cymene (11.9%) and *cis*-chrysanthenyl acetate (4.2%), as main components.

Considering the different groups of compounds, monoterpene hydrocarbons contributed most to the oils obtained from *F. pachyloba* (57.7%) and *F. longistylis* (22.4%), whereas oxygenated monoterpenes formed the main portion of the oils of *F. platycarpa* (33.1%) and *F. isaurica* (18.0%). Comparison of the main constituents of these four species (Table 2) shows that each species has a different set of dominant compounds. However, previous studies of the oils of *Ferulago* species [18] revealed that the three compounds detected in high percentages in this study, namely (*Z*)- β -ocimene, α -pinene and *p*-cymene have also been detected as major components in many other species. Erdurak *et al.* reported the essential oil contents of the fruit and root of *F. isaurica* [19] in 2006. The oils

obtained from the different parts of this species did not show much qualitative resemblance. The major constituents detected, namely α -pinene, limonene, and myrcene in the fruit, and terpinolene and myrcene in the root were not identified in the oil from the aerial parts of *F. isaurica*. Analysis of the fruit oil of *F. longistylis* conducted in 2008 showed 2,3,6-trimethylbenzaldehyde and bornyl acetate as the major compounds, in accordance with the results obtained for the aerial parts of the same species in this study [18].

Chemical profiling of the volatiles may be useful in taxonomical classification. The results presented in this study confirm some specific features of the oil composition of *Ferulago* species in Turkey and contribute to a better knowledge of this genus.

Experimental

Plant materials: Aerial parts of 4 *Ferulago* species were collected by the authors from their natural habitats in different localities of Turkey by random sampling from a single established population, as shown in Table 1; the plants were identified by Prof. Dr. Hayri Duman and voucher specimens were deposited in GAZI and in AEF.

Essential oil isolation: Air dried aerial parts of plants (50 g) were subjected hydrodistillation for 3 h using a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia [20]. The obtained oils were dried over anhydrous sodium sulfate and stored in sealed vials at +4°C in the dark until analyzed and tested. All oils were pleasant smelling, transparent with a faint yellow color.

GC/MS analysis: GC-MS analysis was carried out with an Agilent 5975 GC-MSD system. An Innovax FSC column (60 m x 0.25 mm, 0.25 μ m film thickness) was used with helium as carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and programmed to 220°C at a rate of 4°C/min, and kept constant at 220°C for 10 min and then programmed to 240°C at a rate of 1°C/min. The split ratio was adjusted at 40:1. The injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. The mass range was from *m/z* 35 to 450.

GC analysis: The GC analysis was carried out using an Agilent 6890N GC system. The FID detector temperature was 300°C. To obtain the same elution order with GC/MS, simultaneous autoinjection was used on a duplicate column applying the same operational conditions. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Table 2: Composition of the essential oils of A: *Ferulago pachyloba*, B: *F. platycarpa*, C: *F. isaurica*, and D: *F. longistylis*.

RRI	Compound	A (%)	B (%)	C (%)	D (%)
1032	α -Pinene	9.8	4.2	-	3.5
1035	α -Thujene	0.3	0.1	-	0.1
1076	Camphene	0.8	0.2	-	0.2
1118	β -Pinene	0.6	0.3	-	0.2
1132	Sabinene	6.3	1.6	-	0.4
1159	δ -3-Carene	3.0	-	-	-
1174	Myrcene	1.9	1.7	-	1.4
1176	α -Phellandrene	0.5	-	-	-
1195	Dehydro-1,8-cineole	0.1	-	tr	-
1203	Limonene	2.2	0.6	0.9	0.5
1218	β -Phellandrene	0.2	0.1	-	0.1
1246	(Z)- β -Ocimene	25.7	0.2	-	2.3
1255	γ -Terpinene	3.1	0.3	0.3	1.4
1266	(E)- β -Ocimene	1.9	0.4	-	0.4
1280	<i>p</i> -Cymene	1.2	1.5	0.2	11.9
1286	Isoterpinolene	0.2	-	-	-
1290	Terpinolene	1.2	-	0.2	-
1294	1,2,4-Trimethyl benzene	-	0.8	-	1.8
1355	1,2,3-Trimethyl benzene	-	0.3	-	0.7
1382	<i>cis</i> -Alloocimene	0.2	-	-	-
1429	Perillen	-	0.1	-	-
1439	γ -Campholene aldehyde	-	0.1	-	0.1
1441	(E)-2-Octenal	-	0.1	-	-
1446	2,6-Dimethyl-1,3(E),5(Z),7-octatetraene	0.1	-	-	-
1452	α , <i>p</i> -Dimethylstyrene	-	0.1	0.2	0.2
1476	(Z)- β -Ocimene epoxide	-	tr	-	0.1
1479	δ -Elemene	-	0.1	-	-
1492	Cyclosativene	-	0.1	tr	-
1497	α -Copaene	0.2	0.2	0.3	tr
1499	α -Campholene aldehyde	-	0.6	0.1	0.7
1528	α -Bourbonene	-	-	0.1	-
1535	β -Bourbonene	0.1	0.1	0.5	0.2
1553	Linalool	-	-	0.7	-
1562	Octanol	-	0.1	tr	-
1571	<i>trans-p</i> -Menth-2-en-1-ol	-	0.1	0.3	tr
1582	<i>cis</i> -Chrysanthenyl acetate	-	24.2	-	4.2
1586	Pinocarvone	0.1	-	-	-
1589	β -Ylangene	-	-	0.1	-
1591	Bornyl acetate	0.9	2.1	5.3	12.6
1597	β -Copaene	tr	-	0.1	-
1600	β -Elemene	0.6	0.2	1.2	-
1611	Terpinen-4-ol	-	1.3	4.6	0.4
1612	β -Caryophyllene	4.4	-	-	0.3
1639	<i>trans-p</i> -Mentha-2,8-dien-1-ol	-	-	0.3	-
1639	Cadina-3,5-diene	0.1	-	-	-
1645	<i>cis</i> -Verbenyl acetate	-	-	-	0.3
1648	Myrtenal	-	0.3	0.1	0.2
1650	γ -Elemene	-	-	-	-
1661	<i>trans</i> -Pinocarvyl acetate	0.3	0.9	-	1.0
1670	<i>trans</i> -Pinocarveol	-	0.7	0.4	0.4
1678	<i>cis-p</i> -Mentha-2,8-dien-1-ol	-	-	0.1	-
1683	<i>trans</i> -Verbenol	-	0.7	0.2	-
1687	α -Humulene	0.6	-	0.2	-
1700	<i>p</i> -Mentha-1,8-dien-4-ol (=Limonen-4-ol)	-	-	0.8	-
1704	Myrtenyl acetate	0.1	-	-	-
1704	γ -Murolene	0.2	-	-	-
1706	α -Terpineol	-	-	0.5	-
1719	Borneol	-	0.1	0.3	0.3
1725	Verbenone	-	0.1	tr	0.1
1726	Germacrene D	1.9	-	1.7	-
1738	<i>p</i> -Mentha-1,5-dien-8-ol	-	-	-	0.7
1740	α -Murolene	0.8	-	-	-
1741	β -Bisabolene	-	-	-	-
1742	β -Selinene	-	0.3	-	-
1744	α -Selinene	-	tr	0.2	-
1751	Carvone	-	0.1	0.7	-
1755	Bicyclogermacrene	1.2	-	-	-
1758	<i>cis</i> -Piperitol	-	tr	0.2	-
1763	Naphthalene	-	-	0.2	0.3
1764	<i>cis</i> -Chrysanthenol	-	0.7	-	-
1773	δ -Cadinene	5.6	-	0.3	-
1776	γ -Cadinene	1.2	-	0.2	-
1785	7- <i>epi</i> - α -Selinene	0.2	-	-	-
1786	<i>ar</i> -Curcumene	-	0.1	-	0.4
1797	<i>p</i> -Methyl acetophenone	-	-	0.1	-
1799	Cadina-1,4-diene (=Cubenene)	0.1	-	-	-
1804	Myrtenol	-	0.2	0.1	-
1811	<i>trans-p</i> -Mentha-1(7),8-dien-2-ol	-	-	0.3	-
1827	(E,E)-2,4-Decadienal	-	0.1	0.1	0.1
1838	(E)- β -Damascenone	-	-	0.1	-
1845	<i>trans</i> -Carveol	-	0.4	0.9	0.3
1857	Geraniol	-	-	0.3	-
1864	<i>p</i> -Cymen-8-ol	-	0.3	0.8	0.4
1868	(E)-Geranyl acetone	-	0.1	0.4	0.1
1882	<i>cis</i> -Carveol	-	-	0.2	-
1896	<i>cis-p</i> -Mentha-1(7),8-diene-2-ol	-	-	0.1	-
1900	<i>epi</i> -Cubebol	0.5	-	-	-
1925	2,3,4-Trimethyl benzaldehyde	-	1.5	-	3.1
1941	α -Calacorene	0.1	-	0.1	-
1945	1,5-Epoxy-salvial(4)14-ene	-	-	-	0.1
1957	Cubebol	0.6	-	-	-
1958	(E)- β -Ionone	-	-	0.1	-
1973	Dodecanol	-	0.1	-	-
2008	Caryophyllene oxide	0.5	-	1.7	0.2
2019	2,3,6-Trimethylbenzaldehyde	-	29.8	0.7	32.7
2037	Salvial-4(14)-en-1-one	-	-	0.3	0.1
2050	(E)-Nerolidol	-	-	0.5	-
2069	Germacrene D-4 β -ol	5.3	-	-	-
2071	Humulene epoxide-II	-	0.1	0.2	0.1
2073	<i>p</i> -Mentha-1,4-dien-7-ol	-	-	0.1	-
2080	Cubenol	0.2	-	-	-
2088	1- <i>epi</i> -Cubenol	0.2	-	-	-
2100	Heneicosane	-	0.1	-	-
2103	Guaiol	0.6	-	-	-
2122	Hedycaryol	1.9	-	-	-
2130	Salviadienol	-	-	0.3	0.2
2131	Hexahydrofarnesyl acetone	-	0.3	0.8	-
2144	Spathulenol	1.1	0.7	0.9	-
2148	(Z)-3-Hexen-1-yl benzoate	-	-	-	0.9
2179	Tetradecanol	-	0.2	0.3	-
2187	T-Cadinol	1.8	-	-	-
2200	Docosane	-	0.1	-	-
2209	T-Murolol	1.5	-	-	-
2219	Dimyrcene II-a	-	0.1	0.7	-
2226	Methyl hexadecanoate	-	tr	0.3	-
2250	α -Eudesmol	0.2	-	-	-
2255	α -Cadinol	4.2	-	tr	-
2269	Guaia-6,10(14)-dien-4 β -ol	-	12	0.3	-
2269	Dimyrcene II-b	-	-	0.4	-
2278	Torilenol	-	tr	0.4	-
2296	Myristicine	-	-	3.4	-
2300	Tricosane	-	0.2	0.6	0.1
2324	Caryophylla-2(12),6(13)-dien-5 α -ol (=Caryophylladienol II)	-	-	0.5	0.3
2369	Eudesma-4(15),7-dien-1 β -ol	-	-	1.1	0.3
2384	Hexadecanol	-	-	-	0.2
2384	Farnesyl acetone	-	-	1.0	-
2392	Caryophylla-2(12),6-dien-5 β -ol (=Caryophyllenol II)	-	-	-	0.4
2500	Pentacosane	-	0.7	0.7	0.2
2622	Phytol	tr	0.4	3.9	0.2
2655	Benzyl benzoate	-	-	0.6	0.7
2670	Tetradecanoic acid	-	0.1	0.6	tr
2700	Heptacosane	-	-	0.6	tr
2822	Pentadecanoic acid	-	tr	tr	tr
2900	Nonacosane	-	7.7	25.5	2.7
2931	Hexadecanoic acid	-	3.9	14.8	1.7
	Monoterpene Hydrocarbons	57.7	11.2	1.4	22.4
	Oxygenated Monoterpenes	2.9	33.1	18	21.9
	Sesquiterpene Hydrocarbones	17.3	1.1	5.0	0.9
	Oxygenated Sesquiterpenes	18.6	2.0	7.2	1.7
	Diterpenes	-	0.5	5.0	0.2
	Fatty acid	-	4.0	15.4	1.7
	Others	0.1	42.2	34.3	43.7
	Identified compounds	53.0	67.0	78.0	59.0
	Total	96.6	94.1	86.3	92.5

Identification of components: Identification of the essential oil components was carried out either by comparison of their relative retention times with those of authentic samples or by comparison of their relative retention index (RRI) with a series of *n*-alkanes. Computer matching against commercial (Wiley GC/MS

Library, Adams Library, MassFinder 2.1 Library) [21,22], and in-house “Başer Library of Essential Oil Constituents” built up from genuine compounds and components of known oils, as well as MS literature data [23-25], was used for the identification.

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Essential Oils of *Daucus carota* subsp. *carota* of Tunisia Obtained by Supercritical Carbon Dioxide Extraction

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The essential oils and supercritical CO₂ extracts of wild *Daucus carota* L. subsp. *carota* from two different sites in Tunisia were investigated. The main components of the essential oil of the flowering and mature umbels with seeds from Sejnane were eudesm-7(11)-en-4-ol (8.2 – 8.5%), carotol (3.5 - 5.2%), sabinene (12.0 -14.5%), α -selinene (7.4 – 8.6) and 11- α -(H)-himachal-4-en-1- β -ol (12.7 – 17.4%), whereas the oils from Tunis were predominantly composed of elemicin (31.5 – 35.3%) and carotol (48.0 – 55.7%). The antimicrobial activity of the essential oils were assayed by using the broth dilution method on *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 43300, and clinical strains of *Candida albicans* and *C. tropicalis* 1011 RM. The MIC values obtained were all > 2.5% (v/v).

Keywords: *Daucus carota* L. subsp. *carota*, essential oil, supercritical CO₂, antibacterial activity, antifungal activity.

Daucus carota L. subsp. *carota* (Apiaceae) is native to Europe, Asia and Africa. Traditional medicine uses *D. carota* extracts for hepatic and renal insufficiency as well as for skin disorders, for example burns and furunculosis [1]. Extracts of the wild plants are known to be antioxidative and iron-chelative. Ahmed *et al.*, from the roots of wild *D. carota* subsp. *carota*, isolated three new sesquiterpene daucane derivatives and four known compounds that showed antifungal activity [2]. Also, the methanol extract of the seeds showed antibacterial activity [3]

Carrot seed essential oil is widely used as a flavor ingredient in many foods and as a fragrance in perfumes, cosmetics and soaps. Claimed biological properties of the oil include antimicrobial, hepatocellular regenerator, general tonic and stimulant, cholesterol regulator, and cicatrisant. The essential oil from the aerial parts of the plant showed particular activity against Gram-positive bacteria, but the activity of the oils from different organs of the plant were not

the same, decreasing in the sequence, mature umbel oils > herb oil > flowering umbel oils [4]. The part of the plant, the stage of development as well as the geographical origin can influence significantly the composition of the oils obtained from this species. From literature data it can be seen that leaf, stem, and blooming umbel oils are dominated by monoterpenes and/or sesquiterpenes [5-6]. Oils isolated from umbels or seeds were dominated by sesquiterpenes and phenylpropanoids, β -bisabolene and β -asarone or by (*E*)-methylisoeugenol accompanied by α -pinene and elemicin.

Considerable variations were also found depending on geographical area, especially in the composition of the seed oils. Góra *et al.* [7] and Staniszewska and Kula, [8] showed that oils from Poland were dominated by either sabinene and α -pinene or sabinene, α -pinene and geranyl acetate. Similar results were reported by Mockute and Nivinskiene [9] for Lithuanian carrot seed essential oil. However, carrot seed oil from Turkey was

reported to contain a high content of carotol (67%) and daucene (9%) [10]. Also Glisic *et al.* [1] found in the supercritical extract from seed of carrot from Serbia, carotol as the principal component (30%). For commercial carrot seed oil, which includes oils of all the subspecies of *D. carota*, four compositions can be distinguished. Three of these are dominated by sabinene (32–60%) and either geranyl acetate (32–77%) or carotol (23–77.5%), and the fourth contains these compounds with an identical ratio [6]. The aim of the present work was to investigate the compositions of the volatile oils of *D. carota* subsp. *carota* growing wild in Tunisia at two different sites (Sejnane and Tunis). Carbon dioxide supercritical extracts (SFE) were also produced and their compositions investigated.

D. carota subsp. *carota* essential oil of ripe umbels (with mature seeds) was obtained in yields of 1.0% and 2.0% for HD and SFE, respectively. Thirty-six identified compounds made up 93.2–97.3% of the oil (Table 1).

The oil from mature umbels with seeds from Sejnane (sample1) was composed of sesquiterpene hydrocarbons (36.1 – 37.2%) and oxygenated sesquiterpenes (29.2 – 35.9%). The main components were carotol (3.5 – 5.2%), β -bisabolene (5.5–7.6), α -selinene (7.4–8.6%), eudesm-7(11)-en-4-ol (8.2 – 8.5%), sabinene (12.0 - 14.5%) and 11- α -(H)-himachal-4-en-1- β -ol (12.7 – 17.4%). However, the oil from the mature umbels with seeds from the second site (sample 2) was of the carotol chemotype (48.0–55.7%), with the second main constituent being elemicin (31.5 - 35.3%). This oil is predominantly composed of oxygenated sesquiterpenes (48–55.7%) and phenylpropanoids (31.5- 35.3%). This latter sample did not contain any oxygenated monoterpenes.

It is interesting to note that phenylpropanoids represented 31.5% of the oil in sample 2, but only 4.7 % of sample 1 (data of HD extract). Similar results were reported by Maxia *et al.* [18], who found that the umbels with seed oils obtained from plants of *D. carota* ssp. *carota* from Italy contained 15.2% of phenylpropanoids, which was nearly five times that found in oils obtained from plants growing in Portugal (3.4%). 11 α -H-himachal-4-ene-1 β -ol and eudesm-7(11)-en-4-ol, the main compounds identified in the extracts from site 1 were not identified in the extracts obtained from site 2. The same components were found in the oil from Sardinia [18]. These differences could have several reasons, such as climate, soil or other geographical factors [19], but also to genetic differences probably responsible for the chemical variability in the carrot oil.

Table1: Retention index (RI) and chromatographic area percentages of compounds found in *Daucus carota* subsp. *carota* essential oils from site 1 (A1=HD and B1=SFE) and site 2 (A2=HD and B2=SFE).

RI	Compound	A1	B1	A2	B2
937	α -Pinene ^{a,b,c}	1.0	0.5	1.4	0.4
977	Sabinene ^{a,b,c}	14.5	12.0	1.7	0.5
980	β -Pinene ^{a,b,c}	2.7	2.0	1.0	0.3
992	Myrcene ^{a,b,c}	0.6	0.2	0.2	-
1019	α -Terpinene ^{a,b}	0.6	-	0.2	-
1027	<i>p</i> -Cymene ^{a,b,c}	0.5	-	-	-
1031	Limonene ^{a,b,c}	0.4	0.1	0.4	0.5
1061	γ -Terpinene ^{a,b,c}	0.7	-	-	-
1141	<i>cis</i> -Sabinol ^{a,b}	0.5	-	-	-
1178	Terpinen-4-ol ^{a,b,c}	1.2	0.2	-	-
1191	α -Terpineol ^{a,b}	0.4	-	-	-
1286	Bornyl-acetate ^{a,b,c}	0.5	-	-	-
1351	α -Longipinene ^{a,b}	1.3	1.4	1.3	0.8
1376	α -Copaene ^{a,b}	0.2	1.9	0.2	-
1380	β -Cubebene ^{a,b}	0.2	0.3	0.1	-
1391	β -Elemene ^{a,b}	0.8	0.9	0.2	-
1404	Methyleugenol ^{a,b,c}	0.7	0.5	-	-
1415	<i>cis</i> - α -Bergamotene ^{a,b}	0.3	-	-	-
1418	(<i>E</i>)-Caryophyllene ^{a,b,c}	0.4	0.4	2.2	1.4
1437	<i>trans</i> - α -Bergamotene ^{a,b}	3.8	0.2	0.2	-
1445	(<i>Z</i>)- β -Farnesene ^{a,b}	4.5	5.0	2.6	1.6
1453	α -Humulene ^{a,b,c}	1.3	1.5	-	-
1458	(<i>E</i>)- β -Farnesene ^{a,b}	2.9	3.5	-	-
1480	γ -Muurolene ^{a,b}	1.2	2.0	-	-
1485	Bicyclogermacrene ^{a,b}	2.4	0.2	-	-
1493	δ -Selinene ^{a,b}	1.0	1.2	-	-
1498	α -Selinene ^{a,b}	7.4	8.6	-	-
1509	β -Bisabolene ^{a,b}	5.5	7.6	-	-
1513	Elemicin ^{a,b}	1.4	1.6	31.5	35.3
1524	<i>E</i> (α)-Bisabolene ^{a,b}	1.8	1.9	0.4	0.5
1556	Germinenene B ^{a,b}	0.6	0.6	0.3	0.3
1559	Carotol ^{a,b}	3.5	5.2	48.0	55.7
1630	11- α -(H)-Himachal-4-en-1- β -ol ^{a,b}	12.7	17.4	-	-
1653	α -Cadinol ^{a,b}	1.8	2.3	-	-
1682	α -Asarone ^{a,b}	1.6	2.6	-	-
1694	Eudesm-7(11)-en-4-ol ^{a,b}	8.2	8.5	-	-
Monoterpene hydrocarbons		21.0	14.8	4.9	1.7
Oxygen containing monoterpenes		2.6	0.2	-	-
Sesquiterpene hydrocarbons		36.1	37.2	7.5	4.6
Oxygen containing sesquiterpenes		29.2	35.9	48.0	55.7
Phenylpropanoids		4.7	5.1	31.5	35.3
Total identified		93.6	93.2	91.9	97.3

Identification has been realized by comparing mass spectra (a), retention indices (b), and injection of authentic compound (c).

Extracts obtained by SFE revealed quantitative differences in their composition when compared with those of distilled essential oils. For example, SFE extract contains lower amounts of monoterpenes (15 vs. 23.6% and 1.7 vs. 4.9%) and higher amounts of phenylpropanoids (5.1 vs. 4.7% and 35.3 vs. 31.5%).

Hydrodistillation induces migration of volatile compounds from the inside of the secretory structures to the surface, followed by their subsequent evaporation. Therefore, low molecular weight compounds are easily taken from the vegetable matrix while supercritical CO₂ emulates an organic solvent, improving the extraction of high molecular weight compounds. No differences were noted in the compositions of SFE extracts with changing extraction times.

The MIC and MCC values of the *D. carota* oil, both samples, obtained by the microdilution method were > 2.5 (v/v) against all the test microorganisms. The values for the Italian and Portuguese carrot oils varied depending on the fungal species investigated. *C. neoformans* and dermatophyte strains showed more sensitivity to these oils than *Candida* and *Aspergillus* strains [9]. On the other hand, Jabrane *et al.* [20] found that flower and root oils of Tunisian *D. carota* ssp. *maritimus* had a significant and broad spectrum of activity against Gram-positive and Gram-negative bacteria, the flower oil being more effective than the root oil against *E. coli* (ESLb). Conversely, the root oil was found to be more active than the flower oil towards *S. aureus* (reference and environmental strains). An essential oil is usually a complex mixture of different components and so it is difficult to reduce the antimicrobial effect of the total oil to either one or a few active principles. It cannot be ignored that, in addition to the main compounds, minor compounds could be making a significant contribution to the oil activity [21].

Experimental

Plant material: Umbels of *Daucus carota* L. subsp. *carota* were collected from two different sites in Tunisia from two different bioclimatic zones: Sejnane (humid) (samples 1) and Tunis (sub-humid) (samples 2). Voucher specimens (numbers: COI00033068 and CAG 625) were deposited at the Herbarium of the Department of Botany of the University of Tunis. Vegetal material was air-dried in a hot air-drier at 40°C with forced ventilation for 2 days. Before utilization, the plant material was ground with a Malavasi mill (Bologna, Italy) taking care to avoid overheating.

Hydrodistillation: Hydrodistillation (HD) was performed in a circulatory Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia [11] for 4 h.

SFE extraction: Supercritical CO₂ (purity 99% - Air Liquide Italia, Cagliari, Italy) extractions were performed according Marongiu *et al.* [12] in a laboratory apparatus equipped with a 320 cm³ extraction vessel and two separator vessels of 300 and 200 cm³, respectively connected in series. Experiments were carried out at 90 bar and 40°C in the extraction section. In the first separator, the temperature was set at -10°C and the pressure at the same value as that of the extraction section. The second separator was set at 15 bar and 10°C. Extractions were carried out in a semi batch mode, with batch charging of vegetable matter and continuous flow of solvent. About 180 g of material were charged in each run.

GC/MS analysis: An Agilent Technologies Inc. gas chromatograph (Santa Clara, CA, USA) model 6890N was employed for analysis of the essential oils. It was equipped with a split-splitless injector, an autosampler Agilent model 7683 and an Agilent HP5 fused silica column; 5% phenyl-methylpolysiloxane, 30 m × 0.25 mm i.d., film thickness 0.25 µm. GC conditions used were: programmed heating from 60 to 280°C at 3°C/min, followed by 30 min under isothermal conditions. The injector was maintained at 250°C. Helium was the carrier gas at 1.0 mL/min; the sample (1 µL) was injected in the split mode (1:20). The GC was fitted with a quadrupole mass spectrometer (MS, Agilent model 5973 detector). MS conditions were as follows: ionization energy 70 eV, electronic impact ion source temperature 200°C, quadrupole temperature 100°C, scan rate 1.6 scan/sec, mass range 50-500 u. The software adopted to handle MS and chromatograms was a ChemStation NIST 02 [13] and LIBR (TP) [14]. Mass Spectra Libraries were used as references. Samples were run in chloroform with a dilution ratio of 1:100. Compounds were identified by matching their MS and retention index with those reported in the literature [15]. Moreover, whenever possible, identification was confirmed by injection of an authentic sample of the compound. A quantitative analysis of each oil component (expressed in percentages) was carried out by peak area normalization measurement. The response factors were estimated using standard compounds having the same molecular weight as the compound families that constitute the essential oil (hydrocarbon and oxygenated monoterpenes, hydrocarbon and oxygenated sesquiterpenes).

Antimicrobial activity: The organisms tested in this study were as follow: *Escherichia coli* (ATCC 35218), *Staphylococcus aureus* (ATCC 43300), *Candida albicans* (clinical strain) and *C. tropicalis* (1011 RM) (clinical strain). Bacteria were cultured overnight in Luria-Bertani Broth (LB) and fungi in Sabouraud Dextrose Agar plates. Minimal inhibitory concentration (MIC) values were determined as the lowest essential oil concentration that inhibits visible growth of the isolates after 24-48 h incubation at 37°C. It was measured with the broth dilution method (microdilution using 96-well microplates) [16,17]. Nine different concentrations of each essential oil from 2.5%, v/v to 0.001%, v/v with 10% Tween 80 were used. The bacterial and fungal cultures were diluted with LB broth and RPMI medium, respectively, to obtain 1.0x10⁸ CFU/mL (0.5 MacFarland).

Minimal cidal concentration (MCC) values were determined as the lowest essential oil concentration that kills both bacteria and fungi. It was measured with the

broth dilution method starting from the MIC as the lowest concentration to the maximum one (2.5% v/v). Positive and negative controls were also included.

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Oil Constituents of *Artemisia nilagirica* var. *septentrionalis* Growing at Different Altitudes

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Oils of *Artemisia nilagirica* var. *septentrionalis* plants growing at different altitudes in Himachal Pradesh, India were hydro-distilled and analyzed by GC-GC/MS. The major constituents of the oil show variation with changes in altitude. At lower, middle and higher altitudes, the major constituents of the oil were caryophyllene oxide (28.6%), borneol (35.8%) and camphor (46.9%), respectively. The percentages of α -humulene and *trans*- β -guaiene also increased, but the percentage of sabinene, *trans*-sabinene hydrate, 4-terpineol, caryophyllene oxide and humulene epoxide-II decreased with an increase in altitude. The characteristic compounds observed in the plants from lower altitudes were 2-hexene-1-ol, β -thujone, thujanol, myrtenol and linalyl acetate, while the higher altitude plants were characterized by the presence of α -pinene, β -pinene, limonene, linalool, γ -gurjunene, germacrene-D and farnesol.

Keywords: *Artemisia nilagirica* var. *septentrionalis*, Asteraceae, essential oil constituents.

Artemisia nilagirica var. *septentrionalis* Pamp., syn *A. vulgaris* L. (Family Asteraceae), plants are distributed mostly in the Western Himalayas. The plants produce a light yellow oil. [1]. *A. nilagirica* is used in traditional medicine as a tonic and in the treatment of bronchial troubles [2]. An ointment of *A. nilagirica* oil has been reported as an antifungal agent against dermatomycosis [3,4]. An insecticidal principle, capillin, has also been isolated from the petroleum extract of *A. nilagirica* [5].

The essential oil of *A. nilagirica* is reported to contain camphor, β -eudesmol, 1,8-cineole and borneol [6]. Recently, the composition of the essential oil of *A. nilagirica* var. *septentrionalis* has been reported [7]. In the present study, the oil obtained from *A. nilagirica* var. *septentrionalis* plants growing at different altitudes in Himachal Pradesh were analyzed by GC-GC/MS and thirty-four constituents were identified. In the Shimla, Mandi and Manali samples, the oil yield was 0.25%, 0.56% and 1.0%, respectively. The identified constituents in the oils totaled 23, 17 and 24, constituting 76%, 83% and 93% of the total oil, respectively. At the lower altitudes of Mandi, caryophyllene oxide (28.6%) was the major constituent of the oil followed by methanoazulene (10.9%) and borneol (4.5%). At the middle altitude of Manali, borneol (35.8%) became the major constituent of the oil followed by methanoazulene (14.7%) and

caryophyllene oxide (13.4%). However, at the higher altitude of Shimla, the major oil constituents were quite different. In this case camphor (46.9%) was the major constituent followed by β -caryophyllene (13.3%) and α -humulene (9.7%). The characteristic constituents identified in the oil of plants collected from Mandi were 2-hexene-1-ol, β -thujone, thujanol, myrtenol and linalyl acetate, which were not identified in the oils from the other places. Oil obtained from the plants collected from Manali had only three characteristic constituents, which were α -phellandrene, β -phellandrene and α -thujone. The Shimla materials contained a maximum of seven characteristic constituents in its oil, which were α -pinene, β -pinene, limonene, linalool, γ -gurjunene, germacrene D and farnesol. However, eleven constituents were observed to be common in all the examined samples; these were sabinene, *trans*-sabinene hydrate, camphor, borneol, β -elemene, β -caryophyllene, α -humulene, *trans*- β -guaiene, caryophyllene oxide, methanoazulene and humulene epoxide-II.

It is interesting to note that some of the compounds, such as α -humulene and *trans* β -guaiene, increase in quantity with an increase of altitude, while constituents like sabinene, *trans*-sabinene hydrate, 4-terpineol, caryophyllene oxide and humulene epoxide-II increase in quantity with a decrease in altitude. Although the oil of *A. nilagirica* var. *septentrionalis* has been analyzed during different growth stages in the plants that were

Table 1: Effect of altitude on the oil constituents of *Artemisia nilagirica* var. *septentrionalis*.

Constituents	RI	% oil constituents (dry weight)		
		Mandi (1044 m)	Manali (2050 m)	Shimla (2210 m)
2-Hexene-1-ol	854	2.6	-	-
α -Pinene	939	-	-	0.6
Camphene	953	0.2	-	0.5
Sabinene	967	4.4	0.1	0.1
β -Pinene	981	-	-	1.1
β -Myrcene	987	-	0.3	0.1
α -Phellandrene	1105	-	0.7	-
Limonene	1021	-	-	0.9
<i>p</i> -Cymene	1026	0.7	-	0.2
β -Phellandrene	1031	-	0.2	-
1,8-Cineole	1035	2.7	-	0.5
<i>cis</i> -Sabinene hydrate	1063	1.3	6.8	-
α -Thujone	1089	-	0.1	-
Linalool	1099	-	-	0.2
<i>trans</i> -Sabinene hydrate	1100	1.1	4.0	0.5
Chrysanthenone	1106	0.9	-	0.7
β -Thujone	1114	0.5	-	-
β -Fenchyl alcohol	1117	0.4	-	1.3
Camphor	1144	2.0	0.2	46.9
Thujanol	1166	2.0	-	-
Borneol	1168	4.5	35.8	0.6
4-Terpineol	1175	2.2	0.8	-
Myrtenol	1194	0.7	-	-
Linalyl acetate	1257	1.3	-	-
β -Elemene	1390	3.0	1.1	1.5
β -Caryophyllene	1420	2.3	0.8	13.3
α -Humulene	1456	0.4	0.4	9.7
γ -Gurjunene	1473	-	-	3.6
Germacrene D	1484	-	-	2.6
<i>trans</i> β -Guaiene	1500	0.6	2.1	2.8
Caryophyllene oxide	1579	28.6	13.4	0.5
Methanoazulene	1587	10.9	14.7	2.8
Humulene epoxide II	1606	2.6	1.2	1.2
Farnesol	1697	-	-	0.7
Oil yield		0.56%	1.0%	0.25%

domesticated under sub-tropical north Indian conditions [7], the present study suggests that the yield of oil is low at lower altitude, but increases when the plants grow at higher altitudes. Interestingly, several constituents like *p*-cymene, β -fenchyl alcohol, γ -gurjunene, *trans*- β -guaiene, humulene epoxide-II and farnesol, which were observed in the natural plants, were not observed in the domesticated plants. In sub-tropical conditions, the domesticated plants also developed constituents like

γ -terpinene, bornyl acetate, δ -cadinene and β -eudesmol, which were not observed in the naturally growing plants. However, the major oil constituents in the vegetative plants remained camphor and β -caryophyllene, but the quantity of camphor reduced drastically in the domesticated plants, while that of β -caryophyllene remained almost the same [7].

Experimental

Plant material: Leaves from mature plants of *Artemisia nilagirica* var. *septentrionalis*, before flowering, were collected from different altitudes in Himachal Pradesh such as Shimla (2210 m), Mandi (1044 m) and Manali (2050 m) in June 2005. Plants were deposited in the CIMAP Herbarium, Lucknow. Collected samples were dried in the shade and 100 g of accurately weighed leaves were subjected to hydro-distillation in a Clevenger type apparatus for 5 h to obtain light yellow oil.

GC-GC/MS analysis: GC of essential oils was carried out using a Perkin Elmer AUTO-XL GC, capillary column PE-5 (50 m x 0.32 mm x 0.25 μ film thickness) with the oven temperature programmed from 100°-280°C at 3°/min with an initial hold of 2 min. Injector and detector temperatures were maintained at 220°C and 300°C, respectively. Hydrogen at 10 psi was used as a carrier gas, with a split ratio of 1:50. The GC/MS analysis was carried out in EI mode using a Perkin Elmer Auto XL/ Turbo Mass instrument with the same column and under identical analytical conditions. Helium at 10 psi was used as a carrier gas with a split ratio 1:20. Mass spectral data were analyzed using NIST and Wiley library information.

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Volatile Oil Composition of *Pogostemon heyneanus* and Comparison of its Composition with Patchouli Oil

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The volatile oil of the leaves of *Pogostemon heyneanus* Benth. (Lamiaceae) was analyzed by GC and GC-MS. Twenty-six components representing 96.0% of the oil were identified. The major components of the oil were acetophenone (51.0%), β -pinene (5.3%), (*E*)-nerolidol (5.4%), and patchouli alcohol (14.0%). Comparison of the compositions of the oils of *P. heyneanus* and *P. cablin* (Blanco) Benth. (Patchouli oil) showed wide variation between them. Though 13 sesquiterpenes and oxygenated sesquiterpenes were detected in both oils, their concentrations in the oils differed widely. Acetophenone, benzoyl acetone and (*E*)-nerolidol present in the oil of *P. heyneanus* were not detected in patchouli oil.

Keywords: *Pogostemon heyneanus*, *Pogostemon cablin*, essential oil composition, acetophenone, patchouli alcohol.

Pogostemon Desf. (Lamiaceae) is an aromatic genus, globally represented by 97 species and in India, it has 54 species [1]. *P. heyneanus* Benth., otherwise called 'Indian Patchouli', is reported to occur in India, Indonesia, Malaysia and Sri Lanka [2,3]. However, it is believed to be indigenous to southern India and Sri Lanka [3]. The plant is a strongly aromatic, sparsely pubescent shrub which grows wild in moist, partially shady places in semi-evergreen and evergreen forests. Several years ago, this species was cultivated in a limited area in Java and the essential oil was distilled. Hence, it is also known as 'Java patchouli'. As the odor of the oil was quite different from that of true patchouli oil *i.e.* *P. cablin* oil, the production of the oil was discontinued [4].

While there are several reports on the composition of the essential oil of patchouli *i.e.* *P. cablin* [5-16], there are only three records of the composition of the oil of *P. heyneanus* [17-19]. Venturella [17] investigated the essential oil of *P. heyneanus* var. *patchouli* Pellet. grown in the Palermo Botanical Garden and reported β -pinene, limonene, borneol, β -patchoulene, α -guaiene,

norpatchoulene and patchoulol as the major components of the oil. Maia and Andrade [18] reported that the oil of *P. heyneanus* from the Amazon contained, as its major constituents, patchouli alcohol, α -guaiene, aromadendrene and δ -guaiene (α -bulnesene). Recently, Souza-Filho *et al* [19] analyzed the essential oil of *P. heyneanus* and reported patchouli alcohol, α -bulnesene, α -guaiene, seychellene, α -patchoulene as the principal components. We have analyzed by GC and GC-MS, the essential oils of *P. heyneanus* collected from the Courtallum hills of the southern Western Ghats in Tamil Nadu, India and true patchouli (*P. cablin*), which was procured from the Central Institute of Medicinal and Aromatic Plants, Resource Centre, Bangalore. The compositions of these two oils are compared in this paper.

Hydrodistillation of the dried leaves of *P. heyneanus* yielded 0.8% of a yellow colored essential oil. GC analysis showed more than fifty peaks. GC-MS analysis and GC retention indices of the peaks enabled the identification of 26 components representing 96.0% of the oil. The identified compounds are listed in Table 1

in the order of elution from a GC column. The major components of the oil were acetophenone (51.0%), β -pinene (5.3%), (*E*)-nerolidol (5.4%), and patchouli alcohol (14.0%). The oil contained monoterpenes (11.8%), oxygenated monoterpenes (1.2%), sesquiterpenes (8.4%), oxygenated sesquiterpenes (20.3%), benzenoid compounds (54.1%) and aliphatic compounds (0.2%). As *P. heyneanus* is often confused with *P. cablin* and is often referred to as Indian or Java patchouli, the composition of the oil of *P. heyneanus* was compared with that of patchouli oil. The major components of patchouli oil are α -guaiene, seychellene, α -patchoulene, α -bulnesene and patchouli alcohol. The minor components, such as norpatchoulene, nortetrapatchoulol and α -cedrenal, and the main component patchouli alcohol were reported to be responsible for the characteristic odor of patchouli oil [13]. Though some constituents, mainly sesquiterpenes and oxygenated sesquiterpenes, were found in both the oils, there are considerable and significant differences between the two oils. The compounds, namely, acetophenone, benzoyl acetone and (*E*)-nerolidol, found in the essential oil of *P. heyneanus* were not detected in that of *P. cablin*. Patchouli oil contained higher amounts of patchouli alcohol, α -guaiene, seychellene, α -patchoulene, aciphyllene, α -bulnesene, caryophyllene oxide and pogostol than the oil of *P. heyneanus*. The compounds, namely, cycloseychellene, δ -patchoulene, norpatchoulene, nortetrapatchoulol, patchoulene, (*E,E*)-farnesol and pogostone found in patchouli oil were not detected in the oil of *P. heyneanus*. Patchouli oil has a pleasant and lasting aroma, whereas that of *P. heyneanus* has a strong pungent odor. Thus, the oil of *P. heyneanus* is quite different from patchouli oil in composition and odor.

The oil of *P. heyneanus* in the present study is quite different in composition from that of the earlier studies. Venturella [17] reported the essential oil composition of *P. heyneanus* var. *patchouli*, but this name is synonymous with *P. cablin*, as mentioned in Flora Malesiana [3]. Hence, the species investigated by Venturella was the true patchouli plant and not *P. heyneanus*. Although several compounds reported in the oil of this plant were also present in the oil of *P. cablin*, a high concentration of monoterpenes, and a low concentration of the sesquiterpenes, α -bulnesene and patchouli alcohol showed that the composition of this oil was somewhat abnormal. Another noteworthy feature was the presence of a high concentration of norpatchoulene (9.0%).

The composition of the oil of *P. heyneanus* from the Amazon was similar to that of patchouli oil, with large amounts of patchouli alcohol, α -guaiene,

Table 1: Composition of the essential oils of *Pogostemon heyneanus* and *P. cablin*.

Compounds	RI*	Area %		Detection
		<i>Pogostemon heyneanus</i>	<i>Pogostemon cablin</i>	
α -Pinene	940	2.4	tr	GC, MS
Camphene	953	tr	--	GC, MS
1-Octen-3-ol	975	--	0.1	GC, MS
3-Octanone	976	0.2	--	GC, MS
β -Pinene	979	5.3	tr	GC, MS
Limonene	1030	4.0	tr	GC, MS
Acetophenone	1068	51.0	--	GC, MS
Terpinolene	1090	0.1	--	GC, MS
Linalool	1098	0.2	tr	GC, MS
α -Terpineol	1189	1.0	--	GC, MS
Methyl salicylate	1191	--	tr	GC, MS
δ -Elemene	1341	--	0.8	GC, MS
β -Patchoulene	1385	--	1.8	GC, MS
β -Elemene	1388	--	0.8	GC, MS
Benzoyl acetone ^{ti}	1391	3.1	--	MS
Cyperene	1395	tr	--	GC, MS
Cycloseychellene ^{ti}	1411	--	0.5	MS
(<i>E</i>)-Caryophyllene	1420	2.0	3.0	GC, MS
(<i>E</i>)- α -Bergamotene	1435	tr	--	GC, MS
α -Guaiene	1441	1.7	9.6	GC, MS
Seychellene	1452	1.5	5.8	GC, MS
α -Humulene	1458	0.3	0.6	GC, MS
α -Patchoulene	1465	1.5	6.1	GC, MS
γ -Patchoulene		--	}	MS
Germacrene D	1482	tr	0.1	GC, MS
δ -Patchoulene ^{ti}	1490	--	0.4	MS
Aciphyllene	1500	0.4	2.4	GC, MS
α -Bulnesene	1507	0.7	13.3	GC, MS
7- <i>epi</i> - α -Selinene	1524	0.1	tr	GC, MS
α -Guaiene epoxide	1540	--	tr	GC, MS
(<i>E</i>)-Nerolidol	1557	5.4	--	GC, MS
Norpatchoulene	1567	--	1.2	GC, MS
α -Bulnesene epoxide	1576	0.1	0.5	GC, MS
Caryophyllene oxide	1588	0.3	1.1	GC, MS
Nortetrapatchoulol	1600	--	0.2	GC, MS
Pogostol	1667	0.7	6.2	GC, MS
Patchouli alcohol	1679	14.0	38.3	GC, MS
Pogostone	1702	--	2.1	GC, MS
(<i>E,E</i>)-Farnesol	1722	--	0.3	GC, MS
Patchoulene ^{ti}	1728	--	0.4	MS
		96.0	95.6	

* RI = Retention index on OV-5 Column; tr = < 0.1%; ti = Tentative identification.

aromadendrene and δ -guaiene. Aromadendrene is, however, not reported to be a constituent of patchouli oil. Furthermore, the wild occurrence of *P. heyneanus* in the Amazon has not been reported and the species investigated by Maia and Andrade [18] may be *P. cablin*, as they mentioned it as patchouli plant. Souza-Filho *et al* [19] analyzed the essential oil of *P. heyneanus* from Brazil and the composition of this oil was similar to that of the oil of *P. cablin*. Furthermore, the Brazilian vernacular name 'oriza', given to *P. heyneanus* in this study, actually refers to *P. cablin* [20]. Hence the *Pogostemon* species investigated by Souza-Filho *et al* [19] may have been *P. cablin*. Thus, the composition of the oil of *P. heyneanus* in the present study is quite different from the earlier reports. The high concentration of the aromatic ketone, acetophenone in the oil is noteworthy. Earlier, this compound was reported as a minor compound in the oils of *Cistus ladaniferus* L. (Labdanum oil) [21,22],

Trifolium repens L. [23], *Elsholzia ciliata* (Thunb.) Hyl. [24], *Garcinia dulcis* Kurz. [25], *Rhodiola rosea* L. [26] and in a few *Hypericum* species [27,28].

Of the 97 species in the genus *Pogostemon* distributed throughout the world, so far, *P. cablin* is the only species commercially exploited and cultivated for producing patchouli oil, which is widely used in perfumery. Recently, the essential oils of two other *Pogostemon* species namely, *P. benghalensis* (*P. plectranthoides*) [29] and *P. travancoricus* [30] were investigated and these oils were found to be rich in sesquiterpenes and oxygenated sesquiterpenes. However, the oil of *P. heyneanus* is unique in having as its main constituent acetophenone, which was not reported in the oils of the above mentioned *Pogostemon* species.

Experimental

Plant material: Leaf material of *P. heyneanus* was collected from Courtallum hills, Tirunelveli district, Tamil Nadu, India. The voucher specimens (*R. Murugan* 33) have been deposited in the Herbaria of Madras Christian College (MCCH), Chennai and Foundation for Revitalisation of Local Health Traditions (FRLH), Bangalore, India. The plant was identified by the first author. The identity of the plant specimen was confirmed by matching it with the Type specimen [*Macrae* 1532 (K-isotype)].

Extraction of oil: The leaf sample of *P. heyneanus*, after drying in the shade for 2 weeks, was hydrodistilled in a Clevenger type apparatus for 8 h. The pale yellow oil was collected, dried over anhydrous sodium sulfate and stored in a refrigerator. The oil content was 0.8% (dry weight basis). The true patchouli oil was procured from the Central Institute of Medicinal and Aromatic Plants, Resource Centre, Bangalore.

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Analysis of oil: GC analysis of the essential oil samples was carried out on a Shimadzu GC-2014 Gas Chromatograph using a non-polar, OV-5 column (30 m x 0.25 mm x 0.25 µm film thickness, coated with 5% diphenyl - 95% dimethyl polysiloxane). Helium was used as carrier gas at a linear velocity of 30 cm/sec. and a pressure of 93.6 kPa. The flow rate was 1 mL/min. Temperature programme: 60°C - 240°C at the rate of 3°C/min. Samples of 1 µL dissolved in *n*-hexane were injected. Split ratio used was 1:60. A Shimadzu GC-MS (17A / QP5050) was used, fitted with a CP-Sil 5 CB column (30 m x 0.25 mm x 1 µm film thickness coated with 100% dimethyl polysiloxane). Helium was used as carrier gas at a linear velocity of 45 cm/sec. and a pressure of 64.5 kPa. The flow rate was 1 mL/min. Temperature programme: 60°C - 250°C at the rate of 5°C/min. Samples of 1 µL dissolved in *n*-hexane were injected. Split ratio used was 1:60. MS were recorded at 70eV with a mass range of m/z 40-500. Identification of the components was achieved by comparison of the retention indices (RI) of the GC peaks with those of compounds reported in the literature [6,31,32] and by comparison of the MS of the peaks with those of compounds reported in the literature [9,31,32]. Peak area percentages were obtained from GC-FID responses without the use of an internal standard of correction factors.

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Chemical Composition of Volatile Oils of *Aquilaria malaccensis* (Thymelaeaceae) from Malaysia

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This paper is dedicated to Professor KHC Başer on occasion of his 60th birthday.

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Volatile oils of *Aquilaria malaccensis* Benth. (Thymelaeaceae) from Malaysia were obtained by hydrodistillation and subjected to detailed GC-FID and GC/MS analyses to determine possible similarities and differences in their chemical composition in comparison with the commercial oil. A total of thirty-one compounds were identified compared with twenty-nine identified in the commercial oil. The major compounds identified were 4-phenyl-2-butanone (32.1%), jinkoh-eremol (6.5%) and α -guaiene (5.8%), while the major compounds in the commercial oil were α -guaiene (10.3%), caryophellene oxide (8.6%), and eudesmol (3.2%). The results of the present study showed that more than nine sesquiterpene hydrocarbons were present, which is more than previously reported. Analysis also showed that the number of oxygenated sesquiterpenes in this study were much less than previously reported. Among the compounds detected were α -guaiene, β -agarofuran, α -bulnesene, jinkoh-eremol, kusunol, selina-3,11-dien-9-one, oxo-agarospirol and guaia-1(10),11-dien-15,2-olide.

Keywords: *Aquilaria malaccensis* Benth., volatile oil, sesquiterpene, gas chromatography-flame ionization detector, gas chromatography-mass spectrometry.

Agarwood originates from north-eastern India, Bhutan and parts of south-east Asia, especially Vietnam, Cambodia, Philippines, Malaysia, Indonesia, and Papua New Guinea. Agarwood has had a very long history of use in folk medicine as incense and as an aromatic oil. Agarwood oil, also known as oud, aloeswood oil, eagleswood, kiara and gaharu, is a natural oil obtained from the fragrant resin of several species of *Aquilaria* (Thymelaeaceae) including *A. malaccensis* Benth. (syn. *Aquilaria agallocha*) and *A. crassna*. *A. malaccensis* is included in Appendix II of the Convention on International Trade in Endangered Species (CITES) and is listed as endangered by the Malaysian government [1-3].

In Malaysia, rural villagers and indigenous peoples depend on *A. malaccensis* harvesting to supplement household income. The species has been introduced as a potential income-generating crop to be planted alongside vegetable farms in agroforestry programs during the Ninth Malaysian Plan (2006-2010). The Peninsular Malaysia Forestry Department is striving to develop a uniform grading system for the fragrant resin

and volatile oil which until now is being carried out by most traders by visual inspection and smell.

Agarwood oil has been reported to contain sesquiterpenoids of the eremophilane, spirovetivane, eudesmane, nor-guaiane and prezizaane types, and 2-(2-phenylethyl) chromone derivatives [4-11]. In the present study, we report on the chemical composition of the volatile oils of *A. malaccensis* from Malaysia obtained by hydrodistillation and subjected to detailed GC-FID and GC/MS analyses. GC-FID and GC-MS are the most common analytical methods to-date for compound identification in volatile oils [12]. The chemical composition of the laboratory prepared and commercially obtained oils are compared with the compounds previously reported in agarwood volatile oils [10,11].

The agarwood volatile oil obtained via hydrodistillation (0.2% yield based on dry weight) was dark green. The commercial oil, however, was brown. The general chemical profiles of the oil samples, their chemical composition and retention indices are summarized in

Table 1: Chemical composition of volatile oils of agarwood.

Compounds	Hydrodistilled			Nor Azah <i>et al.</i> [12]	Ishihara <i>et al.</i> [11]			Identification
	DB-1	Lab.	Commercial		Sample I	Sample II	Sample III	
<i>Monoterpene hydrocarbons</i>								
Benzaldehyde	935	3.3						RI,MS
2-Hydroxy-benzaldehyde	1003	0.6						RI,MS
Acetophenone	1066	0.7						RI,MS
4-Phenyl-2-butanone	1210	32.1	3.4	5.8				RI,MS
<i>Sesquiterpene hydrocarbons</i>								
β -Maaliene	1414	0.4	0.7					RI,MS
α -Guaiene	1440	5.8	10.3	0.7	0.1	0.1		RI,MS
Aromadendrene	1443		0.6					RI,MS
γ -Gurjunene	1472	0.7	1					RI,MS
β -Agarofuran	1474		0.5	2.0	0.3	0.2	0.1	RI,MS
β -Selinene	1486	4.9						RI,MS
α -Muurolene	1496	3.4	0.7					RI,MS
γ -Guaiene	1499	1.5	0.8					RI,MS
α -Bulnesene	1503	1.3	1		0.2	0.2		RI,MS
<i>Oxygenated sesquiterpenes</i>								
α -Elemol	1530		3.3					RI,MS
<i>nor</i> -Ketoagarofuran	1555				0.1	0.1	0.1	RI,MS
Epoxybulnesene	1572				0.1			RI,MS
Caryophellene oxide	1600	0.9	8.6					RI
Guaiol	1603	0.6	1.2					RI
Humulene epoxide II	1606	1.7	2.3					RI
1,5-Epoxy- <i>nor</i> -ketoguaiene	1614	1.1	0.6		0.1			RI,MS
10- <i>Epi</i> - γ -eudesmol	1619	1.6	0.8	9.0				RI,MS
Agarospirol	1631	0.9	1.4	5.5			0.1	RI,MS
<i>Epi</i> - α -cadinol	1640		2.9					RI
Jinkoh-eremol	1643	6.5	0.5	7.7	0.4	0.7	0.8	RI
Kusunol	1650	1	0.6		1.7	1	1	RI
α -Eudesmol	1652	0.7	0.9					RI,MS
Bulnesol	1664	1.5	0.6					RI, MS
Dehydrojinkoh-eremol	1673	1.4	1.2				0.1	RI,MS
<i>Epi</i> - α -bisabolol	1678	1.5	1					RI
α -Bisabolol	1683	1.8	0.5					RI
Selina-3,11-dien-9-one	1687	1.3	0.5		0.9	2.1	0.2	RI,MS
Rotundone	1703	0.5			0.2	0.1		RI,MS
Selina-3,11-dien-9-ol	1721				1.2	2.8	0.4	RI,MS
Selina-4,11-dien-14-oic acid	1728				0.6			RI,MS
Selina-3,11-dien-9-al	1735				0.7	0.6	0.8	RI,MS
9,11-Eremophiladien-8-one	1740				1.1	1.2	0.3	RI,MS
Selina-3,11-dien-14-ol	1750				1.2	1.5	0.4	RI,MS
Guaia-1(10),11-dien-9-one	1752				0.1			RI,MS
Selina-4,11-dien-14-al	1758				0.8	0.6	0.4	RI,MS
Guaia-1(10),11-dien-15-ol	1770				0.6	1.2		RI,MS
Selina,3,11-dien-14-oic acid	1775				0.9	0.2		RI,MS
Sinenofuranol	1776						0.2	RI,MS
Dihydrokaranone	1799				1	0.7	0.2	RI,MS
Guaia-1(10), 11-dien-15-al	1806	1.7			3.4	2.5	0.4	RI,MS
Guaia-1(10), 11-dien-15-oic acid	1811	0.6			4.7			RI,MS
Karanone	1812		1.1		0.1	0.1		RI,MS
Oxo-agarospirol	1822	0.3	0.8		1.6	1.4	5.3	RI,MS
Eudesmol	1880	2.1	3.2					RI, MS
2-Hydroxyguaia-1(10),11-dien-15-oic acid	1932				1.8	0.3		RI,MS
<i>n</i> -Hexadecanoic acid	1950	4.2						RI
9-Hydroxyselina-4,11-dien-14-oic acid	1948				0.8			RI,MS
Guaia-1(10),11-dien-15,2-olide	2019		0.5		0.5	0.5	0.2	RI,MS
<i>Others</i>								
2-(2-Phenylethyl) chromone	2296				16.1	17.2	23.6	RI,MS
2(2-(4-Methoxyphenyl)ethyl) chromone	2545				21.2	24.5	33	RI,MS
6-Methoxy-2-(2-(4-methoxy-phenyl) chromone	2949				2.0	3.2	3.7	RI,MS

¹Component are listed in order of their relative content > 0.1 %; ²RI, linear retention indices were determined relative to the retention times on a DB-1 column of a homologous series of C₈-C₂₀ *n*-alkanes. ³Identification: MS, by comparison of the MS with those of the NIST98 library (>90% matching from the library).

Table 1. Over twenty-five compounds were identified in both the laboratory and commercial samples. The laboratory sample was found to contain 42.4% monoterpene compounds, 20.8% hydrocarbon sesquiterpenes and 36.8% oxygenated sesquiterpenes. The commercial oil contained less monoterpene (5.0 %) compared with the laboratory sample, but more sesquiterpene hydrocarbons (22.9%) and oxygenated sesquiterpenes at 72.1%. One possible explanation for the lower content of monoterpenes in the commercial oil could be vaporization during the prolonged extraction time of 3 days. The percentage of sesquiterpene hydrocarbons was essentially equal for both the laboratory sample and the commercial oil.

The major compounds identified in the laboratory sample were 4-phenyl-2-butanone (32.1%), jinkoh-eremol (6.5%), and α -guaiene (5.8%). The commercial oil contained several major compounds, namely α -guaiene (10.3%) caryophellene oxide (8.6%) and eudesmol (3.2%).

Previously reported data [10] on solvent extracted agarwood oil from Vietnam, which ranged in color from green to brown and purple and labelled I, II and III, respectively, are included for comparison. Also included are findings from a recent report indicating 4-phenyl-2-butanone, β -agarofuran, α -agarofuran, agarospirol and 10-*epi*- γ -eudesmol, which were detected in hydrodistilled oils from Peninsular Malaysia [11].

Based on the data obtained from the present study and previous reports [10,11], several compounds in *A. malaccensis* oil can be classified as marker compounds, namely α -guaiene, β -agarofuran, α -bulnesene, jinkoh-eremol, kusunol, selina-3,11-dien-9-one, oxo-agarospirol and guaia-1(10),11-dien-15,2-olide.

Experimental

Plant material: *Aquilaria malaccensis* infected wood from Kelantan Forest (Malaysia) and a sample of commercial oil were procured in September 2008 from

Mazlan Mohamed, an agarwood trader certified by the Forestry Department, Malaysia.

Isolation procedure: Fresh, air dried woods were dried further in an oven at 40°C for 24 h. This was followed by 3 days of soaking in water prior to hydrodistillation in a Pyrex glass Clevenger type apparatus for 12 h. The oils were taken up in *n*-hexane, dried over anhydrous sodium sulfate, purged with nitrogen gas (N₂), and then stored at 4°C in glass amber vials prior to analysis. The commercial oil was isolated by the trader from 25 kg air dried wood chips in a large scale Clevenger-type steel apparatus.

GC-FID and GC-MS analyses: Chemical analyses of agarwood oil were undertaken by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). An Agilent 7890 gas chromatograph coupled to a 5973 quadrupole mass spectrometer equipped with a DB-1 capillary column (30m x 0.25 mm, film thickness 0.25 μ m) and a selective mass detector was used for GC-MS detection; an electron ionization system was set with an ionization energy of 70eV. Helium was the carrier gas, set at a flow rate of 1.0 mL/min. Injector and ion source temperatures were both set at 230°C. Injection volume was 1 μ L (split ratio 20:1) and the oven temperature was programmed from 50°C to 230°C at 3°C/min.

Identification of components: The components were identified on the basis of comparison of their retention indices and mass spectra with published data [13], and by matching with the National Institute of Standards Technology (NIST) libraries. Retention indices were calculated using a homologous series of *n*-alkanes (C8-C22).

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Chemical Composition and Phytotoxic Effects of Essential Oils from Four *Teucrium* Species

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The essential oils of four *Teucrium* species were studied and 131 components, in all, were identified. All oils were rich in sesquiterpenes (50.0-61.9%). Caryophyllene and caryophyllene oxide were the main components of *Teucrium arduini*; germacrene D, δ -cadinene and γ -cadinene predominated in *Teucrium maghrebinum*. Carvacrol and caryophyllene predominated in *Teucrium polium* ssp. *capitatum*, while carvacrol, caryophyllene oxide and caryophyllene were the most abundant components in *Teucrium montbretii* ssp. *heliotropiifolium*. The germination of radish and garden cress was less sensitive to the four essential oils. The radicle elongation, above all, of radish was significantly inhibited by all oils, in particular by the essential oil of *T. arduini*, at the highest doses tested. Among the main components of the oils, monoterpenes resulted the more active compounds.

Keywords: *Teucrium arduini*, *Teucrium maghrebinum*, *Teucrium polium* ssp. *capitatum*, *Teucrium montbretii* ssp. *heliotropiifolium*, essential oils, monoterpenes, radicle elongation, radish, garden cress.

Teucrium is a genus of perennial plants which belongs to the Lamiaceae family: is represented by more than 340 species, distributed mainly in the Mediterranean basin [1]. An unusual feature of this genus compared with other members of Lamiaceae is that the flowers completely lack the upper lip of the corolla. As in other Lamiaceae, the aerial organs of *Teucrium* spp. are covered by an indumentum of glandular and non-glandular trichomes. Plants of this genus are well known in traditional medicine as antispasmodics, diuretics, antidiabetics [2], tonics, diaphoretics, analgesics [3], antiphlogistics, antirheumatics, antiseptics, antihelmintics, carminatives, flavouring [4] and as well as antipyretic and stimulant agents [5].

The genus *Teucrium* is one of the richest sources of diterpenes with a neoclerodane skeleton. More than 220 diterpenes have been described to date, many of which are particularly interesting because of their ecological role as antifeedants against different species of insects and of their role in the medicinal properties of the plants [6]. So far, essential oils have been also reported from the aerial parts of several *Teucrium* species [7-10].

Teucrium arduini L. is an endemic Illyric Balcan species; it is a semi-woody, branchy, erect or ascending, dwarf shrub, 10–40 cm (rarely up to 60 cm) high. Whitish flowers form simple, very dense, up to 16 cm long inflorescences. *T. arduini* grows on calcareous rocky slopes at altitudes between 0 and 1600 m a.s.l. Previous papers reported the biological activities of leaf and flower infusions of this species [11] and the composition of the essential oil [12,13].

T. maghrebinum Greuter et Burdet is a species growing in Algeria and Morocco. The plant is called locally "kayat el gerah" and is used in traditional medicine to treat burns and fevers, as well as an antimicrobial agent [14]. The chemical composition of its essential oil has been reported [8].

Teucrium montbretii Benth. ssp. *heliotropiifolium* (Barbey) Davis is an evergreen dwarf (5–20 cm high) semishrub–chasmophyte with lignified branches, opposite and ovate leaves. The different subspecies of *T. montbretii* show different qualitative contents of neoclerodane diterpenoids [6]; the essential oil of this species was also analyzed [9].

Table 1: Essential oil composition of *T. arduini* (*Ar*), *T. maghrebinum* (*Ma*), *T. montbretii* ssp. *heliotropifolium* (*Mo*) and *T. polium* ssp. *capitatum* (*Po*).

Component	KI ^a	KI ^b	Identification ^c	<i>Ar</i> % ^d	<i>Ma</i> % ^d	<i>Mo</i> % ^d	<i>Po</i> % ^d
Monoterpene hydrocarbons							
α -Thujene	930	1014	LRI, MS		8.9		1.2
α -Pinene	938	1075	LRI, MS, Co-GC		0.2		0.2
Camphene	953	1076	LRI, MS, Co-GC		1.6		0.2
Sabinene	973	1132	LRI, MS		0.3		
β -Pinene	978	1118	LRI, MS, Co-GC				0.3
Myrcene	993	1174	LRI, MS, Co-GC		1.9		
α -Terpinene	1012	1189	LRI, MS, Co-GC		0.5		t
<i>p</i> -Cymene	1025	1278	LRI, MS, Co-GC				0.5
Limonene	1030	1203	LRI, MS, Co-GC		4.4		
(<i>Z</i>)- β -Ocimene	1038	1245	LRI, MS				
Oxygenated monoterpenes							
<i>cis</i> -Linalool oxide, furanoid	1062	1450	LRI, MS	2.8	6.7	3.1	15.0
<i>cis</i> -Sabinene hydrate	1063	1555	LRI, MS			0.1	0.5
<i>trans</i> -Linalool oxide, furanoid	1076	1478	LRI, MS			0.1	0.2
<i>trans</i> -Sabinene hydrate	1093	1474	LRI, MS				0.1
Linalool	1098	1553	LRI, MS, Co-GC	1.6	1.3	2.7	1.0
α -Campholenal	1128	1497	LRI, MS		0.4		
<i>cis</i> -Sabinol	1135	1789	LRI, MS				1.7
Nopinone	1136	1597	LRI, MS		0.1		0.2
<i>cis</i> -Verbenol	1144	1667	LRI, MS		0.4		2.0
Sabina ketone	1155	1652	LRI, MS				0.4
Pinocarvone	1165	1587	LRI, MS		0.6		0.1
Borneol	1167	1719	LRI, MS, Co-GC				0.1
Umbellulone	1175	1656	LRI, MS				0.3
Terpineol-4	1176	1611	LRI, MS, Co-GC		0.4		0.5
α -Terpineol	1189	1706	LRI, MS, Co-GC		0.6	0.2	
Myrtenal	1193	1648	LRI, MS, Co-GC		t		t
Myrtenol	1196	1812	LRI, MS				1.2
<i>cis</i> -Verbenone	1204	1723	LRI, MS		0.3		4.0
<i>trans</i> -Carveol	1217	1845	LRI, MS		0.1		
β -Cyclocitral	1223	1629	LRI, MS		t		0.1
<i>cis</i> -Carveol	1226	1878	LRI, MS				0.6
Cumin aldehyde	1232	1804	LRI, MS				0.1
Carvone	1241	1750	LRI, MS		2.5		0.3
<i>p</i> -Menth-9-en-1-ol	1291	1945	LRI, MS	1.2			
<i>p</i> -Cymen-7-ol	1293	2067	LRI, MS				1.5
Sesquiterpene hydrocarbons							
δ -Elemene	1335	1476	LRI, MS	30.8	51.5	29.4	33.9
α -Longipinene	1351		LRI, MS		0.6		
α -Cubebene	1352	1466	LRI, MS	t	0.8	0.4	0.1
Cyclosativene	1363	1492	LRI, MS				
α -Ylangene	1372	1493	LRI, MS			0.3	
α -Copaene	1377	1503	LRI, MS	0.6	0.8	0.6	1.0
β -Cubebene	1382	1547	LRI, MS	0.7	2.5		0.1
β -Bourbonene	1385	1535	LRI, MS	1.2	1.0	1.8	0.5
β -Elemene	1387	1598	LRI, MS	0.2			0.2
α -Elemene	1396		LRI, MS			0.2	
α -Gurjunene	1408	1529	LRI, MS				1.2
α -Funebrene	1409	1510	LRI, MS				0.2
Caryophyllene	1415	1612	LRI, MS, Co-GC	10.0	4.9	8.2	10.1
Aromadendrene	1422	1628	LRI, MS	0.2	0.6		0.2
β -Gurjunene (Calarene)	1423	1632	LRI, MS		0.3		0.3
<i>epi</i> -Bicyclosesquiphellandrene	1424		LRI, MS	0.6			0.7
β -Cedrene	1430	1638	LRI, MS			0.9	
γ -Elemene	1436	1650	LRI, MS	0.8	0.1		0.1
β -Humulene	1437	1674	LRI, MS		t		0.3
(<i>E</i>)- β -Farnesene	1452	1673	LRI, MS	t	0.3		0.1
α -Humulene	1455	1689	LRI, MS	3.1	0.9	2.8	3.4
<i>allo</i> -Aromadendrene	1463	1661	LRI, MS	1.9	0.5	2.8	0.4
Germacrene D	1477	1726	LRI, MS	5.8	14.3	3.7	3.9
γ -Muurolole	1478	1704	LRI, MS	1.0	0.3	1.0	
(<i>E</i>)- β -Ionone	1482	1957	LRI, MS, Co-GC	1.4	0.5		
<i>ar</i> -Curcumene	1483	1784	LRI, MS				0.2
α -Amorphene	1487	1679	LRI, MS	0.2	0.5	0.5	2.5
<i>cis</i> - β -Guaiene	1490	1694	LRI, MS				1.5
Valencene	1494	1741	LRI, MS			0.5	0.2
Bicyclosesquiphellandrene	1491	1626	LRI, MS	0.2		1.8	0.1
Bicyclogermacrene	1492	1756	LRI, MS	1.9			0.7
α -Muurolole	1503	1740	LRI, MS	0.5		0.3	0.1
γ -Cadinene	1515	1776	LRI, MS	0.5	7.5		0.1
δ -Cadinene	1526	1773	LRI, MS		13.5	2.7	3.1

Table 1 (contd.)

Cadina-1,4-diene	1538	1799	LRI, MS		0.9		0.2
α -Calacorene	1541	1942	LRI, MS		0.3	0.2	0.2
Germacrene B	1554	1856	LRI, MS	t			0.6
Cadalene	1677	2256	LRI, MS		0.4	0.7	1.6
Oxygenated sesquiterpenes					21.4	10.4	20.6
1- <i>endo</i> -Bourbonanol	1520		LRI, MS			0.5	
Ledol	1565	2057	LRI, MS	0.8		1.7	
(<i>E</i>)-Nerolidol	1566	2050	LRI, MS		1.4		
Longipinanol	1572				t		
Germacrene D 4-ol	1577	2069	LRI, MS				3.0
Spathulenol	1578	2150	LRI, MS, Co-GC	5.8	1.8		0.1
Caryophyllene oxide	1580	2008	LRI, MS, Co-GC	7.7	4.0	8.8	5.0
Globulol	1588	2098	LRI, MS				0.2
Viridiflorol	1591	2104	LRI, MS	0.7		2.0	
Widdrol	1600		LRI, MS			1.4	0.1
Humulene epoxide II	1605	2071	LRI, MS			0.9	1.3
Cedrenol	1606	2133	LRI, MS	4.8		0.1	0.1
Torreyol	1645		LRI, MS				6.5
α -Cadinol	1649	2255	LRI, MS		1.9	0.7	4.0
Caryophyllenol II	1650	2396	LRI, MS			3.2	0.1
(<i>E</i>)-Isoelemicin	1659	2403	LRI, MS	1.6			0.2
Patchoulol	1664		LRI, MS			0.5	
Germacrene	1685		LRI, MS		t		
α -Bisabolol	1686	2219	LRI, MS	t	0.8		0.3
(<i>Z,E</i>)-Farnesol	1689	2276	LRI, MS		0.5		0.2
Vulgarol B	1691		LRI, MS			0.8	
<i>cis</i> (<i>Z</i>)- α -Bisabolene-epoxide	1698		LRI, MS				0.2
Phenolic compounds					2.5	3.6	14.3
Thymol	1290	2198	LRI, MS, Co-GC	0.2			0.1
Carvacrol	1297	2239	LRI, MS, Co-GC			13.5	9.6
4-Vinyl guaiacol	1312	2180	LRI, MS	1.8	2.0		0.1
Eugenol	1353	2186	LRI, MS, Co-GC	0.5	1.6	0.8	0.5
Hydrocarbons					10.2	4.3	6.8
α -Ionene	1208		LRI, MS			0.2	
Heptadecane	1700	1700	LRI, MS, Co-GC			0.2	0.1
Pentacosane	2500	2500	LRI, MS	1.0	0.8	1.9	0.7
Hexacosane	2600	2600	LRI, MS	0.2	0.2		
Heptacosane	2700	2700	LRI, MS	4.0	1.5	1.7	1.9
Octacosane	2800	2800	LRI, MS	0.6	0.1	0.5	
Nonacosane	2900	2900	LRI, MS	2.8	1.0	1.2	2.5
Triacotane	3000	3000	LRI, MS	0.4	t		0.1
Hentriacontane	3100	3100	LRI, MS	1.2	0.5	1.3	1.2
Fatty acids					9.7	2.6	13.1
Hexadecanoic acid	1957	2931	LRI, MS, Co-GC	9.3	1.8	10.7	0.1
Heptadecanoic acid	2054	2975	LRI, MS, Co-GC	0.1			
(<i>Z</i>)-9-Octadecenoic acid	2115		LRI, MS, Co-GC	0.1			
(<i>Z,Z</i>)-9,12-Octadecadienoic acid	2122	3157	LRI, MS, Co-GC		0.6	2.4	0.5
Octadecanoic acid	2172	3402	LRI, MS, Co-GC	0.2	0.2		
Others					1.2	1.3	2.9
1-Octen-3-ol	977	1425	LRI, MS	0.5	0.3	0.1	0.1
Octan-3-ol	992	1394	LRI, MS, Co-GC		0.4		
2-Pentylfuran	1002	1244	LRI, MS	t			
Dihydroedulan I	1296		LRI, MS	0.4	0.1		
Dihydroactinidiolide	1486	2354	LRI, MS		0.5	1.6	
13- <i>epi</i> -Manoyl oxide	1963	2388	LRI, MS			0.7	
Manoyl oxide	1994		LRI, MS			0.5	0.1
Kaurene	2048	2399	LRI, MS				0.8
Squalene	2829		LRI, MS	0.3			
Carbonylic compounds					7.6	4.7	3.2
(<i>E</i>)-2-Hexenal	854	1231	LRI, MS		t		
Benzaldehyde	963	1543	LRI, MS, Co-GC		0.2		
1-Octen-3-one	975	1312	LRI, MS		0.3		
Acetophenone	1036	1645	LRI, MS			0.1	
Phenylacetaldehyde	1048	1663	LRI, MS, Co-GC	0.1	0.4	0.1	0.1
<i>p</i> -Methoxyacetophenone	1302	1797	LRI, MS, Co-GC	2.3			1.5
(<i>E</i>)- β -Damascenone	1380	1835	LRI, MS	1.1	1.0	0.4	0.2
(<i>E</i>)- α -Ionone	1419		LRI, MS, Co-GC	0.3			
Hexahydrofarnesylacetone	1845	2131	LRI, MS	3.8	2.8	2.6	0.8
Total amount of compounds					86.2	94	93.4
							92.4

^a: Kovats retention index on HP-5 MS column; ^b: Kovats retention index on HP Innowax; ^c: LRI linear retention index; MS identification based on comparison of mass spectra; Co-GC retention time identical to authentic compounds; ^d: t = trace < 0.05.

Teucrium polium L. ssp. *capitatum* (L.) Arcangeli (syn. *Teucrium capitatum* L.) is widespread diffused in the dry and stony places of Greece and almost all Mediterranean countries [15]. It is a perennial and prostrate species with white-to-bright pink flowers, crenate leaves and ramified indumentum. This plant is used in folk medicine to treat diabetes and intestinal troubles. Aerial part extracts were known for their anti-inflammatory, antibacterial, antihypertensive, hypoglycemic, hypolipidemic, anorexic and antioxidant activities [9]. The species has been reported for its neo-clerodane diterpenoids and essential oils [16].

In continuation of our studies on the possible phytotoxic activity of essential oils from plants collected in the Mediterranean area [9,17-18], we analyzed the chemical composition of the essential oils of four different species of *Teucrium* and carried out *in vitro* experiments in order to verify the possible effects of the essential oils and of their main components on germination and initial radicle elongation of *Raphanus sativus* L. (radish) and *Lepidium sativum* L. (garden cress).

In the four oils, 131 compounds in all were identified (Table 1): 53 for oil of *T. arduini* (86.2% of the total oil), 71 for *T. maghrebinum* (94.0% of the oil), 51 for *T. montbretii* (93.4% of the oil) and 89 for *T. polium* (92.4% of the oil). The components are listed in Table 1 according to their retention indices on HP-5 column and are classified on the basis of their chemical structures into 9 classes.

The oil of *T. arduini* comprised mainly sesquiterpenes (52.2%), particularly sesquiterpene hydrocarbons (30.8%). In particular, 21 sesquiterpene hydrocarbons were present in the oil, with a prevalence of caryophyllene (10.0%), germacrene D (5.8%) and α -humulene (3.1%). In particular, 7 oxygenated sesquiterpenoids were present in the oil, being the main constituents caryophyllene oxide (7.7%), spathulenol (5.8%) and cedrenol (4.8%). Hydrocarbons were quite abundant (10.2%) and were constituted by almost heptacosane (4.0%) and nonacosane (2.8%). Fatty acids (9.7%) were represented by hexadecanoic acid (9.3%), while hexahydrofarnesylacetone (3.8%) was the main constituent among carbonylic compounds (7.6%). Blazevic and coworkers [12] and Kovacevic and coworkers [13] analyzed the essential oil composition of this species: in the both cases, β -caryophyllene and germacrene D were the main compounds, as in the our samples.

In the oil of *T. maghrebinum* the most abundant compounds were germacrene D (14.3%), δ -cadinene (13.5%) and γ -cadinene (7.5%). On the whole, the oil was constituted mainly by sesquiterpenes (61.9%) and

monoterpenes (15.6%). Sesquiterpene hydrocarbons (51.5%) prevailed over oxygen containing sesquiterpenes (10.4%). Twenty-three sesquiterpene hydrocarbons were present in the oil, with a prevalence of germacrene D (14.3%), δ -cadinene (13.5%), γ -cadinene (7.5%) and caryophyllene (4.9%). Among 8 oxygen containing sesquiterpenes, the most abundant was caryophyllene oxide (4.0%). Among monoterpenes, 6 hydrocarbons accounted for the 8.9% of the total oil, with limonene (4.4%) as the main compound, while carvone (2.5%) prevailed among the 12 oxygen containing monoterpenes. The composition of essential oil of *T. maghrebinum*, growing in Algeria, was studied by Formisano and coworkers [8]: germacrene D, δ -cadinene and γ -cadinene were the most abundant compounds.

The oils from *T. montbretii* and *T. polium* from Greece were previously analyzed by us [9]; for the present study the aerial parts of the plants have been collected again in the same place and the GC and GC-MS analyses have been repeated on the new samples. Table 1 shows that results obtained in the present study are similar to those of the previously studies [9], even if the percentages of some components are slightly different.

In the essential oil of *T. montbretii*, the main fraction was constituted by sesquiterpenes (50.0%). Among these, sesquiterpene hydrocarbons (29.4%) prevailed on oxygen containing sesquiterpenes (20.6%). In the first fraction caryophyllene (8.2%), germacrene D (3.7%), *allo*-aromadendrene (2.8%) and α -humulene (2.8%) predominated, while among the 11 oxygen containing sesquiterpenes the most abundant compounds were caryophyllene oxide (8.8%) and caryophyllenol II (3.2%). The phenols were quite abundant (14.3%) and were constituted almost entirely by carvacrol (13.5%). Fatty acids (13.1%) were mainly represented by hexadecanoic acid (10.7%), while linalool (2.7%) was the main constituent of oxygenate monoterpenes (3.1%). The composition of the oil of *T. montbretii* confirmed literature data [9], in which the main fraction was constituted by sesquiterpenes.

As in the other oils studied, in the oil of *T. polium* sesquiterpenes constituted also the main fraction and accounted for the 55.2% of the total oil with a prevalence of sesquiterpene hydrocarbons (33.9%) over oxygen containing sesquiterpenes (21.3%). Among the 30 sesquiterpene hydrocarbons, caryophyllene (10.1%), germacrene D (3.9%), α -humulene (3.4%), δ -cadinene (3.1%) and α -amorphene (2.5%) were the most abundant. In the other fraction, torreyol (6.5%), caryophyllene oxide (5.0%) and α -cadinol (4.0%) prevailed. Monoterpenes contributed for the 16.2% of the oil with a predominance of oxygen containing

monoterpenes (15.0%), particularly *cis*-verbenone (4.0%) and *cis*-verbenol (2.0%).

The phenolic compounds (10.3%) were represented almost entirely by carvacrol (9.6%). The chemical analysis of the essential oil of *T. polium* was also reported by Antunes and coworkers [19] and Cozzani and coworkers [20] with different compositions.

The four essential oils were evaluated for their phytotoxic activity against germination and initial radicle elongation (Table 2) of radish and garden cress, two species usually utilized in biological assays [21]. Also the main components of these oils were assayed for the same activity (Table 3).

The oils affected the germination and the radicle elongation of radish and garden cress in a distinct way. Radicle elongation seemed to be more affected in comparison to germination. The germination of radish did not appear sensitive to the four essential oils: only the essential oil of *T. polium*, at a dose of 1.25 µg/mL, significantly inhibited the germination of radish. The germination of garden cress did not appear sensitive to the four essential oils, too: only the essential oil of *T. arduini*, at the highest dose tested, significantly inhibited the germination of garden cress. The radicle elongation of radish was significantly inhibited by the all oils: particularly, the essential oil of *T. arduini*, at the highest doses tested, inhibited the radicle elongation of radish, and in minor measure, of garden cress. The essential oil of *T. montbretii* inhibited only the radicle elongation of radish, at the dose of 1.25 µg/mL.

The main components, both monoterpenes and sesquiterpenes, were also assayed against germination and radicle elongation of two seeds at four concentrations (10^{-3} - 10^{-6}). Monoterpenes resulted the more active compounds: in fact, limonene was the most active compound, at the highest doses assayed, against both germination and radicle elongation of radish; also carvacrol is very active in inhibiting radicle elongation of garden cress.

Among sesquiterpenes, *allo*-aromadendrene, at 10^{-4} M, inhibited, in a significant way, only the germination of garden cress; caryophyllene, at the lowest dose, inhibited significantly only the germination of radish. On the other hand, caryophyllene oxide promoted the germination of this seed. Radicle elongation of the seeds was never affected.

In previous papers, Kordali and coworkers [22] reported that limonene and β -pinene inhibited seed germination of *Chenopodium album*. Also linalool completely inhibited seed germination of *Cassia occidentalis*.

Kordali and coworkers also reported phytotoxic activity of carvacrol [23].

The different degrees of biological activity could be related to the composition of the essential oils: in particular, the presence of monoterpenoids [18] could explain the biological activity. Although the mechanisms of essential oil action against germination is still unclear, it reported that volatile oils, monoterpenoids and also sesquiterpenoids inhibit cell division and induce structural breaks and decomposition in roots [18,24]. In a previous paper, we demonstrated that essential oils of different species of *Salvia*, rich in sesquiterpenoid compounds, showed a good antigerminative activity, both *in vitro* and *in vivo* [18]. So, both monoterpenoids and sesquiterpenoids appear to be involved in allelopathic effects: some monoterpenoids, as citronellol, linalool, α -pinene and limonene, are potent inhibitors of seed germination and radicle elongation; moreover, sesquiterpenoid compounds, as β -maaliene, α -isocomene, β -isocomene, δ -cadinene, 5-hydroxy-calamenene and 5-methoxy-calamenene were recently shown to inhibit the seedling growth of associated native vegetation, and thus possibly help in successful invasion in the introduced sites [18,25].

Experimental

Plant material: Aerial parts of *T. arduini* and *T. maghrebinum* were cultivated in the Orto Botanico "G. E. Ghirardi", University of Milano, Toscolano (Garda Lake, Italy). Seeds were provided by the Jardin des Plantes, Paris. Plant materials were collected in June 2009, at the full flowering stage. Aerial parts of *T. montbretii* ssp. *heliotropiifolium* were collected in June 2009, at Spoa, on the Karpathos Island of Greece. Aerial parts of *T. polium* ssp. *capitatum* were collected in June 2009, at Gournes, about 15 km east of Heraklion, Crete.

Chemicals: *allo*-Aromadendrene, β -pinene, carvacrol, caryophyllene, caryophyllene oxide, limonene and linalool were purchased from Sigma-Aldrich Co. (Milan, Italy).

Isolation of the essential oils: The air-dried samples were ground in a Waring blender and then subjected to hydrodistillation for 3 h using *n*-hexane as a solvent. The extracts were dried over anhydrous sodium sulphate and then stored in sealed vials, at 20°C, ready for the GC and GC-MS analyses. The hydrodistillation yielded 0.5%, 0.9%, 0.7% and 0.8% of pale yellow oil (on a dry mass basis) for *T. arduini*, *T. maghrebinum*, *T. polium* ssp. *capitatum* and *T. montbretii* ssp. *heliotropiifolium* respectively.

Table 2: Biological activities of essential oils of *T. arduini* (*Ar*), *T. maghrebicum* (*Ma*), *T. montbretii* ssp. *heliotropiifolium* (*Mo*) and *T. polium* ssp. *capitatum* (*Po*) against germination and initial radicle elongation of radish and garden cress, 120 h after sowing. Results are shown as mean \pm standard deviation (SD) of three experiments.

<i>Raphanus sativus</i>								
Doses	Germinated seeds				Radicle elongation (cm)			
	<i>Ar</i>	<i>Ma</i>	<i>Mo</i>	<i>Po</i>	<i>Ar</i>	<i>Ma</i>	<i>Mo</i>	<i>Po</i>
Control	6.2 \pm 1.2	6.2 \pm 1.2	6.2 \pm 1.2	6.2 \pm 1.2	0.5 \pm 0.3	0.5 \pm 0.3	0.5 \pm 0.3	0.5 \pm 0.3
2.5 μ g/mL	6.0 \pm 1.0	3.7 \pm 3.2	6.7 \pm 0.6	5.7 \pm 0.6	0.3 \pm 0.2*	0.4 \pm 0.2	0.4 \pm 0.3	0.4 \pm 0.2
1.25 μ g/mL	5.3 \pm 1.5	5.7 \pm 1.2	6.7 \pm 1.2	3.7 \pm 0.6*	0.3 \pm 0.2*	0.4 \pm 0.2	0.4 \pm 0.2*	0.4 \pm 0.2
0.625 μ g/mL	4.7 \pm 0.6	7.0 \pm 1.0	5.0 \pm 0.0	5.7 \pm 1.5	0.3 \pm 0.2*	0.5 \pm 0.3	0.4 \pm 0.2	0.3 \pm 0.2*
0.25 μ g/mL	6.3 \pm 1.2	5.3 \pm 0.6	4.7 \pm 3.1	4.7 \pm 0.6	0.4 \pm 0.2	0.4 \pm 0.2	0.5 \pm 0.3	0.6 \pm 0.3
0.125 μ g/mL	4.0 \pm 1.7	5.0 \pm 1.0	5.3 \pm 1.2	4.3 \pm 1.2	0.4 \pm 0.1	0.4 \pm 0.3	0.4 \pm 0.2	0.5 \pm 0.3
0.06 μ g/mL	4.3 \pm 1.2	6.3 \pm 0.6	5.0 \pm 1.0	5.0 \pm 1.0	0.3 \pm 0.2	0.2 \pm 0.2***	0.4 \pm 0.2	0.5 \pm 0.2
<i>Lepidium sativum</i>								
Doses	Germinated seeds				Radicle elongation (cm)			
	<i>Ar</i>	<i>Ma</i>	<i>Mo</i>	<i>Po</i>	<i>Ar</i>	<i>Ma</i>	<i>Mo</i>	<i>Po</i>
Control	9.2 \pm 0.8	9.2 \pm 0.8	9.2 \pm 0.8	9.2 \pm 0.8	2.2 \pm 1.2	2.2 \pm 1.2	2.2 \pm 1.2	2.2 \pm 1.2
2.5 μ g/mL	7.7 \pm 0.6*	9.3 \pm 0.6	9.3 \pm 0.6	9.3 \pm 0.6	1.7 \pm 1.0*	2.3 \pm 1.0	2.2 \pm 0.9	2.5 \pm 0.9
1.25 μ g/mL	8.0 \pm 1.0	9.3 \pm 0.6	9.7 \pm 0.6	9.0 \pm 1.7	2.4 \pm 1.0	1.9 \pm 0.9	2.2 \pm 1.1	1.9 \pm 1.1
0.625 μ g/mL	8.0 \pm 2.0	9.3 \pm 0.6	9.3 \pm 0.6	9.3 \pm 0.6	2.3 \pm 1.2	2.4 \pm 1.0	2.5 \pm 0.9	2.2 \pm 1.0
0.25 μ g/mL	10.0 \pm 0.0	8.3 \pm 0.6	9.0 \pm 1.0	8.3 \pm 1.2	2.2 \pm 1.1	2.1 \pm 1.1	2.4 \pm 1.3	1.8 \pm 0.9*
0.125 μ g/mL	9.0 \pm 1.0	8.0 \pm 0.0	9.0 \pm 1.0	9.7 \pm 0.6	2.3 \pm 0.9	1.9 \pm 1.0	2.2 \pm 0.9	2.7 \pm 1.2
0.06 μ g/mL	8.7 \pm 2.3	9.3 \pm 0.6	9.0 \pm 1.0	9.3 \pm 1.2	2.4 \pm 1.1	1.7 \pm 1.2*	2.1 \pm 1.1	2.3 \pm 1.1

Note: *p < 0.05; ** p<0.01; ***p<0.001 vs. control.

Table 3: Biological activities of main constituents of essential oils of *T. arduini*, *T. maghrebicum*, *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* against germination and initial radicle elongation of radish and garden cress, 120 h after sowing. Results are shown as mean \pm standard deviation (SD) of three experiments.

<i>Raphanus sativus</i>								
	Germinated seed				Radicle elongation (cm)			
	[10 ⁻³]	[10 ⁻⁴]	[10 ⁻⁵]	[10 ⁻⁶]	[10 ⁻³]	[10 ⁻⁴]	[10 ⁻⁵]	[10 ⁻⁶]
Control	9.0 \pm 0.6	9.0 \pm 0.6	9.0 \pm 0.6	9.0 \pm 0.6	7.8 \pm 2.0	7.8 \pm 2.0	7.8 \pm 2.0	7.8 \pm 2.0
β -Pinene	8.7 \pm 0.0	8.0 \pm 0.0	9.4 \pm 1.4	8.0 \pm 2.1	5.6 \pm 0.6	6.5 \pm 0.9	5.6 \pm 0.6	6.1 \pm 0.8
Limonene	5.1 \pm 1.4**	5.8 \pm 2.8*	6.2 \pm 4.9	8.3 \pm 2.1	3.9 \pm 0.4**	3.9 \pm 0.5**	8.2 \pm 1.1	6.1 \pm 0.7
Linalool	7.2 \pm 1.4	7.6 \pm 2.1	7.0 \pm 2.1	8.7 \pm 2.8	6.7 \pm 1.1	6.5 \pm 0.7	5.2 \pm 0.7	6.7 \pm 0.6
Carvacrol	7.6 \pm 0.7	9.4 \pm 1.4	9.1 \pm 2.1	8.3 \pm 3.5	7.8 \pm 1.0	8.7 \pm 1.3	8.7 \pm 1.3	9.5 \pm 1.6
allo-Aromadendrene	9.0 \pm 0.0	9.0 \pm 0.0	9.7 \pm 0.6	9.7 \pm 0.6	8.6 \pm 4.5	9.2 \pm 5.0	8.6 \pm 5.4	8.5 \pm 4.5
Caryophyllene	9.7 \pm 0.6	8.3 \pm 0.6	9.0 \pm 1.0	7.7 \pm 0.6*	9.4 \pm 4.7	10.9 \pm 4.6	9.1 \pm 5.0	7.8 \pm 6.3
Caryophyllene oxide	7.7 \pm 1.5	9.0 \pm 1.0	10.0 \pm 0.0*	10.0 \pm 0.0*	8.7 \pm 5.1	9.1 \pm 5.1	8.5 \pm 5.3	7.6 \pm 4.9
<i>Lepidium sativum</i>								
	Germinated seed				Radicle elongation (cm)			
	[10 ⁻³]	[10 ⁻⁴]	[10 ⁻⁵]	[10 ⁻⁶]	[10 ⁻³]	[10 ⁻⁴]	[10 ⁻⁵]	[10 ⁻⁶]
Control	9.2 \pm 0.4	9.2 \pm 0.4	9.2 \pm 0.4	9.2 \pm 0.4	8.1 \pm 1.1	8.1 \pm 1.1	8.1 \pm 1.1	8.1 \pm 1.1
β -Pinene	9.6 \pm 0.0	9.6 \pm 0.0	9.6 \pm 0.0	9.6 \pm 0.0	8.5 \pm 0.8	8.5 \pm 0.8	7.2 \pm 0.8	7.7 \pm 0.8
Limonene	7.3 \pm 3.5*	9.6 \pm 0.0	9.3 \pm 0.7	8.3 \pm 1.4*	7.7 \pm 0.8	5.5 \pm 0.9	6.4 \pm 0.6	5.1 \pm 0.8
Linalool	8.9 \pm 0.0	8.6 \pm 0.7	8.3 \pm 1.4	9.3 \pm 0.7	6.8 \pm 0.7	8.5 \pm 0.9	6.0 \pm 0.7	7.2 \pm 0.8
Carvacrol	8.6 \pm 0.7	8.9 \pm 0.0	9.6 \pm 0.0	8.5 \pm 1.4	2.5 \pm 0.3***	7.2 \pm 0.8	7.7 \pm 0.8	8.9 \pm 0.7
allo-Aromadendrene	9.3 \pm 1.2	8.3 \pm 0.6*	9.0 \pm 0.0	9.7 \pm 0.5	8.2 \pm 2.6	8.3 \pm 3.4	7.0 \pm 3.8	7.7 \pm 3.4
Caryophyllene	9.7 \pm 0.6	9.5 \pm 0.7	8.3 \pm 1.5	9.7 \pm 0.6	6.2 \pm 2.9	7.0 \pm 3.4	7.9 \pm 3.7	8.8 \pm 2.8
Caryophyllene oxide	9.0 \pm 0.0	8.7 \pm 0.6	8.5 \pm 2.1	9.7 \pm 0.6	7.3 \pm 2.4	7.2 \pm 3.6	7.9 \pm 1.4	7.5 \pm 2.8

Note: *p < 0.05; ** p<0.01; ***p<0.001 vs. control.

GC and GC/MS analyses: GC analyses were carried out on a Hewlett Packard Sigma 115 gas chromatograph equipped with FID and a HP 5MS fused silica capillary column (30 m x 0.25 mm i.d.; film thickness: 0.25 μ m). Column temperature: 40°C, with 5 min initial hold, and then to 260°C at 2°C/min, 260°C (20 min); injection mode splitless (1 μ L of a 1:1000 *n*-pentane solution). Injector and detector temperatures were 250°C and 290°C, respectively. Analysis was also run by using a fused silica HP Innowax polyethyleneglycol capillary column (50 m x 0.20 mm, i.d.; 0.25 μ m film thickness). In both cases, carrier gas was He, with flow rate of 1 mL/min. GC-MS analyses were performed on an

Agilent 6850 Ser. II apparatus, fitted with a fused silica DB-5 capillary column (30 m x 0.25 mm i.d.; 0.33 μ m film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy 70 eV; electron multiplier voltage 2,000 V. Mass spectra were scanned in the range 40-500 amu, scan time 5 scans/s. Gas chromatographic conditions were as reported above; transfer line temperature, 295°C.

Identification of components: Most constituents were identified by gas chromatography by comparison of their linear retention indices (LRI) with either those of the literature [26,27] or with those of authentic

compounds available in our laboratories. The linear retention indices were determined in relation to a homologous series of *n*-alkanes (C₈-C₂₈) under the same operating conditions. Further identification was made by comparison of their mass spectra on both columns with either those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature [26,28] and a home made library. Components relative concentrations were obtained by peak area normalization. No response factors were calculated.

Biological assay: A bioassay based on germination and subsequent radicle growth was used to study phytotoxic effects of the essential oils of *T. arduini*, *T. maghrebinum*, *T. montbretii* ssp. *heliotropiifolium* and *T. polium* ssp. *capitatum* on seeds of *Raphanus sativus* L. cv. "Saxa" (radish), and *Lepidium sativum* L. (cress). Seeds of *L. sativum* and *R. sativus* were purchased from Blumen srl, Piacenza, Italy. The seeds were surface-sterilized in 95% ethanol for 15 s and sown in Petri dishes (Ø=90 mm), containing five layers of Whatman

filter paper, impregnated with 7 mL of distilled water (control) or 7 mL of tested solution of the essential oil at the different assayed doses. The germination conditions were 20 ± 1 °C, with natural photoperiod. The essential oils, in water–acetone mixture (99.5:0.5), were assayed at the doses of 2.5, 1.25, 0.625, 0.25, 0.125 and 0.062 µg/mL. The pure compounds, dissolved in water–acetone mixture (99.5:0.5), were assayed at the concentrations of 10⁻⁶ M, 10⁻⁵ M, 10⁻⁴ M, and 10⁻³ M. Controls performed with water–acetone mixture alone showed no appreciable differences in comparison with controls in water alone. Seed germination was observed directly in Petri dishes, each 24 h. Seed was considered germinated when the protrusion of the radicle became evident [29]. After 120 h (on the fifth day), the effects on radical elongation were measured in centimeters. Each determination was repeated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean ± SD of both germination and radical elongation. The Student's t test of independence was applied [30].

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Chemical Constituents and Larvicidal Activity of *Hymenaea courbaril* Fruit Peel

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The chemical compositions of the essential oils from the peel of ripe and unripe fruits of *Hymenaea courbaril* L., obtained by hydrodistillation, were analyzed by GC and GC-MS. The main constituents of the essential oil from the peel of the ripe fruits were the sesquiterpenes α -copaene (11.1%), spathulenol (10.1%) and β -selinene (8.2%), while germacrene-D (31.9%), β -caryophyllene (27.1%) and bicyclogermacrene (6.5%) were the major compounds in the oil from unripe fruits. The essential oils were tested against *Aedes aegypti* larvae and showed LC₅₀ values of 14.8 \pm 0.4 μ g/mL and 28.4 \pm 0.3 μ g/mL for the ripe and unripe fruit peel oils, respectively. From the peel of the ripe fruits, the diterpenes zanzibaric acid and isoozic acid were isolated, along with the sesquiterpene caryolane-1,9 β -diol. To the best of our knowledge, this is the first report of this sesquiterpene in the genus. The structures of all compounds isolated were identified on the basis of their spectral data (IR, MS, 1D- and 2D-NMR) and by comparison with literature spectral data.

Keywords: *Hymenaea courbaril*, Caesalpinoideae, sesquiterpenes, diterpenes, *Aedes aegypti*.

The genus *Hymenaea* (Caesalpinoideae) comprises 14 species [1] and is widely distributed from Central to South America, mainly in the Amazon basin [2]. *H. courbaril* L., known popularly as “jatobá”, is useful for its timber and has been employed in folk medicine as an anodyne, antiseptic, astringent, expectorant, laxative, purgative, sedative, stimulant, tonic, and vermifuge [3]. *Enantio*-labdanoic and *enantio*-halimane type diterpenes have been isolated from *H. courbaril* [2,4-12], but there is no report of essential oils.

Interest in the control of *Aedes aegypti* lies in the fact that it acts as a vector of dengue and dengue

hemorrhagic fever. Dengue is an endemic disease in most of the countries of the Americas and, over the past twenty years, has shown sustained cycles of outbreaks, every 3 to 5 years, and outbreaks are re-emerging in Brazil [13]. There are no effective vaccines, and vector control is the only way to minimize the transmission of the virus. Plant products have been used against the vectors, and plant-derived products can act as larvicides [14]. Essential oils from plants like *Lippia* species [15] and *Croton zehntneri* [16], and various extracts and compounds [17,18] have been documented for larvicidal activity towards *Aedes aegypti*.

Table 1: Volatile components identified in the essential oils from ripe (oil A) and unripe fruit peel (oil B) of *H. courbaril*.

Compound	RI	Oil A (%)	Oil B (%)
δ-Elementene	1333	-	0.1
α-Cubebene	1342	1.7	0.4
α-Ylangene	1364	0.5	0.1
α-Copaene	1371	11.1	4.2
β-Elementene	1384	5.0	-
7-epi-Sesquithujene	1386	-	0.8
Cyperene	1401	-	0.1
β-Caryophyllene	1414	2.0	27.1
β-Copaene	1424	0.6	0.1
α-trans-Bergamotene	1431	-	0.9
Aromadendrene	1432	1.6	-
(Z)-β-Farnesene	1439	-	1.2
cis-Muurolo-3,5-diene	1447	-	0.1
α-Humulene	1450	0.3	4.2
Allo-Aromadendrene	1454	0.6	0.5
γ-Muurolole	1469	7.9	-
Amorpha-4,7(11)-diene	1475	0.3	-
trans-Cadina-1(6),4-diene	1475	-	2.3
Germacrene-D	1482	-	31.9
β-Selinene	1488	8.2	0.6
δ-Selinene	1490	5.7	-
trans-Muurolo-4(14),5-diene	1491	-	0.8
Bicyclogermacrene	1494	-	6.5
γ-Cadinene	1507	2.7	1.1
δ-Amorphene	1512	5.3	0.3
trans-Calamenene	1515	1.1	-
δ-Cadinene	1516	-	3.3
trans-Cadina-1,4-diene	1530	-	0.2
α-Cadinene	1534	-	0.2
α-Calacorene	1535	1.1	-
Germacrene-B	1553	1.8	1.7
Spathulenol	1572	10.1	0.9
Caryophyllene oxide	1577	6.9	2.1
β-Copaen-4α-ol	1583	1.2	-
Globulol	1583	-	0.2
Salvia-4(14)-en-1-one	1587	1.8	-
Humulene epoxide II	1604	1.2	0.3
1-epi-Cubenol	1622	1.3	0.2
Camphoric acid	1631	0.8	-
epi-α-Muurolole	1637	1.1	-
α-Muurolole	1641	0.7	-
Selin-11-en-4α-ol	1649	1.8	-
α-Cadinol	1653	-	0.8
(Z)-14-Hydroxy-caryophyllene	1664	0.4	-
Mustakone	1668	0.9	-
Amorpha-4,9-dien-2-ol	1684	0.4	-
Levomenol	1887	-	0.1
Oxygenated sesquiterpenes		28.6	4.6
Non-oxygenated sesquiterpenes		57.5	88.7
Total		86.1	93.3

As part of a continuous research program on plants from northeast Brazil, this work describes the isolation of the diterpenes zanzibaric acid and isoozic acid, and the sesquiterpene caryolane-1,9β-diol from the peel of the ripe fruits of *H. courbaril*, and the chemical composition of the essential oils from the peel of both ripe and unripe fruits of *H. courbaril*, as well as the evaluation of these oils for their larvicidal activity against *A. aegypti*.

Successive chromatographic treatments of the *n*-hexane extract of the air-dried, ripe, fruit peel of *H. courbaril* afforded the *ent*-labdane diterpene zanzibaric acid [19] and the sesquiterpene caryolane-1,9β-diol [20]; from the ethyl acetate extract, the diterpene isoozic acid was isolated [21].

The chemical composition of the essential oils from the peel of the ripe and unripe fruits of *H. courbaril*, including retention index (RI) values listed in order of elution from the DB-5MS column, and the percentage relative to each constituent is presented in Table 1. From the two analyzed oil samples, a total of 47 compounds were identified, all of which were sesquiterpenes, representing 86.1% and 93.3% of the oils. α-Copaene (11.1%), spathulenol (10.1%), β-selinene (8.2%), γ-muurolole (7.9%) and caryophyllene oxide (6.9%) were the major constituents identified in the essential oil from ripe fruit peel (oil A), while germacrene D (31.9%), β-caryophyllene (27.1%), bicyclogermacrene (6.5%), α-humulene (4.2%), and α-copaene (4.2%) were the prevalent compounds in the essential oil from unripe fruit peel (oil B).

The larvicidal potential of the essential oils was evaluated against *A. aegypti* larvae. The essential oil from the ripe fruit peel (oil A) showed stronger larvicidal activity against third-instar *A. aegypti* larvae (LC₅₀ 14.8 ± 0.4 µg/mL) than the oil from unripe fruit peel (oil B; LC₅₀ 28.4 ± 0.3 µg/mL); this can be justified by the higher concentration of oxygenated sesquiterpenes (28.6%), mainly spathulenol [22], present in the essential oil from mature fruit peel (oil A). *O,O'*-(Thiodi-4,1-phenylene)bis(*O,O*-dimethyl phosphorothioate (Temephos®)) was used as a positive control (LC₅₀ 1.4 ± 0.2 µg/mL).

This study showed the efficacy of the essential oils from the peels of ripe and unripe fruits of *H. courbaril* against *A. aegypti* larvae. Furthermore, it may represent a contribution to mosquito control. The isolation of caryolane-1,9β-diol represents the first report of this sesquiterpene in the genus.

Experimental

Plant material: Fruits of *H. courbaril* were collected in 2007 in Crato County, State of Ceará, northeast Brazil. A voucher specimen (#EAC41026) is deposited at the Herbário Prisco Bezerra, Departamento de Biologia, Universidade Federal do Ceará, Brazil.

Extraction of essential oils: The ripe (636 g) and unripe fruit peels (940 g) of *H. courbaril* were triturated and subjected to hydrodistillation in a Clevenger-type apparatus for 2 h to afford 0.27 g (0.042%) and 0.67 g

(0.071%) of pale yellow oils, respectively. The yields (w/w) were calculated based on the fresh weight of the plant materials. The isolated oils, after drying over anhydrous sodium sulfate (0.20 g) and filtration, were stored in sealed glass vials and maintained under refrigeration before analysis.

Analytical conditions: The essential oils were analyzed by GC/MS on a Shimadzu QP5050A (Shimadzu Corporation, Kyoto, Japan) system equipped with a AOC-20i autosampler under the following conditions: J&W Scientific DB-5MS fused silica capillary column (30 m x 0.25 mm i.d., x 0.25 µm film thickness, composed of 5%-phenyl-95%-methylpolysiloxane) operating in EI mode at 70 eV. Helium (99.999%) was used as the carrier gas at a constant flow of 1.2 mL/min. The injection volume was 0.5 µL (split ratio of 1:100), the injector temperature 250°C and the ion-source temperature 280°C. The oven temperature was programmed from 50°C (isothermal for 1.5 min), with an increase of 4°C/min to 200°C, then 10°C/min to 300°C, ending with a 10 min isothermal period at 300°C. Mass spectra were taken at 70 eV with a scan interval of 0.5 s and fragments from 40 to 500 Da.

Quantitative analysis of the chemical constituents was performed by flame ionization gas chromatography (FID), using a Shimadzu GC-17A (Shimadzu Corporation, Kyoto, Japan) instrument, under the following operational conditions: capillary ZB-5M5 column (5%-phenyl-arylene-95%- methylpolysiloxane fused silica capillary column 30 m x 0.25 mm i.d. x 0.25 µm film thickness), under the same conditions

reported for the GC-MS. Quantification of each constituent was estimated by area normalization (%). Compound concentrations were calculated from the GC peak areas and they were arranged in order of GC elution.

Identification of individual components of the essential oils was performed by computerized matching of the acquired MS with those stored in NIST21 and NIST22 mass spectral library of the GC/MS data system. Retention indices (RI) for all compounds were determined according to literature [23] for each constituent, as previously described [24].

Larvicidal bioassay: Aliquots of the essential oils tested (12.5 to 500 µg/mL) were placed in a beaker (50 mL) and dissolved in DMSO/H₂O 1.5% (20 mL). Fifty instar III larvae of *Aedes aegypti* were delivered to each beaker. After 24 h at room temperature, the number of dead larvae was counted and the lethal percentage calculated. A control using DMSO/H₂O 1.5% was carried out in parallel. For each sample, 3 independent experiments were run [25].

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Caryophyllene Oxide-rich Essential Oils of Lithuanian *Artemisia campestris* ssp. *campestris* and Their Toxicity

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The chemical composition of the essential oils of aerial parts of *Artemisia campestris* ssp. *campestris*, collected from ten different locations in Lithuania is detailed in this paper. The major component in all the oils was caryophyllene oxide (8.5-38.8%), whereas compounds with the caryophyllane skeleton ranged from 10.2 to 44.5%. Other representative constituents were germacrene D ($\leq 15.0\%$), humulene epoxide II ($\leq 8.1\%$), β -ylangene ($\leq 7.7\%$), spathulenol ($\leq 6.8\%$), β -elemene ($\leq 6.8\%$), β -caryophyllene ($\leq 6.2\%$), junenol ($\leq 6.1\%$) and α - or β -pinene ($\leq 5.5\%$). Eighty-seven compounds were identified, comprising 73.6-92.3% of the oils. The chemical composition was highly variable depending on the sample location.

Toxicity of *A. campestris* oils was determined using the brine shrimp (*Artemia* sp.) assay. LC₅₀ values ranging to 20 $\mu\text{g/mL}$ were obtained for three of the oils after 24 hours of exposure. Data of this test revealed that *A. campestris* ssp. *campestris* essential oils with dominant caryophyllene oxide are notably toxic.

Keywords: *Artemisia campestris* ssp. *campestris*, essential oil, toxicity, brine shrimp lethality test, β -caryophyllene, caryophyllene oxide, β -elemene, β -ylangene, humulene epoxide II, junenol.

Artemisia (with up to 500 species) is one of the largest and most widely distributed genus of the Asteraceae. *A. campestris* (tribe Anthemideae, *Dracunculus* section), commonly known as field wormwood or field sagewort, is a perennial plant that is found in temperate regions throughout the northern hemisphere. Field wormwood, common for most parts of Europe, prefers open sites in light (sandy) and medium (loamy) soils. The species is polymorphous and is divided into several subspecies and forms. The plant is known as a medicinal herb with anthelmintic, antiseptic, cholagogue, deobstruent, emmenagogue, stomachic and tonic properties.

Flavonoids and terpenoids are the main secondary metabolites of the genus *Artemisia* [1]. Previous studies of the chemical composition of essential oils of *A. campestris* from different countries gave contrasting results (Table 1, [2-13]). However, the authors worked on plant material from various locations with many environmental variables, and in some of these studies,

the plant parts used were not clearly specified and/or the subspecies was not indicated. Moreover, even for the same subspecies, i.e. *A. campestris* ssp. *glutinosa*, oil compositions varied from one study to another [2-5,12]. In addition, variation in the essential oil composition may occur depending on the phenological stage. This parameter was not always specified in the previous reports.

Furthermore, the literature shows the lack of research concerning the ethno-pharmacology, volatile oil composition and antibacterial activity of *A. campestris* from Lithuania. Thus the aim of this study was to determine the chemical composition and toxicity of the essential oils from the aerial parts of *A. campestris* ssp. *campestris* from various Lithuanian locations. All samples were collected at the full flowering to compare oil compositions of plants at the same phenological stage.

Table 1: Major constituents (%) of *Artemisia campestris* essential oils from various countries, according to the literature.

<i>A. campestris</i> subspecies	Organ	Extract	Main components, (%)	Place of origin	Reference
ssp. <i>glutinosa</i>	not specified	EO	<i>ar</i> -curcumene, caryophyllene oxide, <i>p</i> -cymene, β -pinene and germacrene D	Italy	2
ssp. <i>glutinosa</i>	flowers and leaves		β -pinene (6.9-57.2), germacrene D (0.4-28.6), bicyclogermacrene (1.0-14.5) and myrcene (1.7-11.2)	Italy	3
ssp. <i>glutinosa</i>	aerial parts	EO	γ -terpinene (\leq 46.5), capillene (33.1), 1-phenyl-2,4-pentadiene (\leq 29.7) and spathulenol (\leq 11.3)	France	4
ssp. <i>glutinosa</i> even if ns	aerial parts	EO	(<i>Z,E</i>)-farnesol (10.3), cedrol (5.4) and verbenone (3.8)	Algeria	5
ssp. <i>campestris</i> and <i>borealis</i>	aerial parts	EO	α -pinene (\leq 16.5), β -pinene (\leq 10.7), caryophyllene oxide (\leq 18.2), spathulenol (\leq 18.7), <i>epi</i> -cubenol (\leq 14.2) and 1,8-cineole (\leq 19.2)	North west Italy	1, 6
ssp. <i>maritima</i> <i>Archangelis</i> * ns	aerial parts ?	EO	β -pinene (17.8), cadin-4-en-7-ol (16.4), γ -terpinene (8.7), (<i>Z</i>)- β -ocimene (7.4), aromadendrene (6.7)	Portugal	7
	leaf	EO	β -pinene (24.0-49.8), α -pinene (5.9-12.5), <i>p</i> -cymene (3.4-9.4), limonene (4.9-9.3), spathulenol (1.2-8.9), γ -terpinene (2.0-6.5), eudesmol (1.0-6.4) and (<i>Z</i>)- β -ocimene (0.2-5.5)	Southern Tunisia	8
ns		vapor	α -pinene (41.0), β -pinene (29.7), limonene (6.4) and sabinene (4.5)	Southern Ural	9
ns	leaf	EO	β -pinene (24.2-27.9), <i>p</i> -cymene (17.4-22.3) and α -pinene (4.1-11.0)	South eastern Tunisia	10
ns	aerial parts	EO	spathulenol (9.2), β -pinene (9.1), α -pinene (3.4), limonene (2.5), germacrene D (3.3), 4-hydroxy-9- <i>epi</i> - β -caryophyllene (3.0)	Serbia	11
var. <i>glutinosa</i> Gay ex Bess ns	aerial parts	EO	β -pinene (41.0), <i>p</i> -cymene (9.9), α -terpinene (7.9), limonene (6.5) and myrcene (4.1)	Southern Tunisia	12
	flowers, leaves and stems, separately	EO	α -pinene (23.9, 23.0, 29.2) and spathulenol (23.9, 15.8, 29.2) in the flower, leaf and stem oils, respectively; bicyclogermacrene (12.0) in the flower and β -pinene (12.6) in the leaf oil	Iran	13

EO-essential oils, ns-name of subspecies not indicated, * also referred to as *Artemisia crithmifolia* L.

1. Chemical composition of Lithuanian *Artemisia campestris* essential oils:

The composition of the essential oils of *A. campestris* collected from 10 populations in different parts of Lithuania (east, west and south of the country) and rich in caryophyllene oxide is presented in Table 2. The major component in all the oils was caryophyllene oxide (8.5-38.8%), while the amount of compounds with a caryophyllane skeleton (caryophyllene, its oxide and caryophylla-4(12),8(13)-dien-5 α -ol) ranged from 10.2 to 44.5%.

In four samples (A, E, F and K) out of ten, germacrene D (9.4-15.0%) was the second main constituent. In the other six oils, four compounds were identified as the second principal component, i.e. caryophyllene (5.7-6.2% in oil samples H and J), β -pinene (4.9% in sample C), spathulenol (5.7-6.8% in B and D) and humulene epoxide II (8.1% in G).

The third most dominant components were found to be α - or β -pinene (4.5-5.5% in A, C and D), β -ylangene (7.7% in sample E), germacrene D (4.9-7.5% in G, H and J), humulene epoxide II (5.3% in B), junenol (6.1% in F) and β -elemene (6.8% in K). Caryophyllene oxide, germacrene D, α - or β -pinene and spathulenol have been previously determined in appreciable amounts in field wormwood oils from other countries (Table 1), while humulene epoxide II, junenol, β -elemene and

β -ylangene have not been mentioned before among the main constituents of these oils. Bicyclogermacrene was only determined in minor amounts in this study, while it was a major constituent (up to 14.5%) in *A. campestris* oils from Italy and Iran [3,13].

Eighty-seven identified constituents (one of them tentatively) comprised 73.6-92.3% of the total oils. Chemical analysis showed a multi-component composition of the essential oils of Lithuanian field wormwood. The main fraction was sesquiterpenoids (49.9-79.3%), where amounts of sesquiterpene hydrocarbons and oxygenated sesquiterpenes varied from 16.1 to 51.2% and from 25.0 to 52.6%, respectively. Monoterpene hydrocarbons accounted for only 5.4-19.7%.

2. Toxic activity of *A. campestris* essential oils against *Artemia* sp. nauplii:

Three oils obtained from the studied plant materials were chosen for tests of toxic activity. The test showed that lethality (LC₅₀) of brine shrimp larvae was 15-20 μ g/mL (16.8, 19.5 and 14.9 μ g/mL for samples A, H and K, respectively). The essential oils of *A. campestris* containing 12.1%, 22.1% and 10.2% of caryophyllene oxide, and 9.4%, 5.8% and 9.9% of germacrene D, respectively were toxic enough to kill the shrimps, despite the fact that the extremely toxic ketones (such as α - and β -thujone or artemisia

Table 2: Main constituents (with quantity over 5%) of essential oils, rich in caryophyllene oxide, of *Artemisia campestris* from Lithuania (2003–2007).

Compound	RI ^a	A	B	C	D	E	F	G	H	J	K	Interval
α -Pinene	939	5.3	t	4.5	4.0	6.8	1.1	5.6	1.3	1.5	0.7	t-6.8
β -Pinene	975	3.9	4.3	4.9	5.5	2.3	0.5	0.3	0.2	t	0.3	t-5.5
<i>cis</i> -Pinane	986	1.0	3.6	0.8	1.5	1.2	1.5	6.0	3.3	3.2	1.9	0.8-6.0
β -Elemene	1391	3.2	t	0.3	0.9	0.7	1.3		0.7	0.1	6.8	0-6.8
β -Caryophyllene	1419	3.0	2.0	2.7	1.7	1.6	2.8	6.1	6.2	5.7	4.9	1.6-6.2
β -Ylangene	1420	1.8	1.0	1.5	0.7	7.7	3.1		1.0		6.0	0-7.7
γ -Curcumene+Acoradiene	1480		5.7			4.6	0.8	5.1	1.5	3.1		0-5.7
Germacrene D	1485	9.4	3.5	3.8	3.7	10.1	15.0	7.5	5.8	4.9	9.9	3.5-15.0
Spathulenol	1578	0.9	6.8	4.5	5.7							0-6.8
Caryophyllene oxide	1583	12.1	19.7	14.5	8.5	16.0	18.7	22.0	22.1	38.8	10.2	8.5-38.8
Humulene epoxide II	1608	3.7	5.3	0.7	2.5	2.9	5.2	8.1	5.6	4.1	2.4	0.7-8.1
Junenol	1619	1.6		1.4			6.1	2.9	0.8		4.6	0-6.1
Sum of main constituents (%)		45.9	51.9	39.6	34.7	53.9	56.1	63.6	48.5	61.4	47.7	34.7-63.6
Total (^a including all compounds with quantity $\leq 5.0\%$)		75.2	73.6	79.3	80.1	81.2	85.4	92.3	84.3	81.0	92.0	73.6-92.3
Compounds with caryophyllene skeleton ^b		15.4	21.7	17.2	11.8	17.6	21.5	30.0	28.3	44.5	15.1	11.8-44.5
Monoterpene hydrocarbons ^b		17.1	9.7	15.6	19.7	15.9	5.4	13.3	10.8	8.1	6.8	5.4-19.7
Sesquiterpene hydrocarbons ^b		27.2	16.1	24.7	21.1	34.1	33.0	31.4	25.7	18.4	51.2	16.1-51.2
Oxygenated sesquiterpenes ^b		25.0	42.2	33.2	28.8	26.6	40.8	39.3	40.4	52.6	28.1	25.0-52.6

A-K indicate harvesting localities, in eastern Lithuania: Trakai district, Streva (A, 2003) and Vilnius district, Vievis (B, 2007); in western Lithuania: Palanga city, Sventoji (C, 2005), Klaipeda district, Plikiai (D, 2005), Palanga city, Butinge (E, 2007), Silute district, Pagegiai (F, 2007), Klaipeda district, Karkle (G, 2007), Kretinga city (H, 2007), Klaipeda city (J, 2007) and in south Lithuania: Druskininkai city, Latezeris (K, 2007).

^aRI- retention index on nonpolar column DB-5; t-traces ($\leq 0.05\%$).

^bCompounds with quantity $\leq 5.0\%$:

0-0.5%: α -thujene, camphene, sabinene, *p*-cymene, 6-camphenone, *allo*-ocimene, *neo-allo*-ocimene, *trans*-verbenol, camphor, verbenone, α -cubebene, citronellyl acetate, neryl acetate, geranyl acetate, β -bourbonene, aromadendrene, *cis*-muurola-4(14),4-diene, α -cadinene, nonadecane and eicosane;

up to 1.5%: α -terpinene, β -phellandrene, (*Z*)- β -ocimene, benzene acetaldehyde, (*E*)- β -ocimene, γ -terpinene, terpinolene, linalool, α -campholenal, *trans*-pinocarveol, terpinen-4-ol, α -terpineol, myrtenol, 4(*Z*)-decen-1-ol, *trans*-sabinyl acetate, terpinyl acetate, *ar*-curcumene, bicyclogermacrene, γ -cadinene, *trans*-cadinina-1(2),4-diene, α -calacorene, β -copaen-4- α -ol, *trans*- β -elemenone, *epi*- α -muurolol and phytol;

lower than 3.0%: 1,3-dimethyl benzene, myrcene, γ -terpineol, δ -elemene, α -copaene, (*E*)- β -ionone, α -zingiberene, *trans*- β -guaiene, α -muurolene, (*E*, *E*)- α -farnesene, δ -cadinene, (*E*)-nerolidol, caryophylla-4(12),8(13)-dien-5- α -ol, germacrene-4(15),5,10(14)-trien-1- α -ol and (6*R*, 7*R*)-bisabolone;

up to 5.0%: limonene, bornyl acetate, β -copaene, (*Z*)- β -farnesene, α -humulene, (*E*)- β -farnesene, β -selinene, *trans*-muurola-4(14),5-diene, (*Z*)-nerolidol, unknown 1, salvia-4(14)-en-1-one, *epi*- α -cadinol, α -cadinol and 3-thujopsanone.

Unknown 1 (RI-1580): M^+ 220, 107(100), 135(69), 91(57), 41(37), 79(34), 69 (21), 119(21), 204(16).

ketone) characteristic for *Artemisia* species, were not detected in the investigated oils. The strong toxicity of *A. campestris* evaluated by us can justify why the plant is used in Lithuanian folk medicine, but not as a food or spice.

According to the literature data [14-16], essential oils of other plant species possessing caryophyllene oxide as a major constituent are toxic. Volatile oils of *Acroptilon repens*, containing 36.6% of caryophyllene oxide and 10% of caryophyllene inhibited the growth of Gram-positive bacteria [14]. This sesquiterpene oxide is toxic to ants and inhibits growth of ant-associated fungi [15]. The toxicity of essential oils isolated from *Artemisia* species and containing caryophyllene oxide as a major component showed a high mortality to granary weevil [16]. However, not only compounds present in the greatest proportions are responsible for the total oil activity. The influence of the less abundant constituents and synergetic effects might also be considered.

Experimental

Plant material and oil isolation: The aerial parts (~20 cm, 15-100g) of individuals from various *A. campestris* ssp. *campestris* populations were collected at full

flowering stage in July-August (2003, 2005 and 2007) from ten different localities in Lithuania. Voucher specimens were deposited in the herbaria of the Institute of Botany (BILAS), Vilnius and Vytautas Magnus University, and Kaunas Botanical Garden, and their numbers are: A-68906, B-68919, C-68913, D-68914, E-SS 657, F-SS 658, G-SS 659, H-68918, J-68911 and K-SS 660. Plant material was dried at room temperature (20-25°C). Essential oils of the air-dried aerial parts (leaves and inflorescences) were prepared by hydrodistillation for 2 h using a Clevenger-type apparatus and a mixture of *n*-pentane and diethyl ether (1:1) as a collecting solvent. Pure oils of yellow-grey color were obtained from 80-100g of dry material. Yields ranged from 0.03 to 0.08 %, v/w on a dry mass basis.

GC-MS analysis: Analyses were performed using an HP 5890 chromatograph interfaced to an HP 5971 mass spectrometer (ionization voltage 70 eV, scan time 0.6 s, scan range 35-400 Da) and equipped with a capillary column DB-5 (50 m \times 0.32 mm i. d., film thickness 0.25 μ m). The oven temperature was held at 60°C for 2 min, then programmed from 60 to 160°C at a rate of 5°C/min, held for 1 min, then increased to 250°C at a

rate 10°C/min and finally isothermal at 250°C for 3 min, using He as a carrier gas (1.0 mL/min), split 1:40. Injector and detector temperatures were 250°C. Qualitative analysis was based on a comparison of retention times, indexes and MS with the corresponding data in the literature [17], by co-injection of some terpene references, and from computer MS libraries (Wiley and NBS 54K).

Toxicity test: Toxicity of 3 of the *A. campestris* oils (A, H and K, plant material from different parts of Lithuania) was tested *in vivo*, using brine shrimp *Artemia* sp. (larvae) [18]. The eggs of the shrimps hatch within 48 h to provide larvae (nauplii) in sea water

(31g/L sea salt) at 20-25°C. Then, different concentrations of field wormwood essential oils dissolved in dimethyl sulfoxide (DMSO) were added, and survivors were counted after 24 h. Lethality (LC₅₀) of nauplii was calculated (n=4, with 95% confidence interval). A control test was done with DMSO.

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Comparison of Antibacterial Activity of Natural and Hydroformylated Essential Oil of *Thymus capitatus* Growing Wild in North Sardinia with Commercial *Thymus* Essential Oils

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Thymus capitatus growing wild in Sardinia showed different essential oil composition if grown surrounding Cagliari than in north Sardinia. Here we verify the composition and antimicrobial activity of the oil to make it suitable for the cosmetic and confectionery industries. With the aim of improving the scent and the antimicrobial activity of *T. capitatus* essential oil, a hydroformylation reaction was carried out to transform the unsaturated components of the oil into the corresponding aldehydes. The essential oil of *T. capitatus* exhibited a significant antibacterial activity (MIC 0.125-0.5 mg/mL), and was also found effective on *C. albicans* (MIC 0.125 mg/mL). After hydroformylation, several new irregular terpenoid aldehydes were detected. The perfume of the new terpenic-like aldehydes is very agreeable and, therefore, the acceptability of the aroma is remarkably improved, but the antimicrobial activity was not increased.

Keywords: *Thymus capitatus*, essential oil, hydroformylation, antibacterial activity, carvacrol.

The family *Lamiaceae* shows an intraspecific biodiversity resulting in the heterogeneous composition of the essential oil produced by various species, which is based on genetic polymorphism, but which is also influenced by environmental conditions. In the genus *Thymus*, for example, many species present intraspecific chemotypes [1]. Wild *Thymus* species growing in Sardinia include only two chemotypes, thymol and carvacrol [2-5], which are present only with two species: *T. catharinae* Camarda (ex *T. herba-barona* Loisel) and *T. capitatus* (L.) Hoffmanns & Link [6,7].

T. capitatus (synonyms *Satureja capitata* L., *Coridothymus capitatus* Rehb. and *Thymbra capitata* (L.) Cav.) is a characteristic species of *Lamiaceae* growing wild in the Mediterranean basin. In Italy, it is present in the South of the Italian peninsula (Abruzzo, Molise, Campania, Puglia, Basilicata, Calabria) and in the two biggest islands, Sicily and Sardinia. In Sardinia, *T. capitatus* is present only in a few calcareous northern areas (in few villages around Sassari) and in southern zones surrounding Cagliari. The composition and

activity of its essential oil have been studied by several researchers [8-12]. *T. capitatus* essential oil has been used since ancient times in pharmaceuticals, for food flavoring, cosmetics and perfumery [13].

Thymus species have been studied for their composition and biological activity, but few studies on *T. capitatus* growing wild in Sardinia are present in the literature, but they indicate that the essential oil from *T. capitatus* growing wild around Cagliari has an essential oil composition [3] that is very different from that of plants growing in north Sardinia [2]. The aim of the present study was the characterization of *T. capitatus* essential oil in order to determine whether its composition and antimicrobial activity made it suitable for the cosmetic, fragrance and confectionery industries. Moreover, the essential oil underwent a hydroformylation reaction catalyzed by rhodium complexes to transform unsaturated terpenes into oxygenated compounds (aldehydes), with the aim of giving the essential oil a more pleasant smell and improving its antimicrobial activity.

Essential oil of *T. capitatus* obtained from plants growing wild in a large station located in north Sardinia was analyzed using GC and GC/MS; 36 constituents were identified, with a percentage of identification near to 100%. To be sure that the essential oil composition of the plants from this station remained constant with time, we monitored the essential oil composition for 3 years before the present analyses (Table 1). As shown in Table 1, the major constituent of the oil is carvacrol. Between 2005-2007 there were no substantial differences, at least in the main constituents, but big differences were found in samples collected in 2008. The first three collections were made at the time of maximum blooming (May-June in normal climatic conditions), while the 2008 harvest was made at the beginning of July, during a very dry summer following a prolonged period of drought.

The biggest detectable differences were the presence of 3.8% α -thujene, 1.5% α -pinene, the doubling of the β -myrcene content, and the presence of 8.6% *p*-cymene. Remarkable was the increase of γ -terpinene (6.9%) and the dramatic decrease in carvacrol, that varied from 83% to 57%. These differences may partially be due to the major concentration of α -pinene and *p*-cymene; the different environmental conditions were in favor of the presence of compounds like α -thujene and 1-octen-3-ol.

It is known that *T. capitatus* essential oil is highly effective against bacteria and fungi [2]. With the aim of increasing the efficiency of the antimicrobial activity and to improve the odor of *Thymus* oil we submitted it to a hydroformylation reaction. Because of the unsaturated terpenes present in the oil, the hydroformylation process should furnish a modified oil containing new terpenic-like aldehydes capable of increasing the antimicrobial performance. Table 2 compares the original *T. capitatus* essential oil with the hydroformylated one. After hydroformylation, we found several new irregular terpenoid aldehydes in the modified oil (Table 2). As a consequence of these modifications, the acceptability of the oil's smell was remarkably enhanced due to the new terpenic-like aldehydes; however, the antimicrobial characteristics of the oil did not improve.

The original essential oil was hydroformylated. The modified essential oil was washed and hydrodistilled to obtain the new, pure, modified essential oil. Comparing the analytical data of the original and modified oils, it was evident that the most important variations were: α -thujene, which was present in good percentage in the original oil, but lost about 2% as a result of hydroformylation; compounds like camphene, *trans*-pinene, 1-octen-3-ol, α -phellandrene, β -myrcene,

Table 1: Quantitative and qualitative composition of *Thymus capitatus* essential oil.

Constituents	K.I.	<i>T. capitatus</i> (2005)	<i>T. capitatus</i> (2006)	<i>T. capitatus</i> (2007)	<i>T. capitatus</i> (2008)
α -Thujene	931	--	--	--	3.8
α -Pinene	939	--	--	--	1.5
Camphene	953	tr	--	--	0.3
Heptanol	969	tr	1.7	1.6	--
1-Octen-3-ol	978	--	--	--	1.3
β -Pinene	980	0.1	--	--	--
3-Octanone	988	0.6	0.6	0.7	--
β -Myrcene	991	1.6	1.6	1.7	3.7
3-Octanol	993	0.2	0.4	0.4	0.4
α -Phellandrene	1005	0.2	0.3	0.3	0.5
Δ^3 -Carene	1011	0.1	--	--	0.2
Δ^4 -Carene	--	0.7	0.8	0.8	2.2
<i>p</i> -Cymene	1026	4.5	4.9	5.0	8.6
β -Phellandrene	1031	--	--	--	1.0
1-8-Cineole	1033	0.4	0.2	0.2	tr
α -Ocimene	1050	--	--	--	0.1
γ -Terpinene	1062	2.6	3.4	3.2	6.9
<i>cis</i> -Sabinene-hydrate	1068	--	--	--	0.1
Terpinolene	1088	--	--	--	0.3
<i>trans</i> -Sabinene-hydrate	1097	0.5	0.7	0.7	--
Linalool	1098	1.3	1.2	1.2	1.9
<i>Exo</i> -Fenchol	1117	--	--	--	0.1
Borneol	1165	0.1	--	--	0.7
Nonanol	1171	--	--	--	0.7
Terpinen-4-ol	1177	0.5	1.3	1.2	2.1
Dihydro carveol	1192	--	--	--	0.5
Nerol	1228	--	--	--	0.1
Neral	1240	0.2	--	--	--
Carvone	1242	tr	--	--	0.2
Thymol	1290	0.1	--	--	tr
Carvacrol	1298	83.5	81.2	81.0	57.0
β -Caryophyllene	1418	1.6	1.5	1.4	3.5
α -Caryophyllene	1454	--	--	--	0.2
β -Bisabolene	1509	--	--	--	0.1
Caryophyllene oxide	1581	0.3	--	--	0.5
undentified		0.1	0.2	0.5	1.0
Total		99.2	100	98.7	97.2

β -caryophyllene and linalool were detected only in the original essential oil because these compounds were hydroformylated and transformed into the corresponding aldehydes; 3-octanol and Δ^3 -carene were present in all samples and did not vary significantly in their concentrations; and carvacrol, which showed little variation, probably due to the experimental steps. On the other hand, there are some compounds that are present only in the hydroformylated oils, as for instance the terpinen-4-ol isomer: 4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol and 3-7-dimethyl-7-octenal. Most of them derive from hydroformylation, but also some isomerization reactions were detected during the oil modification process.

The antimicrobial activity screening was carried out on the *Thymus* essential oils and their main components, respectively, in comparison with chlorhexidine gluconate. The essential oil of *T. capitatus* exhibited a significant antibacterial activity (MIC 0.125-0.5 mg/mL), qualitatively similar to but higher than those of oils of other species of *Thymus*; Gram-positive strains

Table 2: Constituents of original essential oil of *Thymus capitatus* and hydroformylated redistilled oil.

Constituents	K.I.	<i>T. capitatus</i> (2008) (original oil)	<i>T. capitatus</i> (2008) hydroformylated (redistilled)
α -Thujene	931	3.8	1.5
α -Pinene	939	1.5	1.9
Camphene	953	0.3	0.1
2,6-dimethyl-3-Octene	nd	--	0.1
3,7-dimethyl 2-Octene	nd	--	0.1
<i>trans</i> -Pinene	973	0.1	--
1-Octen-3-ol	978	1.3	--
β -Pinene	980	--	0.2
isolimonene	983	--	0.1
<i>p</i> -Menth-3-ene	986	--	1.6
β -Myrcene	991	3.7	--
3-Octanol	993	0.3	0.4
2,6-Dimethyl-2- <i>trans</i> -6-Octadiene	nd	--	0.3
α -Phellandrene	1005	0.5	--
Δ^3 -Carene	1011	0.2	--
Δ^4 -Carene	n.d.	2.2	2.4
α -Cymene	1022	8.6	--
β -Phellandrene	1031	1.0	--
α -Ocimene	1050	0.1	--
γ -Terpinene	1062	6.9	6.9
<i>cis</i> -Sabinene-hydrate	1068	0.1	--
Terpinolene	1088	0.3	0.3
Linalool	1098	1.8	--
<i>exo</i> -Fenchol	1117	0.1	--
Citronellal	1153	--	0.4
Borneol	1165	0.7	0.9
Terpinen-4-ol	1177	2.1	2.3
4-Methyl-(1-methylethyl)-3-Cyclohexen-1-ol	n.d.	--	3.5
Dihydrocarveol	1192	0.5	0.2
3-7 dimethyl-7-Octanal	n.d.	--	1.7
Nerol	1228	0.1	--
Carvone	1242	0.2	--
Thymol	1290	tr	0.8
Carvacrol	1298	57.0	54.7
Carvacrol acetate	1371	--	0.2
γ -Caryophyllene	1404	--	0.5
β -Caryophyllene	1418	3.5	0.1
α -Caryophyllene	1454	0.2	--
α - <i>neo</i> -Clovone	1454	--	1.2
β -Bisabolene	1509	0.1	--
α -Caryophyllene oxide	1581	0.5	0.7
Hexadecene	1593	--	0.2
1-Cyclohexylheptene	1637	--	0.1
Heptadecane	1700	--	0.5
undentified		1.0	1.9
Total		99.5	85.8

were more sensitive that the Gram-negative bacteria examined. *T. capitatus* oil was also found effective on *C. albicans*, with a MIC of 0.125 mg/mL. Under the same experimental conditions, chlorhexidine gluconate showed remarkable antimicrobial activity (MIC 0.001-0.016 mg/mL) against all strains tested. Hydroformylation decreased the antimicrobial activity of *T. capitatus* oil; the MICs of the hydroformylated product were 0.25 mg/mL for *S. aureus* and *S. epidermidis*, 0.5 mg/mL for *E. coli* and >0.5 mg/mL for *Ps. aeruginosa*. As regards the antimicrobial activity of the main oil components, carvacrol showed good inhibitory activity against bacterial strains and *Candida* (MIC values 0.062-0.500 mg/mL), whereas linalool, terpinen-4-ol and β -myrcene did not exhibit any antimicrobial activity at concentrations up to 0.500 mg/mL.

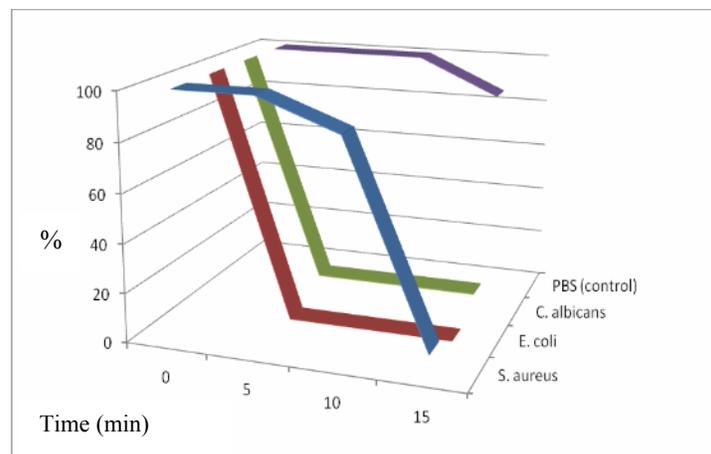
**Figure 1:** Percentages of surviving microorganisms after different exposure times and inhibiting concentrations of essential oil of *T. capitatus*. The percentages are the averages of three experiments.

Figure 1 shows the inactivation kinetics of the killing of standardized inocula of *S. aureus*, *E. coli* and *C. albicans* by inhibitory concentrations of *T. capitatus* oil (0.250 mg/mL for *E. coli*, and 0.125 mg/mL for *S. aureus* and *C. albicans*).

Experimental

Plant materials: Samples of wild *Thymus capitatus* (L.) Hoffmanns. & Link) were collected in 4 consecutive years, in San Michele in Plaiano, one of the largest stations existing around Sassari (north Sardinia). The plants were identified by M. Usai. Voucher specimens have been deposited at the Herbarium S.A.S.S.A. (cumulative identification number: 1078) of the Department of Scienze del Farmaco, University of Sassari. The aerial parts of the plants were randomly collected in June during the blooming period.

Oil distillation and yield: Fresh plant material (5 Kg) was separately hydrodistilled using a Clevenger-type apparatus, according to the Italian Official Pharmacopoeia X [14]. The obtained oil was light yellow. Three replicate samples were distilled simultaneously. Oil floating to the water surface was collected by draining away water, dried over anhydrous sodium sulfate and stored at -20°C (under nitrogen atmosphere) until analyzed. The yield was up to 1.1%.

GC oil analyses: Three replicates of each sample were analyzed by using a Hewlett-Packard Model 5890A GC equipped with a flame ionization detector and fitted with a 60 m x 0.25 mm thickness 0.25 μm AT-5 fused silica capillary column (Alltech). Injection port and detector temperatures were 280°C . The column temperature was programmed from 50°C to 135°C at $5^{\circ}\text{C}/\text{min}$ (1 min), $5^{\circ}\text{C}/\text{min}$ to 225°C (5 min), $5^{\circ}\text{C}/\text{min}$ to 260°C and then held for 10 min. The samples (0.2 μL each), analyzed without dilution (using 2,6-dimethyl-

phenol as internal standard), were injected using a split/splitless automatic injector HP 7673 and with helium as carrier gas. Measurements of peak areas were performed with a HP workstation; the threshold was set at 0, peak width at 0.02. The quantification of each compound was expressed as the absolute weight percentage using internal standard and response factors. The detector response factors (RFs) were determined for key components relative to 2,6-dimethylphenol and assigned to other components on the basis of functional group and/or structural similarity, since oxygenated compounds have lower detectability by F.I.D. than hydrocarbons [15]. The standards purity was checked by GC. Several response factor solutions were prepared that consisted of only 4 or 5 components (plus 2,6-dimethylphenol) to prevent interference due to trace impurities.

GC/MS: GC/MS analyses were carried out with a Hewlett Packard G1800B-GCD system using the same conditions and column described above. The column was connected with the ion source of the mass spectrometer. Mass units were monitored from 10 to 450 at 70 eV. The identification of the components was made by comparison of their retention time with respect to *n*-alkanes (C6-C22). The MS and retention indices (RI) were compared with those of commercial (NIST 98 and WILEY) and home-made library MS built up from authentic samples (analytical standards from Aldrich and Fluka; purity > 97%) and MS literature data [16-21]. The percentage composition of the oil was obtained by the normalization method from the GC peak areas, using correction factors.

Evaluation of antibacterial activity: The antibacterial activity of the essential oils (*Thymus capitatus*, both unmodified and hydroformylated, *T. vulgaris* and *T. serpyllum*) and standard compounds (carvacrol, linalool, β -myrcene and terpinen-4-ol) was determined as minimum inhibitory concentration (MIC) against a panel of bacterial strains, by using chlorhexidine gluconate as reference antimicrobial substance. Microorganisms examined included both Gram-positive (*Staphylococcus aureus* ATCC 25923, CultiLoops[®] Oxoid, Basingstoke, UK; *S. epidermidis* 155U and *Propionibacterium acnes* ATCC 6919, supplied by LCG Promochem, Middlesex, UK) and Gram-negative strains (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853; CultiLoops[®] Oxoid). MICs were determined by using an agar dilution technique, except for *P. acnes*, which was examined by a broth dilution technique. Oils and standards were dissolved at 10% w/v in PEG 200 (Sigma); preliminary tests with this vehicle were performed to ensure that no microorganism inhibition occurred at the used concentrations.

For the agar dilution technique, two-fold serial dilutions of the solutions (0.5 mg/mL to 0.0031 mg/mL) were prepared in triplicate in molten Mueller Hinton Agar (Oxoid) at 45°C and poured into 50 mm Petri dishes. Each dish was inoculated with about 1×10^4 bacteria, applied as a spot of about 5 mm in diameter; after 18-24 h of incubation at 35°C, MICs were recorded as the lowest concentrations which completely inhibited bacterial growth. MICs against *P. acnes* were determined by preparing two-fold serial dilutions (0.5 mg/mL to 0.0031 mg/mL) in triplicate in Brain Heart Infusion (Oxoid) and pouring them into microtiter 96-well plates; each well was inoculated with about 1×10^4 bacteria. MICs were recorded after 72 h of anaerobic incubation (Gas Generating Kit, Anaerobic System BR 038B, Oxoid) as the lowest concentrations which completely inhibited bacterial growth.

Evaluation of antifungal activity: The above mentioned essential oils and standard compounds were also tested against *Candida albicans* ATCC 10231 in an agar dilution test similar to that one previously described for bacteria, by using Sabouraud Dextrose Agar (Oxoid). Agar plates were inoculated with about 10^4 yeast cells from an overnight culture and incubated at 25°C. After 18-24 h, MICs were recorded as the lowest concentrations able to inhibit completely fungal growth.

Killing time test: The exposure times required by inhibitory concentrations of *T. capitatus* essential oil to kill an appropriate microbial inoculum were determined according to a previously described technique [5]. Assays were carried out on *E. coli* ATCC 25922 and *C. albicans* ATCC 10231. Briefly, microorganisms in the logarithmic phase of growth were centrifuged at 1200 g for 15 min, washed in phosphate buffered saline (PBS, pH 7.3) and resuspended in an appropriate volume of essential oil in the same buffer (at a concentration of oil corresponding to the MIC for the tested microorganism) at a density of 5×10^5 - 1×10^6 c.f.u./mL. A control suspension (bacterial in PBS at the same density) was included in each experiment. At time zero and after 5, 10 and 15 mins, 0.5 mL of each suspension was removed, subjected to serial tenfold dilutions in PBS and inoculated onto either MHA or SDA plates. The number of viable microorganisms at each time was evaluated by counting colonies after incubation for 24 h at 35°C, and was expressed as the percentage of the control.

Hydroformylation of *Thymus capitatus* essential oil: [22-24]. All chemicals were purchased from commercial sources and used as received, unless otherwise indicated. Dicarbonylrhodiumacetylacetonate [$\text{Rh}(\text{CO})_2(\text{acac})$] 98% (acacH = acetylacetonate) and triphenylphosphine (PPh_3) were purchased from Sigma-

Aldrich. Hydroformylation experiments were carried out in homemade autoclaves with magnetic stirring. To prevent direct contact with stainless steel, the reaction solution was kept in a glass vessel and the autoclave cap was glass-covered. The reaction products were identified by GC/MS.

In a typical experiment, Rh(CO)₂(acac) (0.02 mmol), PPh₃ (0.04 mmol), and essential oil (3.7 g) were transferred under nitrogen into the autoclave, which was pressurized to 50 bar total pressure (CO/H₂ = 1/1), heated (80°C) and stirred with a magnetic stirrer. After carrying out the reaction for 24 h and cooling at room temperature, the excess CO and H₂ were slowly vented. The essential oil was redistilled in steam to avoid

contamination from the catalyst and analyzed using a Hewlett Packard gas-chromatograph, Mod. G1800 B GCD System, equipped with an Alltech ATTM-5 column (60 m x 0.25 mm x 0.25 μm) and a MS EI detector operating at 70 eV. The components were identified as previously reported and by accurate interpretation of MS.

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Composition and Chemical Variability of the Leaf Oil from Corsican *Juniperus thurifera*. Integrated Analysis by GC(RI), GC-MS and ^{13}C NMR

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The composition of 16 samples of leaf oil from Corsican *Juniperus thurifera* was investigated by integrated techniques, GC, GC-MS and ^{13}C NMR. K-means partitioning and PCA analysis of the data allowed the definition of a main group (14 samples) dominated by limonene (mean = 52.2%, SD = 6.4) and α -pinene (mean = 7.2%, SD = 3.8). Limonene and β -elemol (up to 19.7%) were identified as the major components of two atypic samples.

Keywords: *Juniperus thurifera*, limonene, β -elemol, PCA, ^{13}C NMR, Corsica.

The genus *Juniperus*, consisting of 67 species and 34 varieties, is divided into three sections: *Caryocedrus* (one species: *J. drupacea* Labill.), *Juniperus* (= *Oxycedrus* Spach with 10 species) and *Sabina* (Miller) Spach (56 species). *J. thurifera* L., a member of the *Sabina* section, is constituted of two varieties: var. *thurifera* (from southern Europe, Spain and France) and var. *africana* (from North Africa, Morocco). RAPDs showed that the Moroccan plants (var. *africana*) were barely distinct from var. *thurifera* [1]. According to Adams [1], *J. thurifera* var. *thurifera* has several synonyms, of which *J. hispanica* Mill., *J. sabinoides* Endl., *J. bonatiana* Vis., *J. cinerea* Carrière, *J. thurifera* L. var. *gallica* Coincy, *J. gallica* (Coincy) Rouy, *Sabina foetidissima* (Willd.) Antoine, *S. thurifera* (L.) Antoine, and *S. pseudothurifera* Antoine are retained.

J. thurifera var. *thurifera* is a dioecious species. It is a pyramidal tree, up to 20 m high, which grows in the mountains of central Spain, the Spanish and French Pyrenees and French Alps [1]. In Corsica, it is found on both faces of Monte Cinto, the highest mountain of the island, in Niolu and Asco valley [2,3].

The essential oil of *J. thurifera* is obtained by hydrodistillation of leaves, berries and wood. A few studies have reported on the isolation and characterization of sesquiterpenes from the wood oil [4,5]. However, the chemical composition of the essential oils from leaves, berries and wood has been

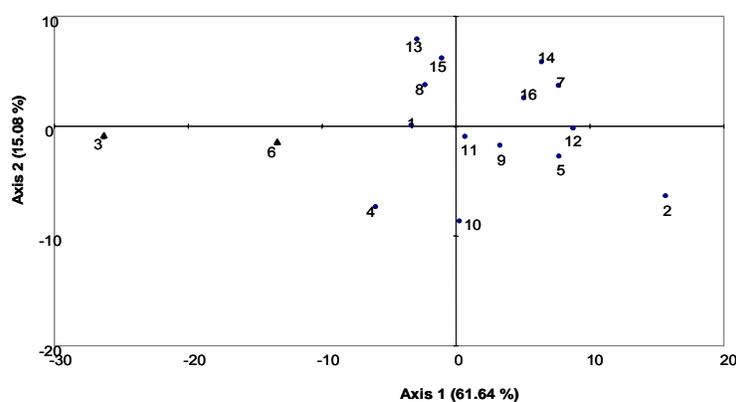
studied less frequently. Only one paper reported the composition of a sample of wood oil, dominated by sesquiterpenes: cedrol (41%), thujopsene (20%), widdrol (16%) and α -cedrene (12%) [6]. Two berry oil samples were characterized by a high content of limonene (88.5% and 84.3%) [7,8]. Concerning the leaf oil, Adams [9, 10] studied the composition of various samples from Morocco, Spain and France. Limonene dominated the composition of the samples from Spain (28.8-61.8%), the Pyrenees (53.0 and 75.1%) and France (French Alps, 52.1%; Corsica, 60.6%). Some samples contained appreciable amounts of linalool (up to 13.4%) and linalyl acetate (up to 13.7%). In contrast, Moroccan oils contained sabinene as the major component in three samples (37.4-45.8%). The composition of the fourth sample was dominated by α -pinene (17.1%), elemol (14.7%) and sabinene (12.2%). It could be pointed out that δ -2-carene was totally absent in the samples of the variety *Africana*, while it accounted for 0.9-4.4% in the samples of the variety *thurifera*.

The aim of this study was to characterize *J. thurifera* var. *thurifera* growing wild in a restricted area in Corsica through the composition of its leaf oil. Leaves were collected from 16 individual trees growing in two locations covering the geographic range of *J. thurifera* in Corsica. The yield of the essential oil isolated by water distillation and calculated from fresh material, varied substantially from sample to sample (0.16-0.89%).

Table 1: Volatile components of the leaf oil from Corsican *Juniperus thurifera* L: average (A) and standard deviation (SD) for samples of the main group, atypical composition of samples 3 and 6.

Compound*	RI		Main group			Sample		Identification mode
	BP-1	BP-20	A % (S.D)	Min %	Max %	No. 3 %	No. 6 %	
1	930	1020	7.2 (3.8)	1.9	15.6	2.1	2.4	RI, MS ¹³ C NMR
2	963	1108	0.3 (0.1)	0.0	0.5	0.1	3.3	RI, MS ¹³ C NMR
3	969	1105	0.5 (0.3)	0.1	1.0	0.2	0.2	RI, MS ¹³ C NMR
4	979	1155	3.0 (0.3)	2.5	3.4	1.0	2.2	RI, MS ¹³ C NMR
5	994	1125	2.0 (0.9)	0.6	4.3	1.0	1.8	RI, MS ¹³ C NMR
6	1004	1142	4.9 (2.9)	0.2	9.6	3.4	2.2	RI, MS ¹³ C NMR
7	1010	1263	0.1 (0.0)	0.1	0.2	0.1	0.1	RI, MS
8	1022	1201	52.2 (6.4)	43.7	67.0	29.7	40.6	RI, MS ¹³ C NMR
9	1077	1275	2.3 (1.2)	0.9	4.9	0.3	0.9	RI, MS ¹³ C NMR
10	1081	1544	5.2 (3.7)	0.8	12.8	2.0	2.4	RI, MS ¹³ C NMR
11	1120	1456	0.2 (0.2)	0.0	0.7	0.1	0.1	RI, MS ¹³ C NMR
12	1148	1698	0.2 (0.2)	0.0	0.6	-	0.4	RI, MS
13	1158	1803	0.4 (0.2)	0.1	0.9	0.3	0.1	RI, MS
14	1161	1598	0.3 (0.1)	0.2	0.5	0.1	1.5	RI, MS ¹³ C NMR
15	1170	1694	0.7 (0.4)	0.1	1.3	0.8	0.9	RI, MS ¹³ C NMR
16	1180	1789	0.2 (0.0)	0.1	0.2	0.2	-	RI, MS
17	1196	1830	0.3 (0.1)	0.1	0.4	0.3	0.5	RI, MS ¹³ C NMR
18	1208	1761	0.2 (0.2)	0.0	0.9	0.3	-	RI, MS ¹³ C NMR
19	1215	1647	0.2 (0.1)	0.0	0.4	0.4	-	RI, MS ¹³ C NMR
20	1225	1729	2.6 (2.1)	0.4	8.5	1.1	1.7	RI, MS ¹³ C NMR
21	1231	1844	0.4 (0.2)	0.1	0.6	0.5	0.4	RI, MS ¹³ C NMR
22	1240	1553	1.3 (0.9)	0.3	2.9	1.0	1.2	RI, MS ¹³ C NMR
23	1269	1575	0.2 (0.2)	0.1	0.7	0.2	0.2	RI, MS ¹³ C NMR
24	1333	1681	0.9 (0.4)	0.3	1.6	1.1	1.0	RI, MS ¹³ C NMR
25	1341	1725	0.1 (0.1)	0.0	0.3	0.2	0.2	RI, MS ¹³ C NMR
26	1359	1748	0.2 (0.1)	0.0	0.5	0.4	0.3	RI, MS ¹³ C NMR
27	1417	1580	0.1 (0.1)	0.0	0.4	0.2	0.1	RI, MS
28	1491	1727	0.1 (0.1)	0.0	0.3	0.2	0.2	RI, MS
29	1505	1752	0.2 (0.2)	0.0	0.6	0.3	0.2	RI, MS
30	1513	1740	0.5 (0.3)	0.0	0.9	0.5	1.0	RI, MS ¹³ C NMR
31	1535	2079	2.5 (2.6)	0.5	10.2	19.7	13.6	RI, MS ¹³ C NMR
32	1597	2021	0.6 (0.8)	0.0	2.8	6.9	0.7	RI, MS ¹³ C NMR
33	1620	2163	0.3 (0.1)	0.2	0.9	1.8	0.9	RI, MS ¹³ C NMR
34	1638	2225	0.6 (0.3)	0.0	2.5	3.8	3.0	RI, MS ¹³ C NMR
35	1641	2216	0.8 (0.4)	0.2	2.8	2.2	0.3	RI, MS ¹³ C NMR

*Order of elution and percentages are given from apolar column. ¹³C NMR (italics) indicate that the corresponding component has been identified by NMR in a chromatography fraction.

**Figure 1:** PCA of 16 leaf oil samples of Corsican *J. thurifera*.

Thirty-five components (26 monoterpenes and 9 sesquiterpenes), accounting for 84.2-99.4% of the whole composition of the samples, were identified (Table 1) using a combination of analytical techniques: GC, GC-MS and ¹³C NMR spectroscopy. The composition of the samples varied substantially. Limonene (29.7-67.0%), β -elemol (0.5-19.7%), α -pinene (1.9-15.6%), linalol (0.8-12.8%), δ -3-carene (0.2-9.6%), and piperitone (0.4-8.5%) were the major constituents. Other monoterpenes, terpinolene (0.3-4.9%), δ -2-carene

(0.6-4.3%), myrcene (1.0-3.4%), linalyl acetate (0.3-2.9%), α -terpinyl acetate (0.3-1.6%), terpineol-4 (0.1-1.5%) and α -terpineol (0.1-1.3%), as well as two sesquiterpenes, α -eudesmol (0.2-2.8%) and γ -eudesmol (0.2-1.8%) were present in moderate amounts. The contents of cedrol and β -eudesmol reached 6.9% and 3.8%, respectively, in one sample.

The 16 compositions were submitted to statistical analysis. Principal Components Analysis (PCA)

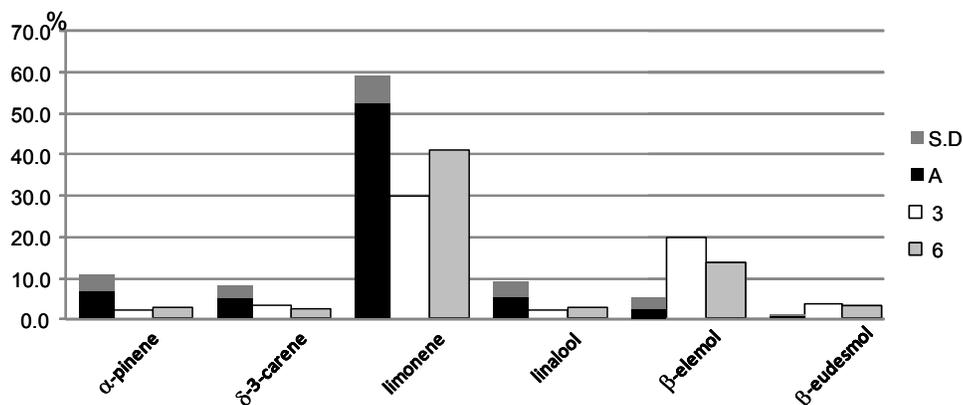


Figure 2: Major components of the main group (A = average, SD = standard deviation) and of the two atypical samples 3 and 6.

(Figure 1) and k-means partitioning were performed on all the terpene data, with individual compounds expressed as a percentage. Although the compositions of the individual samples varied substantially, it was not possible to distinguish groups within the essential oil samples. Therefore, we constituted one major group (14 samples) and differentiated two atypical compositions (Figure 2). For that reason, in Table 1 we reported the mean composition of the 14 samples of the main group, as well as the highest and the lowest values for each component, and the compositions of the two atypical samples n° 3 and n° 6.

All the leaf oil samples of *J. thurifera* var. *thurifera* from Corsica of the main group exhibited limonene as the major component, with a mean value of 52.2% (SD = 6.4) (Table 1, Figure 2). The similarity to oil samples from Spain, the Pyrenees and French Alps is particularly observed for some compositions which afforded α -pinene as the second major component. The sample from Corsica reported by Adams [10] belongs to that group. However, several samples contained fair amounts of δ -3-carene (up to 9.6%) and β -elemol (0.5-5.7% and 10.2% in sample 4), both of these components being scarce in the *J. thurifera* var. *thurifera* leaf oils reported in the literature (0.0-0.8% and 0.3-3.2%). The samples of that group differed drastically from the sabinene-rich oils from Morocco [9,10].

The content of limonene in the two atypical samples was less (40.6 and 29.7%). Conversely, in those samples, β -elemol accounted for 13.6 and 19.7% and the content of cedrol reached 6.9%. It could be pointed out that similar contents of β -elemol and cedrol (14.7 and 4.4%, respectively), were reported in one sample of *J. thurifera* var. *africana* from Morocco, which also contained α -pinene (17.1%) and sabinene (12.2%) as major components [10].

Experimental

Plant material: Leaves from 16 mature trees of *J. thurifera* were collected from 2 locations in Corsica: Asco (1-6) and Niolu (7-16) during March to April 2007.

Essential oil isolation and fractionation: Leaves of individual trees (55-393 g) were hydrodistilled in a Clevenger-type apparatus for 4 h. In order to carry out a detailed analysis, one oil sample (1.3 g) was fractionated by flash chromatography (column: 18 mm id; silica gel, 200-500 μ m, 26 g) and 4 fractions (F1-F4) were eluted with a mixture of solvents of increasing polarity (*n*-pentane:diethyl oxide, 100:0 to 50:50): *n*-pentane, F1, (200 mL, 456 mg); *n*-pentane: Et₂O, 95:5, F2 (200 mL, 52 mg), *n*-pentane:Et₂O, 90:10, F3 (200 mL, 20 mg), *n*-pentane:Et₂O, 50:50, F4 (200 mL, 188 mg). The 4 fractions were analysed by GC(RI) and ¹³C NMR.

Analytical GC: GC analyses were carried out using a Perkin-Elmer Autosystem GC apparatus equipped with 2 flame ionisation detectors, and fused-silica capillary columns (50 m x 0.22 mm i.d., film thickness 0.25 μ m), BP-1 (polydimethylsiloxane) and BP-20 (polyethyleneglycol). Oven temperature was programmed from 60°C to 220°C at 2°C/min and then held isothermal (20 min); detector temperature, 250°C; injector temperature, 250°C (injection mode, split, 1/60); carrier gas, helium (0.8 mL/min). Injected volume: 0.5 μ L of a solution of 50 μ L of the mixture (oil or chromatographic fraction) diluted in 350 μ L of CCl₄.

GC-MS analysis: GC-MS analyses were carried out using a Perkin-Elmer TurboMass detector (quadrupole), directly coupled to a Perkin-Elmer Autosystem XL, equipped with a fused-silica capillary column (60 m x 0.22 mm i.d., film thickness 0.25 μ m), Rtx-1 (polydimethylsiloxane). Carrier gas, helium at 1 mL/min; split, 1/80; injection volume, 0.2 μ L; injector temperature, 250°C; oven temperature programmed

from 60°C to 230°C at 2°C/min and then held isothermal (45 min). Ion source temperature, 150°C; energy ionisation, 70 eV; EI MS were acquired over the mass range 35-350 Da.

¹³C NMR analysis: ¹³C NMR spectra of 5 selected samples and the chromatographic fractions were recorded on a Bruker 400 Avance Fourier Transform spectrometer operating at 100.13 MHz for ¹³C, equipped with a 5 mm probe, in deuteriochloroform, with all shifts referred to internal tetramethylsilane (TMS). ¹³C NMR spectra were recorded with the following parameters: pulse width = 4 μs (flip angle 45°); acquisition time = 2.7 s for 128K data table with a spectral width of 25,000 Hz (250 ppm); CPD mode decoupling; digital resolution = 0.183 Hz/pt. The number of accumulated scans was 3,000 for each sample (around 50 mg of the oil or chromatographic fraction in 0.5 mL CDCl₃).

Identification of components: All 16 samples were submitted to GC(RI) analysis. Among them, 2 samples (N° 11, 15) selected on the basis of their chromatographic profile, were analysed by GC-MS. Five samples (N° 1, 3, 4, 7, 8) were analyzed by ¹³C NMR spectroscopy. Identification of the components

was based: (i) on comparison of their GC retention indices (RI) on polar and apolar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation with those of authentic compounds, and literature data [11]; (ii) on computer matching against laboratory-made (CPN Lab, University of Corsica) and commercial mass spectral libraries [12-15] and (iii) by ¹³C NMR spectroscopy, following the methodology developed and computerised in our laboratories, using home-made software and spectral data library [16-18]. Most of the components were identified by comparison of their spectral data (MS and ¹³C NMR) with those of reference compounds compiled in our laboratory-made spectral data library. In order to confirm the identification of minor components suggested by MS analysis, all the chromatographic fractions obtained from one oil sample (Experimental, second paragraph) were analysed by GC(RI) and ¹³C NMR spectroscopy.

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Combined Analysis by GC (RI), GC-MS and ^{13}C NMR of the Supercritical Fluid Extract of *Abies alba* Twigs

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Two samples (leaves and twigs) of *Abies alba* Miller from Corsica were extracted using supercritical CO₂ and their chemical compositions were compared with those of the essential oils obtained from the same batch of plant material. In total 45 components were identified using combined analysis by GC (RI), GC-MS and ^{13}C NMR. It was observed that the contents of monoterpenes (mainly represented by limonene, α -pinene and camphene) were significantly lower in the supercritical fluid extract (SFE) than in the essential oil (EO). Conversely, the proportions of sesquiterpenes were much higher in CO₂ extracts than in essential oils (around 30% vs 4%). *Cis*-abienol, a diterpene alcohol, was identified only in SFE, and the proportions of this constituent (7.5% and 17.3%) were determined using quantitative ^{13}C NMR since it was underestimated using the standard conditions of GC.

Keywords: *Abies alba* Miller, essential oil, supercritical fluid extract, ^{13}C NMR spectroscopy, gas chromatography, mass spectrometry, *cis*-abienol.

The silver fir (*Abies alba* Miller, family Pinaceae) is widely distributed in many European mountain forests (for example in France, Germany and Poland). In Corsica, the south-western limit of its area of distribution, it constitutes pure fir forests as well as mixed forests with other conifers, particularly *Pinus nigra* ssp. *laricio* [1]. Twigs of *A. alba* produce by either vapour or water distillation an essential oil (EO) whose chemical composition is well documented. Most of the studies concerned plants from the centre and south of Europe (Poland [2], Germany [3], Austria [4], France [5], Montenegro [6], Serbia [7] and Greece [8]). All the investigated samples were characterised by the predominance of limonene (34-55%, Germany [3], Austria [4], France [5], Greece [8]), or β -pinene (20-33%, Montenegro [6], Serbia [7]). Other important components were camphene (15-17%) [4,6,7], α -pinene (11-17%) [5-7] and bornyl acetate (9-14%) [6,7].

Recently, we analysed 53 oil samples from twigs of *A. alba* collected in all the Corsican forests where the tree is growing wild [9]. The results were submitted to chemometric analysis (K-mean's clustering and

Principal Component Analysis) and two groups were distinguished within the oil samples depending on the content of limonene [9].

Extraction by means of carbon dioxide, in the supercritical state, is a good technique for the production of flavors and fragrances from vegetable matter. The extensive use of compressed carbon dioxide to extract either volatiles or aroma substances destined for human nutrition and for the pharmaceutical industry is due to its chemical and physical properties. It is safe, non-toxic, non-combustible, inexpensive and its critical temperature and pressure are not high (31.06 °C; 7.38 MPa) [10]. The aim of the present work was to determine the yield and the chemical composition of the supercritical fluid extract (SFE) of twigs of *A. alba* and to compare them with those of the essential oil obtained by hydrodistillation of the same batch material.

Two samples, each belonging to a cluster previously defined [9], were submitted to supercritical CO₂ extraction and hydrodistillation and their chemical compositions were compared (Table 1). The four oil

Table 1: Compounds identified in the supercritical CO₂ extracts and the essential oils of leaves and twigs of *Abies alba*.

Constituents	I _r _a	I _r _p	Sample 1		Sample 2		Identification
			SFE	EO	SFE	EO	
1 Santene	884	984	tr	3.6	0.1	3.0	RI, MS, ¹³ C NMR
2 Tricyclene	925	1020	0.3	2.1	0.5	2.1	RI, MS, ¹³ C NMR
3 α-Pinene	935	1032	3.4	18.0	2.6	11.4	RI, MS, ¹³ C NMR
4 Camphene	948	1079	1.9	13.7	3.9	15.7	RI, MS, ¹³ C NMR
5 β-Pinene	975	1120	0.4	1.3	2.7	8.1	RI, MS, ¹³ C NMR
6 Myrcène	984	1170	0.4	1.2	0.3	0.9	RI, MS, ¹³ C NMR
7 α-Phellandrene	998	1175	-	-	-	0.2	RI, MS
8 <i>p</i> -Cymene	1013	1270	-	0.1	-	0.1	RI, MS
9 Limonene*	1025	1209	17.7	43.5	5.6	15.6	RI, MS, ¹³ C NMR
10 β-Phellandrene*	1025	1219	0.1	0.2	7.0	14.4	RI, MS, ¹³ C NMR
11 γ-Terpinene	1049	1254	-	0.1	-	0.1	RI, MS
12 Terpinolene	1081	1291	0.1	0.4	0.1	0.5	RI, MS, ¹³ C NMR
13 Linalool	1083	1555	-	-	-	0.4	RI, MS
14 Citronellal	1134	1489	-	0.2	-	0.2	RI, MS
15 Borneol	1153	1713	-	0.1	0.9	1.0	RI, MS, ¹³ C NMR
16 α-Terpineol	1173	1709	-	0.7	-	1.2	RI, MS, ¹³ C NMR
17 Decanal	1188	1498	-	-	0.1	0.1	RI, MS
18 Citronellol	1212	-	-	0.3	0.1	0.2	RI, MS
19 Geraniol	1237	1858	-	0.1	0.3	0.2	RI, MS
20 Linalyl acetate	1243	1564	-	-	0.4	0.1	RI, MS
21 Bornyl acetate	1274	1589	1	1.4	8.1	9.9	RI, MS, ¹³ C NMR
22 Citronellyl acetate	1337	1670	0.7	1.2	1.1	1.1	RI, MS, ¹³ C NMR
23 α-Longipinene	1359	1475	2.3	0.5	2.3	-	RI, MS, ¹³ C NMR
24 Geranyl acetate	1364	1766	-	0.2	1.7	2.0	RI, MS, ¹³ C NMR
25 Dodecanal	1392	1718	-	0.2	1.2	0.4	RI, MS, ¹³ C NMR
26 Longifolene	1413	1575	1.3	0.4	1.7	-	RI, MS, ¹³ C NMR
27 (<i>E</i>)-β-Caryophyllene	1425	1604	5.6	1.5	6.3	0.8	RI, MS, ¹³ C NMR
28 Himachala-2,4-diene	1430	1611	2.6	-	2.4	-	RI, MS, ¹³ C NMR
29 α-Himachalene	1455	1650	0.7	-	0.9	-	RI, MS, ¹³ C NMR
30 α-Humulene	1458	1678	2.2	0.7	2.1	0.4	RI, MS, ¹³ C NMR
31 γ-Curcumene	1472	1685	0.4	-	1.0	-	RI, MS, ¹³ C NMR
32 γ-Himachalene	1477	1706	0.8	-	0.2	-	RI, MS, ¹³ C NMR
33 Germacrene D	1479	-	-	-	-	0.2	RI, MS
34 γ-Humulene	1485	1732	2.0	-	1.6	0.1	RI, MS, ¹³ C NMR
35 β-Selinene	1489	1726	3.8	-	5.2	-	RI, MS, ¹³ C NMR
37 α-Selinene	1498	1732	0.7	-	1.1	-	RI, MS, ¹³ C NMR
38 β-Himachalene	1503	1732	1.3	-	0.9	0.1	RI, MS, ¹³ C NMR
36 γ-Cadinene	1506	1766	1.4	-	0.3	-	RI, MS, ¹³ C NMR
39 β-Sesquiphellandrene	1511	-	-	0.8	-	0.1	RI, MS, ¹³ C NMR
40 δ-Cadinene	1517	1766	1.8	-	0.7	0.1	RI, MS, ¹³ C NMR
41 <i>ar</i> -Himachalene	1530	1729	0.4	-	0.7	-	RI, MS, ¹³ C NMR
42 Longiborneol	1594	2162	-	0.2	2.6	0.7	RI, MS, ¹³ C NMR
43 τ-Cadinol	1639	-	-	-	-	0.3	RI, MS
44 Manoyl oxide	1999	-	-	tr	0.2	-	RI, MS
45 <i>cis</i> -Abienol	2112	-	17.3#	-	7.5#	-	RI, MS, ¹³ C NMR
Total			70.4	92.7	74.2	91.9	
Monoterpenes			25.8	88.4	35.2	88.4	
Sesquiterpenes			27.3	4.1	30.0	3.0	
Diterpenes			17.3	-	7.7	-	
Acyclic non terpenic compounds			-	0.2	1.3	0.5	

^a. Order of elution from apolar column (BP-1). The percentages are those obtained from the apolar column corrected after quantification of *cis*-abienol using ¹³C NMR (correcting factors 0.875 for sample 1 and 0.947 for sample 2). *: percentages measured on polar column. #: percentage measured by ¹³C NMR.

samples were analyzed by GC, in combination with retention indices, GC-MS and ^{13}C NMR spectroscopy. As previously reported [9], qualitative analysis of both EO was easily achieved by combination of GC (RI), GC-MS and ^{13}C NMR and quantitative analysis was performed by GC (FID). Conversely, in the SFE, GC-MS and ^{13}C NMR revealed the occurrence of *cis*-abienol, a diterpene alcohol, bearing the labdane skeleton (Figure 1). The quantitative determination of that alcohol was more accurately achieved using ^{13}C NMR spectroscopy.

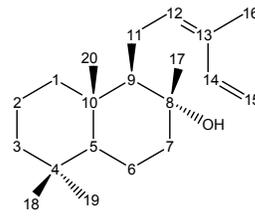
Quantitative determination of *cis*-abienol by ^{13}C NMR spectroscopy. Concerning *cis*-abienol, we observed a bad correlation between the mean intensity of the carbon signals of this compound in both ^{13}C NMR spectra and the percentages measured by GC, the content of the compound being substantially underestimated. In order to overcome this problem, we carried out a quantitative determination of *cis*-abienol using ^{13}C NMR spectroscopy. Quantification of *cis*-abienol in the SFE was achieved using standard quantitative conditions with respect to a fixed amount of diglyme chosen as internal standard. The pulse sequence in the inverse gated decoupling technique used a pulse angle of 90° and a total recycling time (T_R) of 20s, corresponding to $5 \times T_1$ of the longest T_1 of the protonated carbons of *cis*-abienol and of diglyme. Using this experimental procedure, the content of *cis*-abienol in SFE was estimated at 17.3% and 7.5%, respectively, instead of 12.5% and 5.3% evaluated by GC.

Composition of the SFE of *Abies alba* and comparison with EO. Individual components of the SFE and EO of *A. alba*, their retention indices on two columns of different polarities, their relative percentages and the mode of identification are reported in Table 1.

Taking into account the quantitative determination of *cis*-abienol by ^{13}C NMR, the percentages of the other constituents of the SFE, measured by GC (FID), were modified applying a correcting factor of 0.875 and 0.947 for SFE samples 1 and 2, respectively. The two essential oils exhibited quite different compositions; each sample belonged to one of the two groups previously defined [9].

Indeed, in the first oil sample, the main constituent was limonene (43.5%) accompanied by α -pinene (18.0%) and camphene (13.7%), while the second oil sample contained various monoterpene hydrocarbons in appreciable amounts [camphene (15.7%), limonene (15.6%), β -phellandrene (14.4%), α -pinene (11.4%) and bornyl acetate (9.9%)].

Figure 1: Structure and spectroscopic data (chemical shifts $\delta^{13}\text{C}$ and longitudinal relaxation times T_1) of *cis*-abienol.



C	$\delta^{13}\text{C}$	T_1
1	40.08	1.3
2	18.58	1.3
3	41.81	1.2
4	33.27	1.2
5	56.06	2.6
6	20.25	1.2
7	43.95	1.3
8	74.24	4.8
9	62.16	1.7
10	38.93	3.7
11	23.09	1.7
12	133.83	3.5
13	130.83	4.9
14	133.57	2.6
15	113.74	2.7
16	19.89	3.1
17	24.44	2.3
18	33.46	1.7
19	21.55	1.9
20	15.42	3.4

Comparison of the compositions of the SFE and EO led to the observation of noticeable differences:

i) in both SFE samples, the proportions of monoterpene hydrocarbons, such as limonene (17.5 and 5.6%), α -pinene (3.4 and 2.6%) and camphene (1.9% and 3.9%), are significantly less important than in the EO. Moreover, in sample 2, β -phellandrene accounted for 7.0% of the SFE vs 14.4% in the EO.

ii) The proportions of the sesquiterpene hydrocarbons, such as α -longipinene, longifolene, (E)- β -caryophyllene, and α -humulene varied from 1.3% to 6.3% in the two SFE, while they accounted for less than 1.5% in the EO. Moreover, 11 sesquiterpene hydrocarbons, belonging mainly to the selinane, cadinane and himachalane families, were found only in the SFE, with proportions ranging from 0.2% to 5.2%. Therefore, the identified sesquiterpenes accounted for 27.3% and 30.0% in the SFE of sample 1 and sample 2 vs 4.1% and 3.0% in the corresponding EO.

iii) Finally, *cis*-abienol (Figure 1) was found only in the two SFE, in which it accounted for 17.3% and 7.5%.

Experimental

Plant material and extractions: The twigs of *Abies alba* Mill. were collected from 2 trees in the forest of Aitone (Corsica) in July 2006. Hydrodistillation and supercritical CO_2 extraction were carried out from the same batches of air-dried material using a laboratory apparatus described in previous papers [9,11,12]. Hydrodistillation yielded 0.65% and 0.74% for samples 1 and 2, respectively, and supercritical CO_2 extraction yielded 0.30% for both samples.

Spectroscopic analysis: GC (FID), GC-MS and ^{13}C NMR analyses were performed as previously reported [9]. Quantitative determination of *cis*-abienol was carried out by ^{13}C NMR using the standard inverse gated decoupling sequence with the following

parameters: pulse width = 8 μ s (flip angle 90°); acquisition time = 2.7 s for 128K data table with a spectral width of 25000 Hz (250 ppm); total recycling time $T_R=20$ s.

The longitudinal relaxation delays of the ^{13}C nuclei (T_1 values) were determined by the inversion-recovery method, using the standard sequence: $180^\circ\text{-}\tau\text{-}90^\circ\text{-}D_1$, with a relaxation delay D_1 of 20s. Each delay of inversion (τ) was thus taken into account for the computation of the corresponding T_1 using the function $I_p=I_0+p.e^{-\tau/T_1}$

Identification of components: Identification of the individual components was based : (i) by comparison of their GC retention indices (RI) on polar and apolar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation with those of authentic compounds or literature data [13]; (ii) on

computer matching against laboratory-made (Laboratoire CNP, University of Corsica) and commercial mass spectral libraries [14a-14c] and by comparison of spectra with literature data [13,15,16a], and (iii) by ^{13}C NMR spectroscopy, following the methodology developed and computerised in our laboratories, using home-made software and spectral data library [16b,16c].

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Eugenol: A Natural Compound with Versatile Pharmacological Actions

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Eugenol, the major constituent of clove oil, has been widely used for its anesthetic and analgesic action in dentistry. Eugenol exhibits pharmacological effects on almost all systems and our aim is to review the research work that has identified these pharmacological actions. Eugenol possesses significant antioxidant, anti-inflammatory and cardiovascular properties, in addition to analgesic and local anesthetic activity. The metabolism and pharmacokinetics of the compound in humans have been studied. Eugenol has also been used as a penetration enhancer. The compound is a very promising candidate for versatile applications, and the design of new drugs based on the pharmacological effects of eugenol could be beneficial.

Keywords: eugenol, clove oil, pharmacological actions.

Clove (*Syzygium aromaticum*, Myrtaceae) oil contains eugenol, an oily liquid that has proven analgesic and antiseptic properties [1]. Eugenol (C₁₀H₁₂O₂; 2-methoxy-4-(2-propenyl) phenol), is an allyl chain-substituted guaiacol. It is a clear, pale yellow, oily liquid which is slightly soluble in water and soluble in organic solvents. In dental plasters, fillings, and cements either clove oil or eugenol have been used for their topical analgesic properties. In addition to these indications it is used as a local antiseptic and anesthetic. Both clove and eugenol are also used in the fragrance and flavoring industries [2]. In the last few decades, a large number of pharmacological studies on the effects of eugenol on the immune, reproductive, cardiovascular, gastric, central nervous, and urinary systems have been reported, as well as the effects on blood biochemistry [3]. Clove essential oil possesses anti-inflammatory, cytotoxic, insect repellent and anesthetic properties in addition to its established antimicrobial, antioxidant, antifungal and antiviral activities [2].

Analgesic activity: Kurian and coworkers studied the antinociceptive ability of eugenol (100 mg/kg) in several mouse models of pain and found that the effect was more pronounced in the inflammatory phase than the neurogenic phase [4]. Eugenol can alleviate

neuropathic pain [5]. Guenette *et al.*, in their study in male Sprague-Dawley rats, showed that eugenol, at a dose of 40 mg/kg, is capable of prolonging reaction time to thermal stimuli [6]. All these results suggested the possible use of eugenol as an analgesic agent.

Antioxidant effect: The potential use of eugenol as a therapeutic antioxidant has been demonstrated by the studies of Nagababu and coworkers [7]. In their study, the formation of thiobarbituric acid reactive substances (TBARS) was decreased by eugenol at concentrations of 4-15 μ M. Tests with compounds that are reactive with the hydroxyl radical in the presence of eugenol showed that it competes with these reactive compounds for this radical and thus could protect skin damage by UV-light [8]. The antioxidant effect of eugenol-grafted chitosan hydrogel has also been reported [9]. Eugenol can cause significant suppression of lipid peroxidation and low density lipoprotein oxidation. Eugenol-like compounds significantly reduce copper-dependent oxidation of low density lipoprotein, and lipid peroxidation and autooxidation of Fe²⁺ [10]. Electron spin resonance spectroscopic studies by Fujisawa and coworkers showed that eugenol (18.50 μ M per 100 μ M sol) significantly scavenged reactive oxygen species [11]. Ogata *et al.* found that the antioxidant action of eugenol (62.5 – 250 μ M) is mediated through the

inhibition of lipid peroxidation at the level of initiation [12]. Vidhya and coworkers investigated the short and long term antioxidant activities of eugenol (1000 mg/kg) on rat intestine and observed that the compound helps in the removal of toxic materials from the intestine as it caused the induction of glutathione-S-transferases [13]. Antioxidants and free radical scavengers like eugenol have also been monitored as agents for preventing the progress of Parkinson disease. A study demonstrated that eugenol (0.1 - 1.0 μ M/kg) inhibits lipid peroxidation and thereby suppresses 6-hydroxydopamine-induced dopamine depression thereby suggesting its potential use in Parkinson disease [14]. The antioxidant activity of eugenol is comparable with that of butylated hydroxyanisole and butylated hydroxytoluene [15]. Although many *in vitro* studies have been carried out on the antioxidant activity of eugenol, there have been insufficient *in vivo* studies.

Protective effect: Eugenol modulates both the NMDA receptor and superoxide radical and thus seems to provide protection against ischemic injury. Eugenol can attenuate NMDA induced acute neurotoxicity and decrease xanthine/xanthine oxidase produced oxidative neuronal injury [16]. Eugenol (10.7 mg/kg/day) has been found to show an *in vivo* antioxidant property and a better inducing effect on phase II enzymes which counteract the metabolizing enzyme reduction caused by carbon tetrachloride [17]. *In vitro* studies on liver microsomal monooxygenase activities and carbon tetrachloride (CCl₄)-induced lipid peroxidation have shown that eugenol inhibits both [18]. The *in vivo* studies by the same researchers also showed a protective effect of eugenol at doses of 5 and 25 mg/kg against CCl₄ induced hepatotoxicity. The methanolic extract of leaves of *Ocimum suave* (Lamiaceae), which contains eugenol as a major constituent [19], has shown gastric cytoprotective and anti-ulcer effects in experimental Wistar rats [20]. Eugenol pre-treatment (10-100 mg/kg) reduces both the number of gastric ulcers and the gravity of lesions induced by ulcerogenic agents like platelet activating factor and ethanol [21]. The mechanism of the gastroprotective action of eugenol has been established by the studies of Morsy and Fouad [22]. Gastric mucosal lesions, gastric acid outputs and pepsin activity in male Sprague-Dawley rats were markedly reduced by a single oral dose of eugenol (100 mg/kg), used as a pretreatment one hour before indomethacin injection. This pretreatment resulted in a marked increase in mucin concentration, suppression of the rise in gastric mucosal malondialdehyde and total nitrite, and attenuation of the decrease in reduced glutathione. It was further confirmed that the protective action of eugenol is through opening of ATP-sensitive potassium channels and not through the transient receptor potential vanilloid

1. The DNA-protective activity of eugenol has been observed by Yogalakshmi and coworkers in thioacetamide-induced liver injury in rats at a dose of 10.7 mg/kg/day [23]. Eugenol imparts a dose dependent protection against nicotine-induced superoxide mediated oxidative damage at a concentration range of 1-20 μ g/mL [24].

Anesthetic action: A randomized, single-blind study was carried out in 73 subjects to evaluate the topical anesthetic action of clove oil in comparison with benzocaine [1]. The pain scores between the clove gel and the benzocaine groups were not significantly different, but were significantly lower than those of the placebo groups. The study showed that clove gel is a cheap and easily available topical anesthetic which could be used as an alternative to benzocaine gel in dentistry. A reversible, dose dependent anesthesia has also been reported after eugenol administration (5 – 60 mg/kg, iv) in male Sprague–Dawley rats [5]. A clinical study was conducted in 100 adult patients to study the effect of eugenol as an intravenous anesthetic. However, they concluded that it is not advisable to use eugenol except in special circumstances as the incidence of development of venous thrombosis around the site of injection was high [25]. The possible use of eugenol as a local anesthetic has also been demonstrated by the studies of Park and coworkers, who suggested its suitability for non-dental anesthesia as well [26].

Antibacterial, antifungal and antiviral activities: The activity has been studied of *Eugenia aromaticum* on several microorganisms and parasites, including pathogenic bacteria, and herpes simplex and hepatitis C viruses [27]. Burt and coworkers studied the antibacterial activity of clove bud oil and found it to be effective [28]. Eugenol is active against *Neisseria gonorrhoeae*, with a minimum inhibitory concentration of 85-256 mg/L [29]. Moreover, eugenol shows synergistic effects with antibiotics against Gram-negative bacteria [30]. Mytle and coworkers have demonstrated the ability of clove oil to inhibit the growth *Listeria monocytogenes*. A 1% v/w concentration was sufficient for the inhibition of growth at 5°C and 15°C [31]. Eugenol was found to be active against sessile cells in *Candida albicans* biofilms [32]. The compound showed fungicidal activity *in vitro* for exponentially growing *Candida albicans*. The mechanism of this action was found to be through envelope damage [33]. In the *in vivo* study, treatment with eugenol caused a significant reduction in the colony count number in an immunosuppressed rat model of oral candidiasis in comparison with the untreated control group [34]. Chami and coworkers also evaluated eugenol in an immunosuppressed rat model of vaginal candidiasis for its efficacy in the prophylaxis

and treatment of this condition [35]. Eugenol has been demonstrated to have activity against *Penicillium* (100 mg/L), *Aspergillus* (100-140 mg/L) and *Fusarium* species (140-140 mg/L) also [36].

Eugenol can cause damage to viral envelopes of freshly formed virions and can cause inhibition of viral replication at the initial stage [37]. The anti-viral activity of eugenol, clove flower bud extract and clove essential oil against *Herpes simplex* virus has been carried out [37]. Direct inactivation of viruses and inhibition of intracellular and extracellular viruses after replication were observed with eugenol. In another study for antiviral activity against HSV-1 and HSV-2 viruses [38], 50% inhibitory concentration values were 25.6 µg/mL and 16.2 µg/mL for HSV-1 and HSV-2, respectively. Eugenol demonstrated a synergistic action with acyclovir against *in vitro* replication of herpes virus. Topical eugenol therapy was observed to suppress herpes virus induced keratitis in mouse.

Anticonvulsant activity: Zelger and coworkers investigated the anticonvulsant activity of phenyleugenol, benzyleugenol and phenylethyleugenol, which are synthetic derivatives of eugenol [39]. All three compounds showed significant anticonvulsant activity in the maximal electroshock seizure test. Phenyleugenol and benzyleugenol were found to have a high therapeutic index. All these studies indicated that eugenol could be taken as a lead molecule in the development of novel anticonvulsant drugs.

Anti-inflammatory action: Eugenol inhibits cyclooxygenase and thus inhibits prostaglandin H synthase [40]. This can be the result of its competition with arachidonic acid. Eugenol is able to resist the release of proinflammatory mediators like interleukin-1 β , tumor necrosis factor- α and prostaglandin E2 from macrophages and is thus useful for acute inflamed dental pulps and apical periodontitis as an anti-inflammatory agent [41]. Eugenol can also inhibit cyclooxygenase-2 in macrophages. Eugenol caused reduction of both paw and joint swelling in arthritis induced male Sprague-Dawley rats at a dose of 33 mg/kg [42]. An *in vivo* study by Rodrigues and coworkers on the effect of an hydroalcoholic extract of clove on pro-inflammatory cytokines production by macrophages of mice showed that clove oil (200 mg/kg) caused cytokine inhibition due to the presence of eugenol, which imparts an anti-inflammatory activity [43]. The *in vivo* anti-inflammatory activity of eugenol in lipopolysaccharide induced lung injury has been demonstrated on administration of 160 mg/kg, ip [44].

Penetration enhancement: The utility of clove oil (82.6% eugenol) as a penetration enhancer has been

demonstrated by Shen and coworkers [45]. Permeation enhancement was found to be significant both *in vivo* and *in vitro*. The *in vivo* effect observed was comparatively weaker than that observed *in vitro*. A marked increase in drug flux was observed with clove oil in *in vivo* percutaneous absorption studies in rabbit. All the results suggested the potential ability of clove oil, of which eugenol is the major active component, as a penetration enhancer in transdermal drug delivery systems [45]. *In vitro* evaluation of penetration enhancers, including eugenol, has been conducted by Mutalik and coworkers using mouse skin [46]. In their study, eugenol was also found to increase skin retention and solubility of glibenclamide and glipizide. Zhao and coworkers studied the *in vitro* penetration enhancement effect of eugenol on percutaneous absorption of a drug through porcine epidermis [47]. The study showed that eugenol was able to enhance markedly the permeability coefficient of tamoxifen with respect to the control group. The study also found that lipid extraction and increased partitioning of the tamoxifen to the stratum corneum is responsible for the observed permeability enhancement action of eugenol.

Cardiovascular actions: Eugenol (0.2 – 20 µmol) produces dose-dependent, reversible vasodilator responses that are partially dependent on the endothelium [48]. Sensch and coworkers showed that eugenol (60 – 600 µmol/L) exerted negative inotropic effects in heart muscle of Guinea-pig [49]. The effect was found to be comparable with that of nifedipine, a calcium channel blocker. Eugenol (0.006 – 6 mM) causes hypotensive effects in hypertensive rats due to vascular relaxation [50]. The blocking of voltage-sensitive and receptor-operated channels by eugenol is responsible for its smooth muscle relaxant effect and these actions are mediated through endothelial-generated nitric oxide [51]. Choudhary and coworkers observed eugenol to counteract isoproterenol-induced cardiac hypertrophy [52]. They conducted their study in male Wistar rats using a dose of 1 mg/kg twice daily. They showed that serum calcineurin activity *in vitro* was suppressed by eugenol, and isoproterenol-induced oxidative stress and apoptosis were reduced. Eugenol was also found to increase cardiac calcineurin and protein kinase C activity in ventricular tissue to normal values.

Anticancer activity: Significant induction of activity of glutathione S-transferase by eugenol was observed in liver and small intestine [53]. Miyazawa and coworkers showed that due to the presence of a C-4 hydroxy group in their structure, *trans*-isoeugenol and eugenol had significant suppressive effects on the SOS-inducing activity of chemical mutagens such as furylfuramide [54]. The antimutagenic effects of a eugenol derivative,

dehydroeugenol, have also been demonstrated by the same authors. Studies have demonstrated that eugenol provides protection from chemical induced skin cancer [55, 56].

Other pharmacological actions: An antigenotoxic effect of eugenol was studied in humans by Rompelberg and coworkers [57], but no evidence was observed for these effects. Tajuddin and coworkers investigated the effect of a 50% ethanolic extract of clove on sexual function improvement in male Swiss mice (500 mg/kg) and observed an enhanced sexual behavior of the mice [58].

Molecular mechanisms and biochemical changes:

Eugenol showed inhibition of high-voltage-activated calcium channel currents in both capsaicin-sensitive and capsaicin-insensitive dental primary afferent neurons [59]. However, the action of eugenol was unaffected by capsazepine. This suggested that the action is not mediated by transient receptor potential vanilloid 1 activation. N-type calcium currents were inhibited by eugenol in the cell line C2D7. The study showed that high-voltage-activated calcium channel current inhibition in dental primary afferent neurons by eugenol is responsible for its dental analgesic action. The mechanism of anesthetic action of eugenol by inhibition of sodium channel currents has been established by the whole-cell patch-clamp method in rat dental primary afferent neurons [60]. Capsaicin-sensitive and capsaicin-insensitive neurons are both inhibited by eugenol and its irritable action is possibly due to the inhibition of voltage-gated K^+ currents [61]. Yang and coworkers showed that eugenol produces its effects in the sensory nerve endings in the teeth through vanilloid receptor 1, at least partially [62].

The analgesic effect of eugenol is probably due to the inhibition of $Ca(V)2.3$ calcium channels. A study by Chung and coworkers showed that the mechanism of inhibition of $Ca(V)2.3$ calcium channels by eugenol is not involved through transient receptor potential vanilloid 1 and thus appeared to be distinctly different from that of capsaicin [63]. Both capsaicin receptor mediated and capsaicin receptor independent pathways are involved in the activation of Ca^{2+} channels by eugenol [64]. Eugenol has the ability to inhibit pro-inflammatory mediators like nitric oxide synthase, lipoxygenase and cyclooxygenase. Eugenol exerts its analgesia by inhibition of Na^+ currents and the inhibition is independent of the stimulus frequency [65]. Inotropic effects of eugenol have been investigated in rat left ventricular papillary muscles by Damiani and coworkers [66]. The contraction force was depressed without affecting the contractile machinery. Complete blockade of inward Ca^{2+} current was observed with 0.5

mM of eugenol. Xu and coworkers explained that the transient receptor potential vanilloid 3 is expressed by eugenol. It is a warm-sensitive Ca^{2+} -permeable cation channel in the skin, tongue and nose [67].

Eugenol has been reported to potentiate the GABA response. The compound's ability to potentiate GABAA receptors can modulate neural transmission in the brain just like, for example, benzodiazepine and barbiturate [68]. Salah and coworkers showed that eugenol causes effects such as relaxation of rat ileal strip, reduced intestinal transit in rats, and the potentiation of the diarrhea inducing effect of castor oil [69]. They suggested that all these effects are mediated through Ca^{2+} channel modulation. Eugenol accelerates inactivation of the Ca^{2+} current in isolated canine and human ventricular cardiomyocytes [70].

Metabolism and pharmacokinetics: Fischer and coworkers investigated the metabolism of eugenol in male and female healthy volunteers [71]. The study revealed that eugenol is rapidly absorbed and metabolized after oral administration. It is almost completely excreted in the urine within 24 hours. Only less than 0.1% of the administered dose was excreted unmetabolized in urine. Eugenol was found in the urine in the form of conjugates and metabolites. Among the metabolite conjugates, 55% consisted of eugenol-glucuronide and sulfate. The epoxide-diol pathway, allylic oxidation, synthesis of a thiophenol and a substituted propionic acid, and migration of the double bond were also found to be other routes of eugenol metabolism in humans. A study was made by Guénette and coworkers of the pharmacokinetic parameters of eugenol using non-compartmental analysis after gavage administration in male Sprague–Dawley rats in a dose of 40 mg/kg [6]. Plasma $T_{1/2}$ of eugenol was found to be 14.0 hours and blood $T_{1/2}$ was 18.3 hours. Glucuronide and sulfate conjugates of eugenol in urine after eugenol administration were also identified in male Sprague–Dawley rats [5].

Toxicity: The prooxidant activity of eugenol may be responsible for its toxicity [72]. An *in vitro* study by Medeiros and coworkers demonstrated that the binding of lysine to eugenol might lead to protein inactivation and consequently to its toxicity [73]. The insecticidal activity of eugenol also has been reported [2].

A summary is given in Table 1 of the various research carried out to demonstrate the different pharmacological activities of eugenol and clove oil. Further research is required to collect more information about the pharmacological effects of eugenol, and also to find new areas of therapeutic applications. A detailed knowledge of eugenol pharmacology could be utilized

Table 1: Pharmacological studies of eugenol and clove oil.

Activity studied	Experimental	Effect (Dose/Concentration)	Ref.
EUGENOL			
Analgesic	Mice	Significant antinociceptive effect (100 mg/kg)	[4]
	Male Sprague-Dawley rats	Prolonged reaction time (40 mg/kg)	[6]
Antioxidant	Microsomal mixed function oxidase mediated peroxidation	Decreased TBARS, inhibition of oxygen uptake, monooxygenase activity inhibition (4-15 μ M)	[7]
	LDL oxidation	Suppressed oxidation (1.5 μ M)	[10]
	Lipid peroxidation	Significant suppression (0.05 – 0.15 mM)	[10]
	Reactive oxygen scavenging activity	Significant activity (18.50 mU/100 μ M sol)	[11]
	Free radical reaction	Inhibited lipid peroxidation at propagation step (62.5 – 250 μ M)	[12]
	Rat intestine	Induced glutathione-S-transferases (1000 mg/kg)	[13]
	Lipid peroxidation	Inhibited (0.1 -1.0 μ M/kg)	[14]
	DPPH scavenging activity	Activity similar to BHA & BHT (20 μ g/mL)	[15]
	Hydroxyl radical scavenging	Activity greater than quercetin (0.6 μ g/mL)	[15]
	Protective	NMDA neurotoxicity	Prevented acute neuronal swelling and reduced neuronal death (100- 300 μ M)
Carbon tetrachloride intoxicated rat liver		Counteracted metabolic enzyme reduction (10.7 mg/kg/day)	[17]
Liver monooxygenase activity & Carbon tetrachloride induced lipid peroxidation		Inhibition (5 & 25 mg/kg)	[18]
Induced gastric lesions in rat		Reduced number and severity of ulcers observed (10-100 mg/kg)	[21]
Induced ulcer in male Sprague-Dawley rats		Gastroprotective action (100 mg/kg)	[22]
Adult male Wistar rats		Curtailed thioacetamide (TA)-induced hepatic injury (10.7 mg/kg/day)	[23]
Nicotine-induced oxidative damage		Dose dependent protection (1-20 μ g/mL)	[24]
Randomized, single-blind study		Activity similar to benzocaine	[1]
Male Sprague-Dawley rats		Reversible & dose dependent activity (5- 60 mg/kg)	[5]
Antibacterial, Antifungal & Antiviral		<i>Candida albicans</i> biofilms	Active against sessile cells (20-2000 mg/L)
	<i>Candida albicans</i>	Antifungal activity by envelope damage (0.2 %)	[33]
	Oral candidiasis in immunosuppressed rats	Significant reduction in the colony counts (0.5 mL, 24 mM)	[34]
	Vaginal candidiasis in immunosuppressed rats	Reduction in the colony counts of <i>Candida albicans</i> (20 mg/kg/day)	[35]
	<i>Penicillium</i> species	Growth inhibition (100 mg/L)	[36]
	<i>Aspergillus</i> species	Growth inhibition (100-140 mg/L)	[36]
	<i>Fusarium</i> species	Growth inhibition (140-150 mg/L)	[36]
	Anti-herpes simplex virus	Active against HSV-1 and HSV-2 viruses (16.2 and 25.6 μ g/mL)	[38]
	Maximal electroshock seizure test	Synthetic eugenol derivatives showed significant activity	[39]
	Anti-inflammatory	Prostaglandin H synthase inhibition	Inhibited cyclooxygenase (100-200 μ M)
Human macrophages		Resisted the release of proinflammatory mediators & inhibited cyclooxygenase-2	[41]
Penetration enhancer	Male Sprague-Dawley rats	Reduced both paw and joint swelling in induced arthritis (33 mg/kg)	[42]
	Mice	Reduced lipopolysaccharide induced lung inflammation (160 mg/kg, ip)	[44]
	<i>In vitro</i> evaluation in mouse skin	Increased drug flux (5 %)	[46]
Cardiovascular	<i>In vitro</i> penetration through porcine epidermis	Significant enhancement of permeability coefficient of drug (5 %)	[47]
	Rat mesenteric vascular bed	Dose-dependent, reversible vasodilatation (0.2 – 20 μ mol)	[48]
	Guinea-pig heart muscle	Potassium current inhibition (60 – 600 μ mol/L)	[49]
	Hypertensive rats	Vascular relaxation (0.006 – 6 mM)	[50]
	Smooth muscle relaxant effect	Action mediated through endothelial-generated nitric oxide (300 μ M)	[51]
Anticancer	Isoproterenol-induced cardiac hypertrophy in Mice	Oxidative stress and apoptosis were reduced (1 mg/kg twice daily)	[52]
	Swiss mice	Protection against chemically induced skin cancer (30 μ L)	[55]
Anticancer	Swiss mice	Restriction of skin carcinogenesis at dysplastic stage (1.25 mg/kg)	[56]
	Swiss mice	Restriction of skin carcinogenesis at dysplastic stage (1.25 mg/kg)	[56]
CLOVE OIL			
Antioxidant	DPPH scavenging activity	Activity similar to BHA & BHT (0.5 μ g/mL)	[15]
	Hydroxyl radical scavenging	Active more than quercetin (0.2 μ g/mL)	[15]
	Lipid peroxidation	Active more than BHT	[15]
Anti-inflammatory	Mice	Caused cytokine inhibition (200 mg/kg)	[43]
Penetration enhancer	Excised rabbit abdominal skin	Significant increase in drug flux (1-3 %)	[45]
	<i>In vivo</i> percutaneous absorption in rabbit	Increase in drug flux (1-3 %)	[45]
Anticancer	Antimutagenic activity against MNNG, 4NQO, AfB ₁ and Trp-P-1.	Suppressive effect on mutagens (ethyl acetate extract)	[54]
Aphrodisiac	Male Swiss mice	Improved sexual function (500 mg/kg of 50 % ethanolic extract)	[58]

to consider eugenol as a lead molecule for the development of new drugs [74] with enhanced therapeutic efficacy.

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Natural Product Communications

Volume 5 (1-12) 2010 Contents

Number 1

Two New Furanoreomophilane Sesquiterpenoids from <i>Ligularia oligonema</i> Hajime Nagano, Mika Matsushima, Hiroka Yamada, Ryo Hanai, Xun Gong and Chiaki Kuroda	1
Synthesis and Stereochemistry of Occidenol, a 4,5-Dihydro-oxipin-containing Sesquiterpene: a Pericyclic Approach John N. Marx and Abdulaziz Ajlouni	5
A New Dolabrane-type Diterpene from <i>Ceriops tagal</i> Xiao-Wei Ouyang, Xia-Chang Wang, Qing-Xi Yue and Li-Hong Hu	9
Structure and Absolute Configuration of a Diterpenoid from <i>Castanea mollissima</i> Hui-Yuan Gao, Xiao-Bo Wang, Rong-Gang Xi, Bo-Hang Sun, Jian Huang and Li-Jun Wu	13
Canthin-6-one Alkaloids and a Tirucallanoid from <i>Eurycoma longifolia</i> and Their Cytotoxic Activity against a Human HT-1080 Fibrosarcoma Cell Line Katsunori Miyake, Yasuhiro Tezuka, Suresh Awale, Feng Li and Shigetoshi Kadota	17
Qualitative and Quantitative Analysis of the Main Constituents of <i>Radix Ilicis Pubescentis</i> by LC-Coupled with DAD and ESI-MS Detection Wen-Yuan Liu, Feng Feng, Cheng-Xia Yu and Ning Xie	23
New Anti-inflammatory Sterols from the Red Sea Sponges <i>Scalariispongia aqabaensis</i> and <i>Callyspongia siphonella</i> Diaa T. A. Youssef, Amany K. Ibrahim, Sherief I. Khalifa, Mostafa K. Mesbah, Alejandro M. S. Mayer and Rob W. M. van Soest	27
Plakinamine L: A New Steroidal Alkaloid from the Marine Sponge <i>Corticium</i> sp. Maurice Aknin, Amira Rudi, Yoel Kashman, Jean Vacelet and Emile M. Gaydou	33
A New Alkaloid and Flavonoids from the Aerial Parts of <i>Euphorbia guyoniana</i> Tarek Boudiar, Lakhel Hichem, A. Khalfallah, Ahmed Kabouche, Zahia Kabouche, Ignacio Brouard, Jaime Bermejo and Christian Bruneau	35
Apigenin Di- and Trirhamnoside from <i>Asplenium normale</i> in Malaysia Tsukasa Iwashina, Sadamu Matsumoto, Junichi Kitajima, Takehisa Nakamura, Goro Kokubugata, Monica Suleiman and Idoris M. Said	39
A New Prenylated Flavanoid with Antibacterial Activity from Propolis Collected in Egypt Ashraf El-Bassuony and Sameh AbouZid	43
Reverse phase-HPLC Method for Determination of Marker Compounds in NP-1, an Anti-osteoporotic Plant Product from <i>Butea monosperma</i> Varsha Gupta, Anil Kumar Dwivedi, Dinesh Kumar Yadav, Manmeet Kumar and Rakesh Maurya	47
Influence of the Extraction Method on the Yield of Flavonoids and Phenolics from <i>Sideritis</i> spp. (Pirin Mountain tea) Kalina Alipieva, Jasmina Petreska, Ángel Gil-Izquierdo, Marina Stefova, Ljuba Evstatieva and Vassya Bankova	51
A New Phenolic and a New Lignan from the Roots of <i>Juniperus chinensis</i> Chi-I Chang, Chiy-Rong Chen, Wen-Ching Chen, Chao-Lin Kuo and Yueh-Hsiung Kuo	55
Flavones from the Stem of <i>Andrographis paniculata</i> Nees Parvataneni Radhika, Yejella Rajendra Prasad and Koduru Rajya Lakshmi	59
Antioxidant Effects of <i>Glechoma hederacea</i> as a Food Additive Mirjana Milovanovic, Dusan Zivkovic and Biljana Vucelic-Radovic	61
<i>Ocimum basilicum</i> L.: Phenolic Profile and Antioxidant-related Activity H.J. Damien Dorman and Raimo Hiltunen	65
Variation in Antioxidant Activity of Extracts of <i>Acacia confusa</i> of Different Ages Yu-Tang Tung and Shang-Tzen Chang	73
4'-O-Methylglycosylation of Curcumin by <i>Beauveria bassiana</i> Jia Zeng, Nan Yang, Xiu-Min Li, Paul J. Shami and Jixun Zhan	77

The Biotransformation of Aromatic Amino Acids by <i>Phoma macrostoma</i> Yevgeniya Tyagunova and John L. Sorensen	81
Lipoxygenase Inhibitory Activity of 6-Pentadecanoylsalicylic Acid without Prooxidant Effect Isao Kubo, Tae Joung Ha and Kuniyoshi Shimizu	85
Capsaicinoids in the Hottest Pepper Bhut Jolokia and its Antioxidant and Antiinflammatory Activities Yunbao Liu and Muraleedharan G. Nair	91
Anti-diabetes Constituents in Leaves of <i>Smalanthus sonchifolius</i> Xiang Zheng, He Fan, Kang Ting-Guo, Dou De-Qiang, Gai Kuo, Shi Yu-Yuan, Kim Young-Ho and Dong Feng	95
Phytotoxic Chlorophyll Derivatives from <i>Petrorhagia velutina</i> (Guss.) Ball et Heyw Leaves Brigida D'Abrosca, Monica Scognamiglio, Nikolaos Tsafantakis, Antonio Fiorentino and Pietro Monaco	99
Inhibitory Effects of Indirubin Derivatives on the Growth of HL-60 Leukemia Cells Nguyen Manh Cuong, Bui Huu Tai, Dang Hoang Hoan, Tran Thu Huong, Young Ho Kim, Jae-Hee Hyun and Hee-Kyoung Kang	103
The Role of Secreting Structures Position on the Leaf Volatile Organic Compounds of <i>Hypericum androsaemum</i> Claudia Giuliani, Roberto Maria Pellegrino, Bruno Tirillini and Laura Maleci Bini	107
Solvent-free Microwave Extraction of Essential Oils from <i>Laurus nobilis</i> and <i>Melissa officinalis</i>: Comparison with Conventional Hydro-distillation and Ultrasound Extraction Burcu Uysal, Fazli Sozmen and Birsen S. Buyuktas	111
Chemotypes of <i>Pistacia atlantica</i> Leaf Essential Oils from Algeria Nadhir Gourine, Isabelle Bombarda, Mohamed Yousfi and Emile M. Gaydou	115
Misidentification of Tansy, <i>Tanacetum macrophyllum</i>, as Yarrow, <i>Achillea grandifolia</i>: a Health Risk or Benefit? Niko S. Radulović, Polina D. Blagojević, Danielle Skropeta, Aleksandra R. Zarubica, Bojan K. Zlatković and Radosav M. Palić	121
Essential Oil Composition of <i>Achillea clusiana</i> from Bulgaria Antoaneta Trendafilova, Milka Todorova and Antonina Vitkova	129
Composition of Essential Oil of Aerial Parts of <i>Chamomilla suaveolens</i> from Estonia Anne Orav, Janne Sepp, Tiiu Kailas, Mati Müürisepp, Elmar Arak and Ain Raal	133
Effects of Essential Oils on the Growth of <i>Giardia lamblia</i> Trophozoites Marisa Machado, Maria do Céu Sousa, Lígia Salgueiro and Carlos Cavaleiro	137
Anxiolytic Effect and Tissue Distribution of Inhaled <i>Alpinia zerumbet</i> Essential Oil in Mice Tadaaki Satou, Shio Murakami, Mariko Matsuura, Shinichiro Hayashi and Kazuo Koike	143
Biological Activities of <i>Bellis perennis</i> Volatiles and Extracts Neslihan Kavalcıoğlu, Leyla Açıık, Fatih Demirci, Betül Demirci, Hülya Demir and K. Hüsnü Can Başer	147
Antiproliferative Effects of Volatile Oils from <i>Centipeda minima</i> on Human Nasopharyngeal Cancer CNE Cells Miaoxian Su, Peng Wu, Yaolan Li and Hau Yin Chung	151
Stimulating Effect of Aromatherapy Massage with Jasmine Oil Tapanee Hongratanaworakit	157
<u>Review/Account</u>	
Essential Oil Biosynthesis and Regulation in the Genus <i>Cymbopogon</i> Deepak Ganjewala and Rajesh Luthra	163

Number 2

Antimosquito and Antimicrobial Clerodanoids and a Chlorobenzenoid from <i>Tessmannia</i> species Charles Kihampa, Mayunga H.H. Nkunya, Cosam C. Joseph, Stephen M. Magesa, Ahmed Hassanali, Matthias Heydenreich and Erich Kleinpeter	175
Two New Terpenoids from <i>Trichilia quadrijuga</i> (Meliaceae) Virginia F. Rodrigues, Hadria M. Carmo, Raimundo Braz Filho, Leda Mathias and Ivo J. Curcino Vieira	179
Effect of Miconazole and Terbinafine on Artemisinin Content of Shooty Teratoma of <i>Artemisia annua</i> Rinki Jain and Vinod Kumar Dixit	185
A New Triterpenoid Saponin from the Stem Bark of <i>Pometia pinnata</i> Faryal Vali Mohammad, Viqar Uddin Ahmad, Mushtaq Noorwala and Nordin HJ.Lajis	191
27-Hydroxyoleanolic Acid Type Triterpenoid Saponins from <i>Anemone raddeana</i> rhizome Li Fan, Jin-Cai Lu, Jiao Xue, Song Gao, Bei-Bei Xu, Bai-Yi Cao and Jing-Jing Zhang	197
Steroids from the South China Sea Gorgonian <i>Subergorgia suberosa</i> Shu-Hua Qi, Cheng-Hai Gao, Pei-Yuan Qian and Si Zhang	201
Auroside, a Xylosyl-sterol, and Patusterol A and B, two Hydroxylated Sterols, from two Soft Corals <i>Eleutherobia aurea</i> and <i>Lobophytum patulum</i> Dina Yeffet, Amira Rudi, Sharon Ketzinel, Yehuda Benayahu and Yoel Kashman	205

Anti-tuberculosis Compounds from <i>Mallotus philippinensis</i> Qi Hong, David E. Minter, Scott G. Franzblau, Mohammad Arfan, Hazrat Amin and Manfred G. Reinecke	211
Phenolic Derivatives with an Irregular Sesquiterpenyl Side Chain from <i>Macaranga pruinosa</i> Yana M. Syah and Emilio L. Ghisalberti	219
Hexaoxygenated Flavonoids from <i>Pteroxygonum giraldii</i> Yanhong Gao, Yanfang Su, Shilun Yan, Zhenhai Wu, Xiao Zhang, Tianqi Wang and Xiumei Gao	223
Comparative Study of the Antioxidant Activities of Eleven <i>Salvia</i> Species Gábor Janicsák, István Zupkó, Imre Máthé and Judit Hohmann	227
Dibenzocyclooctadiene Lignans from <i>Fructus Schisandrae Chinensis</i> Improve Glucose Uptake <i>in vitro</i> Jing Zhang, Lei Ling Shi and Yi Nan Zheng	231
Honokiol and Magnolol Production by <i>in vitro</i> Micropropagated Plants of <i>Magnolia dealbata</i>, an Endangered Endemic Mexican Species Fabiola Domínguez, Marco Chávez, María Luisa Garduño-Ramírez, Víctor M. Chávez-Ávila, Martín Mata and Francisco Cruz-Sosa	235
Design, Synthesis and Biological Evaluation of Novel Spin-Labeled Derivatives of Podophyllotoxin Jia-qiang Zhang, Zhi-wei Zhang, Ling Hui and Xuan Tian	241
Secondary Metabolites of the Phytopathogen <i>Peronophythora litchi</i> Haihui Xie, Yaoguang Liang, Jinghua Xue, Qiaolin Xu, Yueming Jiang and Xiaoyi Wei	245
Bioassay-guided Isolation of Antibacterial and Cytotoxic Compounds from the Mesophilic Actinomycete M-33-5 Mustafa Urgen, Fatma Kocabaş, Ayşe Nalbantsoy, Esin Hameş Kocabas, Ataç Uzel and Erdal Bedir	249
Aristolactams, 1-(2-C-Methyl-β-D-ribofuranosyl)-uracil and Other Bioactive Constituents of <i>Toussaintia orientalis</i> Josiah O. Odalo, Cosam C. Joseph, Mayunga H.H. Nkunya, Isabel Sattler, Corinna Lange, Gollmick Friedrich, Hans-Martin Dahse and Ute Möllman	253
Salaramides A and B; Two α-Oxoamides Isolated from the Marine Sponge <i>Hippospongia</i> sp. (Porifera, Dictyoceratida) Julia Bensemhoun, Amira Rudi, Yoel Kashman, Emile M. Gaydou, Jean Vacelet and Maurice Aknin	259
Antioxidant Activity and Total Phenolic Content of 24 Lamiaceae Species Growing in Iran Omidreza Firuzi, Katayoun Javidnia, Maryam Gholami, Mohammad Soltani and Ramin Miri	261
Preparation and Characterization of 5'-Phosphodiesterase from Barley Malt Rootlets Jie Hua and Ke-long Huang	265
Volatiles of <i>Callicarpa macrophylla</i>: A Rich Source of Selinene Isomers Anil K. Singh, Chandan S. Chanotiya, Anju Yadav and Alok Kalra	269
Volatile Components of Aerial Parts of <i>Centaurea nigrescens</i> and <i>C. stenolepis</i> Growing Wild in the Balkans Carmen Formisano, Felice Senatore, Svetlana Bancheva, Maurizio Bruno, Antonella Maggia and Sergio Rosselli	273
Compositional Variability in Essential Oil from Different Parts of <i>Alpinia speciosa</i> from India Rajendra C. Padalia, Chandan S. Chanotiya and V. Sundaresan	279
Composition at Different Development Stages of the Essential Oil of Four <i>Achillea</i> Species Grown in Iran Majid Azizi, Remigius Chizzola, Askar Ghani and Fatemeh Oroojalian	283
Characterization of Some Italian Ornamental Thyme by Their Aroma Alessandra Bertoli, Szilvia Sárosi, Jenő Bernáth and Luisa Pistelli	291
Characterization of <i>Szovitsia callicarpa</i> Volatile Constituents Obtained by Micro- and Hydrodistillation Betül Demirci, Nurgün Küçükboyacı, Nezaket Adıgüzel, K. Hüsnü Can Başer and Fatih Demirci	297
Biological Activity of Essential Oils from <i>Aloysia polystachya</i> and <i>Aloysia citriodora</i> (Verbenaceae) against the Soybean Pest <i>Nezara viridula</i> (Hemiptera: Pentatomidae) Jorge O. Werdin González, María M. Gutiérrez, Ana P. Murray and Adriana A. Ferrero	301
Essential Oil from the Underground Parts of <i>Laserpitium zernyi</i>: Potential Source of α-Bisabolol and its Antimicrobial Activity Višnja Popović, Silvana Petrović, Milica Pavlović, Marina Milenković, Maria Couladis, Olga Tzakou, Šemija Duraki and Marjan Niketić	307
Chemical Composition and Antibacterial Activity of the Essential Oil from Fruits of <i>Bursera tomentosa</i> José Moreno, Rosa Aparicio, Judith Velasco, Luis B Rojas, Alfredo Usubillaga and Marcó Lue-Merú	311
Composition and Antioxidant Activity of <i>Inula crithmoides</i> Essential Oil Grown in Central Italy (Marche Region) Laura Giamperi, Anahi Bucchini, Daniele Fraternali, Salvatore Genovese, Massimo Curini and Donata Ricci	315
<i>Foeniculum vulgare</i> Essential Oils: Chemical Composition, Antioxidant and Antimicrobial Activities Maria Graça Miguel, Cláudia Cruz, Leonor Faleiro, Mariana T. F. Simões, Ana Cristina Figueiredo, José G. Barroso and Luis G. Pedro	319
Chemical Variability, Antifungal and Antioxidant Activity of <i>Eucalyptus camaldulensis</i> Essential Oil from Sardinia Andrea Barra, Valentina Coroneo, Sandro Dessi, Paolo Cabras and Alberto Angioni	329

Composition and Anti-Wood-Decay Fungal Activities of the Leaf Essential oil of <i>Machilus philippinensis</i> from Taiwan	
Chen-Lung Ho, Kuang-Ping Hsu, Eugene I-Chen Wang, Chai-Yi Lin and Yu-Chang Su	337
Composition, Cytotoxicity and Antioxidant Activity of the Essential Oil of <i>Dracocephalum surmandinum</i> from Iran	
Ali Sonboli, Mohammad Ali Esmaeili, Abbas Gholipour and Mohammad Reza Kanani	341
Antifungal Activities of <i>Ocimum sanctum</i> Essential Oil and its Lead Molecules	
Amber Khan, Aijaz Ahmad, Nikhat Manzoor and Luqman A. Khan	345
Number 3	
(R)-(-)-Linalyl Acetate and (S)-(-)-Germacrene D from the Leaves of Mexican <i>Bursera linanoe</i>	
Koji Noge, Nobuhiro Shimizu and Judith X. Becerra	351
Three New Insecticidal Sesquiterpene Polyol Esters from <i>Celastrus angulatus</i>	
Shaopeng Wei, Minchang Wang, Zhiqin Ji, Baojun Shi, Shengkun Li and Jiwen Zhang	355
Triterpenoids from Aerial Parts of <i>Glochidion eriocarpum</i>	
Vu Kim Thu, Phan Van Kiem, Pham Hai Yen, Nguyen Xuan Nhiem, Nguyen Huu Tung, Nguyen Xuan Cuong, Chau Van Minh, Hoang Thanh Huong, Trinh Van Lau, Ngo Thi Thuan and Young Ho Kim	361
Identification of Sakurasosaponin as a Cytotoxic Principle from <i>Jacquinia flammea</i>	
Alberto Sánchez-Medina, Luis M. Peña-Rodríguez, Filogonio May-Pat, Gloria Karagianis, Peter G. Waterman, Anthony I. Mallet and Solomon Habtemariam	365
Vasoconstrictor and Inotropic Effects Induced by the Root Bark Extracts of <i>Anthocleista schweinfurthii</i>	
Nadège Kabamba Ngombe, Dibungi T. Kalenda, Joëlle Quetin-Leclercq and Nicole Morel	369
Hydroxylation of Diosgenin by <i>Absidia coerulea</i>	
Ying Zhao, Ling-Mei Sun, Xiao-Ning Wang, Tao Shen, Mei Ji and Hong-Xiang Lou	373
Dibromotyrosine and Histamine Derivatives from the Tropical Marine Sponge <i>Aplysina</i> sp.	
Elena A. Santalova, Vladimir A. Denisenko and Valentin A. Stonik	377
<i>In vitro</i> Inhibitory Activities of Lauraceae Aporphine Alkaloids	
Ericsson David Coy Barrera and Luis Enrique Cuca Suárez	383
Leishmanicidal activity of racemic \pm 8-[(4-Amino-1-methylbutyl)amino]-6-methoxy-4-methyl-5-[3,4-dichlorophenoxy]quinoline	
Angélica P. Isaac-Márquez, James D. McChesney, N.P. Dammika Nanayakara, Abhay R. Satoskar and Claudio M. Lezama-Dávila	387
Insecticidal, Mutagenic and Genotoxic Evaluation of Annonaceous Acetogenins	
Olga Álvarez Colom, Analia Salvatore, Eduardo Willink, Roxana Ordóñez, María I. Isla, Adriana Neske and Alicia Bardón	391
Water-retentive and Anti-inflammatory Properties of Organic and Inorganic Substances from Korean Sea Mud	
Jung-Hyun Kim, Jeongmi Lee, Hyang-Bok Lee, Jeong Hyun Shin and Eun-Ki Kim	395
A Phenethyl bromo ester from <i>Citharexylum fruticosum</i>	
Seru Ganapaty, Desaraju Venkata Rao and Steve Thomas Pannakal	399
New 2-(2-Phenylethyl)chromone Derivatives from the Seeds of <i>Cucumis melo</i> L var. <i>reticulatus</i>	
Sabrin R. M. Ibrahim	403
Phenolic Compounds in Different Barley Varieties: Identification by Tandem Mass Spectrometry (QStar) and NMR; Quantification by Liquid Chromatography Triple Quadrupole-Linear Ion Trap Mass Spectrometry (Q-Trap)	
Kamilla Klausen, Anne G. Mortensen, Bente Laursen, Kim F. Haselmann, Birthe Møller Jespersen and Inge S. Fomsgaard	407
Effect of <i>Cleome arabica</i> Leaf Extract Treated by Naringinase on Human Neutrophil Chemotaxis	
Hamama Bouriche and Juegen Arnhold	415
Two New Carthamosides from <i>Carthamus oxycantha</i>	
Zahid Hassan, Viqar Uddin Ahmad, Javid Hussain, Aqib Zahoor, Imran Nafees Siddiqui, Nasir Rasool and Muhammad Zubair	419
A New Lignan Dimer from <i>Mallotus philippensis</i>	
Nguyen Thi Mai, Nguyen Xuan Cuong, Nguyen Phuong Thao, Nguyen Hoai Nam, Nguyen Huu Khoi, Chau Van Minh, Yvan Vander Heyden, Ngo Thi Thuan, Nguyen Van Tuyen, Joëlle Quetin-Leclercq and Phan Van Kiem	423
Tectone, a New Antihyperglycemic Anthraquinone from <i>Tectona grandis</i> Leaves	
Nivedita Shukla, Manmeet Kumar, Akanksha, Ghufraan Ahmad, Neha Rahuja, Amar B. Singh, Arvind K. Srivastava, Siron M. Rajendran and Rakesh Maurya	427
A Semi-quantitative FIA-ESI-MS Method for the Rapid Screening of <i>Hypericum perforatum</i> Crude Extracts	
Anna Piovani, Raffaella Filippini and Rosy Caniato	431
Free and Bound Cinnamic Acid Derivatives in Corsica Sweet Blond Oranges	
Eric Carrera, Mohamed Vall Ould El Kebir, Camille Jacquemond, François Luro, Yves Lozano and Emile M. Gaydou	435
Antioxidants from Tropical Herbs	
Rasyidah Razab and Azlina Abdul Aziz	441

The Antitumor and Immunostimulating Activities of Water Soluble Polysaccharides from <i>Radix Aconiti</i>, <i>Radix Aconiti Lateralis</i> and <i>Radix Aconiti Kusnezoffii</i> Tingting Gao, Hongtao Bi, Shuai Ma and Jingmei Lu	447
Seasonal Variation in the Leaf Essential Oil Composition of <i>Zanthoxylum clava-herculis</i> growing in Huntsville, Alabama Lauren C. Eiter, Henry Fadamiro and William N. Setzer	457
Supercritical CO₂ Extraction of Essential Oils from <i>Chamaecyparis obtusa</i> Yinzhe Jin, Dandan Han, Minglei Tian and Kyung-Ho Row	461
Variability of the Essential Oil Content and Composition of Chamomile (<i>Matricaria recutita</i> L.) affected by Weather Conditions Beáta Gosztola, Szilvia Sárosi and Éva Németh	465
Acaricidal Activity against <i>Tetranychus urticae</i> and Chemical Composition of Peel Essential Oils of Three Citrus Species Cultivated in NE Brazil Claudio Pereira Araújo Júnior, Claudio Augusto Gomes da Camara, Ilzenayde Araújo Neves, Nicolle de Carvalho Ribeiro, Cristianne Araújo Gomes, Marcílio Martins de Moraes and Priscilla de Sousa Botelho	471
Essential Oil Composition, Antioxidant Capacity and Antifungal Activity of <i>Piper divaricatum</i> Joyce Kelly R. da Silva, Eloísa Helena A. Andrade, Elsie F. Guimarães and José Guilherme S. Maia	477
Chemical Composition, Toxicity and Larvicidal Activity of the Essential Oil from the Whole Plant of <i>Acalypha segetalis</i> from South-West Nigeria Sherifat A. Aboaba, Olapeju O. Aiyelaagbe and Olusegun Ekundayo	481
<u>Review/Account</u>	
Toxicity of Non-protein Amino Acids to Humans and Domestic Animals Peter B. Nunn, E. Arthur Bell (the late), Alison A. Watson and Robert J. Nash	485
Number 4	
Novel Bisabolane Sesquiterpenes from the Marine-derived Fungus <i>Verticillium tenerum</i> Celso Almeida, Somaia Elsaedi, Stefan Kehraus and Gabriele M. König	507
Sesquiterpene Lactones from <i>Inula oculus-christi</i> Mahmoud Mosaddegh, Maryam Hamzeloo Moghadam, Saeedeh Ghafari, Farzaneh Naghibi, Seyed Nasser Ostad and Roger W. Read	511
Biotransformation of α-Cedrol and Caryophyllene Oxide by the Fungus <i>Neurospora crassa</i> Ismail Kiran, Zeynep Durceylan, Neşe Kirimer, K. Hüsni Can Başer, Yoshiaki Noma and Fatih Demirci	515
Two New Diterpene Phenols from <i>Calocedrus decurrans</i> Sheeba Veluthoor, Shujun Li, Rick G. Kelsey, Marc C. Dolan, Nicholas A. Panella and Joe Karchesy	519
Vitamin E Ameliorates High Dose <i>trans</i>-Dehydrocrotonin-Associated Hepatic Damage in Mice Alana Fontales Lima Rabelo, Marjorie Moreira Guedes, Adriana da Rocha Tomé, Patricia Rodrigues Lima, Maria Aparecida Maciel, Silveria Regina de Sousa Lira, Ana Carla da Silva Carvalho, Flávia Almeida Santos and Vietla Satyanarayana Rao	523
A Pentacyclic Triterpene from <i>Litchi chinensis</i> Imran Malik, Viqar Uddin Ahmad, Shazia Anjum and Fatima. Z. Basha	529
Ellagitannins from <i>Geranium potentillaefolium</i> and <i>G. bellum</i> Juan A. Gayosso-De-Lucio, J. Martín Torres-Valencia, Carlos M. Cerda-García-Rojas and Pedro Joseph-Nathan	531
The Quantitative Effects of Temperature and Light Intensity on Phenolics Accumulation in <i>St. John's Wort</i> (<i>Hypericum perforatum</i>) Mehmet Serhat Odabas, Necdet Camas, Cuneyt Cirak, Jolita Radušiene, Valdimaras Janulis and Liudas Ivanauskas	535
Chemical Analysis of the Principal Flavonoids of <i>Radix Hedysari</i> by HPLC Yi Liu, Yuying Zhao, Hubiao Chen, Bin Wang and Qingying Zhang	541
Chemical Composition and Bioactivity of <i>Pleiogynium timorense</i> (Anacardiaceae) Eman Al Sayed, Olli Martiskainen, Jari Sinkkonen, Kalevi Pihlaja, Nahla Ayoub, Abd-El Naser Singab and Mohamed El-Azizi	545
A Chromone from <i>Seseli praecox</i> (Apiaceae) Marco Leonti, Laura Casu, Maria Novella Solinas, Filippo Cottiglia, Pierluigi Caboni, Costantino Floris, Juerg Gertsch and Anna Rita Saba	551
Determination of Chromones in <i>Dysophylla stellata</i> by HPLC: Method Development, Validation and Comparison of Different Extraction Methods Raju Gautam, Amit Srivastava and Sanjay M. Jachak	555
Antimicrobial Coumarins from the Stem Bark of <i>Afraegle paniculata</i> Valerie Beatrice Tsassi, Hidayat Hussain, Bouberte Yemele Meffo, Simeon F. Kouam, Etienne Dongo, Barbara Schulz, Ivan R. Green and Karsten Krohn	559

A Novel Glycoside from <i>Acanthus hirsutus</i> (Acanthaceae) Seval Çapanlar, Nazlı Böke, Ihsan Yaşa and Süheyla Kırmızıgül	563
Stemphol Galactoside, a New Stemphol Derivative Isolated from the Tropical Endophytic Fungus <i>Gaeumannomyces amomi</i> Juangjun Jumpathong, Muna Ali Abdalla, Saisamorn Lumyong and Hartmut Laatsch	567
Suppression of Nitric Oxide Implicated in the Protective Effect of Echinacoside on H₂O₂-Induced PC12 Cell Injury Rong Kuang, Yiguo Sun and Xiaoxiang Zheng	571
Chemical Changes during Fermentation of <i>Abhayarishtha</i> and its Standardization by HPLC-DAD Uma Ranjan Lal, Shailendra Mani Tripathi, Sanjay M. Jachak, Kamlesh Kumar Bhutani and Inder Pal Singh	575
Betaine Yields from Marine Algal Species Utilized in the Preparation of Seaweed Extracts Used in Agriculture Gerald Blunden, Peter F. Morse, Imre Mathe, Judit Hohmann, Alan T. Critchley and Stephen Morrell	581
Differentiation of <i>Symphytum</i> Species Using RAPD and Seed Fatty Acid Patterns Tamer Özcan	587
New Angucyclines and Antimicrobial Diketopiperazines from the Marine Mollusk-Derived Actinomycete <i>Saccharothrix espanaensis</i> An 113 Nataliya I. Kalinovskaya, Anatoly I. Kalinovskiy, Lyudmila A. Romanenko, Pavel S. Dmitrenok and Tatyana A. Kuznetsova	597
Cellulose of <i>Salicornia brachiata</i> Naresh D Sanandiyaa, Kamallesh Prasad, Ramavatar Meena and Arup K Siddhanta	603
<i>In vivo</i> Release of Lectins from the Green Alga <i>Ulva fasciata</i> Pablo Djabayan-Djibeyan, Roslyn Gibbs and Brian Carpenter	607
Essential Oil from Leaves of <i>Lippia dulcis</i> Grown in Colombia Bárbara Moreno-Murillo, Clara Quijano-Céllis, Arturo Romero R. and Jorge A. Pino	613
Analysis of Chemical Constituents of the Volatile Oil from Leaves of <i>Solanum bicolor</i> Alida Pérez Colmenares, Luis B. Rojas, Eilen Arias, Juan Carmona Arzola and Alfredo Usubillaga	615
Compositions and <i>in vitro</i> Anticancer activities of the Leaf and Fruit Oils of <i>Litsea cubeba</i> from Taiwan Chen-Lung Ho, Ou Jie-Ping, Yao-Chi Liu, Chien-Ping Hung, Ming-Chih Tsai, Pei-Chun Liao, Eugene I-Chen Wang, Yi-Lin Chen and Yu-Chang Su	617
GC-MS Analysis, Antibacterial Activity and Genotoxic Property of <i>Erigeron mucronatus</i> Essential Oil Bahlul Z. Awen, C. Ramachandra Unnithan, Subban Ravi and Akoni J. Lakshmanan	621
Nepetalactone Content and Antibacterial Activity of the Essential Oils from Different Parts of <i>Nepeta persica</i> Ali Shafaghath and Khodamali Oji	625
Chemical Composition and Antiradical Activity of the Essential Oil from <i>Satureja intricata</i>, <i>S. obovata</i> and their Hybrid <i>Satureja x delpozoi</i> María J. Jordán, P. Sánchez-Gómez, Juan F. Jiménez, María Quilez and José A. Sotomayor	629
A Chemical Marker Proposal for the <i>Lantana</i> genus: Composition of the Essential Oils from the Leaves of <i>Lantana radula</i> and <i>L. canescens</i> José G. Sena Filho, Haroudo S. Xavier, José M. Barbosa Filho and Jennifer M. Düringer	635
Comparison of the Volatile Constituents of <i>Elsholtzia fruticosa</i> Extracted by Hydrodistillation, Supercritical Fluid Extraction and Head Space Analysis Rikki Saini, Shailja Guleria, Vijay K. Kaul, Brij Lal, Garikapati D. Kiran Babu and Bikram Singh	641
The Effect of Essential Oil Formulations for Potato Sprout Suppression Moses S. Owolabi, Labunmi Lajide, Matthew O. Oladimeji and William N. Setzer	645
<u>Review/Account</u>	
Phytochemicals and Biological Activities of <i>Gentiana</i> Species Jun-Li Yang, Lei-Lei Liu and Yan-Ping Shi	649
Number 5	
A new Bisabolane Derivative of <i>Leontopodium andersonii</i> Stefan Schwaiger, Stefanie Hehenberger, Ernst P. Ellmerer and Hermann Stuppner	667
Eudesmanolides and Methyl Ester Derivatives from <i>Dimerostemma arnottii</i> Sérgio Ricardo Ambrosio, Ricardo Stefani, Vladimir Constantino Gomes Heleno, Márcio Antônio de Menezes, Antonio Gilberto Ferreira, Paulo Gustavo Barboni Dantas Nascimento, Mara Angelina Galvão Magenta and Fernando Batista Da Costa	669
Acid Rearrangement of Epoxy-germacranolides and Absolute Configuration of 1β,10α-Epoxy-salonitenolide Sergio Rosselli, Antonella Maria Maggio, Rosa Angela Raccuglia, Susan L. Morris-Natschke, Kenneth F. Bastow, Kuo-Hsiung Lee and Maurizio Bruno	675
Sesquiterpenes Lactones and Flavonoids from <i>Eremanthus argenteus</i> (Asteraceae) Humberto Takeshi Sakamoto, Eugênio Paceli Laudares, Antônio Eduardo Miller Crotti, Norberto Peporine Lopes, Walter Vichnewski, João Luis Callegari Lopes and Vladimir Constantino Gomes Heleno	681

Constituents of <i>Calamintha ashei</i>: Effects on Florida Sandhill Species	
Marios A. Menelaou, Hidelisa P. Henandez, Francisco A. Macías, Jeffrey D. Weidenhamer, G. Bruce Williamson, Frank R. Fronczek, Helga D. Fischer and Nikolaus H. Fischer	685
Biotransformation of Sesquiterpenoids from Liverworts by Fungi and Mammals	
Yoshinori Asakawa, Toshihiro Hashimoto and Yoshiaki Noma	695
Identification and Functional Characterization of a new Sunflower Germacrene A Synthase (HaGAS3)	
Jens Göpfert, Anna-Katharina Bülow and Otmar Spring	709
Anticancer Activity of Andrographolide Semisynthetic Derivatives	
Vidya Menon and Sujata Bhat	717
Prenylated Pterocarpanes from <i>Erythrina melanacantha</i>	
Wera Hauschild, Patrick B. Chalo Mutiso and Claus M. Passreiter	721
Natural Products from <i>Scorzonera aristata</i> (Asteraceae)	
Manuela Jehle, Johanna Bano, Ernst P. Ellmerer and Christian Zidorn	725
Protective Effect on Human Lymphocytes of Some Flavonoids Isolated from Two <i>Achillea</i> Species	
Ivana Aljančić, Miroslava Stanković, Vele Tešević, Ljubodrag Vujisić, Vlatka Vajs and Slobodan Milosavljević	729
Effects of Caffeoylquinic Acid Derivatives and C-Flavonoid from <i>Lychnophora ericoides</i> on <i>in vitro</i> Inflammatory Mediator Production	
Michel David dos Santos, Guanjie Chen, Maria Camila Almeida, Denis Melo Soares, Glória Emília Petto de Souza, Norberto Peporine Lopes and R. Clark Lantz	733
LC-MS-MS Identification and Determination of the Flavone-C-Glucoside Vicenin-2 in Rat Plasma Samples Following Intraperitoneal Administration of <i>Lychnophora</i> Extract	
Valquiria A. Polisel Jabor, Denis Melo Soares, Andrea Diniz, Glória Emília Petto de Souza and Norberto Peporine Lopes	741
A New Bianthracene C-arabinopyranoside from <i>Senna septentrionalis</i>	
Gizachew Alemayehu, Legesse Adane and Berhanu M. Abegaz	747
An antiproliferative xanthone of <i>Symphonia pauciflora</i> from the Madagascar rainforest	
Ende Pan, Shugeng Cao, Peggy J. Brodie, James S. Miller, Rolland Rakotodrajaona, Fidy Ratovoson, Chris Birkinshaw, Rabodo Andriantsiferana, Vincent E. Rasamison and David G. I. Kingston	751
Computer-aided Structure Elucidation of Neolignans	
Mara B. Costantin, Marcelo J. P. Ferreira, Gilberto V. Rodrigues and Vicente P. Emerenciano	755
New Improvements in Automatic Structure Elucidation Using the LSD (Logic for Structure Determination) and the SISTEMAT Expert Systems	
Bertrand Plainchont, Jean-Marc Nuzillard, Gilberto V. Rodrigues, Marcelo J. P. Ferreira, Marcus T. Scotti and Vicente P. Emerenciano	763
A New <i>ent</i>-Labdane Diterpene Glycoside from the Leaves of <i>Casearia sylvestris</i>	
Wei Wang, Zulfiqar Ali, Xing-Cong Li and Ikhlās A. Khan	771
Triterpene Saponins from the Fruits of <i>Phytolacca rugosa</i> (Phytolaccaceae)	
Elier Galarraga M., Anne-Claire Mitaine-Offer, Juan Manuel Amaro-Luis, Tomofumi Miyamoto, Laurent Pouységu, Stéphane Quideau, Luis B. Rojas and Marie-Aleth Lacaille-Dubois	775
Preliminary Investigation of Naringenin Hydroxylation with Recombinant <i>E. coli</i> Expressing Plant Flavonoid Hydroxylation Gene	
Ilef Limem-Ben Amor, Nidhal Salem, Emmanuel Guedon, Jean-Marc Engasser, Leila Chekir-Ghedira and Mohamed Ghoul	777
Rapid Analysis of the Main Components of the Total Glycosides of <i>Ranunculus japonicus</i> by UPLC/Q-TOF-MS	
Wen Rui, Hongyuan Chen, Yuzhi Tan, Yanmei Zhong and Yifan Feng	783
Constituents of <i>Da-Cheng-Qi</i> Decoction and its Parent Herbal Medicines Determined by LC-MS/MS	
Fengguo Xu, Ying Liu, Rui Song, Haijuan Dong and Zunjian Zhang	789
Pharmacokinetic Comparison in Rats of Six Bioactive Compounds between <i>Da-Cheng-Qi</i> Decoction and its Parent Herbal Medicines	
Fengguo Xu, Ying Liu, Haijuan Dong, Rui Song and Zunjian Zhang	795
Structure Determination of Two New Monocillin I Derivatives	
Jixun Zhan, E. M. Kithsiri Wijeratne and A. A. Leslie Gunatilaka	801
Isolation of Salvianolic acid A, a Minor Phenolic Carboxylic Acid of <i>Salvia miltiorrhiza</i>	
Luyang Lu, Hao Zhang, Yu Qian and Yuan Yuan	805
Antimicrobial Activity of the Dichloromethane Extract from <i>in vitro</i> Cultured Roots of <i>Morinda royoc</i> and Its Main Constituents	
Janetsy Borroto, Ricardo Salazar, Alejandro Pérez, Yemeys Quiros, Martha Hernandez, Noemí Waksman and Reinaldo Trujillo	809
Beauvericin from the Endophytic Fungus, <i>Fusarium redolens</i>, Isolated from <i>Dioscorea zingiberensis</i> and Its Antibacterial Activity	
Lijian Xu, Jihua Wang, Jianglin Zhao, Peiqin Li, Tijiang Shan, Jingguo Wang, Xiaolin Li and Ligang Zhou	811

Phenyl Alkynes Rich Essential Oil of <i>Artemisia capillaris</i> Rakesh K. Joshi, Rajendra C. Padalia and Chandra S. Mathela	815
Essential Oil Composition of the Different Parts of <i>Eryngium aquifolium</i> from Spain Jesús Palá-Paúl, Jaime Usano-Aleman, Joseph J. Brophy, María J. Pérez-Alonso and Ana-Cristina Soria	817
Essential Oil Content and Composition, Nutrient and Mycorrhizal Status of Some Aromatic and Medicinal Plants of Northern Greece Nikitas Karagiannidis, Hellen Panou-Filothou, Diamando Lazari, Ioannis Ipsilantis and Christina Karagiannidou	823
Composition and Antimicrobial Activity of the Essential Oils from Flowers of <i>Senecio othonnae</i>, <i>S. racemosus</i> and <i>S. nemorensis</i> Osman Üçüncü, Nuran Kahriman, Salih Terzioğlu, Şengül Alpay Karaoğlu and Nurettin Yaylı	831
Chemical Composition and Antimicrobial Activity of Essential Oils of <i>Genista ulicina</i> and <i>G. vepres</i> Takiya Lograda, Adel Nadjib Chaker, Jean Claude Chalchat, Messaoud Ramdani, Hafsa Silini, Gilles Figueredo and Pierre Chalard	835
Number 6	
α-Glucosidase Inhibitory Constituents of <i>Linaria kurdica</i> subsp. <i>ericalyx</i> İrfan Aydoğdu, Figen Zihnioğlu, Tamer Karayildirim, Derya Gülcemal, Özgen Alankuş-Çalışkan and Erdal Bedir	841
Synthesis and Insecticidal Activities of New Ether-Derivatives of Celangulin-V Jiwen Zhang, Zhaonong Hu, Hua Yang and Wenjun Wu	845
New Sesquiterpene Lactone and Other Constituents from <i>Centaurea sulphurea</i> (Asteraceae) Hichem Lakhali, Tarek Boudiar, Ahmed Kabouche, Zahia Kabouche, Rachid Touzani and Christian Bruneau	849
Protective Effects of Isoatropicolide Tiglate from <i>Paulownia coreana</i> against Glutamate-induced Neurotoxicity in Primary Cultured Rat Cortical Cells Ill-Min Chung, Eun-Hye Kim, Hyun-Seok Jeon and Hyung-In Moon	851
Antimicrobial and Antiparasitic Abietane Diterpenoids from the Roots of <i>Clerodendrum eriophyllum</i> Francis Machumi, Volodymyr Samoylenko, Abiy Yenesew, Solomon Derese, Jacob O. Midiwo, Frank T. Wiggers, Melissa R. Jacob, Babu L. Tekwani, Shabana I. Khan, Larry A. Walker and Ilias Muhammad	853
Tetranortriterpenoids from <i>Spathelia sorbifolia</i> (Rutaceae) Denise S. Simpson, Stewart McLean, William F. Reynolds and Helen Jacobs	859
A Validated Method for Standardization of the Bark of <i>Clerodendron serratum</i> Arunava Gantait, Payel Roy, Neelesh Kumar Nema, Pradip Kumar Dutta and Pulok Kumar Mukherjee	863
Activity of Extracts and Procesterol from <i>Calotropis gigantea</i> against <i>Entamoeba histolytica</i> Shailendra Singh, Neelam Bharti, Manoj Chugh, Fehmida Naqvi and Amir Azam	867
Ampullosine, a new Isoquinoline Alkaloid from <i>Sepedonium ampullosporum</i> (Ascomycetes) Dang Ngoc Quang, Jürgen Schmidt, Andrea Porzel, Ludger Wessjohann, Mark Haid and Norbert Arnold	869
HPLC - DAD Analysis of Lycorine in Amaryllidaceae Species Gulen Irem Kaya, Derya Cicek, Buket Sarikaya, Mustafa Ali Onur and Nehir Unver Somer	873
Simultaneous HPLC Determination of Three Bioactive Alkaloids in the Asian Medicinal Plant <i>Stephania rotunda</i> Sothavireak Bory, Sok-Siya Bun, Béatrice Baghdikian, Fathi Mabrouki, Sun Kaing Cheng, Riad Elias, Hot Bun and Evelyne Ollivier	877
Impact of Cruciferous Phytoalexins on the Detoxification of Brassilexin by the Blackleg Fungus Pathogenic to Brown Mustard M. Soledade C. Pedras and Ryan B. Snitynsky	883
Flavonoids from <i>Erythrina vogelii</i> (Fabaceae) of Cameroon Muhammad Imran Ali, Zeeshan Ahmed, Alain Francois Kamdem Waffo and Muhammad Shaiq Ali	889
HPLC/DAD Comparison of Sixteen Bioactive Components Between <i>Da-Cheng-Qi</i> Decoction and its Parent Herbal Medicines Fengguo Xu, Ying Liu, Rui Song, Haijuan Dong and Zunjian Zhang	893
Secondary Metabolites of <i>Hypericum confertum</i> and their Possible Chemotaxonomic Significance Cüneyt Çırak, Jolita Radušienė, Valdimaras Janulis and Liudas Ivanauskas	897
Antioxidant Effects of Secondary Metabolites from <i>Geranium psilostemon</i> Didem Şöhretoğlu, Suna Atasayar Sabuncuoğlu, M. Koray Sakar, Hilal Özgüneş and Olov Sterner	899
Bioactive Isoflavones from <i>Dalbergia vacciniifolia</i> (Fabaceae) Ester Innocent, Joseph J. Magadula, Charles Kihampa and Matthias Heydenreich	903
RP-HPLC Analysis and Antidiabetic Activity of <i>Swertia paniculata</i> Jagmohan S. Negi, Pramod Singh, Geeta Joshi née Pant and Mohan S. M. Rawat	907
Antioxidants from the Leaves of <i>Cinnamomum kotoense</i> Kuo-Chen Cheng, Man-Chun Hsueh, Hou-Chien Chang, Alan Yueh-Luen Lee, Hui-Min Wang and Chung-Yi Chen	911

Chemical Constituents and Antimicrobial Activities of <i>Canthium horridum</i> Biao Yang, Guangying Chen, Xiaoping Song, Zhong Chen, Xinming Song and Jing Wang	913
Colon Targeted Curcumin Delivery Using Guar Gum Edwin J. Elias, Singhal Anil, Showkat Ahmad and Anwar Daud	915
Formadienoate-A and B: Two New Long Chained Feruloyl Esters from <i>Clerodendrum formicarum</i> (Lamiaceae) of Cameroon Muhammad Shaiq Ali, Zeeshan Ahmed, Muhammad Imran Ali and Joseph Ngoupayo	919
Free-radical Scavenging Activity of some European Polyporales Kateřina Macáková, Lubomír Opletal, Miroslav Polášek and Věra Samková	923
<i>In vitro</i> Plant Regeneration from Callus of <i>Citrus x monstrosa</i> (Pompia), an Endemic Citrus of Sardinia Daniele Fraternali, Laura Giampieri, Anahi Bucchini, Pierpaolo Cara and Donata Ricci	927
Purification and Biochemical Characterization of Alkaline Serine Protease from <i>Caesalpinia bonducella</i> Hidayatullah Khan, Irshad Ali, Arif-ullah Khan, Mushtaq Ahmed, Zamarud Shah, Ahmad Saeed, Rubina Naz, Mohamad Rais Mustafa and Atiya Abbasi	931
Composition of the Essential Oil of <i>Argania spinosa</i> (Sapotaceae) Fruit Pulp Hicham Harhar, Said Gharby, Mohamed Ghanmi, Hanae El Monfalouti, Dominique Guillaume and Zoubida Charrouf	935
The Volatile Constituents of <i>Salvia leucantha</i> Luis B. Rojas, Tomas Visbal, Marielba Morillo, Yndra Cordero de Rojas, Juan Carmona Arzola and Alfredo Usubillaga	937
Terpenoid Composition of the Essential Oils of <i>Teucrium royleanum</i> and <i>T. quadrifarium</i> Lalit Mohan, Charu C. Pant, Anand B. Melkani and Vasu Dev	939
Influence of Some Environmental Factors on the Essential Oil Variability of <i>Thymus migricus</i> Alireza Yavari, Vahideh Nazeri, Fatemeh Sefidkon and Mohammad Esmail Hassani	943
Chemical Investigations of Essential Oils from Endemic Cupressaceae Trees from New Caledonia Nicolas Lebouvier, Chantal Menut, Edouard Hnawia, Audrey Illinger, Pierre Cabalion and Mohammed Nour	949
Comparative Composition of Four Essential Oils of Oregano Used in Algerian and Jordanian Folk medicine Djemaa Berrehal, Tarek Boudiar, Lakhel Hichem, Assia Khalfallah, Ahmed Kabouche, Ahmad Al-Freihat, Alireza Ghannadi, Ebrahim Sajjadi, Mitra Mehrabani, Jawad Safaei-Ghomi and Zahia Kabouche	957
Chemical Composition and Biological Activities of <i>Santiria trimera</i> (Burseraceae) Essential Oils from Gabon Raphaël Bikanga, Thomas Makani, Huguette Agnani, Louis Clément Obame, Fatouma Mohamed Abdoul-Latif, Jacques Lebibi and Chantal Menut	961
Chemical Composition and Larvicidal Activity of <i>Eugenia triquetra</i> Essential Oil from Venezuelan Andes Flor D. Mora, Jorge L. Avila, Luis B. Rojas, Rosslyn Ramirez, Alfredo Usubillaga, Samuel Segnini, Juan Carmona and Bladimiro Silva	965
Evaluation of Bioactivity of Linalool-rich Essential Oils from <i>Ocimum basilicum</i> and <i>Coriandrum sativum</i> Varieties Ahmet D. Duman, Isa Telci, Kenan S. Dayisoğlu, Metin Digrak, İbrahim Demirtas and Mehmet H. Alma	969
Constituents, Antileishmanial Activity and Toxicity Profile of Volatile Oil from Berries of <i>Croton macrostachyus</i> Yinebeb Tariku, Ariaya Hymete, Asrat Hailu and Jens Rohloff	975
Free Radical Scavenging and Antibacterial Activities, and GC/MS Analysis of Essential oils from Different Parts of <i>Falcaria vulgaris</i> from Two Regions Ali Shafaghat	981
The Effects of Maturity on Chilli Pepper Volatile Components Determined by SDE, GC-MS and HPLC Rong Liu, Ke Xiong, Xiongze Dai, Li Wang, Zhimin Liu and Wentong Xue	985
Number 7	
A DFT Analysis of Thermal Decomposition Reactions Important to Natural Products William N. Setzer	993
Novel Terpenoids from the New Zealand Liverworts <i>Jamesoniella colorata</i> and <i>Bazzania novae-zelandiae</i> Masao Toyota, Ikuko Omatsu, Fumi Sakata, John Braggins and Yoshinori Asakawa	999
Two New C₂₀-Diterpenoid Alkaloids from <i>Delphinium anthriscifolium</i> var. <i>savateri</i> Xiao-Yu Liu, Lei Song, Qiao-Hong Chen, and Feng-Peng Wang	1005
Cytotoxic Activity of Quassinoids from <i>Eurycoma longifolia</i> Katsunori Miyake, Feng Li, Yasuhiro Tezuka, Suresh Awale and Shigetoshi Kadota	1009
Antifungal Activity of Saponin-rich Extracts of <i>Phytolacca dioica</i> and of the Sapogenins Obtained through Hydrolysis Melina Di Liberto, Laura Svetaz, Ricardo L. E. Furlán, Susana A. Zacchino, Carla Delporte, Marco A. Novoa, Marcelo Ascencio and Bruce K. Cassels	1013
New Lupane-type Triterpenoid Saponins from Leaves of <i>Oplopanax horridus</i> (Devil's Club) Pei-Pei Liu, Mo Li, Ting-Guo Kang, De-Qiang Dou and David C Smith	1019

Triterpene Saponins from <i>Cyclamen persicum</i> Ghezala Mihci-Gaidi, David Pertuit, Tomofumi Miyamoto, Jean-François Mirjolet, Olivier Duchamp, Anne-Claire Mitaine-Offer and Marie-Aleth Lacaille-Dubois	1023
Cytotoxic Pentacyclic Triterpenoids from <i>Combretum oliviforme</i> Xiao-Peng Wu, Chang-Ri Han, Guang-Ying Chen, Yuan Yuan and Jian-Ying Xie	1027
Preparative Separation of Four Major Bufadienolides from the Chinese Traditional Medicine, Chansu, Using High-Speed Counter-Current Chromatography Xiu Lan Xin, Junying Liu, Xiao Chi Ma, Qing Wei, Li Lv, Chang Yuan Wang, Ji Hong Yao and Jian Cui	1031
Acetylcholinesterase and Butyrylcholinesterase Inhibitory Compounds from <i>Eschscholzia californica</i> (Papaveraceae) Lucie Cahliková, Kateřina Macáková, Jiří Kuneš, Milan Kurfürst, Lubomír Opletal, Josef Cvačka, Jakub Chlebek and Gerald Blunden	1035
In Vitro Testing for Genotoxicity of Indigo Naturalis Assessed by Micronucleus Test Luca Dominici, Barbara Cerbone, Milena Villarini, Cristina Fatigoni and Massimo Moretti	1039
Metabolites from <i>Withania aristata</i> with Potential Phytotoxic Activity Gabriel G. Llanos, Rosa M. Varela, Ignacio A. Jiménez, José M. G. Molinillo, Francisco A. Macías and Isabel L. Bazzocchi	1043
Bioassay-guided Isolation and Quantification of the α-Glucosidase Inhibitory Compound, Glycyrrhisoflavone, from <i>Glycyrrhiza uralensis</i> Wei Li, Songpei Li, Lin Lin, Hong Bai, Yinghua Wang, Hiroyoshi Kato, Yoshihisa Asada, Qingbo Zhang and Kazuo Koike	1049
Xanthenes, Biflavanones and Triterpenes from <i>Pentadesma grandifolia</i> (Clusiaceae): Structural Determination and Bioactivity Grace Leontine Nwabouloun Djoufack, Karin M. Valant-Vetschera, Johann Schinnerl, Lothar Brecker, Eberhard Lorbeer and Wolfgang Robien	1055
Gmelinoside I, a New Flavonol Glycoside from <i>Limonium gmelinii</i> Zhanar A. Kozhamkulova, Mohamed M. Radwan, Galiya E. Zhusupova, Zharilkasin Zh. Abilov, Saniya N. Rahadilova and Samir A. Ross	1061
2-Arylbenzofuran Neolignans from the Bark of <i>Nectandra purpurascens</i> (Lauraceae) Jaime Rios-Motta and Eliseo Avella	1063
Coumarins from <i>Seseli hartvigii</i> Lin Zhang, Alev Tosun, Masaki Baba, Yoshihito Okada, Lijun Wu and Toru Okuyama	1067
Spartinoxide, a New Enantiomer of A82775C with Inhibitory Activity Toward HLE from the Marine-derived Fungus <i>Phaeosphaeria spartinae</i> Mahmoud Fahmi Elsebai, Stefan Kehraus, Michael Gütschow and Gabriele M. König	1071
The First Total Synthesis of Aspergillusol A, an α-Glucosidase Inhibitor Nisar Ullah and Shamsuddeen A. Haladu	1077
The Effect of a Phytosphingosine-like Substance Isolated from <i>Asterina pectinifera</i> on Involucrin Expression in Mite Antigen-Stimulated HaCaT Cells Gui Hyang Choi, Fazli Wahid and You Young Kim	1081
Chemical Composition of Fatty Acid and Unsaponifiable Fractions of Leaves, Stems and Roots of <i>Arbutus unedo</i> and in vitro Antimicrobial Activity of Unsaponifiable Extracts Mohamed Amine Dib, Julien Paolini, Mourad Bendahou, Laurent Varesi, Hocine Allali, Jean-Marie Desjobert, Boufeldja Tabti and Jean Costa	1085
Poly[3-(3,4-dihydroxyphenyl)glyceric Acid] from <i>Anchusa italica</i> Roots Vakhtang Barbakadze, Lali Gogilashvili, Lela Amiranashvili, Maia Merlani, Karen Mulkiyanyan, Manana Churadze, Antonio Salgado and Bezhana Chankvetadze	1091
New Metabolite from <i>Viburnum dilatatum</i> Bin Wu, Xing Zeng and Yufeng Zhang	1097
Two New Glycosides from <i>Conyza bonariensis</i> Aqib Zahoor, Imran Nafees Siddiqui, Afsar Khan, Viqar Uddin Ahmad, Amir Ahmed, Zahid Hassan, Saleha Suleman Khan and Shazia Iqbal	1099
K⁺_{ATP} Channels-Independent Analgesic Action of <i>Crotalus durissus cumanensis</i> venom Ticiana Praciano Pereira, Adriana Rolim Campos, Luzia Kalyne A. M. Leal, Taiana Magalhães Pierdoná, Marcos H. Toyama, and Helena Serra Azul Monteiro and Alice Maria Costa Martins	1103
Isothymol in Ajowan Essential Oil Chahrazed Bekhechi, Jean Brice Boti, Fewzia Atik Bekkara, Djamel Eddine Abdelouahid, Joseph Casanova and Félix Tomi	1107
GC-MS Analysis of the Essential Oils of Ripe Fruits, Roots and Flowering Aerial Parts of <i>Elaeostelinum asclepium</i> subsp. <i>meoides</i> growing in Sicily Ammar Bader, Pier Luigi Cioni and Guido Flamini	1111
Chemical Composition of the Essential Oil of Leaves and Roots of <i>Ottoa oenanthoides</i> (Apiaceae) from Mérida, Venezuela Janne Rojas, Alexis Buitrago, Luis B. Rojas, Antonio Morales and Shirley Baldovino	1115

Volatile Profiles of <i>Artemisia alba</i> from Contrasting Serpentine and Calcareous Habitats Niko Radulović and Polina Blagojević	1117
Volatile Constituents of Two Rare Subspecies of <i>Thymus praecox</i> Danijela Vidic, Sanja Čavar, Marija Edita Šolić and Milka Maksimović	1123
Antiproliferative and Cytotoxic Effects on Malignant Melanoma Cells of Essential Oils from the Aerial Parts of <i>Genista sessilifolia</i> and <i>G. tinctoria</i> Daniela Rigano, Alessandra Russo, Carmen Formisano, Venera Cardile and Felice Senatore	1127
Chemical Composition and Antibacterial Activity of the Essential Oil of <i>Retrophyllum rospigliosii</i> Fruits from Colombia Clara E. Quijano-Celis, Mauricio Gaviria, Consuelo Vanegas-López, Ina Ontiveros, Leonardo Echeverri, Gustavo Morales and Jorge A. Pino	1133
Essential Oil Composition and Insecticidal Activity of <i>Blumea perrottetiana</i> Growing in Southwestern Nigeria Moses S. Owolabi, Labunmi Lajide, Heather E. Villanueva and William N. Setzer	1135
Chemical Composition, Antibacterial and Antioxidant Activity of the Essential Oil of <i>Bupleurum longiradiatum</i> Baojun Shi, Wei Liu, Shao-peng Wei and Wen-jun Wu	1139
Composition and Antimicrobial and Anti-wood-decay Fungal Activities of the Leaf Essential Oils of <i>Machilus pseudolongifolia</i> from Taiwan Chen-Lung Ho, Pei-Chun Liao, Kuang-Ping Hsu, Eugene I-Chen Wang, Wei-Chih Dong and Yu-Chang Su	1143
<u>Review/Account</u>	
Key Enzymes of Triterpenoid Saponin Biosynthesis and the Induction of Their Activities and Gene Expressions in Plants Chang Ling Zhao, Xiu Ming Cui, Yan Ping Chen and Quan Liang	1147
Number 8	
Phytochemical Investigation of <i>Verbesina turbacensis</i> Kunth: Trypanosome Cysteine Protease Inhibition by (–)-Bornyl Esters Ifedayo V. Ogungbe, Rebecca A. Crouch, William A. Haber and William N. Setzer	1161
Anti-herpetic Activities of Chemical Components from the Brazilian Red Alga <i>Plocamium brasiliense</i> Wilton José Ferreira, Rodrigo Amaro, Diana Negrão Cavalcanti, Claudia Moraes de Rezende, Viveca Antonia Giongo Galvão da Silva, Juliana Eymara Barbosa, Izabel Christina Nunes de Palmer Paixão and Valéria Laneuville Teixeira	1167
Chemical Constituents of the Soft Coral <i>Sarcophyton infundibuliforme</i> from the South China Sea Xue-Ping Sun, Chang-Yun Wang, Chang-Lun Shao, Liang Li, Xiu-Bao Li, Min Chen and Pei-Yuan Qian	1171
Metabolites from the Fungus <i>Phoma</i> sp. 7210, Associated with <i>Aizoon canariense</i> Jingqiu Dai, Hidayat Hussain, Siegfried Dräger, Barbara Schulz, Tibor Kurtán, Gennaro Pescitelli, Ulrich Flörke and Karsten Krohn	1175
Triterpenes from <i>Protium hebetatum</i> Resin Delcio Dias Marques, Ilmar Bernardo Graebner, Telma Leda Gomes de Lemos, Luciana Lucas Machado, João Carlos Costa Assunção and Francisco José Queiroz Monte	1181
Cytotoxicity of 9,11-Dehydroergosterol Peroxide Isolated from <i>Ganoderma lucidum</i> and its Target-related Proteins Ya-Jun Cui, Shu-Hong Guan, Li-Xing Feng, Xiao-Yi Song, Chao Ma, Chun-Ru Cheng, Wen-Bo Wang, Wan-Ying Wu, Qing-Xi Yue, Xuan Liu and De-An Guo	1183
Polar Alkaloids from the Caribbean Marine Sponge <i>Niphates digitalis</i> Erik L. Regalado, Judith Mendiola, Abilio Laguna, Clara Nogueiras and Olivier P. Thomas	1187
A Short Stereoselective Synthesis of Racemic 2-Epicalvine Basem A. Moosa and Shaikh A. Ali	1191
Cytochrome P450 3A4 Inhibitory Activity Studies within the Lycorine series of Alkaloids James McNulty, Jerald J. Nair, Mohini Singh, Denis J. Crankshaw, Alison C. Holloway and Jaime Bastida	1195
Analysis of Amaryllidaceae Alkaloids from <i>Zephyranthes robusta</i> by GC-MS and Their Cholinesterase Activity Lucie Cahliková, Andrea Kulhánková, Klára Urbanová, Irena Valterová, Kateřina Macáková and Jiří Kuneš	1201
Stereochemistry and NMR Data Assignment of Cyclopeptide Alkaloids from <i>Zizyphus oxyphylla</i> Muhammad Nisar, Waqar Ahmad Kaleem, Achyut Adhikari, Zulfiqar Ali, Nusrat Hussain, Inamullah Khan, Mughal Qayum and M. Iqbal Choudhary	1205
Geranylated Flavonols from <i>Macaranga rhizinoides</i> Mulyadi Tanjung, Didin Mujahidin, Euis H. Hakim, Ahmad Darmawan and Yana M. Syah	1209
A New Biflavonyloxymethane from <i>Pongamia pinnata</i> Anindita Ghosh, Suvra Mandal, Avijit Banerji and Julie Banerji	1213
Anti-inflammatory and Gastroprotective Properties of <i>Hypericum richeri</i> Oil Extracts Gordana Zdunić, Dejan Godevac, Marina Milenković, Katarina Šavikin, Nebojša Menković and Silvana Petrović	1215

Production of Flavonoids in Organogenic Cultures of <i>Alpinia zerumbet</i> Cristiane P. Victório, Rosani do Carmo de O. Arruda, Celso Luiz S. Lage and Ricardo M. Kuster	1219
Phenolic Compounds in Leaves of <i>Alchornea triplinervia</i>: Anatomical Localization, Mutagenicity, and Antibacterial Activity Tamara R. Calvo, Diego Demarco, Fabio V. Santos, Helen P. Moraes, Taís M. Bauab, Eliana A. Varanda, Ilce M. S. Cólus and Wagner Vilegas	1225
Phytotoxic Activity of Flavonoids from <i>Dicranostyles ampla</i> Amaya Castro, Charles L. Cantrell, Amber L. Hale and Stephen O. Duke	1233
Flavonoids of <i>Enhydra fluctuans</i> Exhibit Anticancer Activity against Ehrlich's Ascites Carcinoma in Mice Santanu Sannigrahi, Upal Kanti Mazumder, Arijit Mondal, Dilipkumar Pal, Silpi Lipsa Mishra and Souvik Roy	1239
Liquiritigenin Derivatives and Their Hepatoprotective Activity Rashmi Gaur, Sunil Kumar, Priyanka Trivedi, Rajendra Singh Bhakuni, Dnyaneshwar Umrao Bawankule, Anirban Pal and Karuna Shanker	1243
Podophyllotoxin Derivatives Show Activity Against <i>Brontispa longissima</i> Larvae Jing Zhang, Ying-Qian Liu, Liu Yang and Gang Feng	1247
Anthraquinones from the Roots of <i>Prismatomeris tetrandra</i> Cun-Li Zhang, Hua Guan, Peng-Zhou Xi, Tao Deng and Jin-Ming Gao	1251
Inhibitory Effects of Black Pepper (<i>Piper nigrum</i>) Extracts and Compounds on Human Tumor Cell Proliferation, Cyclooxygenase Enzymes, Lipid Peroxidation and Nuclear Transcription Factor-kappa-B Yunbao Liu, Vivek R. Yadev, Bharat B. Aggarwal and Muraleedharan G. Nair	1253
Cinnamoylphenethylamine ¹H-NMR Chemical Shifts: A Concise Reference for Ubiquitous Compounds Hans A. Pedersen, Stine K. Steffensen and Carsten Christophersen	1259
Pro-coagulant Activity of Phenolic Acids Isolated from <i>Blumea riparia</i> Li Huang, Cuiwu Lin, Aiyuan Li, Baoyao Wei, Jianwen Teng and Lue Li	1263
Vascular Effects of a Sulfated Polysaccharide from the Red Marine Alga <i>Solieria filiformis</i> Ana Maria S. Assreuy, Grazielle C. Pontes, Natália V. F. C. Rodrigues, Daniel M. Gomes, Paulo A. Xavier, Glacio S. Araujo, Alexandre H. Sampaio, Benildo S. Cavada, Maria G. Pereira and Wladimir R. L. Farias	1267
Encapsulation and Regeneration of <i>in vitro</i> Derived <i>Zephyranthes grandiflora</i>: an Effective Way for Exchange of Germplasm Moumita Gangopadhyay, Saikat Dewanjee, Dipjyoti Chakraborty and Sabita Bhattacharya	1273
Comparison of Aqueous Plant Extracts Before and After Fermentation with <i>Lactobacillus paracasei</i> LS-2 on Cytokine Induction and Antioxidant Activity Heeson Chon, Gyeomheon Kim and Sungkwon Kim	1277
Volatile Compounds from <i>Tagetes pusilla</i> (Asteraceae) Collected from the Venezuela Andes Diolimar Buitrago, Luis B. Rojas, Janne Rojas and Antonio Morales	1283
Volatile Components of Two Endemic Species from the Apuan Alps (Tuscany, Italy), <i>Centaurea arachnoidea</i> and <i>C. montis-borlae</i> (Asteraceae) Lucia Viegi, Mirko Boracchia, Roberto Cecotti and Aldo Tava	1285
Composition of Essential Oil from Seeds and Cones of <i>Abies alba</i> Anna Wajs, Justyna Urbańska, Ewa Zaleśkiewicz and Radosław Bonikowski	1291
Comparative Analysis of Essential Oil Components of Two <i>Pinus</i> Species from Taibai Mountain in China Yuan Zhang and Zhezhi Wang	1295
Antimicrobial Activity and Volatile Constituents of the Essential Oil of <i>Pulsatilla albana</i> from Iran Ali Shafaghat	1299
Chemical Composition and Antimicrobial Activity of the Essential Oils from <i>Cleome spinosa</i> Megil J. McNeil, Roy B. R. Porter, Lawrence A.D. Williams and Lois Rainford	1301
Virucidal Activity and Chemical Composition of Essential Oils from Aromatic Plants of Central West Argentina Cybele C. García, Eliana G. Acosta, Ana C. Carro, María C. Fernández Belmonte, Renata Bomben, Claudia B. Duschatzky, Marina Perotti, Carola Schuff and Elsa B. Damonte	1307
<i>Neolütsea sericea</i> Essential Oil Attenuates LPS-induced Inflammation in RAW 264.7 Macrophages by Suppressing NF-κB and MAPK Activation Weon-Jong Yoon, Ji-Young Moon, Ji-Yong Kang, Gi-Ok Kim, Nam Ho Lee and Chang-Gu Hyun	1311
Qualitative Analysis of the Smoke-Stream of Different Kinds of Incense by SPME/GC-MS Antonietta Lombardozzi, Morela Strano, Manuela Cortese, Massimo Ricciutelli, Sauro Vittori and Filippo Maggi	1317
Essential Oil Composition and <i>in vivo</i> Volatiles Emission by Different Parts of <i>Coleostephus myconis</i> Capitula Guido Flamini, Pier Luigi Cioni, Simonetta Maccioni and Rosa Baldini	1321
Pesticide and Plasticizer Residues in Citrus Essential Oils from Different Countries Giuseppa Di Bella, Vincenzo Lo Turco, Rossana Rando, Gabriella Arena, Donatella Pollicino, Rosario Rocco Luppino and Giacomo Dugo	1325

Review/Account

Applying New Science for Old Medicines: Targeting Leukocyte-Endothelial Adhesions by Antiinflammatory Herbal Drugs Solomon Habtemariam	1329
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Number 9

Microbial Transformation of (-)-Nopol Benzyl Ether: Direct Dihydroxylation of Benzene Ring Yoshiaki Noma and Yoshinori Asakawa	1339
Structure-Activity Relationships of Sandalwood Odorants: Synthesis of a New Campholene Derivative Iris Stappen, Joris Höfinghoff, Gerhard Buchbauer and Peter Wolschann	1343
Chemical Composition, Olfactory Evaluation and Antimicrobial Activity of Selected Essential Oils and Absolutes from Morocco Juergen Wanner, Erich Schmidt, Stefanie Bail, Leopold Jirovetz, Gerhard Buchbauer, Velizar Gochev, Tanya Girova, Teodora Atanasova and Albena Stoyanova	1349
Chemical Composition and Antimicrobial Activity of Cumin Oil (<i>Cuminum cyminum</i>, Apiaceae) Juergen Wanner, Stefanie Bail, Leopold Jirovetz, Gerhard Buchbauer, Erich Schmidt, Velizar Gochev, Tanya Girova, Teodora Atanasova and Albena Stoyanova	1355
Chemical Composition and Antibacterial Activity of Selected Essential Oils and Some of Their Main compounds Juergen Wanner, Erich Schmidt, Stefanie Bail, Leopold Jirovetz, Gerhard Buchbauer, Velizar Gochev, Tanya Girova, Teodora Atanasova and Albena Stoyanova	1359
Antimicrobial Activities of Single Aroma Compounds Erich Schmidt, Stefanie Bail, Susanne Mirjam Friedl, Leopold Jirovetz, Gerhard Buchbauer, Jürgen Wanner, Zaprjana Denkova, Alexander Slavchev, Albena Stoyanova and Margit Geissler	1365
Essential Oil Composition, Antimicrobial and Cytotoxic Activities of Two Endemic <i>Stachys cretica</i> Subspecies (Lamiaceae) from Turkey Tuba Şerbetçi, Betül Demirci, Çağla Bozkurt Güzel, Şükran Kültür, Mine Ergüven and Kemal Hüsnü Can Başer	1369
Volatile Components of Selected Liverworts, and Cytotoxic, Radical Scavenging and Antimicrobial Activities of Their Crude Extracts Ismiarni Komala, Takuya Ito, Yasuyuki Yagi, Fumihiro Nagashima and Yoshinori Asakawa	1375
Pharmacological Interactions of Essential Oil Constituents on the Viability of Micro-organisms Robyn Lynne Van Zyl, Sammy Tsietsi Seatlholo, Sandy Freda Van Vuuren and Alvaro Viljoen	1381
Antimicrobial Properties of Volatile Phenylpropanes Alexander Pauli and Karl-Heinz Kubeczka	1387
Potential Interaction between the Volatile and Non-volatile Fractions on the <i>In vitro</i> Antimicrobial Activity of Three South African <i>Pelargonium</i> (Geraniaceae) Species Jacqueline Y. Lalli, Alvaro M. Viljoen and Sandy F. Van Vuuren	1395
Encapsulation of Essential Oils within a Polymeric Liposomal Formulation for Enhancement of Antimicrobial Efficacy Sandy F. van Vuuren, Lisa C. du Toit, Ashleigh Parry, Viness Pillay and Yahya E. Choonara	1401
<i>Eupatorium capillifolium</i> Essential Oil: Chemical Composition, Antifungal Activity, and Insecticidal Activity Nurhayat Tabanca, Ulrich R. Bernier, Maia Tsikolia, James J. Becnel, Blair Sampson, Chris Werle, Betül Demirci, Kemal Hüsnü Can Başer, Eugene K. Blythe, Cecil Pounders and David E. Wedge	1409
Raman Optical Activity: A Powerful Technique to Investigate Essential Oil Components Malgorzata Baranska and Katarzyna Chruszcz-Lipska	1417
Microdistillation and Analysis of Volatiles from Eight Ornamental <i>Salvia</i> Taxa Nurhayat Tabanca, Betül Demirci, Jimmy L. Turner, Cecil Pounders, Fatih Demirci, Kemal Hüsnü Can Başer and David E. Wedge	1421
Comparison of Hydrodistillation and Headspace Solid-Phase Microextraction Techniques for Antibacterial Volatile Compounds from the Fruits of <i>Seseli libanotis</i> Krystyna Skalicka-Wozniak, Renata Los, Kazimierz Glowinski and Anna Malm	1427
Volatile Fraction of Lavender and Bitter Fennel Infusion Extracts Christine Tschiggerl and Franz Bucar	1431
Can Glandular Hair Density be a Breeding Marker for <i>Origanum vulgare</i> subsp. <i>hirtum</i> with High Essential Oil Content? Krisztina Szabó, Szilvia Sárosi, Beatrix Cserhádi and Antal Ferenczy	1437
The Influence of Essential Oils on Human Vigilance Eva Heuberger and Josef Ilmberger	1441

Comparison of Liquid-Liquid Partition, HS-SPME and Static HS GC/MS Analysis for the Quantification of (–)-Linalool in Human Whole Blood Samples	
Susanne Mirjam Friedl, Katharina Oedendorfer, Simone Kitzer, Gottfried Reznicek, Guenther Sladek and Eva Heuberger	1447
<i>Salvia officinalis</i> L.: Composition and Antioxidant-related Activities of a Crude Extract and Selected Sub-fractions	
Müberra Koşar, H.J. Damien Dorman, K. Hüsnu Can Başer and Raimo Hiltunen	1453
Developing and Characterizing a Mouse Model of Hepatotoxicity Using Oral Pyrrolizidine Alkaloid (Monocrotaline) Administration, with Potentiation of the Liver Injury by Co-administration of LPS	
Mohamed Sadek Abdel-Bakky, Mohamed A. Hammad, Larry A. Walker and Mohammad K. Ashfaq	1457
Xenobiotic Biotransformation of 4-Methoxy-N-methyl-2-quinolone, Isolated from <i>Zanthoxylum monophyllum</i>	
Raquel Rodriguez-Guzmán, Mohamed M. Radwan, Charles L. Burandt, John S. Williamson and Samir A. Ross	1463
<u>Review/Account</u>	
Volatiles from <i>Thymbra</i> and <i>Thymus</i> species of the Western Mediterranean Basin, Portugal and Macaronesia	
A. Cristina Figueiredo, José G. Barroso and Luis G. Pedro	1465
Essential Oil Composition of Wild Growing Apiaceae from Europe and the Mediterranean	
Remigius Chizzola	1477
Essential Oil and Volatile Components of the Genus <i>Hypericum</i> (Hypericaceae)	
Sara L. Crockett	1493
Terpene Bioconversion – How Does its Future Look?	
Ulrich Krings and Ralf Guenter Berger	1507
Number 10	
Antibacterial and Antifungal Screening of <i>Centaureum pulchellum</i> Crude Extracts and Main Secoiridoid Compounds	
Branislav Šiler, Danijela Mišić, Jasmina Nestorović, Tijana Banjanac, Jasmina Glamočlija, Marina Soković and Ana Čirić	1525
A New Sesquiterpene and other Constituents from <i>Saussurea lappa</i> Root	
Jin-ao Duan, Pengfei Hou, Yuping Tang, Pei Liu, Shulan Su and Hanqing Liu	1531
Terpenoids from <i>Turraeanthus</i> species	
Juliette Catherine Vardamides, Valerie Tedjon Sielinou, Sergi Herve Akone, Augustin Ephrem Nkengfack and Berhanu M. Abegaz	1535
Bioactive Clerodane Diterpenes from Roots of <i>Carex distachya</i>	
Antonio Fiorentino, Brigida D'Abrosca, Severina Pacifico, Angelina Izzo, Grazia D'Angelo and Pietro Monaco	1539
An Unusual Bisanor-clerodane Diterpenoid from <i>Polyalthia simiarum</i>	
Selina Kabir, Mohammad S. Rahman, A. M. Sarwaruddin Chowdhury, Choudhury M. Hasan and Mohammad A. Rashid	1543
Anti-inflammatory Mechanisms of Compounds from <i>Curcuma mangga</i> Rhizomes using RAW264.7 Macrophage Cells	
Kanidta Kaewkroek, Chatchai Wattanapiromsakul and Supinya Tewtrakul	1547
Analysis of MS/MS Fragmentation of Taxoids	
Kouhei Morikawa, Ken Tanaka, Feng Li, Suresh Awale, Yasuhiro Tezuka, Takahiro Nobukawa and Shigetoshi Kadota	1551
New Triterpene Glycosides from <i>Camptosorus sibiricus</i>	
Ning Li, Wan Xiao, Bailing Hou and Xian Li	1557
New Hopane Triterpenes and Antioxidant Constituents from <i>Potentilla fulgens</i>	
Vikas Jaitak, Vijay K. Kaul, Himlata, Neeraj Kumar, Bikram Singh, Jyoti Dhar and Om P. Sharma	1561
Cytotoxic Evaluation of Semisynthetic Ester and Amide Derivatives of Oleanolic Acid	
Shikha Gupta, Komal Kalani, Mohit Saxena, Santosh K. Srivastava, Satyam K. Agrawal, Nitasha Suri and Ajit K. Saxena	1567
Two Ring-A-Aromatized Bile Acids from the Marine Sponge <i>Sollasella moretonensis</i>	
Zhenyu Lu, Ryan M. Van Wagoner, Mary Kay Harper, John N. A. Hooper and Chris M. Ireland	1571
Asporergosterol, A New Steroid from an Algicolous Isolate of <i>Aspergillus oryzae</i>	
Ming-Feng Qiao, Nai-Yun Ji, Xiang-Hong Liu, Fang Li and Qin-Zhao Xue	1575
Two Minor Phytoecdysteroids of the Plant <i>Silene viridiflora</i>	
Nilufar Zokirzhonovna Mamadalieva, Abdulaziz Adilhanovich Janibekov, Jean-Pierre Girault and René Lafont	1579
7-O-Methylvariecolorotide A, a New Spirocyclic Diketopiperazine Alkaloid from a Marine Mangrove Derived Endophytic Fungus, <i>Eurotium rubrum</i>	
Dong-Li Li, Xiao-Ming Li, Peter Proksch and Bin-Gui Wang	1583
Cytotoxic Thiocarbamate Derivatives of Boldine	
Franz A. Thomet, Pablo Pinyol, Joan Villena, Luis J. Espinoza and Patricio G. Reveco	1587
Synthesis and Bioactivity of β-Carboline Derivatives	
Shengkun Li, Bing Yang, Qianliang Zhang, Jiwen Zhang, Junru Wang and Wenjun Wu	1591
Two Acylated Flavonoid Glycosides from the Leaves of <i>Quercus dentata</i>	
Ling-Li Wang, Mei-Xiang Jiang, Sui-Xu Xu, Qi-Shi Sun, Guang-Yao Zeng and Ying-Jun Zhou	1597

Cytotoxicity of Constituents from Mexican Propolis Against a Panel of Six Different Cancer Cell Lines Feng Li, Suresh Awale, Yasuhiro Tezuka and Shigetoshi Kadota	1601
Antihepatotoxic and Antioxidant Activities of Methanol Extract and Isolated Compounds from <i>Ficus chlamydocarpa</i> Jean Hubert Donfack, Christophe Colombe Fotso Simo, Bathelmy Ngamei, Angèle N. Tchana, Philip G. Kerr, Paola Vita Finzi, Giovanni Vidari, Silvana Giardina, Daniela Buonocore, Bonaventure T. Ngadjui, Paul F. Moundipa and Fulvio Marzatico	1607
Anthocyanin-Rich Black Currant Extract Suppresses the Growth of Human Hepatocellular Carcinoma Cells Anupam Bishayee, Erzsébet Háznagy-Radnai, Thomas Mbimba, Péter Sipos, Paolo Morazzoni, Altaf S. Darvesh, Deepak Bhatia and Judit Hohmann	1613
New Coumarin-Hemiterpene Ether Glucosides and a Structurally Related Phenylpropanoic Acid Derivative from <i>Artemisia armeniaca</i> Mahdi Mojarab, Abbas Delazar, Matthias Hamburger and Olivier Potterat	1619
Synthesis of Fluorescent Analogues of the Anticancer Natural Products 4-Hydroxyphenylmethyle Hydantoin and δ-Tocotrienol Mudit Mudit, Fathy A. Behery, Vikram B. Wali, Paul W. Sylvester and Khalid A. El Sayed	1623
Two Pairs of Enantiomeric Neolignans from <i>Lobelia chinensis</i> Jian-Xin Chen, Shen-Hui Huang, Lei Wang, Wei-Li Han, Ying Wang, Dong-Mei Zhang and Wen-Cai Ye	1627
Evaluation and Discrimination of Cortex <i>Magnoliae officinalis</i> Produced in Zhejiang Province (Wen-Hou-Po) by UPLC-DAD-TOF-MS Fingerprint Lin Wang, Ke Yuan, Wei-Wu Yu and Jing Wang	1631
Phenolic Compounds from <i>Eucalyptus gomphocephala</i> with Potential Cytotoxic and Antioxidant Activities Eman Al-Sayed, Olli Martiskainen, Małgorzata Bobrowska-Hägerstrand, Jari Sinkkonen, Kid Törnquist, Kalevi Pihlaja, Nahla Ayoub and Abdel-Nasser Singab	1639
Nutritional Value of the Chilean Seaweeds <i>Cryptonemia obovata</i> and <i>Rhodymenia corallina</i> Jaime Ortiz, Juan Vivanco, Paula Jiménez, Moisés Leiva, Leslie Ramírez and Andrés Bustamante	1643
Volatile Components of <i>Centaurea bracteata</i> and <i>C. pannonica</i> subsp. <i>pannonica</i> growing wild in Croatia Carmen Formisano, Felice Senatore, Svetlana Bancheva, Maurizio Bruno, Antonella Maggio and Sergio Rosselli	1649
Biodiversity of Volatile Organic Compounds from Five French Ferns Françoise Fons, Didier Froissard, Jean-Marie Bessière, Bruno Buatois and Sylvie Rapior	1655
Composition and Chemical Variability of Leaf Oil of <i>Myrtus communis</i> from North-Eastern Algeria Amel Bouzabata, Faffani Boussaha, Joseph Casanova and Félix Tomi	1659
Chemical Composition and Antimicrobial Activity of Essential Oils from <i>Centaurea pannonica</i> and <i>C. jacea</i> Tanja Milošević, Catherine Argyropoulou, Slavica Solujić, Dragana Murat-Spahić and Helen Skaltsa	1663
GC/MS Analysis and Antimicrobial Activity of the Essential Oil of Fresh Leaves of <i>Eucalyptus globulus</i>, and Leaves and Stems of <i>Smyrniolus olusatrum</i> from Constantine (Algeria) Habiba Daroui-Mokaddem, Ahmed Kabouche, Mabrouka Bouacha, Boudjemaa Soumati, Aida El-Azzouny, Christian Bruneau and Zahia Kabouche	1669
Chemical Composition and Antifungal Activity of Essential Oils of <i>Thuja sutchuenensis</i>, a Critically Endangered Species Endemic to China Huaping Lei, Yonggang Wang, Chang Su, Fengyin Liang, Weiwei Su, Mamie Hui, Pangchui Shaw and Yulong Luo	1673
Composition and Antifungal Activities of the Leaf Essential oil of <i>Litsea coreana</i> from Taiwan Chen-Lung Ho, Kuang-Ping Hsu, Yen-Hsueh Tseng, Pei-Chun Liao, Eugene I-Chen Wang, Narumon Jeyashoke, Tzu-Chao Chien, Wei-Chih Dong and Yu-Chang Su	1677
Essential Oil Polymorphism of Wild Growing Hungarian Thyme (<i>Thymus pannonicus</i>) Populations in the Carpathian Basin Zsuzsanna Pluhár, Szilvia Sárosi, Adrienn Pintér and Hella Simkó	1681
<u>Review/Account</u>	
Naturally Occurring Diarylheptanoids Haining Lv and Gaimei She	1687
Number 11	
Alkaline Phosphatase (ALP) Enhancing Iridoid Glucosides from the Indonesian Medicinal Plant <i>Barleria lupulina</i> Retno Widyowati, Yasuhiro Tezuka, Taturou Miyahara, Suresh Awale and Shigetoshi Kadota	1711
Two New Sesquiterpenes from <i>Sarcandra glabra</i> Do Thi Oanh, Pham Thanh Ky, Nguyen Thi Bich Hang, Pham Hai Yen, Tran Hong Hanh, Nguyen Xuan Cuong, Dang Vu Luong, Chau Van Minh and Phan Van Kiem	1717
New Acyclic Diterpenic Acids from Yacon (<i>Smallanthus sonchifolius</i>) Leaves María I. Mercado, María V. Coll Aráoz, Alfredo Grau and César A. N. Catalán	1721

4-Deacetylbaecatin III: a Proposed Biosynthetic Precursor of Paclitaxel from the Bark of <i>Taxus wallichiana</i> Muhammad Nisar, Mughal Qayum, Achyut Adhikari, Inamullah Khan, Waqar Ahamad Kaleem, Zulfiqar Ali and M. Iqbal Choudhary	1727
Chemical Composition of Natural Colophony from <i>Pinus brutia</i> and Comparison with Synthetic Colophony Ahmet C. Gören, Gökhan Bilsel, Alp Hakan Öztürk and Gülaçtı Topçu	1729
Straightforward Approach to the Discrimination of (4R)- and (4S)-β-Isocryptoxanthin from a Conformationally Insensitive CD Band Shinzo Hosoi, Takeyuki Tanaka, Yukiteru Katsumoto, Takashi Maoka, Toshio Fujiwara, Masayuki Yamashita and Manabu Node	1733
Two New Steroidal Saponins, Hylodoside A and Novaeguinoside Y, from the Starfish <i>Leptasterias hylodes reticulata</i> and <i>Culcita novaeguineae</i> (Juvenile) Eleonora V. Levina, Anatoly I. Kalinovsky, Pavel S. Dmitrenok, Ekaterina A. Martyyas and Valentin A. Stonik	1737
New Steroidal Alkaloids from <i>Solanum hypomalacophyllum</i> Alida Pérez Colmenares, Libia Alarcón, Luis B. Rojas, Anne-Claire Mitaine-Offer, Laurent Pouységu, Stéphane Quideau, Thomas Paululat, Alfredo Usubillaga and Marie-Aleth Lacaille-Dubois	1743
Alkaloidal Constituents of <i>Tinospora crispa</i> M. Iqbal Choudhary, Muhammad Ismail, Zulfiqar Ali, Khozirah Shaari, Nordin H. Lajis and Atta-ur-Rahman	1747
Acetylcholinesterase and Butyrylcholinesterase Inhibitory Compounds from <i>Chelidonium majus</i> (Papaveraceae) Lucie Cahliková, Lubomír Opletal, Milan Kurfürst, Kateřina Macáková, Andrea Kulhánková and Anna Hošťálková	1751
Identification of <i>Glycyrrhiza</i> Species by Direct Analysis in Real Time Mass Spectrometry Eriko Fukuda, Masaki Baba, Noriaki Iwasaki, Yoshihiro Uesawa, Kazunori Arifuku, Osamu Kamo, Koji Tsubono and Yoshihito Okada	1755
DPPH-Scavenging Activities and Structure-Activity Relationships of Phenolic Compounds Cheng-Dong Zheng, Gang Li, Hu-Qiang Li, Xiao-Jing Xu, Jin-Ming Gao and An-Ling Zhang	1759
RP-HPLC Analysis of <i>Jirakadyarishita</i> and Chemical Changes during Fermentation Uma Ranjan Lal, Shailendra Mani Tripathi, Sanjay M. Jachak, Kamlesh Kumar Bhutani and Inder Pal Singh	1767
Isoflavones from the Mangrove Endophytic Fungus <i>Fusarium</i> sp. (ZZF41) Zhongjing Huang, Jianxiang Yang, Zhigang She and Yongcheng Lin	1771
HPLC/DAD/MS and Antioxidant Activity of Isoflavone-Based Food Supplements Annalisa Romani, Pamela Vignolini, Annalisa Tanini, Barbara Pampaloni and Daniela Heimler	1775
A New Biisoflavonoid from the Roots of <i>Erythrina variegata</i> Hitoshi Tanaka, Masaru Sudo, Miyuki Hirata, Hideo Etoh, Masaru Sato, Ryozo Yamaguchi, Eiji Sakai, Ih-Sheng Chen and Toshio Fukai	1781
Chemical Constituents of <i>Nepeta distans</i> Javid Hussain, Nausheen Bukhari, Hidayat Hussain, Najeeb U Rehman and Syed Murtaza Hussain	1785
Two new Diarylheptanoids from <i>Alnus nitida</i> Imran N. Siddiqui, Viqar U. Ahmad, Aqib Zahoor, Amir Ahmed, Saleha S. Khan, Afsar Khan and Zahid Hassan	1787
(-)-Sclerotiorin from an Unidentified Marine Fungus as an Anti-meiotic and Anti-fungal Agent Li Bao, Zhenyu Xu, Shu-bin Niu, Michio Namikoshi, Hisayoshi Kobayashi and Hong-wei Liu	1789
Mitrepenin, a New Annonaceous Acetogenin from <i>Mitrephora maingayi</i> Qiang Zhang, Ying-Tong Di, Hong-Ping He, Shun-Lin Li and Xiao-Jiang Hao	1793
Pycnangloside: A New Cerebroside from Bark of <i>Pycnanthus angolensis</i> Valérie Béatrice Tsaassi, Hidayat Hussain, Hélène Tamboue, Etienne Dongo, Simeon F. Kouam and Karsten Krohn	1795
Long Argan Fruit Drying Time is Detrimental for Argan Oil Quality Hicham Harhar, Saïd Gharby, Badr Eddine Kartah, Hanae El Monfalouti, Zoubida Charrouf and Dom Guillaume	1799
Volatiles from Steam-distilled Leaves of Some Plant Species from Madagascar and New Zealand and Evaluation of Their Biological Activity Rosaria Costa, Francesco Pizzimenti, Francesca Marotta, P. Dugo, Luca Santi and Luigi Mondello	1803
Volatile Constituents of Different Parts of <i>Smyrniolus olusatrum</i> from Greece Fotini Papaioannou, Aikaterini Koutsaviti and Olga Tzakou	1809
Volatile Constituents of <i>Senecio pterophorus</i> (African Daisy) DC. from South Africa Oladipupo A. Lawal and Adebola O. Oyediji	1811
Essential Oil Constituents and Biological Activities of <i>Peristrophe bicalyculata</i> and <i>Borreria verticillata</i> Isiaka A. Ogunwande, Tameka M. Walker, Anita Bansal, William N. Setzer and Emmanuel E. Essien	1815
Insecticidal Activity Against <i>Bemisia tabaci</i> Biotype B of Peel Essential Oil of <i>Citrus sinensis</i> var. pear and <i>Citrus aurantium</i> Cultivated in Northeast Brazil Nicolle de Carvalho Ribeiro, Claudio Augusto Gomes da Camara, Flávia de Souza Born and Herbert Álvaro Abreu de Siqueira	1819
Composition and Antimicrobial Activity of the Leaf and Twig Oils of <i>Litsea mashaensis</i> and <i>L. linii</i> from Taiwan Chen-Lung Ho, Eugene I-Chen Wang, Yen-Hsueh Tseng, Pei-Chun Liao, Chien-Nan Lin, Ju-Ching Chou and Yu-Chang Su	1823

Essential Oil of <i>Turnera ulmifolia</i> Leaves from Cuba Jorge A. Pino	1829
Essential Oil of <i>Galinsoga parviflora</i> Leaves from Colombia Jorge A. Pino, Mauricio Gaviria, Juana Quevedo-Vega, Laura García-Lesmes and Clara E. Quijano-Celis	1831
Essential Oil Composition of Three Australian Endemic Species of <i>Darwinia</i> (Myrtaceae) Joseph J. Brophy, Robert J. Goldsack, Jesús Palá-Paúl, Lachlan M. Copeland and Erich V. Lassak	1833
Chemistry and Biological Activity of Essential Oils from <i>Piper clausenianum</i> (Piperaceae) André M. Marques, Anna Léa S. Barreto, Eber M. Batista, José Alexandre da R. Curvelo, Leosvaldo S. M. Velozo, Davyson de L. Moreira, Elsie F. Guimarães, Rosângela Maria A. Soares and Maria Auxiliadora C. Kaplan	1837
Antioxidant Activity and Chemical Composition of Essential Oil from <i>Atriplex undulata</i> Silvana A. Rodriguez and Ana P. Murray	1841

Number 12

Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence Damião P. de Sousa, Franklin F. F. Nóbrega, Camila C. M. P. Santos and Reinaldo N. de Almeida	1847
Kinetic Analysis of Genipin Degradation in Aqueous Solution Paul Slusarewicz, Keng Zhu and Tom Hedman	1853
Microbial Transformation of Marine Halogenated Sesquiterpenes Aurelio San Martin, Juana Roviroso, Alvaro Carrasco, Silvia Orejarena, Jorge Soto-Delgado, Renato Contreras and M. Cristina Chamy	1859
Two New Guaianolides from <i>Amberboa ramosa</i> Muhammad Ibrahim, Rehan Khan and Abdul Malik	1865
Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from <i>Canella winterana</i> Mary H. Grace, Carmen Lategan, Flaubert Mbeunkui, Rocky Graziöse, Peter J. Smith, Ilya Raskin and Mary Ann Lila	1869
Three New 18-Oxygenated <i>ent</i>-Kaurane Diterpenoids from <i>Isodon leucophyllus</i> Hai Bo Zhang, Jian Xin Pu, Yong Zhao, Fei He, Wei Zhao, Li Guang Lou, Wei Lie Xiao and Han Dong Sun	1873
Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber <i>Cucumaria okhotensis</i>: Stimulation of Activity of Mouse Peritoneal Macrophages Dmitry L. Aminin, Alexandra S. Silchenko, Sergey A. Avilov, Vadim G. Stepanov and Vladimir I. Kalinin	1877
Three New Aaptamines from the Marine Sponge <i>Aaptos</i> sp. and Their Proapoptotic Properties Larisa K. Shubina, Tatyana N. Makarieva, Sergey A. Dyshlovoy, Sergey N. Fedorov, Pavel S. Dmitrenok and Valentin A. Stonik	1881
Isolation and Characterization of Crotosparamide, a New Cyclic Nonapeptide from <i>Croton sparsiflorus</i> Rashad Mehmood and Abdul Malik	1885
Two New Lavandulyl Flavonoids from <i>Sophora flavescens</i> Dan Liu, Xiulan Xin, Dong-hai Su, Junying Liu, Qing Wei, Bo Li and Jian Cui	1889
Biotransformation of Naringenin to Eriodictyol by <i>Saccharomyces cerevisiae</i> Functionally Expressing Flavonoid 3' Hydroxylase Ilef Limem-Ben Amor, Alain Hehn, Emmanuel Guedon, Kamel Ghedira, Jean-Marc Engasser, Leila Chekir-Ghedrira and Mohamed Ghoul	1893
Two New 3-C-Carboxylated Flavones from the Rhizomes of <i>Caragana conferta</i> Rehan Khan, Abdul Malik, Shazia Yasmeen and Nighat Afza	1899
Kaempferol Glycosides in the Flowers of Carnation and their Contribution to the Creamy White Flower Color Tsukasa Iwashina, Masa-atsu Yamaguchi, Masayoshi Nakayama, Takashi Onozaki, Hiroyuki Yoshida, Shuji Kawanobu, Hiroshi Ono and Masachika Okamura	1903
Factors Influencing Glabridin Stability Mingzhang Ao, Yue Shi, Yongming Cui, Wentao Guo, Jing Wang and Longjiang Yu	1907
Effect of Different Strains of <i>Agrobacterium rhizogenes</i> and Nature of Explants on <i>Plumbago indica</i> Hairy Root Culture with Special Emphasis on Root Biomass and Plumbagin Production Moumita Gangopadhyay, Saikat Dewanjee, Somnath Bhattacharyya and Sabita Bhattacharya	1913
Fujianmycin C, A Bioactive Angucyclinone from a Marine Derived <i>Streptomyces</i> sp. B6219 Muna Ali Abdalla, Elisabeth Helmke and Hartmut Laatsch	1917
Dioscorealide B from the Traditional Thai Medicine Hua-Khao-Yen Induces Apoptosis in MCF-7 Human Breast Cancer Cells via Modulation of Bax, Bak and Bcl-2 Protein Expression Jiraporn Saekoo, Potchanapond Graidist, Wilairat Leeanansaksiri, Chavaboon Dechsukum and Arunporn Itharat	1921
Inhibition of Protein Tyrosine Phosphatase 1β by Hispidin Derivatives Isolated from the Fruiting Body of <i>Phellinus linteus</i> Yeon Sil Lee, Il-Jun Kang, Moo Ho Won, Jae-Yong Lee, Jin Kyu Kim and Soon Sung Lim	1927

A New Azafluorenone from the Roots of <i>Polyalthia cerasoides</i> and its Biological Activity Kanchana Pumsalid, Haruthai Thaisuchat, Chatchanok Loetchutinat, Narong Nuntasaeen, Puttinan Meepowpan and Wilart Pompimon	1931
Evaluation of Antiviral Activities of Curcumin Derivatives against HSV-1 in Vero Cell Line Keivan Zandi, Elissa Ramedani, Khosro Mohammadi, Saeed Tajbakhsh, Iman Deilami, Zahra Rastian, Moradali Fouladvand, Forough Yousefi and Fatemeh Farshadpour	1935
Hyloglyceride and Hylodiglyceride: Two New Glyceride Derivatives from <i>Hyloidendron gabunensis</i> Awazi Tengu Nyongha, Hidayat Hussain, Etienne Dongo, Ishtiaq Ahmed and Karsten Krohn	1939
Chemical Composition and Bioactivities of the Marine Alga <i>Isochrysis galbana</i> from Taiwan Chi-Cheng Yu, Hsiao-Wei Chen, Mao-Jing Chen, Yu-Ching Chang, Shih-Chang Chien, Yueh-Hsiung Kuo, Feng-Ling Yang, Shih-Hsiung Wu, Jie Chen, Hsiao-Hui Yu and Louis Kuop-Ping Chao	1941
An Efficient Protocol for High-frequency Direct Multiple Shoot Regeneration from Internodes of Peppermint (<i>Mentha x piperita</i>) Sanjog T. Thul and Arun K. Kukreja	1945
Essential Oil Yield and Chemical Composition Changes During Leaf Ontogeny of Palmarosa (<i>Cymbopogon martinii</i> var. <i>motia</i>) Bhaskaruni R. Rajeswara Rao, Dharmendra K. Rajput, Rajendra P. Patel and Somasi Purnanand	1947
Essential Oil Composition of Four Endemic <i>Ferulago</i> Species Growing in Turkey Ceyda Sibel Kılıç, Ayşe Mine Gençler Özkan, Betül Demirci, Maksut Coşkun and Kemal Hüsni Can Başer	1951
Essential Oils of <i>Daucus carota</i> subsp. <i>carota</i> of Tunisia Obtained by Supercritical Carbon Dioxide Extraction Hanen Marzouki, Abdelhamid Khaldi, Danilo Falconieri, Alessandra Piras, Bruno Marongiu, Paola Molicotti and Stefania Zanetti	1955
Oil Constituents of <i>Artemisia nilagirica</i> var. <i>septentrionalis</i> Growing at Different Altitudes Flora Haider, Narendra Kumar, Ali Arif Naqvi and Guru Das Bagchi	1959
Volatile Oil Composition of <i>Pogostemon heyneanus</i> and Comparison of its Composition with Patchouli Oil Ramar Murugan, Gopal Rao Mallavarapu, Kyathsandra Venkataramaiah Padmashree, Ramachandra Raghavendra Rao and Christus Livingstone	1961
Chemical Composition of Volatile Oils of <i>Aquilaria malaccensis</i> (Thymelaeaceae) from Malaysia Saiful Nizam Tajuddin and Mashitah M. Yusoff	1965
Chemical Composition and Phytotoxic Effects of Essential Oils from Four <i>Teucrium</i> Species Laura De Martino, Carmen Formisano, Emilia Mancini, Vincenzo De Feo, Franco Piozzi, Daniela Rigano and Felice Senatore	1969
Chemical Constituents and Larvicidal Activity of <i>Hymenaea courbaril</i> Fruit Peel José Cláudio D. Aguiar, Gilvandete M. P. Santiago, Patricia L. Lavor, Helenicy N. H. Veras, Yana S. Ferreira, Michele A. A. Lima, Ângela M. C. Arriaga, Telma L. G. Lemos, Jefferson Q. Lima, Hugo C. R. de Jesus, Péricles B. Alves and Raimundo Braz-Filho	1977
Caryophyllene Oxide-rich Essential Oils of Lithuanian <i>Artemisia campestris</i> ssp. <i>campestris</i> and Their Toxicity Asta Judzentiene, Jurga Budiene, Rita Butkiene, Eugenija Kupcinskiene, Isabelle Laffont-Schwob and Véronique Masotti	1981
Comparison of Antibacterial Activity of Natural and Hydroformylated Essential Oil of <i>Thymus capitatus</i> Growing Wild in North Sardinia with Commercial <i>Thymus</i> Essential Oils Marianna Usai, Marzia Foddai, Barbara Sechi, Claudia Juliano and Mauro Marchetti	1985
Composition and Chemical Variability of the Leaf Oil from Corsican <i>Juniperus thurifera</i> Integrated Analysis by GC(RI), GC-MS and ¹³C NMR Josephine Ottavioli, Joseph Casanova and Ange Bighelli	1991
Combined Analysis by GC (RI), GC-MS and ¹³C NMR of the Supercritical Fluid Extract of <i>Abies alba</i> Twigs Emilie Duquesnoy, Bruno Marongiu, Vincent Castola, Alessandra Piras, Silvia Porcedda and Joseph Casanova	1995
<u>Review/Account</u>	
Eugenol: A Natural Compound with Versatile Pharmacological Actions Kannissery Pramod, Shahid H. Ansari and Javed Ali	1999

Natural Product Communications

Volume 5 (1-12)

2010

Author Index

Abbasi, A	931	Ansari, SH	1999	Bastida, J	1195	Bucar, F	1431
Abdalla, MA	567,1917	Ao, M	1907	Bastow, KF	675	Bucchini, A	315,927
Abdel-Bakky, MS	1457	Aparicio, R	311	Bauab, TM	1225	Buchbauer, G	1343,1349,1355
Abdelouahid, DE	1107	Arak, E	133	Bawankule, DU	1243	Buchbauer, G	1359,1365
Abdoul-Latif, FM	961	Aráoz, MVC	1721	Bazzocchi, IL	1043	Budiene, J	1981
Abegaz, BM	747,1535	Araujo, GS	1267	Becerra, JX	351	Buitrago, A	1115
Abilov, ZZ	1061	Arbola, JC	615,937	Becnel, JJ	1409	Buitrago, D	1283
Aboaba, SA	481	Arena, G	1325	Bedir, E	249,841	Bukhari, N	1785
AbouZid, S	43	Arfan, M	211	Behery, FA	1623	Bülow, AK	709
Abrosca, BD	99	Argyropoulou, C	1663	Bekhechi, C	1107	Bun, H	877
Açık, L	147	Arias, E	615	Bekkara, FA	1107	Bun, S-S	877
Acosta, EG	1307	Arifuku, K	1755	Bell, EA	485	Buonocore, D	1607
Adane, L	747	Arnhold, J	415	Bella, GD	1325	Burandt, CL	1463
Adhikari, A	1205,1727	Arnold, N	869	Belmonte, MCF	1307	Bustamante, A	1643
Adigüzel, N	297	Arriaga, AMC	1977	Benayahu, Y	205	Butkiene, R	1981
Afsar Khan, A	1099	Arruda, RCO	1219	Bendahou, M	1085	Buyuktas, BS	111
Afza, N	1899	Asada, Y	1049	Bensemhoun, J	259		
Aggarwal, BB	1253	Asakawa, Y	695,999,1339,1375	Berger, RG	1503	Cabalion	949
Agnaniet, H	961	Asencio, M	1013	Bermejo, J	35	Caboni, P	551
Agrawal, SK	1567	Ashfaq, MK	1457	Bernáth, J	291	Cabras, P	329
Aguiar, JCD	1977	Assreuy, AMS	1267	Bernier, UR	1409	Cahliková, L	1035,1201,1751
Ahmad, A	345	Assunção, JCC	1181	Berrehal, D	957	Calvo, TR	1225
Ahmad, G	427	Atanasova, T	1349,1355,1359	Bertoli, A	291	Camara, CAG	1819
Ahmad, S	915	Atta-ur-Rahman	1747	Bessière, JM	1655	Camara, CAGD	471
Ahmad, VU	1099,1787	Avella, E	1063	Bhakuni, RS	1243	Camas, N	535
Ahmad, VU	191,419,529	Avila, JL	965	Bharti, N	867	Campos, AR	1103
Ahmed, A	1099,1787	Ávila, VMC	235	Bhat, S	717	Caniato, R	431
Ahmed, I	1939	Avilov, SA	1877	Bhatia, D	1613	Cantrell, CL	1233
Ahmed, M	931	Awale, S	17,1009,1551,1601,1711	Bhattacharya, S	1273,1913	Cao, BY	197
Ahmed, Z	889,919	Awen, BZ	621	Bhattacharyya, S	1913	Cao, S	751
Aiyelaagbe, OO	481	Aydođdu, I	841	Bhutani, KK	575,177	Çapanlar, S	563
Ajlouni, A	5	Ayoub, N	545,1639	Bi, H	447	Cara, P	927
Akanksha	427	Azam, A	867	Bighelli, A	1991	Cardile, V	1127
Aknin, M	33,259	Aziz, AA	441	Bikanga, R	961	Carmo, HM	179
Akone, SH	1535	Azizi, M	283	Bilsel, G	1729	Carmona, J	965
Alankuş-Çalışkan, Ö	841	Azizi, ME	545	Bini, LM	107	Carpenter, B	607
Alarcón, L	1743			Birkinshaw, C	751	Carrasco, A	1859
Alemaný, JU	817	Baba, M	1067,1755	Bishayee, A	1613	Carrera, E	435
Alemayehu, G	747	Babu, GDK	641	Blagojević, P	1117	Carro, AC	1307
Al-Freihat, A	957	Bader, A	1111	Blagojević, PD	121	Carvalho, ACDS	523
Ali, I	931,1999	Bagchi, GD	1959	Blunden, G	581,1035	Casanova, J	1107,1659,1991,1995
Ali, MI	889,919	Baghdikian, B	877	Blythe, EK	1409	Cassels, BK	1013
Ali, MS	889,919	Bai, H	1049	Böke, N	563	Castola, V	1995
Ali, SA	1191	Bail, S	1349,1355,1359,1365	Bombarda, I	115	Castro, A	1233
Ali, Z	771,1727,1747,1205	Baldini, R	1321	Bomben, R	1307	Casu, L	551
Alipieva, K	51	Baldovino, S	1115	Bonikowski, R	1291	Catalán, CAN	1721
Aljančić, I	729	Bancheva, S	273,1649	Boracchia, M	1285	Cavada, BS	1267
Allali, H	1085	Banerji, A	1213	Born, FS	1819	Cavalcanti, DN	1167
Alma, MH	969	Banerji, J	1213	Boroto, J	809	Cavaleiro, C	137
Almeida, C	507	Banjanac, T	1525	Bory, S	877	Cavar, S	1123
Almeida, MC	733	Bankova, V	51	Botelho, PDS	471	Cecotti, R	1285
Alonso, MJP	817	Bano, J	725	Boti, JB	1107	Célsis, CQ	613
Al-Sayed, E	1639	Bansal, A	1815	Bouacha, M	1669	Cerbone, B	1039
Alves, PB	1977	Bao, L	1789	Boudiar, T	35,849,957	Chaker, AN	835
Amaro, R	1167	Baranska, M	1417	Bouriche, H	415	Chakraborty, D	1273
Ambrosio, SR	669	Barbakadze, V	1091	Boussaha, F	1659	Chalard, P	835
Amin, H	211	Barbosa, JE	1167	Bouzabata, A	1659	Chalchat, JC	835
Aminin, DL	1877	Bardón, A	391	Braggins, J	999	Chamy, MC	1859
Amiranashvili, L	1091	Barra, A	329	Brecker, L	1055	Chang, CI	55
Amor, ILB	777,1893	Barrera, EDC	383	Brodie, PJ	751	Chang, H-C	911
Andrade, EHA	477	Barroso, JG	319,1465	Brophy, JJ	817,1833	Chang, ST	73
Andriantsiferana, R	751	Başer, KHC	147,297,515,1369	Brouard, I	35	Chang, YC	1941
Angioni, A	329	Başer, KHC	1409,1421,1453,1951	Bruneau, C	35,849,1669	Chankvetadze, B	1091
Anil, S	915	Basha, FZ	529	Bruno, M	273,675,1649	Chanotiya, CS	269,279
Anjum, S	529	Bassuony, AE	43	Buatois, B	1655	Chao, LKP	1941

- Charrouf, Z935,1799
 Chávez, M235
 Chen, CR55
 Chen, C-Y911
 Chen, G733,913
 Chen, G-Y1027
 Chen, H541,783
 Chen, HW1941
 Chen, IS1781
 Chen, J1941
 Chen, JX1627
 Chen, M1171
 Chen, MJ1941
 Chen, Q-H1005
 Chen, WC55
 Chen, YL617
 Chen, YP1147
 Chen, Z913
 Cheng, C-R1183
 Cheng, K-C911
 Cheng, SK877
 Chien, SC1941
 Chien, TC1677
 Chizzola, R283,1477
 Chlebek, J1035
 Choi, GH1081
 Chon, H1277
 Choonara, YE1401
 Chou, JC1823
 Choudhary, MI1205,1727,1747
 Chowdhury, AMS1543
 Christophersen, C1259
 Chruszcz-Lipska, K1417
 Chugh, M867
 Chung, HY151
 Chung, I-M851
 Churadze, M1091
 Cicek, D873
 Cioni, PL1111,1321
 Cirak, C535,897
 Ćirić, A1525
 Colmenares, AP615,1743
 Colom, OA391
 Contreras, R1859
 Copeland, LM1833
 Coroneo, V329
 Cortese, M1317
 Coşkun, M1951
 Costa, FBD669
 Costa, J1085
 Costa, R1803
 Costantin, MB755
 Cottiglia, F551
 Couladis, M307
 Crankshaw, DJ1195
 Critchley, AT581
 Crockett, SL1493
 Crotti, AEM681
 Crouch, RA1161
 Cruz, C319
 Cserhádi, B1437
 Cui, J1031,1889
 Cui, XM1147
 Cui, Y1907
 Cui, Y-J1183
 Cuong, NM103
 Cuong, NX361,423,1717
 Curini, M315
 Cvačka, J1035
 D'Abrosca, B1539
 D'Angelo, G1539
 da Silva, VAGG1167
 Dahse, HM253
 Dai, J1175
 Dai, X985
 Damonte, EB1307
 Darmawan, A1209
 Daroui-Mokaddem, H1669
 Darvesh, AS1613
 Daud, A915
 Dávila, CML387
 Davisoylu, KS969
 de Almeida, RN1847
 de Jesus, HCR1977
 de Lemos, TLG1181
 de Rezende, CM1167
 de Sousa, DP1847
 Dechsukum, C1921
 Deilami, I1935
 Delazar, A1619
 Delporte, C1013
 Demarco, D1225
 Demir, H147
 Demirci, B147,297
 Demirci, B1369,1409,1421,1951
 Demirci, F147,297,515,1421
 Demirtas, I969
 Deng, T1251
 Denisenko, VA377
 Denkova, Z1365
 Derese, S853
 Desjobert, JM1085
 Dessi, S329
 Dev, V939
 Dewanjee, S1273,1913
 Dhar, J1561
 Dib, MA1085
 Digrak, M969
 Diniz, A741
 Dixit, VK185
 Djibeyan, PD607
 Djoufack, GLN1055
 Dmitrenok, PS597,1737,1881
 Dolan, MC519
 Dominguez, F235
 Dominici, L1039
 Donfack, JH1607
 Dong, H789,795,893
 Dong, W-C1143
 Dong, WC1677
 Dongo, E559,1795,1939
 Dorman, HJD65,1453
 Dou, D-Q1019
 Dräger, S1175
 du Toit, LC1401
 Duan, J1531
 Dubois, MAL775
 Duchamp, O1023
 Dugo, G1325
 Dugo, P1803
 Duke, SO1233
 Duman, AD969
 Duquesnoy, E1995
 Duraki, S307
 Durceylan, Z515
 Durringer, JM635
 Duschatzky, CB1307
 Dutta, PK863
 Dwivedi, AK47
 Dyshlovoy, SA1881
 Echeverri, L1133
 Eiter, LC457
 Ekundayo, O481
 El Monfalouti, H935
 El Sayed, KA1623
 El-Azzouny, A1669
 Elias, EJ915
 Elias, R877
 Ellmerer, EP667,725
 Elsaedi, S507
 Elsebai, MF1071
 Emerenciano, VP755,763
 Engasser, JM777,1893
 Ergüven, M1369
 Esmaeili, MA341
 Espinoza, LJ1587
 Essien, EE1815
 Etoh, H1781
 Evstatieva, L51
 Fadamiro, H457
 Falconieri, D1955
 Faleiro, L319
 Fan, H95
 Fan, L197
 Farias, WRL1267
 Farshadpour, F1935
 Fatigoni, C1039
 Fedorov, SN1881
 Feng, D95
 Feng, F23
 Feng, G1247
 Feng, L-X1183
 Feng, Y783
 Feo, VD1969
 Ferenczy, A1437
 Ferreira, AG669
 Ferreira, MJP755,763
 Ferreira, WJ1167
 Ferreira, YS1977
 Ferrero, AA301
 Figueiredo, AC319,1465
 Figueiredo, G835
 Filho, JGS635
 Filho, JMB635
 Filho, RB179,1977
 Filippini, R431
 Filotheou, HP823
 Finzi, PV1607
 Fiorentino, A99,1539
 Firuzi, O261
 Fischer, HD685
 Fischer, NH685
 Flamini, G1111,1321
 Floris, C551
 Flörke, U1175
 Foddai, M1985
 Fomsgaard, IS407
 Fons, F1655
 Formisano, C273,1127,1649,1969
 Fouladvand, M1935
 Franzblau, SG211
 Fraternali, D315,927
 Friedl, SM1365,1447
 Friedrich, G253
 Froissard, D1655
 Fronczek, FR685
 Fujiwara, T1733
 Fukai, T1781
 Fukuda, E1755
 Furlán, RLE1013
 Galarraga, ME775
 Ganapaty, S399
 Gangopadhyay, M1273,1913
 Ganjewala, D163
 Gantait, A863
 Gao, CH201
 Gao, HY13
 Gao, J-M1251
 Gao, JM1759
 Gao, S197
 Gao, T447
 Gao, X223
 Gao, Y223
 García, CC1307
 García-Lesmes, L1831
 Gaur, R1243
 Gautam, R555
 Gavia, M1133,1831
 Gaydou, EM33,115,259,435
 Geissler, M1365
 Genovese, S315
 Gertsch, J551
 Ghafari, S511
 Ghani, A283
 Ghanmi, M935
 Ghannadi, A957
 Gharby, S935,1799
 Ghedira, K1893
 Ghedrira, LC777,1893
 Ghisalberti, EL219
 Gholami, M261
 Gholipour, A341
 Ghosh, A1213
 Ghou, M777,1893
 Giamperi, L315,927
 Giardina, S1607
 Gibbs, R607
 Girault, JP1579
 Girova, T1349,1355,1359
 Giuliani, C107
 Glamočlija, J1525
 Glowniak, K1427
 Gochev, V1349,1355,1359
 Godevac, D1215
 Gogilashvili, L1091
 Goldsack, RJ1833
 Gomes, CA471
 Gomes, DM1267
 Gómez, PS629
 Gong, X1
 González, JOW301
 Göpfert, J709
 Gören, AC1729
 Gosztola, B465
 Gourine, N115
 Grace, MH1869
 Graebner, IB1181
 Graidist, P1921
 Grau, A1721
 Graziose, R1869
 Green, IR559
 Guan, H1251
 Guan, S-H1183
 Guedes, MM523
 Guedon, E777,1893
 Guillaume, D935,1799
 Guimarães, EF477,1837
 Gülcehal, D841
 Guleria, S641
 Gunatilaka, AAL801
 Guo, D-A1183
 Guo, KT95
 Guo, W1907
 Gupta, S1567
 Gupta, V47
 Gutiérrez, MM301
 Gütschow, M1071
 Güzel, CB1369
 Ha, TJ85
 Haber, WA1161
 Habtemariam, S365,1329
 Hågerstrand, MB1639
 Haid, M869
 Haider, F1959
 Hailu, A975
 Hakim, EH1209
 Haladu, SA1077
 Hale, AL1233
 Hamburger, M1619
 Hammad, MA1457
 Han, C-R1027
 Han, D461
 Han, WL1627
 Hanai, R1
 Hang, NTB1717
 Hanh, TH1717
 Hao, XJ1793
 Harhar, H935,1799
 Harper, MK1571
 Hasan, CM1543
 Haselmann, KF407

- Hashimoto, T695
 Hassan, Z 419,1099,1787
 Hassanali, A175
 Hassani, ME943
 Hauschild, W721
 Hayashi, S143
 Háznagy-Radnai, E1613
 He, F1873
 He, HP1793
 Hedman, T1853
 Hehenberger, S667
 Hehn, A1893
 Heimler, D1775
 Heleno, VCG669,681
 Helmke, E1917
 Henandez, HP685
 Hernandez, M809
 Heuberger, E1441,1447
 Heyden, YV423
 Heydenreich, M175,903
 Hichem, L35,957
 Hiltunen, R65,1453
 Himlata1561
 Hirata, M1781
 Hnawia, E949
 Ho, C-L1143
 Ho, CL337,617,1677,1823
 Ho, KY95
 Hoan, DH103
 Höfinghoff, J1343
 Hohmann, J227,581,1613
 Holloway, AC1195
 Hong, Q211
 Hongratanaworakit, T157
 Hooper, JNA1571
 Hosoi, S1733
 Hošťálková, A1751
 Hou, B1557
 Hou, P1531
 Hsu, K-P1143
 Hsu, KP337,1677
 Hsueh, M-C911
 Hu, LH9
 Hu, Z845
 Hua, J265
 Huang, J13
 Huang, KL265
 Huang, L1263
 Huang, SH1627
 Huang, Z1771
 Hui, L241
 Hui, M1673
 Hung, CP617
 Huong, HT361
 Huong, TT103
 Hussain, H559,1175,1785,1795
 Hussain, H1939
 Hussain, J419,1785
 Hussain, N1205
 Hussain, SM1785
 Hymete, A975
 Hyun, C-G1311
 Hyun, JH103

 Ibrahim, AK27
 Ibrahim, M1865
 Ibrahim, SRM403
 Illinger, A949
 Ilmberger, J1441
 Innocent, E903
 Ipsilantis, I823
 Iqbal, S1099
 Ireland, CM1571
 Isla, MI391
 Ismail, M1747
 Itharat, A1921
 Ito, T1375
 Ivanauskas, L535,897
 Iwasaki, N1755

 Iwashina, T1903
 Iwashina, T39
 Izquierdo, AG51
 Izzo, A1539

 Jabor, VAP741
 Jachak, SM555,575,1767
 Jacob, MR853
 Jacobs, H859
 Jacquemond, C435
 Jain, R185
 Jaitak, V1561
 Janibekov, AA1579
 Janicsák, G227
 Janulis, V535,897
 Javidnia, K261
 Jehle, M725
 Jeon, H-S851
 Jespersen, BM407
 Jeyashoke, N1677
 Ji, M373
 Ji, N-Y1575
 Ji, Z355
 Jiang, MX1597
 Jiang, Y245
 Jiménez, IA1043
 Jiménez, JF629
 Jiménez, P1643
 Jin, Y461
 Jirovetz, L1349,1355,1359,1365
 Jordán, MJ629
 Joseph, CC175,253
 Joshi, RK815
 Judzentiene, A1981
 Juliano, C1985
 Jumpathong, J567
 Júnior, CPA471

 Kabir, S1543
 Kabouche, A35,849,957,1669
 Kabouche, Z35,849,957,1669
 Kadota, S17,1009,1551,1601,1771
 Kaewkroek, K1547
 Kahriman, N831
 Kailas, T133
 Kalani, K1567
 Kaleem, WA1205,1727
 Kalenda, DT369
 Kalinin, VI1877
 Kalinovskaya, NI597
 Kalinovsky, AI597,1737
 Kalra, A269
 Kamo, O1755
 Kanani, MR341
 Kang, HK103
 Kang, IJ1927
 Kang, J-Y1311
 Kang, T-G1019
 Kaplan, MAC1837
 Karagianis G365
 Karagiannidis, N823
 Karagiannidou, C823
 Karaoğlu, SA831
 Karayildirim, T841
 Karchesy, J519
 Kartah, BE1799
 Kashman, Y33,205,259
 Kato, H1049
 Katsumoto, Y1733
 Kaul, VK641,1561
 Kavalcioğlu, N147
 Kawanobu, S1903
 Kaya, GI873
 Kebir, MVOE435
 Kehraus, S507,1071
 Kelsey, RG519
 Kerr, PG1607
 Ketzinel, S205
 Khaldi, A1955

 Khalfallah, A35,957
 Khalifa, SI27
 Khan, A345,931,1787
 Khan, H931
 Khan, I1205,1727
 Khan, IA771
 Khan, LA345
 Khan, R1865,1899
 Khan, SI853
 Khan, SS1099,1787
 Khoi, NH423
 Kiem, PV361,423,1717
 Kingston, C175,903
 Kılıç, CS1951
 Kim, E-H851
 Kim, EK395
 Kim, G1277
 Kim, G-O1311
 Kim, JH395
 Kim, JK1927
 Kim, S1277
 Kim, YH103,361
 Kim, YY1081
 Kingston, DGI751
 Kiran, I515
 Kirimer, N515
 Kırmızıgül, S563
 Kitajima, J39
 Kitzer, S1447
 Klausen, K407
 Kleinpeter, E175
 Kobayashi, H1789
 Kocabas, EH249
 Kocabaş, F249
 Koike, K143,1049
 Kokubugata, G39
 Komala, I1375
 König, GM507,1071
 Koşar, M1453
 Kouam, SF559,1795
 Koutsaviti, A1809
 Kozhamkulova, ZA1061
 Krings, U1503
 Krohn, K559,1175,1795,1939
 Kuang, R571
 Kubeczka, KH1387
 Kubo, I85
 Küçükboyacı, N297
 Kukreja, AK1945
 Kulhánková, A1201,1751
 Kültür, S1369
 Kumar, M47,427
 Kumar, N1561,1959
 Kumar, S1243
 Kuneš, J1035,1201
 Kuo, CL55
 Kuo, G95
 Kuo, YH1941
 Kuo, YH55
 Kupcinskiene, E1981
 Kurfürst, M1035,1751
 Kuroda, C1
 Kurtán, T1175
 Kuster, RM1219
 Kuznetsova, TA597
 Ky, PT1717

 Laatsch, H567,1917
 Lacaille-Dubois, MA1023,1743
 Lafont, R1579
 Lage, CLS1219
 Laguna, A1187
 Lajide, L645,1135
 Lajis, NH1747
 Lajis, NHJ191
 Lakhali, H849
 Lakshmanan, AJ621
 Lakshmi, KR59
 Lal, B641

 Lal, UR575,1767
 Lalli, JY1395
 Lange, C253
 Lantz, RC733
 Lassak, EV1833
 Lategan, C1869
 Lau, TV361
 Laudares, EP681
 Laursen, B407
 Lavor, PL1977
 Lawal, OA1811
 Lazari, D823
 Leal, LKAM1103
 Lebibi, J961
 Lebouvier, N949
 Leclercq, JQ369,423
 Lee, AYL911
 Lee, HB395
 Lee, J395
 Lee, JY1927
 Lee, KH675
 Lee, NH1311
 Lee, YS1927
 Leeanansaksiri, W1921
 Lei, H1673
 Leiva, M1643
 Lemos, TLG1977
 Leonti, M551
 Levina, EV1737
 Li, S1049
 Li, A1263
 Li, B1889
 Li, DL1583
 Li, F17,1009,1551,1575,1601
 Li, G1759
 Li, HQ1759
 Li, L1171,1263
 Li, M1019
 Li, N1557
 Li, P811
 Li, S355,519
 Li, SI1793
 Li, W1049
 Li, X811,1557
 Li, X-B1171
 Li, XC771
 Li, XM77,1583
 Li, Y151
 Liang, F1673
 Liang, Q1147
 Liang, Y245
 Liao, P-C1143
 Liao, PC617,1677,1823
 Liberto, MD1013
 Lila, MA1869
 Lim, SS1927
 Lima, JQ1977
 Lima, MAA1977
 Lima, PR523
 Lin, C1263
 Lin, CN1823
 Lin, CY337
 Lin, L1049
 Lin, Y1771
 Lira, SRDS523
 Liu, H1531,1789
 Liu, J1031
 Liu, J1889
 Liu, LL649
 Liu, P1531
 Liu, PP1019
 Liu, R985
 Liu, W1139
 Liu, WY23
 Liu, X1183
 Liu, X-H1575
 Liu, X-Y1005
 Liu, Y91,541,789,795,893

Liu, YC	617	Mathias, L	179	Mosaddegh, M	511	Ollivier, E	877
Liu, Y-Q	1247	Matsumoto, S	39	Moundipa, PF	1607	Omatsu, I	999
Liu, Z	985	Matsushima, M	1	Mudit, M	1623	Ono, H	1903
Livingstone, C	1961	Matsuura, M	143	Muhammad, I	853	Onozaki, T	1903
Llanos, GG	1043	Maurya, R	47,427	Mujahidin, D	1209	Ontiveros, I	1133
Loetchutinat, C	1931	Mayer, AMS	27	Mukherjee, PK	863	Onur, MA	873
Lograda, T	835	Mazumder, UK	1239	Mulkijanyan, K	1091	Opletal, L	923,1035,1751
Lombardozi, A	1317	Mbeunkui, F	1869	Murakami, S	143	Orav, A	133
Lopes, JLC	681	Mbimba, T	1613	Murat-Spahić, D	1663	Ordóñez, R	391
Lopes, NP	681,733,741	McChesney, JD	387	Murillo, BM	613	Orjarena, S	1859
Lorbeer, E	1055	McLean, S	859	Murray, AP	1841	Oroojalian, F	283
Los, R	1427	McNeil, MJ	1301	Murria, AP	301	Ortiz, J	1643
Lou, HX	373	McNulty, J	1195	Murugan, R	1961	Ostad, SN	511
Lou, LG	1873	Medina, AS	365	Mustafa, MR	931	Ottavioli, J	1991
Lozano, Y	435	Meena, R	603	Mutiso, PBC	721	Ouyang, XW	9
Lu, J	447	Meepowpan, P	1931	Müüriisepp, M	133	Owolabi, MS	645,1135
Lu, JC	197	Meffo, BY	559	Nagano, H	1	Oyedéji, AO	1811
Lu, L	805	Mehmood, R	1885	Nagashima, F	1375	Özcan, T	587
Lu, Z	1571	Mehrabani, M	957	Naghibi, F	511	Özgünes, H	899
Lucio, JAGD	531	Melkani, AB	939	Nair, JJ	1195	Özkan, AMG	1951
Luis, JMA	775	Mendiola, J	1187	Nair, MG	91,1253	Öztürk, AH	1729
Lumyong, S	567	Menelaou, MA	685	Nakamura, T	39	Pacifico, S	1539
Luo, Y	1673	Menezes, MAD	669	Nakayama, M	1903	Padalia, RC	279,815
Luong, DV	1717	Menković, N	1215	Nalbantsoy, A	249	Padmashree, KV	1961
Luppino, RR	1325	Menon, V	717	Nam, NH	423	Paixão, ICNP	1167
Luro, F	435	Menut, C	949,961	Nam, NH	423	Pal, A	1243
Luthra, R	163	Mercado, MI	1721	Namkoshi, M	1789	Pal, D	1239
Lv, H	1687	Merlani, M	1091	Nanayakara, NPD	387	Palá-Paúl, J	1833
Lv, L	1031	Merú, ML	311	Naqvi, AA	1959	Palić, RM	121
Ma, C	1183	Mesbah, MK	27	Naqvi, F	867	Pampaloni, B	1775
Ma, S	447	Midiwo, JO	853	Nascimento, PGBD	669	Pan, E	751
Ma, XC	1031	Miguel, MG	319	Nash, RJ	485	Panella, NA	519
Mabrouki, F	877	Mihci-Gaidi, G	1023	Nathan, PJ	531	Pannakal, ST	399
Macáková, K	923,1035,1201,1751	Milenković, M	307,1215	Natschke, SLM	675	Pant, CC	939
Maccioni, S	1321	Miloslavjević, S	729	Naz, R	931	Pant, G	907
Machado, LL	1181	Milošević, T	1663	Nazeri, V	943	Paolini, J	1085
Machado, M	137	Milovanovic, M	61	Negi, JS	907	Papaioannou, F	1809
Machumi, F	853	Minh, CV	361,423,1717	Nema, NK	863	Parry, A	1401
Macias, FA	685,1043	Minter, DE	211	Németh, E	465	Passreiter, CM	721
Maciel, MA	523	Miri, R	261	Neske, A	391	Pat, FM	365
Magadula, JJ	903	Mirjolet, JF	1023	Nestorović, J	1525	Patel, RP	1947
Magenta, MAG	669	Mishra, SL	1239	Neves, IA	471	Paúl, JP	817
Magesa, SM	175	Mišić, D	1525	Ngadjui, BT	1607	Pauli, A	1387
Maggi, F	1317	Mitaine-Offer, AC	1023,1743	Ngameni, B	1607	Paululat, T	1743
Maggio, A	273,1649	Miyahara, T	1711	Ngombe, NK	369	Pavlović, M	307
Maggio, AM	675	Miyake, K	17,1009	Ngoupayo, J	919	Pedersen, HA	1259
Mai, NT	423	Miyamoto, T	775,1023	Nhiem, NX	361	Pedras, MSC	883
Maia, JGS	477	Moghadam, MH	511	Niketić, M	307	Pedro, LG	319,1465
Makani, T	961	Mohammad, FV	191	Nisar, M	1205,1727	Pellegrino, RM	107
Makariev, TN	1881	Mohammadi, K	1935	Niu, A	1789	Pereira, MG	1267
Maksimović, M	1123	Mohan, L	939	Nkengfack, AE	1535	Pereira, TP	1103
Malik, A	1865,1885,1899	Mojarrab, M	1619	Nkunya, MHH	175,253	Pérez, A	809
Malik, I	529	Molicotti, P	1955	Nobukawa, T	1551	Perotti, M	1307
Mallavarapu, GR	1961	Molinillo, JMG	1043	Node, M	1733	Pertuit, D	1023
Mallet, AI	365	Möllman, U	253	Noge, K	351	Pescitelli, G	1175
Malm, A	1427	Monaco, P	99,1539	Nogueiras, C	1187	Petreska, J	51
Mamadalieva, NZ	1579	Mondal, A	1239	Noma, Y	515,695,1339	Petrović, S	307,1215
Mancini, E	1969	Mondello, L	1803	Noorwala, M	191	Pierdoná, TM	1103
Mandal, S	1213	Monfalouti, HE	1799	Nour, M	949	Pihlaja, K	545,1639
Manzoor, N	345	Monte, FJQ	1181	Novoa, MA	1013	Pillay, V	1401
Maoka, T	1733	Monteiro, HSA	1103	Nunn, PB	485	Ping, OJ	617
Marchetti, M	1985	Moon, H-I	851	Nuntasaeen, N	1931	Pino, JA	613,1131,1829,1831
Marongiu, B	1955,1995	Moon, J-Y	1311	Nuzillard, JM	763	Pintér, A	1681
Marotta, F	1803	Moosa, BA	1191	Nyongha, AT	1939	Pinyol, P	1587
Marques, DD	1181	Mora, FD	965	Oanh, DT	1717	Piovan, A	431
Márquez, API	387	Moraes, HP	1225	Obame, LC	961	Piozzi, F	1969
Martin, AS	1859	Moraes, MMD	471	Odabas, MS	535	Piras, A	1955,1995
Martino, LD	1969	Morales, A	1115,1283	Odalo, JO	253	Pistelli, L	291
Martins, AMC	1103	Morales, G	1133	Oedendorfer, K	1447	Pizzimenti, F	1803
Martiskainen, O	545,1639	Morazzoni, P	1613	Offer, ACM	775	Plainchont, B	763
Martyyas, EA	1737	Moreira, DL	1837	Offer, ACM	775	Pluhár, Z	1681
Marx, JN	5	Morel, N	369	Okungbe, IV	1161	Poláček, M	923
Marzatico, F	1607	Moreno, J	311	Ogunwande, IA	1815	Pollicino, D	1325
Marzouki, H	1955	Moretti, M	1039	Oji, K	625	Pompimon, W	1931
Masotti, V	1981	Morikawa, K	1551	Okada, Y	1067,1755	Pontes, GC	1267
Mata, M	235	Morillo, M	937	Okamura, M	1903	Popović, V	307
Máthé, I	227,581	Morrell, S	581	Okuyama, T	1067	Porcedda, S	1995
Mathela, CS	815	Morse, PF	581	Oladimeji, MO	645	Porter, RBR	1301
		Mortensen, AG	407				

- Porzel, A869
 Potterat, O1619
 Pounders, C1409,1421
 Pouységu, L775,1743
 Pramod, K1999
 Prasad, K603
 Prasad, YR59
 Proksch, P1583
 Pu, JX1873
 Pumsalid, K1931
 Purnanand, S1947

 Qayum, M1205,1727
 Qi, SH201
 Qian, P-Y1171
 Qian, PY201
 Qian, Y805
 Qiang, DD95
 Qiao, M-F1575
 Quang, DN869
 Quevedo-Vega, J1831
 Quideau, S775,1743
 Quijano-Celis, CE1133,1831
 Quilez, M629
 Quiros, Y809

 Raal, A133
 Rabelo, AFL523
 Raccuglia, RA675
 Radhika, P59
 Radovic, BV61
 Radulović, N1117
 Radulović, NS121
 Radušiene, J535,897
 Radwan, MM1061,1463
 Rahadilova, SN1061
 Rahman, MS1543
 Rahuja, N427
 Rainford, L1301
 Rajendran, SM427
 Rajeswara Rao, BR1947
 Rajput, DK1947
 Rakotodrajaona, R751
 Ramdani, M835
 Ramedani, E1935
 Ramirez, L1643
 Ramirez, MLG235
 Ramirez, R965
 Rando, R1325
 Rao, DV399
 Rao, RR1961
 Rao, VS523
 Rapior, S1655
 Rasamison, VE751
 Rashid, MA1543
 Raskin, I1869
 Rasool, N419
 Rastian, Z1935
 Ratovoson, F751
 Ravi, S621
 Rawat, MSM907
 Razab, R441
 Read, RW511
 Regalado, EL1187
 Rehman, NU1785
 Reinecke, MG211
 Reveco, PG1587
 Reynolds, WF859
 Reznicek, G1447
 Ribeiro, NC1819
 Ribeiro, NDC471
 Ricci, D315,927
 Ricciutelli, M1317
 Rigano, D1127,1969
 Rios-Motta, J1063
 Robien, W1055
 Rodrigues, GV755,763
 Rodrigues, NVFC1267
 Rodrigues, VF179

 Rodríguez, LMP365
 Rodríguez, SA1841
 Rodríguez-Guzmán, R1463
 Rohloff, J975
 Rojas, CMCG531
 Rojas, J1115,1283
 Rojas, LB1115,1283,1743
 Rojas, LB331,615,775,937,965
 Rojas, YC937
 Romanenko, LA597
 Romani, A1775
 Romero, RA613
 Ross, SA1061,1463
 Rosselli, S273,675,1649
 Roviroso, J1859
 Row, KH461
 Roy, P863
 Roy, S1239
 Rudi, A33,205,259
 Rui, W783
 Russo, A1127

 Saba, AR551
 Sabuncuoğlu, SA899
 Saeed, A931
 Saekoo, J1921
 Safaei-Ghomi, J957
 Said, IM39
 Saini, R641
 Sajjadi, E957
 Sakai, E1781
 Sakamoto, HT681
 Sakar, MK899
 Sakata, F999
 Salazar, R809
 Salem, N777
 Salgado, A1091
 Salgueiro, L137
 Salvatore, A391
 Samková, V923
 Samoylenko, V853
 Sampaio, AH1267
 Sampson, B1409
 Sanandiyā, ND603
 Sannigrahi, S1239
 Santalova, EA377
 Santi, L1803
 Santiago, GMP1977
 Santos, CMP1847
 Santos, FA523
 Santos, FV1225
 Santos, MDD733
 Sarikaya, B873
 Sárosi, S291,465,1437,1681
 Sato, M1781
 Satoskar, AR387
 Satou, T143
 Sattler, I253
 Šavikin, K1215
 Saxena, AK1567
 Saxena, M1567
 Sayed, EA545
 Schinnerl, J1055
 Schmidt, E1349,1355,1359,1365
 Schmidt, J869
 Schuff, C1307
 Schulz, B559,1175
 Schwaiger, S667
 Schwob, IL1981
 Scognamiglio, M99
 Scotti, MT763
 Seatlholo, ST1381
 Sechi, B1985
 Sefidkon, F943
 Segnini, S965
 Senatore, F273,1127,1649,1969
 Sepp, J133
 Şerbetçi, T1369
 Setzer, WN457,645,993,1135

 Setzer, WN1161,1815
 Shaari, K1747
 Shafaghāt, A625,981,1299
 Shah, Z931
 Shami, PJ77
 Shan, T811
 Shanker, K1243
 Shao, C-L1171
 Sharma, OP1561
 Shaw, P1673
 She, G1687
 She, Z1771
 Shen, T373
 Shengkun Li, S1591
 Shi, B355,1139
 Shi, LL231
 Shi, Y1907
 Shi, YP649
 Shimizu, K85
 Shimizu, N351
 Shin, JH395
 Shubina, LK1881
 Shukla, N427
 Siddhanta, AK603
 Siddiqui, IN419,1099,1787
 Sielinou, VT1535
 Silchenko, AS1877
 Šiler, B1525
 Silini, H835
 Silva, B965
 Silva, JKRD477
 Simkó, H1681
 Simo, CCF1607
 Simões, MTF319
 Simpson, DS859
 Singab, AEN545
 Singab, AN1639
 Singh, AB427
 Singh, AK269
 Singh, B641,1561
 Singh, IP575,1767
 Singh, M1195
 Singh, P907
 Singh, S867
 Sinkkonen, J545,1639
 Sipos, P1613
 Siqueira, HAA1819
 Skalicka-Wozniak, K1427
 Skaltsa, H1663
 Skropeta, D121
 Sladek, G1447
 Slavchev, A1365
 Slusarewicz, P1853
 Smith, DC1019
 Smith, PJ1869
 Snitynsky, RB883
 Soares, DM733,741
 Soares, RMA1837
 Soest, RWMV27
 Şöhretoğlu, D899
 Soković, M1525
 Šolić, ME1123
 Solinas, MN551
 Soltani, M261
 Solujić, S1663
 Somer, NU873
 Sonboli, A341
 Song, L1005
 Song, R789,795,893
 Song, X913
 Song, X-Y1183
 Sorensen, JL81
 Soria, AC817
 Sosa, FC235
 Soto-Delgado, J1859
 Sotomayor, JA629
 Soumati, B1669
 Sousa, MDC137
 Souza, GEPD733,741

 Sozmen, F111
 Spring, O709
 Srivastava, A555
 Srivastava, AK427
 Srivastava, SK1567
 Stanković, M729
 Stappen, I1343
 Stefani, R669
 Steffensen, SK1259
 Stefova, M51
 Stepanov, VG1877
 Sterner, O899
 Stonik, VA377,1737,1881
 Stoyanova, A1349,1355,1365,1359
 Strano, M1317
 Stuppner, H667
 Su, C1673
 Su, DH1889
 Su, M151
 Su, S1531
 Su, W1673
 Su, Y223
 Su, YC337,617,1143,1677,1823
 Suárez, LEC383
 Sudo, M1781
 Suleiman, M39
 Sun, BH13
 Sun, HD1873
 Sun, LM373
 Sun, QS1597
 Sun, X-P1171
 Sun, Y571
 Sundaresan, V279
 Suri, N1567
 Svetaz, L1013
 Syah, YM219,1209
 Sylvester, PW1623
 Szabó, K1437

 Tabanca, N1409,1421
 Tabti, B1085
 Tai, BH103
 Tajbakhsh, S1935
 Tajuddin, SZ1965
 Tamboue, H1795
 Tan, Y783
 Tanaka, H1781
 Tanaka, K1551
 Tanaka, T1733
 Tang, Y1531
 Tanini, A1775
 Tanjung, M1209
 Tariku, Y975
 Tava, A1285
 Tchana, AN1607
 Teixeira, VL1167
 Tekwani, BL853
 Telci, I969
 Teng, J1263
 Terzioğlu, S831
 Tešević, V729
 Tewtrakul, S1547
 Tezuka, Y17,1009,1551,1601,1711
 Thaisuchat, H1931
 Thao, NP423
 Thomas, OP1187
 Thomet, FA1587
 Thu, VK361
 Thuan, NT361,423
 Thul, ST1945
 Tian, M461
 Tian, X241
 Tirillini, B107
 Todorova, M129
 Tomé, ADR523
 Tomi, F1107,1659
 Tong Di, YT1793
 Topçu, G1729
 Törnquist, K1639

- Tosun, A1067
 Touzani, R849
 Toyama, MH1103
 Toyota, M999
 Trendafilova, A129
 Tripathi, SM575,1767
 Trivedi, P1243
 Trujillo, R809
 Tsaassi, VB1795
 Tsafantakis, N99
 Tsai, MC617
 Tsassi, VB559
 Tschiggerl, C1431
 Tseng, YH1677,1823
 Tsikolia, M1409
 Tsubono, K1755
 Tung, NH361
 Tung, YT73
 Turco, VL1325
 Turner, JL1421
 Tuyen, NV423
 Tyagunova, Y81
 Tzakou, O307,1809

 Üçüncü, O831
 Uesawa, Y1755
 Ullah, N1077
 Unnithan, CR621
 Urbanová, K1201
 Urbaňská, J1291
 Urgen, M249
 Usai, M1985
 Usubillaga, A311,615,937,965
 Usubillaga, A1743
 Uysal, B111
 Uzel, A249

 Vacelet, J33,259
 Vajs, V729
 Valant-Vetschera, KM1055
 Valencia, JMT531
 Valterová, I1201
 Van Vuuren, SF1381,1395,1401
 Van Wagoner, RM1571
 Van Zyl, RL1381
 Vanegas-López, C1133
 Varanda, EA1225
 Vardamides, JC1535
 Varela, RM1043
 Varesi, L1085
 Velasco, J311
 Velozo, LSM1837
 Veluthoor, S519
 Veras, HNH1977
 Vichniewski, W681
 Victório, CP1219
 Vidari, G1607
 Vidic, D1123
 Viegi, L1285
 Vieira, IJC179
 Vignolini, P1775

 Viljoen, A1381,1395
 Villanueva, HE1135
 Villarini, M1039
 Villena, J1587
 Visual, T937
 Vitkova, A129
 Vittori, S1317
 Vivanco, J1643
 Vujišić, L729

 Waffo, AFK889
 Wahid, F1081
 Wajs, A1291
 Waksman, N809
 Wali, VB1623
 Walker, LA853,1457
 Walker, TM1815
 Wang, B541
 Wang, BG1583
 Wang, CY1031
 Wang, C-Y1171
 Wang, EIC337,617,1143
 Wang, EIC1677,1823
 Wang, F-P1005
 Wang, H-M911
 Wang, J1907
 Wang, J811,913,1591,1631
 Wang, L985,1627,1631
 Wang, LL1597
 Wang, M355
 Wang, T223
 Wang, W771
 Wang, W-B1183
 Wang, XB13
 Wang, XC9
 Wang, XN373
 Wang, Y1627,1673
 Wang, YH1049
 Wang, Z1295
 Wanner, J1349,1355,1359,1365
 Waterman, PG365
 Watson, AA485
 Wattanapiromsakul, C1547
 Wedge, DE1409,1421
 Wei, B1263
 Wei, Q1031
 Wei, Q1889
 Wei, S355,1139
 Wei, X245
 Weidenhamer, JD685
 Werle, C1409
 Wessjohann, L869
 Widyowati, R1711
 Wiggers, FT853
 Wijeratne, EMK801
 Williams, LAD1301
 Williamson, GB685
 Williamson, JS1463
 Willink, E391
 Wolschann, P1343
 Won, MH1927

 Wu, B1097
 Wu, L1067
 Wu, LJ13
 Wu, P151
 Wu, SH1941
 Wu, W845,1139,1591
 Wu, W-Y1183
 Wu, X-P1027
 Wu, Z223

 Xavier, HS635
 Xavier, PA1267
 Xi, P-Z1251
 Xi, RG13
 Xiao, W1557
 Xiao, WL1873
 Xie, H245
 Xie, J-Y1027
 Xie, N23
 Xin, X1889
 Xin, XL1031
 Xiong, K985
 Xu, BB197
 Xu, F789,795,893
 Xu, L811
 Xu, Q245
 Xu, SX1597
 Xu, XJ1759
 Xu, Z1789
 Xue, J197,245
 Xue, Q-Z1575
 Xue, W985

 Yadav, A269
 Yadav, DK47
 Yadev, VR1253
 Yagi, Y1375
 Yamada, H1
 Yamaguchi, M1903
 Yamaguchi, R1781
 Yamashita, M1733
 Yan, S223
 Yang, B1591
 Yang, B913
 Yang, FL1941
 Yang, H845
 Yang, J1771
 Yang, JL649
 Yang, L1247
 Yang, N77
 Yao, JH1031
 Yaşa, I563
 Yasmeen, S1899
 Yavari, A943
 Yaylı, N831
 Ye, WC1627
 Yeffet, D205
 Yen, PH361,1717
 Yenesew, A853
 Yoon, W-J1311
 Yoshida, H1903

 Yousefi, F1935
 Yousefi, M115
 Youssef, DTA27
 Yu, CC1941
 Yu, CX23
 Yu, HH1941
 Yu, L1907
 Yu, WW1631
 Yuan, K1631
 Yuan, SY95
 Yuan, Y805,1027
 Yue, Q-X1183
 Yue, QX9
 Yunbao Liu, Y1253
 Yusoff, MM1965

 Zacchino, SA1013
 Zahoor, A419,1099,1787
 Zaleskiewicz, E1291
 Zandi, K1935
 Zanetti, S1955
 Zarubica, AR121
 Zdunić, G1215
 Zeng, GY1597
 Zeng, J77
 Zeng, X1097
 Zhan, J77,801
 Zhang, AL1759
 Zhang, C-L1251
 Zhang, DM1627
 Zhang, H805
 Zhang, HB1873
 Zhang, J231,355,845,1247,1591
 Zhang, JJ197
 Zhang, JQ241
 Zhang, L1067
 Zhang, Q541,1049,1591,1793
 Zhang, S201
 Zhang, X223
 Zhang, Y1097,1295
 Zhang, Z789,795,893
 Zhang, ZW241
 Zhao, CL1147
 Zhao, J811
 Zhao, W1873
 Zhao, Y373,541,1873
 Zheng, CD1759
 Zheng, X95,571
 Zheng, YN231
 Zhong, Y783
 Zhou, L811
 Zhou, YJ1597
 Zhu, K1853
 Zhusupova, GE1061
 Zidorn, C725
 Zihnioglu, F841
 Zivkovic, D61
 Zlatković, BK121
 Zubair, M419
 Zupkó, I227

Natural Product Communications

Volume 5 (1-12) 2010 Keywords Index

- A549 cells 717
Aaptamine 1881
Abhayarishtha 575
Abies alba 1291,1995
Abietane diterpenoids 853
Abietic acid 1729
Absidia coerulea 373
Absolute configuration 675,1733
Absolutes 1349
ABTS^{•+} radical scavenging 441
Acacia confuse 73
Acalypha segetalis 481
Acanthaceae 59,563,1722
Acanthus hirsutus 563
Acaricidal activity 471
Acetate-MVA pathway 163
Acetophenone 1961
1 β -Acetoxy-furanouedism-4(15)-ene 1809
Acetylcholinesterase 1035,1201,1751
Achillea 283
Achillea clusiana 129
Achillea grandifolia 121
Aconitum 447
Actinomycete 249,597
Activity assay 137
Acyclic diterpenes 1721
Acylated flavonoid glycoside 1597
Adhesion molecules 1329
Aedes aegypti 1409
Aedes aegypti 1977
Afraegle paniculata 559
Agrobacterium rhizogenes 1913
Ajowan 1107
Alchornea triplinervia 1225
Aleyrodidae 1819
Algeria 835,1659
Alkaline phosphatase 1711
Alkaloids 17,35,877,1195,1743
Alkyl glycoside 1099
Allelopathy 685
Allium cepa 391
Allosylantirrhinoside 841
4-Allylanisole 1283
Alnus nitida 1787
ALT 1457
Aloysia citriodora 301
Aloysia polystachya 301
ALP 1711
Alpinia speciosa 279
Alpinia zerumbet 143
Alzheimer's disease 1035,1751
Amaryllidaceae 1195,1201
Amaryllidaceae alkaloids 873
Amberbin A 1865
Amberbin B 1865
Amberboa ramose 1865
Ambrosanoli-10(14)-en-11,12-diol 179
Amino acids 81,1643
Amino-dihydroimidazo-pyrimidinone 377
Aminoquinolines 387
Ammoides verticillata 1107
Ampullosine 869
Anacardiaceae 545
Anchusa italica 1091
Andrographis paniculata 59, 717
Andrographolide 717
Anemone raddeana 197
Angucyclines 597
Angucyclinones 1917
Annonaceae 1931
Annonaceae 253,1543
Annonaceous acetogenin 1783
Anofinic acid 1071
Anthocleista schweinfurthii 369
Anthocyanin 1613
Anthraquinones 427,747,1251
Anthriscifolmines 1005
Anti osteoporotic 47
Antibacterial 43,621,809,1387
Antibacterial activity 311,515,625,811,981,
1133, 1255, 1359,1427,1525,1781, 1985,1995
Anticancer 1239,1253,1623
Anticancer activities 617
Anticoagulant 1267
Anticonvulsant activity 1847
Antidepressive activity 431
Antidiabetic activity 907
Antifungal 329,809,1387,1789
Antifungal activity 477,869,1013,1525,1673,
1677,1955
Antihepatotoxic effect 1607
Antihyperglycemic 427
Antihyperglycemic drug 1077
Anti-inflammation 395
Antiinflammatory 253,415,545,1253
Anti-inflammatory activity 27,1215
Anti-larval 201
Antileishmanial 853
Antileishmanial activity 975
Antimalarial 853,1869
Antimalarial activity 1187
Anti-meiotic 1789
Antimicrobial 1369,1493,1803,1815
Antimicrobial 147,403,853,961,
Antimicrobial activities 835,913
Antimicrobial activity 249,307,559,563,1085,
1139,1143,1301,1349, 1355,1365,1395,1663,
1823,1831
Antimicrobials 175,253
Anti-mosquitoes 175
Antimycobacterial activity 1931
Antioxidant 147,227,241,315,477,545,899,
911,1253
Antioxidant activity 73,329,341,441,563,
629,1139, 1301,1339,1543,1561, 1607,1841
Antioxidant status 1213
Antioxidants 61,65,261,1453
Antiplasmodial 1869
Antiproliferative 253
Antiproliferative activity 751
Anti-radical 1759
Antiradical activity 1775
Antirrhide 841
Antirrhinoside 841
Antitumor 241,717
Antitumor activity 447
Antitumor compounds 531
Antiviral against HSV-1 1167
Anti-wood-decay fungal activity 337,1143,
1677,1823
Anxiolytic effect 143
Aphidicolin 1175
Apiaceae 297,307,817,1107,1111,1477,
1669,1951
Apigenin 7-*O*-dirhamnoside 39
Apigenin 7-*O*-dirhamnoside-4'-*O*-rhamnoside
39
Apigenin-7-*O*-[galacturonide (1 \rightarrow 4)-*O*-
glucoside] 1767
Aplysina 377
Apocarotenoids 1043
Apoptosis 151,571,1881,1921
Aporphine alkaloids 383,1747
Apuan Alps 1285
Aquilaria malaccensis 1965
Arbutus unedo 1085
Argan tree 935
Argania spinosa 935,1799
Arishta 575
Aristolactams 253
Aromatherapy 1441
Aromatic and medicinal plants 823
Aromatic glycoside 1099
Artemisia afra 1401
Artemisia alba 1117
Artemisia annua 185
Artemisia armeniaca 1619
Artemisia campestris ssp. *campestris* 1981
Artemisia capillaris 815,1277
Artemisia nilagirica var. *septentrionalis* 1959
Artemisinin 185
2-Arylbenzofuran 1063
Ascaridole 121
Ascophyllum nodosum 581
Aspergillus 1463
Aspergillus cellulosa 695
Aspergillus niger 695,1339
Aspergillus oryzae 1575
Aspergillusol A 1077
Aspleniaceae 39
Asplenium normale 39
Asporergosterol 1575
Asteraceae 1,95,273,419,621,669,681,709,
725,849,1099,1283,1285,1321,1619,1649,
1811,1831,1959
Asterina pectinifera 1081
Asterosaponins 1737
Atriplex undulate 1841
Autonomic arousal 157
Ayurveda 575
Azafluorenone 1931

Bacteria 1381
Baicalein glycosides 1239
Barleria lupulina 1711
Barley 407
Barley malt rootlet 265
Basil 65,969
Bcl-2 1921
Beauveria bassiana 77,801
Beauvericin 811
Behavioral arousal 157
Bellis perennis 147
benzo[de][1,6]-naphthyridine alkaloids 1881
Betaines 581

- Betulaceae 1787
 BHT 729
 Bianthracene-C-arabinopyranoside 747
 Bianthracenes 747
 I3,I18-Biapigenin 1215
 Biflavonones 1055
 Biflavonyloxymethane 1213
 Biisoflavonoid 1781
 Bilariciresinol 423
 Binarigenin 1055
 Bioactivity 649,1055,1171,1591,1941
 Bioassay-guided isolation 811
 Bioassays 1329
 Bioconversion 1507
 Biodiversity 1111
 Biological activity 297,319,969,1061,1375,1737
 Biorational pesticides 845
 Biosynthesis 1147
 Biotransformation 81,373,515,695,1339,1463,1507,1859
 Bisabolane derivative 667
 α -Bisabolol 307,465
 Bisabolol-oxide A 465
 Bisabolol-oxide B 465
 Biseryvarin A 1781
 Bitter orange 1819
 Black currant 1613
 Blood 1263
Blumea perrottetiana 1135
Blumea riparia 1263
 Boldine 1587
Bonellia 365
 Boraginaceae 1091
 Bornyl acetate 937
 Bornyl esters 1161
Borreria verticillata 1815
 Botanicals 65,1453
 Brassicaceae 883
 Brassilexin 883
 Brassinin 883
 Breeding 1437
 Brine shrimp lethality test 245,1981
Brontispa longissima 1247
 Bryophytes 999
 Bufalin 1031
Bupleurum longiradiatum 1139
Bursera delpechiana 351
Bursera linanoe 351
Bursera tomentosa 311
 Burseraceae 311,351,961,1181
 Butyrylcholinesterase 1035,1751

 C₂₀-diterpenoid alkaloids 1005
 Cabreuva oil 1359
 δ -Cadinene 613
 α -Cadinol 337,515,1143,1823
 τ -Cadinol 337,1677,1823
Caesalpinia bonducella 931
 Caesalpinaceae 175
 Caesalpinoideae 1977
 Caffeic acid 227,435
 Caffeic acid derivatives 725
 Caffeic acid-derived polymer 1091
 Caffeoylquinic acids 733
Calamintha ashei 685
Callicarpa macrophylla 269
Callitris neocaledonica 949
Callitris sulcata 949
 Callus 235
 Callus culture 927
Callyspongia siphonella 27
Calocedrus decurrans 519
Calotropis gigantea 867
 Camalexin 883
 Camphor 283,935,1417
Camptosorus sibiricus 1557
Candida 345
Canella winterana 1869
 Canonical correlation analysis 943

 Canthin-6-one 17
Canthium horridum 913
 Capillene 815
 Caprifoliaceae 1097
 Caprolactam 1061
 Capsaicin 91
 Capsaicinoids 1253
Caragana conferta 1899
 β -Carboline alkaloids 1591
 Carbonyl ene reaction 993
 3-C-carboxylated flavones 1899
Carex distachya 1539
 Carnation 1903
 Carotenoid 1733
 Carthamosides 419
Carthamus oxyacantha 419
 Carvacrol 957,1985
 β -Caryophyllene 613,939,961,1365,1815,1829,1981
 Caryophyllene oxide 273,515,1365,1649,1981
Casearia sylvestris 1771
Castanea mollissima 13
 CAT 899
 CBMN 729
 Cedarwood oil 1359
 α -Cedrol 515
 67/548/CEE Directive 1317
 Celastraceae 845
Celastrus angulatus 355
 Cell adhesion 1329
 Cell culture 1935
 Cell viability 1127
 Cellulose 603
 cembrane diterpenoid 1171
Centaurea arachnoidea 1285
Centaurea bracteata 1649
Centaurea jacea 1663
Centaurea montis-borlæ 1285
Centaurea nigrescens 273
Centaurea pannonica subsp. *Pannonica* 1649
Centaurea pannonica 1663
Centaurea stenolepis 273
Centaurea sulphurea 849
Centaurium pulchellum 1525
Centipeda minima 151
 Cepharanthine 877
Ceratitis capitata 391
 Cerebroside 1795
Ceriops tagal, 9
 Chá de Bugre 771
Chamaecyparis obtuse 461
 Chamazulene 465
Chamomilla recutita 465
Chamomilla suaveolens 133
 Chansu 1031
Chelidonium majus 1751
 Chemical compositions 1941
 Chemical markers 635,939
 Chemical variation 329
 Chemiluminescence 923
 Chemosyndrom 465
 Chemosystematics 725
 Chemotaxis 415
 Chemotaxonomic marker 1091
 Chemotype 115,465,943,1681
 Chenopodiaceae 603,1841
Chenopodium ambrosioides 645
 Chiclero's ulcer 387
 Chirality 1417
 Chloranthaceae 1717
 Chlorobenzene 175
 Chromenes 211
 Chromones 551,555,859
 Cichorieae 725
 1,8-Cineole 279,283,457,617,935,1421,1659,1669,1815
 Cinnamic acid 1365
Cinnamomum camphora 1803
Cinnamomum kotoense 911

 Cinnamoylphenethylamine 1259
 Cinobufagin 1031
 Cinobufotalin 1031
 Circular dichroism 1733
cis-Abienol 1995
cis-Ocimene 311
Citharexylum fruticosum 399
 Citral 163,617
Citrus aurantium 471
 Citrus essential oils 1325
Citrus sinensis 435
Citrus sinensis var. *Mimo* 471
Citrus sinensis var. *Pera* 471
Citrus x monstrosa 927
 Cleomaceae 1301
Cleome spinosa 1301
 Clerodane 1539
 Clerodane diterpenoids 999,1543
Clerodendron serratum 863
Clerodendrum ertophyllum 853
Clerodendrum formicarum 919
 Clovamide 1259
 Clove oil 1999
 Clusiaceae 751
 Cluster analysis 943
 CNE 151
¹³C NMR 763,1107,1687,1991,1995
 Coagulant 1263
Coleostephus myconis 1321
 Colon cancer 915
 Colophony 1729
Combretum oliviforme 1027
Commiphora myrrha 1359
 Comparative evaluation 641
 Competitive inhibitor 85
 Compositae 511,1531
 Computer assisted structure elucidation 763
 Computer-aided analysis 755
 Cone 1291
 Confetin A 1899
 Confetin B 1899
 Conformational analysis 1733
 Contact toxicity 301
Conyza bonariensis 1099
 α -Copaene 613
 Coriander 969
 Corsica 1991
Cortex Magnoliae officinalis 795,1893,1631
Corticium sp. 33
 Coumarin 559,1067,1619
 COX-2 383
 Creamy white flower 1903
 Crosslinking 1853
Crotalus durissus cumanensis venom 1103
Croton macrostachyus 975
Croton sparsiflorus 1885
 Crotosparamide 1885
Cryptonemia obovata 1643
 Crystallography 511
Cucumaria okhotensis 1877
Cucumis melo 403
 Cucurbitaceae 403
 Cu-cucurmin 1935
 Cultivar 291
 Cumenic aldehydes 1355
Cuminum cyminum 1767
Cunninghamella 1463
 Cupressaceae 55,461,519,949
Curcuma mangga 1547
 Curcumin 77,915,1935
 Curzerene 1669,1809
 Cutaneous leishmaniasis 387
 Cyanidin 1613
 Cyanidin-3-O-rutinoside 1613
Cyclamen persicum 1023
 Cyclamiretin A 1023
 Cyclic peptide 1885
 Cyclization 675

- Cyclo-(D-leucyl-*trans*-4-hydroxy-L-proline) 597
 Cyclo-(L-leucyl-*trans*-4-hydroxy-L-proline) 597
 Cyclo-(L-phenylalanyl-*cis*-4-hydroxy-D-proline) 597
 Cycloartane glycosides 1557
 Cyclooxygenase enzyme 91
 Cyclopeptide alkaloids 1205
 Cyclophosphamide 447
Cymbopogon 163
Cymbopogon martinii var. *mottii* 1947
 Cytochrome P450 3A4 1195
 Cytochrome P450 695
 Cytokines 733
 Cytokinins 1945
 Cytotoxic activity 249,341,1027,1167,1183, 1187,1369,1557,1587
 Cytotoxicity
 Cytotoxicity 9,33,201,253, 365,373,551,675, 903,975,1009,1023, 1567,1601,1613,1771, 1815,1869,1873,1889,1931
Da-Cheng-Qi decoction 789,795,893
Dalbergia vacciniifolia 903
 DART-MS 1755
Darwinia fascicularis ssp. *fascicularis* 1833
Darwinia peduncularis 1833
Darwinia procera 1833
Daucus carota 1955
 Davanone 1365
 4'-Deacetylgriseusin A 249
 4-Deacetylbaicatin III 1727
 (*E*)-2-Decenal 1655
 Deglycosylation 415
 Degradation 1853
 Dehydroxyisoflavones 903
 9,11-Dehydrogosterol peroxide 1183
 Delphinidin 1613
Delphinium anthriscifolium var. *savatieri* 1005
 Dendrolasin 269
 Dengue virus 1307
 Densitometry 863
 Density functional theory 993
 2-Deoxy-2-ribose 1639
 2-Deoxypolypodine B-3- β -D-glucoside 1579
 Detoxification 883
 Devil's Club 1019
 Diabetes 1927
 Diarylheptanoids 1687,1787
 Dibenzocyclooctadiene lignans 231
Dicranostyles ampla 1233
 Different developmental stage 1673
 Different growth ages 73
 Dihydroagarofuran derivatives 845
 Dihydrocapsaicin 91
 Dihydroflavonol 215
 Dihydromyricetin 1233
 Dihydrooxipin 2
 Dihydroxymethyleneamino propanoic acid 259
 3 β ,4 β -Dihydroxypregnan-16-one 179
 6,8-di-*C*- β -glucosylapigenin 741
 Dihydro- β -agarofuran 355
 Diketopiperazine alkaloid 1583
 Diketopiperazines 597
Dimerostemma 669
Dimerostemma arnottii 669
 Dimethoxy-*p*-cymene 1135
 Dimethyl-*S-trans*-marmin 559
Dioscorea zingiberensis 811
 Dioscorealide B 1921
 Diosgenin 373
 Direct analysis in real time-MS 1755
 Discriminant analysis 1631
 Diterpenes 519,1535,1977
 Diterpenic resin acids 1729
 Diterpenoid 13,1873
 Diversity 1681
 DNA damage 1127
 2D NMR 763
 Dolabrane 9
 DPPH 261,545,729,923,1561,1639,1759
Dracocephalum surmandinum 341
 Drimane sesquiterpenes 999,1869
 Drying time 1799
Dumortiera hirsute 1375
Dysophylla stellata 555
 ECH 571
 Echinacoside 571
 Egonic acid 1063
 Egyptian propolis 43
 Ehrlich's ascites carcinoma 1239
Elaeoselinum asclepium subsp. *Meoides* 1111
 Electrospray ionization 1551
 β -Elemene 1981
 β -Elemol 1991
Eleutherobia aurea 205
 Ellagitannins 531
Elsholtzia fruticosa 641
 Enantiomers 1417,1623
 Encapsulation 1273
endo-Fenchyl acetate 279
 Endophyte 1175
 Endophytic fungus 567,811,1575,1583
Enhydra fluctuans 1239
Entamoeba histolytica 867
ent-Clorodanoids 175
ent-Kaurane 1873
ent-Labdane diterpenoid 771
 Environment 465
 Environmental factors 943
epi-Calocedrin 55
 Epicalvine 1191
 Epicuticular wax 1721
 Epileptic seizure 1847
 Epimerization 1055
epi- β -Santalol 1343
 Epoxygermacranolides 675
Eremanthus argenteus 681
 Ergolide 511
Erigeron mucronatus 621
 Erigeside E 1099
 Erigeside F 1099
Eryngium aquifolium 817
Erythrina melanacantha ssp. *Melanacantha* 721
Erythrina variegata 1781
Erythrina vogelii 889
Escherichia coli 249,777
Eschscholzia californica 1035
 ESIMS 1737
 ESI-MS/MS 1639
 Essential cumin oil 1355
 Essential oil 115,121,129,133,137,143,163, 269,279,283,291,301,307,311,315,319,329, 337,341,457,471,481,613,615,625,629,815, 817,823,831,835,8937,939,957,961,965,969, 975,981,1107,1111,1115,1117,1123,1127,1133, 1135,1139,1143,1161,1283,1285,1291,1295, 1299,1301,1307,1311,1321,1349,1427,1431, 1437,1465,1477,1493,1663,1669,1673,1677, 1681,1809,1811,1815,1819,1823,1829,1831, 1833,1837,1841,1951,1955,1981,1985,1995,1961
 Essential oil composition 1947
 Essential oil constituent 1381,1959
 Essential oil interaction 1395
 Essential oil yield 1947
 Essential oils 111,461,645,949,1401,1847,1969
 Ethyl-amino-imidazolyl-propylcarbamate 377
 6-Ethoxydihydrochelerithrine 1751
 6-Ethoxydihydrodrosanguinarine 1751
 Etiolated wheat coleoptile bioassay 1043
Eucalyptus camaldulensis 329
Eucalyptus globules 1401,1669
Eucalyptus gomphocephala 1639
 Eudesmanes 669,1717
 Eudesmanolides 675
 β -Eudesmol 273,1143,1677,1823
Eugenia triquetra 965
 Eugenol 345,477,1999
Eupatorium capillifolium 1409
Euphorbia guyoniana 35
 Euphorbiaceae 35,215,361,423,481,1209,1885
Eurotium rubrum 1583
Eurycoma longifolia 17,1009
exo-Fenchyl acetate 279
 Expert systems 763
 Expression 1329
 Extract screening 431
 Extractive rate 893
 Extracts 1395,1941
 F3'H from *Gerbera hybrid* 1893
 Fabaceae 721,747,889,903,1213,1939
 Fagaceae 1597
Falcaria vulgaris 981
 Fatty acid 587,1085
 Fatty acids 1643,1663
 Fermentation 1277,1767
 Ferns 1655
 Ferric reducing activity 441
Ferulago isaurica 1951
Ferulago longistylis 1951
Ferulago pachyloba 1951
Ferulago platycarpa 1951
 Ferulic acid 435
 FIA-ESI-MS 431
Ficus chlamydocarpa 1607
 Filicinic acid 1655
 Fingerprint 1631
 Fir 1291
 Flacourtiaceae 771
 Flavanone 3 hydroxylase 777
 Flavanone glycoside 1899
 Flavone 399,1213
 Flavone glycosides 39,841
 Flavonoid 1893
 Flavonoid C-glycoside 741
 Flavonoid glycosides 223,783
 Flavonoid hydroxylation 777
 Flavonoids 35,59,65,223,541,685,725,729,849 1219,1225,1233,1601,1607,1759
 Flavonol 215,1209
 Flavonols 415,1061,1903
 Flower heads 129
 Fluorescent labeling 1623
Foeniculum vulgare 1431
Foeniculum vulgare 319
 Food additive 61
 Food chain 485
 Forensic chemistry 1317
 Formadienoate-A 919
 Formadienoate-B 919
 Formulations 645
 Fragmentation 1551
 Fragrance 1441
 FRAP 261
 Free radical scavenging 65,1453
 Free radicals 1607
 Free-radical scavengers 923
Fructus Aurantii Immaturus 795,893
 Fruit dehulling 1799
Frullania 1375
Fucus serratus 581
 Fujianmycin C 1917
 Fumigant toxicity 301
 Fungal secondary metabolites 1175
 Fungi 81
 Furanoremophil-1-one 1809
 Furanoeremophilane 1
 Furocoumarin 551
Fusarium redolens 811
Fusarium sp. 1771
Gaemannomyces amomi 567
 Gaillardin 511
Galinsoga parviflora 1831
 Gallium-curcumin 1935

- Ganoderma lucidum* 1183
 Garden cress 1969
 Gas chromatography mass spectrometry 1995
 Gas chromatography-flame ionization detector 1965
 Gas chromatography-mass spectrometry 1965
 Gastroprotective activity 1215
 GC 147,297,1107,1111
 GC analysis 1951
 GC/MS 147,641,985,1085,1201,1285, 1421,1441
 GC-FID 831
 GC-MS 143,291,297,457,461,621,831,1123, 1317,1427,1431,1649
 GC-MS analysis 107,1951
 Gene expression 1147
 Genetic engineering 1507
 Genipin 1853
Genista sessilifolia 1127
Genista tinctoria 1127
Genista ulicina 835
Genista vepres 835
 Genisteae 835
 Genotoxicity 621,1039
Gentiana 649
 Gentianaceae 369
 Geraniaceae 899
 Geraniol 163,1947
Geranium 899
Geranium bellum 531
Geranium potentillaefolium 531
 Geranyl acetate 163
 Geranylnerol derivatives 1721
 Germacranolides 675
 Germacrene 1117
 (S)-(-)-Germacrene D 351
 Germacrene A synthase 709
 Germacrene-D 311,817,939
Giardia lamblia 137
 Glabranol A 1717
 Glabranol B 1717
 Glabridin 1907
 Glandular hair 1437
 Glandular trichome 709
Glechoma hederacea 61
 Glibenclamide 1103
 Globulol 1669
Glochidion eriocarpum 361
 Glochieriol 361
 Glochieriosides C-E 361
 β -Glucan 407
 Glucose uptake 231
 α -Glucosidase 1049
 α -Glucosidase inhibitor 1077
 Glutamate-induced toxicity 851
 Glyceride derivatives 1939
 Glycoalkaloids 1743
 Glycyrrhisoflavone 1049
Glycyrrhiza glabra 1243
Glycyrrhiza inflata 1755
Glycyrrhiza uralensis 1049
 Gmelinoside I 1061
 Griseusin A 249
 Guaianolides 1865
 Guar gum 915
 Guttiferone 751

 H/D-exchange 1055
 HaCaT cell 1081
 Hairy root 1913
 Halophyte 603
 Headspace 1123
 HeLa cells 1639
 Heliantheae 669
Helianthus annuus 709
 Hemolytic activity 975
 Hemostasis 1263
 Hepatocellular carcinoma 1613
 Hepatoprotective 1243

 Hepatotoxicity 519,1457
 HepG₂ 1613
 HepG2 cells 1039
 Herbal medicines 431,523,1329
 Herbs 65,441,1453
 Herpes simplex virus 1307
 Herpes simplex virus type 1 1935
Heterosiphonia japonica 1575
 Hexadecanoic acid 1649
 Hexahydrofarnesyl acetone 273
 (Z)-3-Hexenal 1421
 (Z)-3-Hexen-1-ol 1829
Hibiscus tiliaceus 1583
 Hierarchical cluster analysis 1631
 High performance liquid chromatography 1081,1453
Hippospongia 259
 Hirsutosoide 563
 Hispidin 1927
 Histochemistry 107
 HL-60 103
 HMBA 1071
 Honokiol 235
 HPLC 47,95,435,541,555,897,985,1049
 HPLC analysis 419
 HPLC/DAD 893
 HPLC-DAD 877
 HPLC-MS/MS 789
 HPLC-PDA-ESI/MS/MS 545
 HPTLC 863
 HSCCC 1031
 HSV-1 1935
 Human cancer cell lines 1567
 Human leukocyte elastase (HLE) 1071
 Human monocytic leukemia (THP-1) cell line 975
 Human parasitosis 137
 Human whole blood 1441
 Humic acid 395
 Humic substances 395
 α -Humulene 1961
 Humulene epoxide II 1981
 Hydration 801
 Hydrodistillation 111,291,297,461,641
 Hydroformylation 1985
 Hydrolysis 801
 Hydroxybutenolide 859
 Hydroxycinnamic acids 435
 Hydroxydimethoxycyclohexenone 245
 Hydroxy-hydroxyphenyl-propionamide 245
 20-Hydroxyecdysone 20,22-monoacetone-25-acetate 1579
 4-Hydroxy-2-isopropylphenyl)ethanoic acid 55
 10 α -Hydroxyl-artemisinic acid 1531
 Hydroxylation 1893
 Hydroxyl radical 923
 5-hydroxymethyl furfural 575
 27-Hydroxyoleanolic acid 197
Hylodendron gabunensis 1939
 Hylodiglyceride 1939
 Hyloglyceride 1939
Hymenaea courbaril 1977
 Hyperforin 897
 Hypericaceae 107,1493
 Hypericins 897
Hypericum 1493
Hypericum androsaemum 107
Hypericum confertum 897
Hypericum perforatum 431,535
Hypericum richeri 1215
 Hypoglycemic 545

 IFRA 1317
 IL-1 β 1457
 Illumination 1907
 Imidazole alkaloids 377
 Immunomodulation 447
 Immunosuppression 447
in vitro Culture 927

in vitro Regeneration 927
in vivo Functional characterization 709
in vivo Volatile emission 1321
 Incense 1317
 Incorporation of ¹⁴C leucine 607
Indigo naturalis 1039
Indigofera tinctoria 1039
 Indirubin 103
 Indirubin-3'-oxime 103
 Induction 1147
 Inflammation 415,1311
 Infrageneric classification 897
 Inos 571
 Inotropic action 369
 Insect repellent 935
 Insecticidal activity 355,1247,1301
 Insecticide 1135
 Integrated bioprocesses 1507
 Interaction 1381
 Internodes 1945
Inula crithmoides 315
Inula oculus-christi 511
in-vitro Drug release 915
 Involutrin 1081
 Iridoid glucosides 1711
 Iridoids 841
 Iron ion chelating ability 1775
 Irregular sesquiterpenyl group 215
 Isoatropicolide tiglate 851
Isochrysis galbana 1941
 β -Isocryptoxanthin 1733
Isodon leucophyllus 1873
 Isoflavone 1771,1795
 Isoliquiritigenin 1243
 Isonymphaeol-D 43
 isoquinoline alkaloids 869,1035,1751
 Isothymol 1107
 Italy 1285

Jacquinia flammea 365
Jasminum sambac 157
 Jiamizioside E 1097
 Jirakadyarishta 1767
 Junenol 1981
 Junin virus 1307
 Juniper berries oil 1359
Juniperus asheii 1359
Juniperus chinensis 55
Juniperus communis 1359
Juniperus thurifera 1991

 Kaempferol 1597
 Kaempferol 3-O-glycosides 1903
 α -Kessyl acetate 297
 Key enzyme, activity 1147
 Kinetics 1953
 Kumatakenin 1063

 Labdane 1535
 Labiatae 555,1465,1873
 Lachnophyllum cumulene 621
 Lactic acid bacteria 1277
 Lactone glycosides 783
 Lamiaceae 65,261,341,625,641,685,919,937, 939,943,957,1465,1785
Laminaria digitata 581
Laminaria hyperborean 581
 Landik 1711
Lantana 635
Lantana camara 1567
Lantana canescens 635
Lantana radula 635
 Larvicidal activity 477,481,965
Laserpitium zernyi 307
 Lauraceae 337,383,1053,1143,1677,1823
Laurencia claviformis 1859
Laurus nobilis 111
 lavandulyl flavanone 1889
Lavendula angustifolia 1431

- Layer-by-layer self-deposition 1401
 LC/ESI-SRM 869
 LC-DAD 23
 LC-ESI-MS 23
 LC-MS/MS 1551
 LC-MS-MS 741
 Leaf biomass yield 1947
 Leaf ontogeny 1947
 Leaves 115
 Lectins 607
 Leguminosae 1781
Leishmania amazonensis 1837
Leishmania mexicana 387
 Leishmanicidal activity 387
Leontopodium andersonii 667
Leptospermum scoparium 1803
Leptosphaeria maculans 883
 Leukemia 103
Libanotis pyrenaica 1427
 Licochalcone A 1755
 Licorice 1049
 Light intensity 535
 Lignan 423,1623
Ligularia oligonema 1
 Limonene 457,965,981,1291,1421,1811,1991
Limonium gmelinii 1061
 Limonoid 859
 Linalool 345,859,965,969,1123,1441,1837
 Linalyl acetate 1123
 (R)-(-)-Linalyl acetate 351
Linaria kurdica subsp. *Eriocalyx* 841
 Linarin 841
 Linarin 841
 Lindenane 1717
 Linoleic acid 85
 Lipid Peroxidation 91
 Lipopolysaccharide 1457
 Liposomes 1401
 Lipxygenase 85
Lippia 635
Lippia dulcis 613
Lippia multiflora 645
 Liquiritigenin 1243
Litchi chinensis 529
Litsea coreana 1677
Litsea cubeba 617
Litsea linii 1823
Litsea mashaensis 1823
 Liverworts 999
Lobelia chinensis 1627
Lobophytum patulum 205
 Long chained feruloyl esters 919
 Longifolene 1365
 5-LOX 383
 LSD 763
 Lup-12,20(29)-diene-3,27-diol 519
 Lutein 1043
 Luteolin-4'-O-glucoside-7-O-galacturonide 1767
Lychnophora ericoides 733
 Lycorine 873,1195
 lysosomal activity 1877

 Macapruinosins A-C 215
Macaranga pruinosa 215
Macaranga rhizinoides 1209
 Macarhizinoidin A 1209
 Macarhizinoidin B 1209
 Macaronesia 1465
 Maceration 51
Machilus philippinensis 337
Machilus pseudolongifolia 1143
 Macrophages 1877
 Madagascar 1803
Magnolia dealbata 235
Magnolia officinalis 1631
 Magnolidaceae 235
 Magnolol 235
 Mallotophilippen 211
Mallotus philippensis 423

Mallotus philippinensis 211
 Mangrove endophytic fungus 1771
 Mangrove plant 1583
Marchantia 1375
 Marine alga 607
 Marine fungus 507,1789
 Marine natural product 1571
 marine sponge 1881
 Marine sponge 259,1187
 Marine streptomycetes 1917
 Marker compounds 47
 Massage aromatherapy 157
Mastigophora dieladlos 1375
Matricaria suaveolens 133
 MC3T3-E1 cells 1711
 MCF-7 1921
Melaleuca alternifolia 1401
 Melanoma cells 1127
 Meliaceae 179
Melissa officinalis 111
 Menispermaceae 1747
Mentha x piperita 1945
 Menthofurans 685
 Menthol 1417
 Menthone 1299
 MEP pathway 163
 Metformin 427
 Methicillin resistant *Staphylococcus aureus* 249,1781
 Method validation 23,863,877
 2-Methoxy-8-methyl-naphthalene-1,4-dione 1115
 4-Methoxyquinolone 1463
 Methyl eugenol 345,477
 Methyl ethyl-trimethyl benzene 315
 4'-O-Methylglycosylation 77
 Methyl-ribofuranosyl-uracil 253
 Miconazole 185
 Microbial biotransformation 77
 Microdistillation 297,1421
 Micronucleus test 1039
 Micropropagation 235
 Microwave assisted extraction 51
 Mite antigen 1081
 Mitrogenin 1783
Mitrephora maingayi 1783
 Mixture analysis 1755
 Modeling 535
 Modified Mosher's method.
 Mollioside 13
 Mollissin 13
 Monocillin 1 801
 Monocrotaline 1457
 Monoterpene 1167
 Monoterpenes 1969
Morinda royoc 809
 Morindone 809
 Morocco 1349
 Mosquito control 1409
 Mouse 143
 mRNA expression 1547
 MRSA 1463,1781
 MS 407,1111
 MS/MS 783
 MTPA esters 801
 MTT assay 511
Mucor plumbeus 1859
 Mud 395
 Multi-components 795
 Multiple shoot regeneration 1945
 Mushrooms 923
 Mutagenicity 1225
Mycobacterium tuberculosis 211
 Mycorrhiza 823
 Myrcene 1811
 Myricitin-3-O- α -rhamnoside 1233
 Myristicaceae 1795
Myrocarpus fastigiatus 1359
 Myrrh oil 1359

 Myrtaceae 965,1639,1669,1833
 Myrtenyl acetate 1833
Myrtus communis 1659

 N-Acetylcysteine 519
 Nasopharyngeal carcinoma 151
Natrii Sulfas 795,893
 Natural flavors 1507
 Natural products 81,137,485,507
n-Decanal 1677
Nectandra purpurascens 1063
Neocallitropsis pancheri 949
 Neolignans 755,1063,1627
Neolitsea sericea 1311
 Neophytadiene 481
 Nepatanol 1785
Nepeta distans 1785
Nepeta persica 625
 Nepetalactone 625
 Nerolidol 1365
 (E)-Nerolidol 1655,1837
 Neuroprotective 851
Neurospora crassa 515
 Neutrophil 415
 New Zealand 1803
N-formylsylimilobine 2-O- β -D-glucopyranoside 1747
 NF- κ B 571
Niphates digitalis 1187
 Nitidone A 1787
 Nitodone B 1787
 Nitric oxide 1547
 Nitrene cycloaddition 1191
 Nitroxides 241
 NMR 191,205,259,407,419,529,551,667,755, 1259,1539,1737,1881
 NMR analysis 99
 NMR data assignment 1205
n-Nonane 311
 NO 571
 Nociception 1103
 Nonacosane 1649
 Non-protein amino acid 485
 Non-volatile 1395
 (-)-Nopol benzyl ether 1339
 Novel oil 587
 Nuclear factor-K β 1311
 Nuclear transcription factor-kappa-B 1253
 Nucleobases 1187
 Nucleosides 1187
 Obtusilactone A 911
 Occidenol 5
 Occidentalol 5
Ocimum basilicum 65
Ocotea macrophylla 383
 1-Octen-3-ol 1421,1655
 Oil extracts 1215
 Oil quality 1799
 Oil yield 1117
 Oleanolic acid 191,863
 Oleanolic acid derivatives 1567
 Olfactory stimulation 1441
Oplopanax horridus 1019
 Orange fruit cultivars 435
 Organogenesis 927
 Organ-specific accumulation 1055
Origanum glandulosum 957
Origanum syriacum var. *syriacum* 957
Origanum vulgare subsp. *Hirtum* 1437
Ormenis multicaulis 1349
Ottoa oenanthoides 1115
 Ovicidal activity 301
 Oxoamides 259
 (-)-4-oxonopol 1339
 (-)-4-oxonopol-2',4'-dihydroxybenzyl ether 1339
 2-Oxo-14,15-bisnor-3,11E-kolavadien-13-one 1543
 Oxygenated monoterpenes 943

- Pacifenol 1859
 Pacifidiene 1859
 Palm oil 1623
 Palmarosa 1947
 Palustric acid 1729
Pancreatium maritimum 873
 Papaveraceae 1035,1751
 Patchouli alcohol 1961
Paulownia coreana 851
 PCA 1991
p-Coumaric acid 435
p-Cymene 1811,315,957
p-Cymene derivatives 519
 Peak origination 789
Pelargonium 1349
Pelargonium 1395
Pelargonium absolute 1349
 6-Pentadecanoylsalicylic acid 85
Pentadesma 1055
 Pentagalloyl glucose 899
 Pepper 985
 Perilla aldehydes 341
Peristrophe bicalyculata 1815
Peronophythora litchi 245
 Pesticides 1325
Petrorhagia velutina 99
 PGE₂ 733
Phaeosphaeria spartinae 1071
 Pharmacokinetics 1441
 Pharmacokinetics 795
 pharmacological actions 1999
 α -Phellandrene 1811
Phellinus linteus 1927
 Phenethyl bromo ester 399
 Phenolic acid derivatives 1601
 Phenolic acids 407,1263
 Phenolic amide 1259
 Phenolic compounds 1759
 Phenolic constituents 897
 Phenolic content 73
 Phenolic glycoside 1097
 Phenolics 535,575,1639
 Phenyl alkynes 815
 2-Phenylethanol 1655
 Phenyl propanoic acid derivative 1619
 Phenylethanoid glycoside 563
 Phenylethyl-chromones 403
 Phenylmethylene hydantoin 1623
 Phenylpentadiyne 815
 Phenylpropanes 1387
 Phenylpropanoid glycerides 1601
 Pheophorbides 99
 Pheophytin 99
 Phloroglucinols 211
Phoma lingam 883
Phoma macrostoma 81
Phoma sp. 1175
 Phosphodiesterase 265
 Photoinduced phytotoxicity 99
 Photooxidation 859
 Phtytoceuticals 319
 Phyllocladene isomer 817
 5,7'-Physcion-physcion-10'-C- α -arabinopyranoside 747
 Physiological activity 1687
 Phytoalexin 883
 Phytoecdysteroids 1579
 Phytol 1815
Phytolacca rugosa 775
 Phytolaccaceae 775
 Phytolaccagenin 1013
 Phytosphingosine 1081
 Phytotoxicity 1043,1233,1539
 Pictet reaction 1591
 Pineapple weed 133
 Pinene 965,1417
 α -Pinene 143,481,961,981,1659,1729,1833
Pinus 1729
Pinus armandii 1295
Pinus tabulaeformis 1295
Piper clausenianum 1837
Piper divaricatum 477
 Piperaceae 477,1837
 Piperidines 1191
 Piperine 1253
 Piperitenone 1299
Pistacia atlantica 115
 Plakinamine 33
 plant growth hormones 1945
 Plant metabolites 391
 Plant pathogens bioautography 1409
 Plant product NP-1 47
 Plasmid stability 1893
 Plasticizers 1325
 Platelet aggregation 383
Pleiogonium timorensis 545
Pleurothyrium cinereum 383
Plocamium brasiliense 1167
 Plumbagin 1913
 Plumbaginaceae 1061
Plumbago indica 1913
 PM-ATPase 345
 Podocarpaceae 1133
 Podophyllotoxin 1247
 Podophyllotoxin 241
Pogostemon cablin 1961
Pogostemon heyneanus 1961
 Poly[3-(3,4-dihydroxyphenyl)glyceric acid] 1091
 Poly[oxy-1-carboxy-2-(3,4-dihydroxy-phenyl)ethylene].
Polyalthia cerasoides 1931
Polyalthia simiarum 1543
 Polygonaceae 223
 Polyhydroxysteroid glycosides 1737
 Polymer-coating 1401
 Polyphenolic content 441
 Polyphenols 227,435,733
 Polyporales 923
 Polysaccharide 447,1941
Pometia pinnata 191
 Pompeia 927
Pongamia pinnata 1213
Porella perrottetiana 1375
 Porifera 259
 Portugal 1465
 Potato sprouting 645
 Potentene A 1561
 Potentene B 1561
Potentilla fulgens 1561
 Prenylated flavonoids 43,721,889
 Primulaceae 1023
 Principal component analysis 115
Prismatomeris tetrandra 1251
 Proanthocyanidin 407
 Proanthocyanidin content 73
 Procersterol 867 Antiamoebic activity 867
 Procoagulant 1263
 pro-IL-1 β 1941
 Propolis 1601
 Prostaglandin E₂ 1547
 protein tyrosine phosphatase 1 β 1927
 Proteomic 1183
Protium hebetatum 1181
 Pteridophytes 1655
 Pterocarpanes 721
Pteroxygonum giraldii 223
 Pulchellin C 511
 Pulegone 1299
Pulsatilla albana 1299
 Purification and characterization of protease 931
p-Vinyl guaiacol 273
 Pycnanthos 1795
Pycnanthus anglonensis 1795
 Pyrolytic syn elimination 993
 Pyrrolizidine alkaloids 1457
 QSAR 1387
 Quality control 23
 Quantification 555
 Quantitative analysis 541,1775,1803
 Quantitative determination 907
 Quassinoids 1009
 Quercetagenin 61
 Quercetin 1215
Quercus dentata 1597
 Radical scavenging activity 515
 Radicle elongation 1969
 Radish 1969
Radix et Rhizoma Rhei 795,893
Radix Hedysari 541
Radix ilicis pubescentis 23
 Ranunculaceae 197,1299
Ranunculus japonicus 783
 RAPD 587
Raphanus sativus 99
 Rat plasma 741
Ravensara aromatica 1803
 Red alga 1575
 Red blood cells 1035,1751
 Red marine alga 1267
 Red Sea sponges 27,1623
 Repellence 301
 Resibufogenin 1031
 Reticuline 1035
 Retro-aldol reaction 993
 Retro-electrocyclic 5
Retrophyllum rospigliosii 1133
 Rhodesain 1161
Rhodymenia coralline 1643
 Ribonucleotides 265
Ribes nigrum 1613
 Ring-A-aromatized bile acids 1571
 ROA 1417
 Root 1531
 ROS formation 1877
Rosa x centifolia 1349
 Rosaceae 1561
 Rosmarinic acid 227,1453
Rosmarinus officinalis 1349
 Rottlerin 211
 RP-HPLC 907
 Rubiaceae 1251
 Rutaceae 457,559
 Rutin 1219
 Sabinene 1669,1811
Saccharomyces cerevisiae 709
Saccharothrix espanaensis An 113 597
 Sage 1453
 Sakurososaponin 365
Salicornia brachiata 603
Salmonella typhimurium 391
Salvia 227,1421
Salvia leucantha 937
Salvia miltiorrhiza 805
Salvia officinalis 1453
 Salvanolic acid A 805
 Salvanolic acid B 805
 Sandalwood odor 1343
 β -Santalol 1343
Santiria trimera 961
 Sapindaceae 191,529
 Saponins 365,1013
 Sapotaceae 935
Sarcandra glabra 1717
Sarcophyton infundibuliforme 1171
Satureja intricata 629
Satureja obovata 629
Satureja x delpozoi 629
Saussurea lappa 1531
Scalarispongia aqabaensis 27
Schisandra chinensis 231
 (-)-Sclerotiorin 1789
 Scopoletin 315
 Scorzonerae 725

- Scrophulariaceae 841
 Seasonal variation 457
 Seaweed extracts 581
 Seaweeds 1643
 Secoiridoid glycosides 1525
 Secokaurane 13
 Secondary metabolites 649
 Secretory structures 1225
 Seed 587,1291
 Selectivity index 975
 α -Selinene 269
 β -Selinene 269
 Semisynthetic derivatives 717
Senecio nemorensis 831
Senecio othonnae 831
Senecio pterophorus 1811
Senecio racemosus 831
Senna septemtrionalis 747
 Separation 805
Sepedonium 869
 Serine protease 931
 serjanic acid 775
 Serpentinophyte 1117
 Sesamin 911
Seseli hartvigii 1067
Seseli libanotis 1427
Seseli praecox 551
 Sesquiceneol 817
 Sesquiterpene 5,667,1531,1717,1965
 Sesquiterpene lactone 151, 669,681,849,851, 1721
 Sesquiterpene synthase 709
 Sesquiterpenes 685,1663,1681,1977
 Sesquiterpenoids 1,695
 SFE 641
 SFME 111
 Shelf life 1799
Sideritis 51
Silene viridiflora 1579
 Simaroubaceae 17
 Similarity analysis 1631
 Sinapic acid 435
 SISTEMAT 755,763
 Smallanthaditerpenic acid 95
Smallanthus sonchifolius 95,1721
Smyrniolum olusatrum 1669,1809
 SOD 899
 Sodium alginate 1273
 Soft corals 205,1171
 Solanaceae 615,1743
Solanum 615,1743
Solanum bicolor 615
Solanum hypomalacophyllum 1743
Solanum tuberosum 645
 Solid cultures 245
 Solid-state CD / TDDFT calculations 1175
Solieria filiformis 1267
Sollasella moretonensis 1571
 Solvent 1907
 Solvent-free microwave extraction 111
Sophora flavescens 1889
 Southern green stink bug 301
 Soy extracts 1775
 Spartinoxide 1071
Spathelia sorbifolia 859
 Spathulenol 273
 SPE 1431
 Spectroscopic characterization 919
 SPME 291,1317,1427
 Sponge 377,1571
 Spreading 1877
 Stability 1907
Stachys cretica ssp. *Lesbiaca* 1369
Stachys cretica ssp. *Trapezuntica* 1369
 Standardization 575,1329
 Starfish 1737
 Stathmin 1183.
 Static headspace 291,1441
 Stemphol galactoside 567
Stephania rotunda 877
Stephanitis pyrioides 1409
 Stereochemistry 1067
 Stereoselective synthesis 1191
Sternbergia 873
 Steroid 201,1795
 Steroidal alkaloid 33
 Sterol synthesis inhibitor 185
 Sterols 205
 Stilbene 215
 Stimulating effect 157
 Strawberry anthracnose 1409
 Strawberry tree 1085
Streptomyces griseus 249
 Streptozotocin 427
 Stress 1441
 Structure-activity relationships 1247,1567,1601,1759,1847
 Structure-odor relationships 1343
Subergorgia suberosa 201
 Sucrose 1273
 Sulfated polysaccharide 1267
 Supercritical CO₂ 461,1955
 Supercritical fluid extract 1995
 Superoxide 545,1639
 Sustained attention 1441
 Sweet orange 1819
Swertia paniculata 907
 Sylvestin 771
Symphonia pauciflora 751
Symphytum 587
 Synergism 1381
 Synseeds 1273
 Synthesis 1587
Szovitsia callicarpa 297
 Tagalsin O 9
Tagetes pusilla 1283
Tanacetum macrophyllum 121
 Taxaceae 1727
 Taxoids 1551
 Taxonomy 587
Taxus wallichiana 1727
 T-cadinol 1321
Tectona grandis 427
 Temperature 535
 TEO 345
 Terbinafine 185
 Terpene 1417,1785
 Terpenes 1291,1507,1847
 Terpenoids 129,179,999,1375
 γ -Terpinene 815,1833
 Terpinen-4-ol 279
 α -Terpineol
Tessmannia martiniana var. *martiniana* 175
Tessmannia martiniana var. *pauloi* 175
 Tetrahydropalmitine 877
 Tetranortriterpenoids 859
Tetranoychus urticae 471
Teucrium arduini 1969
Teucrium maghrebinum 1969
Teucrium montbretii ssp. *Heliotropiifolium* 1969
Teucrium polium ssp. *Capitatum* 1969
Teucrium quadrifarium 939
Teucrium royleanum 939
 Theophrastaceae 365
 Thiocarbamates 1587
Thuja sutchuenensis 1673
 α -Thujone 121,283
Thymbra 1465
 Thyme 1681
 Thymol 957,1681
 Thymol methyl ether 1409
Thymus 1465
Thymus capitatus 1985
Thymus migricus 943
Thymus pannonicus 1681
Thymus praecox ssp. *polytrichus* 1123
Thymus praecox ssp. *skorpilii* 1123
Thymus vulgaris 291
Tinospora crispa 1747
 Tirucallane 1181
 Tirucallanoid 17
 Tissue cultures 1219
 Tissue distribution 143
 Tocopherols 1643
 δ -Tocotrienol 1623
 Total glycosides 783
 Total phenol 261
 Total phenolics 923,1775
 Total synthesis 1077
Toussaintia orientalis 253
 Toxicity 481,485,1981
 Toxin 485
 Traditional Chinese medicine 789
trans-Anethole 1283
trans-Dehydrocrotonin 519
 Translucent glands 107
trans-Pinocarveol 1669
 Tricalysioside U 771
Trichilia quadrijuga 179
 Trichome density 1437
 Trigalloyl glucose 899
 Trigonelline 581
 Triterpene 529
 triterpene glycosides 1877
 Triterpene saponins 775,1023
 Triterpenes 1055,1181,1561
 Triterpenoid 1027
 Triterpenoid saponin 191,361,1019
 Triterpenoid saponins 197,1147
 Trypanosomiasis 1161
Turnera ulmifolia 1829
 Turneraceae 1829
Turraeanthus longipes 1535
Turraeanthus mannii 1535
 Type 2 diabetes mellitus 231
 Tyrosol 81

 U-937 733
 Ultrasound-assisted extraction 51,111
Ulva fasciata 607
 Umbelliferae 981,1809
 Unsaponifiable fraction 1085
 UPLC/Q-TOF 783
 UPLC-DAD-TOF-MS 1631

 Validation 555
 Vasoconstriction 369
 Vasodilatation 1267
 Verbenaceae 269,613,853
 Verbeneol 1417
Verbesina turbacensis 1161
 Vernoniaceae 681
Verticillium tenerum 507
 Verticinol A 507
 Verticinol B 507
Viburnum dilatatum 1097
 Vicenin-2 733,741
 Virucidal activity 1307
 Vitamin E 519
 VOC 1655
 Vogeol 889
 Vogliol 889
 Volatile 985,1381
 Volatile components 273,1649
 Volatile oil 151,1965
 Volatile organic compounds 107,1655
 Volatiles 147,1291,1427,1465,1493

 Water retention 395
 Wen-Hou-Po 1631
 Western Mediterranean 1465
 Whitefly 1819
 wild Moroccan chamomile 1349
Withania aristata 1043

 Xanthones 751,907,1055

XRD 603
Xylopinine 877

Yacon 95,1721
Yeast 1381
Yield variations 581

β -Ylangene 1981

Zanthoxylum clava-herculis 457
Zephyranthes grandiflora 1273
Zephyranthes robusta 1201
Zhejiang Province 1631

Zingiberaceae 279,1219
 α -Zingiberene 1815
Zizyphus oxyphylla 1205

Natural Product Communications

Manuscripts in Press Volume 5, Number 12 (2010)

Essential Oil Composition of *Vismia macrophylla* Leaves (Guttiferae)

Janne Rojas, Alexis Buitrago, Luis Rojas and Antonio Morales

Traditional Medicine in Syria: Folk Medicine in Aleppo Governorate

Amal Alachkar, Ahmad Jaddouh, Muhammad Salem Elsheikh, Anna Rita Bilia and Franco Francesco Vincieri

Chemical Variability of Essential Oils in Natural Populations of *Cupressus dupreziana*

Messaoud Ramdani, Takia Lograda, Pierre Chalard, Jean Claude Chalchat and Gilles Figueredo

Antimicrobial Activities of Indole Alkaloids from *Tabernaemontana catharinensis*

Maria Rita Furquini Medeiros, Luiz Afonso de Melo Prado, Vanessa Colnaghi, Sérgio Souza Figueiredo, Juliana Coppede, Juliana Martins, Giovana Maria Lanchoti Fiori, Nilce Maria Martinez-Rossi, Rene Oliveira Belebani, Silvia Helena Taleb Contini, Paulo Sérgio Pereira and Ana Lúcia Fachin

Composition of a Monoterpenoid-rich Essential Oil from the Rhizome of *Zingiber officinale* from North Western Himalayas

Suphla Gupta, Pankaj Pandotra, Gandhi Ram, Rajneesh Anand, Ajai Prakash Gupta, Mohd. Kashif Husain, Yashbir Singh Bedi and Gopal Rao Mallavarapu

Authentication of Chinese Crude Drug Gecko by DNA Barcoding

Hai-Feng Gu, Yun Xia, Rui Peng, Bang-Hui Mo, Li Li and Xiao-Mao Zeng

Oxyresveratrol Protects Against DNA Damage Induced by Photosensitized Riboflavin

Manusannunt Chatsumpun, Taksina Chuanasa, Boonchoo Sritularak and Kittisak Likhitwitayawuid

Comparative Biochemical Characterization of 5'-Phosphodiesterase and Phosphomonoesterase from Barley Malt Sprouts

Suncica Beluhan and Vladimir Maric

Chemical Composition of the Essential Oil of *Croton gossypifolius* from Venezuela

Alfrica I. Suárez, Marly Oropeza, Luís Vásquez, Stephen Tillett and Reinaldo S. Compagnone

Terpenoid Compositions and Antioxidant Activities of Two Indian Valerian Oils from the Khasi Hills of North-east India

Jayashankar Das, Ashiho A. Mao and Pratap J. Handique

Volatile Constituents of *Festuca nigrescens*, *Phleum alpinum* and *Poa alpina* from N.W. Italian Alpine Pastures

Aldo Tava, Roberto Cecotti, Maris Grecchi, Luca Falchero, Mauro Coppa and Giampiero Lombardi

Asimafoetidol: a New Sesquiterpenoid Coumarin from the Gum Resin of *Ferula assa-foetida*

Debasish Bandyopadhyay, Manas Banerjee, Subrata Laskar and Bidyut Basak

Comparison of *Eucalyptus cinerea* Essential Oils Produced by Hydrodistillation and Supercritical Carbon Dioxide Extraction

Tavleen S. Mann, Garikapati D. Kiran Babu, Shailja Guleria and Bikram Singh

Leaf Essential Oil of *Manekia naranjoana* (Piperaceae) from Costa Rica and its Cytotoxic Activity

Carlos Chaverri, Cecilia Díaz and José F. Cicció

The Essential Oil of *Artemisia scoparia* from Tajikistan is Dominated by Phenylpropanoids

Farrukh S. Sharapov and William N. Setzer

Bioactive Isocoumarins from a Terrestrial *Streptomyces* sp. ANK302

Dhafer Saber Zinad, Khaled A. Shaaban, Muna Ali Abdalla, Md. Tofazzal Islam, Anja Schüffler and Hartmut Laatsch

Chemical Composition of Essential Oil of *Senecio coincoyi*, an Endemic Species of the Central Iberian Peninsula

Carlos Arrabal, Felipe Martínez García, María Paz Arraiza and Silvia Guerrero García

Composition, Antioxidant and Antimicrobial Activities of the Seed Essential Oil of *Calocedrus formosana* from Taiwan

Chen-Lung Ho, Eugene I-Chen Wang, Pei-Chun Liao and Yu-Chang Su

New Stress Metabolite from *Bulbophyllum kwangtungense*

Jianbo Chen, Huifang Zhang, Li Chen and Bin Wu

In vitro Antioxidant Activities of Maillard Reaction Products Produced in the Steaming Process of *Polygonum multiflorum* Root

Zhenli Liu, Yuanyan Liu, Zhimao Chao, Zhiqian Song, Chun Wang and Aiping Lu

Quinone Reductase Inducing Activity of the Dichloromethane/Ethanol Extract of the Roots of *Pulsatilla chinensis*

Dan Wang, Ling Han and Zengjun Guo

Targets of Red Grapes: Oxidative Damage of DNA and Leukaemia Cells

Jaouad Anter, Noriluz de Abreu-Abreu, Zahira Fernández-Bedmar, Myriam Villatoro-Pulido, Ángeles Alonso-Moraga and Andrés Muñoz-Serrano

5-Methoxyaristololactam I, the First Natural 5-Substituted Aristololactam from *Asarum ichangense*

Bai-Bo Xie, Ming-Ying Shang, Kuo-Hsiung Lee, Xuan Wang, Katsuko Komatsu and Shao-Qing Cai

A New Eudesmane Sesquiterpene from *Pluchea arguta*

Nikhat Saba, Rasheeda Khatoon, Viqar Uddin Ahmad and Saleha Suleman Khan

Chemical Composition of the Essential Oil from *Carramboa littlei* (Asteraceae)

Yndra Cordero de Rojas, Luis B. Rojas and Alfredo Usubillaga

2-Undecanone Rich Leaf Essential Oil from *Zanthoxylum armatum*

Deepa Bisht and Chandan S. Chanotiya

Inhibition of Protein Tyrosine Phosphatase 1β by Hispidin Derivatives Isolated from the Fruiting Body of <i>Phellinus linteus</i>	
Yeon Sil Lee, Il-Jun Kang, Moo Ho Won, Jae-Yong Lee, Jin Kyu Kim and Soon Sung Lim	1927
A New Azafluorenone from the Roots of <i>Polyalthia cerasoides</i> and its Biological Activity	
Kanchana Pumsalid, Haruthai Thaisuchat, Chatchanok Loetchutinat, Narong Nuntasaeen, Puttinan Meepowpan and Wilart Pompimon	1931
Evaluation of Antiviral Activities of Curcumin Derivatives against HSV-1 in Vero Cell Line	
Keivan Zandi, Elissa Ramedani, Khosro Mohammadi, Saeed Tajbakhsh, Iman Deilami, Zahra Rastian, Moradali Fouladvand, Forough Yousefi and Fatemeh Farshadpour	1935
Hyloglyceride and Hylodiglyceride: Two New Glyceride Derivatives from <i>Hylodendron gabunensis</i>	
Awazi Tengu Nyongha, Hidayat Hussain, Etienne Dongo, Ishtiaq Ahmed and Karsten Krohn	1939
Chemical Composition and Bioactivities of the Marine Alga <i>Isochrysis galbana</i> from Taiwan	
Chi-Cheng Yu, Hsiao-Wei Chen, Mao-Jing Chen, Yu-Ching Chang, Shih-Chang Chien, Yueh-Hsiung Kuo, Feng-Ling Yang, Shih-Hsiung Wu, Jie Chen, Hsiao-Hui Yu and Louis Kuop-Ping Chao	1941
An Efficient Protocol for High-frequency Direct Multiple Shoot Regeneration from Internodes of Peppermint (<i>Mentha x piperita</i>)	
Sanjog T. Thul and Arun K. Kukreja	1945
Essential Oil Yield and Chemical Composition Changes During Leaf Ontogeny of Palmarosa (<i>Cymbopogon martinii</i> var. <i>motia</i>)	
Bhaskaruni R. Rajeswara Rao, Dharmendra K. Rajput, Rajendra P. Patel and Somasi Purnanand	1947
Essential Oil Composition of Four Endemic <i>Ferulago</i> Species Growing in Turkey	
Ceyda Sibel Kılıç, Ayşe Mine Gençler Özkan, Betül Demirci, Maksut Coşkun and Kemal Hüsnü Can Başer	1951
Essential Oils of <i>Daucus carota</i> subsp. <i>carota</i> of Tunisia Obtained by Supercritical Carbon Dioxide Extraction	
Hanan Marzouki, Abdelhamid Khaldi, Danilo Falconieri, Alessandra Piras, Bruno Marongiu, Paola Molicotti and Stefania Zanetti	1955
Oil Constituents of <i>Artemisia nilagirica</i> var. <i>septentrionalis</i> Growing at Different Altitudes	
Flora Haider, Narendra Kumar, Ali Arif Naqvi and Guru Das Bagchi	1959
Volatile Oil Composition of <i>Pogostemon heyneanus</i> and Comparison of its Composition with Patchouli Oil	
Ramar Murugan, Gopal Rao Mallavarapu, Kyathsandra Venkataramaiah Padmashree, Ramachandra Raghavendra Rao and Christus Livingstone	1961
Chemical Composition of Volatile Oils of <i>Aquilaria malaccensis</i> (Thymelaeaceae) from Malaysia	
Saiful Nizam Tajuddin and Mashitah M. Yusoff	1965
Chemical Composition and Phytotoxic Effects of Essential Oils from Four <i>Teucrium</i> Species	
Laura De Martino, Carmen Formisano, Emilia Mancini, Vincenzo De Feo, Franco Piozzi, Daniela Rigano and Felice Senatore	1969
Chemical Constituents and Larvicidal Activity of <i>Hymenaea courbaril</i> Fruit Peel	
José Cláudio D. Aguiar, Gilvandete M. P. Santiago, Patrícia L. Lavor, Helenicy N. H. Veras, Yana S. Ferreira, Michele A. A. Lima, Ângela M. C. Arriaga, Telma L. G. Lemos, Jefferson Q. Lima, Hugo C. R. de Jesus, Pérciles B. Alves and Raimundo Braz-Filho	1977
Caryophyllene Oxide-rich Essential Oils of Lithuanian <i>Artemisia campestris</i> ssp. <i>campestris</i> and Their Toxicity	
Asta Judzentiene, Jurga Budiene, Rita Butkiene, Eugenija Kupcinskiene, Isabelle Laffont-Schwob and Véronique Masotti	1981
Comparison of Antibacterial Activity of Natural and Hydroformylated Essential Oil of <i>Thymus capitatus</i> Growing Wild in North Sardinia with Commercial <i>Thymus</i> Essential Oils	
Marianna Usai, Marzia Foddai, Barbara Sechi, Claudia Juliano and Mauro Marchetti	1985
Composition and Chemical Variability of the Leaf Oil from Corsican <i>Juniperus thurifera</i> Integrated Analysis by GC(RI), GC-MS and ¹³C NMR	
Josephine Ottavioli, Joseph Casanova and Ange Bighelli	1991
Combined Analysis by GC (RI), GC-MS and ¹³C NMR of the Supercritical Fluid Extract of <i>Abies alba</i> Twigs	
Emilie Duquesnoy, Bruno Marongiu, Vincent Castola, Alessandra Piras, Silvia Porcedda and Joseph Casanova	1995
<u>Review/Account</u>	
Eugenol: A Natural Compound with Versatile Pharmacological Actions	
Kannissery Pramod, Shahid H. Ansari and Javed Ali	1999

Natural Product Communications

2010

Volume 5, Number 12

Contents

<u>Original Paper</u>	<u>Page</u>
Anticonvulsant Activity of the Linalool Enantiomers and Racemate: Investigation of Chiral Influence Damião P. de Sousa, Franklin F. F. Nóbrega, Camila C. M. P. Santos and Reinaldo N. de Almeida	1847
Kinetic Analysis of Genipin Degradation in Aqueous Solution Paul Slusarewicz, Keng Zhu and Tom Hedman	1853
Microbial Transformation of Marine Halogenated Sesquiterpenes Aurelio San Martin, Juana Roviroso, Alvaro Carrasco, Silvia Orejarena, Jorge Soto-Delgado, Renato Contreras and M. Cristina Chamy	1859
Two New Guaianolides from <i>Amberboa ramosa</i> Muhammad Ibrahim, Rehan Khan and Abdul Malik	1865
Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from <i>Canella winterana</i> Mary H. Grace, Carmen Lategan, Flaubert Mbeunkui, Rocky Graziose, Peter J. Smith, Ilya Raskin and Mary Ann Lila	1869
Three New 18-Oxygenated <i>ent</i>-Kaurane Diterpenoids from <i>Isodon leucophyllus</i> Hai Bo Zhang, Jian Xin Pu, Yong Zhao, Fei He, Wei Zhao, Li Guang Lou, Wei Lie Xiao and Han Dong Sun	1873
Immunomodulatory Action of Monosulfated Triterpene Glycosides from the Sea Cucumber <i>Cucumaria okhotensis</i>: Stimulation of Activity of Mouse Peritoneal Macrophages Dmitry L. Aminin, Alexandra S. Silchenko, Sergey A. Avilov, Vadim G. Stepanov and Vladimir I. Kalinin	1877
Three New Aaptamines from the Marine Sponge <i>Aaptos</i> sp. and Their Proapoptotic Properties Larisa K. Shubina, Tatyana N. Makarieva, Sergey A. Dyshlovoy, Sergey N. Fedorov, Pavel S. Dmitrenok and Valentin A. Stonik	1881
Isolation and Characterization of Crotosparsamide, a New Cyclic Nonapeptide from <i>Croton sparsiflorus</i> Rashad Mehmood and Abdul Malik	1885
Two New Lavandulyl Flavonoids from <i>Sophora flavescens</i> Dan Liu, Xiulan Xin, Dong-hai Su, Junying Liu, Qing Wei, Bo Li and Jian Cui	1889
Biotransformation of Naringenin to Eriodictyol by <i>Saccharomyces cerevisiae</i> Functionally Expressing Flavonoid 3' Hydroxylase Ilef Limem-Ben Amor, Alain Hehn, Emmanuel Guedon, Kamel Ghedira, Jean-Marc Engasser, Leila Chekir-Ghedrira and Mohamed Ghoul	1893
Two New 3-C-Carboxylated Flavones from the Rhizomes of <i>Caragana conferta</i> Rehan Khan, Abdul Malik, Shazia Yasmeen and Nighat Afza	1899
Kaempferol Glycosides in the Flowers of Carnation and their Contribution to the Creamy White Flower Color Tsukasa Iwashina, Masa-atsu Yamaguchi, Masayoshi Nakayama, Takashi Onozaki, Hiroyuki Yoshida, Shuji Kawanobu, Hiroshi Ono and Masachika Okamura	1903
Factors Influencing Glabridin Stability Mingzhang Ao, Yue Shi, Yongming Cui, Wentao Guo, Jing Wang and Longjiang Yu	1907
Effect of Different Strains of <i>Agrobacterium rhizogenes</i> and Nature of Explants on <i>Plumbago indica</i> Hairy Root Culture with Special Emphasis on Root Biomass and Plumbagin Production Moumita Gangopadhyay, Saikat Dewanjee, Somnath Bhattacharyya and Sabita Bhattacharya	1913
Fujianmycin C, A Bioactive Angucyclinone from a Marine Derived <i>Streptomyces</i> sp. B6219 Muna Ali Abdalla, Elisabeth Helmke and Hartmut Laatsch	1917
Dioscorealide B from the Traditional Thai Medicine Hua-Khao-Yen Induces Apoptosis in MCF-7 Human Breast Cancer Cells via Modulation of Bax, Bak and Bcl-2 Protein Expression Jiraporn Saekoo, Potchanapond Graidist, Wilairat Leeanansaksiri, Chavaboon Dechsukum and Arunporn Itharat	1921

Continued inside backcover