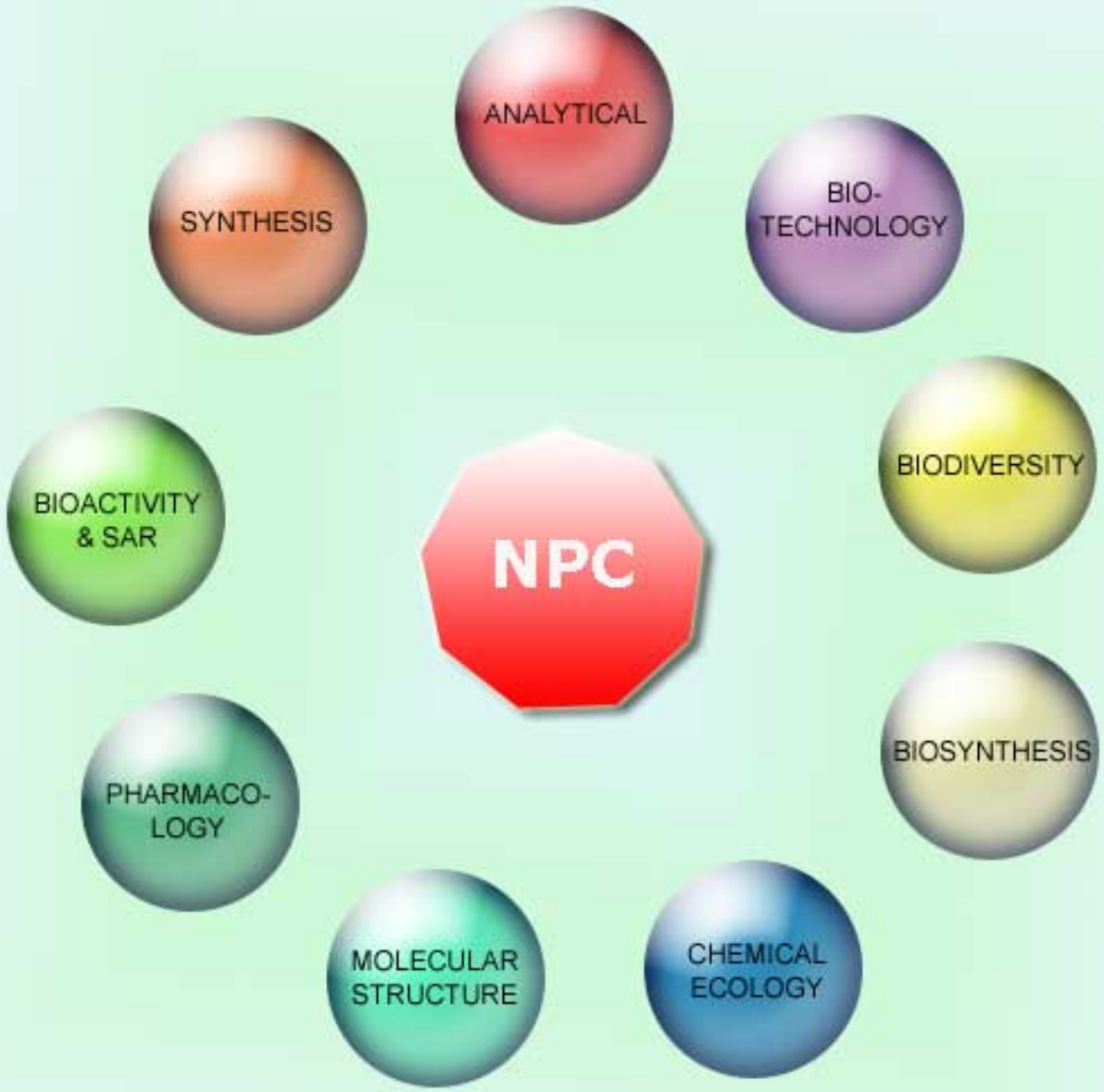
NATURAL PRODUCT COMMUNICATIONS

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



This Issue is Dedicated to
Professor Francesco De Simone
on the Occasion of his 72nd Birthday

Volume 4. Issue 12. Pages 1615-1798. 2009
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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Natural Product Communications Vol. 4 (12) 2009
Published online (www.naturalproduct.us)

Editorial

In Honor of the 72nd Birthday of Professor Francesco De Simone

It is our great privilege and pleasure to introduce this issue, which is dedicated to Prof. Francesco De Simone, Professor of Pharmaceutical Technology, Faculty of Pharmacy, Department of Pharmaceutical Science, University of Salerno, on the occasion of his 72nd birthday.

Prof. De Simone has made many outstanding contributions to the field of natural products science, particularly for the conservation of biodiversity through drug discovery. His research has been based on various aspect of modern pharmacognosy, offering valuable contributions to phytochemistry, food chemistry, and, more recently, pharmaceutical technology applied to herbal drug preparations.

During his distinguished and productive career, spanning over 45 years, Francesco De Simone has either authored or co-authored over 200 peer-reviewed scientific publications, 15 book chapters, some patent applications, and a great number of contributions in congress proceedings.

Prof. De Simone, during his research activity, has paid particular attention to the enhancement of the value of traditional medicine in Latin American countries, including the conservation of their cultural and natural value. His efforts have represented an excellent opportunity to improve collaboration and to re-enforce networks between Italian and Latin American research groups.

This issue, on the occasion of his 72nd birthday on June 27, 2009, represents a tribute to his outstanding scientific contributions and an opportunity to express congratulations and best wishes from all of us, colleagues and friends. Our personal thanks go also to all the authors, mostly of them Prof. De Simone's friends, and reviewers who have contributed to the success of this special issue.

Nunziatina De Tommasi
Alessandra Braca
Pawan K. Agrawal

Natural Product Communications Vol. 4 (12) 2009
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Preface

It is a pleasure for me, as a colleague and friend, to send wishes to Prof. Francesco De Simone on the occasion of his 72^{ns} birthday. The first time I met Prof. De Simone was in October 1969, when I entered, as a student, the Institute of Medicinal and Toxicological Chemistry of the University of Naples. A strong friendship was quickly created between us: I have always appreciated the ecleptic knowledge of Prof. De Simone and his open minded vision of the world.

From the beginning of the 1980s and for almost 20 years, we have been collaborating in very productive scientific work, studying the phytochemistry of medicinal plants from all over the world, but mainly from South America. During this period, mainly due to his efforts, a generation of highly able scientists has been created operating in the field of natural products.

Apart from the high scientific value of Prof. De Simone as a chemist, I also recognize his ability in organizing and promoting science. From the end of the 1980s Prof. De Simone has been the main player in the activation of the Faculty of Pharmacy at the University of Salerno, which is now a well organized and active Faculty. Prof. De Simone also deserves great credit for his efforts as Dean in the periods 1994-1999 and 2004-2008.

Prof. Francesco De Simone's research has focused on all major aspects of natural product science, including isolation, structure elucidation, and the biological evaluation of natural compounds in medicinal plants, foods and cosmetics.

To Francesco, a very outstanding scientist, sincere thanks for his contribution to Phytochemistry!

Prof. Cosimo Pizza
Chairman of Italian Society of Phytochemistry
University of Salerno, Department of Pharmaceutical Sciences
Via Ponte Don Melillo 84084 Fisciano (SA), Italy

A Friend Named Ciccio

I met for the first time Professor Francesco De Simone (friends call him 'Ciccio') in the Spring of 1995 when he, as Dean of the Faculty of Pharmacy, asked me to move from University of Naples to the University of Salerno. I was somewhat sceptical, but, at the same time, I was impressed by his ability to list all the good opportunities that a such change could mean for me and the Faculty of Pharmacy. At that time, the newly established (1992) Faculty of Pharmacy of University of Salerno was very young and, consequently, quite small. I trusted him and now the Faculty include 75 tenure track professors and conducts a relevant educational role in the University of Salerno. Thus, I have to acknowledge his foresight. In the 1996 Professor De Simone was one of founders of the Department of Pharmaceutical Sciences and he has actively participated to its scientific development. Professor De Simone is always lavish of ideas and indefatigable energy in all projects, either national or international, in which he is involved. A characteristic feature of Ciccio's attitude is to see today what is going to be tomorrow and I appreciate his opinions not only as colleague but mainly as a friend.

Prof. Aldo Pinto
Head of Department
University of Salerno, Department of Pharmaceutical Sciences,
Via Ponte don Melillo, 84084 Fisciano (SA), Italy

Natural Product Communications Vol. 4 (12) 2009
Published online (www.naturalproduct.us)

I first met Francesco De Simone at the end of Seventies, when I was a CNR fellow associated to the research group of the late Prof. Minale at the University of Napoli. It was right at the time when he started to move his research interest towards Phytopharmacy and Phytochemistry. He was very active in that field at the Faculty of Pharmacy in Naples where he built a strong research team and established a powerful network of cooperation in several south American countries. Our lives joined again at the University of Salerno in the early 90's. Here he assumed several institutional roles and played a major role in the birth and development of the Faculty of Pharmacy, of which he has been Dean in the years 1994-2000 and 2004-2008. Francesco De Simone is a very active and extremely motivated scientist who pleads with fervour the cause of pharmacognosy and phytochemistry and can confer his enthusiasm to every style of audience. His travels have taken him all over the world and it is not without reason that he is a regular guest at meetings and symposia. He has himself organized many congresses and meetings. A large number of students have carried out their doctoral dissertations in his lab and under his supervision. His lab has also seen a multitude of visiting scientists from all over the world, including a high proportion from developing countries – due recognition of his endeavour to aid less privileged people.

It is with great pleasure and honour that I express to my colleague and friend Francesco De Simone my best wishes for his 72nd birthday.

Prof. Raffaele Riccio
President, Division of Organic Chemistry, Italian Chemical Society
Dean, Faculty of Pharmacy, University of Salerno
Via Ponte don Melillo
84084 Fisciano Italy

One of the most ambitious project in Prof. De Simone career was to promote scientific collaborations and exchanges between Italy and Latin American countries in the field of phytochemistry, food chemistry and pharmacognosy. With this purpose in the 90's he had the intuition to found the SILAE (Società Italo-Latinoamericano di Etnomedicina, Italian-Latin American Society of Ethnomedicine) (1992).

Since the day SILAE was founded its objective has been set to contribute to the close examination of the themes of great interest and actuality in the context of the relationships between Latin America and European Union. In addition to this it aimed to individualize new ways of collaboration among its member countries, also in the amplest frame of the European continent to sign under such accords with intergovernmental organisms.

The work of SILAE is related to the organization of Congresses that are held every other year in Italy and in Latin American countries, and that this year came to the XVIIIth edition. These congresses represent a landmark for the research, the development, the teaching and the training of all the researchers interested in the dietary and the officinal use of medicinal plants.

Our most grateful thanks to Prof. De Simone for the work done by him to strengthen friendships and scientific exchange between Italian and Latin American institutions and investigators.

Prof. Massimo Curini
University of Perugia,
Department of Drug Chemistry and Technology (Organic Chemistry Section),
Via del Liceo 1, 06123 Perugia, Italy
Prof. Wagner Vilegas
Universidade Estadual Paulista (UNESP),
Chemistry Institut of Araraquara, Caixa Postal 335,
14801-970 Araraquara, Brazil
Presidents of SILAE (Italian-Latin American Society of Pharmacognosy)

Natural Product Communications
2009
Volume 4, Number 12

Contents

Continued Overleaf

Review/Account

Manuscripts in Press **1797**

Cummulaive Index

LIST OF AUTHORS

NPC Natural Product Communications

2009
Vol. 4
No. 12
1615 - 1619

Terpenoid Content of the Antarctic Soft Coral *Alcyonium antarcticum*

Emiliano Manzo, Maria Letizia Ciavatta*, Genoveffa Nuzzo and Margherita Gavagnin

Istituto di Chimica Biomolecolare, CNR, Via Campi Flegrei, 34, I 80078, Pozzuoli, Na, Italy

lciavatta@icb.cnr.it

Received: August 14th, 2009; Accepted: September 23rd, 2009

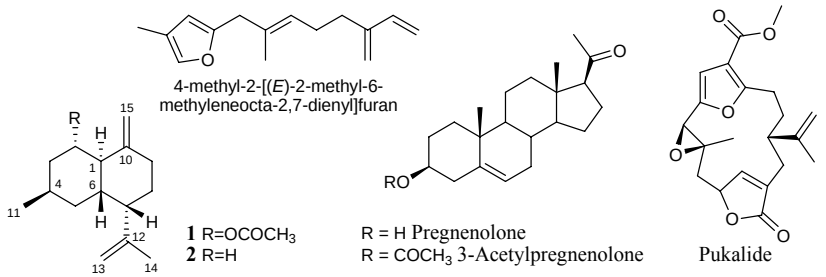
Chemical investigation of the soft coral *Alcyonium antarcticum*, collected off Terra Nova Bay, resulted in the isolation of two closely related sesquiterpenes, alcyonicene (**1**) and deacetoxy-alcyonicene (**2**), along with known terpenoid compounds. The structure elucidation of the new molecules, possessing a rare bulgarane skeleton, has been made mainly by NMR techniques.

Keywords: Marine invertebrate, soft coral, Antarctic benthos, sesquiterpenes, bulgarane, *Alcyonium antarcticum*.

The Antarctic benthic community has been regarded with major interest only recently, due to the interest of the scientific studies in tropical and temperate waters. In spite of the low temperature, the pronounced seasonality and limitation of food reserves, it appears very rich and stable. Cnidarians represent an ecologically important group in Antarctic invertebrates and have provided several products with interesting bioactivities [1]. Among Antarctic cnidarians, the soft corals belonging to the genera *Clavularia* [2], *Gersemia* [3] and *Alcyonium* [4] are the most studied group and were found to be especially rich in terpenes and steroids. These chemical compounds possess feeding-deterrence and ichthyotoxic activity against predators, and some of them have been found to be cytotoxic to several human tumor cell lines [5].

In this paper we report the first chemical investigation of *Alcyonium antarcticum*, which has resulted in the isolation of two new closely related sesquiterpenes, alcyonicene (**1**) and deacetoxy-alcyonicene (**2**), along with three known compounds, 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan [6], pregnenolone [7], pregnenolone-3-acetate [7] and pukalide [8]. The structures of compounds **1** and **2**, which exhibited a rare bulgarane skeleton [9], never described from the marine environment, were determined by means of spectroscopic methods. The known metabolites were identified by comparison of their NMR and mass spectral data with those reported in the literature [6-8].

The molecular formula $C_{17}H_{26}O_2$ of alcyonicene (**1**) was deduced by the sodiated molecular peak at *m/z* 285.1824 $[M+Na]^+$ in the HRESIMS spectrum and indicated five degrees of unsaturation. The ^{13}C NMR spectrum disclosed five sp^2 and twelve sp^3 carbons. Four olefinic carbon signals [(δ 149.5, s, C-10), (147.5, s, C-12), (112.9, t, C-13), (105.2, t, C-15)] were attributed to two 1,1-disusbstituted double bonds, accounting for two unsaturations. The presence of an acetyl group satisfying the third unsaturation degree was suggested by an intense IR band at 1731 cm^{-1} and confirmed by the carboxyl and methyl signals [δ 170.7 (*C*OCH_3) and δ 21.5 (CO*C*H_3)] in the ^{13}C NMR spectrum. Accordingly, the 3H acetyl singlet at δ 2.01 (COC*H*$_3$) was observed in the ^1H NMR spectrum. The remaining two unsaturations required by the molecular formula were thus attributed to two rings, indicating the presence of a bicyclic sesquiterpene skeleton. The ^1H NMR spectrum exhibited a 1H multiplet at δ 5.15 (ddd, J = 4.7, 10.8, and 10.8 Hz, H-2) that was assigned to the proton linked to an oxygenated carbon (δ_C 70.2), and four 1H broad singlets at δ 4.91 (H-13a), 4.89 (H-13b), 4.75 (H-15a), and 4.46 (H-15b) due to the protons of two exomethylene groups. In the high-field region of the ^1H NMR spectrum, two signals at δ 1.82 (br s, H_3-14) and 1.05 (d, J = 7.3 Hz,



Structures of compounds isolated from the Antarctic soft coral *Alcyonium antarcticum*.

Table 1: NMR spectroscopic data for alcyonicene (**1**) and deacetoxyalcyonicene (**2**).

	1			**2**	
Position	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m	HMBC	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m
1	46.9, CH	2.40, m	H-2, H-6	42.3, CH	2.08, br t (11.5)
2	70.2, CH	5.15*ax*, ddd (4.7,10.8,10.8)	H-1, H_2-3	23.9, CH_2	1.65, m 1.41, m
3	37.0, CH_2	1.92*eq*, m, 1.48*ax*, ddd (4.4,10.8,15.8)	H-2, H_3-11, H-4, H_2-5	n.d.	1.41, m
4	27.5, CH	2.13, m,	H_3-11	27.4, CH	2.04, m
5	35.7, CH_2	1.66*ax*, ddd (4.4,13.1,17.5) 1.25*eq*, m	H_3-11	n.d.	1.25, m
6	39.5, CH	1.78, m	H-1, H-7	38.6, CH	1.70, m
7	44.7, CH	2.31*eq*, br dd (5.3, 5.3)	H-6, H_2-8, H_2-13, H_3-14	45.4, CH	2.20, br t (5.18)
8	32.8, CH_2	1.86, m 1.72, m	H-7	31.9, CH_2	1.83, m 1.71, m
9	33.5, CH_2	2.38, m 2.18, ddd 3.9,13.1,13.1)	H_2-8, H_2-15	32.6, CH_2	2.40, ddd (2.9,13.1,13.1) 2.17, dt (3.9,13.1)
10	149.5, C	---	H_2-15, H-1, H_2-9	151.5, C	
11	18.2, CH_3	1.05, d (7.3)	H-4	17.9, CH_3	0.97, s
12	147.5, C	---	H_2-13, H_3-14	147.7, C	
13	112.9, CH_2	4.91, br s 4.89, br s	H_3-14	112.5, CH_2	4.89 br s 4.85 br s
14	26.6, CH_3	1.82, s	H-7, H_2-13	26.2, CH_3	1.81, s
15	105.2, CH_2	4.75, br s 4.46, br s	H_2-9, H-1	104.3, CH_2	4.69, br s 4.58, br s
OAc	170.7, C 21.5, CH_3	--- 2.01, s	H_3-17, H-2		

[a] Bruker 300 MHz, δ values are reported in ppm referred to *C*DCl_3 (δ_c 77.4).
[b] Assignments deduced by DEPT sequence.
[c] Bruker 400 MHz, δ values are reported in ppm referred to C*H*Cl_3 (δ_H 7.26).
n.d. not detected.

H_3-11), each integrating for three protons, were assigned to a vinyl methyl and to a secondary sp^3 methyl, respectively. The remaining signals integrating for twelve protons were attributed to four methylenes and four methines. The ^{1}H-^{1}H COSY experiment showed the presence in the molecule of a single spin system, H-1/H_2-9 sequence (Table 1), which was consistent with the decaline framework of a cadinene carbon skeleton [9-13]. Diagnostic HMBC correlations observed between *C*OCH_3 and H-2; C-10 and H-1, H_2-9, and H_2-15; C-7 and both H_2-13 and H_3-14, allowed the connection of the substituents, as depicted in formula **1**. Analysis of HSQC and HMBC spectra of **1** confirmed this hypothesis and aided us to assign all the proton and carbon values, as reported in Table 1.

Careful analysis of the vicinal proton coupling constants (Table 1), NOE difference experiments and the ^{13}C NMR values led us to establish the relative stereochemistry of alcyonicene (**1**). In particular, irradiation of the proton at δ 5.15 (H-2), in the ^{1}H-^{1}H homodecoupling experiment, simplified the signal at δ 2.40 to a large doublet (11.6 Hz) suggesting the presence of a *trans*-fused ring (Figure 1). Accordingly, no NOE effect was detected between the junction protons at δ 2.40 (H-1) and δ 1.78 (H-6). The relative configuration at C-4 was suggested by the high-field shifted value of C-11 (δ 18.2),

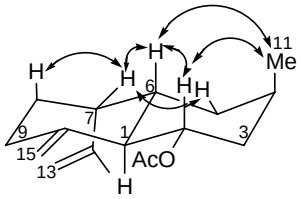


Fig. 1 NOE correlations for alcyonicene (**1**)

consistent with an axial orientation of the methyl at C-4. This was in agreement with the spectroscopic data reported in the literature for related cadinene models exhibiting at C-4 either the equatorial methyl (i.e. cadinane: $\delta_{C\text{-}11}$ 23.05 [10]) or the axial methyl (i.e. xenitorin A: $\delta_{C\text{-}11}$ 18.3 [11]; 8-*epi*-xenitorin A: $\delta_{C\text{-}11}$ 18.1 [12]). Diagnostic NOE effects were observed between H_3-11, H-6 and H-2 thus inferring the axial orientation for all of them (Fig. 1). The relative configuration of C-7 was deduced by the multiplicity of the H-7 signal (δ 2.31, br dd, J = 5.3 and 5.3 Hz), consistent with its equatorial orientation. This suggestion was further supported by a series of NOE effects observed between H-7 and H-6, H-8ax and H-5*eq* confirming the proposed stereochemistry (Figure 1).

Alcyonicene (**1**) was thus characterized as possessing a *trans*-fused decaline system with the isopropenyl chain at C-7 axially oriented, as occurs in the bulgarane subgroup of the cadinene sesquiterpene class [9,13].

In order to establish the absolute stereochemistry, we hydrolyzed compound **1** and applied the modified Mosher's method to the corresponding alcohol. Unfortunately, every attempt to obtain the hydroxyl derivative of **1** was unsuccessful due to its rapid degradation under different hydrolysis conditions. Thus the absolute stereochemistry remains undetermined.

Deacetoxy-alcyonicene (**2**) was isolated in trace amount from the extract (see Experimental). The molecular formula $C_{15}H_{24}$ was deduced by both the molecular peak at *m/z* 204 in the EIMS and the ^{13}C NMR spectrum. The proton and carbon resonances of **2** were very similar to those of **1**, indicating the presence of the same carbon framework. The only difference was the lack of the acetoxy substituent at C-2 in compound **2** with respect to compound **1**. Accordingly, in deacetoxy-alcyonicene (**2**), C-2 was a methylene rather than an oxygenated methine (in **2**: δ_C 23.9, δ_H 1.65/1.41; in **1**: δ_C 70.2, δ_H 5.15). ^1H and ^{13}C NMR values of **2** were attributed by careful analysis of 2D NMR experiments and also by comparison with the main metabolite **1** (see Table 1). The relative stereochemistry of **2** was suggested to be the same as **1** by biogenetic considerations.

Feeding-deterrence and ichthyotoxic activities of the main metabolite **1**, as well as of the known compounds here isolated, 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan, pregnenolone, pregnenolone-3-acetate, and pukalide, were preliminarily evaluated by conducting assays with *Carassius auratus* [14] and *Gambusia affinis* [15].

Among the compounds tested, pukalide showed feeding-deterrence against *C. auratus* at a concentration of 50 μg/cm^2. Feeding deterrence against the carnivorous fish, *Canthigaster solandri*, has been previously reported for a derivative of pukalide, isolated from a soft coral and its prey, the aeolid mollusc *Phyllodesmium guamensis* [16]. All compounds were also tested in antimicrobial assays [17], but no significant activity was evidenced.

This work represents the first chemical study of the Antarctic soft coral *A. antarcticum*, which was characterized by the presence of a rich terpenoid content. The new compounds **1** and **2** exhibit the rare bulgarane skeleton previously described only in essential oils from *Mentha piperita* [18] and *Juniperus oxycedrus* [19].

Experimental

General experimental procedures: Silica-gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were recorded on a Biorad FTS 155 FT-IR spectrophotometer. HPLC separation was performed using a Shimadzu liquid chromatograph LC-10AD equipped with an UV SPD-10A wavelength detector. NMR experiments were conducted at ICB-NMR Service Centre. 1D and 2D NMR spectra were acquired in $CDCl_3$, (shifts are referenced to the solvent signal) on a Bruker Avance-400 operating at 400 MHz, using an inverse probe fitted with a gradient along the Z-axis and a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the Z-axis.^{13}C NMR spectra were obtained on a Bruker DPX-300 operating at 300 MHz using a dual probe. High and low resolution ESIMS were recorded on a Micromass Q-TOF MicroTM coupled with a HPLC

Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW (resolution specification 5000 FWHM, deviation <5 ppm RMS in the presence of a known lock mass). GC-MS was carried out on an ion-trap MS instrument in EI mode (70 eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by a 5% diphenyl (30 m x 0.25 mm x 0.25 um) column using helium as gas carrier.

Animal material: The soft coral *Alcyonium antarcticum* was collected in January 2002 during the XVII Italian Campaign in Antarctica off Terra Nova Bay (Stazione M. Zucchelli) and immediately frozen and transferred to ICB. The soft coral was classified by Dr Stefano Schiapparelli (Dipartimento per lo Studio del Territorio e delle sue Risorse, University of Genova). A voucher specimen is stored at ICB under the code AlcyAA.

Extraction and isolation: The frozen *A. antarcticum* (dry weight, 112 g) was chopped and then extracted exhaustively with Me_2CO (400 mL x 4) using ultrasound. After filtration and evaporation *in vacuo* of the organic solvent, the residue was subsequently extracted with Et_2O (200 mL x 4) and *n*-BuOH (100 mL x 4). The evaporation of Et_2O and *n*-BuOH extracts gave two oily residues (3.8 g and 2.2 g, respectively). The ethereal extract was subjected to silica-gel column chromatography using as eluent a gradient of light petroleum and Et_2O, $CHCl_3$ and finally MeOH to give five fractions: fr. I (550 mg), fr. II (20 mg), fr. III (890 mg), fr. IV (810 mg), and fr. V (400 mg). Fraction I was subjected to silica gel column purification (light petroleum/diethyl ether gradient) to give the known 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan (50 mg) and deacetoxy-alcyonicene (**2**, 0.5 mg). Fr. II was further purified by HPLC *n*-phase (*n*-hexane/EtOAc, 95:5, Kromasil analytical column, flow rate 1 mL/min) to give pure alcyonicene (**1**, 6 mg). Fr III was purified by silica-gel column chromatography yielding 45 mg of pregnenolone-3-acetate. Fr.IV gave, after filtration on a Sephadex LH-20 column, pregnenolone (1.0 mg). Finally, the more polar fraction (V) was subjected to silica-gel column chromatography and TLC to afford pure pukalide (7.0 mg). The known compounds, 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan, 3-acetyl-pregnenolone, pregnenolone and pukalide were identified by comparison of the spectroscopic data (^{1}H NMR, ^{13}C NMR, MS and $[\alpha]_D$) with those reported in the literature.

Alcyonicene (**1**)
(0.006 g, 0.16%)
$[\alpha]_D$: +33.9 (*c* 0.6, $CHCl_3$).
Rf : 0.5 (light petroleum -Et_2O, 95:5).
IR (KBr): 3100, 2938, 2878, 1731, 1645, 1445, 1245, 892 cm^{-1}.
^{1}H NMR and ^{13}C NMR: Table 1.
HRMS-ESI: *m/z* [M + Na$^+$] calcd for $C_{17}H_{26}O_2Na$: 285.1831; found: 285.1824.

Deacetoxy-alcyonicene (**2**)
(0.0005 g, 0.013%)
$[\alpha]_D$: -2.8 (*c* 0.05, $CHCl_3$).
Rf : 1 (Light petroleum).
IR (KBr): 3100, 2938, 2878, 1645, 1445, 1245, 892 cm^{-1}.
GC-EIMS (EI, 70 eV): *m/z* (%) = 204.2 [M$^+$] (10), 189.3 (20).

Biological assays: Ichthyotoxicity tests against the mosquito fish, *Gambusia affinis* (Baird & Girard, 1853), were conducted according to literature procedures [15]. All the isolated metabolites were assayed at 10 ppm. Feeding-deterrence tests against gold fish, *Carassius auratus*, were conducted according to literature procedures [16]. All the compounds were assayed at 50 μg/cm^2.

The antimicrobial activity against *Escherichia coli* DH5a and *Staphylococcus aureus* ATCC6538P was determined. Activity was studied by the broth microdilution method [17]. The test was carried out in a 96-well, flat-bottomed, microtitration plate. Plates were incubated at 37°C without shaking, and reading was performed following 24 h of incubation by optical density (OD) determination with a spectrophotometer; plates were shaken for 5 min and the OD values at 492 nm of each well were read with a microtiter plate reader (Thermolabsystems Multiskan MK3). All experiments were performed in triplicate at concentration of 100 μg/mL.

Acknowledgments - The authors kindly acknowledge Dr S. Schiapparelli (University of Genova) for taxonomic identification of the cnidarian, Dr S. Costigliola for technical support and Mr A. Crispino for collection of biological material. This research has been partially supported by the Italian National Programme for Antarctic Research and by Project MIUR PRIN 2007 “Sostanze naturali ed analoghi sintetici con attività antitumorale”.

References

[1] Avila C, Taboada S, Nunez-Pons L. (**2008**) Antarctic marine chemical ecology: what is next?. *Marine Ecology*, ***29***, 1-71.

[2] McClintock JB, Baker BJ. (**2001**) *Marine Chemical Ecology*. CRC Marine Science Series Press, Boca Raton.

[3] Slattery M, Hamann MT, McClintock JB, Perry TL, Puglisi MP, Yoshida WY. (**1997**) Ecological roles for water-borne metabolites from Antarctic soft corals. *Marine Ecology Progress Series*, ***161***, 133-144.

[4] (a) Rodriguez-Brasco MF, Seldes AM, Palermo JA. (**2001**) Paesslerins A and B: Novel tricyclic sesquiterpenoids from the soft coral *Alcyonium paessleri*. *Organic Letters*, ***3***, 1415-1417; (b) Carbone M, Nunez-Pons L, Castelluccio F, Avila C, Gavagnin M. (**2009**) Illudalane sesquiterpenoids of the Alcyopterosin series from the Antarctic marine soft coral *Alcyonium grandis*. *Journal of Natural Products*, ***72***, 1357-1360.

[5] Mellado GG., Zubia E, Ortega MJ, Lopez-Gonzalez PJ. (**2005**) Steroids from the Antarctic octocoral *Anthomastus bathyproctus*. *Journal of Natural Products*, ***68***, 1111-1115.

[6] Cimino G., De Rosa S, De Stefano S, Sodano G. (**1984**) A new furanosesquiterpene from the Mediterranean alcyonacean *Alcyonium palmatum*. *Journal of Natural Products*, ***47***, 877-878.

[7] Szendi Z, Forgò P, Sweet F. (**1995**) Complete ^{1}H and ^{13}C NMR spectra of pregnenolone. *Steroids*, ***60***, 442-446.

[8] Missakian MG, Burreson BJ, Scheuer PJ. (**1975**) Pukalide, a furanocembranolide from the soft coral *Sinularia abrupta*. *Tetrahedron*, ***31***, 2513-2515.

[9] Bordoloi M, Shukla VS, Nath SC, Sharma RP. (**1989**) Naturally occurring cadinenes. *Phytochemistry*, ***28***, 2007-2037.

[10] Kruk C, Cox HC, de Leeuw JW. (**1988**) Assignments of the ^{1}H and ^{13}C NMR resonances of cadinanes and bicadinane by means of two-dimensional shift-correlated NMR techniques. *Magnetic Resonance in Chemistry*, ***26***, 228-235.

[11] Duh C-Y, Chien S-C, Song P-Y, Wang S-K, El-Gamal AA, Dai C-F. (**2002**) New cadinene sesquiterpenoids from the Formosan soft coral *Xenia puerto-galerae*. *Journal of Natural Products*, ***65***, 1853-1856.

[12] Shen Y-C, Tzeng G-L, Chen M-H, Khalil AT. (**2007**) Three new cadinene derivatives from *Xenia puerto-galerae*. *Journal of the Chinese Chemical Society*, ***54***, 1347-1352.

[13] Koster FH, Wolf H, Kluge H. (**1986**) Stereoisomeric ε-dienes of the cadalane series: synthesis and configurational correlation. *Liebigs Annalen der Chemie*, 78-92.

[14] Cimino G, De Rosa S, De Stefano S, Sodano G. (**1982**) The chemical defense of four Mediterranean nudibranchs. *Comparative Biochemistry and Physiology*, ***73B***, 471-474.

[15] Coll JC, La Barre S, Sammarco PW, Williams TW, Bakus GJ. (**1982**) Chemical defences in soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef: A study of comparative toxicities. *Marine Ecology Progress Series*, ***8***, 271-278.

[16] (a) Wylie CR, Paul VJ. (**1989**) Chemical defenses in three species of *Sinularia* (Coelenterata, Alcyonacea): effects against generalist predators and the butterflyfish *Chaetodon unimaculatus* Bloch. *Journal of Experimental Marine Biology and Ecology*, ***129***, 141-160; (b) Slattery M, Avila C, Starmer J, Paul VJ. (**1998**) A sequestered soft coral diterpene in the aeolid nudibranch *Phyllodesmium guamensis*. *Journal of Experimental Marine Biology and Ecology*, ***226***, 33-49.

[17] Schwalbe R, Steel-Moore L, Goodwin AC. (**2007**). Antimicrobial Susceptibility Testing Protocols. Taylor & Francis Eds.

[18] Vlakhov R, Holub M, Herout V. (**1967**) Terpenes. CLXXXV. Structure of two hydrocarbons of cadalene type isolated from *Mentha piperita* oil of Bulgarian origin. *Collection of Czechoslovak Chemical Communications*, ***32***, 822-829.

[19] De Pascual TJ, Barrero AF, San Feliciano A, Caballero MC. (**1977**) Componentes de las arcestidas de *Juniperus oxycedrus*. *Anales de Quimica*, ***73***, 1527-1528.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1621 - 1630

Phytotoxic Activity of *Salvia x jamensis*

Angela Bisio[a*], Daniele Fraternale[b], Gianluca Damonte[c], Enrico Millo[c], Anna Paola Lanteri[a], Eleonora Russo[a], Giovanni Romussi[a], Brunella Parodi[a], Donata Ricci[b] and Nunziatina De Tommasi[d]

[a]*Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari*, *University of Genoa*, *Genoa*, *Italy*

[b]*Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura*, *University of Urbino*, *Urbino*, *Italy*

[c]*Dipartimento di Medicina Sperimentale e Centro di Eccellenza per la Ricerca Biomedica*, *University of Genoa*, *Genoa*, *Italy*

[d]*Dipartimento di Scienze Farmaceutiche*, *University of Salerno*, *Salerno*, *Italy*

bisio@dictfa.unige.it

Received: August 3rd, 2009; Accepted: October 7th, 2009

A study has been carried out on the surface exudate of *Salvia x jamensis*, which showed a significant phytotoxic activity against *Papaver rhoeas* L. and *Avena sativa* L.. Bioguided separation of the exudate yielded active fractions from which 3β-hydroxy-isopimaric acid (**1**), hautriwaic acid (**2**), betulinic acid (**3**), 7,8β-dihydrosalviacoccin (**4**), isopimaric acid (**5**), 14α-hydroxy-isopimaric acid (**7**), 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide (**8**), cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone, **9**) and two new neoclerodane diterpenes (**6** and **10**) were isolated. The structures of **6** and **10** were identified as 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide and 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide respectively on the basis of spectroscopic data analysis. All compounds, but **7**, **8** and **10**, were active in inhibiting the germination of the tested species.

Keywords: *Salvia*, diterpenes, triterpenes, flavonoids, phytotoxic activity.

Phytotoxic chemicals originated by aerial parts of the plants are known to be released into the environment by different ways as foliar leaching, volatilization or residue decomposition [1,2]. Many of these compounds are found in the complex secretion product originated in the epidermal secretory structures [3]. The surface exudate of *Salvia x jamensis* J. Compton (Lamiaceae), whose platelet antiaggregating activity we described previously [4], showed a good antigerminative activity against *Papaver rhoeas* L. and *Avena sativa* L. (Final germination [5] 0.4±0.7% against *Papaver* and 0.0 % against *Avena* at 5 mg/L).

Isolation and identification of the compounds. Bioguided separation of the exudate with column chromatography yielded active fractions (Table 1). From fractions III and IV, 3β-hydroxy-isopimaric acid (**1**), hautriwaic acid (**2**), betulinic acid (**3**), 7,8β-dihydrosalviacoccin (**4**), isopimaric acid (**5**), 14α-hydroxy-isopimaric acid (**7**), 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide (**8**), cirsiliol (5,3',4'-trihydroxy-6,7-dimethoxyflavone, **9**) and two new neoclerodane diterpenes (**6** and **10**) were isolated.

Table 1: Effect of the various fractions of *Salvia x jamensis* exudate on the Final germination (maximum average percentage of seeds that germinated during the experiment) and early growth of *Papaver rhoeas* L. and *Avena sativa* L. at various concentrations. Data are expressed as mean (± S.D.) of three triplicates. Significant differences among the means were evaluated using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Fraction groups of *S. x jamensis*	*Papaver rhoeas* L.			*Avena sativa* L.		
	2 mg/L	5 mg/L	10 mg/L	2 mg/L	5 mg/L	10 mg/L
I	26.7±2.1^{c}	6.3±0.6^{b}	6.0±1.7^{b}	50.3±2.5^{c}	26.3±2.5^{c}	20.0±2.0^{c}
II	22.7±3.1^{c}	8.7±2.3^{b}	6.7±1.2^{b}	31.7±2.5^{b}	13.7±2.5^{b}	9.7±2.1^{b}
III	6.7±2.5^{a}	0.0^{a}	0.0^{a}	5.0±2.0^{a}	0.0^{a}	0.0^{a}
IV	15.0±3.6^{b}	0.0^{a}	0.0^{a}	6.0±1.7^{a}	0.0^{a}	0.0^{a}

Table 2: NMR spectral data for compound **6**: δ values, $CDCl_3$, ^{13}C NMR at 125 MHz, ^1H NMR at 500 MHz.

C	^{13}C	^1H (*J* in Hz)	HMBC correlations of the C
1	26.6	1.61 ddd (5.3, 12.0, 12.4), 1.80 m	-
2	23.3	2.52, 2.57 both m	-
3	135.6	6.78 m	1.80, 2.52, 2.57
4	139.5	-	4.47
5	49.3	-	1.80, 1.84, 2.07, 4.47, 6.78
6	26.2	1.84, 2.10 both m	1.97, 3.88, 4.47
7	19.4	1.97, 2.07 both m	-
8	42.9	3.53 dd (4.2, 12.0)	2.68
9	40.8	-	0.91, 2.68, 3.53
10	77.3	-	0.91, 1.80, 2.52, 2.57, 2.68
11	40.3	1.67, 2.68 both m	0.91, 3.53
12	71.9	5.38 dd (6.2, 11.2)	1.67
13	125.4	-	1.67, 5.38, 6.48, 7.47
14	109.7	6.48 br s	5.38
15	144.8	7.47 br s	7.52
16	140.7	7.52 br s	5.38, 6.48
17	176.0	-	3.53
18	168.9	-	4.47
19	73.7	4.47 d (6.8), 3.88 dd (2.0, 6.8)	-
20	21.0	0.91 s	1.67, 2.68, 3.53

Compounds **1**, **3**, **4**, **5**, **7**, **8** were previously isolated from this plant [4] and compounds **2** and **9** were identified by comparison of their physical and spectroscopic data with those published in the literature [6,7].

IR absorption bands at 3495 (-OH groups), 1755, 1725 (two lactone rings), and 3121, 1502, 868 (β-substituted furan ring) cm^{-1} and 20 carbon resonances in the ^{13}C NMR suggested for **6** an oxygenated clerodane diterpenoid structure. By inspection of ESI-MS, HR-MS, COSY, HSQC and HMBC spectra it was possible to assign all the protons and carbons (Table 2) as belonging to the planar structure **6** (Figure 1). ROESY correlation H-8/H-12 showed that these protons are on the same side. The lack of correlation between H8 and CH_3-20 suggested that they are on opposite sides. ROESY correlations CH_3-20/CH_2-19 showed that these groups are on the same side. ROESY correlations of H-1 α axial (δ_H = 1.61) with CH_3-20 and H-19 *endo* could be explained by a 5α,10β *trans*-fused structure and thus by a β orientation of the 10-OH. From these results, **6** is 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide. A stereoisomer of **6** was isolated from *Salvia haenkei* [8].

IR absorption bands at 3560, 3520 (-OH groups), 1755, 1720 (two lactone rings), and 3130, 1500, 875 (β-substituted furan ring) cm^{-1} and 20 carbon

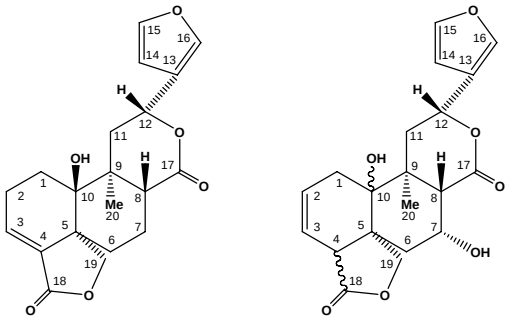


Figure 1: Structure of compound **6** and of compound **10**.

Table 3: NMR spectral data for compound **10**: δ values, $CDCl_3$, ^{13}C NMR at 125 MHz, ^1H NMR at 500 MHz.

C	^{13}C	^1H (*J* in Hz)	HMBC correlations of the C
1	30.1	2.22 m, 2.63 nd (7.9)	6.00
2	126.3	6.00 m	2.22
3	123.2	5.98 m	2.22
4	51.8	2.88 br s	5.12, 6.00
5	46.4	-	2.19, 5.12
6	35.0	1.95 dd (13.0, 3.0), 2.19 m	4.15, 5.12
7	65.7	4.56 br s ($W_{1/2}$ = 8)	2.19, 3.37
8	45.1	3.37 br s	1.28, 1.57, 2.19, 2.76
9	41.0	-	1.28, 1.57, 2.22, 2.76, 3.37
10	74.7	-	1.28, 2.22, 2.76, 3.37
11	40.9	1.57 dd (11.6, 13.5), 2.76 dd (5.5, 13.5)	1.28, 3.37, 5.32
12	72.2	5.32 dd (5.5, 11.6)	1.57, 2.76
13	124.1	-	1.57, 5.32, 6.45, 7.44
14	108.9	6.45 br s	5.32, 7.48
15	144.2	7.44 br s	6.45, 7.48
16	140.1	7.48 br s	5.32, 6.45, 7.44
17	176.8	-	3.37
18	175.1	-	5.12
19	73.7	4.15 d (8.6), 5.12 d (8.6)	2.19
20	24.8	1.28 s	1.57, 2.76, 3.37

resonances in the ^{13}C NMR suggested an oxygenated clerodane diterpenoid structure for **10**. By inspection of ESI-MS MS, HR-MS, COSY, HSQC and HMBC spectra it was possible to assign all the protons and carbons (Table 3) and to get the planar structure for **10**. ROESY correlation H-8/H-12 showed that these protons are on the same side. The lack of correlation between H8 and CH_3-20 suggested that they are on opposite sides. The axial orientation of the hydroxyl group at C-7 was indicated by the small *J* of the H-7 (br s). The strong deshielding effect on the *exo* H-19 (δ_H = 5.12) as compared to the *endo* H-19 (δ_H = 4.15), the chemical shift of CH_3-20 (δ_H = 1.28), which is similar to that of **8** [4] and ROESY correlations CH_3-20/CH_2-19 showed that these last groups and the 7-OH are on the same α side. The relative stereochemistry of C-4 and C-10 could not

Table 4: *Papaver rhoeas* L. germination indices after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. Percent germination data were arcsin-square root transformed for analysis to meet the requirements of the test. Retransformed data are presented in the results. Significant differences among the means were evaluated by one-way ANOVA, using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Index	Compound	compound concentration (mg/L)			
		2	5	10	20
Total germination (G_T)	**1**	15.7 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	13.3 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	20.7 ^{a}	12.0 ^{b}	6.3 ^{b}	0.0 ^{a}
	5	87.0 ^{c}	84.7 ^{c}	31.3 ^{c}	11.3 ^{b}
	6	20.3 ^{a}	11.3 ^{b}	6.0 ^{b}	0.0 ^{a}
	7	74.7 ^{c}	78.0 ^{c}	78.4 ^{d}	78.4 ^{c}
	8	78.0 ^{c}	80.0 ^{c}	71.7 ^{d}	79.7 ^{c}
	9	44.3 ^{b}	15.6 ^{b}	5.5 ^{b}	0.0 ^{a}
	10	81.7 ^{c}	82.3 ^{c}	82.3 ^{d}	83.0 ^{c}
	control	83.0 ^{c}	83.0 ^{c}	83.0 ^{d}	83.0 ^{c}
Speed of germination (S)	**1**	2.39 ± 0.22 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	1.80 ± 0.23 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	1.50 ± 0.23 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	2.98 ± 0.13 ^{a}	1.60 ± 0.14 ^{b}	0.87 ± 0.12 ^{a}	0.0 ^{a}
	5	13.66 ± 0.12 ^{e}	13.24 ± 0.11 ^{c}	4.29 ± 0.34 ^{b}	1.45 ± 0.14 ^{b}
	6	2.97 ± 0.07 ^{a}	1.46 ± 0.23 ^{b}	0.77 ± 0.04 ^{a}	0.0 ^{a}
	7	11.68 ± 0.07 ^{c}	12.62 ± 0.49 ^{c}	12.18 ± 0.46 ^{c}	12.26 ± 0.58 ^{c}
	8	12.56 ± 0.24 ^{d}	12.92 ± 0.14 ^{c}	11.81 ± 0.31 ^{c}	12.75 ± 0.44 ^{c}
	9	6.24 ± 0.33 ^{b}	2.06 ± 0.31 ^{b}	0.66 ± 0.29 ^{a}	0.0 ^{a}
	10	12.72 ± 0.07 ^{d}	12.83 ± 0.16 ^{c}	12.87 ± 0.14 ^{d}	12.86 ± 0.17 ^{c}
	control	12.95 ± 0.24 ^{d}	12.95 ± 0.24 ^{c}	12.95 ± 0.24 ^{d}	12.95 ± 0.24 ^{c}
Speed of accumulated germination (AS)	**1**	8.2 ± 1.0 ^{b}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	5.27 ± 0.76 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	4.21 ± 0.81 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	9.65 ± 0.59 ^{b}	4.51 ± 0.43 ^{b}	2.70 ± 0.32 ^{a}	0.0 ^{a}
	5	47.38 ± 0.18 ^{e}	45.85 ± 0.39 ^{c}	12.7 ± 1.3 ^{b}	3.85 ± 0.29 ^{b}
	6	9.65 ± 0.66 ^{b}	3.91 ± 0.86 ^{b}	2.04 ± 0.29 ^{a}	0.0 ^{a}
	7	40.6 ± 1.6 ^{d}	44.9 ± 2.6 ^{c}	42.2 ± 1.4 ^{c}	42.5 ± 2.9 ^{c}
	8	45.1 ± 1.1 ^{e}	46.43 ± 0.59 ^{c}	43.4 ± 1.8 ^{c}	45.1 ± 1.1 ^{c}
	9	19.4 ± 1.3 ^{c}	5.91 ± 0.88 ^{b}	1.40 ± 0.60 ^{a}	0.0 ^{a}
	10	43.85 ± 0.25 ^{e}	44.24 ± 0.20 ^{c}	44.56 ± 0.42 ^{c}	44.07 ± 0.49 ^{c}
	control	44.7 ± 1.1 ^{e}	44.7 ± 1.1 ^{c}	44.7 ± 1.1 ^{c}	44.7 ± 1.1 ^{c}
Coefficient of the rate of germination (CRG)	**1**	12.28 ± 0.15 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	11.77 ± 0.10 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.72 ± 0.13 ^{a}	0.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	12.03 ± 0.09 ^{a}	11.78 ± 0.04 ^{c}	11.79 ± 0.28 ^{c}	0.0 ^{a}
	5	12.52 ± 0.03 ^{a}	12.48 ± 0.04 ^{d}	11.85 ± 0.13 ^{c}	11.50 ± 0.16 ^{b}
	6	12.10 ± 0.26 ^{a}	11.52 ± 0.07 ^{b}	11.49 ± 0.23 ^{c}	0.0 ^{a}
	7	12.46 ± 0.10 ^{a}	12.63 ± 0.13 ^{d}	12.42 ± 0.07 ^{d}	12.48 ± 0.10 ^{c}
	8	12.56 ± 0.07 ^{a}	12.59 ± 0.04 ^{d}	12.62 ± 0.10 ^{d}	12.59 ± 0.04 ^{c}
	9	11.96 ± 0.09 ^{a}	11.56 ± 0.10 ^{b}	10.98 ± 0.11 ^{b}	0.0 ^{a}
	10	12.47 ± 0.04 ^{a}	12.48 ± 0.02 ^{d}	12.49 ± 0.01 ^{d}	12.46 ± 0.03 ^{c}
	control	12.49 ± 0.07 ^{a}	12.49 ± 0.07 ^{d}	12.49 ± 0.07 ^{d}	12.49 ± 0.07 ^{c}

be determined. From these results **10** is 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide. For both the molecules the whole structure was confirmed by tandem ESI-ion trap mass spectrometry in HPLC-MS and MS^2 experiments conducted as described below. The HR mass spectrometry analysis was performed in DIA (direct infusion analysis) in the negative ion mode to assess the elemental composition.

Dose–response studies. The activity of the isolated compounds on the germination of *Papaver rhoeas* L. and *Avena sativa* L. is described by the data reported in Table 4 and 5. In respect of both species, germination was measured to be significantly low at all the concentrations of **1**, **2**, **3**, **4**, **5**, **6**, and **9** and the decrease exhibited a strong reciprocal correlation with the increasing concentration. At the highest compound concentration (20 mg/L), germination capacity, calculated by means of the Total germination index (G_T) of *Papaver* seeds [9,10] (Table 4), was significantly lower than control after exposure to **1**, **2**, **3**, **4**, **6** and **9**. **5** resulted less active, while **7**, **8** and **10** were not active. At the lowest tested concentration (2 mg/L), G_T values of *Papaver* (Table 4) showed the lowest delay in inhibiting

Table 5: *Avena sativa* L. germination indices after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. Percent germination data were arcsin-square root transformed for analysis to meet the requirements of the test. Retransformed data are presented in the results. Significant differences among the means were evaluated by one-way ANOVA, using the Tukey's honest significant difference test. Values with the same letters in a column are not significantly different at the 0.05 probability level. The letters are given following increasing values.

Index	Compound	compound concentration (mg/L)			
		2	5	10	20
Total germination (G_T)	**1**	27.6 ^{b}	12.6 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	21.3 ^{b}	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	11.3 ^{a}	11.3 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	32.3 ^{b}	10.6 ^{a}	3.3 ^{b}	0.0 ^{a}
	5	73.4 ^{c}	73.0 ^{b}	28.7 ^{c}	9.7 ^{b}
	6	13.2 ^{a}	13.0 ^{a}	4.9 ^{b}	0.0 ^{a}
	7	72.7 ^{c}	79.7 ^{b}	74.4 ^{d}	74.8 ^{d}
	8	73.7 ^{c}	73.0 ^{b}	74.7 ^{d}	72.4 ^{d}
	9	78.0 ^{c}	77.0 ^{b}	70.0 ^{d}	52.3 ^{c}
	10	79.3 ^{c}	78.0 ^{b}	79.4 ^{d}	82.4 ^{e}
	control	83.0 ^{c}	83.0 ^{b}	83.0 ^{d}	83.0 ^{e}
Speed of germination (S)	**1**	4.52 ± 0.53 ^{a}	1.85 ± 0.15 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	3.36 ± 0.40 ^{a}	1.64 ± 0.24 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	1.79 ± 0.19 ^{a}	1.71 ± 0.05 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	4.94 ± 0.47 ^{a}	1.67 ± 0.33 ^{a}	0.50 ± 0.08 ^{a}	0.0 ^{a}
	5	21.2 ± 1.3 ^{b}	23.79 ± 0.48 ^{c}	4.65 ± 0.27 ^{b}	1.58 ± 0.15 ^{b}
	6	2.14 ± 0.44 ^{a}	1.90 ± 0.14 ^{a}	0.77 ± 0.26 ^{a}	0.0 ^{a}
	7	24.4 ± 1.0 ^{c}	24.4 ± 1.2 ^{c}	23.4 ± 1.3 ^{d}	23.0 ± 1.0 ^{d}
	8	24.97 ± 0.95 ^{c}	24.3 ± 2.5 ^{c}	23.09 ± 0.51 ^{d}	23.78 ± 0.56 ^{d}
	9	19.25 ± 0.53 ^{b}	18.82 ± 0.71 ^{a}	15.42 ± 0.87 ^{c}	8.72 ± 0.50 ^{c}
	10	24.40 ± 0.40 ^{c}	24.09 ± 0.24 ^{c}	24.17 ± 0.51 ^{d}	24.61 ± 0.35 ^{e}
	control	24.72 ± 0.36 ^{c}	24.72 ± 0.36 ^{c}	24.72 ± 0.36 ^{d}	24.72 ± 0.36 ^{e}
Speed of Accumulated Germination (AS)	**1**	16.2 ± 2.4 ^{a}	6.07 ± 0.47 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	11.8 ± 1.9 ^{a}	5.3 ± 1.0 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	6.21 ± 0.80 ^{a}	5.67 ± 0.32 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	16.5 ± 1.7 ^{a}	5.7 ± 1.2 ^{a}	1.68 ± 0.32 ^{a}	0.0 ^{a}
	5	80.6 ± 6.5 ^{c}	91.9 ± 1.9 ^{c}	16.3 ± 1.1 ^{b}	5.52 ± 0.86 ^{a}
	6	7.7 ± 1.8 ^{a}	6.01 ± 0.67 ^{a}	2.57 ± 0.85 ^{a}	0.0 ^{a}
	7	95.1 ± 3.6 ^{d}	91.9 ± 5.6 ^{c}	89.7 ± 5.7 ^{d}	87.1 ± 5.3 ^{c}
	8	98.0 ± 3.4 ^{d}	94 ± 10 ^{c}	87.6 ± 3.1 ^{d}	91.4 ± 3.6 ^{c}
	9	70.9 ± 2.3 ^{b}	70.2 ± 3.0 ^{b}	55.8 ± 3.7 ^{c}	30.2 ± 2.0 ^{b}
	10	95.1 ± 1.7 ^{d}	93.2 ± 1.2 ^{c}	93.6 ± 1.8 ^{d}	95.0 ± 1.5 ^{c}
	control	94.4 ± 1.9 ^{d}	94.4 ± 1.9 ^{c}	94.4 ± 1.9 ^{d}	94.4 ± 1.9 ^{c}
Coefficient of the rate of germination (CRG)	**1**	12.57 ± 0.11 ^{a}	12.07 ± 0.34 ^{a}	0.0 ^{a}	0.0 ^{a}
	2	12.38 ± 0.17 ^{a}	12.04 ± 0.25 ^{a}	0.0 ^{a}	0.0 ^{a}
	3	12.51 ± 0.32 ^{a}	12.37 ± 0.12 ^{a}	0.0 ^{a}	0.0 ^{a}
	4	12.32 ± 0.12 ^{a}	12.47 ± 0.02 ^{a}	12.10 ± 0.56 ^{b}	0.0 ^{a}
	5	14.66 ± 0.19 ^{b}	15.05 ± 0.02 ^{c}	12.55 ± 0.27 ^{b}	12.58 ± 0.40 ^{b}
	6	12.47 ± 0.63 ^{a}	12.15 ± 0.42 ^{a}	12.37 ± 0.39 ^{b}	0.0 ^{a}
	7	15.20 ± 0.11 ^{b}	14.95 ± 0.21 ^{c}	14.96 ± 0.19 ^{d}	14.85 ± 0.31 ^{c}
	8	15.16 ± 0.05 ^{b}	15.13 ± 0.21 ^{c}	14.92 ± 0.01 ^{d}	15.06 ± 0.22 ^{c}
	9	14.13 ± 0.12 ^{b}	14.07 ± 0.15 ^{b}	13.70 ± 0.22 ^{c}	12.76 ± 0.09 ^{b}
	10	14.89 ± 0.06 ^{b}	15.01 ± 0.02 ^{c}	14.91 ± 0.07 ^{d}	14.84 ± 0.03 ^{c}
	control	14.94 ± 0.10 ^{b}	14.94 ± 0.10 ^{c}	14.94 ± 0.10 ^{d}	14.94 ± 0.10 ^{c}

germination for **1**, **2**, **3**, **4** and **6**.G_T values of *Avena* seeds (Table 5) gave similar results, with the exception of that related to **9**. As G_T index doesn't consider slackening in germination [9], germination progress was evaluated by means of the three indices used by Chiapusio [9] and Allaie [10], i.e. Speed of germination (S), Speed of accumulated germination (AS) and Coefficient of the rate of germination (CRG). For both *Papaver rhoeas* and *Avena sativa*, all calculated indices were significantly influenced by the different concentrations of **1**, **2**, **3**, **4**, **5**, **6** and **9** (Tables 4 and 5). At all the tested concentrations against *Papaver* and *Avena*, **1**, **2**, **3**, **4**, and **6**, gave S and AS values significantly lower than those obtained for the other compounds, as found for G_T index. Moreover, as well as for G_T values, S values of **1**, **2**, **3**, **4**, and **6** against *Papaver* showed the lowest delay in inhibiting germination at the lowest concentration (2 mg/L), while AS indicated that only **2** and **3** showed the lowest delay. As S considers the number of germinated seeds between two exposure times and AS considers the cumulative number of germinated seeds at each exposure time [9], S index appears to be the most sensitive [9,10]. This difference was not noted with *Avena* results (Table 5).

Table 6: LC_{50} and LC_{90} for *Papaver* and *Avena* germination inhibition after exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations, calculated by linear interpolation between two adjacent values. Data are means (± S.D.) of three replicates. LC_{50} and LC_{90} are expressed as 95% confidence interval. (NC= not calculated)

Compound	*Papaver rhoeas* L.						*Avena sativa* L.					
	LC_{50} (mg/L)			LC_{90} (mg/L)			LC_{50} (mg/L)			LC_{90} (mg/L)		
1	1.23	±	0.15	3.41	±	0.47	1.50	±	0.29	6.7	±	1.3
2	1.19	±	0.15	3.13	±	0.73	1.35	±	0.21	6.3	±	1.4
3	1.16	±	0.15	2.80	±	0.87	1.16	±	0.18	5.4	±	4.4
4	1.33	±	0.14	8.3	±	2.9	1.64	±	0.31	6.6	±	3.2
5	8.96	±	0.53		NC		8.50	±	0.41		NC	
6	1.32	±	0.17	7.8	±	3.0	1.19	±	0.24	7.5	±	3.4
7		NC			NC			NC			NC	
8		NC			NC			NC			NC	
9	2.30	±	0.76	8.7	±	3.8		NC			NC	
10		NC			NC			NC			NC	

The values of CRG of all compounds were similar to those obtained for the other considered germination indices against both *Papaver* and *Avena* (Tables 4 and 5) at the highest compound concentration used (20 mg/L); the differences from the other germination indices, observed at the lower tested concentrations, are probably ascribable to the lower sensitivity of this index in respect to the other indices [9].

From the dose-response studies, LC_{50} and LC_{90} [11] were determined for both the species (Table 6) and proved to be consistent with the results obtained previously: the lowest values of LC_{50} and LC_{90} against *Papaver* and *Avena* were yielded by **1**, **2**, **3**, **4** and 6, whereas LC_{50} and, consequently LC_{90}, for **7**, **8** and **10** could not be calculated. It can be highlighted that LC_{90} values registered for *Avena* were doubled with respect to those found for *Papaver*. Results for **5** and **9** were consistent with those obtained for the other considered parameters.

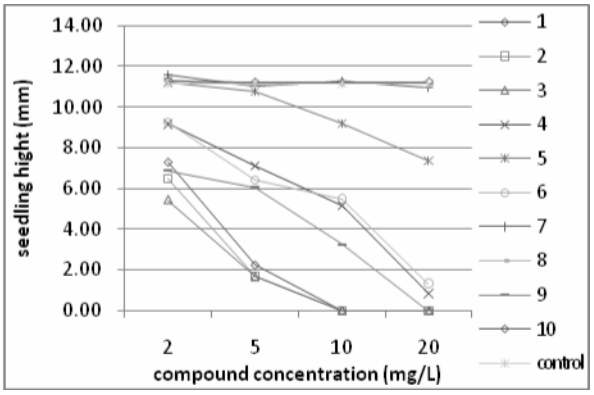
Growth experiments. In addition to germination, even the subsequent growth of the seedlings of the both species was reduced at the various concentrations of several *Salvia x jamensis* metabolites (Figure 2). **1**, **2**, **3**, **4** and **6** significantly reduced the seedling height, the cotyledon length, the root length and the chlorophyll content of *Papaver rhoeas* L. and *Avena sativa* L. at the highest tested concentration (20 mg/L); at the same concentration **9** was active only against *Papaver*, while **5** was less active than the other compounds, but effective against the both species. **7**, **8** and **10** were inactive. The evaluation of seedling fresh weight and dry weight of both *Papaver* and *Avena* seedlings at the highest concentration (20 mg/L) gave similar results (Figure 3). **1**, **2**, **3**, **4**, **6** and **9** significantly reduced the fresh weight of *Papaver* seedlings; **5** showed the same activity of **1**, **2**, **3**, **4**, **6** and **9** was less active; **7**, **8** and **10** were inactive on *Avena* seedlings.

On the whole, the tested compounds differed greatly in their biological activity. **7**, **8** and **10** were essentially inactive, whereas **1**, **2**, **3**, **4** and **6** were phytotoxic against both the tested species, whereas **9** was active only against *Papaver*. **5** had significant inhibitory activity on germination of both *Papaver* and *Avena*, but its effect on the subsequent growth of these species was significantly lower than those of the other active compounds.

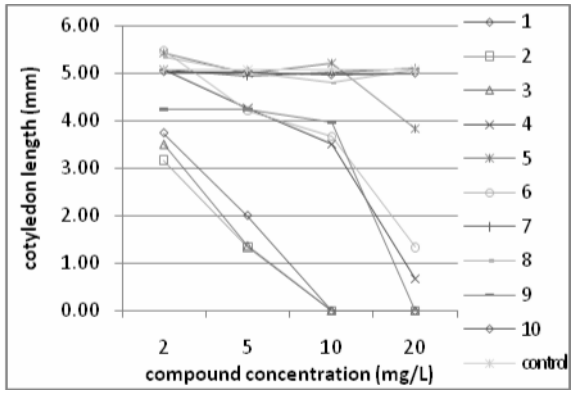
The phytotoxic activity of some of the considered diterpenoidic as well as flavonoidic compounds is consistent with literature where phytotoxicity has been described for these types of substances, and particularly for clerodane and ent-labdane diterpenes [12,13].

To our knowledge this is the first report on phytotoxic activity of isopimarane derivatives. However, it is clear from the present study that structure-activity relationships need to be deeply investigated, as clerodane and isopimarane structures resulted present in both active and inactive compounds.

Triterpenes are usually considered to be not highly phytotoxic, but are thought to be co-solubilizant agents of other bioactive compounds [13]. Nevertheless, the phytotoxic activity of betulinic acid against other dicotyledon and monocotyledon species was previously described [14-16]. The presence of



(a)



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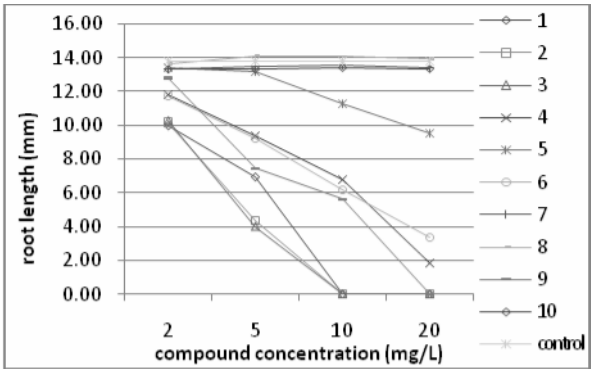
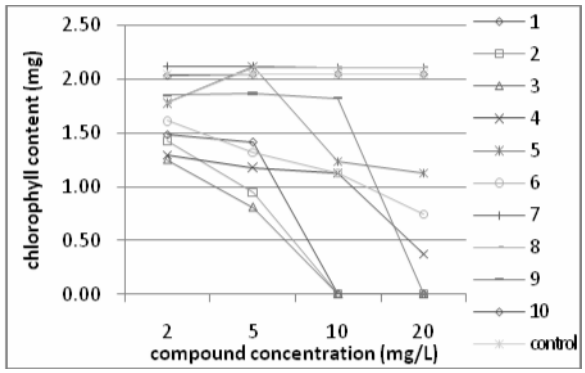
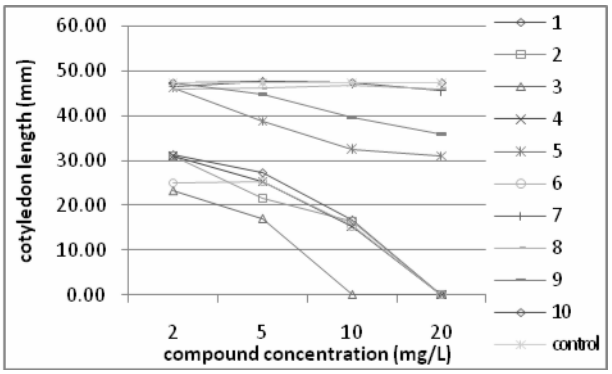


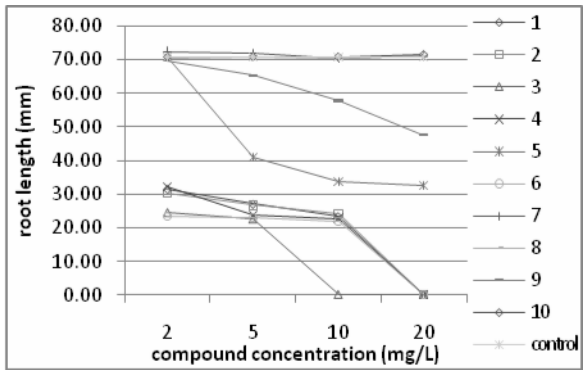
(c)



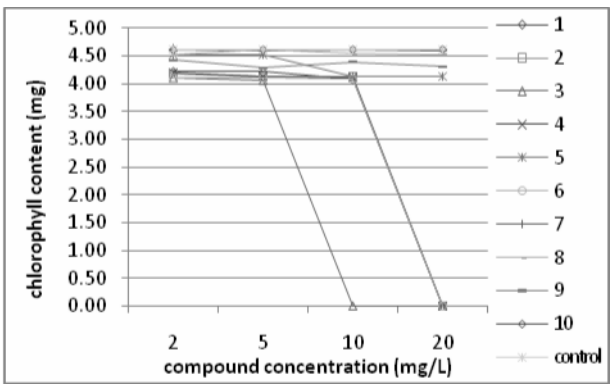
(d)



(e)



(f)



(g)

Figure 2: *Papaver rhoeas* L. and *Avena sativa* L. seedlin growth parameters after ten days exposure to *Salvia x jamensi* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwai acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, 4 isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod 3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod 3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16 epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19- diolide, **10**) at various concentrations. *Papaver rhoeas* L.: (a seedling height; (b) cotyledon length; (c) root length; (d chlorophyll content, fresh weight. *Avena sativa* L.: (e) cotyledo length; (f) root length; (g) chlorophyll content, fresh weigh Standard deviation bars are omitted for clarity.

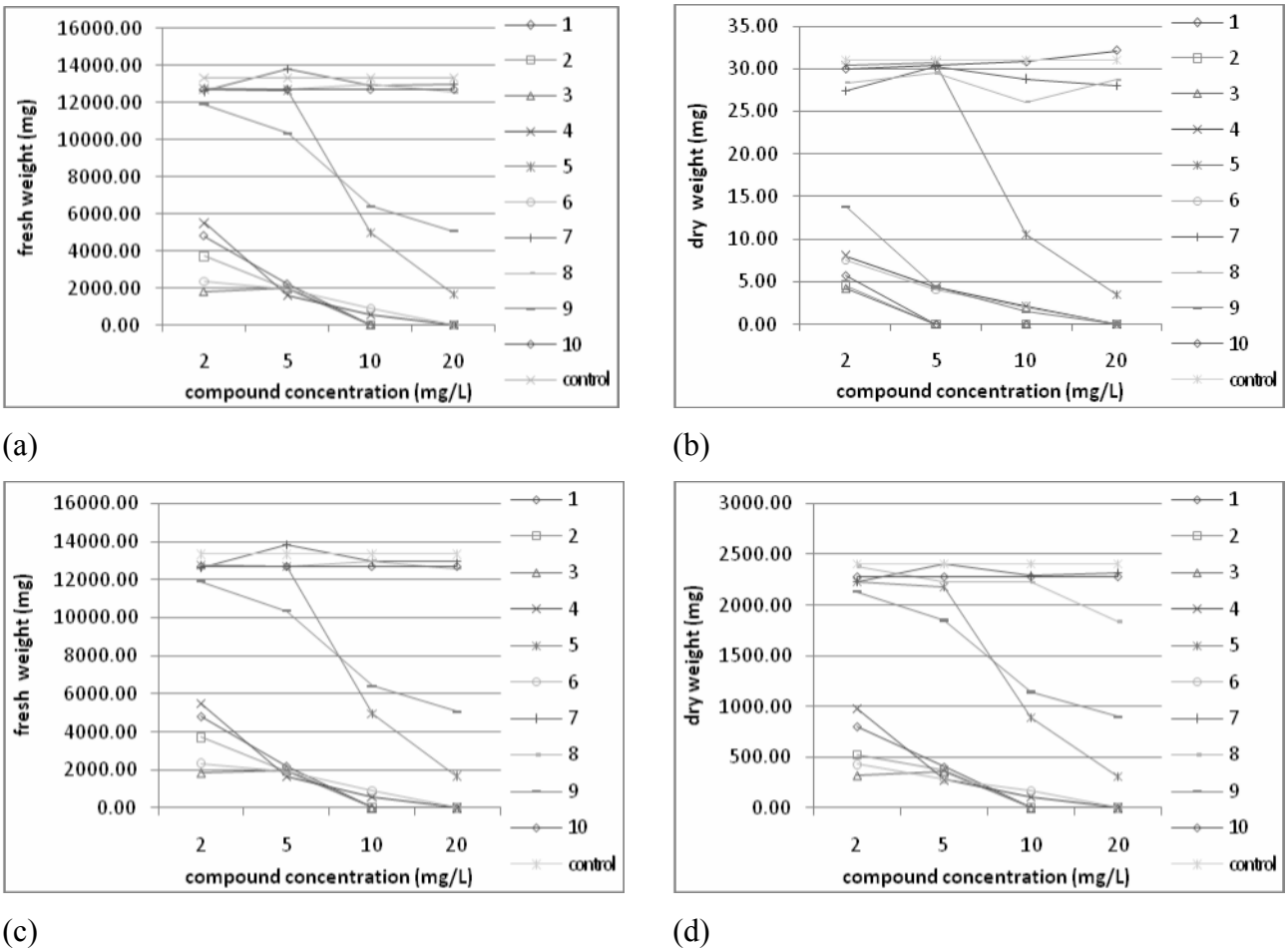


Figure 3: *Papaver rhoeas* L. and *Avena sativa* L. seedling fresh and dry weight after ten days exposure to *Salvia x jamensis* isolated compounds (3β-hydroxy-isopimaric acid, **1**; hautriwaic acid, **2**; betulinic acid, **3**; 7,8β-dihydrosalviacoccin, **4**; isopimaric acid, **5**; 15,16-epoxy-10β-hydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **6**; 14α-hydroxy-isopimaric acid, **7**; 15,16-epoxy-7α,10β-dihydroxy-clerod-3,13(16),14-trien-17,12;18,19-diolide, **8**; cirsiliol, **9**; 15,16-epoxy-7α,10-dihydroxy-clerod-2,13(16),14-trien-17,12;18,19-diolide, **10**) at various concentrations. *Papaver rhoeas* L.: (a) seedling fresh weight; (b) seedling dry weight. *Avena sativa* L.: (c) seedling fresh weight; (d) seedling dry weight. Standard deviation bars are omitted for clarity.

ursolic acid in the exudate of *S. jamensis* [4] may be useful for the allelopathic activity of these active liphophilic compounds since it was suggested that ursolic acid and other natural detergents, which are released from a source plant, enhance the solubilization of allelopathic lipids via micellization [17].

These laboratory bioassays will be followed by greenhouse studies to verify whether these effects take place also in the natural environment [12].

Experimental

General Experimental Procedures: Melting points are uncorrected and were measured on a Tottoli melting point apparatus (Büchi). Silica gel 60 (Merck 230-400 mesh) were used for column chromatography; aluminum sheets of silica gel 60 F_{254} (Merck 0.2 mm thick) with $CHCl_3$/MeOH/HCOOH (10:0.5:0.1) as an eluent were used for TLC and the spots were detected by spraying 50% H_2SO_4, followed by heating. HPLC-MS and MS^2 experiments were performed on an 1100 MSD HPLC-MS iontrap system (Agilent Technologies, Palo Alto, CA USA) equipped with a G1313A autosampler and diode array detector coupled with an electrospray ion source. The HPLC eluents were A: water with 0.1% formic acid and B: methanol with 0.05% formic acid. Starting from a A:B composition of 80:20 the linear gradient reached the 100% of B concentration in 30 min at a flow rate of 0.2 mL/min. The column employed was a Zorbax C18 150x2.1 mm ID, 3.5 μm particle size. Semi-preparative HPLC was carried out using a Waters W600 pump (Waters Corporation, Milford, USA) equipped with a Rheodyne Delta 600 Injector (with a 100 μL loop) and a Waters 2414 Refractive Index detector. The semi-preparative reversed-phase chromatography was performed at room temperature on a chemically

bonded stationary phase, 10μm μBondapack C18 column (7.8x300 mm ID) (Waters). The elution mixture (helium-degassed) was composed of CH_3OH/H_2O 40:60. The flow rate was 2.0 mL/min. The preparative HPLC was conducted on a Shimadzu LC8A system using the same eluents employed in the HPLC-MS analysis. The linear gradient from 30% to 90% of B in 40 minutes was performed at 15 ml/min on a 250x21.2 mm ID column (Phenomenex Luna C18). Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. UV spectra were obtained by a HP 8453 diode array spectrophotometer (Hewlett Packard, USA). IR spectra were recorded on a Perkin-Elmer 1310 spectrophotometer. NMR experiments were performed on a Bruker DRX-500 spectrometer. The standard pulse sequence and phase cycling were used for all 2D experiments: DQF-COSY, HSQC, HMBC, and ROESY spectra. The ROESY spectra were acquired with t_{mix}=400 ms. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. ESI-MS measures were carried out in the negative ion mode within a mass range containing the expected m/z signals. The acquisition parameters were set time by time in DIA (direct infusion analysis) to obtain the optimal signal to noise ratio for each molecule. The HR mass spectrometry analyses were performed in DIA (direct infusion analysis) in the negative ion mode on a QSTAR XL system (Applied Biosystems, Toronto Canada) to assess the elemental composition. The calculations were performed using a utility integrated in the dedicated software and the calculated elemental formulas were unambiguous for both the compounds.

Plant Material: Aerial parts of *S. x jamensis* J. Compton were obtained from Centro Regionale di Sperimentazione ed Assistenza Agricola (Albenga. Italy). The species has been identified by Dr. Gemma Bramley and a voucher specimen is deposited in Kew Herbarium (K).

Commercial seeds of *Papaver rhoeas* L. (La Semeria-www.Lasemeria.it- Italy) and *Avena sativa* L. (Il Monastero-Sementi-Italy), chosen as model species of dicotyledonous and monocotyledonous plants [10], were used. Damaged and undersized seeds were discarded; the seeds to be assayed were selected based on the uniformity of size. Germination of *Papaver* and *Avena* was tested before use and it was about 83% and 80% respectively.

Extraction and isolation of the active components: Extraction of leaf surface constituents was performed as previously described [4]. The exudate (18 g) was chromatographed in portions of 1.5 g on Sephadex LH-20 column (60x3 cm) using $CHCl_3$/MeOH (7:3) as an eluent to give, in order of elution, four fraction groups: fraction group I (3g) with waxy compounds (from 0 to 170 mL), fraction group II (3g) with very crude ursolic acid (from 170 mL to 220 mL), fraction group III (12 g) with the mixture of **1-8** and **10** (from 220 mL to 290 mL) and fraction group IV (0.2 g) (from 295 mL to 365 mL) with **9**. Fraction group III was chromatographed in portions of 4 g on a silica gel column (40 x 4 cm), eluting with mixtures of *n*-hexane/$CHCl_3$ [50:50 (4.1 L), 40:50 (2.1 L), 33:67 (1.0 L), 25:75 (1.0 L), 10:90 (11.0 L], then with $CHCl_3$ (8.3 L), and then with $CHCl_3$/MeOH [95:5 (3.4 L)]. Elution with *n*-hexane/$CHCl_3$ (40:50) (from 0.2 L to 2.1 L) and with *n*-hexane/$CHCl_3$ (33:67) and 25:75 afforded fractions with **5** (crystallized from MeOH: 2.05 g). Elution with *n*-hexane/$CHCl_3$ (10:90) afforded at first fractions with **3** (from 1.0 to 1.4 L; crystallized from EtOH: 0.2 g), followed by fractions with ursolic acid (from 1.6 to 2.7 L; crystallized from EtOH: 1.4 g), fractions with **4** (from 2.7 to 3.0 L; crystallized from $CHCl_3$/MeOH: 0.14 g), a mixture of **6** and **10** (from 3.1 to 3.8 L), and finally fractions with **8** (from 3.8 to 11.0 L; crystallized from $CHCl_3$/MeOH: 0.58 g). Elution with $CHCl_3$ afforded fractions with **7** (from 3.5 to 6.0 L; crystallized from MeOH/H_2O: 0.25 g). Elution with $CHCl_3$/MeOH 95:5 afforded at first fractions with very crude **2** (from 0.1 to 0.9 L; purified with semi-preparative RP-HPLC (20 mg), then fractions with **1** (from 1.1 to 2.0 L**,** crystallized from MeOH/H_2O: 50 mg). The mixture of **6** and **10** was purified by preparative RP-HPLC yielding 13 mg of **6** and 17 mg of **10**. Fraction group IV (0.2 g) was crystallized from $CHCl_3$/MeOH yielding 36 mg of **9**.

Germination and growth test: *Dose–response studies* The seeds were surface sterilized in 2% sodium hypochlorite under vacuum for 20 min, rinsed 3 times in sterile distilled water, and dipped for 24 h in sterile distilled water for imbibition prior to the germination trial. The seeds were then equidistantly placed in 9 cm and 15 cm diameter Petri dishes respectively for *Papaver* and *Avena* (50 seeds per Petri dish, 3 replicates per treatment) lined with three layers no. 1 Whatman sterilized filter paper. Standard solutions of 2, 5, 10 and 20 μg/mL of each compound were obtained by dissolving each weighed compound in DMSO and diluting with the

appropriated quantity of sterile distilled water. A similar treatment with distilled water, containing the same DMSO concentration, served as control. The filter paper was treated with the above solutions, and the Petri dishes were sealed with parafilm and placed in a growth chamber at 25±2°C under 16h/8h light/dark photoperiod and light intensity of 65 µmol m^{-2} s^{-1}. Emergence of the radicle (≥1 mm) was used as an index of germination and it was recorded daily in replicate. Germination counts were conducted for a period of 10 days. Seed germinability was assessed by the total germination of the seeds at the end of the test [10]; germination progress or germination rate was evaluated by the calculation of other indices, i.e. speed of germination, speed of accumulated germination and coefficient of the rate of germination [9,10]. The lethal concentrations needed to reduce germinability by 50% (LC_{50}) and by 90% (LC_{90}) were calculated by linear regression between the two adjacent values respectively comprehending the 50% and the 10% response of the final cumulative percentage of germination at the end of tests [5] for each compound [11].

Growth experiments: In another set of experiments, the effects of the same solutions of *Salvia x jamensis* compounds plus control solution were studied on the early growth of *Papaver rhoeas* L. and *Avena sativa* L., using the same assay procedure. After 10 days, seedling growth, in terms of seedlings height, cotyledons length and roots length were recorded with callipers. Fresh weights and dry weights of the seedlings were also measured.

Chlorophyll content: Chlorophyll was extracted from seedlings using dimethyl sulphoxide following the method of Lichtenthaler [18] and estimated using the equation of Arnon [19]. Chlorophyll content was expressed on the basis of the fresh weight of the tissue.

Statistical analysis: All the activities were studied in triplicate. Significant differences were statistically evaluated using analysis of variance (one-way ANOVA) and the Tukey test on untransformed data. For analysis of variance, percent germination data (Total germination) were arcsine-square root transformed to meet the requirements of the test [10]. For all tests, statistical significance was set at $P<0.05$. SYSTAT (Version 8.0 – SPSS Inc., 1998) software was used for statistical analysis of the data.

Compound 6

MP: >260ºC (dec.).
$[\alpha]_D^{25}$: -53.1 (*c* 0.1, CH_3OH).
IR (KBr): 3495, 3121, 1755, 1725, 1502, 868 cm^{-1}.
UV/Vis λ_{max} (CH_3OH) nm (log ε): 207 (4.04), 239 (3.09).
^1H NMR and ^{13}C NMR: Table 2.
HR-MS: *m/z* 357.1352 $[M-H]^-$ (3.88 ppm error).

Compound 10

MP: >230ºC (dec.).
$[\alpha]_D^{25}$: -125.0 (*c* 0.1, $CHCl_3$).
IR (KBr): 3560, 3520, 3130, 1755, 1720, 1500, 875 cm^{-1}.
UV/Vis λ_{max} (CH_3OH) nm (log ε): 207 (3.72).
^1H NMR and ^{13}C NMR: Table 3.
HR-MS: *m/z* 373.1303 $[M-H]^-$ (4.21 ppm error).

Acknowledgments –We wish to thank Dr. Annalisa Salis and Dr. Andrea Armirotti for their technical assistance.

References

[1] Chou CH. (**2006**) Introduction to allelopathy. In *Allelopathy – A Physiological Process with Ecological Implications*; Reigosa MJ, Pedrol N, Gonzáles L Eds.; Springer: Dordrecht, The Netherlands, pp. 1-10.

[2] Field B, Jordàn F, Osbourn A. (**2006**) First encounters – deployment of defence-related natural products. *New Phytologist*, ***172***, 193-207.

[3] Duke SO. (**1994**) Glandular Trichomes - A Focal Point of Chemical and Structural Interactions. *International Journal of Plant Sciences*, ***155***, 617–620.

[4] Bisio A, Romussi G, Russo E, De Tommasi N, Mascolo N, Alfieri A, Bonito MC, Cicala C. (**2008**) Platelet Antiaggregating Activity and Chemical Constituents of *Salvia x jamensis* J. Compton. *Natural Product Communications*, ***3***, 881-884.

[5] Jefferson LV, Pennacchio M. (**2003**) Allelopathic effects of foliage extracts from four Chenopodiaceae species on seed germination. *Journal of Arid Environments*, ***55***, 275-285.

[6] Jolad SD, Hoffman JJ, Schram KH, Cole JR, Tempesta MS, Bates RB. (**1982**) Constituents of *Eremocarpus setigerus* (Euphorbiaceae). A new diterpene, Eremone, and Hautriwaic Acid. *Journal of Organic Chemistry*, ***47***, 1356-1358.

[7] Iinuma M, Matsuura S, Kusuda K. (**1980**) ^{13}C-Nuclear Magnetic Resonance (NMR) Spectral Studies on Polysubstituted Flavonoids. I. ^{13}C-NMR spectra of flavones. *Chemical and Pharmaceutical Bulletin*, ***28***, 708-716.

[8] Almaza G, Balderrama L, Labbé C, Lavaud C, Massiot G, Nuzillard JM., Connolly JD, Farrugia LG, Rycroft DS. (**1997**) Clerodane Diterpenoids and an Ursane Triterpenoid from *Salvia haenkei*. Computer-assisted Structural Elucidation. *Tetrahedron*, ***53***, 14719-14728.

[9] Chiapusio G, Sanchez AM, Reigosa MJ, Gonzalez L, Pellissier F. (**1997**) Do germination indices adequately reflect allelochemical effects on the germination process? *Journal of Chemical Ecology*, ***23***, 2445-2453.

[10] Allaie RR, Reshi Z, Rashid I, Wafai BA. (**2006**) Effect of aqueous leaf leachate of *Anthemis cotula* - An alien invasive species on germination behaviour of some field crops. *Journal of Agronomy and Crop Science*, ***192***, 186-191

[11] Dayan FE, Romagni JG, Duke SO. (**2000**) Investigating the mode of action of natural phytotoxins. *Journal of Chemical Ecology*, ***26***, 2079-2094.

[12] Vyvyan JR. (**2002**) Allelochemicals as leads for new herbicides and agrochemicals. *Tetrahedron*, ***58***, 1631-1646

[13] Seigler DS. (**2006**) Basic pathways for the origin of allelopathic compounds. In *Allelopathy – A Physiological Process with Ecological Implications*; Reigosa MJ, Pedrol N, Gonzáles L Eds.; Springer: Dordrecht, The Nederlands, pp. 11-61.

[14] Chaudhuri PK, Srivastava R, Kumar S, Kumar S. (**2004**) Phytotoxic and antimicrobial constituents of *Bacopa monnieri* and *Holmskioldia sanguinea. Phytotherapy Research*, ***18***, 114-117.

[15] Ohara S, Ohira T. (**2003**) Plant growth regulation effects of triterpenoid saponins. *Journal of Wood Science, **49***, 59-64.

[16] Macías FA, Simonet AM, Esteban MD. (**1994**) Potential allelopathic lupane triterpenes from bioactive fractions of *Melilotus messanensis*. *Phytochemistry*, ***36***, 1369-1379.

[17] Fischer NH, Williamson GB, Tanrisever N, De la Pena A, Weidenhamer JD, Jordan ED, Richardson DR. (**1989**) Allelopathic actions in the Florida scrub community. *Biologia Plantarum, **31***, 471-478.

[18] Lichtenthaler HK. (**1987**) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology*, ***148***, 350-382.

[19] Arnon D. (**1949**) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. *Plant Physiology*, ***24***, 1-15.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1631 - 1636

A New β-D-Glucopyranosyl 2-Oxo-urs-12-en-28-oate from the Cameroonian Plant *Combretum bracteatum*

Germain Ntchatcho[a], Luisella Verotta[b], Paola Vita Finzi[a], Giuseppe Zanoni[a] and Giovanni Vidari[a,*]

[a]*Dipartimento di Chimica Organica, Università di Pavia, Via Taramelli 10, 27100 Pavia, Italy*

[b]*Dipartimento di Chimica Organica e Industriale, Università di Milano, Via Venezian 21, 20133 Milano, Italy*

vidari@unipv.it

Received: September 8th, 2009; Accepted: November 5th, 2009

From the Cameroonian plant *Combretum bracteatum* (Laws.) Engl. and Diels a new compound, β-D-glucopyranosyl 3β,19α-dihydroxy-2-oxo-urs-12-en-28-oate, was isolated along with three known ursane and oleane triterpenes and two galactopyranosyl lipids. This represents the first example of a naturally occurring 2-oxo-urs-12-enoic acid derivative.

Keywords: *Combretum bracteatum*, Combretaceae, glucopyranosyl 2-oxo-ursenoate, triterpenoids, galactopyranosyl lipids.

Amongst the about 20 genera comprising the Combretaceae, the genus *Combretum* is the largest, with about 370 species of trees, shrubs, and llanas, roughly 300 of which are native to tropical and southern Africa [1], about 5 to Madagascar, some 25 to tropical Asia and approximately 40 to tropical America. The genus is absent from Australia. Several species are used in African and Indian traditional medicine [2]. Combretastatins [3], found in the South African bushwillow (*C. caffrum*) and presumably other species of this genus, are probably the most important compounds isolated so far from *Combretum*, which is under study for the therapy of tumors, including anaplastic thyroid cancer for which there is little or no approved treatment at present. Triterpenes and their glycosides, phenanthrenes, bibenzyls, stilbenes, flavonoids, and tannins, in addition to other aromatic compounds, are the most representative secondary metabolites of this genus. Some of them display anticancer, antimicrobial, hepatoprotective, anti-inflammatory, anti-cholinesterase, antiprotozoal, and anti-HIV properties, among others [4].

C. bracteatum (Laws.) Engl. and Diels is a scandent shrub of wooded savannahs [5] recorded from south Nigeria, west Cameroon and Fernando Po to Zaïre. The flowers are showy, yellowish and red. The plant has been brought into cultivation. The flowers are sucked by children and visited by sun-birds to obtain the nectar, and the hollow stems are used in Nigeria for tapping palms for wine. In Cameroonian folk medicine the plant is used for curing headache, diarrhea, and fever. In an investigation of 62 plants from tropical Africa aimed at the discovery of new naturally occurring bioactive substances, *C. bracteatum* exhibited promising herbicidal and fungicidal activities [6].

In continuation of our studies on Cameroonian plants [7], we describe in this paper the results of the first phytochemical survey of extracts from *C. bracteatum*, reporting the isolation and structure elucidation of a new triterpene glucoside, 2β,19α,23-trihydroxy-2-oxo-12-ursen-28-oate (**1**), along with the known glucosyl triterpenes **2**-**4**, rutin, phytol, and two known galactopyranosyl lipids **5** and **6**. The triterpenes and rutin were isolated from a methanol extract of dried leaves, whereas the remaining compounds were found in an acetone extract of fresh leaves. The structures of these molecules were elucidated from spectroscopic data.

Compound **1** was obtained as a colorless solid. The infrared (IR) spectrum indicated the presence of hydroxyl groups (3600–3200 cm^{-1}), carbonyl and

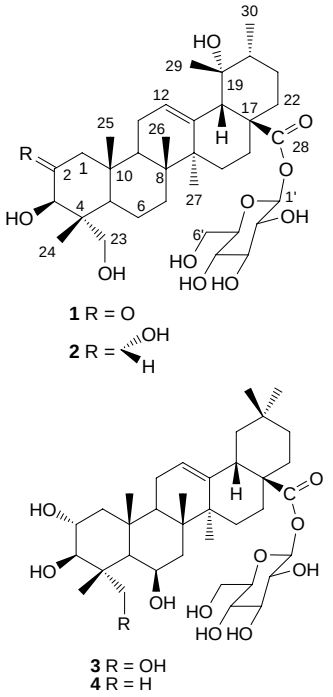


Figure 1: Triterpenes **1**-**4** isolated from *Combretum bracteatum*.

carboxylic ester groups (1725–1715 cm^{-1}), and a double bond (1648 cm^{-1}). The compound displayed a pseudo-molecular ion peak at *m*/*z* 687.3735 $[M+Na]^+$ in the HRESIMS spectrum (positive ion mode), consistent with the molecular formula $C_{36}H_{56}O_{11}$ (calcd. for $C_{36}H_{56}O_{11}$·Na 687.3720), and the counting of protons and carbons in the NMR spectra. The ^1H NMR spectrum of **1** showed five aliphatic methyl singlets at δ 0.59, 0.80, 0.93, 1.22, and 1.42, a doublet at δ 0.95 (3H, d, J = 6.6 Hz) for a secondary methyl group, and a triplet at δ 5.31 (1H, J = 3.0 Hz) assignable to an olefinic hydrogen.

The ^{13}C and DEPT NMR spectra exhibited signals for six methyls, ten methylenes, comprising two oxymethylenes at δ 62.7 and 65.6, eleven methines, including one olefinic and six oxymethines, and nine quaternary carbons, comprising one olefinic, one ester carbonyl carbon at δ 178.9, and one ketone carbonyl carbon at δ 214.2. Five oxymethines and one oxymethylene were assignable to a hexose unit and the remaining 30 carbons were consistent with a triterpene aglycone moiety. The secondary methyl signal on ring E at δ 0.95 provided a most useful indicator for the presence of an urs-12-ene skeleton [8]. In addition, the signals in the ^{13}C NMR spectrum at δ 129.3 (d) and 140.1 (s) were characteristic for a C-12/C-13 trisubstituted double bond in the ursene-type structure [9]. On the other hand, a singlet at δ 2.54 (1H), attributable to the bridgehead H-18,

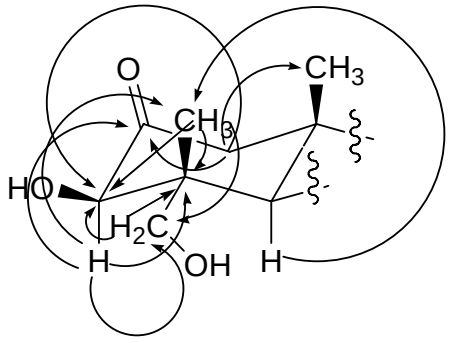


Figure 2: Selected HMBC correlations of compound **1**

suggested the presence of a 19-*O*-substituted urs-12-ene skeleton [10,11]. The tertiary hydroxyl group at C-19 was assigned to the α position by analogy with the ^{13}C NMR chemical shifts of similarly substituted triterpene saponins [10,11], while the ester carbonyl group was attached to C-17 on the basis of a HMBC correlation to H-18. Furthermore, the ^1H NMR spectrum of **1** also revealed a two-proton AB quartet, centered at δ 3.42, assignable to diastereotopic hydroxymethylene hydrogens, which showed cross-peak correlations with a carbon signal at δ 65.6 in the HSQC spectrum. This information, along with an upfield shifted methyl carbon signal at δ 13.8 attributable to axial C-24 and a downfield quaternary carbon resonance at δ 44.6, assignable to C-4 [11], strongly suggested the location of this primary hydroxyl substituent at C-23. This assumption could be confirmed by connectivities observed in the HMBC spectrum (Figure 2) between this carbon signal and a hydroxymethine proton H-3 (δ 4.40) and Me-24 (δ 0.59) resonances, and between the hydroxymethylene protons and C-3 and C-4 signals. The ketone was placed at the C-2 position, due to the fact that H-3 resonated as a sharp singlet, while the H_2-1 protons gave rise to an isolated AB pattern at δ 2.18 and 2.37 (J_{AB} = 12 Hz), and showed long-range correlations with the methyl carbon at δ 17.5 (C-25) in the HMBC spectrum: The stereochemistry of the secondary hydroxyl group was confirmed by NOE experiments. In particular, on irradiation of the signal of the H-3 proton at δ 4.40, the NOE effect was observed on H_2-23, H-5, and axial αH-1 (δ 2.18) protons. Furthermore, irradiation of the signal of the Me-24 protons at δ 0.59 gave NOE enhancement for the Me-25 protons at δ 0.93, whereas, on irradiation of the last signal, the NOE effect was observed for Me-24, Me-26 (δ 0.80) and βH-1 (δ 2.37) protons. These data clearly indicated the equatorial β-configuration of the C-3 hydroxyl group, as indicated in structure **1**.

Table 1. ^{13}C NMR spectral data of compound **1** (75 MHz).[a,b]

C	δ	C	δ	C	δ
1	54.6 t	13	140.1 s	25	17.5 q
2	214.2 s	14	43.2 s	26	17.5 q
3	78.8 d^{+}	15	29.4 t	27	24.9 q
4	44.6 s	16	27.5 t*	28	178.9 s
5	48.5 d°	17	49.9 s	29	27.3 q
6	19.7 t	18	55.3 d	30	16.9 q
7	33.5 t	19	73.9 s	1’	96.1 d
8	41.9 s	20	43.2 d	2’	74.1 d
9	48.4 d°	21	26.8 t*	3’	78.1 d^{+}
10	38.5 s	22	38.5 t	4’	71.4 d
11	25.0 t	23	65.6 t	5’	78.5 d^{+}
12	129.3 d	24	13.8 q	6’	62.7 t

[a]Chemical shifts (δ, ppm) are relative to the central line (δ_C 49.86) of the MeOH-d_4 solvent signal. [b]Multiplicities determined by DEPT sequences. $^{+,\circ,*}$Assignments indicated by the same symbol can be interchanged.

The ^{13}C NMR data also allowed the assignment of the pyranose form of glucose to the hexose unit of glycoside **1** [12], while the value of the coupling constant of the H-1’ doublet at δ 5.33 (J = 7.8 Hz) in the ^{1}H NMR spectrum, indicated the anomeric hydrogen to be α-oriented, thus establishing the β-configuration for the glucopyranosyl moiety. The chemical shift value of its anomeric carbon (δ 96.1) supported the fact that compound **1** was an ester glucoside [13]. The long-range coupling discernible in the HMBC spectrum of **1** between the C-28 carbonyl carbon (δ 178.9) and the anomeric hydrogen of the sugar moiety (δ 5.33) further supported the linkage site of the glucopyranosyl unit at C-28. Finally, acid hydrolysis of **1** gave D-glucose, identical with an authentic sample (TLC, GC, optical rotation).

The complete assignment of all the carbons of triterpene **1** was determined (Table 1) with the help of DEPT, ^{1}H–^{1}H COSY, HSQC, and NOE and followed by analysis of the HMBC spectral data. On the basis of all the above information, compound **1** was established to be a new ursane-type triterpene glucoside, and the structure of the natural product was formulated as β-D-glucopyranosyl 3β,19α-dihydroxy-2-oxo-urs-12-en-28-oate. This is a rare structure and, to the best of our knowledge, represents the first naturally occurring 2-oxo-urs-12-enoic acid derivative.

The structures of compounds **2**, **3**, and **4** were readily discernible from both the ^{1}H- and ^{13}C-NMR spectra as those of the known 19-α-hydroxyursane derivative nigaichigoside F1 (β-D-glucopyranosyl 2α,3β,19α, 23-tetrahydroxyurs-12-en-28-oate) [10,11], and the oleane-type triterpenoids chebuloside II (β-D-glucopyranosyl 2α,3β,6β,23-tetrahydroxyolean-12-en-28-oate) [4,14-16], and 28-β-D-glucopyranosyl-6β-hydroxy maslinate (β-D-glucopyranosyl 2α,3β,6β,-trihydroxyolean-12-en-28-oate) [4,16], respectively, the spectral data for which were in accordance with those published in the literature. This constitutes the second reported isolation of compounds **2** and **4** in the Combretaceae, where they were first described in *C. quadrangulare* [17] and in *C. laxum* [4], respectively, whereas chebuloside II (**3**) has been reported to occur both in *C. laxum* [4] and *C. quadrangulare* [8]. Interestingly, compounds **3** and **4** have been reported to show minimal inhibitory concentration (MIC) values in the range of 100-200 μg/mL against the fungal strains *Candida albicans*, *C. krusei*, and *Cryptococcus neoformans* [4]. Moreover, nigaichigoside F1 (**2**), at 25 μg/mL, exhibited 53% lipid peroxidation (LPO) inhibitory activity and weakly inhibited the COX-2 isozyme [11].

Compounds **5** and **6** (Figure 3), isolated from the acetone extract of fresh leaves of *C. bracteatum* were identified as the important chloroplast membrane glycolipids 1,2-di-*O*-α-linolenoyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol and 1,2-di-*O*-α-linolenoyl-3-*O*-[α-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl]-*sn*-glycerol, respectively, on the basis of complete spectral analyses, comparison with literature NMR data [18-21], and methanolysis products (D-galactose, methyl linolenate).

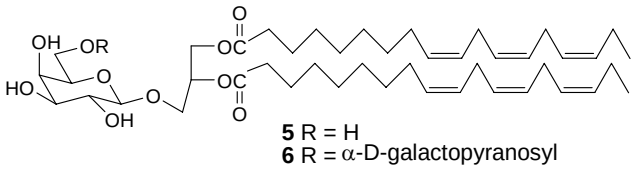


Figure 3: Galactopyranosyl lipids **5** and **6** isolated from *Combretum bracteatum*.

It is the first finding of metabolites of this type in plants of the genus *Combretum*. It is noteworthy that these glycolipids were found to inhibit the activities of replicative DNA polymerases *in vitro* and also the proliferation of human cancer cells [20-22].

Experimental

General experimental procedures: Melting points (uncorrected) were determined using a Fisher Johns melting point apparatus. Optical rotations were read on a Perkin-Elmer 241 digital polarimeter. IR spectra were recorded on a FT-IR Perkin Elmer Paragon 100 PC spectrometer as either neat films on NaCl discs or as KBr pellets. ^{1}H and ^{13}C NMR spectra were

determined in the indicated solvent on a Bruker AV 300 spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C), respectively. ^{1}H and ^{13}C chemical shifts (δ, ppm) are relative to the solvent signals used as references [$CDCl_3$: δ_C (central line of t) 77.16; residual $CHCl_3$ in $CDCl_3$: δ_H 7.26; CD_3OD: δ_C (central line of septuplet) 49.86; residual CH_3OD in CD_3OD: δ_H 3.34). The abbreviations s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad are used throughout; coupling constants (*J*) are reported in Hz. The number of H-atoms attached to each C-atom was determined by DEPT experiments. COSY, DEPT, HSQC, HMBC spectra were recorded using standard pulse sequences. Positive ion mode ESIMS analysis was performed on a Thermo-Finnigan LCQ Advantage MAX ion trap spectrometer, equipped with XCalibur 1.4 software. HRESIMS were acquired on a Bruker-Daltonics Apex II FT-ICR mass spectrometer. TLC was performed on either 0.25 mm silica gel 60 (GF_{254}, Merck) or RP-18 (F_{254}S, Merck), aluminum-supported plates. Compounds were visualized either under UV light (254 and 366 nm) or by spraying with a 0.5% solution of vanillin in H_2SO_4-EtOH (4:1), followed by charring. Preparative flash column chromatography (FCC) was performed on either Kieselgel 60 (40–63 μm, Merck) or reversed phase LiChroprep RP-18 (25–40 μm, Merck). FID-GC analyses were carried out on a Hewlett-Packard mod. 5890 II gas chromatograph equipped with a HP-5 (Crosslinked 5% PH ME Siloxane) capillary column (25 m, ID = 0.32 mm, f.t. = 0.25 μm). Helium was used as the carrier gas. Injector and detector temperatures were 260 and 290°C, respectively. Sil-Prep® (HMDS:TMCS:Py, 3:1:9), used for preparing the TMS derivatives of sugars, was purchased from Grace (Deerfield, IL, USA). Reagent grade solvents, redistilled just before use, were used for extraction; HPLC grade solvents were employed for chromatographic separations. Semi-preparative HPLC separations were performed with a dual Jasco PU-2080 *Plus* pumps system, using a home-made 2.5 × 28 cm, RP-18 (15 μm particle size) column, with a flow rate of 8 mL min^{-1}, and monitoring at 215 nm.

Plant material: The different parts of *C. bracteatum* used in this experiment were collected in July 2002 from a site near Nkoelon, located in the rain forest of South West Cameroon. The material was identified by comparison with an authentic sample by R. Y. Kongor, herbarium curator of the Limbe Botanical and Zoological Gardens, Limbe, Cameroon. A voucher sample of the leaves has been deposited with the number GN-CB-02 at the Department of Organic Chemistry of the University of Pavia.

Extraction and isolation: Fresh leaves of *C. bracteatum* were cut into pieces and then part (85 g) was extracted by percolation successively with CH_2Cl_2 (3 x 300 mL), Me_2CO (3 x 300 mL), Me_2CO–H_2O, 3:1 (2 x 200 mL), and MeOH–H_2O, 3:1 (2 x 200 mL), each time for 8 h at room temperature. The solvents were evaporated under reduced pressure to afford extracts A1 (0.99 g), A2 (10.45 g), A3 (1.86 g), and A4 (0.46 g), respectively. Another part of the leaves (33 g) was air-dried, ground, and extracted by percolation successively with CH_2Cl_2, MeOH, and MeOH–H_2O 3:1, as above, to give, after evaporation, extracts B1 (0.12 g), B2 (6.53 g), and B3 (0.67 g), respectively. Subsequently, only the most abundant fractions, A2 and B2, were examined. Part of mixture B2 (5.23 g) was subjected to FCC by reversed phase (200 g). Elution was performed with a gradient of MeOH–H_2O, starting from a mixture of 3:1, v/v, and increasing the MeOH proportion regularly every 200 mL, until a final mixture of MeOH–H_2O, 7:1, v/v, collecting a total of 20 fractions (50 mL each). The fractions were combined on the basis of similar TLC profiles (RP-18, MeOH–H_2O 4:1) and evaporated to give 5 residues denoted from B2-A to B2-E. A large amount of chlorophyll remained adsorbed at the top of the column and was not recovered. B2-A (300 mg) contained mainly sugars and tannins and was discarded. B2-B (110 mg) was further separated on a RP-18 column. Elution with MeOH–H_2O (1:1) gave 3-*O*-rhamnoglucosylquercetin (rutin, 5 mg) [23], followed by nigaichigoside F1 (**2**) [10,11], 8 mg. B2-C (172 mg), B2-D (0.72 g), and B2-E (0.42 g) were dissolved in 9, 36, and 20 mL MeOH, respectively, and the corresponding solutions, 1.5 mL of each elution, were successively separated by HPLC over the indicated semi-preparative reversed phase column. Elution was performed with the following standard program: H_2O–MeOH, 4:1 (2 min) → H_2O–MeOH, 1:3 (60 min) → H_2O–MeOH, 1:9 (100 min) → MeOH (120 min). Eighteen fractions were collected (from B2-CDE-1 to B2-CDE-18). B2-CDE-1, eluted after 39.5 min, was constituted by nigaichigoside F1 (**2**) [10,11], 22 mg; B2-CDE-4 (20 mg), eluted after 45.5 min, corresponded to β-D-glucopyranosyl 3β,19α-dihydroxy-2-oxo-urs-12-en-28-oate (**1**). Chebuloside II (**3**) [4,14-16], 38 mg, was contained in B2-CDE-5, eluted after 57.4 min. The last identified compound was β-D-glucopyranosyl

2α,3β,6β,-trihydroxyolean-12-en-28-oate (**4**) [4,16], 17 mg, contained in B2-CDE-11, eluted after 71 min.

A2 (9 g) was treated with MeOH, in which only a part dissolved. After separation of the insoluble fraction, containing mainly chlorophyll and carotenes, the solution was evaporated, and the residue (A2-A, 5.2 g) was separated over a RP-18 column (150 g). Elution with MeOH gave 8 fractions (from A2-A-1 to A2-A-8). Further chromatography of A2-A-3 (0.20 g) over a RP-18 column (20 g) eluted with MeOH gave 11 sub-fractions (from A2-A-3/1 to A2-A-3/11). A2-A-3/3–5 contained unidentified glycolipids, while combined A2-A-3/6–7 gave (*E*)-phytol (10 mg) [24], identical with an authentic sample. Further CC of combined A2-A-3/9–11 over silica gel eluted with a gradient of MeOH in EtOAc (from 0:100 to 20:80) afforded glycolipids **5** (12 mg) and **6** (10 mg) [18–21]. NMR spectroscopy revealed the presence of **5** and **6**-type glycolipids also in A2-A-4 and A2-A-5.

β-D-Glucopyranosyl 3β,19α-dihydroxy-2-oxo-urs-12-en-28-oate (**1**)

Colorless solid
MP: 184-187°C.
$[\alpha]_D^{20}$: +25.52 (*c* 0.3, MeOH)
R_f : 0.66 (RP18, MeOH-H_2O, 4:1).
IR (KBr): 3600-3200, 2928, 2870, 1715, 1648, 1074 cm^{-1}.
^1H NMR (MeOD): 0.59 (3H, s, H_3-24), 0.80 (3H, s, H_3-26), 0.93 (3H, s, H_3-25), 0.95 (3H, d, J = 6.6 Hz, H_3-30), 1.02 (1H, m, H_a-15), 1.22 (3H, s, H_3-29), 1.25–1.45 (m, H-5, H_2-6, H-20, H_a-21), 1.42 (3H, s, H_3-27), 1.5–1.9 (m, H-7, H_b-15, H_a-16, H_b-21, H_2-22), 1.9–2.10 (2H, m, H_2-11), 2.18 (1H, d, J = 12.0 Hz, αH-1), 2.37 (1H, d, J = 12.0 Hz, βH-1), 2.55 (1H, s, H-18), 2.64 (td, J =13.0, 4.6 Hz, H_b-16), 3.25–3.40 (4H, m, H-2', H-3', H-4', H-5'), 3.42 (2H, ABq, H_2-23), 3.68 (1H, dd, J = 12.0, 4.5 Hz, H_a-6'), 3.82 (1H, dd, J = 12.0, 2.0 Hz, H_b-6'), 4.40 (1H, s, H-3), 5.31 (1H, t, J =3.0, H-12), 5.33 (1H, d, J = 7.8 Hz, H-1').
^{13}C NMR: Table 1.
Positive ion ESI-FT-ICR-MS: *m/z* $[M + Na]^+$ calcd for $C_{36}H_{56}O_{11}Na$: 687.3720; found: 687.3735.

Acid hydrolysis of glucoside 1 and sugar identification: A solution of compound **1** (3 mg) in 2N aqueous CF_3COOH (1 mL) was heated for 2 h at 80°C. After extraction with $CHCl_3$, the aqueous layer was repeatedly evaporated to dryness with MeCN and the residue was analyzed by TLC on silica gel. It coeluted with glucose on comparison with standard sugars (solvent system: *n*-BuOH: toluene: pyridine: H_2O, 5:1:3:3). A solution of the residue (about 1 mg) in H_2O (1.5 mL) showed a positive optical rotation, as expected for D-glucose. The aqueous solution was then lyophilised and the resulting residue was treated with 200 μL Sil-Prep® for 30 min at 60°C. Solvents were evaporated at 60°C under N_2; the reaction product was dissolved in 100 μL *n*-hexane and analysed by GC. After injection, the oven temperature was programmed at a rate of 10°C/min from 60 to 280°C. The sample from the hydrolysis of **1** coeluted with the TMS derivatives of glucose: α-anomer at 18.8 min, β-anomer at 19.43 min.

Acknowledgments – We thank Prof. Mariella Mella and Prof. Giorgio Mellerio for NMR and mass spectral measurements, respectively. Financial support from the Italian MIUR (Grants COFIN) and the University of Pavia (Grant FAR) is acknowledged.

References

[1] Wickens GE. (**1973**) *Flora of Tropical East Africa*. *Combretaceae*., Whitefriars Press Ltd, London and Tonbridge, UK.

[2] Rogers CB, Verotta L. (**1996**) Chemistry and biological properties of the African Combretaceae. In *Chemistry and Pharmacological Properties of African Medicinal Plants*. Hostettmann K, Chinyanganga F, Maillard M, Wolfender J-L (Eds). University of Zimbabwe Publications, Harare, Zimbabwe.

[3] Cirla A, Mann J. (**2003**) Combretastatins: from natural products to drug discovery. *Natural Product Reports*, ***20***, 558-564.

[4] Bisoli E, Garcez WS, Hamerski L, Tieppo C, Garcez FR. (**2008**) Bioactive pentacyclic triterpenes from the stems of *Combretum laxum*. *Molecules*, ***13***, 2717-2728 and references cited therein.

[5] Burkill HM. (**1985**) *The Useful Plants of West Tropical Africa*, Vol ***1***. Royal Botanic Gardens, Kew, UK.

[6] Higashi O, Takagaki T, Koshimizu K, Watanabe K, Kaji M, Hoshino J, Nishida T, Huffman MA, Takasaki H, Jato J, Muanza DN. (**1991**) Biological activities of plant extracts from tropical Africa. *African Study Monographs*, ***12***, 201-210.

[7] Ngueyem TA, Brusotti G, Marrubini G, Grisoli P, Dacarro C, Vidari G, Vita Finzi P, Caccialanza G. (**2008**) Validation of use of a traditional remedy from *Bridelia grandis* (Pierre ex Hutch) stem bark against oral Streptococci. *Journal of Ethnopharmacology*, ***120***, 13-16.

[8] Johns SR, Lamberton, JA, Morton TC, Suarez H, Willing RI. (**1983**) Triterpenes of *Lantana tiliaefolia*. 24-Hydroxy-3-oxours-12-en-28-oic acid, a new triterpene. *Australian Journal of Chemistry*, ***36***, 2537-2547.

[9] Durham DG, Liu X, Richards RME. (**1994**) A triterpene from *Rubus pinfaensis*. *Phytochemistry*, ***36***, 1469-1472.

[10] Seto T, Tanaka T, Tanaka O, Naruhashi N. (**1984**) β-Glucosyl esters of 19α-hydroxyursolic acid derivatives in leaves of *Rubus* species. *Phytochemistry*, ***23***, 2829-2834.

[11] Bowen-Forbes CS, Mulabagal V, Liu Y, Nair MG. (**2009**) Ursolic acid analogues: non-phenolic functional food components in Jamaican rasberry fruits. *Food Chemistry*, ***116***, 633-637.

[12] Agrawal PK. (**1992**) NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry*, ***31***, 3307-3330.

[13] Boyd J, Turvey JR. (**1978**) Identification by ^{13}C-n.m.r. spectroscopy of oligosaccharides derived from alginic acid. *Carbohydrate Research*, ***61***, 223-226.

[14] Kunu, AP, Mahato, SB. (**1993**) Triterpenoids and their glucosides from *Terminalia chebula*. *Phytochemistry*, ***32***, 999-1002.

[15] Adnyana IK, Tezuka Y, Awale S, Banskota AH, Tran KQ, Kadota S. (**2000**) Quadranosides VI–XI, six new triterpene glucosides from the seeds of *Combretum quadrangulare*. *Chemical & Pharmaceutical Bulletin*, ***48***, 1114-1120.

[16] Gallo MBC, da Silva FC, Vieira PC, Fernandes JBF, da Silva MF das GF. (**2006**) New natural products from *Siphoneugena densiflora* Berg (Myrtaceae) and their chemotaxonomic significance. *Journal of Brazilian Chemical Society*, ***17***, 279-288.

[17] Adnyana IK, Tezuka Y, Banskota AH, Xiong Q, Tran KQ, Kadota S. (**2000**) Quadranosides I–V, new triterpene glucosides from the seeds of *Combretum quadrangulare*. *Journal of Natural. Products*, ***63***, 496-500.

[18] Olugbade TA, Adesanya SA. (**2000**) Prieurianoside, a protolimonoid glucoside from the leaves of *Trichilia prieuriana*. *Phytochemistry*, ***54***, 867-870.

[19] Hauksson JB, Bergqvist MHJ, Rilfors L. (**1995**) Structure of digalactosyldiacylglycerol from oats. *Chemistry and Physics of Lipids*, ***78***, 97-102.

[20] Morimoto T, Nagatsu A, Murakami, N, Sakakibara J, Tokuda H, Nishino H, Iwashima A. (**1995**) Anti-tumour-promoting glyceroglycolipids from the green alga *Chlorella vulgaris*. *Phytochemistry*, ***40***, 1433-1437.

[21] Wang R, Furumoto T, Motoyama K, Okazki K, Kondo A, Fukui H. (**2002**) Possible antitumor promoters in *Spinacia oleracea* (spinach) and comparison of their contents among cultivars. *Bioscience, Biotechnology, and Biochemistry*, ***66***, 248-254.

[22] Kuriyama I, Musumi K, Yonezawa Y, Takemura M, Maeda N, Iijima H, Hada T, Yoshida H, Mizushina Y. (**2005**) Inhibitory effects of glycolipids fraction from spinach on mammalian DNA polymerase activity and human cancer cell proliferation. *The Journal of Nutritional Biochemistry*, ***16***, 594-601.

[23] Lallemand, JY, Duteil M. (**1977**) ^{13}C n.m.r. spectra of quercetin and rutin. *Organic Magnetic Resonance*, ***9***, 179-180.

[24] Goodman RA, Oldfield E, Allerhand A. (**1973**) Assignments in the natural-abundance carbon-13 nucler magnetic resonance spectrum of chlorophyll a and a study of segmental motion in neat phytol. *Journal of the American Chemical Society*, ***95***, 7553-7555.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1637 - 1638

Cyclohexanones from *Mimulus glabratus* and *M. luteus*

Marisa Piovano[a], Juan Garbarino[a], Lamberto Tomassini[b] and Marcello Nicoletti[c,*]

[a]*Universidad Técnica Federico Santa María, Av. España 1680, Valparaíso, Chile*

[b]*Dipartimento di Biologia Vegetale, Università "La Sapienza", P.le A. Moro 5, I-00185, Roma, Italy*

[c]*Dipartimento di Fisiologia Generale e Farmacologia, Università "La Sapienza", Roma, Italy*

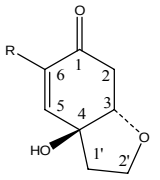
marcello.nicoletti@uniroma1.it

Received: August 24th, 2009; Accepted: October 5th, 2009

The phytochemical study of *Mimulus glabratus* A.Gray allowed the isolation of two cyclohexenones: the new compound 6-chlorohalleridone **1** and halleridone **2**. Halleridone was also identified in *Mimulus luteus* L., together with dihydroalleridone **3**, the naphtoquinone α-dunnione **4**, ursolic acid and β-sitosterol.

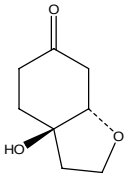
Keywords: *Mimulus luteus*, *Mimulus glabratus*, Cyclohexanones, Halleridone, Dihydrohalleridone, 6-Chlorohalleridone.

Mimulus (Scrophulariaceae) is a large (ca. 120 species) herbaceous genus typical of North-Western America, but also widely present in Asia and South America [1a]. In particular, the species *Mimulus glabratus* A.Gray and *M. luteus* L. can be found in humid zones of Central and Southern Chile [1b,1c], wherein the plant samples we examined were collected. *M. luteus* is also cultivated for its beautiful yellow flowers. The phytochemical studies carried out in the past years on leaves and flowers of *Mimulus* species showed the occurrence of several flavonoids [1d-1f]. In this article we report a new phytochemical study of the whole plants of *Mimulus glabratus* and *M. luteus*.

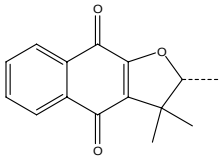


1 R = Cl 6-Chlorohalleridone

2 R = H Halleridone



3 Dihydrohalleridone



4 α-dunnione

The main constituents of the EtOAc soluble fraction obtained from *Mimulus glabratus* were separated by column chromatography, affording two cyclohexenones: 6-chlorohalleridone **1** and halleridone **2**. Halleridone **2** has been previously isolated from *Halleria lucida* [2a] and from *Forsythia suspensa* (with the name rengyolone) [2b], while the structure of **1** is novel and elucidated predominantly by means of NMR spectroscopy. In particular, the ^{1}H NMR spectrum of **1** appeared almost identical to that of **1**, except for the evident absence of the olefinic signal at position 6 and for the consequent loss of the large coupling in the signal of proton 5 ($J_{5,6}$= 10.0 Hz, for **2**) [2a,2b]. In the ^{13}C NMR spectrum of **1**, the carbon corresponding to position 6 was a quaternary olefinic carbon, indicating the occurrence of an additional substituent which was supposed to be a halogen atom. The EI mass spectrum indicated the presence of a chlorine atom, observed not only for the molecular ion peak at *m/z* 189/191 but also for daughter ions at *m/z* 144/146 (M^+ - C_2H_4O) and at *m/z* 102/104 (M^+ - $C_4H_6O_2$). Compound **1** was therefore assigned the structure of 6-chlorohalleridone. In the ^{1}H-NMR spectrum **1** the existence of a long-range W coupling (CH-C-CH, J = 1.5 Hz) between H-3 and H-5 is noteworthy. As in the structure of **2**, the planarity of this zig-zag configuration rules out the possibility of a *trans* junction between the two rings [2a]. Moreover, although cyclohexylethanoids are often isolated as racemic mixtures, the specific rotation value

registered for compound **1** ($[\alpha]^{20}_D$= +13.5) indicates, at least, an enantiomeric excess of the form (+)-**1**, corresponding to the formula reported in the Figure.

Following an identical separation procedure, *Mimulus luteus*, afforded halleridone, **2**, and its hydrogenated derivative dihydrohalleridone **3**, previously obtained as synthetic derivative of **2** [3a] and isolated as a natural product (with the name cleroindicine C) from *Clerodendrum indicum* [3b]. In addition, three other compounds were obtained: the naphthoquinone α-dunnione **4**, first isolated from *Streptocarpus dunnii* Mast. and later from several Scrophulariaceae [3c,3d], together with ursolic acid and β-sitosterol.

Experimental

General procedures: Optical rotations were measured at room temperature in $CDCl_3$ using a JASCO DIP-370 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 MHz NMR spectrometer. MS spectra were run on a EI-MS spectrometer HP 5989A. ICRMS spectra were obtained by electrospray ionisation with an instrument APEX II 4.7. TLC were performed on Si gel F_{254} (Merck) and plates were visualized using 2N H_2SO_4 spray.

Plant material: *M. glabratus* was collected near the source of the river Rio Tolten, Villarrica, IX region, Chile. *M. luteus* was collected near the river Rio Aconcagua, Portillo (V region, Chile). Both plants were collected in December. Their identities were confirmed by Prof. Mélica Muñoz-Schick, at Museo Nacional de Historia Natural of Santiago. Voucher specimens are deposited in the Herbarium of the Universidad F. Santa María (UTSM n. 325, 329).

Extraction and isolation: Fresh whole plants of *M. glabratus* (0.5 kg) and *M. luteus* L. (0.8 kg) were extracted twice with EtOH at room temperature, filtered and concentrated to dryness. The residues (5.2 g and 6.5 g respectively) were partitioned between H_2O and EtOAc (1:1). In *M. glabratus* the organic phase was chromatographed on silica gel, using *n*-hexane with increasing proportions of EtOAc as eluent, obtaining pure **1** and **2**. The same procedure on *M. luteus* allowed the isolation of **1**, **3** and **4**, together with ursolic acid and β-sitosterol. All known compounds were identified by analysis of spectroscopic data and direct comparison, but **4** was identified by comparison of NMR and physico-chemical data with literature.

6-Chlorohalleridone (**1**)

Amorphous powder.
$[\alpha]^{20}_D$: +13.5 (*c* 0.4, $CHCl_3$).
Rf: 0.6 (EtOAc–*n*-Hexane, 3:2).
IR ν_{max} ($CHCl_3$): 3420, 1680 cm^{-1}.
UV λ_{max} (MeOH) nm (log ε): 234 (3.37).
^{1}H NMR ($CDCl_3$): 2.28 (1H, ddd, *J* = 12.7, 8.3, 5.8 Hz, H-1'a), 2.36 (1H, ddd, *J* = 12.7, 8.3, 6.8 Hz, H-1'b), 2.82 (1H, dd, *J* = 17.1, 5.3 Hz, H-2a), 2.92 (1H, dd, *J* = 17.1, 5.3 Hz, H-2b), 3.95 (1H, dt, *J* = 8.3, 6.8 Hz, H-2'a), 4.08 (1H, dt, *J* = 8.3, 5.8 Hz, H-2'b), 4.22 (1H, dt, *J* = 5.3, 1.5 Hz, H-3), 6.85 (1H, d, *J* = 1.5 Hz, H-5).
^{13}C NMR ($CDCl_3$): 38.9, C-2; 39.1, C-1'; 65.3, C-2'; 76.3, C-4; 79.9, C-3; 131.9, C-6; 142.6, C-5; 187.7, C-1.
MS (EI, 70 eV) *m/z* (%): 189 [M + H$^+$] (37), 191 (12), 144 (100), 146 (32), 102 (63), 104 (20).
ICRMS *m/z* calcd for $C_8H_9{}^{35}ClO_3$ 188.0240, found 188.0243; for $C_8H_9{}^{37}ClO_3$ 190.0210, found 190.0212.

References

[1] (a) Melchior M. (**1964**) In *Engler's Syllabus der Pflanzenfamilien*. 12th edn., Vol. **2**, Gebr. Borntrager, Berlin, 450; (b) Hoffman AI. (**1978**) *Flora silvestre de Chile-zona central*. Ed.Fundacion C.Gay, Santiago de Chile, 226; (c) Von Bohlen C. (**1995**) El genero *Mimulus* L. (Scrophulariaceae) en Chile. *Gayana Botanica*, ***52***, 7-28; (d) Harborne JB. (**1965**) Plant polyphenols. XV. Flavonols as yellow flower pigments. *Phytochemistry*, ***4***, 647-657; (e) Bloom M, Vickery RK. (**1973**) Pattern partitioning of floral flavonoids in the *Mimulus luteus* complex. *Phytochemistry*, ***12***, 165-167; (f) Bohm BA. (**1992**) Exudate flavonoids of *Mimulus lewisii*. *Biochemical Systematics and Ecology*, ***20***, 591.

[2] (a) Messana I, Sperandei M, Multari G, Galeffi C, Marini-Bettolo GB. (**1984**) A cyclohexadienone and a cyclohexenone from *Halleria lucida*. *Phytochemistry*, ***23***, 2616-2619; (b) Endo K, Hikino H. (**1984**) Structures of rengyol, rengyoxide and rengyolone, new cycloexylethane derivatives from *Forsythia suspensa* fruits. *Canadian Journal of Chemistry*, ***62***, 2011-2014;

[3] (a) Nishino C, Kobayashi K, Fukushima M. (**1988**) Halleridone, a cytotoxic constituent from *Cornus controversa*. *Journal of Natural Products*, ***51***, 1281-1282; (b) Tian J, Zhao QS, Zhang HJ, Lin ZW, Sun HD. (**1997**) New cleroindicins from *Clerodendrum indicum*. *Journal of Natural Products*, ***60***, 766-769; (c) Price JR, Robinson R. (**1938**) Natural coloring matter of the naphthalene group. *Nature*, ***142***, 147-148; (d) Chamy MC, Jimenez I, Piovano M, Garbarino JA, Didyk B. (**1993**) Diterpenoids of *Calceolaria* Species. Secondary metabolites from *Calceolaria sessilis*. *Boletin de la Sociedad Chilena de Quimica*, ***38***, 187-190, and references therein.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1639 - 1642

New Flavonoid Glycosides from *Vernonia ferruginea*

Nicola Malafronte[a], Maria Sabina Pesca[a], Angela Bisio[b], Luis Morales Escobar[c] and Nunziatina De Tommasi[a*]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy*

[b]*Dipartimento di Chimica e Tecnologie Farmaceutiche e Alimentari, University of Genoa, Via Brigata Salerno 13, 16147, Genova, Italy*

[c]*Istituto de Investigaciones Quimicas, Universidad Mayor de San Andes, Calle 27, esq. A. Bello, Cota Cota Campus Universitario, HQ, Casilla 303, La Paz, Bolivia*

detommasi@unisa.it

Received: July 17th, 2009; Accepted: October 8th, 2009

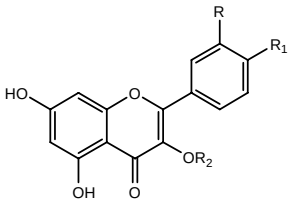
Four new flavonoid glycosides, quercetin 3-*O*-β-D-xylopyranosyl-(1-4)-β-D-glucopyranoside, quercetin 3-*O*-β-D-apiofuranosyl-(1-4)-β-D-glucopyranoside, kaempferol 3-*O*-β-D-apiofuranosyl-(1-4)-β-D-glucopyranoside, and kaempferol 4'-methyl ether 3-*O*-β-D-xylopyranoside, along with other known flavonoids and phenolic derivatives, were isolated from the leaves of *Vernonia ferruginea* Less. Their structures were established on the basis of detailed spectral analysis.

Keywords: *Vernonia ferruginea*, Asteraceae, flavonoids, quinic acid.

The genus *Vernonia* (Asteraceae) comprises tropical and sub-tropical species widespread through both hemispheres [1]. Previous phytochemical studies on this genus led to the isolation and characterization of flavonoids, steroidal glycosides, and sesquiterpenes [2-5]. In our continuing studies on the chemistry of *Vernonia*, we selected *V. ferruginea* Less. a species native to Bolivia, where it is used traditionally for the preparation of anti-inflammatory remedies.

The aim of our work was a phytochemical investigation of the aerial parts of the species and herein we report the isolation and structural characterization of four new flavonoid glycosides, obtained from the methanol extract, on the basis of extensive spectroscopic and spectrometric analysis (1D-NMR, 2D-NMR, ESI-MS).

The negative-ion ESI-MS of **1** showed a pseudomolecular ion peak at *m/z*: 595 $[M-H]^-$, together with ion fragments at *m/z* 463 $[M-H-132]^-$ and 301 $[M-H-132-162]^-$. These data, the elemental analysis, the ^{13}C NMR spectrum (Table 1) showing a total of 26 signals, and the DEPT data indicated that **1** had the molecular formula $C_{26}H_{28}O_{16}$. Analysis of its NMR spectra suggested a flavonoid skeleton.



	R	R_1	R_2
1	-OH	-OH	Xyl-(1->4)-glc
2	-OH	-OH	Api-(1->4)-glc
3	-H	-OH	Api-(1->4)-glc
4	-H	$-OCH_3$	Xyl

The ^1H NMR spectrum (Table 1) indicated a 5,7-dihydroxylated pattern for ring A (two *meta*-coupled doublets at δ 6.25 and 6.44, J = 2.0 Hz) and a 3',4'-dihydroxylation pattern for ring B (ABX system signals at δ 6.91, d, J = 7.8 Hz; 7.68, dd, J = 7.8, 1.8 Hz; 7.78, d, J = 1.8 Hz), allowing the aglycone to be identified as quercetin [4]. The ^1H NMR spectrum of **1** showed also signals ascribable to sugar moieties. Two anomeric protons arising from the sugar moieties appeared at δ 5.38 (1H, d, J = 8.0 Hz) and 4.80 (1H, d, J = 7.5 Hz), which correlated respectively with signals at δ 102.4, and 105.5 ppm in the HSQC spectrum. The network of all proton chemical shifts of the sugar residues was derived from a combination of 1D-TOCSY, DQF-COSY,

Table 1: ^{1}H and ^{13}C NMR data of compound **1** and **2** (CD_3OD)[a].

	1		**2**	
	δ_H	δ_C	δ_H	δ_C
2	-	158.4	-	158.6
3	-	135.7	-	135.8
4	-	180.0	-	180.0
5	-	163.0	-	163.4
6	6.25 d (2.0)	99.0	6.24 d (2.0)	99.1
7		165.0		165.2
8	6.44 d (2.0)	94.5	6.38 d (2.0)	94.5
9		158.8		158.9
10		106.0		105.8
1'		123.0		123.0
2'	7.78 d (1.8)	117.3	7.80 d (1.8)	117.4
3'	-	146.0	-	146.1
4'	-	150.0	-	150.3
5'	6.91 d (7.8)	116.0	6.91 d (7.5)	116.1
6'	7.68 d (7.8, 1.8)	123.2	7.70 d (7.5, 1.8)	123.5
1"	5.38 d (8.0)	102.4	5.42 d (8.0)	102.1
2"	3.58 dd (8.0, 9.0)	72.5	3.56 dd (8.0, 9.0)	72.5
3"	3.50 t (9.0)	76.0	3.52 t (9.0)	75.7
4"	3.68 t (9.0)	78.6	3.70 t (9.0)	78.2
5"	3.45 m	75.4	3.49 m	75.1
6"a	3.91 dd (5.0, 12.0)	62.1	3.96 dd (5.0, 12.0)	61.9
6"b	3.74 dd (3.5,12.0)		3.71 dd (3.5,12.0)	
1'"	4.80 d (7.5)	105.5	5.36 d (3.0)	110.6
2'"	3.35 dd (7.5,9.0)	74.6	4.09 br s	77.9
3'"	3.42 d (9.0)-	77.0	-	80.7
4'"	3.47 m	70.9	3.77 d (10.0)	75.2
5'"a	3.24 dd (12.0, 5.0)	66.5	4.12 d (10.0)	66.2
5'"b	3.89 dd (12.0, 3.0)		3.67 br s	

[a] Coupling pattern and coupling constants (*J* in Hz) are in parentheses.

HSQC, and HMBC experiments. The ^{13}C NMR spectroscopic data for the sugar moieties indicated that the monosaccharides were in pyranose forms. The chemical shifts, the multiplicity of the signals, the coupling constant and the magnitude in the ^{1}H NMR spectrum, as well as the ^{13}C NMR data, indicated the presence of one xylopyranosyl and one glucopyranosyl moiety with β-configuration at the anomeric carbon [5]. The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolyzate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic samples prepared in the same manner. The lower field shift of the C-4" (78.6 ppm) signal suggested the substitution pattern of the glycosyl moiety. Unequivocal information could be obtained from the 2D NMR spectra; the HMBC experiment indicated connections between δ 5.38 (H-1") and 135.7 (C-3); δ 4.80 (H-1'") and 78.6 (C-4"). Thus, the structure of **1** was determined as quercetin 3-*O*-β-D-xylopyranosyl-(1→4)-β-D-glucopyranoside.

Compound **2** showed the molecular formula $C_{26}H_{28}O_{16}$ by negative-ion ESI-MS (*m/z* 595 $[M-H]^-$), ^{13}C and ^{13}C DEPT NMR data, and the elemental analysis. The ^{1}H NMR spectrum of **2** (Table 1) had signals for the quercetin aglycone and sugar units. Comparison of NMR spectral data of **2** with those of compound **1** revealed that **2** differed from **1** in the sugar chain. The identity of the monosaccharides and the sequence of the oligosaccharide chain were determined by analysis of a combination of 1D-TOCSY, DQF-COSY, HSQC, and HMBC NMR spectra. The results of 1D-TOCSY combined with those of DQF-COSY and ^{13}C NMR experiments allowed us to identify the two sugar units as one β-D-glucopyranose (δ 5.42) and one β-D-apiofuranose (δ 5.36). The apiofuranosyl ring configuration was also confirmed by comparing ^{1}H-^{1}H scalar coupling constants with those reported for methyl apiofuranosides and DL-apioses and by NOE data [6]. The 2D NOESY spectrum of **1** contained cross peaks between H-2 and the protons of the hydroxymethyl group, and H-2 and H-4b, indicating that H-2, the hydroxymethyl group and H-4b are found on the same face of the ring for this sugar, thus confirming its structure as β-D-apiose. The absence of any glycosidation shift for the β-D-apiofuranosyl moiety as obtained from HSQC data suggested that this sugar was a terminal unit, while glycosidation shifts were observed for C-4_{glc} (78.2 ppm). A cross peak due to long-range correlation (HMBC) between C-3 (135.8 ppm) of the aglycone and H-1_{glc} (δ 5.42) indicated that glucose was the residue linked to C-3; a cross peak between C-4_{glc} (78.2 ppm) and H-1_{api} (δ 5.36) confirmed the sugar sequence. On the basis of all this evidence, compound **2** was identified as quercetin 3-*O*-β-D-apiofuranosyl-(1→4)-β-D-glucopyranoside.

Compound **3** had the molecular formula $C_{26}H_{28}O_{15}$, as obtained from NMR (Table 3) and ESI-MS data (*m/z* 579 $[M-H]^-$). Analysis of the NMR data of compound **3** (Table 2) and comparison with those of **2** showed that they possessed the same saccharide chain at C-3, while the compounds are based on different aglycons. The spectral data of compound **3** for the aglycon were typical of ~~a~~ kaempferol [7]. From these results, the structure of **3** was kaempferol 3-*O*-β-D-apiofuranosyl-(1→4)-β-D-glucopyranoside.

The molecular formula $C_{21}H_{20}O_{10}$ was assigned to compound **4** by the ESI-MS ($[M-H]^-$ peak at *m/z* 431), the ^{13}C NMR, and the ^{13}C DEPT data. The ^{1}H NMR spectrum of **4** (Table 2) was very similar to that of **3** except for exhibiting signals which could be ascribed to kaempferol [8], and methoxyl moieties, along with those of one anomeric proton, which was identified with the help of DQF-COSY and 1D-TOCSY as one xylose (δ 5.35, *J* = 7.8 Hz). The configuration of the xylose unit was determined as reported for compound **1**. The assignments of all

Table 2: ^{1}H and ^{13}C NMR data of compounds **3** and **4** (CD_3OD)[a].

	3		**4**	
	δ_H	δ_C	δ_H	δ_C
2	-	159.3	-	159.3
3	-	134.8	-	136.0
4	-	180.5	-	180.0
5	-	163.6	-	163.5
6	6.40 d (1.7)	99.6	6.50 d (2.0)	99.4
7		165.0		165.7
8	6.55 d (1.7)	96.8	6.70 d (2.0)	94.5
9		158.9		158.9
10		105.5		105.8
1'		123.0		122.0
2'	7.88 d (8.5)	130.0	7.89 d (8.0)	129.0
3'	6.93 d (8.5)	117.8	7.10 d (8.0)	115.7
4'	-	160.0	-	161.0
5'	6.93 d (8.5)	117.8	7.10 d (8.0)	115.7
6'	7.88 d (8.5)	130.0	7.89 d (8.0)	129.0
OMe			3.87 s	57.8
1"	5.34 d (8.0)	102.1	5.35 d (7.8)	104.5
2"	3.56 dd (8.0, 9.0)	72.5	3.45 dd (9.0, 7.8)	74.0
3"	3.52 t (9.0)	75.7	3.50 dd (9.0, 9.0)	77.3
4"	3.70 t (9.0)	78.2	3.53 m	69.9
5"	3.49 m	75.1	3.36 dd (11.0, 5.0)	66.3
6"a	3.96 dd (5.0, 12.0)	61.9	3.86 dd (11.0, 2.5)	
6"b	3.71 dd (3.5,12.0)			
1'"	5.36 d (3.0)	111.3		
2'"	4.09 br s	77.9		
3'"	-	80.7		
4'"	3.77 d (10.0)	75.2		
5'"a	4.12 d (10.0)	66.2		
5'"b	3.67 br s			

[a]Coupling pattern and coupling constants (*J* in Hertz) are in parentheses.

protonated carbons were accomplished by interpretation of the HSQC spectrum, while HMBC experiment correlations indicated connections between δ 5.35 (H-1") and 136.0 (C-3), δ 3.87 (OMe) and 161.0 (C-4'). From these results, the structure of **4** was concluded to be kaempferol 4'-methyl ether 3-*O*-β-D-xylopyranoside.

The known compounds astragalin [9], quercetin 3-*O*-β-D-glucopyranoside [4], roseoside, 7,8-dihydro-6α-hydroxy-α-ionol 9-*O*-β-D-glucopyranoside [8], 4,5-di-*O*-caffeoylquinic acid, 3,4,5-tri-*O*-caffeoyl-quinic acid [13], and rutin were identified by means of 1D- and 2D-NMR spectroscopy, ESI-MS analysis, and a comparison of their data with those reported in the literature.

Experimental

General experimental procedures: Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. Elemental analysis was obtained from a Carlo Erba 1106 elemental analyzer. UV spectra were recorded on a Perkin-Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer using the UXNMR software package was used for NMR experiments. ESIMS (negative mode) were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma-Aldrich, St. Louis, Mo, USA) and NTS (Naturstoffe reagent)-PEG (Polyethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Waters 515 pumping system equipped with a Waters R401 refractive index detector and Waters U6K injector, using a C_{18} □-Bondapak column (30 cm x 7.8 mm) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses were performed using a Dani GC 1000 instrument.

Plant material: The leaves of *Vernonia ferruginea* Less were collected in Cotapata, Bolivia, in 2002, and identified by Ing. Rossi de Michel of the Herbario Nacional de Bolivia, where a voucher specimen is deposited.

Extraction and isolation: The air-dried powdered leaves of *V. ferruginea* (170 g) were defatted with *n*-hexane and extracted successively by exhaustive maceration (3 x 1 L, for 48 h) with $CHCl_3$, $CHCl_3$-MeOH 9:1, and MeOH. The extracts were concentrated under reduced pressure to afford 3.0, 1.8, and 5.5 g of dried residues, respectively. A portion of the MeOH extract (5 g) was partitioned between *n*-BuOH and H_2O to give a *n*-BuOH soluble portion (1.8 g); this residue was chromatographed over a Sephadex LH-20 column (100 cm x 5 cm) with MeOH as the eluent. A total of 90 fractions were collected (10 mL each). These were combined according to TLC analysis [silica 60 F_{254} gel-coated glass sheets with *n*-BuOH-AcOH-H_2O (60:15:25) and $CHCl_3$-MeOH-H_2O (40:9:1)] to give 8 pooled fractions (A-H).

Fraction C (80 mg) was purified by RP-HPLC using MeOH-H_2O (2:3) to give roseoside (12 mg, t_R= 12 min) and 7,8-dihydro-6α-hydroxy-α-ionol 9-*O*-β-D-glucopyransiode (6 mg, t_R= 16 min). Fraction E (100 mg) was purified by RP-HPLC using MeOH-H_2O (2:3) to give dicaffeoyl quinic acid (18 mg, t_R= 21 min) and tricaffeoyl quinic acid (20 mg, t_R= 23 min). Fraction F (90 mg) was purified by RP-HPLC using MeOH-H_2O (5.6:4.4) to give compounds **1** (11 mg, t_R= 20 min), **4** (4 mg, t_R= 34 min), and astragalin. Fraction G (110 mg) was purified by RP-HPLC using MeOH-H_2O (1.2:1.3) to give compounds **1** (3 mg, t_R= 20 min), **2** (7 mg, t_R= 18.5 min), **3** (4.5 mg, t_R= 22 min), and rutin (15 mg, t_R= 16 min). Fraction I

(35 mg) was identified as quercetin-3-*O*-β-D-glucopyranoside.

Quercetin 3-*O*-β-D-xylopyranosyl-(1→4)-β-D-glucopyranoside (**1**)
Yellow amorphous powder.
$[\alpha]_D$: -20 (*c* 0.1, MeOH).
UV λ_{max} (MeOH) nm 258, 269 sh, 355.
^{1}H NMR and ^{13}C NMR (CD_3OD): Table 1.
ESIMS: *m/z* 595 $[M-H]^-$, 463 $[M - H\text{-}132]^-$, 301 $[M–H\text{-}132\text{-}162]^-$.
Anal. Calcd for $C_{26}H_{28}O_{16}$: C, 52.35; H, 4.73. Found C, 52.39; H 4.74.

Quercetin 3-*O*-β-D-apiofuranosyl-(1→4)-β-D-glucopyranoside (**2**)
Yellow amorphous powder.
$[\alpha]_D$: -64.5 (*c* 0.1, MeOH).
UV λ_{max} (MeOH) nm 254, 300 sh, 354.
^{1}H NMR and ^{13}C NMR (CD_3OD): Table 1.
ESIMS: *m/z* 595 $[M - H]^-$, 301 $[M – H\text{-}132\text{-}162]^-$
Anal. Calcd for $C_{26}H_{28}O_{16}$: C, 52.35; H, 4.73. Found C, 52.41; H 4.75.

Kaempferol 3-*O*-β-D-apiofuranosyl-(1→4)-β-D-glucopyranoside (**3**)
Yellow amorphous powder.
$[\alpha]_D$: - 42.5(*c* 0.1, MeOH).
UV λ_{max} (MeOH) nm 263, 300 sh, 351.
^{1}H NMR and ^{13}C NMR (CD_3OD): Table 2.
ESIMS: *m/z* 579, $[M - H]^-$, 447, $[M – H\text{-}132]^-$, 285 $[M – H\text{-}132\text{-}162]^-$.

Kaempferol 4'-methyl ether 3-*O*-β-D-xylopyranoside (**4**)
Orange amorphous powder.
$[\alpha]_D$: + 32 (*c* 0.1, MeOH).
^{1}H NMR and ^{13}C NMR (CD_3OD): Table 2.
ESIMS: *m/z* 431 $[M - H]^-$.

Acid hydrolysis of compounds 1-4: A solution of each compound (**1-4**, 2.0 mg) in 1 N HCl (1 mL) was stirred at 80°C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60°C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm x 25 m). Temperatures of the injector and detector were 200°C for both. A temperature gradient system was used for the oven, starting at 100°C for 1 min and increasing up to 180°C at a rate of 5°C/min. Peaks of the hydrolysate were detected by comparison with retention times of D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Acknowledgements - Authors are grateful to Ing. Rossi de Michel of the Herbario Nacional de Bolivia for the help in collecting the plant material.

References

[1] De Matteis M. (**2002**) Cytotaxonomic analysis of South American species of *Vernonia* (Vernonieae: Asteraceae). *Botanical Journal of the Linnean Society*, ***139***, 401–408.

[2] (a) Igile G, Oleszek W, Jurzysta M, Aquino R, De Tommasi N, Pizza C. (**1995**) Vemoniosides D and E, two novel saponins from *Vernonia amygdalina*. *Journal of Natural Products*, ***58***, 1438-1443; (b) Miserez F, Potterat O, Marston A, Mungai GM, Hostettmann K. (**1996**) Flavonol glycosides from *Vernonia galamensis* ssp. *nairobiensis*. *Phytochemistry*, ***43***, 283-286.

[3] (a) Sanogo R, Germanò MP, De Tommasi N, Pizza C, Equino R. (**1998**) Vernoniosides and an androstane glycoside from *Vernonia kotschyana*. *Phytochemistry*, ***47***, 73-78; (b) Cioffi G, Sanogo R, Diallo D, Romussi G, De Tommasi N. (**2004**) New compounds from an extract of *Vernonia colorata* leaves with anti-inflammatory activity. *Journal of Natural Products*, ***67***, 389-394.

[4] Fico G, Braca A, De Tommasi N, Tomè F, Morelli I. (**2001**) Flavonoids from *Aconitum napellus* subsp. *neomontanum*. *Phytochemistry*, ***57***, 543-546.

[5] De Tommasi N, Piacente S, Gacs-Baitz E, De Simone F, Pizza C, Aquino, R. (**1998**) Triterpenoid saponins from *Spergularia ramosa*. *Journal of Natural Products*, ***61***, 323-327.

[6] Ishii T, Yanagisawa M. (**1998**) Synthesis, separation and NMR spectral analysis of methyl apiofuranosides. *Carbohydrate Research*, ***313***, 189-192.

[7] Hasan A, Ahmed I, Jay M, Voirin B. (**1995**) Flavonoid glycosides and an anthraquinone from *Rumex halepensis*. *Phytochemistry*, ***39***, 1211-1213.

[8] Agrawal PK. (**1989**) *Carbon-13 NMR of Flavonoids*. Elsevier Science, Amsterdam, 294-364.

[9] Romussi G, Caviglioli G, Pizza C, De Tommasi N. (**1993**) Triterpene saponins and flavonoid from *Quercus laurifolia* Michx. *Archives Pharmazie*, ***326***, 525-528.

[10] De Tommasi N, Piacente S, De Simone F, Pizza C. (**1996**) Constituents of *Cydonia vulgaris*: Isolation and structure elucidation of four new flavonol glycosides and nine new a-ionolderived glycosides *Journal of Agriculture and Food Chemistry*, ***44***, 1676-1681.

[11] Mahmood N, Moore PS, De Tommasi N, De Simone S, Colman S, Hay AJ, Pizza C. (**1993**) Inhibition of HIV-infection by caffeoylquinic acid-derivatives *Antiviral Chemistry and Chemotherapy*, ***4***, 235-241.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1643 - 1644

Phytochemical Profile of *Iris tenax* Extract

Antonella Piccin[a], Mauro Serafini[a] and Marcello Nicoletti[b]

[a]*Department of Plant Biology, University "Sapienza" of Rome, Italy*

[b]*Department of Physiology and Pharmacology, University "Sapienza" of Rome, Italy*

mauro.serafini@uniroma1.it

Received: August 8th, 2009; Accepted: October 26th, 2009

A phytochemical profile of a botanical product (TM) from *Iris tenax* has been produced focused on flavonoids and iridals, leading to a useful fingerprint of the tested extract based on HPLC and NMR data.

Keywords: *Iris tenax*, iridals, flavonoids, HPLC, NMR.

The rapid increase in the introduction of new species and novel foods in the botanicals market has led to the urgent call for analytical data in order to determine the actual content of these products. Although in some cases information can be obtained from the literature, this often lacks uniformity and covers a low percentage of the used species.

The genus *Iris* (Iridaceae) (sword lilies), consisting of 150-300 species, is mainly native to temperate zones of the North Hemisphere. Many species boast economic value, for example *I. germanica*, *I. pseudoacorus* and *I. florentina*. This current work concerns the production of a phytochemical profile for *I. tenax*, which has recently been introduced in the homeopathic market.

The most prominent constituents of *Iris* species are flavonoids [1] and iridals. Iridals are mainly monocyclic triterpenoids featuring a homofarnesyl side chain and are characteristic of lipid extracts of various sword lilies [2]. More than 30 iridals have been isolated from Iridaceae species and classified on the basis of hydroxylation and further cyclization [3]. Iridals can also be found as their C-3 fatty acid esters and glycosides [4].

Iridals are important precursors of irones, the pleasant smelling terpenoids producing the violet fragrance of stored dry rhizomes of certain *Iris* species important in the perfume industry. However, several medicinal uses have been reported, for example as a strong purgative and emetic in *Rhizoma Iridis* from *I. pseudoacorus*, and as a pacifier during teething in *I. germanica* and *I. florentina* [2]. Furthermore, rhizome extracts of *I. pseudoacorus* are prescribed in homeopathic medicine as a soothing remedy for different forms of psychic ailments and hyperacidity of the stomach. *I. tenax* is also used in homeopathic medicine for the treatment of gastro-intestinal pains and gastric reflux.

Previous phytochemical studies of *I. tenax* were confined to the paper chromatographic determination of flavonoids in the rhizomes [5]. This present study was focused on the phytochemical profile of a mother tincture (TM) of *I. tenax*, a sword lily native to the Pacific Coast of North America. The TM is commercialized by Boiron ® and obtained by extraction of fresh rhizomes of *I. tenax* with ethanol 65%, v/v, according to Prèparations homèopathiques (1965) of the French Pharmacopoeia, X ed. [6]. The analysis was based on flavonoids and iridals, since these compounds are considered reliable markers of *Iris* species. The flavonoids kaempferol, quercetin and saponaretin (= apogenin 6-*C*-glucoside) were identified in *I. tenax* TM by HPLC analysis by direct comparison with reference samples. The iridals isoiridogermanal and (6*S*,10*R*,11*R*)-18,19-epoxy-10-deoxyiridal were identified by HPLC comparison with authentic samples, obtained from rhizomes of *I. germanica* in a previous study [7-9]. As confirmation, iridals were also separated and analyzed by NMR spectroscopy, leading to the identification of iridobelamal A [7,10,11].

Since the NMR fingerprint is a valuable method of identification of botanicals, the ^{1}H NMR fingerprint of the more studied and used *I. germanica* was compared with that of *I. tenax*. Both showed the presence of the same main classes of products, but relevant additional peaks for the first species were observed in the aromatic part, due to the occurrence of several flavonoids, and in the region near to 5 ppm, attributable to additional iridals. Therefore, on the basis of these results, NMR fingerprints can be used to distinguish the two species [12,13].

Experimental

Plant material: TM was obtained from Boiron®, as the result of the extraction of fresh rhizomes of *I. tenax* Douglas ex Lindley with ethanol 65% (v/v). *I. germanica* L. rhizomes were collected in the Botanical Garden of the University "Sapienza" of Rome, and used to obtain iridal standards, whereas flavonoid standards came from Extrasynthese s.n.c. TM of *I. tenax* (700 mL) was evaporated to dryness, the residue extracted twice with diethyl ether (100 mL), and the extract evaporated to afford a dense mobile residue (6.7 g); this was separated by CC on Si gel in toluene-EtOAc (9:1), to give pure iridals, identified by HPLC analysis and comparison of physical and spectroscopic data (^{1}H and ^{13}C NMR) with those in the literature and with authentic samples from *I. germanica.* NMR analyses were performed using a Bruker AM 400 spectrometer operating at 400 and 100 MHz for ^{1}H and ^{13}C NMR, respectively.

HPLC analyses: HPLC measurements were carried out on a Waters 1525 apparatus with a binary pump. Chromatographic data were processed with Breeze 2 software (Waters**).** Injection volume loop: 20 μL. Stationary phase: Bondapack C18 125A. Flow rate: 1.00 mL min^{-1}; UV-VIS detector: 214 nm; elution program: 8 min isocratic, 45% acetonitrile/55% water; 22 min linear gradient to 100 % CH_3CN; 10 min isocratic 100% CH_3CN. All assignments were confirmed by co-injection with the standard solutions.

References

[1] Tsukasa I, Shunji O. (**1998**) Flavonoids of the genus *Iris*; structures, distribution and function (review). *Annals of the Tsukuba Botanical Garden*, ***17***, 147-183.

[2] Homoepathic Pharmacopoeia of the United States. 9th Ed. (**1998**).

[3] Miyake Y, Ito H, Yoshida T. (**1997**) Identification of iridals as piscicidal componemts of Iridaceous plants and their conformations associated with CD spectra. *Canadian Journal of Chemistry*, ***75***, 734-741.

[4] Marner FJ. (**1997**) Iridals and cycloiridals. Products of an unusual squalene metabolism in sword lilies (Iridaceae) *Current Organic Chemistry*, ***1***, 153-186.

[6] Pharmacopée française (**2002**) X Ed. Groupe Liaisons, Rueil.

[7] Bonfils JP, Marner FJ, Sauvaire Y. (**1998)** An epoxidized iridal from *Iris germanica* var. 'Rococò'. *Phytochemistry*, ***48***, 751-753.

[8] Ito H, Onoue S. (**1999**) Iridal-type triterpenoids with ichthyotoxic activity from *Belamcanda chinensis. Journal of Natural Products*, ***62***, 89-93.

[9] Piccin A. (unpublished data).

[10] Takahashi K, Hoshino Y, Suzuki S, Hano Y, Nomura T. (**2000**) Iridals from *Iris tectorum* and *Belamcanda chinensis. Phytochemistry*, ***53***, 925-929.

[11] Fang R, Houghton PJ, Luo C, Hylands PJ. (**2007**) Isolation and structure determination of triterpenes from *Iris tectorum*. *Phutochemistry*, ***68***, 1242-1247.

[12] Marner FJ. Singab ANB, Al-Azizi MM, Al-Emary NA, Schafer M. (**2002**) Iridal glycosides from *Iris spuria* (Zeal) cultivated in Egypt *Phytochemistry*, ***60***, 301-307.

[13] Webster GK, Marsden I, Pommerening CA, Tyrakowski CM, Tobias B. (**2009**) Determination of relative response factors for chromatographic investigations using NMR spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, ***49***, 1261-1265 (and references therein).

NPC Natural Product Communications

2009
Vol. 4
No. 12
1645 - 1650

New Phenolic Glycosides from *Securinega virosa* and Their Antioxidant Activity

Rokia Sanogo[a], Antonio Vassallo[b], Nicola Malafronte[b], Salvatore Imparato[b], Alessandra Russo[c], and Fabrizio Dal Piaz[b*]

[a]*Departement Medicine Traditionelle (DMT), INRSP, B.P. 1746, Bamako, Mali*

[b]*Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy*

[c]*Dipartimento di Chimica Biologica, Chimica Medica e Biologia Molecolare, Università di Catania, v.le A. Doria 6, 95125 Catania, Italy*

fdalpiaz@unisa.it

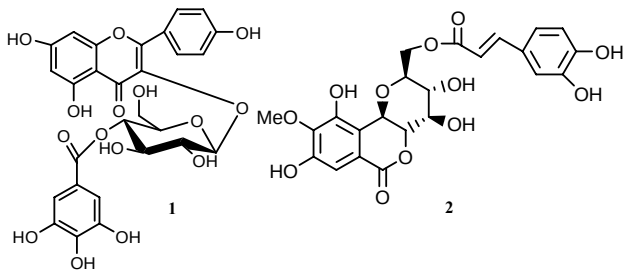
Received: July 31st, 2009; Accepted: September 15th, 2009

One new flavonoid glycoside, 3-*O*-kaempferol 4-*O*-(galloyl)-β-D-glucoside, one new bergenin derivative, 11-*0*-caffeoylbergenin, along with other known flavonoids and phenolic derivatives, were isolated from the leaves of *Securinega virosa*. Their structures were established on the basis of detailed spectral analysis. *In vitro* biological analysis of the isolated compounds showed that they were able to quench DPPH radicals and had a direct scavenging activity on superoxide anion. Kaempferol 3-*O*-(4-galloyl)-β-D-glucopyranoside (**1**), 11-*0*-caffeoylbergenin (**2**), and glucogallin (**6**) exhibited the highest antioxidant capacity, being also able to modulate hydroxyl radical formation more efficiently than the other compounds, acting as direct hydroxyl radical scavengers and chelating iron.

Keywords: *Securinega virosa*, Euphorbiaceae, flavonoids, phenolic compounds, antioxidant activity.

Securinega virosa (Roxb. ex Willd) A Juss., syn. *Flueggea virosa* (Roxb. ex Willd) Voigt, is a small tree widely distributed in Mali [1,2]. It is used in traditional medicine for many diseases, including diarrhea, rheumatism, malaria, liver disease, inflammation and pain. Extracts of the plant are used for the expulsion of worms and in the treatment of bilharziasis, and for other urinary and genital tract disorders [2]. Some biological activities have already been confirmed. Extracts of the aerial parts were shown to have antispasmodic activity against both histamine and acetylcholine induced spasms, and increased phenobarbitone sleeping time [3]; the extracts also demonstrated antitumor activity against KB, L1210 and P388 cell lines [4]. Alkaloids isolated from the plant caused hypotension in cats and relaxation of rabbit gut smooth muscle [5].

The present paper deals with the antioxidant bioassay-guided fractionation of *S. virosa* methanol extract, leading to the isolation and structural characterization of one new flavonoid (**1**), one new



1 **2**

bergenin derivative (**2**), together with other known compounds, including quercetin 3-*O*-β-D-glucopyranoside (**3**), corilagin (**4**), acalyphidin M (**5**), glucogallin (**6**), geraniin (**7**), and bergenin (**8**).

Compound **1** was isolated as a yellow amorphous powder. Its molecular formula was established as $C_{28}H_{24}O_{15}$ by means of the ESI-MS ([M-H]$^-$ peak at *m/z* 599), and by ^{13}C, ^{13}C-DEPT NMR, and elemental analysis. Analysis of the 600 MHz NMR spectra suggested a flavonoid skeleton for compound **1**. The ^{1}H NMR spectrum (Table 1) indicated a 5,7-dihydroxylated pattern for ring A (two *meta*-coupled

Table 1: ^{1}H- and ^{13}C- NMR spectroscopic data of compound **1** (CD_3OD, 600 MHz)[a].

position	δ_H	δ_C
2	-	159.0
3	-	134.2
4	-	180.0
5	-	163.0
6	6.3 d (1.7)	101.8
7		165.0
8	6.46 d (1.7)	95.6
9		158.7
10		104.8
1'		123.0
2'	7.88 d (8.5)	129.4
3'	6.93 d (8.5)	117.5
4'	-	160.0
5'	6.93 d (8.5)	117.5
6'	7.88 d (8.5)	129.4
3-*O*-Glc'''	5.40 d (7.5)	102.0
2"	3.47 dd (7.5, 9.0)	73.6
3"	3.55 t (9.0)	77.0
4"	4.05 t (9.0)	73.6
5"	3.39 m	76.2
6"a	3.83 dd (5.0, 12.0)	62.7
6"b	3.61 dd (3.5,12.0)	
galloy*l*1'''	-	122.0
2'''	7.07 s	110.0
3'''	-	147.8
4'''	-	139.6
5'''	7.07 s	147.8
6'''	-	110.0
COO	-	169.7

[a] Coupling pattern and coupling constants (*J* in Hertz) are in parentheses.

doublets at δ 6.46 and 6.33, *J* = 1.5 Hz) and a 4'-hydroxylation pattern for ring B (ABX system signals at δ 6.93, d, *J* = 8.0 Hz; 7.88, d, *J* = 8.0 Hz), allowing the aglycon to be recognized as kaempferol [6]. The ^{1}H NMR spectrum of **1** also showed signals ascribable to sugar moieties and a galloyl residue (Table 1). One anomeric proton arising from the sugar moiety appeared at δ 5.40 (1H, d, *J* = 7.5 Hz), which correlated with the signal at δ 102.0 in the HSQC spectrum. All the ^{1}H and ^{13}C NMR signals of **1** were assigned using 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments. Complete assignments of proton and carbon chemical shifts of the sugar portion were accomplished by DQF-COSY and 1D-TOCSY experiments and allowed identifying the sugar as a β-glucopyranosyl unit.

The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilylated, and the GC retention times of each sugar were compared with those of authentic sugar samples prepared in the same manner. The lower field shifts of H-4" (δ 4.05) suggested the substitution pattern of a galloyl moiety. Unequivocal information could be obtained by the 2D-NMR spectra; the HMBC experiment indicated connections between δ 5.40 (H-1") and 134.2 (C-3); and δ 4.05 (H-4") and 169.7 (COO). Thus, the structure of **1** was determined as kaempferol 3-*O*-(4-galloyl)-β-D-glucopyranoside.

Table 2: ^{1}H- and ^{13}C- NMR spectroscopic data of compound **2** (CD_3OD, 600 MHz)[a].

position	δ_H	δ_C
2		81.8
3	3.47 t (9.0)	71.9
4	3.84 t (9.0)	75.5
4a	4.05	81.2
6	-	166.0
6a	-	119.7
7	7.11 s	111.0
8	-	149.5
9	-	152.3
10	-	142.3
10a	-	117.3
10b	4.99 d (10.0)	74.2
11a	4.08 dd (5.0, 12.0)	64.5
11b	4.24 dd (3.0, 12.0)	
OMe	3.94 s	60.9
caffeoyl		
1	-	125.0
2	6.70 d (1.8)	114.3
3	-	149.7
4	.	146.0
5	6.45 d (8.0)	116.5
6	6.68 dd (8.0, 1.8)	123.5
α	6.37 d (16.0)	115.0
β	7.42 d (16.0)	146.8
COO		169.0

[a] Coupling pattern and coupling constants (*J* in Hertz) are in parentheses.

The molecular formula $C_{23}H_{22}O_{12}$ for compound **2** was determined from the ESI-MS ion $[M-H]^-$ at *m/z* 489, and from the ^{13}C, ^{13}C-DEPT NMR and elemental analyses. The ^{1}H- and ^{13}C-NMR spectra (see Table 2) indicated that the compound was a bergenin derivative [3]. The ^{1}H NMR spectrum showed a low-field signal at δ 7.11 (1H, s) due to the aromatic proton of the bergenin nucleus [7]. All the ^{1}H and ^{13}C NMR signals of **2** were assigned using COSY, 1D-TOCSY, HSQC, and HMBC experiments. In particular, the COSY spectrum, together with the 1D-TOCSY experiments, allowed the identification of the spin system of a glucosyl unit.

The presence of one caffeoyl moiety was shown in the ^{1}H NMR spectrum by the signals at δ 6.70 (1H, d, *J* = 1.8 Hz), δ 6.68 (1H, dd, *J* = 8.0, 1.8 Hz), and δ 6.45 (1H, d, *J* = 8.0 Hz). The HSQC spectrum showed esterification shifts for C-11 (δ 64.5). An unambiguous determination of the sequence and linkage sites was obtained from HMBC experiment, which showed cross peak correlations between δ 4.08 (H-11) and 169.0 (C-1'), δ 4.99 (H-10b) and 142.3 (C-10), 119.7 (C-6a), 81.8 (C-2), and δ 4.05 (H-4a) and 71.9 (C-3), 117.3 (C-10a), and 166.0 (C-6).

Therefore, the structure 11-*O*-caffeoylbergenin was assigned to compound **2**.

Compounds **3-8** were identified by means of 1D- and 2D-NMR spectroscopy and ESI-MS analysis; a comparison of their data with those reported in the literature led to their identification as quercetin 3-*O*-β-D-glucopyranoside (**3**) [6], corilagin (**4**) [7], acalyphidin M (**5**) [8], glucogallin (**6**) [9], geraniin (**7**) [10], and bergenin (**8**) [11].

Preliminary *in vitro* biological analysis indicated that compounds **1**-**7** were able to quench DPPH radicals and exhibited a direct scavenging activity on the superoxide anion; this radical was in fact produced by the reduction of β-mercaptoethanol, excluding the Fenton-type reaction and the xanthine/xanthine oxidase system (Table 3).

Kaempferol 3-*O*-(4-galloyl)-β-D-glucopyranoside (**1**), 11-*0*-caffeoylbergenin (**2**), corilagin (**4**), acalyphidin M (**5**), and glucogallin (**6**) exhibited the highest antioxidant capacity. The biological activity of the kaempferol derivatives, corilagin, glucogallin and corilagin is reported in the literature [12-14].

Table 3: Scavenger effect on DPPH stable radical and superoxide anion of methanol fractions and compounds **1-8** isolated from *C. senegalensis*

Compound	DPPH test	Effect on $O_2^{-}\cdot$
	[a]IC_{50} (μg/μL) ± [b]SD	
A	253 ± 14	80.0 ± 4.3
B	209 ± 48	57.0 ± 1.3
C	110 ± 17	8.7 ± 0.5
D	53 ± 5	2.3 ± 0.04
E	88 ± 14	1.8 ± 0.5
F	61 ± 23	1.6 ± 0.05
G	6.4 ± 0.5	0.4 ± 0.03
1	3.7 ± 0.9	0.2 ± 0.04
2	2.6 ± 0.1	0.65 ± 0.03
3	12.9 ± 0.5	0.07 ± 0.01
4	4.8 ± 1.1	0.4 ± 0.01
5	1.15 ± 0.08	0.5 ± 0.01
6	2.15 ± 0.04	0.2 ± 0.01
7	6.8 ± 0.9	0.1 ± 0.03
8	168 ± 34	67.0 ± 0.03
[c]Trolox	96 ± 2	
[d]SOD		89 ± 15

[a]concentration that inhibited radicals by 50%. [b]n = 6.
[c]Trolox (50 μM) and [d]superoxide dismutase (SOD) (80 mU/mL) were used as standards; the results are expressed as % of inhibition.

Although both $O_2^{-}\cdot$ and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by the OH· radical, which is generated by the reaction between $O_2^{-}\cdot$ and H_2O_2 in the presence of transition metal ions [15]. In fact, the OH· radical can react with a number of target molecules, including proteins, membrane lipids, and DNA.

Table 4: Effect of methanol fractions and compounds **1-8** isolated from *S. virosa* (100 μg/mL) on DNA cleavage induced by the photolysis of H_2O_2 and metal chelating activity.

Compound	Supecoiled DNA (% of native DNA)	Ferrozine assay [a]IC_{50} (μg/μL) ± [b]SD
scDNA	100	–
A	10.0 ± 0.6*	–
B	14.0 ± 0.9*	–
C	20.0 ± 0.7*	–
D	46.7 ± 1.4*	–
E	46.7 ± 1.4*	–
F	55.0 ± 4.7*	114 ± 20
G	63.3 ± 1.6*	13 ± 3
1	63.3 ± 1.6*	7 ± 2
2	82.6 ± 7.5	7 ± 1
3	–	232 ± 20
4	52.5 ± 2.3*	85 ± 4
5	94.0 ± 3.6	13 ± 3
6	53.2 ± 5.2*	27 ± 3
7	97.1 ± 3.6*	32 ± 5
8	79.0 ± 2.6*	333 ± 4
[c]DMSO	73.2 ± 3.4*	
[d]DTPA		75.1 ± 2.1*

The hydroxyl radicals generated by the photolysis of H_2O_2 inhibited the supercoiled DNA (SCDNA). Each value represents the mean ± SD of 3 experiments. *Significant vs. supercoiled DNA ($p<0.001$).
[a]concentration that inhibited the ferrozine-Fe^{2+} formation by 50%.
[b]ν = 6. [c]DTPA (5 μM) and [d]DMSO (1mM) were used as standardS;
[c]the result is expressed as % inhibition.

Based on the data obtained from this study, compounds **1**, **2** and **6** might also be able to modulate hydroxyl radical formation more efficiently than other compounds acting as direct scavengers and chelating iron. These natural compounds exhibited a more efficient protection against DNA strand scission induced by OH· radicals generated by UV-photolysis of H_2O_2 (Table 4), and showed metal chelating activity capturing ferrous ions before ferrozine, with IC_{50} values (concentration that inhibited the ferrozine-Fe_{2+} by 50%) of 7.2, 7.3 and 27 μg/mL, respectively (Table 4). These data also suggest that the biological effect of *S. virosa* observed from ethnopharmacological studies is due in part to the antioxidant action of its active components.

Experimental

General experimental procedures: Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. Elemental analysis was obtained from a Carlo Erba 1106 elemental analyzer. UV spectra were recorded on a Perkin-Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer using the UXNMR software package was used for NMR experiments. ESI-MS (negative mode) were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by

spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma-Aldrich, St. Louis, Mo, USA) and NTS (Naturstoffe reagent)-PEG (Poliethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Waters 515 pumping system equipped with a Waters R401 refractive index detector and Waters U6K injector, using a C_{18} μ-Bondapak column (30 cm x 7.8 mm) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses were performed using a Dani GC 1000 instrument.

Plant material and chemicals: The leaves of *Securinega virosa* were collected in Bandiagara, Mali, in 2005 and identified by Prof. N'Golo Diarra of the Departement Medicine Traditionelle (DMT), Bamako, Mali where a voucher specimen is deposited.

pBR322 plasmid DNA, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), and 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma Aldrich Co (St. Louis, USA); β-nicotinamide-adenine dinucleotide (NADH) was obtained from Boehringer Mannheim GmbH (Germany). All other chemicals were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA).

Extraction and isolation: The air-dried powdered leaves of *Securinega virosa* (300 g) were defatted with *n*-hexane and extracted successively by exhaustive maceration (3 x 1 L, for 48 h) with $CHCl_3$, $CHCl_3$-MeOH 9:1, and MeOH. The extracts were concentrated under reduced pressure to afford 7, 4, 6, and 10 g of dried residues, respectively. A portion of the MeOH extract (6 g) was partitioned between *n*-BuOH and H_2O to give a *n*-BuOH soluble portion (2.5 g); this residue was chromatographed over a Sephadex LH-20 column (100 cm x 5 cm) with MeOH as the eluent. A total of 95 fractions were collected (10 mL each). These were combined according to TLC analysis [silica 60 F_{254} gel-coated glass sheets with *n*-BuOH-AcOH-H_2O (60:15:25) and $CHCl_3$-MeOH-H_2O (40:9:1)] to give 8 pooled fractions (A-H). Fraction D (90 mg) was purified by RP-HPLC using MeOH-H_2O (1:1) to give compounds **1** (6 mg, t_R= 11 min) and **3** (5 mg, t_R= 13 min). Fraction E (58 mg) was purified by RP-HPLC using MeOH-H_2O (3:7) to give compounds **2** (7 mg, t_R= 12 min) and **8** (22 mg, t_R= 10 min). Fraction F (70.5 mg) was purified by RP-HPLC using MeOH-H_2O (25:75) to give compounds **6** (12.5 mg, t_R= 8 min) and **4** (10.8 mg, t_R= 11 min). Fraction H yielded compound **7** (29.2 mg). Fraction G (100 mg) was chromatographed over a Sephadex LH-20 column (25 cm x 2 cm) with MeOH as the eluent, yielding compound **5** (14.0 mg).

Kaempferol 3-*O*-(4-galloyl)-β-D-glucopyranoside (1)

Yellow amorphous powder.
$[\alpha]_D$: -27 (*c* 0.1, MeOH).
^{1}H NMR (600 MHz, CD_3OD): Table 1.
^{13}C NMR (600 MHz, CD_3OD): Table 1.
ESIMS: *m/z* 599 $[M - H]^-$.
Anal. Calcd for $C_{28}H_{24}O_{15}$: C, 56.01; H, 4.03. Found C, 54.49; H 4.05.

11-*O*-Caffeoylbergenin (2)

Yellow amorphous powder.
$[\alpha]_D$: -42.5(*c* 0.1, MeOH).
MP: 210-212ºC
^{1}H NMR (600 MHz, CD_3OD): Table 2.
^{13}C NMR (600 MHz, CD_3OD): Table 2.
ESIMS: *m/z* 489 $[M - H]^-$.
Anal. Calcd for $C_{23}H_{22}O_{12}$: C, 56.33; H, 4.52. Found C, 56.38; H 4.57.

Acid hydrolysis of compound 1: A solution of compound **1** (2.0 mg) in 1 N HCl (1 mL) was stirred at 80°C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60°C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm x 25 m). Temperatures of the injector and detector were 200°C for both. A temperature gradient system was used for the oven, starting at 100°C for 1 min and increasing up to 180°C at a rate of 5°C/min. Peaks of the hydrolysate were detected by comparison with retention times of an authentic sample of D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine

Antioxidant activity in cell-free systems

Quenching of DPPH: The free radical-scavenging capacity of extracts, fractions and pure compounds was tested by their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) [16]. The reaction mixture contained 86 μM DPPH and

different concentrations of the natural compounds in 1 mL of ethanol. After 10 min at room temperature the absorbance at λ = 517 nm was recorded. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Scavenger effect on superoxide anion: Superoxide anion was generated *in vitro* as described by Paoletti *et al*. [17]. The assay mixture contained, in a total volume of 1 mL, 100 mM triethanolamine-diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, and 10 mM β-mercapto-ethanol; some samples contained the natural compounds at different concentrations. After 20 min incubation at 25°C, the decrease in absorbance was measured at λ = 340 nm. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

DNA cleavage induced by hydrogen peroxide UV-photolysis: The experiments were performed, as previously reported [18], in a volume of 20 μL containing 33 μM in bp of pBR322 plasmid DNA in 5 mM phosphate saline buffer (pH 7.4), and the natural compounds at different concentrations. Immediately prior to irradiating the samples with UV light, H_2O_2 was added to a final concentration of 2.5 mM. The reaction volumes were held in caps of polyethylene microcentrifuge tubes, placed directly on the surface of a transilluminator (8000 μW cm^{-1}) at 300 nm. The samples were irradiated for 5 min at room temperature. After irradiation, 4.5 μL of a mixture, containing 0.25% bromophenol blue, 0.25% xylen cyanol FF, and 30% glycerol, were added to the irradiated solution. The samples were then analyzed by electrophoresis on a 1% agarose horizontal slab gel in Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 μg/mL; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

Metal chelating activity: The chelating of ferrous ions by fractions and pure compounds was estimated by the ferrozine assay [19]. Briefly, natural compounds were added to a solution of 0.15 mM $FeSO_4$. The reaction was initiated by the addition of 0.5 mM ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 mins. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. The results were expressed as percentage inhibition of the ferrozine-Fe^{2+} complex formation. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

References

[1] Adjanohoun EJ, Aké Assi L, Floret JJ, Guindo S, Koumaré M, Ahyi AMR, Raynal J. (**1985**) Contribution aux études ethnobotaniques et floristiques au Mali. Médecine traditionnelle et pharmacopée. ACCT Ed, Paris, 250 p.

[2] Kerharo J, Adam JC. (**1974**) *Pharmacopée sénégalaise traditionnelle*: *Plantes médicinales et toxicologiques*. Paris Vigot et Frères Ed, 1011p.

[3] Bhakuni DS, Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN. (**1969**) Screening of Indian plants for biological activity. Part II. *Indian Journal of Experimental Biology*, ***7***, 250-262.

[4] Tatematsu H, Mori M, Yang TH, Chang JJ, Lee TTY, Lee KH. (**1991**) Cytotoxic principles of *Securinega virosa*: virosecurinine and viroallosecurinine and related derivatives. *Journal of Pharmaceutical Science*, ***80***, 325-327.

[5] Paris RR, Moyse H, Le Men J. (**1955**) Alkaloids from new Euphorbiaceae: *Flueggea virosa*. *Annales Pharmaceutiques Francaises*, ***13***, 245-250.

[6] Fico G, Braca A, De Tommasi N, Tomè F, Morelli I. (**2001**) Flavonoids from *Acontuym napellus* subsp. n*eomontanum. Phytochemistry*, ***57***, 543-546.

[7] Nawwar MAM, Hussein SAM, Merfort I. (**1994**) NMR spectral analysis of polyphenolics from *Punica granatum. Phytochemistry*, ***36***, 793-798.

[8] Liu Y, Cui C, Zhao M, Wang J, Luo W, Yang B, Jiang Y. (**2008**) Identification of phenolics in the fruit of emblica (*Phyllanthus emblica* L.) and their antioxidant activities. *Food Chemistry*, ***109***, 909-915.

[9] Kashiwada Y, Nonaka G, Nishioka I. (**1984**) Isolation and characterization of new dimeric and trimeric procyanidins. *Chemical & Pharmaceutical Bulletin*, ***32***, 3461-3470.

[10] Adesina SK, Idowu O, Ogundaini AO, Oladimeji H, Olugbade TA, Onawunmi GO, Pias M. (**2000**) Antimicrobial constituents of the leavs of *Acalypha wilkesiana* and *Acalypha hispida*. *Phytotherapy Research*, ***14***, 371-374.

[11] Lee YY, Jang DS, Jin JL, Yun-Choi HS. (**2005**) Anti-platelet aggregating and anti-oxidative activities of 11-*O*-(4'-*O*-methylgalloyl)-bergenin a new compound isolated from *Crassula cv*. 'Himaturi'. *Planta Medica*, ***71***, 776-777.

[12] Bors W, Michel C. (**1999**) Antioxidant capacity of flavanols and gallate esters: pulse radiolysis studies. *Free Radical Biology & Medicine*, ***27***, 1413-1426.

[13] Kinoshita S, Inoue Y, Nakama S, Ichiba T, Aniya Y. (**2007**) Antioxidant and hepatoprotective actions of medicinal herb, *Terminalia catappa* L. from Okinawa Island and its tannin corilagin. *Phytomedicine*, ***14***, 755-762.

[14] Maldonado PD, Chánez-Cárdenas ME, Pedraza-Chaverrí J. (**2005**) Aged garlic extract, garlic powder extract, S-allylcysteine, diallyl sulfide and diallyl disulfide do not interfere with the antibiotic activity of gentamicin. *Phytotherapy Research*, ***19***, 252-254.

[15] Halliwell B, Gutteridge JM. (**1995**) The definition and measurement of antioxidants in biological systems. *Free Radical Biology & Medicine*, ***18***, 125-126.

[16] Latté KP, Kolodziej H. (**2004**) Antioxidant properties of phenolic compounds from *Pelargonium reniforme*. *Journal of Agricultural and Food Chemistry*, ***52***, 4899-4902.

[17] Paoletti F, Aldinucci D, Mocalli A, Caparrini A. (**1986**) A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Analytical Biochemistry*, ***154***, 536-541.

[18] Russo A, Acquaviva R, Campisi A, Sorrenti V, Di Giacomo C, Virgata G. (**2000**) Bioflavonoids as antiradicals, antioxidants and DNA cleavage protectors. *Cell Biology and Toxicology*, ***16***, 91-98.

[19] McGown EL, Rusnak MG, Lewis CM, Tillotson JA. (**1982**) Tissue ascorbic acid analysis using ferrozine compared with the dinitrophenylhydrazine method. *Analytical Biochemistry*, ***119***, 55-61.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1651 - 1656

Characterization of Flavonoid and Naphthopyranone Derivatives from *Eriocaulon ligulatum* using Liquid Chromatography Tandem Mass Spectrometry

Lourdes Campaner dos Santos[a,*], Marcelo Aparecido da Silva[a], Clenilson Martins Rodrigues[a], Virginia Carbone[b], Assunta Napolitano[b], Carla Bassarello[b], Angela Mari[b,c], Sonia Piacente[c], Cosimo Pizza[c] and Wagner Vilegas[a]

[a]*São Paulo State University - UNESP, Institute of Chemistry, Organic Chemistry Department, CP 355, CEP 14800-900, Araraquara, SP - Brazil*

[b]*Centro di Spettrometria di Massa Proteomica e Biomolecolare, Istituto di Scienze dell'Alimentazione del CNR, Via Roma, 52 a/c, 83100, Avellino, Italy*

[c]*Università degli Studi di Salerno, Dipartimento di Scienze Farmaceutiche, via Ponte Don Melillo, 84084 Fisciano (SA) - Italy*

loursant@iq.unesp.br

Received: July 30th, 2009; Accepted: October 27th, 2009

Liquid chromatography-electrospray ionization multistage ion trap mass spectrometry (LC-ESI-IT-MS^n) was used to analyze the secondary metabolites in the methanol extract of the capitulae of *Eriocaulon ligulatum*. The major components were mono- and diglycosides of flavonoids and naphthopyranones. Eleven compounds, including four new flavonol glycosides, were identified based on their fragmentation patterns in MS experiments and on NMR analysis of the isolated compounds. The described data may contribute to a better understanding of the taxonomic classification of the Eriocaulaceae family.

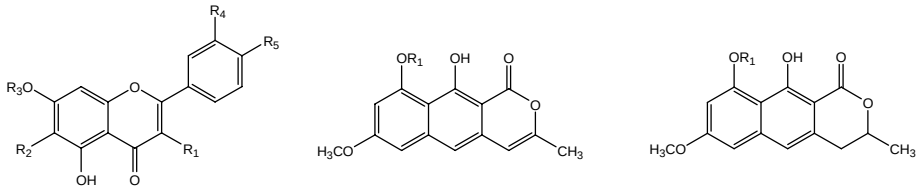
Keywords: LC/MS/MS, *Eriocaulon ligulatum*, flavonoids, naphthopyranones.

Eriocaulaceae is a pantropical, predominantly herbaceous monocotyledonous family, comprising around 1100 species in 11 genera [1a,1b]. They are common and diagnostic components of the herbaceous rocky outcrop vegetation of Brazil called "campos rupestres", which flourishes at an altitude above 900 m. *Eriocaulon ligulatum* (Vell.) L.B.Smith, locally named "botão-dourado" (golden button), is exported to Europe, Japan and North America as an ornamental flower, representing an important source of income to the population of Minas Gerais State, Brazil. Scientific research on *Eriocaulon* is limited and very little is known about its chemistry. Previous phytochemical studies on *Eriocaulon* led to the isolation of flavonoids [1c,1d], tocopherol and naphthopyranones [1e].

The pre-eminence of flavonoids as chemical markers among the secondary metabolites of plants is well known. Flavonoids have been widely used in chemotaxonomy because their patterns tend to be specific, they are relatively stable and their biosynthesis/accumulation is largely independent of environmental influence [2].

Naphtopyranones are a class of natural metabolites, present only in some genera of the family Eriocaulaceae, displaying antitumor, antileukemic, antiviral [3a], mutagenic and cytotoxic activities [3b]. Naphthopyranones are compounds used for chemotaxonomic purposes in the Eriocaulaceae family.

In the present study, an investigation was carried out of the composition of the methanolic extract obtained from the capitulae of *E. ligulatum* using an analytical approach based on high-performance liquid chromatography coupled to electrospray negative ionization multistage ion trap mass spectrometry (HPLC/ESI-ITMSn), and to ultraviolet-diode array detection (UV-DAD). Using this analytical approach eleven compounds were identified, belonging to the naphthopyranone and flavonoid classes. Among



1-7, **11** **8**, **10** **9**

Compound	R_1	R_2	R_3	R_4	R_5	MW
1	OH	OCH_3	β-D-Glc-(1→6)-β-D-Glc	OH	OH	656
2	H	OCH_3	β-D-Glc-(1→6)-β-D-Glc	OH	OH	640
3	OH	OCH_3	6-Vanilloyl-β-D-Glc-(1→6)-β-D-Glc	OH	OH	806
4	OH	OCH_3	β-D-Glc-(1→6)-β-D-Glc	OH	OCH_3	670
5	OH	OCH_3	β-D-Glc	OH	OH	494
6	OH	OCH_3	Glc-Glc-Rha	OH	OH	802
7	H	OCH_3	β-D-All	H	OH	462
8	β-D-All-(1→6)-β-D-Glc	-	-	-	-	596
9	β-D-Glc	-	-	-	-	436
10	β-D-Glc	-	-	-	-	434
11	H	OCH_3	OH	H	OH	300

Figure 1: Compounds identified in the methanol extract of *E. ligulatum* by HPLC/ESI-ITMS^n and NMR analyses.

these, four new flavonol glycosides were isolated and fully characterized by ESI-MS and NMR analysis. Furthermore, to the best of our knowledge, this is the first report on the presence of 9,10-dihydroxy-7-methoxy-3-(*R*)-methyl-1H-naphtho[2,3c]pyran-1-one-9-*O*-β-D-allopyranosyl (1→6) glucopyranoside and 9,10-dihydroxy-7-methoxy-3-(*R*)-methyl-1H-3,4-dihydronaphtho[2,3c] pyran-1-one-9-*O*-β-D-gluco-pyranoside in the genus *Eriocaulon*.

The methanol extract of *E. ligulatum* capitulae was separated and analyzed by HPLC/ESI-ITMS^n and HPLC/UV-DAD. Several experiments were performed to establish suitable HPLC conditions. The best results were obtained using a C_{18} RP BDS Hypersil column eluted with a gradient of acetonitrile/water acidified with acetic acid. The HPLC-UV-DAD and HPLC-MS/MS^n analyses of compounds occurring in the methanol extract of *E. ligulatum* allowed the detection of flavonoid (quercetin, luteolin and apigenin) and naphthopyranone derivatives (Table 1 and Figure 1).

A methoxyquercetin derivative was detected at the retention time (t_R) of 27.37 min in the total ion current (TIC) profile. The relative mass spectrum exhibited a peak at *m/z* 493 and the MS^2 spectrum one at *m/z* 331, due to the flavone aglycon, originating by the loss of a hexose unit from the precursor ion. NMR data of the isolated compound **5** suggested that it was 6-methoxyquercetin-7-*O*-glucoside, also known as patulitrin [4a].

The UV absorption spectra of compounds **1** and **4** were nearly identical to that of compound **5**, while the MS/MS spectra showed some differences. In

Table 1: Chromatographic, UV and mass spectral characteristics of the compounds identified in methanol extract of *E. ligulatum* capitulae.

Compound	R_t (min)	[M-H]	Major MS^2 and MS^3 fragments	UV spectra λ_{max} (nm)
1	24.45	655	640, 493, 331, 316	235, 345
2	26.63	639	477, 315, 300	235, 335
3	26.75	805	655, 493, 331	235, 340
4	27.04	669	345, 330, 302, 287	235, 345
5	27.37	493	331, 316	235, 345
6	28.70	801	655, 493,331	235, 335
7	31.13	461	446, 299, 284	235, 270,335
8	33.87	595	433, 271, 256	275, 385
9	36.72	435	273, 258	260, 360
10	38.85	433	271, 256	280, 380
11	42.13	299	284	235, 270, 335

particular, the fragmentation pattern exhibited by compound **1** was coherent with a 6-methoxyquercetin core supporting a two hexosyl moiety, while the MS/MS experiment on compound **4** showed a product ion at *m/z* 345, due to the contemporary elimination of two sugar units [M- H-162-162]$^-$, and an ion a *m/z* 330, due to the loss of a methyl group from the 6-methoxyquercetin core. Therefore, it could be considered that, on each compound, the two sugars were linked to each other otherwise a change in the UV spectrum would have been observed. The ^{1}H NMR spectrum of the isolated compound **1** showed one uncoupled proton at δ 6.72 and one methoxy group at δ 3.88 and the typical pattern (δ 7.74, d, J = 1.5 Hz; δ 7.60, dd, J = 8.0, 1.5 Hz; δ 6.88, d, J = 8.0 Hz) of a 1',3',4'-trisubstituted ring B of a flavonoid skeleton. Indeed, the HMBC correlations between the methoxy signal at δ 3.88 and the carbon resonance at δ 133.7, and between the proton signal at δ 6.72 and the carbon resonances at δ 133.7, 158.7, 153.8, and 107.0 (C-6, C-7, C-9, C-4a, respectively) allowed us to locate the methoxyl group at position 6 and the singlet at position 8, confirming for the

aglycon the structure of 6-methoxyquercetin. Two anomeric proton signals at δ 5.06 and 4.40 (*J* = 7.5 Hz) were also evident. On the basis of 1D TOCSY and 2D NMR experiments it was possible to deduce that the disaccharide chain was made up of two (1->6) linked β-D-glucopyranosyl units. HMBC correlation between the anomeric signal of glucose (δ 5.06) and the carbon resonance at δ 158.7 (C-7) of the aglycon allowed us to establish that the sugar portion was linked to C-7. A correlation between the anomeric proton signal at δ 4.40 (H-1_{glcII}) and the carbon resonance at δ 70.5 (C-6_{glcI}) confirmed the interglycosidic linkage. Thus **1** was characterized as 6-methoxyquercetin-7-*O*- β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside.

Analysis of NMR data of compound **4** in comparison with those of **1** clearly showed that the difference between the two compounds should be confined to the occurrence of a methoxy group instead of an hydroxy group on ring B of the flavonoid skeleton. On the basis of the HMBC correlations between the carbon resonance at δ 150.8, and the proton signals at δ 7.77 (d, *J* = 1.5, H-2'), δ 7.74 (dd, *J* = 1.5, 8.0, H-6'), 7.06 (d, *J* = 8.0, H-5'), and 4.02 (s, OC<u>H</u>$_3$) the additional methoxy group was located at the C-4′ position. Thus compound **4** was assigned the structure 6,4'-dimethoxyquercetin-7-*O*- β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside. The monoglucoside was found earlier in the methanol extract of *E. ligulatum*. [1e].

Another methoxyquercetin diglycoside derivative (**3**) was identified at t_R 26.75 min in the TIC profile. The corresponding mass spectrum showed a peak at *m/z* 805, while the product ion spectrum for this compound revealed the presence of a fragment ion at *m/z* 655, corresponding to [M-H-150]$^-$, followed by two fragment ions at *m/z* 493 and *m/z* 331, due to the loss of one and two hexose moieties, respectively. The loss of 150 Da could be attributed to a vanilloyl unit linked to the 6-methoxyquercetin-*O*-diglycoside. In the NMR spectrum of compound **3** the hydrogen signals at δ 7.68 (dd, *J* = 8.0, 1.5 Hz), δ 6.95 (d, *J* = 8.0 Hz), and δ 7.59 (d, *J* = 1.5 Hz), typical of an aromatic trisubstituted AMX system, together with the two *ortho* hydroxy and methoxy aromatic carbon signals (δ 148.4 and 151.1), and a carbonyl signal (δ 168.8) confirmed the presence of a vanilloyl moiety. Moreover, the presence of a ROESY correlation between the methoxy group at δ 4.00 and the signal at δ 7.59 (H-2') supported the location of the methoxy group at C-3'. Analysis of NMR data of the sugar portion confirmed the occurrence of a β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside moiety and showed a downfield shift for H_2-6glcII (δ 4.12 and 4.25) and C-6glcII (δ 64.7), as expected for an esterification. The HMBC correlation between the signals at δ 4.12 and 4.25 and the carbon resonance at δ 168.8 (<u>C</u>=O) confirmed the location of the vanilloyl moiety at C-6 of the second glucose unit. Thus compound **3** was identified as 6-methoxyquercetin-7-*O*-(6'''-vanilloyl)-β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside.

In the total ion current chromatogram a triglycoside methoxyquercetin derivative (**6**) exhibiting a precursor ion at *m/z* 801 (t_R 28.70 min) was also identified. The MS^2 experiment in product ion scan mode of the deprotonated molecule [M–H]$^-$ produced ions at *m/z* 655 [M-H-146]$^-$, due to the loss of a deoxyhexose unit, and at *m/z* 493 [M–146–162–H]$^-$ and *m/z* 331 [M-H-146-162-162]$^-$, corresponding to the sequential elimination of two hexose moieties. Considering that Vilegas [4b] and Dokkedal [4c] found that the only 6-deoxyhexose present in the methanolic extracts of *Paepalanthus argenteus* var. *argenteus*, *P. vellozioides* and *P. latipes* (Eriocaulaceae) was rhamnose, we can suppose that compound **6** was a 6-methoxyquercetin-*O*-diglycosylrhamnoside [4b,4c]. According to this hypothesis and to the best of our knowledge, this would be the first report of a flavonoid with a rhamnose unit in an *Eriocaulon* species. Unfortunately, several attempts made to isolate this compound failed due to its occurrence as a very minor constituent.

A luteolin derivative (**2**) was also found at t_R 26.63 min in the TIC profile. The MS/MS spectrum of the ion at *m/z* 639, corresponding to the deprotonated molecule [M-H]$^-$, showed product ions at *m/z* 477 and *m/z* 315, indicative of the loss of one and two hexose units (162 Da), respectively, from a methoxyluteolin aglycon. Analysis of NMR data of **2** in comparison with those of **1** clearly showed that the two compounds differed by the absence of the 3-OH group in **2**. Thus compound **2** was identified as 6-methoxyluteolin-7-*O*- β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside, which has not been reported before. In our previous papers we reported the isolation and characterization of 6-methoxyluteolin-7-*O*-allopyranoside and 6-methoxyluteolin-7-*O*-glucopyranoside from the capitulae and scapes of *E. ligulatum* [4d,5]. The peak at t_R 42.13 min in the TIC profile was assigned to 6-methoxyapigenin (**11**) [4d].

A 6-methoxyapigenin derivative (**7**) was also found at t_R 31.13 min in the TIC profile, displaying a peak at *m/z* 461, was identified as 6-methoxyapigenin-7-*O*-β-D-allopyranoside [4d].

In addition, we have detected hexoside derivatives of three naphthopyranones, whose structures were different from those of the two previously found in this plant [4d]. In particular, at t_R 38.85 min in the TIC profile there was a peak that corresponded to the 9,10-dihydroxy-7-methoxy-3-(*R*)-methyl-1H-naphtho[2,3c]pyran-1-one-9-*O*-β-D-glucopyranoside (**10**) [6a]. This hypothesis was confirmed by comparison of our NMR data with those published and by the MS analyses, here performed for the first time. In particular, the MS/MS spectrum of the ion at *m/z* 433, corresponding to the deprotonated molecule $[M-H]^-$, showed a product ion at *m/z* 271, due to the loss of a hexose unit, while an MS^3 experiment, conducted on the ion at *m/z* 271, produced a signal at *m/z* 256, due to the loss of a methyl group. In the same chromatogram, at t_R 33.87 min, there was compound with a molecular weight of 596 Da, 162 Da higher than that of compound **10**. On the basis of MS and NMR data, this peak could be attributed to the 9,10-dihydroxy-7-methoxy-3-(*R*)-methyl-1H-naphtho[2,3c]pyran-1-one-9-*O*-β-D-allopyranosyl-(1→6) glucopyranoside (**8**), previously isolated from *Paepalanthus* spp. [6b], but never reported in the genus *Eriocaulon*. The mass spectrum of the peak at t_R 36.72 min in the TIC profile exhibited a molecular ion at *m/z* 435. The MS^2 spectrum of the $[M-H]^-$ ion showed the ion $[M-H-162]^-$ at *m/z* 273; this was isolated into the ion trap and subjected to an MS^3 experiment producing a signal at *m/z* 258, due to a loss of a methyl group. On the basis of this mass spectrometric behaviour, very similar to that described for compound **10**, and of the NMR data, this compound could be identified as 9,10-dihydroxy-7-methoxy-3-(*R*)-methyl-1H-3,4-dihydronaphtho [2,3c]pyran-1-one-9-*O*-β-D-glucopyranoside (**9**) [6c]. To the best of our knowledge, this is the first report of this naphthopyranone in a species of *Eriocaulon*. Moreover, the UV spectra of compounds **8-10** presented absorption maxima in the typical bands of naphthopyranone derivatives (bands 260-280 and 360-385 nm) [6d,6e].

Analyses using HPLC/ESI-$ITMS^n$ and HPLC/UV-DAD resulted in the identification of eleven compounds (flavonoid and naphthopyranone derivatives) from the methanol extract of *E. ligulatum* capitulae. characterized as. The described data contribute to a better understanding of the taxonomic classification of the Eriocaulaceae family, in particular to establish that *E. ligulatum* shows a phenolic profile closely related to some species of *Paepalanthus* [6f].

Experimental

General: NMR experiments were performed in CD_3OD on a Bruker DRX-600 spectrometer. Optical rotations were measured on a Jasco DIP 1000 polarimeter.

Plant material: Capitulae of *E. ligulatum* were collected in May 1999 in Diamantina city, Minas Gerais State, Brazil and authenticated by Professor Dr Paulo Takeo Sano of the São Paulo University (USP), SP. A voucher specimen (SANO 2978) was deposited at the Herbarium of the IB-USP.

Sample preparation: The methanol extract of capitulae of *E. ligulatum* was prepared as reported in our previous work [4d]. The extract (1 g) was dissolved in methanol (10 mL) and the mixture was centrifuged for 5 min at 3200 rpm. The supernatant was filtered through an Iso-Disk P-34, 3 mm diameter PTFE membrane, 0.45 μm pore size (Supelco, Bellefonte, PA).

Isolation of compounds by HPLC/UV: Part of the methanol extract of *E. ligulatum* capitulae (80 mg) was separated by HPLC using an Agilent HP 1100 apparatus. The mobile phase consisted of water (eluent A) and acetonitrile (eluent B), both containing 0.5% of acetic acid. After a 5 min hold at 5%, the percentage of solvent B was increased from 5% (B) to 60% (B) in 60 min; to 95% (B) in 10 min, followed by 5 min of maintenance. Elution in a Vydac C18 semipreparative column (250 mm x 10 mm, 5 μm) (Separations Group, Hesperia, CA, USA) at a flow rate of 3 mL/min yielded pure compounds **1** (2.4 mg, t_R = 22.61 min), **2** (2.0 mg, t_R = 23.93 min), **3** (1.2 mg, t_R = 24.74 min), **4** (1.2 mg, t_R = 25.10 min), **5** (1.5 mg, t_R = 26.01 min), **7** (12 mg, t_R = 28.74 min), **8** (16 mg, t_R = 31.04 min), **9** (1.3 mg, t_R = 35.17 min), **10** (2.8 mg, t_R = 36.49 min) and **11** (6 mg, t_R = 39.89 min). Detection was performed at 280 nm.

HPLC/ESI-$ITMS^n$ and HPLC/UV-DAD analyses: The methanol extract of *E. ligulatum* capitulae was analyzed by HPLC using a SURVEYOR MS micro HPLC (Thermo Finnigan, San José, CA, USA) in an Hypersil BDS RP18 column (250 x 2.1 mm, 5 μm) (Thermo, Bellefonte, PA, USA) at a flow rate of 200

μL/min. Mobile phase and chromatographic conditions were the same as those described above. The column effluent was split into two by means of a "T junction" placed after the chromatographic column and analyzed "on-line" both by ESI/MS and UV-DAD; 80% of the effluent sent to the UV-DAD detector; 20% of the effluent was analyzed by ESI/MS in negative ion mode using a Finnigan LCQ DECA XP Plus ion trap instrument (Thermo Finnigan, San Jose, CA) equipped with Xcalibur software. The capillary voltage was set at -4 V, the spray voltage at 4.5 kV and the tube lens offset at -5 V. The capillary temperature was 275°C. Data were acquired in MS^1 and MS^n scanning mode and recorded in the 50-1500 m/z range. Total ion current (TIC) profile was produced by monitoring, during the chromatographic run, the intensity of all the ions produced and acquired in every scan.

6-Methoxyquercetin-7-*O*- β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside (**1**)

$[\alpha]_D$: + 35.0 (*c* 0.1, MeOH).
UV: Table 1.
^{1}H NMR (600 MHz, CD_3OD): δ 7.74 (1H, dd, *J* = 1.5 Hz, H-2'), 7.60 (1H, dd, *J* = 8.0, 1.5 Hz, H-6'), 6.88 (1H, d, *J* = 8.0 Hz, H-5'), 6.72 (1H, s, H-8), 3.88 (3H, s, 6-OC<u>H</u>$_3$), 5.06 (1H, d, *J* = 7.5 Hz, H-1glcI), 4.40 (1H, d, *J* = 7.5 Hz, H-1glcII), 4.21 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcI), 3.92 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.89 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcII), 3.75 (1H, m, H-5glcI) 3.69 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcI), 3.67 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.56 (1H, dd, *J* = 9.0, 9.0 Hz, H-3 glcI), 3.48 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcI), 3.38 (1H, dd, *J* = 9.0, 9.0 Hz, H-3glcII), 3.33 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcII), 3.30 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcII), 3.28 (1H, m, H-5glcII).
^{13}C NMR (150 MHz, CD_3OD): δ 178.2 (C, C-4), 158.7 (C, C-7), 153.8 (C, C-5, C-8a), 149.7 (C, C-2), 149.5 (C, C-4'), 146.4 (C, C-3'), 138.0 (C, C-3), 133.7 (C, C-6), 124.5 (C, C-1'), 122.6 (CH, C-6'), 117.0 (CH, C-2'), 116.5 (CH, C-5'), 107.0 (C, C-4a), 105.1 (CH, C-1glcII), 102.8 (CH, C-1glcI), 96.2 (CH, C-8), 78.0 (CH, C-3glcII, C-5glcII), 77.6 (CH, C-3glcI), 77.3 (CH, C-5glcI), 75.2 (CH, C-2glcI, C-2glcII), 71.6 (CH, C-4glcII), 71.5 (CH, C-4glcI), 70.5 (CH_2, C-6glcI), 62.8 (CH_2, C-6glcII), 62.3 (6-O<u>C</u>H_3).
ESI-MS: Table 1.

6-Methoxyluteolin-7-*O*-β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside (**2**)

$[\alpha]_D$: + 32.0 (*c* 0.1, MeOH).
UV: Table 1.
^{1}H NMR (600 MHz, CD_3OD): δ 7.60 (1H, dd, *J* = 1.5 Hz, H-2'), 7.52 (1H, dd, *J* = 8.0, 1.5 Hz, H-6'), 6.88 (1H, d, *J* = 8.0 Hz, H-5'), 6.74 (1H, s, H-8), 6.67 (1H, s, H-3), 3.88 (3H, s, 6-OC<u>H</u>$_3$), 5.05 (1H, d, *J* = 7.5 Hz, H-1glcI), 4.40 (1H, d, *J* = 7.5 Hz, H-1glcII), 4.22 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcI), 3.92 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.89 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcII), 3.74 (1H, m, H-5glcI) 3.67 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcI), 3.67 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.56 (1H, dd, *J* = 9.0, 9.0 Hz, H-3 glcI), 3.48 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcI), 3.38 (1H, dd, *J* = 9.0, 9.0 Hz, H-3glcII), 3.32 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcII), 3.30 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcII), 3.28 (1H, m, H-5glcII).
^{13}C NMR (150 MHz, CD_3OD): δ 184.5 (C, C-4), 166.8 (C-2), 164.4 (C-5), 161.0 (C, C-7), 155.1 (C, C-9), 152.0 (C, C-4'), 148.5 (C, C-3'), 133.8 (C, C-6), 124.2 (C, C-1'), 121.4 (CH, C-6'), 118.7 (CH, C-5'), 116.0 (CH, C-2'), 106.1 (C, C-3), 105.7 (C, C-4a), 105.1 (CH, C-1glcII), 102.2 (CH, C-1glcI), 96.5 (CH, C-8), 78.0 (CH, C-3glcII, C-5glcII), 77.5 (CH, C-3glcI), 77.4 (CH, C-5glcI), 75.2 (CH, C-2glcI, C-2glcII), 71.6 (CH, C-4glcII), 71.5 (CH, C-4glcI), 70.5 (CH_2, C-6glcI), 62.6 (CH_2, C-6glcII), 62.3 (6-O<u>C</u>H_3).
ESI-MS: Table 1.

6-Methoxyquercetin-7-*O*- (6"'-vanilloyl)-β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside (**3**)

$[\alpha]_D$: + 44.8 (*c* 0.1, MeOH).
UV: Table 1.
^{1}H NMR (600 MHz, CD_3OD): δ 7.74 (1H, dd, *J* = 1.5 Hz, H-2'), 7.68 (1H, dd, *J* = 8.0, 1.5 Hz, H-6""), 7.61 (1H, dd, *J* = 8.0, 1.5 Hz, H-6'), δ 7.59 (1H, dd, *J* = 1.5 Hz, H-2""), 6.95 (1H, d, *J* = 8.0 Hz, H-5""), 6.88 (1H, d, *J* = 8.0 Hz, H-5'), 6.74 (1H, s, H-8), 3.88 (3H, s, 6-OC<u>H</u>$_3$), 5.04 (1H, d, *J* = 7.5 Hz, H-1glcI), 4.39 (1H, d, *J* = 7.5 Hz, H-1glcII), 4.27 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcII), 4.20 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcI), 4.12 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcII), 4.00 (3H, s, 3""-OC<u>H</u>$_3$), 3.92 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.84 (1H, m, H-5glcII), 3.75 (1H, m, H-5glcI) 3.69 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcI), 3.56 (1H, dd, *J* = 9.0, 9.0 Hz, H-3 glcI), 3.48 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcI), 3.37 (1H, dd, *J* = 9.0, 9.0 Hz, H-3glcII), 3.33 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcII), 3.32 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcII).
^{13}C NMR (150 MHz, CD_3OD): δ 178.2 (C, C-4), 168.8 (C=O), 158.4 (C, C-7), 153.8 (C, C-5), 153.2 (C, C-8a), 151.1 (C, C-4""), 149.9 (C, C-2), 149.5 (C, C-4'), 148.4 (C, C-3""), 146.2 (C, C-3'), 137.7 (C, C-3), 133.6 (C, C-6), 125.3 (C, C-6""), 124.5 (C, C-1'), 122.4 (C, C-1""), 122.6 (CH, C-6'), 117.0 (CH, C-2'), 116.3 (CH, C-5'), 115.9 (CH, C-5""),

113.7 (CH, C-2""), 106.7 (C, C-4a), 105.0 (CH, C-1glcII), 102.6 (CH, C-1glcI), 96.4 (CH, C-8), 78.0 (CH, C-3glcII), 77.6 (CH, C-3glcI), 77.3 (CH, C-5glcI), 75.6 (C, C-5glcII), 75.2 (CH, C-2glcI), 75.0 (CH, C-2glcII), 72.0 (CH, C-4glcII), 71.5 (CH, C-4glcI), 70.5 (CH_2, C-6glcI), 64.7 (CH_2, C-6glcII), 62.2 (6-O$\underline{C}H_3$), 56.2 (3""-O$\underline{C}H_3$)..
ESI-MS: Table 1.

6,4'-Dimethoxyquercetin-7-*O*-β-D-glucopyranosyl-(1->6)- β-D-glucopyranoside (**4**)

$[\alpha]_D$: + 38.5 (*c* 0.1, MeOH).
UV: Table 1.
^{1}H NMR (600 MHz, CD_3OD): δ 7.77 (1H, dd, *J* = 1.5 Hz, H-2'), 7.74 (1H, dd, *J* = 8.0, 1.5 Hz, H-6'), 7.06 (1H, d, *J* = 8.0 Hz, H-5'), 6.72 (1H, s, H-8), 4.02 (3H, s, 4'-OC$\underline{H}_3$), 3.90 (3H, s, 6-OC$\underline{H}_3$), 5.07 (1H, d, *J* = 7.5 Hz, H-1glcI), 4.42 (1H, d, *J* = 7.5 Hz, H-1glcII), 4.21 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcI), 3.92 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.86 (1H, dd, *J* = 12.0, 2.0 Hz, H-6b glcII), 3.75 (1H, m, H-5glcI), 3.69 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcI), 3.67 (1H, dd, *J* = 12.0, 4.5 Hz, H-6a glcI), 3.54 (1H, dd, *J* = 9.0, 9.0 Hz, H-3 glcI), 3.48 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcI), 3.38 (1H, dd, *J* = 9.0, 9.0 Hz, H-3glcII), 3.34 (1H, dd, *J* = 9.0, 9.0 Hz, H-4glcII), 3.30 (1H, dd, *J* = 7.5, 9.0 Hz, H-2glcII), 3.28 (1H, m, H-5glcII).
^{13}C NMR (150 MHz, CD_3OD): δ 178.2 (C, C-4), 158.7 (C, C-7), 153.8 (C, C-5, C-8a), 149.7 (C, C-2), 150.8 (C, C-4'), 146.2 (C, C-3'), 137.4 (C, C-3), 133.6 (C, C-6), 123.7 (CH, C-6'), 123.2 (C, C-1'), 118.3 (CH, C-2'), 117.5 (CH, C-5'), 106.7 (C, C-4a), 105.1 (CH, C-1glcII), 102.5 (CH, C-1glcI), 96.2 (CH, C-8), 78.0 (CH, C-3glcII, C-5glcII), 77.5 (CH, C-3glcI), 77.3 (CH, C-5glcI), 75.2 (CH, C-2glcI, C-2glcII), 71.6 (CH, C-4glcII), 71.5 (CH, C-4glcI), 70.5 (CH_2, C-6glcI), 62.5 (CH_2, C-6glcII), 62.3 (6-O$\underline{C}H_3$), 58.3 (4'-O$\underline{C}H_3$).
ESI-MS: Table 1.

Acknowledgments - The authors gratefully acknowledge the financial support of the FAPESP Program which provided the fellowship to C.M.R. and the project grant to L.C.S. We also thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and FUNDUNESP for grants to W.V. and L.C.S. and the Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for a fellowship to M.A.S.

References

[1] (a) Giulietti AM, Scatena VL, Sano PT, Parra LR, Queiroz LP, Harley RM, Menezes NL, Yseppon AMB, Salatino A, Salatino ML, Vilegas W, Santos LC, Ricci CV, Bonfim MCP, Miranda EB. (**2000**) In *Monocots*: *Systematics and evolutions*. (Ed). CSIRO Publishing, Austrália. 580-588; (b) Sano PT. (**2004**) *Actinocephalus* (Koern.) Sano (*Paepalanthus* sect. *Actinocephalus*), a new genus of Eriocaulaceae, and other taxonomic and nomenclatural changes involving *Paepalanthus* Mart. *Taxon*, ***53*****,** 99-107; (c) Bate-Smith EC, Harborne JB. (**1969**) Quercetagetin and patuletin in *Eriocaulon. Phytochemistry*, ***8***, 1035-1037; (d) Ho JC, Chen CM. (**2002**) Flavonoids from the aquatic plant *Eriocaulon buergerianum. Phytochemistry*, ***61***, 405-408; (e) Silva MA, Santos LC, Olive*ira,* APS, Sannomiya M, Sano PT, Varanda EA, Vilegas W. (**2007**) Flavovoids and naphthopyranone from *Eriocaulon ligulatum* and their mutagenicity activity. *Chemical & Pharmaceutical Bulletin*, ***55***, 1635-1639.

[2] Markham KR. (**1989**), Flavones, flavonols and their glycosides In: *Methods in Plant Biochemistry,* Vol*. 1, Plant Phenolics*. Dey PM, Harborne JB (Ed.). Academic Press, London UK. 197–235.

[3] (a) Hill RA. (**1986**) Naturaly occuring isocoumarins. *Fortschritte der Chemie Organischer Naturstoffe*, ***49*****,** 1-78; (b) Varanda EA, Raddi MSG, Dias FL, Araújo MCP, Gibran SCA, Takahashi CS, Vilegas W. (**1997**) Evaluation of the genotoxic potential of the isocoumarin paepalantine *in vivo* and *in vitro* mammalian systems*. Teratogenesis Carcinogenesis and Mutagenesis, **17***, 85-95.

[4] (a) Jung PE, Kim Y, Kim J. (**2000**) Acylated flavonol glycosides from the flower of *Inula britannica*. *Journal of Natural Products*, ***63***, 34-36; (b) Vilegas W, Nehme CJ, Dokkedal AL, Piacente S, Rastrelli L, Pizza C. (**1999**) Quercetagetin 7-methyl ether glycosides from *Paepalanthus velloziodes* and *Paepalanthus latipes*. *Phytochemistry*, ***51***, 403-409; (c) Dokkedal AL, Santos LC, Sano PT, Vilegas W. (**2008**) Chemistry in Eriocaulaceae. *Zeitschrift Naturforschung C*, ***63***, 169-175; (d) Santos LC, Rodrigues CM, Silva MA, Coelho RG, Sannomiya, M, Vilegas W. (**2005**) Chemical pofile of *Eriocaulon ligulatum* (Vell.) LB Smith (Eriocaulaceae). *Biochemical Systematics and Ecology*, ***33***, 1159-1161.

[5] Santos LC, Silva MA, Rodrigues CM, Rodrigues J, Rinaldo D, Sannomiya M, Vilegas W. (**2005**) Fast preparative separation of flavones from capitula of *Eriocaulon ligulatum* (Vell.) L.B. Smith. (Eriocaulaceae) by high-speed countercurrent chromatography (HSCCC). .*Revista de Ciências Farmacêuticas Básica Aplicada*, ***26***, 101-103.

[6] (a) Jing-Jing F, Guan Y, Wen-Liang C, Wei-Min Z. *(***2008**) Antibacterial phenolic components from *Eriocaulon buergerianum*. *Phytochemistry, **69***, 1279-1286; (b) Andrade, FDP, Rastrelli L, Pizza C, Sano PT, Vilegas W. (**2002**) Flavonol glycosides and a naphthopyranone glycoside from *Paepalanthus macropodus* (Eriocaulaceae). *Biochemical Systematics and Ecology, **30***, 275-277; (c) Vilegas W, Dokkeddal AL, Piacente, S, Rastrelli L, Pizza C. (**1999**) New naphthopyranone glycosides from *Paepalanthus vellozioides* and *Paepalanthus latipes, Journal of Natural Products*, ***62***, 746-749; (d) Piacente S, Santos LC, Mahmood N, Zampelli A, Pizza C, Vilegas W. (**2001**) Naphthopyranone glycosides from *Paepalanthus microphyllus. Journal of Natural Products*. ***64***, 680-682; (e) Vilegas W, Santos LC, Alécio AC, Pizza C, Piacente S, Pauw E, Sano PT. (**1998**) Naphthopyranone glycoside from *Paepalanthus vellozioides* and *Paepalanthus latipes*, *Phytochemistry **49*****,** 207-210; (f) Giulietti AM, Hensold N. (**1990**) Padrões de distribuição geográfica do gênero Eriocaulaceae. *Acta Botanica Brasilica*, ***4***, 133-158.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1657 - 1659

Effect of Flavonoids from *Exellodendron coriaceum* (Chrysobalanaceae) on Glucose-6-Phosphatase

Diana Carrasco[a,*], Jeannette Méndez[a], Alessandra Braca[b], Marinella De Leo[b], Freddy González-Mujica[c] and Sandra Duque[d]

[a]*Laboratorio de Productos Naturales, Escuela de Química, Facultad de Ciencias, Universidad Central de Venezuela, Apartado Postal 47102, Caracas 1020-A, Venezuela*

[b]*Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 33, 56126, Pisa, Italy*

[c]*Instituto de Medicina Experimental, Sección de Bioquímica Médica, Facultad de Medicina, Universidad Central de Venezuela. Caracas, Venezuela*

[d]*Laboratorio de Bioensayos José María Benitez .Grupo de Productos Naturales, Escuela de Química, Facultad de Ciencias, Universidad Central de Venezuela. Caracas, Venezuela*

diana.carrasco@ciens.ucv.ve

Received: July 30th, 2009; Accepted: November 10th, 2009

From the *n*-butanol extract of the aerial parts of *Exellodendron coriaceum* (Benth.) Prance the flavonoids quercetin-3-*O*-β-D-galactopyranoside (**1**), quercetin-3-*O*-α-L-arabinopyranoside (**2**), quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside (**3**), and quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**4**) were isolated. Additionally from this extract three flavonoids were isolated and partially characterized as quercetin glycosides. All these compounds were tested for their hypoglycemic activity using the glucose-6-phosphatase microsomal hepatic system. The flavonoids inhibited the activity of the enzyme when intact microsomes were used, the highest percentage of inhibition being 65%. To the best of our knowledge, this is the first report of chemical and biological activity of *E. coriaceum*.

Keywords: *Exellodendron coriaceum*, Chrysobalanaceae, flavonoids, glucose-6-phosphatase, hypoglycemic activity.

Chrysobalanaceae is a family of flowering plants, consisting of 17 genera and approximately 400 species of trees and shrubs that grow mainly in tropical and subtropical regions. In Venezuela, there are 7 genera in this family [1], and our group has studied the phytochemistry and biological activity of species from 5 of these genera [2, 3]. The results have shown these plants to be rich in flavonoids and to have different biological properties, such as cytotoxic, antibacterial, hypotensive, antiviral, leishmanicidal, antioxidant, and hypoglycemic activity [4]. These findings encouraged us to continue our research on the Chrysobalanaceae family. In the present work we analyzed *Exellodendron coriaceum* (Benth.) Prance, one of 5 species in the genus, which are distributed in Brazil and in the south of Venezuela. This is the first species of *Exellodendron* to be studied.

We report here the isolation of seven flavonoids from the *n*-butanol extract of the aerial parts of *E. coriaceum*, of which compounds **1**-**4** were characterized as quercetin-3-*O*-β-D-galactopyranoside (**1**), quercetin-3-*O*-α-L-arabinopyranoside (**2**), quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside (**3**), and quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**4**), while three compounds, due to the small amounts isolated, could not be fully identified, but were partially characterized as derivatives of methoxylated and/or glycosylated quercetin with glucose, galactose, or arabinose as sugar moieties, according to data from ESI-MS analysis. Flavonoids **3** and **4** are being reported for the first time in the family. All compounds were tested for potential hypoglycemic activity in the rat liver microsomal glucose-6-phosphatase (G-6-Pase) bioassay, which might be a useful method to

determine substances that reduce glycaemia. The G-6-Pase (EC 3.1.3.9) catalyzes the last step of glycogenolysis and gluconeogensis, and is constituted by a catalytic subunit and three transporters: T1 for glucose-6-phosphate, T2 for phosphate, and T3 for glucose. The enzyme and transporters are potential targets for antidiabetic therapy [5].

All flavonoids evaluated inhibited the G-6-Pase activity in intact microsomes, but only two of them (Que + 2 *O*Me + 162 + 132 and Que + 162 + 162) in the disrupted system. Phlorizin was used as a positive control (28% inhibition), because it is a well known inhibitor of T1. According to the G-6-Pase model [6], when a compound inhibits G-6-Pase activity of disrupted microsomes, it is acting on the catalytic subunit; on the other hand, if the inhibition occurs in intact microsomes, the compound is affecting any one of the G-6-Pase components, more probably the T1 transporter. Our results suggest that the flavonoids from the *n*-butanol extract of *E. coriaceum* inhibit one of the transporters, probably T1. In general, most of the compounds studied in the present work behaved in a similar way as phlorizin.

Quercetin-3-*O*-α-L-arabinopyranoside (**2**) and quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranoside (**3**) had the lowest inhibitory effect on G-6-Pase in intact microsomes. The percentage of inhibition was higher (52%) for quercetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**4**) and quercetin with a methoxyl and 2 hexose units (possibly glucose and/or galactose). Comparing these values with the ones obtained for **3** and quercetin with two hexoses, respectively, it could be suggested that the presence of galactose increases the inhibitory effect of the flavonoid glycoside; the same applies to a methoxyl substituent. The strongest inhibitory effect was observed for quercetin containing two methoxyl substituent and two glycosyl moieties (with 162 and 132 uma, respectively). This result supports the statement about the effects of a methoxy group and galactose unit on the inhibitory effect of these glycosides. Compounds **1**-**4** and methoxylated quercetin with two hexoses increased the G-6-Pase activity in the disrupted microsomes. This effect could be due to modifications in the organelle membranes that expose the active center of the enzyme as a consequence of the joint action of histones and the glycosylated flavonoids with anphipatic characteristics.

Our results on the inhibition of the G-6-Pase activity by flavonoids from *E. coriaceum* are in conformity with previous results on the inhibitory effects of flavonoids from *Licania densiflora* (Chrysobalanaceae) on the activity of the G-6-Pase enzyme. From the methanol extract of *L. densiflora* leaves quercetin and quercetin 3-*O*-α-L-rhamnopyranoside were isolated, both of which showed inhibition of the enzyme [7].

So far, the results indicate that the presence of quercetin seems to have an important role in the inhibition of the G-6-Pase activity. Further studies are needed to establish a more specific structure-activity relationship.

Experimental

General: All 1D and 2D experiments were performed on a Bruker Avance II 250 MHz spectrometer. The NMR spectra were recorded in CD_3OD. ESI-MS spectra were recorded on an ion trap Advantage LCQ Thermo Finnigan spectrometer, San Jose, C.A. Column chromatography was performed on Sephadex LH-20. For preparative RP-HPLC a Shimadzu LC-8A was used. Column: Waters μ-Bondapak C_{18} (7.8 x 300 mm; Waters, Milford, MA). TLC was performed on silica gel 60 F_{254} aluminum plates (Merck, Darmstadt, Germany). TLC plates were sprayed with a saturated solution of ceric sulfate in 65% sulfuric acid (Sigma-Aldrich, Milano, Italy) and heated at 120°C. Naturstoff reagent (1% 2-aminoethyldiphenylborinate in MeOH) specific for flavonoids was also used.

Plant material: The aerial parts and fruits of *E. coriaceum* (Benth.) Prance were collected near the Sipapo River, Amazonas State in October 1998, and were identified by Prof. Anibal Castillo. A voucher specimen is deposited at Fundación Instituto Botánico de Venezuela (voucher number 6969 VEN 309885) Universidad Central de Venezuela.

Microsome purification and G-6-Pase assay: Liver microsomes were purified as described by Marcucci *et al.* [8] from rats fasted overnight. The microsomal fraction was resuspended in 0.25 mM sucrose, 1mM $MgCl_2$, and 5 mM HEPES, pH 6.5, to give a final protein concentration of 1 mg/mL and frozen at -80°C until use. Proteins were estimated using a modification of the Lowry method [9]. Enzymatic assays were performed by the method of Burchell *et al.* [10], with intact and disrupted (histone treated) microsomes.

In order to study the effect of each flavonoid, each one was added to the G-6-Pase assay at a final concentration of 160 μg/mL. The final concentration of DMSO in the control and experimental assays was 0.5%. All the microsomes used were at least 95% intact, as determined by the hydrolysis of mannose-6-phosphate [9].

Extraction and isolation: The oven dried aerial parts and fruits of *E. coriaceum* (1389 g) were defatted with *n*-hexane and successively extracted for 48-72 h with $CHCl_3$, $CHCl_3$-MeOH (9:1), MeOH, and MeOH-H_2O (1:1) (4 x 1.5 L) to give 17.9 g, 18.0 g, 70.8 g, and 21.2 g of the respective residue. A portion of the methanolic extract (25 g) was partitioned between *n*-BuOH and H_2O. After evaporation of the solvent, the *n*-butanol residue (7.14 g) was chromatographed on Sephadex LH-20 using MeOH as eluent. Fractions of 9 mL were collected and grouped into 20 major fractions by TLC with *n*-BuOH-AcOH-H_2O (60:15:25). Fraction 10 (58.8 mg) was identified as **3**. Fraction 11 (158.2 mg) was submitted to RP-HPLC with MeOH-H_2O (2:3), flow rate: 2 mL/min, to give compound **3** (1.8 mg) and a flavonoid characterized as quercetin + OMe + 162 + 162. Fraction 13 (119.6 mg) was submitted to RP-HPLC with MeOH-H_2O (2:3), flow rate: 2 mL/min, to give compound **4** (4.2 mg) and a flavonoid, characterized as quercetin + 162 + 162. Fraction 15 (98.1 mg) was chromatographed over RP-HPLC with MeOH-H_2O (45:55), flow rate: 2mL/min, to give a flavonoid characterized as quercetin + 2 OMe + 162 + 132. Finally, fraction 18 (53.3 mg) was subjected to RP-HPLC with MeOH-H_2O (2:3), flow rate: 2 mL/min, to give compounds **1** (4.7 mg) and **2** (5.8 mg).

Identification of the isolated compounds was achieved by means of spectroscopic (1D- and 2D-NMR) and ESI-MS methods, and by comparison with literature data [11,12].

Acknowledgments – This work was supported by grants PG-03-005741-2004 from the Consejo de Desarrollo Científico y Humanístico de la Universidad Central de Venezuela.

References

[1] Toledo CL, Kubitzi K, Prance GH. (**1982**) *Flora de Venezuela* vol. IV. Caracas, Ediciones Fundacion Ambiental.

[2] Braca A, Bilia AR, Mendez J, Morelli I. (**1999**) Three flavonoids from *Licania densiflora*. *Phytochemistry*, ***51***, 1125-1128.

[3] Carrasco D. (**2009**) Estudio fitoquímico y análisis de la actividad biológica de *Exellodendron coriaceum*. Una aproximación a la quimiotaxonomía de la familia Chrysobalanaceae. Tesis Doctoral, Universidad Central de Venezuela. Caracas.

[4] Braca A, Bilia AR, Mendez J, Pizza C, Morelli I, De Tommasi N. (**2003**) Chemical and biological studies on *Licania* genus. *Studies in Natural Products Chemistry*. Atta U-Rahman (Ed.), Elsevier, Amsterdam. ***28***, 35-67.

[5] McCormack JG, Westergaard N, Kristiansen M, Brand CL, Lau J. (**2001**) Pharmacological approaches to inhibit endogenous glucose production as a means of anti-diabetic therapy. *Current Pharmaceutical Design*, ***7***, 1451-1474.

[6] Burchell A, Waddell ID. (**1991**) The molecular basis of the hepatic microsomal glucose-6-phosphatase. *Biochimica and Biophysica Acta*, ***1092***, 129-137.

[7] Fernandez M. (**1996**) Fitoquímica del extracto polar de *Licania densiflora* (Chrysobalanaceae). Actividad hipoglicemiante. Trabajo Especial de Grado, Universidad Central de Venezuela.

[8] Marcucci OL, González-Mujica F, Perez-Ayuso E. (**1983**) Alterations of liver nuclear envelopes accompanying thioacetamide administration in rats. *Acta Cientifica Venezolana*, ***34***, 109-117.

[9] Marwell M, Hass S, Bieber L, Tolbert N. (**1978**) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein sample. *Analytical Biochemistry*, ***87***, 206-210.

[10] Burchell A, Hume R, Burchell B. (**1988**) A new micro-technique for the analysis of the human hepatic microsomal glucose-6-phosphatase system. *Clinica Chimica Acta*, ***173***, 183-192.

[11] dos Santos PM, Schripsema J, Kuster R. (**2005**) Flavonoides *O*-glicosilados de *Croton campestres* St. Hill (Euphorbiaceae). *Brazil Journal of Pharmacognosy*, ***15***, 321-325.

[12] Flamini G, Bulleri C, Morelli I, Manunta A. (**2000**) A new flavonoid glycoside from *Centaura horrida*. *Journal of Natural Products*, ***63***, 662-663.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1661 - 1664

Phenolics in Aerial Parts of Persian Clover *Trifolium resupinatum*

Bogdan Janda[a], Anna Stochmal[a], Paula Montoro[b], Sonia Piacente[b] and Wieslaw Oleszek[a*]

[a]*Biochemistry Department, Institute of Soil Science and Plant Cultivation, State Research Institute, ul. Czartoryskich 8, 24-100 Pulawy, Poland*

[b]*Dipartimeto di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte Don Melillo, 84084 Fisciano, Salerno, Italy*

wo@iung.pulawy.pl

Received: July 30th, 2009; Accepted: September 16th, 2009

The nutritional quality of Persian clover (*Trifolium resupinatum*), an important pasture crop, depends not only on a high protein content but also on the occurrence of animal health and welfare promoting phytochemicals. Nine phenolic constituents present in the aerial parts of this species were isolated and their structures confirmed by NMR and ESI-MS analyses. The compounds included two chlorogenic acids, four quercetin and two kaempferol glycosides, as well as the isoflavone formononetin-7-glucoside. The concentration of isoflavone was low, not exceeding 1.2 mg/g of dry matter. The concentration of flavonols ranged between 5.9 and 11.8 mg/g, depending on the sampling dates, with the highest concentration occurring in the first cut. A similar trend in the concentration was found for chlorogenic acids, which ranged from 2 mg/g in summer to 7.3 mg/g in spring.

Keywords: Persian clover, *Trifolium resupinatum*, flavonols, phenolic acids, composition.

The genus *Trifolium* (Papilionoidae-Trifollieae) includes about 250-300 species, which are distributed both in temperate and subtropical regions [1]. Out of this number, only a few species have gained economic significance as pasture crops. These include *Trifolium pratense* L., *T. repens* L., *T. resupinatum* L., *T. incarnatum* L., *T. hybridum* L., *T. pannonicum* Jacq., *T. subterraneum* L., *T. fragiferum* L. and *T. medium* L. Some of these species have been characterized for secondary metabolites, such as triterpene saponins [2-5], flavonoids [1,6], isoflavones [7,8] and cyanogenic glucosides [9,10].

The recent survey of seeds of 57 species of *Trifolium* showed a big diversity in secondary metabolite profiles [11]. Similar diversity can be also be found in aerial parts of *Trifolium* species [12], suggesting that some species can be recognized as important sources of natural products for food, feeding stuffs and cosmetic industries.

T. resupinatum (Persian clover, Reversed clover, Shaftal clover) has some importance as an annual pasture crop. The species originates from the Mediterranean and Middle East (Iran, Afganistan) region, where it has been cultivated for centuries. In temperate regions it has been cultivated since the beginning of the twentieth century, but problems exist with seed reproduction. Recent work on genetic selection has resulted in new varieties, which give the same yield of dry matter per hectare as the original Mediterranean populations, but also are able to produce satisfactory yield of seeds [13]. In spite of the wide geographical distribution and its usefulness as a pasture crop, little is known about the secondary metabolites of this species and their possible significance in animal nutrition [1]. Thus, the aim of the present work was to isolate and identify the major phenolic constituents of the green parts of this species.

In our previous work on phenolic content of 57 *Trifolium* species, three subspecies of *T. resupinatum* were classified into clusters high in phenolic acids (0.4-1.0% of dry matter), with some flavonoids, but free of cloveamids [12]. One of the subspecies contained low level of isoflavones.

Separation of *T. resupinatum* phenolics using liquid chromatography allowed us to distinguish a number of compounds, which were classified into three groups: two phenolic acids, six flavonols and one isoflavone. These were separated into individual components using low pressure preparative chromatography.

Two phenolic acids showed similar UV spectra and MS pseudomolecular ions at *m/z* 353 and fragmentations patterns characteristic for chlorogenic acids (caffeic and quinic acids). From NMR and MS data, compound **1** was shown to be 3-caffeoylquinic acid, known as chlorogenic acid, and compound **2** 4-caffeoylquinic acid, known as crypto-chlorogenic acid, with the second molecule being the dominant.

The six isolated flavonoids, based on their UV, MS and NMR spectra, were identified as quercetin and kaempferol derivatives, namely quercetin 3-*O*-β-D-glucopyranosyl-1→2-β-D-galactopyranoside (**3**), quercetin 3-*O*-β-D-glucopyranosyl-1→3-α-L-rhamnopyranosyl-1→6-β-D-galactopyranoside (**4**), kaempferol 3-*O*-β-D-glucopyranosyl-1→2-β-D-galactopyranoside (**5**), kaempferol 3-*O*-β-D-glucopyranosyl-1→3-α-L-rhamnopyranosyl -1→6-β-D-galactopyranoside (**6**), quercetin 3-O-β-D-glucopyranoside (**7**), quercetin 3-*O*- (6"-malonyl)-β-D-glucopyranoside (**8**), and formononetin 7-*O*-β-D-glucopyranoside (**9**). Compounds **3** and **5** are constituents of *Panax notoginseng* [14], while the triglycosides (**4**) and (**6**) occur in the leaves of tea (*Camellia sinensis)* [15]. Formononetin 7-*O*-β-D-glucopyranoside (**9**) is an isoflavone commonly occurring in plants belonging to the Leguminosae family [16,17].

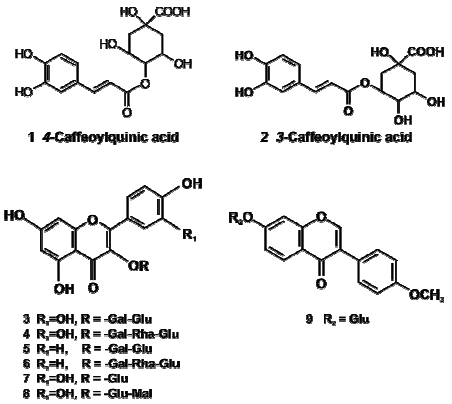


Figure 1: Chemical structures of phenolics isolated from *T. resupinatum* aerial parts.

Table 1: Concentration of phenolics in aerial parts of *T. resupinatum* var. Irain [three sampling dates (cuts) during one vegetation season].

Compounds	Cut 1* (mg/g)	Cut 2 (mg/g)	Cut 3 (mg/g)
1	0.54±0.08	0.48±0.09	0.35±0.05
2	6.74±0.43	3.24±0.59	1.68±0.58
3	0.75±0.07	0.27±0.03	0.24±0.03
4	0.89±0.07	0.36±0.05	0.54±0.06
5	0.72±0.09	0.35±0.05	0.20±0.02
6	1.80±0.20	1.59±0.23	1.56±0.14
7	5.18±0.41	2.55±0.28	2.20±0.24
8	2.53±0.35	1.10±0.13	1.13±0.18
9	1.18±0.08	0.52±0.05	0.58±0.08
Total	**20.33**	**10.46**	**8.48**

*Cut 1 – 6 June, Cut 2 – 4 July, Cut 3 -15 August 2007

The isolated compounds were used to develop an HPLC method for their determination in the plant material. These standard compounds were used both for the localization of phenolics in the HPLC profile, as well as for the preparation of standard curves for their determination. All nine isolated compounds were successfully separated by gradient elution (Figure 2).The concentration of isolated phenolics was measured in the aerial parts collected three times during the growing season. As shown in Table 1,

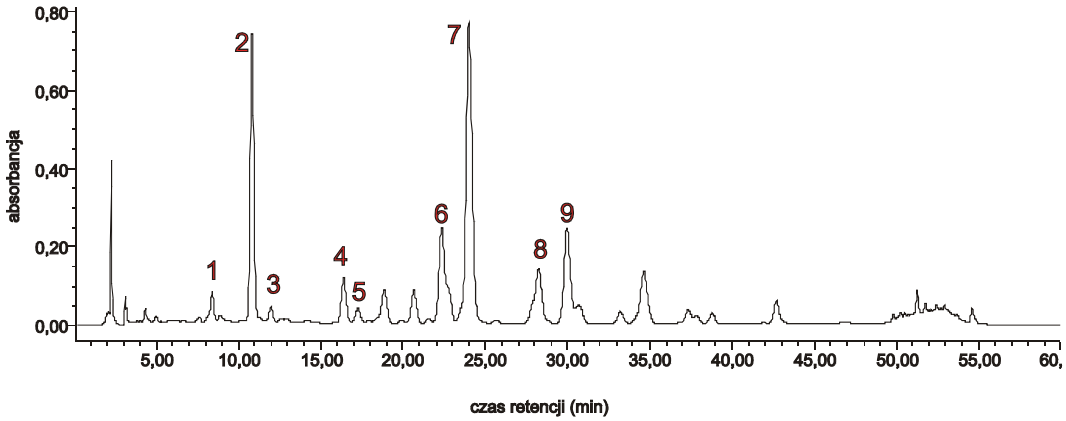


Figure 2: HPLC profile of *T. resupinatum* var. Ira phenolics.

the concentration of phenolics was highest in the first, spring collected material. The concentration of all three groups: chlorogenic acids, flavonols and isoflavone was double in spring samples as compared with the following two sampling dates. This remains in a good agreement with previous findings, showing successive decrease in the total flavonoid concentration in alfalfa aerial parts, during three growing seasons [18].

The overall concentration of phenolics in the aerial parts was quite high in a June sampled material and was about 2% of dry matter, dropping down by a half in the next two sampling dates.

The concentration of phenolics in the present research was very similar to the data obtained previously for three *T. resupinatum* subspecies [12]. The high flavonol concentration and thus their high antioxidant capacity, improves the nutritional value of this plant when used as feedstuff.

Experimental

Plant materials: Seeds of *T. resupinatum* L. var. Majus Boiss. were obtained from Genebank, Zentralinstitute fűr Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany (Herbarium Voucher TRIF 81/83). Seeds of *T. resupinatum* L. var. Ira were obtained from Bartazek Breeding Station, Poland. Seeds were planted in the field on small plots 2 x 2 m at IUNG Experiment Station. Plants of *T. resupinatum* var. Majus Boiss were collected at THE early flowering stage, freeze dried, powdered and used for isolation of phenolics.

Spectroscopic analysis: ESI-MS were recorded on a Mariner Biospectrometry workstation (PerSeptive Biosystems). NMR experiments were performed on a Bruker DRX-600 spectrometer equipped with a Bruker 5 mm TCI CryoProbe at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma Aldrich) and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC spectra. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using the Bruker XWIN-NMR software.

Extraction and separation of phenolics: Phenolics and saponins were extracted with 70% aqueous MeOH and purified according to a previously described procedure [19]. The purified phenolic fraction was loaded onto a preparative column (400 x 30 mm i.d., LiChroprep RP-18, 40-60 μm, Merck), which was washed with water and then with increasing concentrations of MeOH in water (linear gradient, Beckman Gradient Former) and 10 mL fractions were collected in a fraction collector. Fractions were analyzed by TLC (DC-Alufolien Cellulose, Merck, solvent 15% OHAc) and those showing similar profiles were combined and evaporated (34 fractions). The fractions were analyzed by HPLC (Waters with 996 PAD detector, 616 pump and Millenium software) and, based on HPLC profiles, isocratic systems were optimized for each fraction to purify individual compounds. The purification was performed on preparative column (400 x 20 mm i.d., LiChroprep RP-18, 25-40 μm, Merck) using an isocratic system (CH_3CN/1%H_3PO_4). Nine compounds were isolated:

3-caffeoylquinic acid (chlorogenic acid) (1)

UV/Vis λ_{max} (MeOH) nm: 323.
Rt min.: 8.2
^{1}H NMR (600 MHz, CD_3OD): 1.95 (1H, dd, *J* = 9.0, 14.0 Hz, H-6ax), 2.13 (2H, m, H-2eq, H-6eq), 2.20 (1H, dd, *J* = 4.0, 15.0 Hz, H-2ax), 3.63 (1H, dd, *J* = 3.0 and 9.0 Hz, H-4), 4.14 (1H, ddd, *J* = 3.0, 9.0, 9.0 Hz, H-5), 5.34 (1H, ddd, *J* = 3.0, 3.0, 4.0 Hz, H-3), 6.30 (1H, d, *J* = 16.0 Hz, H-8'), 6.76 (1H, d, *J* = 8.0 Hz, H-5'), 6.93 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 7.04 (1H, d, *J* = 2.0 Hz, H-2'), 7.58 (1H, d, *J* = 16.0 Hz, H-7').
^{13}C NMR (150 MHz, CD_3OD): 36.7 (C-2), 41.5 (C-6), 68.3 (C-5), 73.0 (C-3), 74.8 (C-4), 75.4 (C-1), 115.1 (C-2'), 115.8 (C-8'), 116.4 (C-5'), 122.9 (C-6'), 127.9 (C-1'), 146.7 (C-3'), 146.8 (C-7'), 149.4 (C-4'), 169.0 (C-9'), 178.3 (C-7).
ESI-MS/MS: *m/z* 707 $[2M-H]^-$, 353 $[M-H]^-$, 179 [caffeic acid-H]$^-$, 191 [quinic acid-H]$^-$.

4-caffeoylquinic acid (crypto-chlorogenic acid) (2)

UV/Vis λ_{max} (MeOH) nm: 243, 328.
Rt min.: 10.9
^{1}H-NMR (600 MHz, CD_3OD): 2.00 (1H, dd, *J* = 9.0, 14.0 Hz, H-6ax), 2.06 (1H, ddd, *J* = 3.0, 14.0 Hz, H-2eq), 2.17 (1H, dd, *J* = 4.0 and 14.0 Hz, H-2ax), 2.20 (1H, ddd, *J* = 4.0, 14.0 Hz, H-6eq), 4.23 (1H, ddd, *J* = 4.0, 9.0, 9.0 Hz, H-5), 4.37 (1H, ddd, *J* = 3.0, 3.0, 4.0 Hz, H-3), 4.80 (1H, dd, *J* = 3.0, 9.0 Hz, H-4), 6.37 (1H, d, *J* = 16.0 Hz, H-8'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.96 (1H, dd, *J* = 2.0, 8.0 Hz, H-6'), 7.06 (1H, d, *J* = 2.0 Hz, H-2'), 7.65 (1H, d, *J* = 16.0 Hz, H-7').
^{13}C NMR (150 MHz, CD_3OD): 38.4 (C-2), 42.7 (C-6), 65.6 (C-5), 69.6 (C-3), 76.6 (C-1), 79.3 (C-4),

115.1 (C-2'), 115.3 (C-8'), 116.5 (C-5'), 123.0 (C-6'), 127.8 (C-1'), 146.7 (C-3'), 147.1 (C-7'), 149.6 (C-4'), 168.9 (C-9'), 177.4 (C-7)
ESI-MS/MS: *m/z* 353 $[M-H]^-$, 335 $[M-H_2O-H]^-$, 179 [caffeic acid-H]$^-$, 161 [caffeoyl-H]$^-$, 135 [caffeoyl-CO_2-H]$^-$.

Determination of phenolics in plant material: Dried samples (200 mg) were extracted with 80% aqueous MeOH (20 mL, 20 min by boiling). Extracts were condensed to remove MeOH and loaded onto C18 Sep-Pak cartridges, preconditioned with water. Cartridges were washed first with water (10 mL) and phenolics were washed out with 40% aq. MeOH (10 mL), condensed and dissolved in 1 mL of MeOH for HPLC analyses. Separations were performed on a RP-18 column (4.6 x 250 mm; Eurospher 100, 10 μm, Säulentechnik, Germany) using a gradient system of A → 100%B (where A: 10% CH_3CN in1%H_3PO_4 and B: 40% CH_3CN in 1%H_3PO4) at a flow rate of 1mL/min for 50 min. All samples were analyzed 5 times.

References

[1] Bisby FA, Buckingham J, Harborne JB. (**1994**) *Phytochemical dictionary of the Leguminoseae*. Chapman and Hall, London, 1-1051.

[2] Oleszek W, Jurzysta M. (**1986**) Isolation, chemical characterization and biological activity of red clover (*Trifolium pratense* L.) root saponins. *Acta Societatis Botanicorum Poloniae*, ***55***, 247-252.

[3] Sakamoto S, Kofuji S, Kuroyanagi M, Ueno A, Sekita S. (**1992**) Saponins from *Trifolium repens*. *Phytochemistry*, ***31***, 1773-1777.

[4] Jurzysta M, Price KR, Ridout C, Fenwick GR. (**1989**) The structure of four triterpenoid saponins isolated from the seeds of *Trifolium incarnatum. Acta Societatis Botanicorum Poloniae*, ***58***, 575-582.

[5] Simonet AM, Stochmal A, Oleszek W, Macias FA. (**1999**) Saponins and polar compounds from *T. resupinatum. Phytochemistry*, ***51***, 1065-1067.

[6] Carlsen SCK, Mortensen AG, Oleszek W, Piacente S, Stochmal A, Fomsgaard IS.(**2008**) Variation in flavonoids in leaves, stems and flowers of white clover cultivars. *Natural Product Communications, **3***, 1299-1306.

[7] Tava A, Pecetti L, Bertoli A, Piano E. (**2006**) Oestrogenic isoflavone content in natural strains of subterranean clover (*Trifolium subterraneum* L.) from Sardinia. *Natural Product Communications*, ***1***, 557-562.

[8] Francis CM, Millington AJ Bailey ET. (1967) The distribution of oestrogenic isoflavones in the genus *Trifolium*. *Australian Journal of Agriculture Research*, ***18***, 47-54.

[9] Butler GW. (**1965**) The distribution of cyanoglucosides linamarin and lotaustralin in higher plants. *Phytochemistry*, ***4***, 127-131.

[10] Stochmal A, Oleszek W. (**1997**) The changes in cyanogenic glucosides in white clover (*Trifolium repens* L.) during the growing season. *Journal of Agricultural and Food Chemistry*, ***11***, 4333-4336.

[11] Oleszek W, Stochmal A. (**2002**) Triterpene saponins and flavonoids in the seeds of *Trifolium* species. *Phytochemistry*, ***61***, 165-170.

[12] Oleszek W, Stochmal A, Janda B. (**2007**) Concentration of isoflavones and other phenolics in the aerial parts of *Trifolium* species. *Journal of Agricultural and Food Chemistry* ***55***, 8095-8100.

[13] Kaszuba J. (**1984**) Cultivation of Persian clover for seeds and green pasture. *Bulletin of IHAR*, 1-11, (in Polish).

[14] Wang XY, Wang D, Ma XX, Zhang YJ, Yang CR. (**2008**) Two new dammarane-type bidesmosides from the fruit pedicels of *Panax notoginseng*. *Helvetica Chimica Acta*, ***91***, 60-66.

[15] Scharbert S, Holzmann N, Hofmann T. (**2004**) Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse D. *Journal of Agricultural and Food Chemistry*, ***52***, 3498-3508.

[16] Swinny EE, Ryan KG. (**2005**) Red clover *Trifolium pratense* L. phytoestrogens: UV-B radiation increases isoflavone yield, and postharvest drying methods change the glucoside conjugate profiles. *Journal of Agricultural and Food Chemistry*, ***53***, 8273-8278.

[17] Klejdus B, Mikelova R, Petrlova J, Potesil D, Adam V, Stiborova M, Hodek P, Vacek J, Kizek R, Kuban V. (**2005**) Determination of isoflavones in soy beans by fast column high-performance liquid chromatography coupled with UV-visible diode-array detection. *Journal of Chromatography A*, ***1084***, 71-79.

[18] Stochmal A, Oleszek W. (**2007**) Seasonal and structural changes of flavones in alfalfa (*Medicago sativa*) aerial parts. *Journal of Food, Agriculture and Environment*, ***5***, 170-174.

[19] Stochmal A, Piacente S, Pizza C, De Riccardis F, Leitz R, Oleszek W. (**2001**) Alfalfa (*Medicago sativa* L.) flavonoids. 1. Apigenin and luteolin glycosides from aerial parts *Journal of Agricultural and Food Chemistry*, ***49***, 753-758.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1665 - 1670

Prunus spinosa Fresh Fruit Juice: Antioxidant Activity in Cell-free and Cellular Systems

Daniele Fraternale[a], Laura Giamperi[a], Anahi Bucchini[a], Piero Sestili[b], Marco Paolillo[b] and Donata Ricci[a]*

[a]*Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura – Sez. Biologia Vegetale Università degli Studi di Urbino "Carlo Bo", Via Bramante, 28 – 61029, Urbino (PU), Italy*

[b]*Dipartimento di Scienze Biomolecolari – Sez. Scienze Motorie e della Salute Università degli Studi di Urbino "Carlo Bo", Via i Maggetti 26, Loc. Sasso, – 61029, Urbino (PU), Italy*

donata.ricci@uniurb.it

Received: June 26th, 2009; Accepted: September 16th, 2009

The antioxidant activity was assessed of fresh juice from *Prunus spinosa* L. fruit (Rosaceae) growing wild in Urbino (central Italy) by using different cell-free *in vitro* analytical methods: 5-lipoxygenase test, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging, and oxygen radical absorbance capacity (ORAC). Trolox was used as the reference antioxidant compound. In the 5-lipoxygenase and DPPH tests the fresh fruit juice of *P. spinosa* showed good antioxidant activity when compared with Trolox, while the ORAC value was 36.0 μmol eq. Trolox /g of fruit. These values are in accord with data reported in the literature for small fruits such as *Vaccinium*, *Rubus* and *Ribes*. The antioxidant capacity in cell-free systems of *P. spinosa* juice has been compared with its cytoprotective – *bona fide* antioxidant activity in cultured human promonocytes (U937 cells) exposed to hydrogen peroxide. The antioxidant activity of red berries has been correlated with their anthocyanin content. The results of this study indicate that the three most representative anthocyanins in *P.spinosa* fruit juice (cyanidin-3-rutinoside, peonidin-3-rutinoside and cyanidin-3-glucoside) are likely to play an important role in its antioxidant properties.

Keywords: *Prunus spinosa* L., fruit juice, antioxidant activity, oxidative stress, cytotoxicity.

Prunus spinosa L. (Rosaceae), known as "blackthorn" or "sloe", is a wild shrub native to Scotland and commonly found in European deciduous forests and temperate countries of Asia, especially in central, north, west and south Anatolia [1]. It is resistant to cold, drought, and calcareous soils and represents one of the ancestors of *P. domestica* [2]. The fruits are bluish black, bloomy, globular drupes, 5-7 mm with green astringent flesh; they are popularly called "sloes" and despite their succulent appearance are far too bitter for human consumption, except as flavoring in liqueurs and wine. In the Marche region (central Italy), *P. spinosa* fruits are used for the preparation of a wine named "Lacrima di Spino Nero" [3].

The medicinal properties of blackthorn fruit extracts (purgative, diuretic, detoxicant) render them suitable for the preparation of natural medicines [2]. Anthocyanins were detected in sloe fruits by Werner *et al.* [4], Ramos and Macheix [5], Casado-Redin *et al.* [6], and Deineka *et al.* [7]; cyanidin-3-glucoside, cyanidin-3-rutinoside, peonidin-3-glucoside and peonidin-3-rutinoside were identified as the main anthocyanins, whereas caffeoil-3'-quinic acid was the most abundant hydroxycinnamic derivative. Different quercetin glycosides were also detected.

Anthocyanins are widely distributed among fruits and vegetables and have been reported to be absorbed unmodified from the diet [8], and to be incorporated in cell cultures, both in the plasma membrane and in the cytosol [9]. They are one of the main classes of flavonoids, contribute significantly to the antioxidant activities of the compounds [10], and are well known for their ability to give red, blue, and purple colors to plants. Anthocyanins can potentially interact with biological systems, conferring enzyme-inhibiting, antibacterial, cardiovascular protection and antioxidant effects [11,12]. Wang and Lin [13] found

a strong correlation between antioxidant capacity, total phenols and anthocyanins, while, on the other hand, some investigations also indicated that anthocyanins may be less significantly correlated with the antioxidant properties [14-17].

It has been proposed, on the basis of experimental data, that anthocyanins may exert therapeutic activities on human diseases associated with oxidative stress, for example coronary heart disease and cancer [18]. These effects have been related mainly to the antioxidant properties of anthocyanins, as demonstrated by experiments both *in vitro* and *in vivo* [19-22].

Various mechanisms have been proposed to explain the antioxidant activity of anthocyanins, such as their ability to scavenge free radicals [19-22], to chelate metal ions [20], to inhibit lipoprotein oxidation [19,23,24], and to form complexes with DNA [25].

As it is generally accepted that oxidative stress plays a role in a number of chronic and degenerative pathologies [26], including the above mentioned ones, considerable effort is being devoted to the search for naturally-occurring antioxidants from edible plants and fruits to prevent the onset and counteract the progression of these maladies [26-28]. In this light, this study has been aimed to investigate whether the natural microfiltered juice of the fresh fruit of *P. spinosa* (PJ), rich in anthocyanins, was effective either in acting as an antioxidant in established cell-free systems or in protecting cultured human promonocytes against the oxidative insult caused by hydrogen peroxide. It is worth noting that only seed extracts [29] and aqueous extracts (thea) from dried fruits of *P. spinosa* [30] have been tested by other groups for their antioxidant activity.

The results obtained in the first set of experiments, involving the three different acellular *in vitro* assays (namely DPPH, 5-lipoxygenase and ORAC), showed that PJ exerts a significant antioxidant capacity as compared with that of the established antioxidant Trolox (Figure 1A, 1B).

In many fruits and vegetables, the antioxidant activity can be attributed to the level of total polyphenols [31-33]. Therefore, the total polyphenol levels were measured for PJ, and a value of 83.5 ± 2.5 mg/g DW was found. One of the dominant classes of polyphenols in fruits are anthocyanins [34]. Tzulcher *et al.* [35] reported a positive correlation between total polyphenols, anthocyanin content and antioxidant activity of the fruit juice of *Punica granatum*. Similarly Ranilla *et al.* [36] found a positive correlation between antioxidant activity and anthocyanin content in seed coats from Brazilian and Peruvian cultivars of *Phaseolus vulgaris*. The total level of anthocyanins in our juice was 55.1 $\pm$5.6 mg/g DW.

In accordance with data reported in the literature for different fruits and vegetables, the total polyphenol and anthocyanin content appeared to contribute significantly to the antioxidant activity of sloe fruits, which indeed are rich in cyanidin-3-rutinoside (53.5%), peonidin-3-rutinoside (32.4%) and cyanidin-3-glucoside (11.4%) [7].

Elisia *et al.* [37] found that cyanidin-3-glucoside showed marked antioxidant efficacy in both *in vivo* and *in vitro* models, as compared with 13 other anthocyanins tested; moreover, anthocyanins, as compared to other classic natural antioxidants, have been recognised as potent inhibitors of lipid peroxidation [38].

Results shown in Figure 1A indicate that PJ exerted a remarkable antioxidant activity using the lipoxygenase assay, in which Trolox was less active. Again, using the DPPH assay PJ exerted a significant antioxidant activity, although lower than that displayed by Trolox.

Oki *et al.* [39] reported that anthocyanins are the most likely contributors to the DPPH scavenging activity of mulberry mature fruits. Interestingly these fruits contain fairly high amounts of cyanidin-3-glucoside and cyanidin-3-rutinoside [39], which are well represented in our juice.

PJ also displayed good antioxidant capacity (36.0 μmol Trolox equivalents/g of fruits), as measured with ORAC (Figure 1B). These data are in accordance with previously published ORAC values [37] for other small fruits such as *Vaccinium*, *Rubus* and *Ribes*, which displayed Trolox equivalent values ranging from 33.3 to 78.8 μmol/g fresh weight [37]. Monagas *et al.* [40] observed that the ORAC values in the grape skin were very close to the anthocyanins content, once again suggesting that the antioxidant capacity was mainly due to this type of compounds**.**

The second set of experiments was aimed at assessing the antioxidant activity of PJ in oxidatively-

Fig. 1A

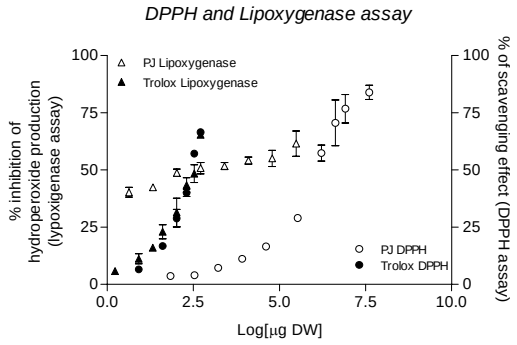


Fig. 1B

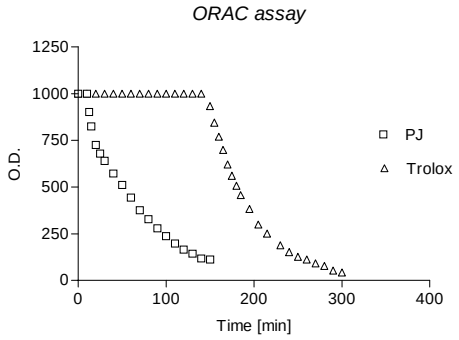


Figure 1A: Antioxidant capacity of PJ in lipoxygenase and DPPH assays. Results represent the percent inhibition of hydroperoxides production and the scavenging effect on DPPH radicals as a function of sample concentration. Trolox (closed symbols) was used as reference. The results represent the means +/- S.E.M. from 3 separate determinations.

Figure 1B: FL fluorescence decay curves induced by AAPH in the presence of 0.05 mg/mL of PJ and of 5 μM Trolox.

injured cultured cells. For this purpose, the effect of the juice on the cytotoxic response of U937 promonocytic cells exposed to H_2O_2 (Figure 2) was investigated. PJ was utilized in the same way in cell-free determinations, and the concentrations selected for this set of experiments(up to 0.01% v/v) were not cytotoxic *per se* (Figure 2, dotted line). Under the selected exposure conditions (see the Experimental section), treatment with 0.3 mM H_2O_2 caused a significant reduction of surviving cells (Figure 2) and, according to previously published observations [26,41-42], the mode of cell death was mainly necrotic. According to the data obtained in cell-free experiments, which indicate a valuable antioxidant activity for PJ, the juice was capable of significantly protecting cells from the oxidative insult in a dose-response fashion (Figure 2).

In conclusion *Prunus spinosa* fruits and juice could be considered as a valuable source of antioxidant

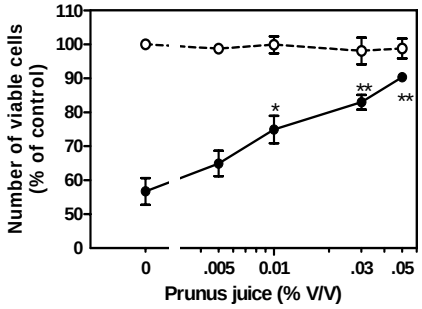


Figure 2: Effect of PJ on the cytotoxic response of U937 cells exposed to H_2O_2.
Cells were treated with 0.3 mM H_2O_2 in the absence or presence of increasing concentrations of PJ, as detailed in the Experimental section. The number of viable cells was determined 48 h post-challenge growth with the Trypan blue exclusion assay. The effect of PJ alone on the viability of U937 cells is also shown (open circles-dotted line). Results represent the means ± S.E.M. from three-five separate experiments, each performed in duplicate. * P <.01 and ** P < .001 (unpaired *t* test) compared to H_2O_2 alone -treated cells.

compounds for nutritional supplementation, as well as of herbal medicine.

Experimental

Plant material: *P. spinosa* fruits were collected in Urbino, Marche, central Italy, at 500 m above sea level, in November 2007 and identified by D. Fraternale. A voucher specimen is deposited in the herbarium of the Botanical Garden of the University of Urbino (P.S.F. 163).

Preparation of juice: PJ was prepared as follows: 10g of sloe pulp was crushed in a mortar, filtered over Whatman No 1 paper under vacuum, and the residue squeezed until exhaustion. The resulting juice, 6 mL, was then centrifuged at 2500 rpm for 10 min. All these steps were performed at ice bath temperature. The supernatant from the centrifugation step was recovered, microfiltered, aliquoted and immediately stored at – 20°C.

Total polyphenol content: Total content of polyphenolic compounds in fresh juice was determined by the Prussian Blue method described by Hagerman and Butler [43], with slight modifications. The optical density of the mixture was determined at 720 nm (Jasco V-530 spectrophotometer). Quercetin (Sigma) was used as standard to construct a calibration curve.

Total anthocyanin content (TA): Total anthocyanin content of the fruit juice of sloe was measured using the pH differential method reported by Elisia *et al.* [37] and Tzulker *et al.* [35]. Absorbancies were read

at 510 and 700 nm against a blank cell containing distilled and deionized water.

DPPH assay: Radical scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [44]. The absorbance decrease at 517 nm was recorded after 10 min and the percent decrease (corrected for the control, without antioxidant agents added) was taken as an index of the antioxidant capacity. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was used as positive control and purchased from Sigma.

Lipoxygenase test: Inhibition of lipid peroxide formation was evaluated by the 5-lipoxygenase (purchased from Sigma) test in the sample and positive controls. The activity of the enzyme was assayed spectrophotometrically according to Holman. This method was modified by Sud'ina *et al.* [45]. The formation of hydroperoxides from linoleic acid was observed spectrophotometrically at 235 nm at 20°C.

ORAC: The original method of Cao *et al.* [46] was used with slight modifications. Fluorescein (3^{l},6^{l}-dihydroxy-spiro[isobenzofuran-1[3*H*],9^{l}[9*H*]-xanthen]-3-one) (purchased from Sigma) was chosen as fluorescent probe instead of B-phycoerythrin (B-PE) [47]. The area under the curve (AUC) of fluorescence decay was proportional to the antioxidant capacity of the sample, and a comparative evaluation with Trolox was performed. The fluorescence was measured every 10 sec at 37°C using a JASCO FP-6200 spectrofluorometer at 485 nm excitation, and 520 nm emission until zero fluorescence was detected. The ORAC value was calculated with a Spectra Manager for Windows 95/NT (Spectra Analysis) program.

Cell culture and treatments: Cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2. Human promonocytic U937 cells were cultured in suspension in RPMI 1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin), 1.2 mM glutamine and 10% fetal bovine serum. For experiments, cells were resuspended at a number of 4 x 10^5 cells/treatment condition in 2 mL of Saline A (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L $NaHCO_3$ and 0.9 g/L glucose), prewarmed at 37°C. PJ was added to the cultures and equilibrated for 20 min before treatment with freshly prepared H_2O_2 (0.3 mM for 1 h). Cells were then washed and grown in extract- and H_2O_2–free culture medium.

Cytotoxicity assays: The cytotoxic response was evaluated with the trypan blue exclusion assay after 48 h post-challenge growth in complete culture medium: this time interval allows the quantification of the extent of growth arrest and cell death caused by the oxidant in treated cultures *vs* logarithmically growing control cells.

Briefly, after the post-challenge growth stage, an aliquot of each experimental sample's cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a hemocytometer. Results are expressed as percent ratio of viable (unstained) cells in treated *vs* untreated samples.

References

[1] Kumarasamy Y, Cox PJ, Jaspars M, Nahar L, Sarker SD. (**2003**) Cyanogenic glycosides from *Prunus spinosa* (Rosaceae). *Biochemical Systematics and Ecology*, ***31***, 1063-1065.

[2] Aparajita M, Juan Pedro M, Itziar A (**2002**) Population genetic analysis of European *Prunus spinosa* (Rosaceae) using chloroplast DNA markers. *American Journal of Botany*, ***89***, 1223-1228.

[3] Bollettino Ufficiale della Regione Marche. (**2002**) Anno XXXIII, n**.** ***36***, 20 maggio.

[4] Werner DJ, Maness EP, Ballinger WE. (**1989**) Fruit anthocyanins in three sections of *Prunus* (Rosaceae). *Horticultural Science*, ***24***, 488-489.

[5] Ramos T, Macheix JJ. (**1990**) Phenols of blackthorn (*Prunus spinosa* L.) fruit. *Plant Medicine and Phytotherapy*, ***24***, 14-20.

[6] Casado-Redin I, Fernandez M, Vega FA. (**1992**) Polyphenols in *Prunus spinosa* L. fruits during ripening period. *Bulletin de Liaison-Groupe Polyphenols*, ***16***, 108-111.

[7] Deineka VI, Deineka LA, Sirotin AA. (**2005**) Anthocyans from fruit of certain plants of the genus *Prunus. Chemistry of Natural Compounds*, ***41***, 230-231.

[8] Cao G, Muccitelli HU, Sanchez-Moreno C, Prior RL. (**2001**) Anthocyanins are adsorbed in glycated forms in elderly women: a pharmacokinetic study. *American Journal of Clinical Nutrition*, ***73***, 920-926.

[9] Youdim KA, Martin A, Joseph JA. (**2000**) Incorporation of the elderberry anthocyanins by endothelian cells increases protection against oxidative stress. *Free Radical Biology and Medicine*, ***29***, 51-60.

[10] Lapidot T, Harel SS, Akiri B, Granit R, Kanner J. (**1999**) pH-dependent forms of red wine anthocyanins as antioxidants. *Journal of Agricultural and Food Chemistry*, ***47***, 67-70.

[11] De Groot H, Rauen U. (**1998**) Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundamental and Clinical Pharmacology*, ***12,*** 249-255.

[12] Cowan MM. (**1999**) Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, ***12***, 564-582.

[13] Wang SY, Lin S. (**2000**) Antioxidant activity in fruits and leaves of blackberry, raspberry and strawberry varies with cultivar and development stage. *Journal of Agricultural and Food Chemistry*, ***48***, 140-146.

[14] Frankel EN, Waterhouse AL, Teissedre PL. (**1995**) Principal phenolic phytochemicals in selected California wines and their antioxidant activity in inhibiting oxidation of human low-density lipoproteins. *Journal of Agricultural and Food Chemistry*, ***43***, 890-894.

[15] Burns J, Gardner PT, O'Neil J, Crawford S, Morecroft I, McPhail DB, Lister C, Matthews D, MacLean MR, Lean ME, Duthie GG, Crozier A. (**2000**) Relationship among antioxidant activity, vasodilation capacity, and phenolic content of red wines. *Journal of Agricultural and Food Chemistry*, ***48***, 220-230.

[16] Amou A, Makris DP, Kefalas P. (**2002**) Correlation of pigment and flavanol content with antioxidant properties in selected aged regional wines from Greece. *Journal of Food Composition and Analysis*, ***15***, 655-665.

[17] Kallithraka S, Mohdalya AAA, Makris DP, Kefalas P. (**2005**) Determination of major anthocyanin pigments in Hellenic native grape varieties (*Vitis vinifera* sp.): association with antiradical activity. *Journal of Food Composition and Analysis*, ***18***, 375-386.

[18] Duthie GG, Duthie SJ, Kyle JAM. (**2000**) Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. *Nutrition Research Reviews*, ***13***, 79-106.

[19] Ramirez –Tortosa C, Andersen OM, Gardner PT, Morrice PC, Wood SG, Duthie SJ, Collins AR, Duthie GG. (**2001**) Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats. *Free Radical Biology and Medicine*, ***31***, 1033-1037.

[20] Aherne SA, O' Brien M. (**2000**) Mechanism of protection by the flavonoids, quercetin and rutin, against tert-butyl-hydro-peroxide- and menadione-induced DNA single strand breaks in CACO-2 cells. *Free Radical Biology and Medicine*, ***29***, 507-514.

[21] Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T. (**1996**) Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. *Biochemical Pharmacology*, ***52***, 1033-1039.

[22] Wang CJ, Wang JM, Lin WL, Chu CY, Chou FP, Tseng TH. (**2000**) Protective effects of *Hibiscus* anthocyanins against tert-butyl-hydroperoxide-induced hepatic toxicity in rats. *Food and Chemical Toxicology*, ***38***, 411-416.

[23] Tsuda T, Kato Y, Osawa T. (**2000**) Mechanism for the peroxynitrite scavenging activity by anthocyanins. *FEBS Letters*, ***484***, 207-210.

[24] Kerry NL, Abbey M. (**1997**) Red wine and fractionated phenolic compounds prepared from red wine inhibit low density lipoprotein oxidation *in vitro*. *Atherosclerosis*, ***135***, 93-102.

[25] Sarma AD, Sharma R. (**1999**) Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage. *Phytochemistry*, ***52***, 1313-1318.

[26] Sestili P, Martinelli C, Ricci D, Fraternale D, Bucchini A, Giamperi L, Curcio R, Piccoli G, Stocchi V. (**2007**) Cytoprotective effect of preparation from various parts of *Punica granatum* L. fruits in oxidatively injured mammalian cells in comparison with their antioxidant capacity in cell free systems. *Pharmacological Research*, ***56***, 18-26.

[27] Kovacic P, Thurn LA. (**2005**) Cardiovascular toxicity from the perspective of oxidative stress, electron transfer, and prevention by antioxidants. *Current Vascular Pharmacology*, ***3,*** 107-117.

[28] Urquiaga I, Leighton F. (**2000**) Plant polyphenol antioxidants and oxidative stress. *Biological Research*, ***33***, 55-64.

[29] Kumarasamy Y, Cox PJ, Jaspars M, Nahar L, Sarker SD. (**2004**) Comparative studies on biological activities of *Prunus padus* and *Prunus spinosa*. *Fitoterapia*, ***75***, 77-80.

[30] Kiselova Y, Ivanova D, Chervenkov T, Gerova D, Galunska B, Yankova T. (**2006**) Correlation between the *in vitro* antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. *Phytotherapy Research*, ***20***, 961-965.

[31] Sellappan S, Akoh CC, Krewer G. (**2002**) Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *Journal of Agricultural and Food Chemistry*, ***50***, 2432-2438.

[32] Rababah TM, Ereifej KI, Howard L. (**2005**) Effect of ascorbic acid and dehydration on concentration of total phenolics, antioxidant capacity, anthocyanins, and color in fruits. *Journal of Agricultural and Food Chemistry*, ***53***, 4444-4447.

[33] Solomon A, Golubowiez S, Yablowiez Z, Grossman S, Bergman M, Gottlieb HE, Altman A, Kerem Z, Flaishman MA. (**2006**) Antioxidant activities and anthocyanin content of fresh fruits of common fig (*Ficus carica* L.). *Journal of Agricultural and Food Chemistry*, ***54***, 7717-7723.

[34] Rapisarda P, Tomaino A, Lo Cascio R, Bonina F, De Pasquale A, Saija A. (**1999**) Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. *Journal of Agricultural and Food Chemistry*, ***47***, 4718-4723.

[35] Tzulker R, Glazer I, Bar-Ilan I, Holland D, Aviram M, Amir R. (**2007**) Antioxidant activity, polyphenol content, and related compounds in different fruit juice and homogenates prepared from 29 different pomegranate accessions. *Journal of Agricultural and Food Chemistry*, ***55***, 9559-9570.

[36] Ranilla LG, Genovese MI, Lajolo FM. (**2007**) Polyphenols and antioxidant capacity of seed coat and cotyledon from Brazilian and Peruvian bean cultivars (*Phaseolus vulgaris* L.). *Journal of Agricultural and Food Chemistry*, ***55***, 90-98.

[37] Elisia I, Hu C, Popovich DG, Kitts DD. (**2007**) Antioxidant assessment of an anthocyanin- enriched blackberry extract. *Food Chemistry*, ***101***, 1052-1058.

[38] Zafra-Stone S, Yasmin T, Bagchi M, Chatterjee A, Vinson JA, Bagchi D. (**2007**) Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition and Food Research*, ***51***, 675-683.

[39] Oki T, Kobayashi M, Nakamura T, Okuyama A, Masuda M, Shiratsuchi H, Suda I. (**2006**) Changes in radical-scavenging activity and components of mulberry fruit during maturation. *Journal of Food Science*, ***71***, 18-21.

[40] Monagas M, Hernandez-Ledesma B, Gómez-Cordovés C, Bartolomé B. (**2006**) Commercial dietary ingredients from *Vitis vinifera* L. leaves and grape skins: antioxidant and chemical characterization. *Journal of Agricultural and Food Chemistry*, ***54***, 319-327.

[41] Palomba L, Sestili P, Columbaro M, Falcieri E, Cantoni O. (**1999**) Apoptosis and necrosis following exposure of U937 cells to increasing concentrations of hydrogen peroxide: the effect of the poly(ADP-ribose)polymerase inhibitor 3-aminobenzamide. *Biochemical Pharmacology*, ***58***, 1743-1750.

[42] Tommasini I, Sestili P, Cantoni O. (**2002**) Delayed formation of hydrogen peroxide mediates the lethal response evoked by peroxynitrite in U937 cells. *Molecular Pharmacology*, ***61***, 870-878.

[43] Hagerman AE, Butler LG. (**1994**) Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Methods in Enzymology*, ***234***, 429-429.

[44] Mellors A, Tappel AL. (**1966**) The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. *Journal of Biological Chemistry*, ***241***, 4353-4356.

[45] Sud'ina GF, Mirzoeva OK, Pushkareva MA, Korshunova GA, Sumbatyan NV, Varfolomeev SD. (**1993**) Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. *FEBS Letters*, ***329***, 21-24.

[46] Cao G, Alessio HM, Culter R. (**1993**) Oxygen-radical absorbance capacity assay for antioxidants. *Free Radical Biology and Medicine*, ***14***, 303-311.

[47] Ou B, Hampsch-Woodill M, Prior L. (**2001**) Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry*, ***49***, 4619-4626.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1671 - 1674

ESI-MS, ESI-MS/MS Fingerprint and LC-ESI-MS Analysis of Proathocyanidins from *Bursera simaruba* Sarg Bark

Mariateresa Maldini, Paola Montoro, Sonia Piacente and Cosimo Pizza

Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte don Melillo, 84084, Fisciano (Sa), Italy

pmontoro@unisa.it

Received: September 2nd, 2009; Accepted: October 6th, 2009

Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry was used to investigate the presence of proanthocyanidins (PAs) in the methanolic extract of *B. simaruba* bark. Additionally, an LC-ESI-MS qualitative study was performed by using a monolithic stationary phase. The fragmentation pattern obtained evidenced the presence in *B. simaruba* bark of PAs belonging to the series of polymers of epicatechin, along with their glycosilated derivatives.

Keywords: *Bursera simaruba* Sarg, proanthocyanidins, ESI-MS, LC-MS.

Bursera simaruba Sarg. (Burseraceae), (red gumbolimbo bark, indio desnudo), is a commonly diffused tree in Venezuela, Belize and in Central America [1]. In these countries it is traditionally used as an antidote to poisonwood sap, to treat insect bites, sunburn, rashes, skin sores, internal infections, fevers, colds and flu [2]. In addition, an interesting anti-inflammatory activity of this plant has been recently reported [3, 4]. The bark of this species is reported to be a rich source of phenolic compounds, in particular it is a good source of lignans with cytotoxic activity as yatein, β-peltatin-*O*-β-D-glucopyranoside, hinokinin and bursehernin [5]. In the present work, in the same extract, epicatechin was isolated, and identified by comparison of spectroscopic and spectrometric data with those present in literature [6]. In addition, we have evidenced the presence of proanthocyanidins, for the first time, in the methanolic extract of *B. simaruba* bark by mass spectrometry analyses.

Proanthocyanidins (condensed tannins, PAs) consisting of oligomers and polymers of flavan-3-ol units are the most widely distributed type of tannins in the plant kingdom. Dietary PAs are hypothesized to be beneficial, possibly due to their antioxidant properties and their ability to complex with macromolecules and metal ions, and they are supposed to play a role in anti-inflammatory activity of several plants [7]. Antinflammatory activity probably is related to their antioxidant and scavenging activities [8] and to their inhibition of arachidonic acid metabolism via both cyclo-oxygenase and lypoxygenase pathways [9]. Due to the complexity of PAs structures we have proposed to rationalise the presence of these compounds on the basis of ESI-MS and ESI-MS/MS profiles.

Direct flow injection/electrospray ionization/ion trap tandem mass spectrometry was used to investigate polyphenolic compounds in the methanolic extract of *B. simaruba*. In a second stage analytical HPLC-ESI-MS and HPLC-ESI-MS/MS was developed.

PAs are very complex to separate with conventional C18 stationary phases. In our study we performed the analysis by using a monolithic C18 column, a stationary phase described to produce better resolution in different analytical fields [10]. Monoliths are rod-shaped continuous bed silica or polymeric materials, which offer an alternative to conventional particle-packed columns for analytical and preparative liquid chromatography.

ESI-MS and ESI-MS/MS fingerprint. In order to obtain qualitative information on proanthocyanidins in *B. simaruba* extract, a sample rich of these compounds was prepared and directly injected into ESI source of the mass spectrometer. Analysis of proanthocyanidins was performed in negative ion mode since it has been demonstrated for this class of

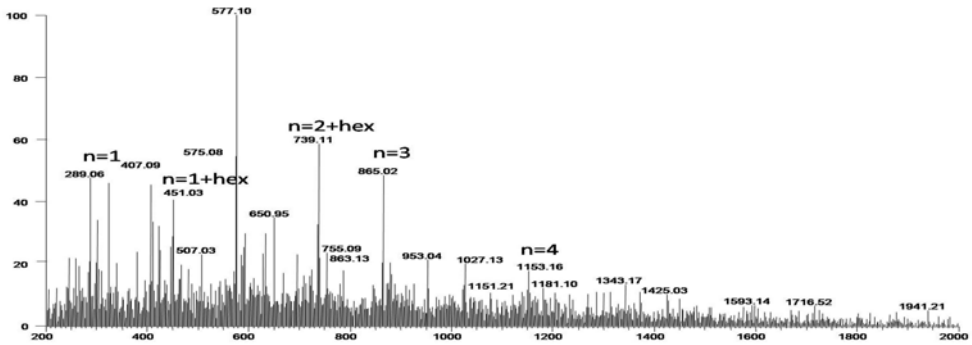


Figure 1: Typical deprotonated ESI-IT-MS fingerprint of methanolic extract of *Bursera simaruba* bark obtained in negative ion mode. n= number of epicatechin units,hex=hexose unit.

compound that negative ionization is more sensitive and selective than the positive one [11]. Figure 1 shows the ESI-MS fingerprint obtained at normal masses scan indicating the $[M-H]^-$ ions of the proanthocyanidins. The fragmentation pattern obtained evidenced the presence of glycosilated proanthocyanidins and polymeric proanthocyanidins. Together with the ion relative to epicatechin at *m/z* 289, a first series of abundant ions separated by 288 Da corresponding to ion peaks of dimeric (*m/z* 577), trimeric (*m/z* 865) and tetrameric (*m/z* 1153) procyanidins, respectively, were observed. Mass spectra also showed ions corresponding to glycosilated epicatechin (*m/z* 451) and glycosilated dimeric proanthocyanidin (*m/z* 739).

In order to verify the results described above, second-order ESI-IT-MS/MS experiments for dimeric proanthocyanidin and glycosilated dimeric proanthocyanidin were performed.

The MS^2 spectrum of the ion at *m/z* 577 (Figure 2) showed major product ions at values of 451, 425, 407 and 289 *m/z*. The ion at *m/z* 451 was derived from loss of 126 mass unit corresponding to the heterocyclic ring fission (HRF) fragmentation. The ion at *m/z* 425 $[M-152-H]^-$ was due to retro-Diels-Alder fragmentation (RDA). RDA yielded the product ion at *m/z* 407 $[M-152-18-H]^-$ due to the neutral loss of the water. Finally, the ion at *m/z* 289 was attributed to Quinone methide (QM) fragmentation $[M-288-H]^-$. This pattern of fragmentation is in accordance with those reported by Rodrigues *et al*. [11]. The product ion spectrum of the glycosilated dimeric procyanidin was also investigated. The MS^2 spectrum of the ion at *m/z* 739 (Figure 3) showed major product ions at values of 587, 577, 451, 435 and 289 *m/z*.

The ion at *m/z* 577 was derived from the neutral loss of the sugar moiety $[M-162-H]^-$ followed by the loss

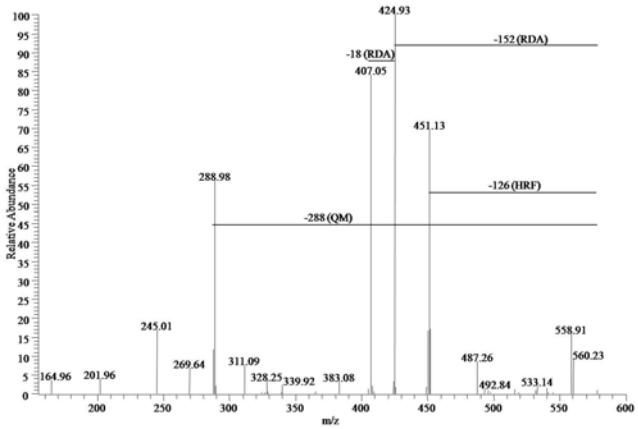


Figure 2: MS/MS spectrum of the ion at *m/z* 577.

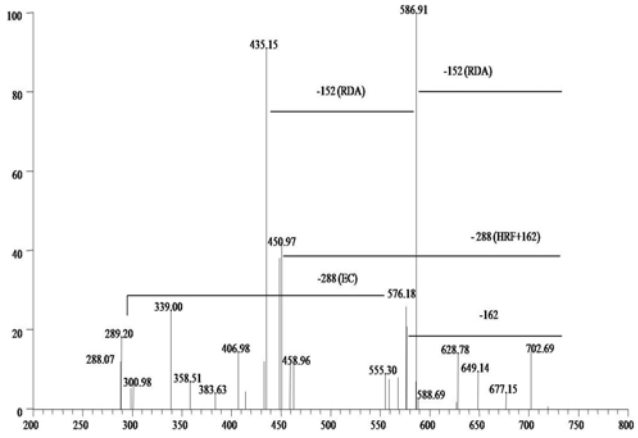


Figure 3: MS/MS spectrum of the ion at *m/z* 579.

of epicatechin unit at *m/z* 289 $[M-288-H]^-$ relative to QM fragmentation. The ions at *m/z* 587 and 435 were due to two subsequent retro Diels-Alder (RDA) fragmentations consecutive $[M-152-H]^-$ and $[M-152-152-H]^-$ respectively. Finally the ion at *m/z* 451 was derived from the loss of the ring A and the sugar unit due to HRF fragmentation. This fragmentation pattern allowed us to suppose the location of the sugar moiety only at C-5 or C-7 of ring A. Figure 4 shows the fragmentation pathway proposed for the

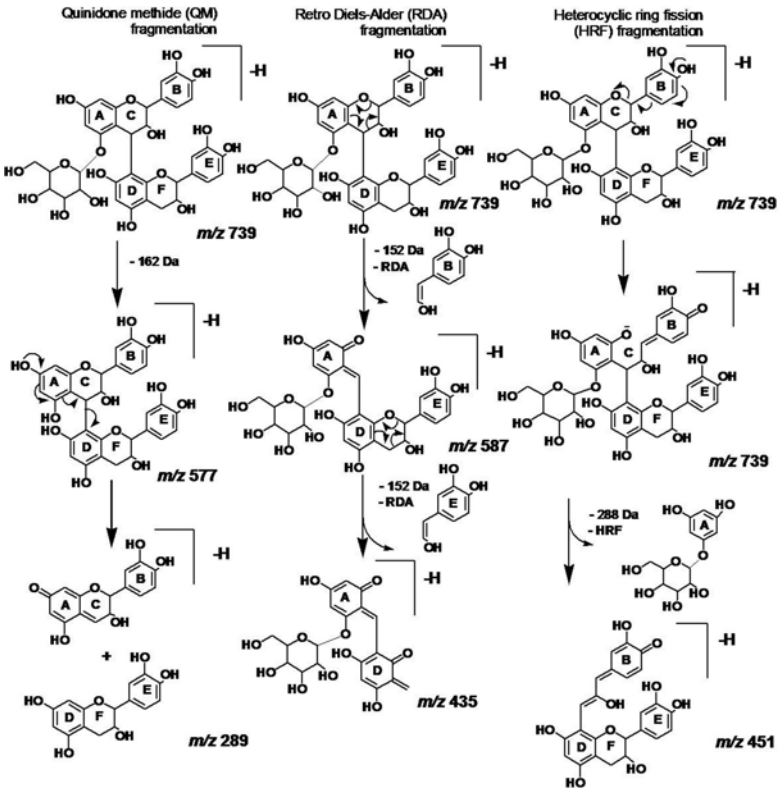


Figure 4: Main fragmentation pathways proposed for the monoglycosylated epicatechin dimer identified in the methanolic extract of *B. simaruba* barks.

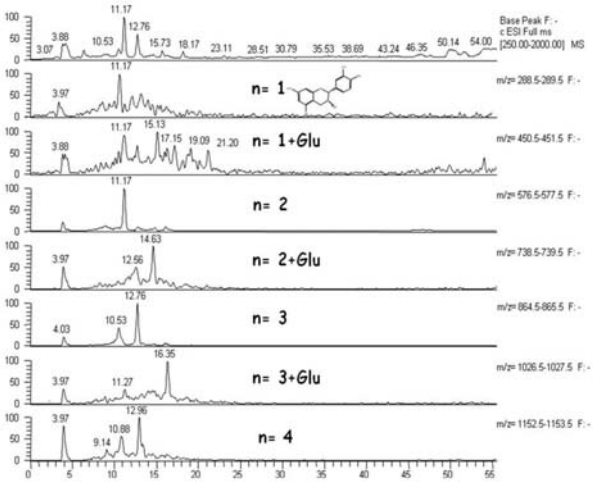


Figure 5: LC-ESI-MS RICs (Reconstructed Ion Chromatograms) of a sample enriched in proanthocyanidins from *B. simaruba* bark.

glycosilated dimeric proanthocyanidin occurring in *B. simaruba*.

LC-ESI-MS analysis: In order to realise a qualitative analysis on the epicatechin derivatives in *B. simaruba* bark methanolic extract, MS experiments were performed by using an LC-MS system equipped with an ES source and an Ion Trap analyser. Negative ion electrospray LC-ESI-MS analysis obtained in the acquisition range of 250-2000 amu (atomic mass unity), total ion current (TIC) profile and reconstructed ion chromatograms (RICs) of the methanolic extract of *B simaruba* bark are shown in Figure 5. LC-ESI-MS analysis confirmed the presence of compounds related to epicatechin.

The 250-2000 *m/z* range allowed to confirm the presence of oligomers with to four epicatechin units, but the occurence of oligomers based on a major number of epicatechins cannot be excluded. The profiles obtained for monomers appear more complex if compared to those obtained for oligomers, maybe for the occurrence of product ions generated by source fragmentation of oligomers.

Experimental

Materials: HPLC grade methanol (MeOH), acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Merck (Merck KGaA, Darmstadt, Germany). HPLC grade water (18mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., Bedford, MA). The reagents used for the extractions, of analytical grade, were purchased from Carlo Erba (Rodano, Italy). Column chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Plant material: Plant material was collected in Belize in February 1999 and authenticated by

Professor M.J. Balick. Voucher specimens were dried and deposited at the New York Botanical Garden (NY, USA).

Extraction and sample preparation: The air-dried plant material (267,8 g of *B. simaruba* bark) was extracted for three days, two times, at room temperature, using solvents of increasing polarity as petroleum ether 1 L, chloroform 1 L and methanol 1 L. The solvents were removed from the filtered extracts under vacuum at 30 °C in a rotary evaporator obtaining respectively 1 g of dried petroleum ether extract; 2 g of chloroform extract and 20 g of methanol extract. Part of the methanol extract (3 g) was fractionated initially on a 100×5.0 cm Sephadex LH-20 column, using CH_3OH as mobile phase, and 120 fractions (8mL each) were obtained. Fractions grouped on the basis of their chromatographic homogeneity (preliminary MS investigation) were analyzed by ESI-MS. Fraction 32 consisted in pure epicatechin, identified by NMR and ESI-MS. A sample enriched in proanthocyanidins was prepared by combining fractions (32-120) containing proanthocyanidins.

Equipment: IT-ESI-MS, IT-ESI-MS/MS and LC-ESI-MS analysis were performed using a Thermo Finnigan Spectra System HPLC coupled with an LCQ Deca ion trap. Chromatography was performed on an RP C18 monolithic column Onyx.

ESI-MS and ESI-MS/MS analyses: Full scan ESI-MS and collision induced dissociation (CID) ESI-MS/MS analyses of sample were performed on a Thermo Electron (San Josè, CA, USA) LCQ Deca IT spectrometer equipped with an ion trap analyser. Sample was infused directly into the source at a flow rate of 5 μL/min. The capillary voltage was -4 V, the spray voltage was 5 kV, the capillary temperature was 270°C, sheath gas (nitrogen) flow rate 80 (arb) and auxiliary gas flow rate was 5(arb). Data were acquired in the negative ion MS and MS/MS modes.

LC-ESI-MS analysis: For qualitative LC-ESI-MS, a gradient elution was performed on an Onyx Monolithic C18 column (Phenomenex, USA), 100 x 4.6 mm, by using a mobile phase A represented by water acidified with TFA (0.05%) and a mobile phase B represented by acetonitrile acidified with TFA (0.05%). The gradient started from 0% of eluent B remained to achieve the 80% of solvent B in 55 min. The flow (250 μL min^{-1}) generated by chromatographic separation was directly injected into the electrospray ion source. The mass spectrometer was operating in the negative ion mode under the following conditions: capillary voltage -7 V, spray voltage 5 kV, tube lens offset 10 V, and capillary temperature 280°C and sheath gas (nitrogen) flow rate 80 (arb). MS spectra were acquired and elaborated using the software provided by the manufacturer.

References

[1] Badillo VM, Schnee L, Benitez de RC. (**1985**) *Clave de las Familias de Plantas Superiores de Venezuela*, 7th ed., Caracas.

[2] Arvigo R, Balik M. (**1993**). Rainforest remedies. In: *One Hundred Healing Herbs of Belize*. Lotus Press, Twin Lakes, WI.

[3] Carretero ME, Lòpez-Perez JL, Abad MJ, Bermejo P, Tillet S, Israel A, Noguera-P B. (**2008**) Preliminary study of the anti-inflammatory activity of hexane extract and fractions from *Bursera simaruba* (Linneo) Sarg. (Burseraceae) leaves *Journal of Ethnopharmacology*, *116*, 11-15.

[4] Sosa S, Balick MJ, Arvigo R, Esposito RG, Pizza C, Altinier G, Tubaro A. (**2002**) Screening of the topical anti-inflammatory activity of some Central American plants. *Journal of Ethnopharmacology*, *81*, 211-215.

[5] Maldini M, Montoro P, Piacente S, Pizza C. (**2009**) Phenolic compounds from *Bursera simaruba* Sarg. bark: phytochemical investigation and quantitative analysis by tandem mass spectrometry. *Phytochemistry*, *122*, 430-433.

[6] Roy B, Dutta S, Chowdhary A, Basak A, Dasgupta S. (**2008**) Design, synthesis and RNase A inhibition activity of catechin and epicatechin and nucleobase chimeric molecules. *Bioorganic & Medicinal Chemistry Letters*, *18*, 5411-5414.

[7] Benavides A, Montoro P, Bassarello C, Piacente S, Pizza C. (**2006**) Flavonoids and isoflavonoids from *Gynerium sagittatum.* *Journal of Pharmaceutical and Biomedical Analysis*, *40*, 639-647.

[8] Goncalves, C, Dinis T; Batist, MT. (**2005**) Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: a mechanism for anti-inflammatory activity. *Phytochemistry, 66*, 89-98.

[9] Zhang W, Liu H, Xie K, Yin L, Li Y, Kwik-Uribe CL, Zhu X. (**2006**) Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of cyclooxygenase-2 in endotoxin-treated monocytic cells. *Biochemical and Biophysical Research Communications*, *345*, 508-515.

[10] Ikegami T, Tanaka N. (**2004**) Monolithic columns for high-efficiency HPLC separations. *Current Opinion in Chemical Biology*, *8*, 527-533.

[11] Rodrigues CM, Rinaldo D, dos Santos L, Montoro P, Piacente S, Pizza C, Hiruma-Lima CA, Brito ARMS, Vilegas W. (**2007**) Metabolic fingerprinting using direct flow injection electrospray ionization tandem mass spectrometry for the characterization of proanthocyanidins from the barks of *Hancornia speciosa*. *Rapid Comunications in Mass Spectrometry*, *21*, 1907-1914.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1675 - 1678

Chemical Composition and Antimycobacterial Activity of the Essential Oil from *Anemia tomentosa* var. *anthriscifolia*

Shaft Corrêa Pinto[a], Gilda Guimarães Leitão[a], Danilo Ribeiro de Oliveira[a], Humberto Ribeiro Bizzo[b], Daniela Fernandes Ramos[c], Tatiane Silveira Coelho[c], Pedro Eduardo A. Silva[c], Maria Cristina S. Lourenço[d] and Suzana Guimarães Leitão[e,*]

[a]*Universidade Federal do Rio de Janeiro, Núcleo de Pesquisas de Produtos Naturais, CCS, Bloco H, Ilha do Fundão, 21941-590, Rio de Janeiro, Brazil*

[b]*Embrapa Agroindústria de Alimentos, Avenida das Américas 29501, 23020-470, Rio de Janeiro, RJ, Brazil*

[c]*Universidade Federal do Rio Grande, FURG, Laboratório de Micobactérias, Rio Grande, RS, Brazil*

[d]*Instituto de Pesquisa Clínica Evandro Chagas, Plataforma de Bioensaios II, FIOCRUZ, 21045-900, Rio de Janeiro, Brazil*

[e]*UFRJ, Faculdade de Farmácia, Departamento de Produtos Naturais e Alimentos, CCS, Bloco A, 2o andar, Ilha do Fundão, 21941-590, Rio de Janeiro, RJ, Brazil*

sgleitao@pharma.ufrj.br

Received: August 2nd, 2009; Accepted: October 12th, 2009

The essential oil from *Anemia tomentosa* var. *anthriscifolia* showed *in vitro* activity against *Mycobacterium tuberculosis* (MIC 100 μg/ml) and therefore was characterized by gas chromatography (GC) and by gas chromatography coupled with mass spectrometry (GC-MS). The major constituents of this essential oil were triquinane sesquiterpenes: silphiperfol-6-ene (14.7%), (–)-*epi*-presilphiperfolan-1-ol (30.6%), presilphiperfol-7-ene (3.9%), cameroonan-7α-ol (4.4%), prenopsan-8-ol (1.9%) and presilphiperfolan-8-ol (8.3%), suggesting the existence of different chemotypes for this species. The essential oil was fractionated by column chromatography and its major constituent and fractions were assayed against *Mycobacterium tuberculosis* and *M. smegmatis*. (–)-*epi*-Presilphiperfolan-1-ol exhibited an MIC of 120 μg/ml against *M. tuberculosis* H37Rv.

Keywords: *Anemia tomentosa*, triquinane sesquiterpenes, *Mycobacterium tuberculosis,* Pteridophyta, tuberculosis.

Ferns are abundant in the fossil record and today include about 11,000 species, placing this group as one of the largest, after flowering plants. They are the most diverse in form and habit, and their diversity is greatest in the tropics, where approximately 75% of its population is distributed [1]. The genus *Anemia* occurs mainly in the Americas [2]. In Brazil, species from this genus are found in the central and southeast regions, where many of them are endemic [3a]. *Anemia tomentosa* (Savigny) Swartz var. *anthriscifolia* (Schrader) Mickel occurs in rocky regions and has very aromatic leaves. This variety is predominant over the four other known varieties [3b]. The leaves of *A. tomentosa* var. *tomentosa* are used as a digestive aid, expectorant and antigripal [4]. In previous studies, the essential oil of *A. tomentosa* var. *anthriscifolia* showed antimicrobial activity and mosquito repellent activity [5a,5b]. The present investigation deals with the chemical and antimycobacterial studies on essential oil of *A. tomentosa* var. *anthriscifolia*.

The essential oil was characterized by gas chromatography (GC) and by gas chromatography coupled with mass spectrometry (GC-MS) analysis. Sixty compounds were detected in the chromatogram. Thirty substances were identified by mass spectrometry and retention indices (Table 1), of which, the major components were triquinane sesquiterpenes such as silphiperfol-6-ene (14.7%), (–)-*epi*-presilphiperfolan-1-ol (30.6%), presilphiperfol -7-ene (3.9%), cameroonan-7α-ol (4.4%), prenopsan-8-ol (1.9%) and presilphiperfolan-8-ol (8.3%), which accounted for a total of 63.8 % of the total substances in the oil.

The biosynthetic origin of the triquinane sesquiterpenes from the caryophyllenyl ion was originally proposed by Bohlmann [6a,6b] based on the co-occurrence of the triquinane sesquiterpenes isocomene, modhephene, silphiperfolenes and silphinenes along with caryophyllene in *Silphium perfoliatum* L. (Asteraceae) [6a]. The co-occurrence of isocomene, modhephene, and caryophyllene in *Isocoma wrightii* (Asteraceae) was first noted by Zalkow *et al*. [6c,6d]. Additional evidence came from the report of the co-occurrence of silphiperfolenes and silphinenes with presilphiperfolanol sesquiterpenes in *Flourensia heterolepis* (Asteraceae) [6b]. Subsequently, Weyerstahl *et al*. described the presence of sesquiterpene skeletons of the type silphiperfolane, presilphiperfolane, isocomene, modhephene and caryophyllene in the essential oil of *Echinops giganteus* var. *lelyi* C. D. Adams (Asteraceae). Three new types of triquinane skeletons - cameroonane, prenopsane and nopsane - were elucidated. Based on these data, Weyerstahl proposed a biosynthetic route that covered all of those constituents, indicating a possible interrelationship between them [7]. The similarity between the compositions of essential oils from *Echinops giganteus* var. *lelyi* and from *A. tomentosa* var. *anthriscifolia* gives additional support to the biosynthetic correlation proposed by Weyerstahl. The essential oil from *A. tomentosa* var. *anthriscifolia* (Anemiaceae) is the second one that presents a large amount of triquinane sesquiterpenes in its composition biosynthetically correlated between them.

The essential oils from the plants of *A. tomentosa* have already been described in the literature. Studies from Juliani *et al*. with plants collected in Argentina described α-bisabolol as its major constituent [3b], while Santos *et al*. described isoafricanol as the main sesquiterpene from the oil obtained from a specimen collected in Rio de Janeiro, Brazil [8a]. In both studies, the presence of triquinane sesquiterpenes was not described. These data suggest the existence of different chemotypes for this species.

The essential of *A. tomentosa* var. *anthriscifolia* oil was fractionated by liquid column chromatography to access the antimycobacterial activity of its major constituent, (–)-*epi*-presilphiperfolan-1-ol, and fractions. By this procedure, 18 fractions were generated (A1 to A18) and characterized by GC-MS (Table 2). The triquinane sesquiterpene (–)-*epi*-presilphiperfolan-1-ol [16] was isolated in fraction A9 with 99% purity (by GC-FID) (Table 2) which showed lower antimycobacterial activity (MIC of 120 μg/mL) than the essential oil. The antimycobacterial activities of fractions A1 to A-18 are shown in Table 2. Fraction A1, consisting of unsaturated hydrocarbon compounds, mainly α-guaiene and silphiperfol-6-ene, showed an MIC of 25 μg/mL.

Table 1: Chemical composition of the essential oil from *A. tomentosa var. anthriscifolia*.

Compound	$RI_{calc.}$	$RI_{lit.}$	%	IM[#]
α-Pinene	938	939	0.1	1, 2
trans-Sabinol	1142	1142	0.6	1, 2
trans-2-Caren-4-ol	1149	-	0.2	2
Pinocarvone	1166	1165	0.2	1, 2
p-Menta-1,5-dien-8-ol	1170	1170	0.1	1, 2
cis-Pinocamphone	1178	1175	0.1	1, 2
Thymol	1293	1290	0.1	1, 2
Silphiperfol-5-ene	1327	1329	0.6	1, 2
Presilphiperfol-7-ene	1334	1337	3.9	1, 2
7-*epi*-Silphiperfol-5-ene	1346	1348	1.6	1, 2
α-Cubebene	1348	1351	0.1	1, 2
Silphiperfol-4,7(14)-diene	1359	1361	0.1	1, 2
Longicyclene	1370	1374	1.0	1, 2
Silphiperfol-6-ene	1378	1379	14.7	1, 2
α-Isocomene	1388	1388	0.1	1, 2
β-Elemene	1393	1391	0.2	1, 2
(*E*)-Caryophyllene	1421	1419	0.6	1, 2
α-Guaiene	1437	1440	5.2	1, 2
α-Muurolene	1482	1480	0.1	1, 2
Cameroonan-7-α-ol	1509	1512	4.4	1, 2
(–)-*epi*-Presilphiperfolan-1-ol	1518	-	30.6	3
Silphiperfolan-7-β-ol	1523	1521	0.7	1, 2
Nopsan-4-ol	1529	1531	1.0	1, 2
Silphiperfolan-6-β-ol	1546	1548	1.1	1, 2
Prenopsan-8-ol	1575	1576	1.9	1, 2
Presilphiperfolan-8-ol	1584	1586	8.3	1,2
di-*epi*-α-Cedrene epoxide	1591	-	0.4	2*
β-Atlantol	1610	1608	0.7	1, 2
Caryophylla-4(14),8(15)-dien-5-α-ol	1638	1641	1.0	1, 2
Ishwarone	1681	1682	0.5	1, 2
α-Bisabolol	1687	1686	0.8	1, 2
Monoterpene hydrocarbons			0.1	
Oxygen containing monoterpenes			1.2	
Sesquiterpene hydrocarbons			28.2	
Oxygen containing sesquiterpenes			51.4	
Total identified			81.0	

[#]Identification methods: 1- retention indices; 2- Wiley library; 3- ^{1}H and ^{13}C-NMR [8]. *tentative identification.

Haermers *et al*. [9a] reported the high lipophilicity of substances as an important feature for antimycobacterial activity. Since the cell wall of mycobacteria contain lipophilic substances such as mycolic acid, more lipophilic substances are likely to penetrate more easily into the cell [9b]. This may partially explain why the fraction containing the unsaturated sesquiterpenes (A1) showed higher activity against the *M. tuberculosis*. However, lipophilicity is not the only requirement for antimycobacterial activity, since A3, also containing unsaturated sesquiterpenes (but also unidentified oxygenated sesquiterpenes), displayed a higher MIC than A1. β-bisabolene and caryophyllene oxide, which were identified in fraction A3, were not

Table 2: Composition (GC-MS/GC-FID) of the fractions from the essential oil of *A. tomentosa* var. *anthriscifolia* and their minimum inhibitory concentration (MIC, in μm/mL) against *Mycobacterium tuberculosis* (H37Rv) and *M. smegmatis* (mc^2155).

Fraction	Identified Constituents (%)	MIC	
		H37Rv	mc^2 155
A1	α-Guaiene (17.3), Silphiperfol-6-ene (39.6)	50	200
A3	Silphiperfol-5-ene (1.8), Presilphiperfol-7-ene (8.5), Silphiperfol-6-ene (48.0), α-Isocomene (1.0), β-Elemene (0.5), *E*-Caryophyllene (2.3), α-Guaiene (17.0), α-Muurolene (0.4), β-Bisabolene (5.0), Caryophyllene oxide (0.7)	100	200
A5	Pinocarvone (0.5), Presilphiperfol-7-ene (0.3), Silphiperfol-6-eno (0.1), Cameroonan-7-α-ol (18.9), Silphiperfolan-7-β-ol (2.1), Prenopsan-8-ol (7.3), Presilphiperfolan-8-ol (31.3)	100	200
A7	(–)-*epi*-Presilphiperfolan-1-ol (89.0), Prenopsan-8-ol (2.0), di-*epi*-α-Cedrene epoxide (t), Cameroonan-7-α-ol (t), Ishwarone (t)	100	200
A8	(–)-*epi*-Presilphiperfolan-1-ol (91.0)	100	200
A9	(–)-*epi*-Presilphiperfolan-1-ol (99.0)	120	-
A18	*trans*-Sabinol (1.6), (–)-*epi*-Presilphiperfolan-1-ol (0.9), Silphiperfolan-7-β-ol (1.4), Silphiperfolan-6-β-ol (31.2)	R	-

t < 0.1%; R – resistant; - not tested

detected in the crude oil and may be artifacts that formed during essential oil fractionation procedures [10a,10b]. Fractions A5 to A8, containing sesquiterpene alcohols, showed the same MIC as the crude oil. Fractions A10 to A17 showed similar chromatographic profiles by TLC and GC, consisting basically of (–)-*epi*-presilphiperfolan-1-ol in different degrees of purity (always above 80%, data not shown) and therefore were not screened for antimycobacterial activity nor fully characterized by GC-MS. The major compound of fraction A18 is silphiperfolan-6β-ol (31.2%, Table 2), which is an isomer of silphiperfolan-6α-ol, a triquinane sesquiterpene isolated from the red algae *Laurencia majuscula* [11]. The latter showed an MIC of 120 μg/mL, corroborating our results for this fraction, which was inactive at 100 μg/mL towards *M. tuberculosis* H37RV (Table 2). In addition to the susceptible strain of *M. tuberculosis* (H37Rv), the essential oil fractions from *A. tomentosa* var. *anthriscifolia* were assayed against *M. smegmatis* (mc^2155), one environment mycobacterial species (Table 2). The assayed fractions, as well as (–)-*epi*-presilphiperfolan-1-ol, were active against this strain only at 200 μg/mL. In fact, environmental species show higher resistance profiles than classical pathogens due to lower permeability or increased drug efflux.

To the best of our knowledge, this is the first report of the antimycobacterial activity of the essential oil from *A. tomentosa* var. *anthriscifolia*. From comparison with chemical data published for this variety, the occurrence of chemotypes is being suggested.

Experimental

General procedures: Gas chromatography analyses were performed with a HP 5890 Series II gas chromatograph equipped with a FID detector and an HP-5 (5% phenyl/95% polydimethylsiloxane) fused silica capillary column (25 m x 0.2 mm, film thickness 0.33 μm) using hydrogen as carrier gas (1.0 mL min^{-1}). The injector temperature was 250ºC and the column oven programmed was 60–240ºC at 3ºC min^{-1}. The detector (FID) was operated at 280ºC. The GC/MS was performed with an Agilent 5973MSD coupled to an Agilent 6890 gas chromatograph, using helium as carrier gas, and the same column and oven conditions as above. Transfer line temperature was 240ºC, ion source was at 230ºC, EIMS, 70 eV. Constituents of the oil were identified by comparing the experimental gas chromatographic retention indices RI and MS fragmentation pattern with corresponding reference data [12a,12b]. A standard solution of *n*-alkanes (C_7–C_{26}) was used to obtain the retention indices.

Plant material and extraction: Aerial parts of *A. tomentosa* var. *anthriscifolia* were collected on a rocky hillside of Vila Velha, Espírito Santo State, Brazil. The plant was identified by Dr. Claudine Mynssen from the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, and a voucher specimen (RB438912) is deposited at the herbarium.. The essential oils from fresh aerial parts were obtained by hydrodistillation in a Clevenger-type apparatus for 2 hours, yielding 0.3 % of a light yellow essential oil.

CC separations: The column (h=54cm; Ø=1.5cm) was packed with silica gel 60 (230-400 Mesh ASTM) as the stationary phase. Mixtures of hexane/ethyl acetate (100:0 to 95:5, v/v) of increasing polarity were used as eluent. The sample of essential oil (1.5 g) to be separated into components was dissolved in a small amount of hexane. This solution is loaded onto the column.

Antimycobacterial tests: Samples were screened against *Mycobaterium tuberculosis* strain H37Rv (ATCC - 27294), *Mycobacterium smegmatis* (mc^2 155), using the resazurin (redox) bioassay [13-15]. The final concentration of the essential oil, substances and fractions was either 200 µg/mL or 100 µg/mL. Media plus bacteria with and without rifampicin were used as controls. In brief, the assay is accomplished in microplates (96 wells) using resazurin as indicator of cellular viability. The minimal inhibitory concentration (MIC) was determined (starting from 200 µg/mL in 1:2 serial dilutions).

Acknowledgments - This work was supported by CNPq (MCT- CNPq/MS-SCTIE-DECIT. 410475/2006-8, and fellowship) and FAPERJ (E-26/111.614/2008). We are indebted to Centro Nacional de Ressonância Magnética Nuclear Jiri Jones, UFRJ, Rio de Janeiro, for the use of NMR equipment, and to Dr. Claudine Mynssen from the Instituto de Pesquisas Jardim Botânico do Rio de Janeiro for plant identification. Collaborative work was performed under the auspices of the Iberoamerican Program for Science and Technology (CYTED), Project X.11:PIBATUB.

References

[1] Raven PH, Evert RF, Eichhorn SE. (**2007**) Biology of Plants. Worth Publishers, New York.

[2] Smith AR, Pryer KM, Schuettpelz E, Korall P, Schneider H, Wolf PG. (**2006**) A classification for extant ferns. *Taxon*, ***55***, 705–731.

[3] (a) Santos MG, Sylvestre LS. (**2006**) Aspectos florísticos e econômicos das pteridófitas de um afloramento rochoso do Estado do Rio de Janeiro, Brasil. *Acta Botanica Brasilica*, ***20***, 115-124; (b) Juliani HR, Zygadlo JA, Scrivanti R, Sota E, Simon JE. (**2004**) The essential oil of *Anemia tomentosa* (Savigny) Sw. var. *anthriscifolia* (Schard.) Mickel. *Flavour and Fragrance Journal*, ***19***, 541-543.

[4] Martínez GJ. (**2005**) Recolección y Comercialización de Plantas Medicinales en el Departamento Santa María, Provincia de Córdoba, Argentina. *Acta Farmacéutica Bonaerense*, ***24***, 575-84.

[5] (a) Demo M, Oliva MM, Lopez ML, Zunino MP, Zygadlo J. (**2005**) Antimicrobial Activity of Essential Oils Obtained from Aromatic Plants of Argentina. *Pharmaceutical Biology*, ***43***, 129-134; (b) Gillij YG, Gleiser RM, Zygadlo JA. (**2008**) Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. *Bioresource Technology*, ***99***, 2507-2515.

[6] (a) Bohlmann F, Jakupovic J. (**1980**) Neue Sesquiterpen-Kohlenwasserstoffe mit anomalen Kohlenstoffgerüst aus Silphium-arten. *Phytochemistry*, ***19***, 259-265; (b) Bohlmann F, Zdero C, Jakupovic J, Robinson H, King RM. (**1981**) Eriolanolides, eudesmanolides and a rearranged sesquiterpene from *Eriophyllum* species. *Phytochemistry*, ***20***, 2239-2244; (c) Zalkow LH, Harris III RN, Van Derveer D, Bertrand JA. (**1977**) Isocomene: a novel sesquiterpene from Isocoma Wrightii. X-Ray crystal structure of the corresponding diol. *Journal of the Chemical Society*, *Chemical Communications*, ***13***, 456-457; (d) Zalkow LH, Harris III RN, Van Derveer D (**1978**) Modhephene: a sesquiterpenoid carbocyclic [3.3.3] propellane. X-Ray crystal structure of the corresponding diol. *Journal of the Chemical Society*, *Chemical Communications*, ***10***, 420-421.

[7] Weyerstahl P, Marschall H, Seelmann I, Jakupovic J. (**1998**) Cameroonane, Prenopsane and Nopsane, Three New Tricyclic Sesquiterpene Skeletons. *European Journal of Organic Chemistry*, ***1998***, 1205-1212.

[8] (a) Santos MG, Rocha LM, Carvalho ES, Kelecom A. (**2005**) Isoafricanol, um sesquiterpeno incomum encontrado na Pteridófita Anemia tomentosa var. anthriscifolia. *Revista Brasileira de Plantas Medicinais*, ***8***, 71-75; (b) Pinto SC, Leitão GG, Bizzo HR, Martinez N, Dellacassa E, Santos-Junior FM, Costa FLP, Amorim MB, Leitao SG. (**2009**) (–)-*epi*-Presilphiperfolan-1-ol, a new triquinane sesquiterpene from the essential oil of Anemia tomentosa var. anthriscifolia (Pteridophyta). *Tetrahedron Letters*, ***50***, 4785-4787.

[9] (a) Haermers A, Leysen DC, Bollaert W, Zhang M, Pattyn SR. (**1990**) Influence of N substitution on antimycobacterial activity of ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, ***34***, 496-497; (b) Palomino JC, Leão SC, Ritacco V. (**2007**) Tuberculosis 2007 – from basic science to patient care. Bélgica, Brasil e Argentina: TuberculosisTextbook.com.

[10] (a) Harborne JB. (**1998**) Chapter 3: Terpenoids In *Phytochemical Methods: a guide to modern techniques of plant analysis*. Chapman & Hall, London, 107-138; (b) Mockutë D, Bernotienë G, Judþentienë A. (**2005**) Storage-induced changes in essential oil composition of *Leonurus cardiaca* L. plants growing wild in Vilnius and of commercial herbs. *Chemia*, ***16***, 29-32.

[11] König GM, Wright AD, Franzblau SG. (**2000**) Assesment of antimycobacterial activity of a series of mainly marine derived natural products. *Planta Medica*, ***66***, 337-342.

[12] (a) Adams RP. (**2001**) *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy*. Allured, Carol Stream, Illinois; (b) Wiley Registry of Mass Spectral Data (**1994**) 6th Edition, Wiley Interscience, New York.

[13] Leitão SG, Castro O, Fonseca EN, Julião LS, Tavares ES, Leo RRRT, Vieira RC, Oliveira DR, Leitão GG, Martino V, Sülsen V, Barbosa YAG, Pinheiro DPG, Silva PEA, Teixeira DF, Neves-Junior I, Lourenço MCS. (**2006**) Screening of Central and South American plant extracts for antimycobacterial activity by the Alamar Blue test. *Brazilian Journal of Pharmacognosy*, ***16***, 6-11.

[14] Franzblau SG, Witzig RS, Mclaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH. (**1998**) Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the Microplate Alamar Blue Assay. *Journal of Clinical Microbiology*, ***36***, 362-366.

[15] Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. (**2002**) Resazurin microtiter assay plate: Simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, ***46***, 2720-2722.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1679 - 1683

Antimicrobial Activity of *Inga fendleriana* Extracts and Isolated Flavonoids

Luisa Pistelli[1*], Alessandra Bertoli[1], Cecilia Noccioli[1], Jeanette Mendez[2], Rosa Anna Musmanno[3], Tiziana Di Maggio[3] and Grazietta Coratza[3]

[1]*Dipartimento di Scienze Farmaceutiche*, *Università di Pisa*, *Italy*

[2]*Grupo de Productos Naturales*, *Centro de Quimica Organica*, *Escuela de Ciencias*, *Universitat Central de Venezuela*, *Venezuela*

[3]*Dipartimento di Biologia Molecolare*, *Università degli Studi di Siena*, *Italy*

luipi@farm.unipi.it

Received: August 7th, 2009; Accepted: November 4th, 2009

The EtOAc and *n*-BuOH extracts of *Inga fendleriana* inhibited Gram-positive, but not Gram-negative bacteria; a narrow spectrum of activity against *Staphylococcus epidermidis* was detected. The MIC values of the extracts ranged from 125 to 850 μg/mL. Quercetin 3-methylether, myricetin 3-*O*-rhamnoside and tricetin showed antibacterial activity against the same bacterial strains with MICs in the range from 31 to 250 μg/mL. In time-kill kinetic studies, the flavonoids showed bactericidal effects at the concentrations corresponding to four times the MICs.

Keywords: *Inga fendleriana*, Fabaceae, antimicrobial activity, myricitrin, quercetin 3-methylether, tricetin.

The interest in medicinal plants has been increased during the last decades as they are potential sources of therapeutic substances. In particular, the emergence of microbial resistance to the available antibiotics [1-3] and the unexpected side effects of synthetic compounds have increased the need for new substances with antimicrobial properties. Studies of the antimicrobial activity of plant extracts and phytochemicals may lead to the discovery of new antibiotics useful to treat infectious diseases caused by resistant microorganisms. Extracts of various medicinal plants have been reported to possess antimicrobial activity [4-10] and their constituents with these properties have actively been investigated as alternatives to synthetic compounds [11]. Among these, flavonoids seem to be potent candidates because they show broad pharmacological activities and are widely distributed in many edible plants and beverages [12]. Plants belonging to the Fabaceae [13] are rich in flavonoids, some of which show defence functions against pathogenic microorganisms.

Inga fendleriana Benth. is a tropical tree restricted to the Andean rain forests in Venezuela and Bolivia, where it is known as "Guama peludo" or "Guama negro" [14]. The literature reports flavonol glycosides [15], pipecolic acid derivatives [16], and the triterpenes, betulinic acid and lupeol [17] as constituents of *Inga* species.

Many species of *Inga* have been used in folk medicine as astringents in diarrhea and dysentery, as a diuretic herb, as a lotion for arthritis and rheumatism, to treat furunculosis, and to aid in the treatment of wounds. The pulp of the fruits of some species is used for cleaning teeth and secretions from the eyelids [18]. The fruits of some species, especially *I. edulis* Mart., are edible and known as Ice Cream Beans; they are very popular in the local markets of Central and South America for their sweet flavor.

No data have been reported on the antimicrobial activity of *I. fendleriana* and so the aim of this study was to investigate the antimicrobial activity of extracts of this species and its main flavonoid constituents in order to support its traditional use.

Table 1: HPLC-UV-ESI-MS analyses of *I. fendleriana* extracts (μg/mg).

Compounds	EtOAc extract	*n*-BuOH extract
Quercetin 3-methylether	4.54	-
Myricetin 3-O-rhamnoside	85.55	2.34
Tricetin	5.88	-

Table 2: MIC values (μg/mL) of *I. fendleriana* extracts and isolated compounds.

Bacterial strains	EtOAc extract	*n*-BuOH extract	Quercetin–3 methyl ether	Myricetin-3-*O*-rhamnoside	Tricetin
Staphylococcus epidermidis 14990	500	250	62.5	62.5	31.25
Staphylococcus epidermidis SM 1	250	500	62.5	31.25	31.25
Staphylococcus aureus 25923	500	500	250	125	250
Staphylococcus epidermidis 27	500	500	125	125	250
Staphylococcus epidermidis 29	250	500	62.5	62.5	250
Staphylococcus epidermidis 30	850	500	125	125	250
Staphylococcus epidermidis 33	250	500	62.5	125	125
Staphylococcus epidermidis 34	250	500	62.5	62.5	31.25
Staphylococcus epidermidis 35	250	500	125	250	125
Staphylococcus epidermidis 36	250	500	125	250	125
Staphylococcus epidermidis 37	125	125	31.25	31.25	31.25
Staphylococcus epidermidis 38	500	850	125	250	125

From the aerial parts of *I. fendleriana*, myricitrin (**1**), quercetin 3-methylether (**2**) and tricetin (**3**) were isolated and characterized. The quantification of these compounds in the extracts was performed by LC-UV-ESI-MS analyses (Table 1). Each extract and the main isolated constituents (**1-3**) were tested for their antimicrobial activity using a range of bacterial strains.

The tested samples were arbitrarily considered as active when MICs were 1000 μg/mL or lower for at least one bacterial strain. On this basis, only the EtOAc and *n*-BuOH extracts were active; both showed a narrow range of activity to Gram-positive strains, but no inhibitory effect against the Gram-negative ones.

The MIC values of the two extracts against the *Staphylococcus* strains are reported in Table 2. Both extracts had MICs ranging from 850 to 125 μg/mL but the MIC values of the EtOAc extract were generally slightly lower than those of the *n*-BuOH extract. All the tested pure compounds also showed antibacterial activity against the selected strains, with MIC values ranging from 31.25 to 250 μg/mL (Table 2). Their pattern of activity was similar; indeed the strains, except for *S. epidermidis* SM29, exhibited similar behavior of either sensitivity or resistance to the substances; this strain was sensitive to quercetin 3-methylether and myricitrin (MIC 62.5 μg/mL), but was resistant to tricetin (MIC 250 μg/mL). The DMSO, at the used concentrations, did not produce any inhibition of bacterial growth.

For more accurate evaluation of the antibacterial activity of the substances, time-kill assays were performed using the strains with MICs of 62.5 μg/mL or lower. In these experiments, each compound was used at concentrations corresponding to 1xMIC, 2xMIC, and 4xMIC. The results obtained with *S. epidermidis* 14990 are reported in Figure 1; the other strains showed a similar behavior.

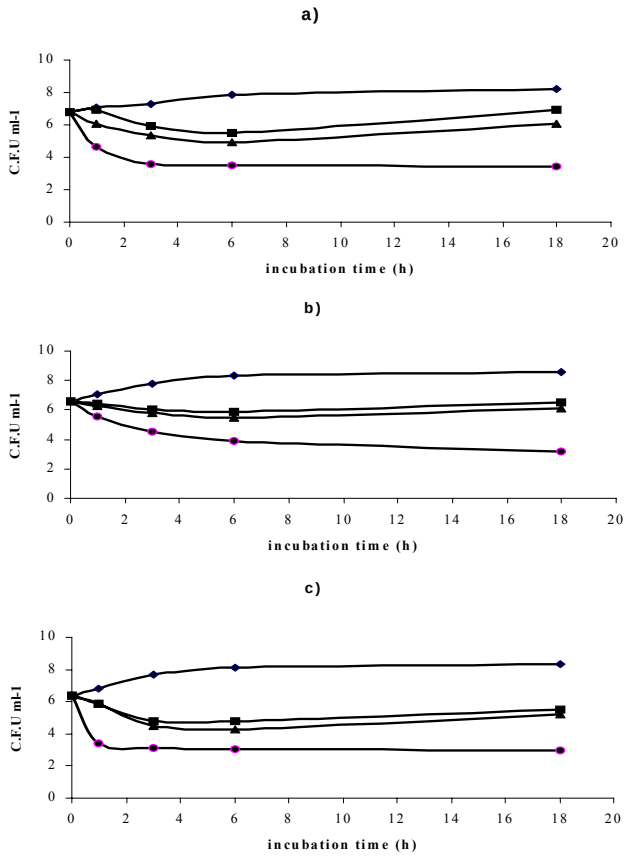


Figure 1: Killing curves of *S.epidermidis* ATCC 14990. (a) quercetin 3-methylether; (b) myricetin 3-*O*-rhamnoside; (c) tricetin. (υ) control; (ν) 1 x MIC; (σ) 2 x MIC; (λ) 4 x MIC.

All the tested compounds showed a bacteriostatic effect at 1x MIC and 2x MIC, as the bacteria remained at the inoculum density after over night incubation; a transient killing (1/2 log units) during the 6 hours exposure period was observed; this effect was followed by regrowth (to > 10^6 CFU/mL) over night This was not due to mutations: In all cases the MICs for isolates recovered after regrowth were the same as those for the initial isolates. At four times the MICs, the compounds produced a bactericidal effect that resulted in over 3 $\log_{10}$ killing by 18 h.

In conclusion the EtOAc and *n*-BuOH extracts from *I. fendleriana* show a narrow spectrum of antibacterial activity against *Staphylococcus* strains. The isolated flavonoids (quercetin 3-methylether, myricitrin and tricetin) are also active against the same strains. The MICs of the extracts are lower than those expected from just the flavonoids tested. The results suggest that these substances may contribute to the antimicrobial activity of the two extracts, but they are not the only ones responsible.

This is the first report on the antibacterial activity of tricetin and apparently this substance has the largest spectrum of activity. It inhibited at 31.2 μg/mL four bacterial strains, while myricitrin and quercetin 3-methylether inhibited two and one, respectively.

The *I. fendleriana* extracts and their isolates, although not active against Gram-negative organisms, displayed an inhibitory effect *in vitro* against clinical isolated *Staphylococci*, which confirms the traditional use of some species of *Inga* to treat forunclosis and to clean teeth and the eyelids.

The antimicrobial properties of the extracts and flavonoids from *I. fendleriana* against clinical isolates of *Staphylococcus epidermidis* could suggest their use to sanitize medical devices, since this bacterial species, actually not considered pathogenic, is responsible for some infections caused by the use of contaminated medical instruments.

Experimental

Plant material: *Inga fendleriana* Benth. aerial parts were collected in the National Park Henri Pittier (Maracay, Venezuela) in January 1999. A voucher specimen was authenticated and deposited (MY Cardoso *et al.* 2667).

Extraction and isolation: Dried and powered *I. fendleriana* aerial parts (600 g) were extracted in a Soxhlet apparatus with light petroleum, chloroform and methanol. The extracts were concentrated under reduced pressure at 40°C, to obtain the residues E (4.29 g), C (8.16 g) and M (27.94 g). The methanolic extract was partitioned with EtOAc and then with *n*-BuOH to obtain the corresponding residues A (4.7 g) and B (7.1 g). Myricitrin (47 mg), quercetin 3-methylether (8 mg) and tricetin (20 mg) were obtained as pure compounds from the EtOAc residue by comparison of their NMR spectroscopic data with those reported in the literature [19-22] or by direct comparison with authentic samples. NMR spectra were measured on a Bruker AC-200 spectrometer, using TMS as internal standard.

Chemicals: Myricetin 3-*O*-rhamnoside, quercetin 3-methylether, and tricetin were included in a home-made data base of natural compounds isolated and characterised by NMR and MS techniques in our laboratory. The standard compounds contained small quantities of impurities, which, although low in amount, could easily be detected by HPLC-PDA-MS. Therefore, all these compounds were analysed and purified by HPLC in the same gradient conditions used for the analyses of the samples in order to guarantee their purity (>98%) before using as reference material. Acetonitrile, acetic acid and methanol were HPLC grade [Baker (Netherlands)]. HPLC-water was purified by a Milli-Q Plus system (Millipore Milford, MA, USA).

Sample preparation for HPLC analyses: Three samples of each extract of *I. fendleriana* were dissolved in methanol (20 mg/mL) and filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene filter (PTFE, 0.45 μm, 25 mm) before HPLC injections. Injected volume of the extracts: 20 μL.

HPLC-PDA-ESI-MS analyses: The HPLC system consisted of a Surveyor Thermofinnigan liquid chromatography pump equipped with an analytical Lichrosorb RP-18 column (250 x 4.6 mm i.d., 5 μm, Merck), a Thermofinnigan Photodiode Array Detector and a LCQ Advantage mass detector.

The analyses were carried out with a linear gradient using water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) with 0.1% HCOOH at flow rate of 0.7 mL/min in the following conditions: from 15:85 v/v (B-A) to 30:70 (B-A) in 25 min for 15 min, then to 70:30 v/v (B-A) in 25 min for 15 min and then conditioning to the initial condition (15:85 v/v B-A) for 10 min. The total analytical run time was 65 min for each sample.

The spectral data from the PDA detector were collected during the whole run in the range 210-700 nm and the peaks were detected at 260 nm. An aliquot (20 μL) of each sample was analyzed in triplicate.

In order to confirm the presence of the analytes in the EtOAc and *n*-BuOH extracts, LC-ESI-MS analyses

Table 3: The linear regression equations, HPLC-UV and correlation coefficients for the analyzed compounds in *Inga fendleriana* extracts.

Compound	*Concentration Range*	*Regression equations*
Myricetin 3-*O*-rhamnoside	5.6–112 μg mL^{-1}	y= 0.99906+9.2428E-5x(*r*=0.9998)
Quercetin 3-methylether	2.4–120 μg mL^{-1}	y=-6.296E-3+2.5567E-5x(*r*=0.9997)
Tricetin	41.5-860 μg mL^{-1}	y= 0.77755+5.944E-5x(*r*=0.9998)

were performed using the same chromatographic conditions and these specific ESI values: sheath gas flow-rate 72 psi, auxiliary gas flow 10 psi, capillary voltage –16 V and capillary temperature 200°C. Full scan spectra from m/z 200 to 700 in the positive ion mode were obtained.

The identification of each constituent was carried out by the comparison of the peaks in the extracts with the retention time, UV and MS spectra of the authentic samples previously injected in the same chromatographic condition.

Calibration curves for the reference compounds (1-3): The dosage of the analysed constituents (**1-3**) was performed by the external standard method, using 6 levels of concentration for each compound; the respective linear regression equations and their correlation coefficients for the marker compounds are shown in Table 3. An aliquot (20 μL) of each standard compound was analyzed in triplicate under the same conditions used for the analyses of the sample extracts by HPLC-PDA-ESI-MS. The standard solutions for the authentic samples (**1-3**) were prepared in methanol.

Bacteria and media: *Escherichia coli* 11303, 15597, 25922, 25404, 23739, 12435 and 15669 were obtained from the American Type Culture Collection; Strain MS 1 was a clinical isolate; and strain O21 was obtained from the Istituto Sieroterapico Milanese. *Salmonella typhimurium* 15277, 13311 and 19585 were purchased from the American Type Culture Collection, and *S. schwarzengrund* and *S. infantis* were clinical isolates. *Pseudomonas aeruginosa* 27853 and 10145 were obtained from the American Type Culture Collection, whereas strains 153, 769 and 776 were clinical isolates. *Staphylococcus epidemidis* 14990 came from the American Type Culture Collection and strain SM1 was a clinical isolate. *S. aureus* was obtained from the American Type Culture Collection. *S. epidermidis* strains 27, 29, 30, 33-38 were clinical isolates.

The used media were: Nutrient Agar (Difco) for maintenance of the strains, Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) (Difco) supplemented with 25 mg of Ca^{2+}/L, 12.5 mg of Mg^{2+}/L, and 2% NaCl for antimicrobial assays [23-24].

Antibacterial assays: The extracts and the pure compounds were dissolved in dimethylsulfoxide (DMSO) to give a concentration of 20 mg/mL and were sterilized by filtration through a Millipore filter (0.2 μm). Further dilutions of the stock solutions were prepared in sterile water.

Minimum inhibitory concentration: MICs were determined by the agar dilution method, as follows: the MHA medium was poured into the plates and allowed to solidify, then a strip of medium in the shape of a ditch was removed. Melted Mueller Hinton Agar containing appropriate dilutions of each stock solution was used to fill the gap. The final concentrations used for the extracts ranged from 1000 to 31.25 μg/mL, while the final concentrations of the pure substances ranged from 500 to 7.8 μg/mL. Table 2 reported the MICs (μg/mL) of the tested samples. Controls were carried out using DMSO (5% v/v) in order to assay toxicity due to the solvent. The inoculum suspensions were prepared using overnight broth cultures of the test organisms which were diluted to achive a turbidity corresponding to 0.5 McFarland (approximately 1 x 1 x 10^8 CFU/mL). Using a calibrated loop, 2 μL of each inoculum was streaked onto the plate perpendicularly to the strip so that each streak crossed it. The plates were incubated for 48 h at 37°C. The lowest concentration at which no visible growth occurred on the strip was defined as the MIC. All experiments were repeated twice with three triplicates.

Time-kill assays: Time-kill kinetic studies were performed in MHB, with an inoculum of 5 x 10^6 CFU/mL, in a final volume of 10 mL. The substances were used at final concentrations corresponding to 1 x MIC, 2 x MIC, 4 x MIC. The bacterial suspensions were incubated at 37°C for 18 h. Bacterial growth was followed by taking samples (100 μL) from the cultures at 0, 1, 3, 6, and 18 h. The samples were ten-fold serially diluted with peptone saline; 10 μL of the

samples (diluted and undiluted) were spread on Mueller Hinton Agar plates (4 plates per dilution) and the Colony Forming Units were counted after incubation at 37°C for 48 h. A 99.9% reduction of the original inoculum was interpreted as indicative of a bactericidal effect. The controls of bacterial growth and the DMSO inhibitory effect were respectively inoculated in MHB alone and inoculated MHB with DMSO (2.5 % v/v).

References

[1] Barker KF. (**1999**) Antibiotic resistance: a current perspective. *British Journal of Clinical Pharmacology*, ***48***, 109-124.

[2] Chambers HF. (**2001**) The changing epidemiology of *Staphylococcus aureus?*. *Emerging Infectious Diseases Journal*, ***7***, 178-182.

[3] Normark BH, Normark S. (**2002**) Evolution and spread of antibiotic resistance. *Journal of Internal Medicine*, ***252***, 91-106.

[4] Basile A, Sorbo S, Giordano S, Ricciardi L, Ferrara S, Monetano D, Castaldo Capobianchi R, Vuotto ML, Ferrara L. (**2000**) Antibacterial and allelopathic activity of extract from *Castanea sativa* leaves. *Fitoterapia*, ***71***, S110-S116.

[5] Bisignano G, Sanogo R, Marino A, Aquino R, D'Angelo V, Germanò MP, De Pasquale R , Pizza C. (**2000**) Antimicrobial activity of *Mitracarpus scaber* extract and isolated constituents. *Letters in Applied Microbiology*, ***30***, 105-108.

[6] Puupponen-Pimia R, Nohynek L, Meier C, Kahkonen M, Heinonen M, Hopia A, Oksman-Caldentey KM. (**2001**) Antimicrobial properties of phenolic compounds from berries. *Journal of Applied Microbiology*, ***90***, 494-507.

[7] Savat A, Antonacci L, Fortunato RH, Suarez EY, Godoy HM. (**2001**) Screening of some plants from northern Argentina for their antimicrobial activity. *Letters in Applied Microbiology*, ***32***, 293-297.

[8] Urza A, Mendoza L. (**2003**) Antibacterial activity of fresh flower heads of *Chrysantemum coronarium*. *Fitoterapia*, ***74***, 606-608.

[9] Martini ND, Katerere DRP, Eloff JN. (**2004**) Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *Journal of Ethnopharmacology*, ***93***, 207-212.

[10] Pistelli L, Bertoli A, Morelli I, Menichini F, Musmanno RA, Di Maggio T, Coratza G. (**2005**) Chemical and antibacterial evaluation of *Hypericum triquetrifolium* Turra. *Phytotherapy Research*, ***19***, 787-791.

[11] Sato M, Fujiwara S, Tsuchiya H, Fujii T, Iinuma M, Tosa H, Ohkawa Y, (**1996**) Flavones with antibacterial activity against cariogenic bacteria. *Journal of Ethnopharmacology*, ***54***, 171-176.

[12] Havsteen B (**1983**) Flavonoids, a class of natural products of high pharmacological potency. *Biochemical Pharmacology*, ***32***, 1141-1148.

[13] Ebel J, Grisebach H (**1988**) Defence strategies of soybean against fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. *TIBS (Trends in Biochemical Sciences)*, ***13***, 23-27.

[14] Pennington TD, Revelo N. (**1997**) In *The Genus Inga.* London: Royal Botanic Gardens Kew. pp. 610-613.

[15] Harborne JB. (**1997)** In *The Genus Inga,* Botany. London: Royal Botanic Gardens Kew. pp. 71-89.

[16] Kite GC. (**1997**) In *The genus Inga Botany*. London: Royal Botanic Gardens Kew. pp. 35-70.

[17] Kingston DGI, Munjal RC. (**1978**) "Plant Anticancer Agents. VIII. Constituents of *Inga punctata*", *Lloydia*, ***41***, 499-500.

[18] Vivot E, Muñoz JD, Cruañes MC, Tapia MJ, Schmeda Hirschmann G, Martinez E, Di Sapio O, Gattuso M, Zacchino S. (**2001**) Inhibitory activity of xanthine-oxidase and superoxide scavenger properties of *Inga verna* subsp. *affinis*. Its morphological and micrographic characteristics. *Journal of Ethnopharmacology*, ***76***, 65-71.

[19] Connolly JD, Hill RA. (**1991**) In: Charlwood BV, Banthorpe DV, (Eds), (2nd ed.), *Methods in Plant Biochemistry*, ***7***, Academic Press, London, 331-357.

[20] Pistelli L, Bertoli A, Giachi I, Manunta A. (**1998**) Flavonoids from *Genista ephedroides*. *Journal of Natural Products* ***61,*** 1404-1406.

[21] Agrawal PK, Bansal MC. (**1989**) *Carbon-13 NMR of Flavonoids.* In: Agrawal PK (Ed.) Elsevier, Amsterdam.

[22] Zhong XN, Otsuka H, Ide T, Hirata E, Takushi A, Takeda Y. (**1997**) Three flavonol glycosides from leaves of *Myrsine seguinii*. *Phytochemistry*, ***46***, 943-946.

[23] Amsterdam D (**1996**) Susceptibility testing of antimicrobials in liquid media. In *Antibiotics in Laboratory Medicine.* Lorian V. (Ed.)., Williams & Wilkins, 52-111.

[24] National Committee for Clinical Laboratory Standars. (**2003**) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically , Approved Standard 6th ed. NCCLS document. M07 –A6. National Committee for Clinical laboratory Standards, Wayne, PA.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1685 - 1690

Rosmarinus officinalis L.: Chemical Modifications of the Essential oil and Evaluation of Antioxidant and Antimicrobial Activity

Giorgio Pintore[a], Mauro Marchetti[b], Mario Chessa[a], Barbara Sechi[b], Nadia Scanu [a], Giuseppe Mangano[a] and Bruno Tirillini[c]

[a]*Dipartimento Farmaco Chimico Tossicologico, Università di Sassari, Via Muroni 23/A, 07100 Sassari, Italy*

[b]*Istituto di Chimica Biomolecolare, CNR, Traversa La Crucca, 3 - Regione Baldinca - 07040 Li Punti, Sassari, Italy*

[c]*Istituto di Botanica, Università di Urbino, via Bramante 28, I-61029 Urbino, Italy*

pintore@uniss.it

Received: August 9th, 2009; Accepted: October 19th, 2009

Rosmarinus officinalis essential oil was separated into its hydrocarbon and oxygenated fractions. The major compounds in the hydrocarbon fraction were α-pinene (44.2%), camphene (24.5%), and limonene (11.7%), while in the oxygenated fraction they were 1,8-cineole (37.6%), camphor (16.5%), and bornyl acetate (21.4%). The hydrocarbon fraction was submitted to a hydroformylation process and the antioxidant activity of the product was screened by the DPPH and β-carotene/linoleic acid tests. The hydroformylated fraction maintained the antioxidant activity of the whole oil. The MIC (minimal inhibitory concentration) and the MBC (minimal bactericidal concentration) of the essential oil, hydrocarbon, oxygenated and hydroformylated fractions were also tested on several microorganisms. *Aeromonas sobria* and *Candida* strains were the most susceptible micro-organisms. The hydroformylated fraction exhibited a MBC against *Candida* strains resistant to the other fractions.

Keywords: *Rosmarinus officinalis*, hydrocarbon fraction, oxygenated fraction, hydroformylation, antioxidant, antimicrobial.

The essential oil of *Rosmarinus officinalis* L. has been the subject of several studies and there are many research papers dealing with its chemical composition [1], biological and pharmacological activities [2]. The utilization of this plant is well known in traditional medicine as a tonic, astringent and diuretic. Also important are the antioxidant [3] and antimicrobial properties of the oil [2].

In consideration of the relatively high abundance of this shrub in Sardinia, our research group has performed several studies on the qualitative and quantitative composition of the essential oil with respect to the seasonal collection time, altitude and geographical distribution in the island [4].

The oil was separated into two fractions, the hydrocarbon fraction and the oxygenated fraction. These were characterized and the hydrocarbon phase was submitted to a hydroformylation process, with the aim of comparing the biological activities of the unmodified oil with those of the transformed one.

Hydrodistillation of the plant material gave yellowish oil with a yield of 0.65%. Twenty –four compounds (12 in the oxygenated fraction and 12 in the hydrocarbon fraction) were identified. The compositions of these fractions are given in Table 1, the compounds being listed in order of their elution from an HP-5 column.

The major compounds in the hydrocarbon fraction were α-pinene (44.2%), camphene (24.5%) and limonene (11.7%), whereas in the oxygenated fraction the major compounds were 1,8-cineole (37.6%), bornyl acetate (21.4%), and camphor (16.5%).

Table 1: Percentage composition of the oil of *Rosmarinus officinalis*.

Hydrocarbon Fraction			*Oxygenated Fraction*		
Compound*	**RI****	**Area%**	**Compound***	**RI****	**Area%**
α-Pinene	941	44.2	1,8-Cineole	1032	37.6
Camphene	952	24.5	Linalool	1109	2.5
β-Pinene	983	6.3	α-Campholenal	1132	0.3
Myrcene	1002	3.8	Camphor	1145	16.5
α-Phellandrene	1010	0.8	*trans*-Pinocamphone	1164	0.4
α-Terpinene	1012	0.7	Borneol	1168	9.3
o-Cymene	1022	3.8	Isopinocamphone	1174	2
Limonene	1033	11.7	Terpinen-4-ol	1180	2
γ-Terpinene	1064	0.9	α-Terpineol	1193	2.3
Terpinolene	1093	0.7	Myrtenol	1198	0.5
(*Z*)-Caryophyllene	1409	1.9	Verbenone	1215	5.4
Humulene	1456	0.7	Bornyl acetate	1284	21.2
Total		**100.0**	**Total**		**100.0**

*compounds listed in order of elution; **retention indices relative to *n*-alkane series on a HP-5 column

Table 2: Results of the hydroformylation.

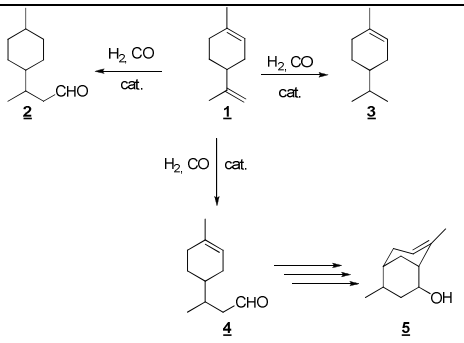
Components before hydroformylation		Components after hydroformylation		
Compound*	**Area%**	**Compound***		**Area%**
1 – Limonene	11.7	**2**	3-(4-Methylcyclo-hexyl)butanal	9.6
		3	*p*-1-Menthene	1.0
		5	4,8-Dimethylbicyclo [3.3.1]non-7-en-2-ol	0.4
6 – β-Pinene	6.3	**7 + 8**	2-((1*S*,2*R*,5*S*)-6,6-Dimethylbicyclo[3.1.1]heptan-2-yl)-ethanal (**7**) and 2-((1*S*,2*S*,5*S*)-6,6-Dimethylbicy-clo[3.1.1]heptan-2-yl)ethanal (**8**)	4.1
		9	2-((1*S*,5*S*)-6,6-Di-methylbicyclo[3.1.1]heptan-2-yl)ethanol	0.7
10 – α-Phellandrene	0.8	**11**	2-(4-Isopropylcyclo-hexyl)ethanal	0.6
12 – Myrcene	3.8	**13**	4,8-Dimethylnon-7-enal	1.0
		14	8-Methyl-4-methyl-enenon-7-enal	1.2
		15	(*Z*)-4,8-Dimethyl-nona-3,7-dienal	0.9
		17	4-(4-Methylpent-3-enyl)cyclopent-1-enecarbaldehyde	0.4
18 – Camphene	24.5	**19**	2-((1*R*,2*S*,4*S*)-3,3-Dimethylbicyclo[2.2.1]heptan-2-yl)ethanal	11.0
		20	2-((1*R*,2*R*,4*S*)-3,3-Dimethylbicyclo[2.2.1]heptan-2-yl)ethanal	10.9
21 – Humulene	0.7	**22**	(1*E*,5*E*)-1,5,8,8-Tetramethylcycloundeca-1,5-diene	0.3
23 – (*Z*)-Caryophyllene	1.9	**24**	(*Z*)-2-(6,10,10-Tri-methylbicyclo[7.2.0]undec-5-en-2-yl)-ethanal	0.9
25 – α-Pinene	44.2	**26**	*cis*-Pinane	0.3
Total	**93.9**	**Total**		**43.3**

The hydroformylation reaction on the hydrocarbon fraction gave an outcome of several compounds, as summarized in schemes 1-8.

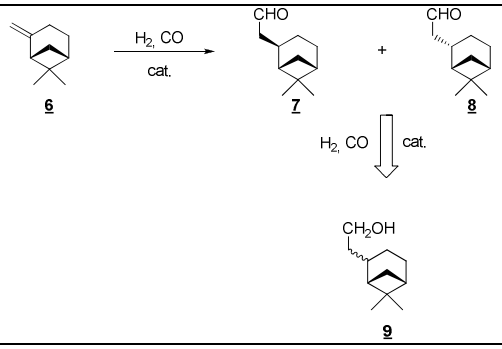
From the results it is evident that external double bonds were more reactive than internal ones. In addition, we observed that hydrogenation of double bonds and reductions of aldehydes occurred under hydroformylation conditions. Scheme 1 shows the outcome of the hydroformylation reaction for limonene (**1**), a component of the hydrocarbon fraction of the oil. Only the most reactive double bond of the molecule (the external one) was transformed to give an aldehyde (**4**), while the internal double bond was preferentially hydrogenated to mentene (**3**). The aldehyde (**4**) underwent rearrangement to (**5**) under hydroformylation conditions. β-Pinene (**6**), in scheme 2, under hydroformylation conditions gave aldehydes (**7**) and (**8**), which were partially reduced to alcohol (**9**).

α-Phellandrene (**10**) was hydroformylated and hydrogenated (scheme 3) at the same time to give the aldehyde (**11**). Hydroformylation occurred at the external double bond and hydrogenation at the double bond in the ring.

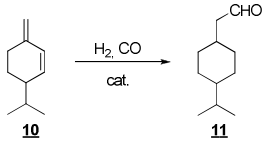
Myrcene (**12**) (scheme 4) underwent hydroformylation to give aldehydes (**13**), (**14**) and (**15**). The aldehyde (**13**) is the result of hydrogenation of the methylene group at position 3, and hydroformylation of the double bond at position 1. This effect is well known and occurs in conjugated dienes with two external double bonds. The aldehyde (**14**) is produced by hydroformylation of myrcene (**12**) at position 1, without hydrogenation of the methylene group at position 3. As an outcome of this reaction, we found an isomerization product (**15**) derived from hydroformylation of the double bond at position 1 and isomerization of the double bond at position 3. Aldehyde (**16**) is the result of hydroformylation of the aldehyde (**14**) at the external double bond. This product is highly reactive and, under hydroformylation conditions, cyclization occurs to form (**17**). Hydroformylation of camphene (**18**) (scheme 5) gave aldehydes (**19**) and (**20**), derived from the exocyclic double bond.



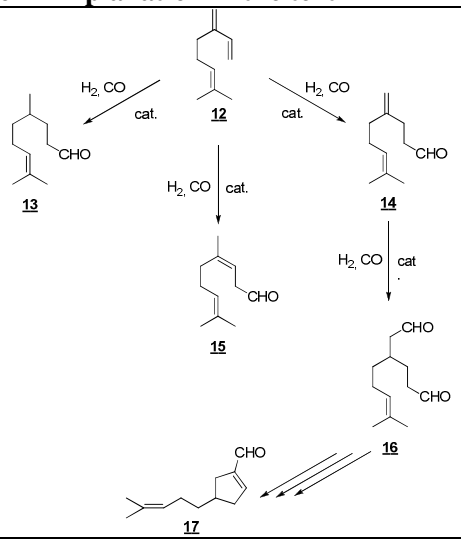
Scheme 1- Explanation in the text



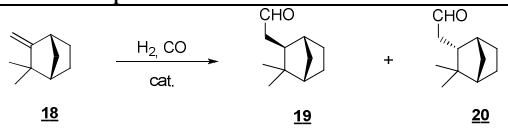
Scheme 2- Explanation in the text



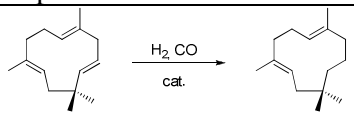
Scheme 3 - Explanation in the text



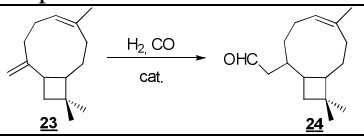
Scheme 4 - Explanation in the text



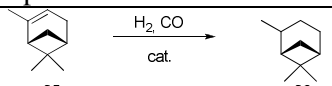
Scheme 5 - Explanation in the text



Scheme 6 - Explanation in the text



Scheme 7 - Explanation in the text



Scheme 8 - Explanation in the text

Partial hydrogenation occurred when humulene (**21**) was hydroformylated (scheme 6) to give the hydrocarbon (**22**), but no trace of aldehydes was detected. Caryophyllene (**23**) (scheme 7), when hydroformylated, gave only one aldehyde (**24**), derived from the reaction of the exocyclic double bond. β-Pinene (**25**), under hydroformylation conditions (scheme 8), gave only the hydrogenated product (**26**) and no aldehydes.

The samples were screened for their antioxidant activity by two complementary test systems: DPPH free radical-scavenging and β-carotene/linoleic acid systems. *R. officinalis* essential oil and its hydroformylated fraction notably reduced the concentration of DPPH free radicals, with no significant difference between them ($P < 0.001$). The values ranged from 55.3 ± 6.5 to 61.1 ± 5.7, respectively and were double the value of Trolox (30.2 ± 3.6).

The lipid peroxidation inhibitory activity of the essential oils and the hydroformylated fraction in the β-carotene bleaching test were consistent with data obtained in the DPPH test. *R. officinalis* essential oil (75.5 ± 8.7) and its hydroformylated fraction (70.3 ± 7.5) performed almost as well as BHT (66.5 ± 5.8).

Table 3: Antimicrobial activity expressed as minimal inhibitory concentration (MIC) and minimal bactericida concentration (MBC).

Microorganism	origin	Essential oil		Hydrocarbon fraction		Oxygenated fraction		Hydroformylated fraction	
		MIC %	MBC%	MIC %	MBC%	MIC %	MBC%	MIC %	MBC%
Aeromonas hydrophila	Fp	2.5^{e}	2.5^{e}	0.15^{a}	>2.5	2.5^{e}	2.5^{e}	2.5^{e}	>2.5
Aeromonas sobria	Fp	0.6^{bc}	>2.5	>2.5	>2.5	0.3^{a}	0.3^{a}	0.3^{a}	0.3^{a}
Candida albicans	Fp	1.25^{d}	1.25^{d}	0.15^{a}	>2.5	0.15^{a}	0.15^{a}	2.5^{e}	2.5^{e}
Candida glabrata	Fp	0.15^{a}	>2.5	0.15^{a}	>2.5	0.15^{a}	0.6^{bc}	2.5^{e}	2.5^{e}
Candida parapsilosis	Fp	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	2.5^{e}	>2.5
Candida tropicalis	Fp	0.15^{a}	>2.5	0.15^{a}	>2.5	0.15^{a}	1.25^{d}	2.5^{e}	2.5^{e}
Enterococcus faecalis	ATCC 24212	>2.5	>2.5	>2.5	>2.5	2.5^{e}	2.5^{e}	>2.5	>2.5
Escherichia coli	ATCC 35218	>2.5	>2.5	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5	>2.5
Pseudomonas aeruginosa	Fp	>2.5	>2.5	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5	>2.5
Salmonella tiphymurium	ATCC 14028	1.25^{d}	>2.5	>2.5	>2.5	>2.5	>2.5	2.5	>2.5
Staphylococcus aureus	ATCC 43300	0.6^{bc}	2.5^{e}	>2.5	>2.5	0.6^{bc}	2.5^{e}	2.5^{e}	2.5^{e}
Staphylococcus epidermidis	Fp	2.5^{e}	>2.5	>2.5	>2.5	1.25^{d}	2.5^{e}	2.5^{e}	2.5^{e}
Vibrio parahaemolyticus	Sb	>2.5	>2.5	>2.5	>2.5	1.25^{d}	2.5^{e}	>2.5	>2.5
Candida albicans s1221	Fp	1.25^{d}	1.25^{d}	1.25^{d}	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	2.5^{e}
Candida albicans s 1234	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	0.5^{ab}	0.5^{ab}	1.25^{d}
Candida albicans s 2314	Fp	1.25^{d}	1.25^{d}	0.15^{a}	>2.5	0.15^{a}	0.15^{a}	>2.5	>2.5
Candida glabrata s 1256	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	1.25^{d}
Candida glabrata s 1324	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	0.5^{ab}
Candida glabrata s 2167	Fp	0.15^{a}	2.5^{e}	0.15^{a}	>2.5	0.15^{a}	0.6^{bc}	2.5^{e}	2.5^{e}
Candida parapsilosis s 4323	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	0.5^{ab}
Candida parapsilosis s 4454	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	1.25^{d}	0.5^{ab}	1.25^{d}
Candida parapsilosis s 4563	Fp	0.9^{c}	1.5^{d}	0.9^{c}	>2.5	0.5^{ab}	0.5^{ab}	0.5^{ab}	1.25^{d}
Candida parapsilosis s 4578	Fp	2.5^{e}	2.5^{e}	2.5^{e}	>2.5	2.5^{e}	>2.5	2.5^{e}	>2.5
Candida tropicalis s 6651	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	>2.5	0.5^{ab}	>2.5
Candida tropicalis s 6834	Fp	1.25^{d}	1.25^{d}	>2.5	>2.5	0.5^{ab}	2.5^{e}	0.5^{ab}	1.25^{d}
Candida tropicalis s 8456	Fp	0.9^{c}	2.5^{e}	0.9^{c}	>2.5	0.5^{ab}	>2.5	0.5^{ab}	>2.5
Candida tropicalis s 8790	Fp	0.15^{a}	2.5^{e}	0.15^{a}	>2.5	0.15^{a}	1.25^{d}	2.5^{e}	2.5^{e}

Values having different letters are significantly different from each other using corrected Tukey's LSD test (P<005). Fp = from patients. Sb = soil-borne
The s number were our archieve number.

The mean values for antimicrobial activity are summarized in Table 3. The oil of *R. officinalis* exhibited good bacteriostatic effects against *C. glabrata* (s 2167) and *C. tropicalis* (s 8456) (MIC = 0.15%), *A. sobria* and *S. aureus* (MIC = 0.6%), and *C. parapsilosis* and *C. tropicalis* (MIC = 0.9%). The oil exhibited poor bactericidal activity in general, but there was good bactericidal activity against *Candida* strains, ranging from 1.25 to 2.5%. The hydrocarbon oil fraction exhibited good bacteriostatic effects against *A. hydrophila* (0.15%) and some *Candida* strains (from 0.9% to 2.5%), but the oil had no bactericidal activity.

The oxygenated oil fraction showed bacterostatic effects against all the micro-organisms tested, except *S. typhymurium*. The most susceptible was *A. sobria* (0.3%). Even the bactericidal activity was very good against *A. sobria* (0.3%) and most *Candida* strains. The hydroformylated fraction was less active than the oxygenated oil fraction, but more active than the hydrocarbon fraction. The most susceptible microorganism was *A. sobria* (MIC and MBC = 0.3%). This fraction exhibited positive MBCs for all the microorganisms tested, although some *Candida* strains were resistant. The hydroformylated oil fraction exhibited higher bactericidal activities against some *Candida* strains than either the oil or the untreated fraction tested, such as *C glabrata* (s 1324) (MBC = 0.5 %) and *C. parapsilosis* (s 4563) (MBC = 0.5 %). Hydroformylation of the hydrocarbon oil fraction could, therefore, improve the bactericidal and bacteriostatic action of *R. officinalis* essential oil against some strains otherwise resistant.

Experimental

Oil distillation and yield: *R. officinalis* L. was collected in May and June 2008 in Sardinia (Monte Doglia) near Alghero. Voucher specimens have been authenticated by Prof. Pintore and deposited in the Herbarium SASSA of the Department of Drug Science, University of Sassari under the accession codes 1091.

Fresh plant material was submitted to hydrodistillation using a Clevenger-type apparatus for 1.5 h, yielding a mean of 0.65% of oil calculated on the dry weight. The oil was dried over anhydrous sodium sulfate and stored in sealed vials under

refrigeration prior to analysis. A fraction of the oil was submitted to column chromatography using silica gel as stationary phase. This separation was performed in order to collect the two component groups of the oil, the hydrocarbon fraction and the oxygenated fraction. Each elution was made using 1.5 g of oil eluting with 300 mL of *n*-hexane first and then with 300 mL of ethyl acetate. Subsequent evaporation of the solvent gave the two oil fractions.

Hydroformylation: In a typical run [5-6], the glass vial inside the autoclave was filled under nitrogen purge with the catalytic complex (molar ratio substrate/catalyst=500/1) $Rh(CO)_2(acac)$ with PPh_3 (ratio catalyst/PPh_3 = 0.5) and the substrate (5.0 mmol referred to limonene) in toluene (10 mL). The reactor was closed and pressurized with syngas ($p(CO) = p(H_2)$ = 40 atm) and heated to 80°C for 6 h. After cooling at 25°C, the residual gases were released and the reaction products were characterized, as described elsewhere.

GC analyses: *R. officinalis* essential oil was analyzed using a Varian 3300 instrument equipped with a FID and either an HP-InnoWax capillary column (30 m x 0.25 mm, film thickness 0.17 μm), working from 60°C (3 min) to 210°C (15 min) at 4°C/min, or a HP-5 capillary column (30 m x 0.25 mm, film thickness 0.25 μm) working from 60°C (3 min) to 300°C (15 min) at 4°C/min; injector and detector temperatures, 250°C; carrier gas, helium (1 mL/min); split ratio, 1:10.

GC/MS: Analyses were carried out using a Hewlett Packard 5890 GC/MS system operating in the EI mode at 70 eV, using the two above mentioned columns. The operating conditions were analogous to those reported in the GC analyses section. Injector and transfer line temperatures were 220°C and 280°C, respectively. Helium was used as the carrier gas; flow rate 1 mL/min; split ratio, 1:10.

The identification of the components was made for both columns by comparison of their retention time with respect to *n*-alkanes (C_6-C_{22}). The MS and RI were compared with those of commercial (NIST 98 and WILEY) and home-made library MS built up from pure compounds and MS literature data. The percentage composition of the oil was obtained by the normalization method from the GC peak areas, without using correction factors.

DPPH assay: The hydrogen atom- or electron-donation abilities of the samples were measured by the bleaching of a purple-colored methanol solution of DPPH. This spectrophotometric assay uses the stable radical, 2,2'-diphenylpicrylhydrazyl (DPPH), as a reagent [7,8]. Trolox (1 mM) was used as a synthetic reference. Inhibition of free radical DPPH in percent (I %) was calculated in the following way:

$$I\ \% = (A_{blank} - A_{sample}/ A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Tests were carried out in triplicate.

β-Carotene/linoleic acid assay: In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [9]. Butylated hydroxytoluene (BHT) was used as positive control. Antioxidative capacities of the extracts were compared with those of the BHT and blank.

Antioxidant activity was calculated as percent inhibition of oxidation versus control sample without sample added, using the equation:

$$\%\ \text{antioxidant activity} = 100 \times [1 - (A_s^0 - A_s^{120})/(A_c^0 - A_c^{120})]$$

where A_s^0 was the absorbance of sample at 0 min, A_s^{120} was the absorbance of sample at 120 min, A_c^0 was the absorbance of control sample at 0 min, and A_c^{120} was the absorbance of control sample at 120 min. Tests were carried out in triplicate.

Micro-organisms: Antimicrobial screening was performed using the general qualitative assay described by Barry [10-11]. A total of 27 strains of bacteria and yeast were investigated; 22 were isolated from patients, one from environmental sources and 4 from the American Type Culture Collection. The bacteriostatic and bactericidal activities were determined by measuring the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of the essential oil performed in microtiter plates using a bacterial inoculum (taken from Luria Berani broth after overnight culture) with a turbidity equivalent to 0.5 MacFarland standard. Values of MICs and MBCs are expressed as percent v/v of total oil and culture medium used as diluent. All micro-organism species were tested in triplicate.

Bacterial species were: *Aeromonas hydrophila* (from patients) (Fp), *Aeromonas sobria* (Fp), *Candida albicans* (Fp), *C. glabrata* (Fp), *C. parapsilosis* (Fp), *C. tropicalis* (Fp), *Enterococcus faecalis* (ATCC 24212), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (Fp), *Salmonella typhymurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 43300), *S. epidermidis* (Fp), and *Vibrio parahaemolyticus* (soil-borne). The follow strains of *Candida* were also tested: *C. albicans* (s 1221, s 1234 and s 2314), *C. glabrata* (s 1256, s 1324 and s 2167), *C. parapsilosis* (s 4323, s 4454, s 4563 and s 4578), *C. tropicalis* (s 6651, s 6834, s 8456 ands 8790).

Statistical analysis: Analyses of variance (Anova) followed by LSD post hoc determination were performed. All computations utilized the statistical software SPSS v. 13.

Acknowledgements - The work was financially supported by Fondazione Banco di Sardegna. The authors gratefully acknowledge Miss Paola Manconi for her technical assistance in the GC-MS analysis. The authors are grateful to Prof. Stefania Zanetti of Dipartimento di Scienze Biomediche, University of Sassari, for the bioassay data.

References

[1] Pintore G, Usai M, Bradesi P, Juliano C, Boatto G, Tomi F, Chessa M, Cerri R, Casanova J. (**2002**) Chemical composition and antimicrobial activity of *Rosmarinus officinalis* L. oils from Sardinia and Corsica. *Flavour and Fragrance Journal*, ***17***, 15-19.

[2] Angioni A, Barra A, Cereti E, Barile D, Coisson JD, Arlorio M, Dessi S, Coroneo V, Cabras P. (**2004**) Chemical composition, plant genetic differences, antimicrobial and antifungal activity investigation of the essential oil of *Rosmarinus officinalis* L. *Journal of Agricultural and Food Chemistry*, ***52***, 3530-3535.

[3] Garbarino JA, Troncoso N, Delpiano P, Carvajal L, Russo A. (**2006**) Antioxidant activity analysis for the selection of *Rosmarinus officinalis* L.. *Natural Product Communications*, ***1***, 1123-1128.

[4] Flamini G, Cioni PL, Macchia M, Ceccarini L. (**2002**) Main agronomic productive characteristics of two ecotypes of *Rosmarinus officinalis* L. and chemical composition of their essential oils. *Journal of Agricultural and Food Chemistry*, ***50***, 3512-3517.

[5] Marchetti M, Sechi B, Usai M. (**2004**) Idroformilazione di oli esenziali: un metodo di valorizzazione di essenze agrumarie di scarto. *La Chimica & l' Industria*, ***86***, 52-56.

[6] Paganelli S, Ciappa A, Marchetti M, Scrivanti A, Matteoli U. (**2006**) Hydroformylation of *m*-diisopropenyl-benzene and 1-isopropyl-3-isopropenyl-benzene for the preparation of the fragrance Florhydral®. *Journal of Molecular Catalyst A: Chemical*, ***247***, 138-144.

[7] Burits M, Bucar F. (**2000**) Antioxidant activity of *Nigella sativa* essential oil. *Phytotherapy Research*, ***14***, 323-328.

[8] Cuendet M, Hostettmann K, Potterat O. (**1997**). lridoid glucosides with free radical scavenging properties from *Fagraea blumei*. *Helvetica Chimica Acta*, ***80***, 1144-1152.

[9] Burda S, Oleszek W. (**2001**). Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry*, ***49***, 2774-2779.

[10] Barry AL. (**1986**) *Procedure for testing antimicrobial agents in agar media: theoretical considerations*. Lorian V. (Ed.), Baltimore.

[11] Deriu A, Sechi LA, Zanetti S. (**2008**) *Rosmarinus officinalis* L.: valutazione attività antimicrobica dell'olio essenziale puro e sue modificazioni semisintetiche. *L'Igiene Moderna*, ***130***, 35-44.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1691 - 1692

Constituents of the Polar Extracts from Algerian *Pituranthos scoparius*

Mostefa Dahia[a]*, Laura Siracusa[b], Hocine Laouer[c] and Giuseppe Ruberto[b]*

[a]*Faculté des Sciences de la Nature et de la Vie, Université de Djelfa, 17000 Djelfa, Algeria*

[b]*Istituto di Chimica Biomolecolare del CNR, Via P. Gaifami, 18 – 95126 Catania, Italy*

[c]*Laboratory of Natural Resources Valorization, Department of Biology, Ferhat Abbas University, 19000 Setif, Algeria*

mdahia2002@yahoo.fr; giuseppe.ruberto@icb.cnr.it

Received: July 31st, 2009; Accepted: November 2nd, 2009

The methanolic and aqueous extracts of *Pituranthos scoparius* (Coss. & Dur.) Benth. & Hook. (Apiaceae) have been analyzed and fifteen metabolites were identified: two cinnamic acids (5-*O*-caffeoyl quinic acid and 5-feruloyl quinic acid), and thirteen known flavonoids (vicenin-2, six quercetin and six isorhamnetin *O*-glycosylated derivatives). 5-*O*-caffeoylquinic acid was the main component, while, of the flavonoids, the isorhamnetin derivatives were present to a greater extent.

Keywords: *Pituranthos scoparius*, Apiaceae, cinnamic acids, flavonoids, LC-UV-Vis-DAD-ESI-MS.

Pursuing our phytochemical investigation of wild plants of the Mediterranean basin, we here report the results of the analyses of *Pituranthos scoparius* (Coss. & Dur.) Benth. & Hook., a medicinal plant of the Saharan territory [1a]. *P. scoparius* has been analyzed for its content of volatile components [1b,1c], isocoumarins [1d] and antibacterial glycosylated flavonoids [1e]. The same flavonoid derivatives have partly been identified in *P. chlorantus* [1f] and *P. tortuosus* [1g].

We here report the compositional study (Table 1) of the polar extracts of *P. scoparius*, obtained with methanol and water, which represent the most similar parts for polarity to folk medical preparations (water infusion). The analyses have been carried out through the use of the LC-UV-Vis-DAD-MS technique to determine the qualitative and quantitative phytochemical profile. First of all, we focused on two components that showed a UV-Vis spectrum diagnostic for cinnamic acids. A detailed analysis of the fragmentation pattern of the compounds led to their identification as 5-*O*-caffeoylquinic acid and 5-*O*-feruloylquinic acid [2a]. The remaining components of the extracts showed UV-Vis and mass spectra ascribable to glycosylated flavonoids. Acid hydrolysis [2b] on a small aliquot of the methanolic extract showed three components identified as quercetin, isorhamnetin and vicenin-2 [2c,2d].

Table 1: Polyphenol derivatives identified in the methanolic and aqueous extracts of *P. scoparius*.

Peak #	$[M-H]^-$ *(m/z)*	Identification	mg/kg dry wt.[b]
1	353	5-*O*-Caffeoylquinic acid	12.9 (M/A)
2	594	Apigenin 6,8 di-*C*-glucoside (Vicenin-2)	2.9 (M/A)
3	367	5-*O*-Feruloylquinic acid	2.4 (M/A)
4	757	Quercetin 3-*O*-gentobiosyl-pentoside[a]	*t*[c] (M/A)
5	625	Quercetin 3-*O*-sophoroside	*t*[c] (M)
6	625	Quercetin 3-*O*-gentobioside	0.3 (M/A)
7	771	Isorhamnetin 3-*O*-gentobiosyl-pentoside[a]	3.3 (M/A)
8	639	Isorhamnetin 3-*O*-sophoroside	0.09 (M)
9	595	Quercetin 3-*O*-glucosyl-pentoside	1.7 (M/A)
10	639	Isorhamnetin 3-*O*-gentobioside	3.7 (M/A)
11	609	Quercetin 3-*O*-neohesperidoside	0.3 (M/A)
12	609	Isorhamnetin 3-*O*-glucosyl-pentoside[a]	2.2 (M)
13	623	Isorhamnetin 3-*O*-rutinoside	0.23(M/A)
14	463	Quercetin 3-*O*-glucoside	1.5 (M)
15	477	Isorhamnetin 3-*O*-glucoside	1.0 (M/A)

[a] Correct isomer not identified. [b] Sum of the two extracts, M = methanol, A=Water; [c] t = trace (< 0.05).

Table 1 shows that 5-*O*-caffeoylquinic acid is the main component, whereas among the flavonoids the isorhamnetin derivatives are present in greater amounts compared with the quercetin ones. Finally, owing to the large difference in yields of the two extracts (0.2 and 4% for the aqueous and methanolic extracts, respectively), it is interesting to report that the same amount of extracts (100 mg) contain comparable amounts of components. This is important to establish the effectiveness of an extract normally used for the biological tests. The high

abundance of flavonoid glycosides and cinnamic acids may explain the effectiveness of this plant as a remedy against fever and rheumatism, as it is known that these metabolites possess anti-inflammatory activities [3a,3b].

Experimental

Plant material and extraction: *Pituranthos scoparius* was collected at the flowering stage (October) in 2004 at the Ain Diss station (Southern Algeria) and dried in the dark at room temperature. The dried aerial parts (1 kg) were ground and extracted (3x) with *n*-hexane, EtOAc, and MeOH to afford 15 g, 12 g, and 40 g extractives, respectively, after solvent evaporation. Finally, the plant residue was suspended in water and extracted three times, to give after filtration, a reddish solution (1.2 L), which was loaded onto a column filled with Amberlite XAD 16 (200 g, 20-60 mesh), conditioned with methanol (500 mL) and water (500 mL), respectively. The water solution was discarded, the column was then washed with water (500 mL) and eluted with methanol (600 mL). The methanolic solution was evaporated to dryness to give a dark red syrup (2 g).

LC-UV-Vis-DAD-MS analyses: LC-UV-Vis-DAD-MS experiments were performed on a Waters instrument equipped with a 1525 Binary HPLC pump, a Micromass ZQ with a ESI Z-spray source operating in negative mode, and a 996 Photo Diode Array Detector (DAD). Chromatographic runs were performed using a reverse-phase column (Alltima C_{18} totally end-capped, 250 x 4.6 mm, 5 µm particle size, Alltech, Italy). Both polar extracts were analysed using solvent system B (2.5% HCOOH in CH_3CN) in solvent system A (2.5% HCOOH in water) with the following gradient: 0 min: 5% B; 10 min: 15% B; 30 min: 25% B; 35 min: 30% B; 50 min: 90% B; then kept for 7 min at 100% B. Solvent flow rate: 1 mL/min, temperature: 25°C (column oven Hitachi L-2300, VWR , Milan, Italy), injector volume: 20 µL. DAD analyses were run in the range between 800 and 190 nm. Total ion current (TIC) chromatograms were acquired in negative mode, with a cone voltage of 20 V in the mass range between 100 and 1500 *m/z* units. Capillary voltage: 2.75 kV; source temperature: 150°C; desolv. temperature: 280°C; gas flow (L/h): 400 (desolv.) and 210 (cone). Data were processed by MassLynx v. 4.00 software (Waters, Milan, Italy).

HPLC-DAD quantification: HPLC analyses were carried out on a Dionex instrument equipped with a P580 binary pump, a PDA-100 Photodiode Array detector, a TCC-100 Thermostatted Column Compartment and an ASI-100 Automated Sample Injector. Collected data were processed through a Chromeleon Management System v. 6.70. Quantification was carried out using chlorogenic acid and quercetin 3-*O*-glucoside as standards. Analyses were carried out in triplicate.

Acknowledgements - The authors are grateful to Dr Alice Sperlinga for her contribution to this work whilst preparing her degree thesis. This work was financially supported by Consiglio Nazionale delle Ricerche (C.N.R. – Rome, Italy), RS-Oran, Algerie and Ministère de l'Enseignement Supérieur et de la Recherche Scientifique (MESRS – Alger, Algerie).

References

[1] (a) Quezel P, Santa S. (**1962-63**) *Nouvelle flore de l'Algérie et des régions désertiques méridionales.* Vol. 2, Edition du Centre National de la Recherche Scientifique (CNRS), 15 Quai Anatole France, 75007 Paris, 643-666 ; (b) Vernin G, Lageot C, Chiglione C, Dahia M, Parkanyi CJ. (**1999**) GC/MS analysis of the volatile constituents of the essential oils of *Pituranthos scoparius* (Coss. et Dur.) Benth. et Hook. from Algeria. *Journal of Essential Oil Research*, ***11***, 673-676; (c) Verite P, Nacer A, Kabouche Z, Seguin E. (**2004**) Composition of seed and stem essential oils of *Pituranthos scoparius* (Coss. & Dur.) Schinz. *Flavour and Fragrance Journal*, ***19***, 562-564; (d) Hamada H, Mohammed B, Massiot G, Long C, Lavaud C. (**2004**) Alkylated isocoumarins from *Pituranthos scoparius. Natural Products Research*, ***18***, 409-413; (e) Benmekhbi L, Kabouche A, Kabouche Z, Ait-Kaki B, Touzani R, Bruneau C. (**2008**) Five glycosylated flavonoids from the antibacterial butanolic extract of *Pituranthos scoparius*. *Chemistry of Natural Compounds*, ***44***, 639-641; (f) Touil A, Rhouati S, Creche J. (**2006**) Flavonoid glycosides from *Pituranthos chloranthus*. *Chemistry of Natural Compounds*, ***42***, 104-105; (g) Singab A, Khalifa T, Mahran GH, Okada Y, Matsumaru Y, Nishino H, Okuyama T. (**1998**) A new flavonoid glycoside from *Pituranthos tortuosus* Desf, Benth & Hook. *Natural Medicine* (Tokyo), ***52***, 191-194.

[2] (a) Clifford MN, Johnston KL, Knight S, Kuhnert N. (**2003**) Hierarchical scheme for LC-MS. Identification of chlorogenic acids. *Journal of Agricultural and Food Chemistry*, ***51***, 2900-2911; (b) Nuutila AM, Kammiovirta K, Oksman-Caldentey K-M. (**2002**) Comparison of methods for the hydrolysis of flavonoids and phenolic acids from onion and spinach for HPLC analysis. *Food Chemistry*, ***76***, 519-525; (c) Niessen WMA. (**2006**) LC-MS analysis of plant phenols in *liquid chromatography-mass spectrometry*, 3rd ed, CRC Press, Chapter 15, 413-437, and references therein; (d) Stobiecki M, (**2000**) Application of mass spectrometry for identification and structural studies of flavonoid glycosides *Phytochemistry*, ***54***, 237-256;

[3] (a) Yen C, Hsieh P, Hwang T, Lan Y, Chang F, Wu Y. (**2009**) Flavonol glycosides from *Muehlenbeckia platyclada* and their anti-inflammatory activity. *Chemical & Pharmaceutical Bulletin*, ***57***, 280-282 ; (b) dos Santos M, Almeida MC, Lopes NP, Petto de Souza GE. (**2006**) Evaluation of the anti-inflammatory, analgesic and antipyretic activities of the natural polyphenol chlorogenic acid. *Biological & Pharmaceutical Bulletin*, ***29***, 2236-2240.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1693 - 1696

Verbascoside isolated from *Lepechinia speciosa* has inhibitory Activity against HSV-1 and HSV-2 *in vitro*

Fernanda O. Martins[a#], Patricia F. Esteves[b#], Gabriella S. Mendes[a], Nanci S. Barbi[c], Fabio S. Menezes[d]* and Maria T.V. Romanos[a] ([#] authors have equally contributed to this paper)

[a]*Laboratório Experimental de Drogas Antivirais e Citotóxicas (LEDAC), Departamento de Virologia doInstituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), CCS, Bloco I, Rio de Janeiro, Brasil*

[b]*Laboratório de Estudo Químico de Espécies de Lamiifloreae, Faculdade de Farmácia/UFRJ, CCS, Bloco I, Rio de Janeiro, Brasil*

[c]*Departamento de Análises Clínicas e Toxicológicas/UFRJ, CCS, Bloco I, Rio de Janeiro, Brasil*

[d]*School of Pharmacy and Pharmaceutical Sciences – Trinity College Dublin – University of Dublin, Dublin 2, Ireland*

desouzaf@tcd.ie

Received: July 26th, 2009; Accepted: September 20th, 2009

Verbascoside has been isolated form *L. speciosa* after several different chromatographic methods. After its purification, the structure has been unequivocally established using modern spectroscopic techniques. As for the antiviral activity, the maximum non toxic concentration has been established and this concentration has been used in the anti herpes assay, *in vitro*. Mechanism of action for this molecule regarding the anti-herpes activity has been studied encompassing the following assays: virucidal activity, cellular receptor assay, penetration assay and intracellular assay, in order to understand the activity for this natural product.

Keywords: HSV-1, HSV-2, antiviral activity, verbascoside, *Lepechinia speciosa*.

Plants of the genus *Lepechinia* (Lamiaceae family) are used in traditional medicine for the treatment of uterine infections [1a], uterine tumors [1b], gastrointestinal ailments [1c] and diabetes mellitus control [1d,2]. In addition, vasorelaxant [3a] spasmolytic [3b,3c], antimicrobial [1a,4a], cytotoxic [bb] and antioxidant [5] activities have been also reported. In this work, the antiviral activity of a compound isolated from *Lepechinia speciosa* was analysed. The focus was on herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) infections. HSV-1 is widespread in the human population and commonly causes infections of the skin or mucosal surfaces, principally associated with oral diseases and occasionally it can cause serious diseases such as encephalitis and ocular infections. HSV-2 is commonly associated to sexually transmitted diseases, being the main cause of genital ulceration worldwide causing severe infections in neonates and immunocompromised patients and it is a cofactor in human immunodeficiency virus type 1 (HIV-1) transmission. The drug of choice for treating these diseases is acyclovir [6], but the widely use for the treatment and suppressive therapy of herpes simplex virus infections led to the appearance of acyclovir-resistant strains as a result of long-term therapy [7]. Therefore, new strategies should be developed as alternative therapy. In this study, we showed the antiviral activity of a verbascoside (Figure 1) isolated from *Lepechinia speciosa* against herpes simplex virus types 1 and 2 with results obtained for *in vitro* analysis.

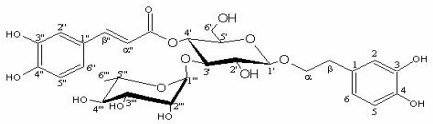


Figure 1: Chemical structure of verbascoside.

There are several pharmacological properties already described for verbascoside, such as vasorelaxant [8a]

Table 1: Cytotoxicity and anti-HSV activity of verbascoside from *Lepechinia speciosa*.

Compound	CC_{50}[a] µg/mL	EC_{50}[b] µg/mL		SI[c]	
		HSV-1[d]	HSV-2[e]	HSV-1	HSV-2
Verbascoside	>200	58	8.9	>3.4	>22.4
Acyclovir*	>200	0.8	1.38	>250	>144.9

[a]Concentration which caused a 50% reduction in the number of viable cells. [b]Concentration required to achieve 50% protection against virus-induced cytopathic effects. [c]Selective index (CC_{50}/EC_{50}). [d]Herpes simplex virus type 1. [e]Herpes simplex virus type 2.*Standard compound

and antiviral activity against respiratory syncytial virus [8b,8c] but the antiviral activity against Herpes virus have never been studied for this molecule. Experiments to evaluate the cytotoxic concentration of the verbascoside and acyclovir were performed before the antiviral activity assay. No change of the cellular morphology was observed when the cells were exposed to the compounds at concentration of 200µg/mL. The cellular viability was not significantly affected by the addition of the verbascoside and acyclovir at the concentration of 200 µg/mL that presented 94% and 100% of viable cells, respectively.

It was observed that verbascoside exhibit a dose dependent activity and EC_{50} values were 58 µg/mL to HSV-1 and 8.9 µg/mL to HSV-2. The selective index (SI) of the verbascoside was higher than 3.4 and 22.4 to HSV-1 and HSV-2, respectively (Table 1). It was not possible to determine the exact CC_{50} values of verbascoside and acyclovir, once the higher concentration used was 200 µg/mL. In contrast of aciclovir, whose activity is only observed in one stage of viral cycle, verbascoside was capable to act in different stages of both viruses replication.

In order to determine the mechanism of action of verbascoside against HSV-1 and HSV-2 infections, experiments were performed to evaluate their inhibitory effect at different stages of virus infection into cells. As observed in Figure 2, verbascoside acted in different stages when comparing both viruses. Against HSV-1, it showed virucidal action, demonstrating that one of the mechanisms of action was the ability to interact with the viral particles and to prevent the viral adsorption. In this step, verbascoside was able to inactivate the HSV-1 particles at 82.2%. Inhibitory activity was also observed in intracellular step showing 82.2% of viral inhibition. Against HSV- 2, verbascoside interacted with cellular receptors avoiding 92% the virus attachment and also inhibiting 92% the penetration step. These results are important due to the fact that the onset of viral infection is an attractive target for therapy because the virus can be blocked before establishing the infection. It should be noticed that the verbascoside mechanism of action was different against HSV-1and HSV-2, showing specific activity. This molecule has previously been isolated in our group from another medicinal plant and non-toxic effects have been shown using different methodologies [9a,9b].

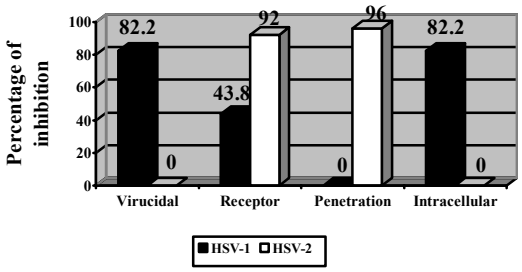


Figure 2: Mechanism of action of the verbascoside from *Lepechinia speciosa*. Virucidal effect - virus particles were treated with verbascoside, at concentrations of 200 µg/mL. The samples were incubated at 37°C for 2h, diluted, and then inoculated in Vero cell monolayers. Receptor – verbascoside was added to Vero cells at 1h pre-infection. Penetration – verbascoside was added to Vero cells after 1h post-infection. Intracellular – verbascoside was added to Vero cells 2h post-infection. After incubation for 48h at 37°C, the supernatants were collected and virus titers in treated and untreated cells were determined and the activity was expressed as percentage of inhibition (PI).

Conclusion: Despite acyclovir is the drug of choice for treating the HSV infections, new antiviral agents exhibiting different mechanisms of action are urgently needed, mainly due to the high number of acyclovir-resistant strains. Verbascoside showed to be a very versatile and potent compound against HSV-1 and 2, being capable to inhibit different steps of viral replication differently from acyclovir.

Experimental

Cells and Virus: Vero cells (African green monkey kidney) were grown in Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50 µg/mL gentamicin, 2.5 µg/mL fungizone and 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C in 5% CO_2 atmosphere. Herpes simplex virus type 1 and type 2 strains isolated from typical oral and genital lesions, respectively, at the Departamento de Virologia, Universidade Federal do Rio de Janeiro (UFRJ), Brazil. The isolates were typed by polymerase chain reaction (PCR) using specific primers to identify HSV-1 and HSV-2 [10] and propagated in a Vero cell culture. The titres were assessed by the cytopathic

end-point assay and were expressed as 50% tissue culture infective dose ($TCID_{50}$) per mL. The virus suspensions were stored at -70°C until use.

Extraction and isolation of the compound: Aerial parts of *Lepechinia speciosa* (St. Hill) Epling were collected in the National Park of Itatiaia, Rio de Janeiro, Brazil. Its authenticity was confirmed by Dr Regina Braga de Moura, (Universidade Estácio de Sá). Voucher specimens (RFA-28365) were deposited in the Herbarium of Universidade Federal do Rio de Janeiro (Brazil) .

The dried and powdered aerial parts of *L. speciosa* were extracted with ethanol at room temperature for at least 24 h, and this was repeated until exhaustion of the material. The ethanol extract was concentrated under reduced pressure, suspended in water and then submitted to a liquid-liquid partition procedure with different solvents [11]. The ethyl acetate (FAE) fraction (1.4 g) was fractionated over a silica gel column starting with dichloromethane and increasing proportions of methanol. Fraction 28, eluted with EtOAc: MeOH (6:1) yielded 212 mg of verbascoside (15.14% w/w).

Cytotoxicity assay

Verbascoside from aerial parts of *L. speciosa* and acyclovir were dissolved in dimethyl sulfoxide (DMSO). Stock solutions were prepared in water at 400 μg/mL and sterilized by filtration using a 0.22 μm Millipore membrane filter. The cytotoxicity assay was performed by incubating triplicate Vero cell monolayers cultivated in 96-well microplates with two-fold serial dilutions of compounds for 48 h at 37°C in 5% CO_2 atmosphere. The morphological alterations of the treated cells were observed in an inverted optical microscope (Leitz) and the maximum non-toxic concentrations (MNTC) were determined [12a]. Cellular viability was further evaluated by the neutral red dye-uptake method [12b]. The 50% cytotoxic concentration (CC_{50}) was defined as the compound concentration which caused a 50% reduction in the number of viable cells.

Antiviral activity assay: Vero cell monolayers cultivated in 96-well microtitrer plates were treated with verbascoside or acyclovir at the MNTC (200μg/mL). Immediately after treatment, logarithmical dilutions of HSV-1 or HSV-2 suspensions were added to treated and untreated cell cultures. After 48 h incubation at 37°C in 5% CO_2 atmosphere, the virus titres in treated and untreated cells were determined. The antiviral activity was expressed as percentage inhibition (PI) [12c] using antilogarithmic $TCID_{50}$ values as follows: PI = [1 - (antilogarithmic test value/antilogarithmic control value)] x 100. The percentage of inhibition is deduced by the viral titre, which is calculated through a statistic test (Reed & Muench). The dose-response curve was established starting from the MNTC, and the 50% effective concentration (EC_{50}) was defined as the concentration required to achieve 50% protection against virus-induced cytopathic effects. The selective index (SI) was determined as the ratio between CC_{50} and EC_{50}.

Mechanism of action studies

Virucidal assay: 100 μl of HSV-1 or HSV-2 suspension were added to either 900 μl of verbascoside, at concentration of 200μg/mL in MEM-Eagle, or MEM-Eagle without serum (control), according to Chen *et al*. [12d]. The samples were incubated at 37°C for 2 h, diluted, and then inoculated in Vero cell monolayers. After 48 h incubation as before, the residual titres of the treated and untreated viruses were determined and expressed as percentage inhibition (PI).

Cellular receptor assay: Verbascoside was added to Vero cell monolayers before infection (pretreatment) in order to evaluate the effect of the compounds on cell receptors. Vero cell monolayer was pretreated with verbascoside, at concentration of 200μg/mL, for 1 h at 4°C. After this time, the cells were washed three times with MEM- Eagle for removing sample, and the treated and untreated cells were inoculated with 100 $TCID_{50}$/mL of HSV-1 or HSV-2. After incubating the cells at 37°C for 48 h, the supernatant was collected and virus titres in treated and untreated cells were determined and the activity was expressed as PI.

Penetration assay: Vero cell monolayers were inoculated with 100 $TCID_{50}$/mL of HSV-1 or HSV-2 and incubated for 1 h at 4°C. After this period, the monolayers were washed with culture medium and verbascoside, at concentration of 200μg/mL, was added. The cultures were immediately shifted to 37°C to allow the penetration of the particles into the cells for another hour. After incubation, the monolayer was washed, MEM- Eagle was added, and the cultures incubated at 37°C for 48 h. After incubating, the supernatant was collected and virus titres in treated and untreated cells were determined and the activity was expressed as PI.

Intracellular assay: Vero cell monolayers were inoculated with 100 $TCID_{50}$/mL of HSV-1 or HSV-2 and incubated at 37°C for two hours. The cells were washed and verbascoside at concentration of 200μg/mL or MEM-Eagle (control) were added and the cultures incubated at 37°C for 16 h. After incubation, the cells were washed to remove the compound before the release of viral particles. MEM-Eagle was added and the cultures incubated for 32 h at 37°C. After incubating, the supernatant was collected and virus titres in treated and untreated cells were determined and the activity was expressed as PI.

Acknowledgements - The authors thank Soluza dos Santos Gonçalves for the technical assistance. This study was supported in part by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and, Fundação Carlos Chagas de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Brazil.

References

[1] (a) Dimayuga RE, Garcia SK, Nielsen PH, Carsten C. (**1991**) Carnosol: A diterpene antibiotic from *Lepechinia hastata*. *Journal of Ethnopharmacology*, ***31***, 43-48; (b) Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. (**2000**) Plants used against cancer- an extension of the work of Jonathan Hartwell. *Journal of Ethnopharmacology*, ***73***, 347-377; (c) Azevedo JGA, Lopez JLM, Cortes, AM. (**2005**) *In vitro* anti-*Vibrio cholerae* activity of essential oil from *Lepechinia caulenscens*. *Fitoterapia*, ***76***, 104-107; (d) Román-Ramos R, Flores-Sáenz JL, Partida-Hernández G, Lara-Lemus A, Alarcón-Aguilar F. (**1991**) Experimental study of the hypoglycemic effect of some antidiabetic plants. *Archivos de Investigacion Medica*, ***22***, 87-93.

[2] Alarcon-Aguilara FJ, Roman-Ramos R, Perez-Gutierez S, Aguilar-Contreras A, Contrera-Weber CC, Flores-Saenz S. (**1998**) Study of the anti-hiperglycemic effect of plants used as antidiabetics. *Journal of Ethnopharmacology*, ***61***, 101-110.

[3] (a) Aguirre-Crespo F, Vergara-Galicia J, Villalobos-Molina R, López-Guerrero JL, Navarrete-Vázquez G, Estrada-Soto S. (**2006**) Ursolic acid mediates the vasorelaxant activity of *Lepechinia caulescens* via NO release in isolated rat thoracic aorta. *Life Sciences*, ***79***, 1062-1068; (b) Rodriguez-Lopez V, Salazar L, Estrada S. (**2003**) Spasmolytic activity of several extracts obtained from some Mexican medicinal plants. *Fitoterapia*, ***74***, 725-728; (c) Estrada-Soto S, Rodrígues-Avilez A, Castañeda-Avila C, Castillo-España P, Navarrete-Vásquez G, Hernández L, Aguirre-Crespo F. (**2007**) Spasmolytic action of Lepechinia caulescens is through calcium channel blockade and NO release. *Journal of Ethnopharmacology*, ***114***, 364-370.

[4] (a) Rojas R, Bustamante B, Bauer J. (**2003**) Antimicrobial activity of selected Peruvian medicinal. *Journal of Ethnopharmacology*, ***88***, 199-204; (b) Jonathan LT, Che CT, Pezzuto JM. (**1989**) 7-*O*-Methylhorminone and other cytotoxic diterpene quinones from *Lepechinia bullata*. *Journal of Ethnopharmacology*, ***31***, 43-48.

[5] Parejo I, Caprai E, Bastida J, Viladomat F, Jauregui O, Codina C. (**2004**) Investigation of *Lepechinia graveolens* for its antioxidant activity and phenolic composition. *Journal of Ethnopharmacology*, ***94***, 175-184.

[6] Schaeffer HJ, Beauchamp L, de Miranda P, Elion GB, Bauer DJ, Collins P. (**1978**) 9-(2-Hydroxyethoxymethyl) guanine activity against viruses of the herpes group. *Nature* (London), ***272***, 583-585.

[7] Christophers J, Sutton RN. (**1987**) Characterisation of acyclovir-resistant and -sensitive clinical herpes simplex virus isolates from an immunocompromised patient. *Journal of Antimicrobial Chemotherapy*, ***20***, 389-398.

[8] (a) Hennebelle T, Sahpaz S, Joseph H, Bailleul F. (**2008**) Ethnopharmacology of *Lippia alba* - *Journal of Ethnopharmacology*, ***116***, 211–222; (b) Chen JL, Blanc P, Stoddart CA, Bogan M, Rozhon EJ, Parkinson N, Ye Z, Cooper R, Balick M, Nanakorn W, Kernan, MR. (**1998**) New iridoids from the medicinal plant *Barleria prionitis* with potent activity against respiratory syncytial virus. *Journal of Natural Products*, ***61***, 1295-1297; (c) Kernan MR, Amarquaye A, Chen JL, Chan J, Sesin DF, Parkinson N, Ye Z, Barrett M, Bales C, Stoddart CA, Sloan B, Blanc P, Limbach C, Mrisho S, Rozhon EJ. (**1998**) Antiviral phenylpropanoid glycoside from the medicinal plant *Markhamia lutea*. *Journal of Natural Products*, ***61***, 564-570.

[9] (a) Falcão DQ, Costa ER, Kuster RM, Nielloud F, Vian L, Menezes FS. (**2007**) Evaluation of cytotoxicity, phototoxicity and genotoxicity from *Calceolaria chelidonioides* (Scrophulariaceae) flowers ethanol extract. *Planta Medica*, ***73***, 477; (b) Falcão DQ, Costa ER, Kuster RM, Nielloud F, Vian L, Menezes FS. (**2007**) The obtention of an oil/water nanoemulsion containing the *Calceolaria chelidonioides* flowers ethanol extract and its skin hydration power. *Planta Medica*, ***73***, 631.

[10] Markoulatos P, Georgopoulou A, Siafakas N, Plakokefalos E, Tzanakaki G, Kourea-Kremastinou J. (**2001**) Laboratory diagnosis of common herpesvirus infections of the central nervous system by a multiplex PCR assay. *Journal of Clinical Microbiology*, ***39***, 4426-4432.

[11] Menezes FS; Falcão DQ, Mendonça-Filho RFW, Silveira CS, Rennó MN, Rodrigues VP, Minto ABM, Moreira DL, Matheus ME, Fernandes PD, Kaplan MAC. (**2005**) Chemical and pharmacological survey on Brazilian medicinal plants using ethnopharmacology information as a tool. *Acta Horticulturae*, ***675***, 89-95.

[12] (a) Walker WE, Waisbren BA, Martins RR, Batayias GE. (**1971**) A method for determining sensitivities of antiviral drugs *in vitro* for possible use as clinical consultation. *American Journal of Clinical Pathology*, ***56***, 687-692; (b) Neyndorff HC, Bartel DL, Tufaro F, Levy JG. (**1990**) Development of a model to demonstrate photosensitizer-mediated viral inactivation in blood. *Transfusion*, ***30***, 485-490; (c) Nishimura T, Toku K, Fukuyasu H. (**1977**) Antiviral compounds. XII. Antiviral activity of aminohydrazones of alkoxyphenil substituted carbonyl compounds against influenza virus in eggs and mice. *Kitasato Archives of Experimental Medicine*, ***50***, 39-46; (d) Chen M, Griffith P, Lucia HL, Hsiung GD. (**1988**) Efficacy of S26308 against guinea pig cytomegalovirus infection. *Antimicrobial Agents Chemotherapy*, ***32***, 678-683.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1697 - 1700

Magnoflorine and Phenolic Derivatives from the Leaves of *Croton xalapensis* L. (Euphorbiaceae)

Carolina Arevalo[a], Cinzia Lotti[b], Anna Lisa Piccinelli[b], Mariateresa Russo[c], Ines Ruiz[a] and Luca Rastrelli[b,*]

[a]*Departamento de Control Químico*, *Facultad de Farmacia*, *Universidad Nacional Autonoma – Tegucigalpa*, *Honduras*

[b]*Dipartimento di Scienze Farmaceutiche*, *Università degli Studi di Salerno*, *Via Ponte don Melillo*, *84084 Fisciano (SA)*, *Italy*

[c]*Dipartimento di Scienze e Tecnologie Agro-Forestali ed Ambientali*, *Università Mediterranea di Reggio Calabria*, *Italy*

rastrelli@unisa.it

Received: August 17th, 2009; Accepted: October 16th, 2009

The alkaloid magnoflorine **1**, has been isolated for the first time from *Croton xalapensis* (Euphorbiaceae), in addition two phenylpropenols derivates, 3,4-dimethoxy-(*E*)-cinnamyl alcohol **2** and 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3**, 3,4,5-trimethoxycinnamic acid **4**, gallic acid **5**, methyl gallate **6** and 3,4-dihydroxybenzoic acid **7** have been also found; these compounds were identified by spectroscopic analysis particularly, 2D NMR and ESI-MS/MS techniques. The high concentration of magnoflorine, calculated with quantitative HPLC, of the aqueous extract, probably contributes to the remarkable medicinal properties of this plant. In addition this is the first phytochemical study on *Croton xalapensis* to be reported.

Keywords: Euphorbiaceae, *Croton xalapensis*, magnoflorine, phenylpropenol derivatives.

Croton (Euphorbiaceae) is one of the largest genera of flowering plants, with nearly 1300 species of herbs, shrubs, and trees that are ecologically prominent and often important elements of secondary vegetation in the tropics and subtropics worldwide [1]. The *Croton* species of plants, are used in South America as folk medicines for the treatment of wounds, inflammation and cancer [2]. The genus *Croton* is one of the richest sources of alkaloids with aporphine, proaporphine and morphinandienone skeletons [3a-3c], in addition, flavonoids [4], lignans [5], phenols [6] and diterpenes with the clerodane skeleton [7a-7d] are also commonly found in this genus. The major constituents of the essential oils from leaves, inflorescences and stalks were monoterpenes [8,9]. *C. xalapensis,* commonly named "china native", is a small tree that grows in the forests of southern Honduras. Literature phytochemical studies on *C. xalapensis* have not been reported; this is the first chemical investigation of *C. xalapensis* leading to the isolation of 7 compounds including the alkaloid magnoflorine and representative phenolic compounds. The leaves of *C. xalapensis* are used as a decoction to treat wounds, infections, malaria, fever, gastrointestinal disease and diabetes in the Honduran folk medicine. Mature trees of genus *Croton* produce a blood-red to yellowish orange colored sap, or latex, which is highly regarded for its ability to speed the healing of wounds. Due to this blood-like appearance, the latex is termed "sangre de drago" in Spanish and "dragon's blood" in English [10]. Over the last two decades a chemical examination of this latex has led to the isolation of the major wound healing constituent of *C. lechleri*, which has been identified as the benzylisoquinoline-derived alkaloid taspine, which results from the conversion of magnoflorine [11a-11c]. The leaves of *C. xalapensis* were extracted in order to investigate its chemical constituents; a part of the methanol extract was partitioned between *n*-butanol and water. The water soluble portion was chromatographed on a RP-18 column and a fraction obtained was further purified by RP-HPLC to yield the aporphine, magnoflorine **1**. The *n*-butanol soluble portion was

fractionated by Sephadex LH-20 to yield pure compounds 3,4-(*E*) dimethoxycinnamyl alcohol **2**, 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3**, 3,4,5-trimethoxycinnamic acid **4**, gallic acid **5**, methyl gallate **6** and 3,4-dihydroxybenzoic acid **7**. The structure of all compounds were unambiguously established by comparison of physical and spectroscopic properties including mass spectrometry, 1D (^{1}H, ^{13}C) and 2D (HSQC, HMBC and COSY) NMR spectroscopy with those reported in literature [12a-12d].

Compound **1** was identified as magnoflorine with the physical and the MS and NMR data [13,14]. The quantitative analysis of magnoflorine from C. *xalapensis* leaves was performed by HPLC. The concentrations of compound in the extract, calculated from the experimental peak areas by interpolation to standard calibration curve was 139.58 mg/g dry weight. This compound was found to inhibit the copper-mediated (Cu^{2+}) oxidation of LDL, as well as of glycated and glycoxidated LDL by increasing the lag time of conjugated diene formation and preventing the generation of thiobarbituric acid reactive substances (TBARS) [15]. These results suggest that magnoflorine may be useful for preventing the oxidation of various LDL forms. Magnoflorine is reported to have multiple pharmacologies as a neuromuscular blocking agent (AChR blocking agent), lipoxygenase inhibitor [16], as well as cytotoxic [17], immunosuppressive [18] and antimicrobial activities [12d].

Compound **3**, was isolated as a yellow amorphous solid and its molecular formula was determined by HRESIMS to be $C_{11}H_{14}O_4$. The ^{1}H and ^{13}C NMR spectra suggested a similar skeleton to that of **2**. The concerted interpretation of the ^{1}H-NMR and COSY spectra allowed us to evidenced the observation of H-2/H-6 *meta*-coupling (J=1.5 ppm); the position of the substituents were deduced as occurring at C-3 and C-4 and C-5 (two methoxyl groups and one hydroxyl group) using HMBC connectivities between H-2 and H-6 with C-7 (131.6 ppm). The coupling constant (J_{AB}=15.9 Hz) of the olefinic protons suggested a *trans* double bond. These data suggest that compound **3** is identical with 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol reported from *Ferula sinica* [14] without ^{13}C NMR data. Thus we report the NMR data here for compound **3** in CD_3OD.

Alkaloids have been proposed as chemotaxonomic markers in the infraspecific classification of the genus *Croton* [3a]. Aporphine alkaloids are widely distributed among members of *Croton* genus but this is the first report of the occurrence of magnoflorine in *Croton xalapensis*.

Experimental

General Experimental Procedure: A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for ^{1}H and at 150.86 MHz for ^{13}C, was used for NMR experiments; chemical shifts are expressed in δ (parts per million) referring to the solvent peaks δ H 3.34 and δ C 49.0 for CD_3OD; coupling constants, J, are in Hertz. DEPT, ^{13}C, DQF-COSY, HSQC, and HMBC NMR experiments were carried out using the conventional pulse sequences as described in the literature. Electrospray ionization mass spectrometry (ESIMS) was performed using a Finnigan LCQ Deca instrument from Thermo Electron (San Jose, CA) equipped with Xcalibur software. Instrumental parameters were tuned for each investigated compound: capillary voltage was set at 3 V, the spray voltage at 5.10 kV and a capillary temperature of 220°C and the tube lens offset at - 60 V was employed; specific collision energies were chosen at each fragmentation step for all the investigated compounds, and the value ranged from 15-33% of the instrument maximum. Data were acquired in the MS1 scanning mode (*m*/*z* 150-700). All compounds were dissolved in MeOH : H_2O (1:1) and infused in the ESI source by using a syringe pump; the flow rate was 5 μL/min. Chromatography was performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden) employing MeOH as solvent. Column chromatography was carried out employing Silica gel RP18 (0.040–0.063 mm; Carlo Erba) and MeOH:H_2O gradients. HPLC separations were performed on a Waters 590 series pumping system equipped with a Waters R401 refractive index detector and a Kromasil C18 column (250 x 10 mm i.d., 10 μm, Phenomenex). HPLC-grade methanol was purchased from Sigma Aldrich (Milano, Italy). HPLC-grade water (18 mΩ) was prepared by a Milli-Q50 purification system (Millipore Corp., Bedford, MA). TLC analysis was performed with Macherey-Nagel precoated silica gel 60 F_{254} plates. Quantitative HPLC analysis was performed on Agilent 1100 series system consisting of a G-1312 binary pump, a G-1328A Rheodyne injector (20 μL loop), a G-1322A degasser, and a G-1315A photodiode array detector, equipped with a 250 x 4.6 mm C-18 Thermo column.

Plant Material: The leaves of *C. xalapensis* were collected in Lange, Valle, Honduras, in January 2004. The plant was identified by Dr. Cirilo Nelson. A

voucher specimen was deposited in the herbarium of the Botanical Department of the Universidad Nacional Autonoma de Honduras, Tegucigalpa, Honduras (Voucher No. 214)

Extraction and Isolation Procedure of Compounds 1-7: Dried and powdered leaves (500 g) of *C. xalapensis* were extracted for a week, three times, at room temperature using solvents of increasing polarity; namely, petroleum ether, chloroform, and methanol. Part (8 g) of the MeOH extract (220 g) was partitioned between *n*-butanol and water. The H_2O soluble portion was chromatographed on a RP-18 column using MeOH-H_2O (from 60% to 20% of MeOH). The fraction obtained (3 g) was successively purified by RP-HPLC on a 250 mm x 10 mm, Kromasil C18, Phenomenex column at a flow rate of 3.0 mL/min with MeOH-H_2O (20:80) to yield magnoflorine **1** (2.3 g). The *n*-butanol soluble portion (5 g) was chromatographed on a Sephadex LH-20 column (100 cm X 5.0 cm) using CH_3OH as mobile phase with a flow rate of 1 mL/min; 60 fractions collected of 8 mL each and monitored by TLC [Si-gel plates, using solvent system *n*-BuOH-AcOH-H_2O (60:15:25)]. TLC plates were developed using UV 254 nm, 366 nm, $Ce(SO_4)/H_2SO_4$. Using the above criteria the 60 fractions were combined into 4 major fractions (1-4). Fraction 1 and 2 (2.0 g) were purified by RP-HPLC on a 250 mm x 10 mm, Kromasil C18, Phenomenex column at a flow rate of 3.0 mL/min with MeOH-H_2O (20:80) to yield pure compound 3,4-(*E*)-dimethoxycinnamyl alcohol **2** (18.5 mg) and 3,4,5-trimethoxycinnamic acid **4** (2.4 mg). Fractions 3 and 4 (2.4 g) were purified by RP-HPLC at the same chromatographic conditions described for earlier fractions to yield pure compounds 3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol **3** (12.1 mg), gallic acid **5** (23.8 mg), methyl gallate **6** (11.1 mg) and 3,4-dihydroxybenzoic acid **7** (6.9 mg).

Magnoflorine (**1**)

Yellow amorphous solid.
UV λ_{max} (MeOH): 230.2, 278.6, 310.4 nm.
^{1}H and ^{13}C NMR were similar to literature [13].
ESI-MS *m/z* 342 $[M-H]^-$; MS/MS *m/z* 297 $[(M - H)-45]^-$.

3,4-dimethoxy-(*E*)-cinnamyl alcohol (**2**)

Yellow amorphous solid
^{1}H and ^{13}C NMR were similar to literature [12c].
ESI-MS *m/z* 193 $[M-H]^-$.

3,4-dimethoxy-5-hydroxy-(*E*)-cinnamyl alcohol (**3**)

Yellow amorphous solid.
UV λ_{max} (MeOH): 280.0, 254.2 nm.
^{1}H NMR (CD_3OD): δ 6.66 (1H, d, *J* = 1.5, H-2), 6.51 (1H, d, *J* = 1.5, H-6), 6.50 (1H, d, *J* = 15.6, H-7), 6.27 (1H, dt, *J* = 15.6 and 6.8, H-8), 4.31 (1H, d, *J* = 6.8, H-9), 3.89 (3H, s, 3-OMe), 3.87 (3H, s, 4-OMe). ^{13}C NMR (CD_3OD): 130.1 (C-1), 110.9 (C-2), 148.1 (C-3), 141.3 (C-4), 147.8 (C-5), 102.8 (C-6), 131.7 (C-7), 127.5 (C-8), 62.4 (C-9), 56.2 (3-OMe), 56.1 (4-OMe). ESI-MS *m/z* 209 $[M-H]^-$.

Trimethoxycinnamic acid (**4**)

Yellow amorphous solid.
^{1}H and ^{13}C NMR were similar to literature [12a].
ESI-MS *m/z* 237 $[M- H]^-$.

Gallic Acid (**5**)

White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m*/ 169 $[M-H]^-$.

Methyl Gallate (**6**)

White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m*/*z* 183 $[M-H]^-$

3,4-Dihydroxy Benzoic Acid (**7**)

White amorphous solid.
^{1}H and ^{13}C NMR were consistent with the literature [12a].
ES-MS, *m*/*z* 153 $[M-H]^-$

***C. xalapensis* Infusion preparation:** The aqueous extract of *C. xalapensis* was obtained by pouring 50 mL of boiling distilled water on 1.5 g of dried leaves and steeping it for 10 min; then the infusion was filtered through filter paper and freeze-dried. The yield of the lyophilized aqueous extract was 447 mg (29.8% of dried leaves). The extractions were performed in triplicate.

HPLC Quantitative Analysis: Quantitative HPLC of the *C. xalapensis* infusion was carried out using a isocratic solvent, MeOH-H_2O (20:80). Detection wavelength was 280 and 320 nm, the flow rate of 1.0 mL/min and the injection volume was 20 μL. Magnoflorine was identified by comparing the retention time of the peak in the aqueous extract with that of the standard compound.

Quantification: The lyophilized infusion was diluted a volume of 10 mL in a volumetric flask. Quantification was performed by reporting the

measured integration area in the calibration equation of the corresponding standard of magnoflorine. The linearity of responses for magnoflorine (**1**) was determined on six level of concentration with three injections for each level. The concentration of magnoflorine calculated from the experimental peak areas by interpolation to standard calibration curves was 139.58 mg/g in dry.

References

[1] Webster GL. (**1993**) A provisional synopsis of the sections of the genus *Croton* (Euphorbiaceae). *Taxon*, ***42***, 793-823.

[2] Marini Bettolo R, Scarpati ML, (**1979**) Alkaloids of *Croton draconoides*. *Phytochemistry*, ***3***, 520.

[3] (a) Farnsworth NR, Blomster NR, Messmer WM, King JC, Persinos GJ, Wilkes JD. (**1969**) A phytochemical and biological review of the genus *Croton*. *Lloydia*, ***32***, 1-28; (b) Southon IW, Buckingham J. (**1989**) *Dictionary of Alkaloids.* Chapman & Hall, London; (c) Del Castillo CH, De Simone F, De Feo V. (**1996**) Proaporphine alkaloids from *Croton ruizianus* Muell.-Arg. (Euphorbiaceae). *Biochemical Systematics and Ecology*, ***5***, 463-464.

[4] Amaral ACF, Barnes RA. (**1998**) Alkaloids of *Croton celtidifolius*. *Planta medica*, ***63***, 485.

[5] Pieters L, De Bruyne T, Claeys M, Vlietinck A, Calomme M, Vanden Berghe D. (**1993**) Isolation of a dihydrobenzofuran lignan from South American dragon's blood (*Croton* spp.) as an inhibitor of cell proliferation. *Journal of Natural Products*, ***56***, 899-906.

[6] Ahmat N, Said IM, Latip J, Din LB, Syah YM, Hakim EH. (**2007**) New prenylated dihydrostilbenes from *Croton laevifolius. Natural Product Communications*, ***2***, 1137-1140.

[7] (a) Tane P, Tatsimo S, Connolly JD. (**2004**) Crotomacrine, a new clerodane diterpene from the fruits of *Croton macrostachyus*. *Tetrahedron Letters*, ***45***, 6997-6998; (b) Júnior SFP, Lucia M. Conserva LM, and José Maria Barbosa Filho JM. (**2006**) Clerodane diterpenes from *Croton* species: distribution and a compilation of their ^{13}C NMR spectral data. *Natural Product Communications*, ***1***, 319-344; (c) Suárez AI, Chavez K, Monache FD, Vasquez L, Delannoy DM, Orsini, G, Compagnone RS. (**2008**) New 3,4-seco *ent*-kaurenes from *Croton caracasana* flowers. *Natural Product Communications*, ***3***, 319-322; (d) Bayor MT, Ayim JSK, Marston G, Phillips RM, Shnyder SD, Wheelhouse RT,Colin W. Wright CW. (**2008**) A cytotoxic diterpenoid from *Croton membranaceus*, the major constituent of anticancer herbal formulations used in Ghana, *Natural Product Communications*, ***3***, 1875-1878.

[8] Santos HS, Santiago GMP, de Oliveira JPP, Arriaga AM, Marques DD, Lemos TLG. (**2007**) Chemical Composition and Larvicidal Activity against *Aedes aegypti* of Essential Oils from *Croton zehntneri*. *Natural Product Communications*, ***2***, 1233-1236

[9] William N. Setzer WN (**2006**) Chemical compositions of the bark essential oils of *Croton monteverdensis* and *Croton niveus* from Monteverde, Costa Rica. *Natural Product Communications*, ***1***, 567-572.

[10] Cobo PB. (**1956**) In: Mateos, Ed.: *Biblioteca de Autores Españoles* (*Series*), Madrid, Vols. ***91-92***.

[11] (a) Persinos Perdue GJ, Blomster RN, Blake DA, Farnsworth NR. (**1979**) South American plants. II. Taspine, isolation and anti-inflammatory activity. *Journal of Pharmaceutical Sciences*, ***68***, 124-126; (b) Porras-Reyes BH, Lewis WH, Roman J, Simchowitz L, Mustoe TA. (**1993**) Enhancement of wound healing by the alkaloid taspine defining mechanism of action. *Proceedings of the Society for Experimental Biology and Medicine*, ***203***, 18-25; (c) Vaisberg AJ, Milla M, del Carmen Planas M, Cordova JL, Rosas de Agusti E, Ferreyra R, del Carmen Mustiga M, Carlin L, Hammond GB. (**1989**) Taspine is the cicatrizant principle in sangre de drago extracted from *Croton lechleri. Planta Medica*, ***55***, 140-143.

[12] (a) Nunez Selles A, Velez Castro H, Aguero-Aguero J, Gonzalez-Gonzalez J, Naddeo F, De Simone F, Rastrelli L. (**2002**) Isolation and quantitative analysis of phenolic antioxidants, free sugars, and polyols from mango (*Mangifera indica* l.) stem bark aqueous decoction used in Cuba as a nutritional supplement. *Journal of Agricultural and Food Chemistry*, ***50***, 762-766; (b) Barbosa-Filho JM, Da-Cunha EVL, Lopes-Cornelio M, Da Silva C Gray AI. (**1997**) Cissaglaberrimine, an aporphine alkaloid from *Cissampelos glaberrima. Phytochemistry*, ***44***, 959-961; (c) Bao-Ning S, Yoshihisa T, Hong-Quan D, Bei C. (**1999**) Phenylpropanol derivatives from *Morina chinensis*. *Journal of Natural Products*, ***62***, 1363-1366; (d) Ahmed AA. (**1991**) Daucanes and other constituents from *Ferula sinaica. Phytochemistry*, ***30***, 1207-1210.

[13] Kim JP, Jung MY, Kim JP, Kim SY. (**2000**) Antiphotooxidative activity of protoberberines derived from *Coptis japonica* makino in the chlorophyll-sensitized photooxidation of oil. *Journal of Agricultural and Food Chemistry*, ***48***, 1058-1063.

[14] Han Q, Jang B, Mei S, Ding G, Sun H, Xie J, Liu Y. (**2001**) Constituents from the roots of *Semiaquilegia adoxoides*. *Fitoterapia*, ***72***, 86-88.

[15] Hung TM, Lee JP, Min BS, Choi JS, Na M, Zang X, Ngoc TM, Lee I, Bae K. (**2007**) Magnoflorine from coptidis Rhizoma protects high densitty lipoprotein during pxidant stress. *Biological and Pharmaceutical Bullettin*, ***30***, 1157-1160.

[16] Misik V, Bezakova L, Malekova L, Kostalova D. (**1995**) Lipoxygenase inhibition and antioxidant properties of protoberberine and aporphine alkaloids isolated from *Mahonia aquifolium. Planta Medica*, ***61***, 372-373.

[17] Chen IS, Chen, JJ, Duh CY, Tsai IL, Chang CT. (**1997**) New aporphine alkaloids and cytotoxic constituents of *Hernandia nymphaeifolia. Planta Medica*, ***63***, 154-157.

[18] Mori H, Fuchigami M, Inoue N, Nagai H, Koda A, Nishioka I. (**1994**) Principle of the bark of *Phellodendron amurense* to suppress the cellular immune response. *Planta Medica*, ***60***, 445-449.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1701 - 1706

The Cytotoxic Properties of Natural Coumarins Isolated from Roots of *Ferulago campestris* (Apiaceae) and of Synthetic Ester Derivatives of Aegelinol

Sergio Rosselli[a], Antonella Maria Maggio[a], Nicoletta Faraone[a], Vivienne Spadaro[b], Susan L. Morris-Natschke[c], Kenneth F. Bastow[d], Kuo-Hsiung Lee[c] and Maurizio Bruno[a,*]

[a] *Dipartimento Chimica Organica "E. Paternò"*, *Università di Palermo*, *Viale delle Scienze*, *Parco d'Orleasn II*, *90128 Palermo*, *Italy*

[b]*Dipartimento di Scienze Botaniche*, *Università degli Studi di Palermo*, *Via Archirafi 38*, *90123 Palermo*, *Italy*

[c]*Natural Products Research Laboratories*, [d]*Division of Medicinal Chemistry*, *Eshelman School of Pharmacy*, *University of North Carolina at Chapel Hill*, *Chapel Hill, North Carolina 27599*, *USA*

bruno@dicpm.unipa.it

Received: June 25th, 2009; Accepted: August 17th, 2009

Grandivittin (**1**), agasyllin (**2**), aegelinol benzoate (**3**) and felamidin (**20**), four natural coumarins isolated from *Ferulago campestris*, and several synthetic ester derivatives of aegelinol (**4**) were tested against four tumor cell lines. Some of them were shown to be marginally cytotoxic against the A549 lung cancer cell line.

Keywords: Apiaceae, *Ferulago campestris*, coumarins, aegelinol derivatives, cytotoxicity.

Since ancient times, *Ferulago* species have been well known as folk medicines due to their several biological properties, as sedatives, tonics, digestive remedies and aphrodisiacs, and also in the treatment of intestinal worms and hemorrhoids [1]. Furthermore, they are also useful against ulcers and snake bites, as well as for headaches and diseases of the spleen [2]. The gum (*galbanum*) obtained by incision of the roots of several species [3] is additionally used as a spice and fragrance in perfumes. *F. campestris* (Besser) Grec., (*F. galbanifera* (Mill) Kock. = *Ferula ferulago* L.), commonly known as *finocchiazzo*, is an annual or perennial herb with small flowers, widespread in the Mediterranean area. Our phytochemical investigation of the roots of this species, collected in Sicily, resulted in the isolation of several pyranocoumarins and one furanocoumarin [4]. Their stereochemical assessment was reported, as well as the antibacterial and antioxidant activities of the three most abundant constituents [grandivittin (**1**), agasyllin (**2**) and aegelinol benzoate (**3**)] and of the hydrolysis product [aegelinol, (**4**)] [4].

Decursinol (**5**) and decursin (**6**) are the enantiomers of aegelinol (**4**) and grandivittin (**1**), respectively, and were isolated from species of the genus *Angelica* [5,6]. They possess significant cytotoxic activity [7,8], and furthermore, decursinol (**5**), when administered orally, shows an antinociceptive effect in a dose-dependent manner [9] and high inhibitory activity toward AChE *in vitro* [10]. Decursin (**6**) and decursinol angelate (**7**), the enantiomer of agasyllin (**2**), showed *in vitro* cytotoxic and protein kinase C activating activities [11], as well as platelet anti-aggregatory effects [12]. Moreover, they possess anti-oxidant and hepatoprotective properties in rats [13], as well as antitumor [14,15] and antibacterial [16] activities. Furthermore, all three pyranocoumarins (**5**-**7**) exhibited significant neuroprotective properties [17]. In contrast, apart from the recently reported antibacterial and antioxidant activities [4], to the best of our knowledge, no other biological properties have been published for compounds **1**-**4**. Consequently, as part of our ongoing research on compounds with cytotoxic activity [18-21], we decided to prepare

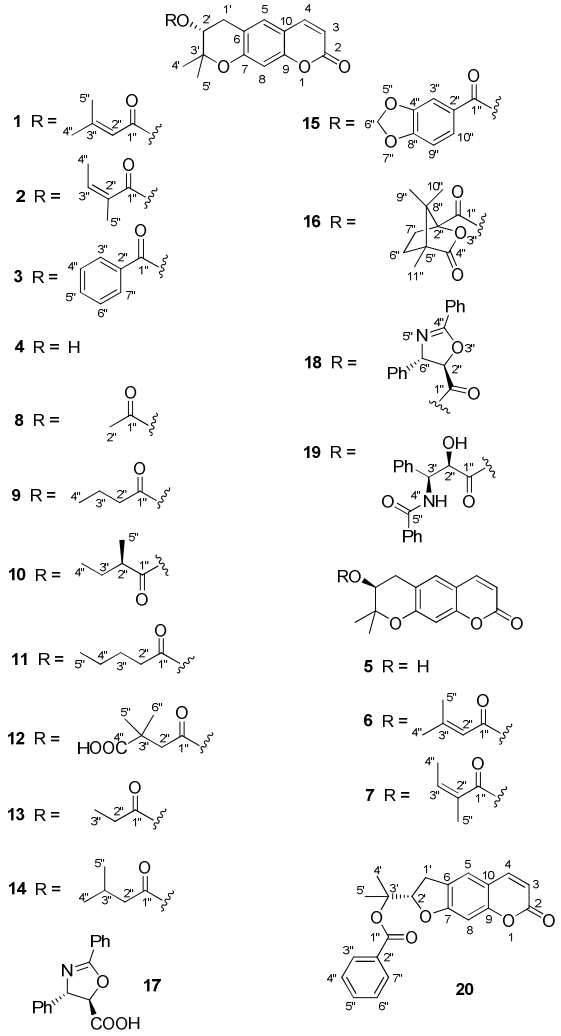


Figure 1: Structures of compounds **1-20**.

some C-2' ester derivatives of aegelinol (**4**) and to test the natural and synthetic compounds against various tumor cell lines.

The ester chains were chosen on the basis of our previous observations [19,20] that certain acyloxy groups were able to enhance biological properties. The three natural esters (**1**-**3**) were hydrolyzed under basic conditions to give aegelinol (**4**) [21]. Treatment of aegelinol (**4**) with triethylamine (TEA) or 4-(dimethylamino)pyridine (DMAP) in CH_2Cl_2 and either various anhydrides (acetic, butyric, (2*R*)-2-methylbutyric, valeric, 2,2-dimethylsuccinic) or acyl chlorides (propionyl, isovaleryl, piperonyl, (1*S*)-camphanyl) gave the esters **8**-**16**.

According to a previously reported procedure [22], the commercially available (2*R*,3*S*)-3-phenylisoserine hydrochloride was converted to compound **17**, which was then esterified with aegelinol (**4**). Acidic hydrolysis of the resulting ester (**18**) gave compound **19**, with the same side chain as paclitaxel.

Compounds **1**-**4**, **8**-**16**, **18**, **19** and felamidin (**20**), a natural coumarin co-occurring in the same plant [4], were screened against a panel of human tumor cell lines including A549 (lung), PC-3 (prostate), KB (nasopharyngeal), and KB-VIN (multidrug-resistant KB subline) in order to explore their anticancer properties. The results against the A549 tumor cell line are shown in Table 5. Except for felamidin (**20**), which was marginally cytotoxic against KB-VIN [EC_{50} = 14.9 μM; doxorubicin: EC_{50} = 1.7 μM], none of the compounds were active against the other three tumor cell lines. The identity of the ester side chain was important to the cytotoxic activity, as only benzoyl (**3**), piperonyl (**15**), isovaleryl (**14**), and 3,3-dimethylacrylyl (**1**) led to slightly active ester derivatives of aegelinol (**4**), the inactive parent coumarin. However, the coumarin backbone was also important as felamidin (**20**), with a benzoate ester, but different coumarin skeleton, was active. Our compound (grandivittin, **1**) seems to have a worse cytotoxic activity towards PC-3 cell line than its enantiomer (decursin, **6**), showing a moderate activity (EC_{50} = 25.0 μM, 96h) [8]. On the other hand, compound **1** has a better response against the A549 tumor cell line (EC_{50} = 15.2 μM, 72h).

Table 1: ^{1}H NMR spectroscopic data of compounds **8**-**13** in $CDCl_3$, δ_H *J*(Hz).

H	8	9	10	11	12	13
3	6.23 (1H) d (9.6)	6.22 (1H) d (9.3)	6.22 (1H) d (9.3)	6.23 (1H) d (9.3)	6.22 (1H) d (9.6)	6.22 (1H) d (9.3)
4	7.59 (1H) d (9.6)	7.58 (1H) d (9.3)	7.58 (1H) d (9.3)	7.59 (1H) d (9.3)	7.82 (1H) d (9.6)	7.58 (1H) d (9.3)
5	7.16 (1H) s	7.15 (1H) s	7.15 (1H) s	7.15 (1H) s	7.35 (1H) s	7.15 (1H) s
8	6.79 (1H) s	6.78 (1H) s	6.79 (1H) s	6.79 (1H) s	6.74 (1H) s	6.77 (1H) s
1′a	3.19 (1H) dd (17.4, 4.8)	3.18 (1H) dd (17.4, 4.8)	3.18 (1H) dd (17.1, 5.1)	3.18 (1H) dd (17.4, 4.8)	3.19 (1H) dd (17.1, 5.1)	3.18 (1H) dd (17.1, 5.1)
1′b	2.85 (1H) dd (17.4, 4.8)	2.83 (1H) dd (17.4, 4.8)	2.82 (1H) dd (17.1, 5.1)	2.84 (1H) dd (17.4, 4.8)	2.89 (1H) dd (17.1, 5.1)	2.83 (1H) dd (17.1, 5.1)
2′	5.05 (1H) t (4.8)	5.05 (1H) t (4.8)	5.03 (1H) t (5.1)	5.05 (1H) t (4.8)	5.09 (1H) t (5.1)	5.05 (1H) t (5.1)
4′	1.38 (3H) s	1.37 (3H) s	1.37 (3H) s	1.37 (3H) s	1.37 (3H) s	1.36 (3H) s
5′	1.35 (3H) s	1.35 (3H) s	1.36 (3H) s	1.36 (3H) s	1.36 (3H) s	1.34 (3H) s
2"	2.07 (3H) s	2.30 (2H) t (7.5)	2.37 (1H) m	2.29 (2H) t (7.2)	2.59 (2H) s	2.33 (2H) q (7.6)
3"a		1.61 (2H) m	1.61 (1H) m	1.59 (2H) m		1.11 (3H) t (7.6)
3"b			1.44 (1H)m			
4"		0.91 (3H) t (7.4)	0.84 (3H) t (7.4)	1.60 (2H) m		
5"			1.11 (3H) d (7.0)	0.88 (3H) t (7.2)	1.28 (3H) s	
6"					1.28 (3H) s	

Table 2: ^{1}H NMR spectroscopic data of compounds **14-16**, **18**, **19** in $CDCl_3$, δ_H *J* (Hz).

H	14	15	16	18	19
3	6.22 (1H) d (9.3)	6.25 (1H) d (9.6)	6.24 (1H) d (9.6)	6.23 (1H) d (9.6)	6.23 (1H) d (9.6)
4	7.58 (1H) d (9.3)	7.59 (1H) d (9.6)	7.59 (1H) d (9.6)	7.54 (1H) d (9.6)	7.59 (1H) d (9.6)
5	7.15 (1H) s	7.17 (1H) s	7.17 (1H) s	7.11 (1H) s	7.52 (1H) s
8	6.78 (1H) s	6.85 (1H) s	6.79 (1H) s	6.76 (1H) s	6.80 (1H) s
1′a	3.18 (1H) dd (17.1, 4.8)	3.29 (1H) dd (17.4, 4.8)	3.28 (1H) dd (17.4, 4.8)	3.24 (1H) dd (17.4, 4.8)	3.14 (1H) dd (16.5, 5.1)
1′b	2.83 (1H) dd (17.1, 4.8)	2.99 (1H) dd (17.4, 4.8)	2.96 (1H) dd (17.4, 4.8)	2.89 (1H) dd (17.4, 4.8)	2.92 (1H) dd (16.5, 5.1)
2′	5.04 (1H) t (4.8)	5.26 (1H) t (4.8)	5.15 (1H) t (4.8)	5.24 (1H) t (4.8)	5.10 (1H) t (5.1)
4′	1.37 (3H) s	1.46 (3H) s	1.42 (3H) s	1.45 (3H) s	1.43 (3H) s
5′	1.35 (3H) s	1.42 (3H) s	1.39 (3H) s	1.42 (3H) s	1.34 (3H) s
2"	2.19 (2H) d (6.9)			4.90 (1H) d (5.7)	4.63 (1H) d (1.8)
3"	2.08 (1H) m	7.39 (1H) s			5.70 (1H) dd (9.0, 1.8)
4"	0.91 (3H) d (6.6)				
5"	0.91 (3H) d (6.6)				
6"a		6.04 (2H) s	2.37 (1H) ddd (13.3, 10.5, 4.2)	5.44 (1H) d (5.7)	
6"b			2.04 (1H) ddd (13.3, 10.8, 4.5)		
7"a			1.87 (1H) ddd (13.3, 10.8, 4.2)		
7"b			1.67 (1H) ddd (13.3, 10.5, 4.5)		
9"		6.82 (1H) d (8.1)	0.83 (3H) s		
10"		7.60 (1H) d (8.1)	0.78 (3H) s		
11"			1.06 (3H) s		
Ar				8.10-8.04 (2H)	7.77-7.73 (2H)
Ar				7.60-7.20 (8H)	7.48-7.28 (8H)
NH					7.00 (1H) d (9.0)

Table 3: ^{13}C NMR spectroscopic data of compounds **8-13** in $CDCl_3$.

C	8	9	10	11	12	13
2	161.2 C	161.3 C	161.3 C	161.3 C	161.2 C	161.3 C
3	113.3 CH	113.4 CH	113.4 CH	113.4 CH	113.3 CH	113.3 CH
4	143.1 CH	143.2 CH	143.2 CH	143.2 CH	143.1 CH	143.2 CH
5	128.6 CH	128.6 CH	128.6 CH	128.6 CH	128.6 CH	128.7 CH
6	115.6 C	115.8 C	115.8 C	115.8 C	115.6 C	115.8 C
7	156.3 C	156.4 C	156.4 C	156.4 C	156.3 C	156.4 C
8	104.7 CH	104.8 CH	104.7 CH	104.8 CH	104.7 CH	104.7 CH
9	154.2 C	154.2 C	154.2 C	154.2 C	154.2 C	154.2 C
10	112.9 C	112.9 C	112.9 C	113.0 C	112.9 C	112.9 C
1′	27.7 CH_2	27.8 CH_2	27.9 CH_2	27.8 CH_2	27.7 CH_2	27.7 CH_2
2′	70.2 CH	70.0 CH	69.9 CH	70.0 CH	70.2 CH	70.1 CH
3′	76.4 C	76.6 C	76.6 C	76.6 C	76.4 C	76.6 C
4′	24.9 CH_3	25.1 CH_3	25.1 CH_3	26.8 CH_3	24.9 CH_3	26.7 CH_3
5′	23.1 CH_3	23.1 CH_3	22.9 CH_3	23.1 CH_3	23.1 CH_3	25.0 CH_3
1"	170.4 C	173.1 C	176.1 C	173.3 C	172.2 C	173.9 C
2"	21.0 CH_3	36.3 CH_2	41.0 CH	34.1 CH_2	44.7 CH_2	27.8 CH_2
3"		18.5 CH_2	26.6 CH_2	25.0 CH_2	41.5 C	23.0 CH_3
4"		13.6 CH_3	11.5 CH_3	22.2 CH_2	180.4 C	
5"			16.6 CH_3	13.7 CH_3	25.3 CH_3	
6"					25.3 CH_3	

Table 4: ^{13}C NMR spectroscopic data of compounds **14-16**, **18**, **19** in $CDCl_3$.

C	14	15	16	18	19
2	161.3 C	161.2 C	161.1 C	161.2 C	161.1 C
3	113.4 CH	113.0 CH	113.7 CH	113.5 CH	113.4 CH
4	143.2 CH	143.1 CH	143.0 CH	143.1 CH	143.0 CH
5	128.6 CH	128.7 CH	128.5 CH	128.6 CH	128.6 CH
6	115.8 C	115.7 C	115.0 C	114.9 C	114.9 C
7	156.4 C	156.4 C	156.2 C	156.8 C	156.8 C
8	104.7 CH	104.8 CH	104.8 CH	104.7 CH	104.6 CH
9	154.2 C	154.0 C	154.3 C	154.2 C	154.3 C
10	112.9 C	113.4 C	113.0 C	112.9 C	112.9 C
1′	27.8 CH_2	27.9 CH_2	27.8 CH_2	27.9 CH_2	27.9 CH_2
2′	70.0 CH	70.8 CH	71.9 CH	72.0 CH	72.0 CH
3′	76.5 C	76.7 C	76.6 C	76.6 C	76.5 C
4′	25.7 CH_3	25.0 CH_3	25.0 CH_3	25.1 CH_3	25.1 CH_3
5′	23.1 CH_3	23.4 CH_3	23.4 CH_3	23.5 CH_3	23.5 CH_3
1"	172.5 C	165.1 C	166.8 C	169.7 C	172.4 C
2"	43.4 CH_2	123.5 C	90.8 C	82.9 CH	73.4 CH
3"	25.1 CH	108.1 CH			54.6 CH
4"	22.4 CH_3	147.8 C	177.7 C	164.2 C	
5"	22.3 CH_3		54.7 C		166.3 C
6"		101.9 CH_2	30.7 CH_2	74.7 CH	
7"			28.9 CH_2		
8"		152.0 C	54.1 C		
9"		109.5 CH	16.6 CH_3		
10"		125.7 CH	16.5 CH_3		
11"			9.6 CH_3		
Ar				142.2-126.4	138.6-126.9

Table 5: Growth-inhibitory activity of **1-4**, **8-16**, **18-20** against A549 tumor cell line replication.

Compound	EC_{50} (μM)	Compound	EC_{50} (μM)
1	15.2	**13**	NA
2	NA	**14**	18.5
3	13.6	**15**	19.2
4	NA	**16**	NA
8	NA	**18**	NA
9	NA	**19**	NA
10	NA	**20**	11.6
11	NA	doxorubicin	0.9
12	NA		

Experimental

General experimental procedures: Optical rotations were determined on a JASCO P-1010 digital polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance series 300 MHz spectrometer, using the residual solvent signal (δ = 7.27 in ^{1}H and δ = 77.00 in ^{13}C for $CDCl_3$) as reference. ^{13}C NMR signal multiplicities were determined by DEPT spectra. ESI-MS were obtained with an Applied Biosystem API-2000 mass spectrometer. Merck silica gel (70-230 mesh), deactivated with 15% H_2O, was used for column chromatography.

Plant material: The roots of *Ferulago campestris* (Besser.) Grec. (700 g) were collected at Alimena, Palermo province, Italy in July 2007 and identified by Professor F. M. Raimondo, Department of Botanic Sciences, University of Palermo (Italy). Voucher specimens were deposited at the Herbarium of the Botanical Gardens of Palermo (Italy) under the number PAL 07-621 (Raimondo, Schimmenti & Scafidi).

Extraction and isolation: The extraction of the roots and the isolation of phytochemicals were performed as previously reported [4].

Synthesis of aegelinol (4): The esters **1-3** (500 mg, about 1.5 mmol) were added to a solution of KOH in dioxane (75 mL, 16.8 g, 0.3 mol, 4 M). The reaction mixture was refluxed for 0.5 h, and was monitored by TLC (2:3 EtOAc-light petroleum). After cooling, the reaction mixture was quenched and portion-wise acidified with 10% H_2SO_4 solution. The solution was extracted with dichloromethane, dried over Na_2SO_4 and evaporated *in vacuo*. Compound **4** was purified by crystallization (EtOAc/*n*-hexane) to obtain white crystals (340 mg, 93 %) [23].

Esterification-General procedure: Aegelinol (**4**, 20 mg) was dissolved in 10 mL of dry CH_2Cl_2 and added to 1 equiv of DMAP (12 mg), 25 equiv of TEA (0.3 mL), and the appropriate acyl chloride/anhydride (4 equiv) at room temperature under an argon atmosphere. After stirring overnight, the reaction was subjected to the usual workup by adding H_2O and extracting with EtOAc. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. Generally, the residue was purified by column chromatography (Si gel, 4:1 light petroleum-EtOAc as eluent). This procedure gave the following ester derivatives.

Compound 8

White solid (90% yield).
$[\alpha]_D^{25}$: –69.8 (*c* 1.09 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{16}H_{16}O_5$.
ESI MS (positive mode) *m/z* (%): 327 $[M+K]^+$ (24), 311 $[M+Na]^+$.

Compound 9

White solid (82% yield).
$[\alpha]_D$: –62.9 (*c* 3.65 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{18}H_{20}O_5$.
ESI MS (positive mode) *m/z* (%): 355 $[M+K]^+$ (10), 339 $[M+Na]^+$ (100), 317 $[M+H]^+$ (5).

Compound 10

White solid (85% yield).
$[\alpha]_D$: –61.7 (*c* 1.29 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (24), 353 $[M+Na]^+$ (100).

Compound 11

White solid (95% yield).
$[\alpha]_D$: –59.1 (*c* 3.21 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (17), 353 $[M+Na]^+$ (100).

Compound 12

White solid (75% yield).
$[\alpha]_D$: –38.0 (*c* 0.59 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{20}H_{22}O_7$.
ESI MS (positive mode) *m/z* (%): 411 $[M+K]^+$ (13), 295 $[M+Na]^+$ (100).

Compound 13

White solid (81% yield).
$[\alpha]_D$: –80.8 (*c* 1.98 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 1.
^{13}C NMR ($CDCl_3$): Table 3.
$C_{17}H_{18}O_5$.
ESI MS (positive mode) *m/z* (%): 341 $[M+K]^+$ (37), 325 $[M+Na]^+$ (100), 303 $[M+H]^+$ (8).

Compound 14

White solid (80% yield).
$[\alpha]_D$: –51.7 (*c* 3.38 $CHCl_3$).
^{1}H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4
$C_{19}H_{22}O_5$.
ESI MS (positive mode) *m/z* (%): 369 $[M+K]^+$ (10), 353 $[M+Na]^+$ (100).

Compound 15

White solid (78% yield).
$[\alpha]_D$: –43.3 (*c* 0.46 $CHCl_3$).
^{1}H NMR ($CDCl_3$): see Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{22}H_{18}O_7$.
ESI MS (positive mode) *m/z* (%): 433 $[M+K]^+$ (52), 417 $[M+Na]^+$ (100).

Compound 16

White solid (70% yield).

$[\alpha]_D$: –86.5 (*c* 0.42 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{24}H_{26}O_7$.
ESI MS (positive mode) *m/z* (%): 465 $[M+K]^+$ (9), 449 $[M+Na]^+$ (100).

Synthesis of compound 18: Aegelinol (**4**, 20 mg) was dissolved in 5 mL of dry CH_2Cl_2, and to this solution were added 1.2 equiv. of DCC and 2 equiv. of oxazoline **17**. After stirring overnight at room temperature, the reaction mixture was filtered and extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by column chromatography (Si gel, 7:3 light petroleum-EtOAc as eluent) to give compound **18** (95% yield).

Compound 18

White solid.
$[\alpha]_D$: –89.7 (*c* 0.35 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{30}H_{25}O_6N$.
ESI MS (positive mode) *m/z* (%): 518 $[M+Na]^+$ (5), 496 $[M+H]^+$ (100).

Synthesis of compound 19: Compound **18** (20 mg), dissolved in CH_2Cl_2 (5 mL), was stirred at room temperature with 2 equiv. of *p*-toluene-sulfonic acid. After stirring overnight at room temperature, the reaction was neutralized with saturated aqueous $NaHCO_3$, diluted with water (10 mL), and extracted 3 times with $CHCl_3$ (15 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure, leaving a residue, which was purified by column chromatography (Si gel, 4:1 light petroleum-EtOAc as eluent) to give compound **19** (72% yield).

Compound 19

White solid.
$[\alpha]_D$: –46.5 (*c* 0.13 $CHCl_3$).
^1H NMR ($CDCl_3$): Table 2.
^{13}C NMR ($CDCl_3$): Table 4.
$C_{30}H_{27}O_7N$.
ESI MS (positive mode) *m/z* (%): 552 $[M+K]^+$ (18), 536 $[M+Na]^+$ (100), 514 $[M+H]^+$ (20).

***In vitro* cytotoxicity assay:** All stock cultures were grown in T-25 flasks. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates with compounds added from DMSO-diluted stock. The plates were incubated for an additional 72 h after attachment and drug addition, and the assay was terminated by 10% TCA. Then, 0.4% SRB dye in 1% HOAc was added to stain the cells for 10 min. Unbound dye was removed by repeated washing with 1% HOAc and the plates were air dried. Bound stain was subsequently solved with 10 mM trizma base, and the absorbance read at 515 nm. Growth inhibition of 50% (EC_{50}) was calculated as the drug concentration that caused a 50% reduction in the net protein increase in control cells during the drug incubation. The mean EC_{50} is the concentration of agent that reduces cell growth by 50% under the experimental conditions and is the average from at least three independent determinations. Variation between replicates was no more than 5% of the mean. The following human tumor cell lines were used in the assay: A549 (non-small cell lung cancer), PC-3 (prostate cancer), KB (nasopharyngeal carcinoma), KB-VIN (vincristine-resistant KB subline). All cell lines were obtained from either the Lineberger Cancer Center (UNC-CH) or from ATCC (Rockville, MD). Cells propagated in RPMI-1640 supplemented with 10% FBS, penicillin-100 IU/mL, streptomycin-1μg/mL, and amphotericin B-0.25μg/mL, were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Acknowledgments - This work was supported by Italian Government project PRIN "Sostanze naturali ed analoghi sintetici con attività antitumorale" (M. Bruno) and in part by a NIH grant from the National Cancer Institute, CA-17625 (K.H. Lee).

References

[1] Boulus L. (**1983**) Medicinal plants of North-Africa MI; Algonae: Michigan, USA, p. 183.

[2] Demetzos C, Perdetzoglou D, Gazouli M, Tan K, Economakis C. (**2000**) Chemical analysis and antimicrobial studies on three species of *Ferulago* from Greece. *Planta Medica*, ***66***, 560-563.

[3] Naves Y R. (**1967**) Études sur les matières végétales volatiles CCIII (1). Présence de *n*-undécatriènes-1,3,5 dans 1'huile essentielle de la gomme-résine de *Galbanum. Bulletin de la Société Chimique de France*, 3152-3154.

[4] Basile A, Sorbo S, Spadaro, V, Bruno M, Maggio A, Faraone N, Rosselli S. (**2009**) Antimicrobial and antioxidant activities of coumarins from the roots of *Ferulago campestris* (Apiaceae). *Molecules*, ***14***, 939-952.

[5] Hata K, Sano K. (**1966**) The constitution of decursin, a new coumarin isolated from the root of *Angelica decursiva* Fr. et Sav. (Umbelliferae). *Tetrahedron Letters*, 1461-1465.

[6] Konoshima M, Chi HJ, Hata K. (**1967**) Coumarins from the root of *Angelica gigas* Nakai. *Chemical and Pharmaceutical Bulletin*, ***16***, 1139-1140.

[7] Itokawa H, Yun Y, Morita H, Takeya K, Lee SR. (**1994**) Cytotoxic coumarins from roots of *Angelica gigas* Nakai. *Natural Medicines*, ***48***, 334-335.

[8] Yim D, Singh R, Agarwal C, Lee S, Chi H, Agarwal R. (**2005**) A novel anticancer agent, decursin, induces G_1 arrest and apoptosis in human prostate carcinoma cells. *Cancer Research*, ***65***, 1035-1044.

[9] Choi SS, Han KJ, Lee JK, Lee HK, Han EJ, Kim DH, Suh H-W. (**2003**) Antinociceptive mechanisms of orally administered decursinol in the mouse. *Life Sciences*, ***73***, 471-485.

[10] Kang SY, Lee KY, Sung SH, Park M.J, Kim YC. (**2001**) Coumarins isolated from *Angelica gigas* inhibit acetylcholinesterase: Structure-activity relationships. *Journal of Natural Products*, ***64***, 683-685.

[11] Ahn KS, Sim WS, Lee IK, Seu YB, Kim IH. (**1997**) Decursinol angelate: A cytotoxic and protein kinase C activating agent from the root of *Angelica gigas*. *Planta Medica*, ***63***, 360-361.

[12] Yong YL, Lee S, Jing LJ, Hye SYC. (**2003**) Platelet anti-aggregatory effects of coumarins from the roots of *Angelica genuflexa* and *A. gigas*. *Archives of Pharmacal Research*, ***26***, 723-726.

[13] Lee S, Lee YS, Jung SH, Shin KH, Kim BK, Kang SS. (**2003**) Antioxidant activities of decursinol angelate and decursin from *Angelica gigas* roots. *Natural Product Sciences*, ***9***, 170-173.

[14] Lee S, Yeon SL, Sang HJ, Kuk HS, Kim BK, Sam SK. (**2003**) Anti-tumor activities of decursinol angelate and decursin from *Angelica gigas*. *Archives of Pharmacal Research*, ***26***, 727-730.

[15] Hyeon HK, Sung SB, Jin SC, Han H, Kim IH. (**2005**) Involvement of PKC and ROS in the cytotoxic mechanism of anti-leukemic decursin and its derivatives and their structure-activity relationship in human K562 erythroleukemia and U937 myeloleukemia cells. *Cancer Letters*, ***223***, 191-201.

[16] Lee S, Shin DS, Ju SK, Oh KB, Sam SK. (**2003**) Antibacterial coumarins from *Angelica gigas* roots. *Archives of Pharmacal Research*, ***26***, 449-452.

[17] Kang SY, Lee KY, Sung SH, Kim YC. (**2005**) Four new neuroprotective dihydropyranocoumarins from *Angelica gigas*. *Journal of Natural Products*, ***68***, 56-59.

[18] Rosselli S, Maggio A, Eiroa C, Formisano C, Bruno M, Irace C, Maffettone C, Mascolo N. (**2008**) Cytotoxic activity of diterpenoids isolated from the aerial parts of *Elaeoselinum asclepium* (L.) Bertol. subsp. *meoides* (Desf.) Fiori (Apiaceae). *Planta Medica*, ***74***, 1285-1287.

[19] Bruno M, Rosselli S, Maggio, A, Raccuglia RA, Bastow KF, Lee KH. (**2005**) Cytotoxic activity of natural and synthetic guaianolides. *Journal of Natural Products*, ***68***, 1042-1046.

[20] Bruno M, Rosselli S, Maggio, A, Raccuglia RA, Bastow KF, Wu CC, Lee KH. (**2005**) Cytotoxic activity of some natural and synthetic sesquiterpene lactones. *Planta Medica*, ***71***, 1176-1178.

[21] Rosselli S, Bruno M, Maggio, A, Bellone G, Chen TH, Bastow KF, Lee KH. (**2007**) Cytotoxyc activity of some natural and synthetic kauranes. *Journal of Natural Products*, ***70***, 347-352.

[22] Kopczacki P, Gumulka M, Massnyk M, Grabarczyk H, Nowak G, Daniewski WM. (**2001**) Synthesis and antifeedant properties of *N*-benzoylphenylisoserinates of *Lactarius* sesquiterpenoid alcohols. *Phytochemistry*, ***58***, 775-787.

[23] Erdelmeier CAJ, Sticher O. (**1985**) Coumarins derivatives from *Eryngium campestre. Planta Medica*, ***51***, 407-409.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1707 - 1710

Rosmarinus officinalis Extract Inhibits Human Melanoma Cell Growth

Alessandra Russo[a*], Laura Lombardo[b], Nicolas Troncoso[c], Juan Garbarino[d] and Venera Cardile [b]

[a]*Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, V.le A. Doria 6, 95125 Catania, Italy*

[b]*Department of Physiological Sciences, University of Catania, V.le A. Doria 6, 95125 Catania, Italy*

[c]*Lo Vicuña & Cia., www.lo vicuna.cl, Santiago, Chile*

[d]*Department of Chemistry, University T.F. Santa Maria, Casilla 110-V, Valparaiso, Chile*

alrusso@unict.it; ales0303@libero.it

Received: July 10th, 2009; Accepted: October 30th, 2009

Rosmarinus officinalis L. is receiving increasing attention due to its anti-inflammatory and antioxidative constituents. Our recent studies showed that *R. officinalis* extract, containing 31.7 % of carnosic acid, was able to counteract the deleterious effects of UV-R, by protecting plasmid DNA from hydroxyl radicals generated by UV-A. In this work, we evaluated the effects of this extract on pBR322 DNA cleavage induced by nitric oxide, and the growth inhibitory activity against two human melanoma cell lines, M14 and A375. The extract showed a protective effect on plasmid DNA damage, and at concentrations of 10-80 μg/mL was able to reduce significantly ($p<0.001$) the growth (MTT assay) of both melanoma cell lines. In addition, our results indicate that apoptotic cell demise is induced in M14 and A375 cells. No statistically significant increase in LDH release was observed in melanoma cells, correlated to a fragmentation of genomic DNA, determined by COMET assay.

Keywords: *Rosmarinus officinalis* L., nitric oxide, melanoma, cancer cells, DNA damage.

UV-R is considered to be the major etiological factor in skin cancer. In humans, both UV-B (280–320 nm) and UV-A (320–400 nm) can cause gene mutations and suppress immunity. These biological events can lead to skin cancer, including melanoma. Melanoma is an aggressive, therapy-resistant malignancy of melanocytes [1]. Of the major forms of skin cancer, it carries the highest risk of mortality from metastasis. It is, therefore, of primary interest to search for new therapeutic agents that are able to prevent and contrast this aggressive tumor. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by both UV-A and UV-B contribute to inflammation, immunosuppression, gene mutation and carcinogenesis. Therefore, substances able to inhibit these reactive species could be used in the prevention of skin cancer, including melanoma [1].

Rosmarinus officinalis L. (rosemary) is a typical Mediterranean species, but is now cultivated all over the world. Usually the plant is clonally propagated because of the poor germinability of its seeds and the genetic diversity of the seedlings [2a]. *R. officinalis* is used as a folk medicine around the world, as well as in cosmetics. In medicine, the extract is receiving increasing attention due to its anti-inflammatory and antioxidative constituents [2b]. Our recent studies evidenced that *R. officinalis* extract, containing 31.7% of carnosic acid, [2b], 0.4% of rosmarinic acid [2b], and 5.9% of carnosol (unpublished data), was able to counteract the deleterious effects of UV-R, protecting plasmid DNA from hydroxyl radicals generated by UV-A [2b]. In this work, we evaluated the effect of this extract on pBR322 DNA cleavage induced by nitric oxide, and its growth inhibitory activity against two human melanoma cell lines (M14 and A375).

It has been reported that NO liberated following UV-R irradiation plays a significant role in initiating erythema and inflammation [3]. NO can combine with UV-induced superoxide to form peroxynitrite, which exists in equilibrium with peroxynitrous acid. These reactive nitrogen species are very toxic, and can cause DNA damage, nitrosylation of tyrosine residues in proteins, and initiate lipid peroxidation, all of which interfere with cellular function [4a].

Table 1: Effect of *R. officinalis* methanolic extract on Angeli's salt-mediated DNA damage.

Treatments	UD of supercoiled DNA (% of native DNA)
scDNA	100
Angeli's salt	
0.2 mM	12 ± 15*
extract	
200 μg/mL	56 ± 4.7*°
400 μg/mL	91 ± 2.6*°
800 μg/mL	94 ± 3.6*°

The values are expressed as densitometric units (D.U.) obtained by scanning the agarose gel electrophoresis photos.
Each value represents the mean ± SD of three experiments, performed in duplicate. *Significant vs. supercoiled DNA ($p<0.001$); °significant vs. Angeli's salt treated DNA ($p<0.001$).

Various compounds in foods, as well as in medicinal plants, have been widely used for wound-healing, anti-aging, and disease treatments of skin. The biological activity of these compounds has been correlated, in part, to their capacity to counteract the oxidative and nitrosative stress. Our results suggest that the *R. officinalis* extract could also act in this way in skin protection. In fact, sample, at concentrations of 200-800 μg/mL, exhibited protection against plasmid DNA damage induced by Angeli's salt, a NO donor [4b], which in our experimental conditions produced a significant decrease in the scDNA band intensity (Table 1). The treatment of plasmid DNA with extract alone did not change the migration pattern (data not shown).

In this study, we also tested the effect of this natural product on melanoma cells M14 and A375, and the results obtained show that the extract inhibited the growth of cancer cells. In fact, the results, summarized in Figure 1, show that the extract used at non toxic concentration in normal cells (data not shown), exhibited a dose dependent inhibitory effect on both human melanoma cells examined. In particular, in M14 and A375 cells treated with our sample at 40 μg/mL concentration for 72 h, the cell vitality was 65% and 42%, respectively. In cutaneous cells, there was a homeostatic relationship between cell proliferation and apoptosis. Alterations in either cell proliferation or cell death can lead to a loss of growth control, and thus play a major role in the process of tumorigenesis. Defects of apoptotic pathways influence also drug resistance, and because of these defects chemotherapy often fails [5]. Recent studies have suggested that the resistance of human melanoma to apoptosis is an important mechanism underlying this cancer's aggressiveness and its poor response to chemotherapeutic agents [5]. The induction of apoptosis in tumor cells is considered very useful in the management and therapy of cancer, including melanoma [5]. It is thus considered

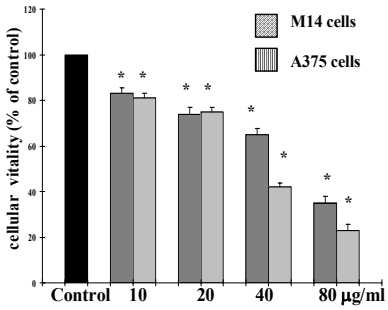


Figure 1: Cell growth, assayed using MTT test, of M14 and A375 cells untreated and treated with the methanolic extract of *R. officinalis* at different concentrations for 72 hours. Stock solution of the extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p<0.001$).

important to screen apoptotic inducers from plants, either in the form of extracts or as components isolated from them. Necrosis results in a disruption of the cytoplasmic membrane and the necrotic cells release cytoplasmic LDH and other cytotoxic substances into the medium. We therefore examined the membrane permeability of the treated cells and the existence of LDH in their culture medium. No statistically significant increase in LDH release was observed in M14 and A375 cells treated with the extract at 10-40 μg/mL concentrations. Conversely, a significant increase in LDH was observed at 80 μg/mL (Table 2).

Table 2: Lactate dehydrogenase (LDH) release, expressed as percentage of LDH released into the cell medium with respect to total LDH in M14 and A375 cells untreated and treated with the methanolic extract of *R. officinalis* at different concentrations for 72 hours. Stock solution of extract was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone.

Treatments	**% LDH released**
M14	
control	4.7 ± 0.9
extract	
20 μg/mL	5.8 ± 0.7
40 μg/mL	4.8 ± 0.6
80 μg/mL	40 ± 3.6*
A375	
control	5.8 ± 15
extract	
20 μg/mL	6.6 ± 1.2
40 μg/mL	5.1 ± 2.6
80 μg/mL	35 ± 1.6*

Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p<0.001$).

Nuclear DNA fragmentation was analyzed using the COMET assay, a sensitive method for detecting DNA strand breaks in individual cells and a versatile tool that is highly efficacious in human bio-monitoring of natural compounds [6a]. The COMET assay also allows the differentiation between apoptotic and necrotic cells based on the DNA fragmentation pattern [6b]. The COMET pattern significantly differs between

Table 3: COMET assay of genomic DNA of M14 and A375 cells untreated and treated with extract from *R. officinalis* at different concentrations for 72 h.

Treatments	TDNA	TMOM
M14 cells		
Control extract	17.7±3.0	86±3.1
20 μg/mL	145±5.0*	1113±11*
40 μg/mL	183±2.3*	1667±41*
80 μg/mL	68±5.5*	256±10*
A375 cells		
Control extract	12.1±3.0	69±4.3
20 μg/mL	173±3.0*	1165±5.3*
40 μg/mL	288±5.8*	2519±12*
80 μg/mL	94±5.9*	331±15*

The values were expressed as TDNA (percentage of the fragmented DNA) and TMOM, the product of TD (distance between head and tail) and TDNA. Each value represents the mean ± SD of three experiments, performed in quadruplicate. *Significant vs. control untreated cells ($p<0.001$).

apoptotic and control cultures, as well as between apoptotic and necrotic ones. Quantification of the COMET data is reported in Table 3 as TDNA, the percentage of the fragmented DNA, and TMOM that is defined as the product of the percentage of DNA in the tail of the COMET and TD value (distance between head and tail). The results clearly demonstrate DNA damage in cells exposed to extract of *R. officinalis* for 72 h, but this natural product produced a high increase in both TDNA and TMOM at concentrations of 20 and 40 μg/mL. These results seem to confirm a programmed cell death, because data in the literature [6c] indicate that only comets with high values of TMOM and TD can be related to apoptosis. On the other hand, previous studies have shown that carnosic acid, the major polyphenol in *R. officinalis*, inhibits the proliferation of different cancer cells [6d,6e,7a], and induces apoptosis in HL-60 cells [7a].

Taken together, our present results suggest that the methanolic extract of *R. officinalis,* because of its ability to counteract nitric oxide–mediated plasmid DNA damage, could be useful in the prevention of cell damage correlated to UV-R, such as dermatitis, premature aging and skin cancer. In addition, this study provided the first evidence that the extract of *R. officinalis* attenuates the growth of human cancer cells by triggering an apoptotic process. In fact, a high DNA fragmentation (COMET assay), not correlated to LDH release, a marker of membrane breakdown, occurred in melanoma cells exposed to the methanolic extract of *R. officinalis* in concentrations of 20-40 μg/mL. Although the molecular mechanism by which apoptosis is induced by this extract remains to be confirmed, the results reported here suggest its possible use as a novel therapeutic agent for melanoma cancer treatment in association with classic drugs with the aim to reduce their toxicity.

Experimental

***Rosmarinus officinalis* sample:** This came from the special collection of more than 160 individual plants used by BOTANE Ltd., and was collected at Goodwood, Canada. The accession was clonally propagated in order to maintain genetic uniformity, and was planted in November 2001 at Illapel, Chile [2b]. The fresh rosemary sample was dried at 40ºC in a forced air circulation oven (MemmertULM500). The leaves were manually separated and ground in a vertical hammer mill (Peruzzo Milly model 35.010) at 12,000 rpm to 0.8 mm mesh. Samples were mixed with 500 mL methanol and stirred for 15 h at 20°C, in the dark. After stirring and filtering under vacuum, the filtrate was evaporated to dryness in a Rotavapor. The samples were extracted again for 6 days (two times) [2b], and the filtrate was evaporated to dryness in a Rotavapor [yield 55.4g (22.9%)]. The content of carnosic acid, rosmarinic acid and carnosol was 31.7%, 0.41% [2b] and 5.93%, respectively.

Analysis of DNA single-strand breaks induced by Angeli's salt: The experiments were performed [7b] by incubating pBR322 plasmid DNA in 100 mM sodium phosphate buffer, pH 7.4, containing 0.1 mM DTPA, 0.15 mM Angeli's salt (prepared in 0.01 N NaOH), an appropriate amount of HCl to neutralize the NaOH present in the solution of Angeli's salt, and the extract at different concentrations at 37°C for 1 h (final volume 10 μL, final pH 7.5). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 h. Gel was stained in ethidium bromide (1 μg/mL; 30 min) and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

Effects on human tumor cell lines: M14 human melanoma cells was obtained from the American Type Culture Collection (Rockville, MD, USA) and were grown in RPMI containing 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 25 μg/mL fungizone. A375 human melanoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2.0 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL fungizone. The cells

were plated at a constant density to obtain identical experimental conditions in the different tests, thus to achieve a high accuracy of the measurements. After 24 h incubation at 37°C in a humidified 5% carbon dioxide atmosphere to allow cell attachment, the cells were treated with different concentrations of the methanolic extract of *R. officinalis* for 72 h under the same conditions. Stock solution of the natural product was prepared in ethanol and the final concentration of this solvent was kept constant at 0.25%. Control cultures received ethanol alone.

MTT bioassay: Cellular growth was determined using the MTT assay on 96-well microplates, as previously described [7b]. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm., and the results were reported as % of control.

Lactate dehydrogenase (LDH) release: Lactate dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation, as previously reported [7b]. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium.

DNA analysis by COMET assay: The presence of DNA fragmentation was examined by single cell gel electrophoresis (COMET assay), as previously reported [7b]. At the end of the electrophoretic run, the "minigels" were neutralized in 0.4 M Tris-HCl, pH 7.5, stained with ethidium bromide and scored using a fluorescence microscope (Leica, Wetzlar, Germany) interfaced with a computer. Software (Leica-QWIN) allowed the analysis and quantification of DNA damage by measuring: a) tail length (TL), intensity (TI) and area (TA); b) head length (HL), intensity (HI) and area (HA). These parameters are employed by the software to determine the level of DNA damage as: i) the percentage of the fragmented DNA (TDNA), and ii) tail moment (TMOM) expressed as the product of TD (distance between head and tail) and TDNA.

Statistical analysis: Statistical analysis of results was performed by using one-way ANOVA, followed by Dunnett's post-hoc test for multiple comparisons with control. All statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA). Each value represents the mean ± SD of three separate experiments performed in quadruplicate.

Acknowledgements - The authors would like to thank Professor Peter Fiedler for proofreading the manuscript.

References

[1] Russo PAJ, Halliday GM. (**2006**) Inhibition of nitric oxide and reactive oxygen species production improves the ability of a sunscreen to protect from sunburn, immunosuppression and photocarcinogenesis. *British Journal of Dermatology*, ***155***, 408-415.

[2] (a) Flamini G, Cioni PL, Morelli I, Macchia M, Ceccarini L. (**2002**) Main agronomic-productive characteristics of two ecotypes of *Rosmarinus officinalis* L. and chemical composition of their essential oils. *Journal of Agricultural and Food Chemistry*, ***50***, 3512-3517; (b) Garbarino J, Troncoso N, Delpiano P, Carvajal L, Russo A. (**2006**) Antioxidant activity analysis to the selection of *Rosmarinus officinalis* L. *Natural Product Communications*, ***1***, 1123-1128.

[3] Deliconstantinos G, Villiotou V, Stavrides JC. (**1996**) Nitric oxide and peroxynitrite released by ultraviolet B-irradiated human endothelial cells are possibly involved in skin erythema and inflammation. *Experimental Physiology*, ***81***, 1021-1033.

[4] (a) Russo A, Acquaviva R, Vanella L. (**2008**) Nitric oxide and skin: effect of natural compounds. In: *Recent Research Developments in Chemistry and Biology of Nitric Oxide*, Pandalai SG. (Ed.), Research Signpost, pp. 149-179; (b) Amatore C, Arbault S, Ducrocq C, Hu S, Tapsoba I. (**2007**) Angeli's Salt (Na(2)N(2)O(3)) is a precursor of HNO and NO: a voltammetric study of the reactive intermediates released by Angeli's salt decomposition. *Chemical and Medicinal Chemistry*, ***2***, 898-903.

[5] Johnstone RW, Ruefli AA, Lowe SW. (**2002**) Apoptosis: a link between cancer genetics and chemotherapy. *Cell*, ***108***, 153-164.

[6] (a) Aruoma OI. (**1999**) Antioxidant action of plant foods: use of oxidative DNA damage as a tool for studying antioxidant efficacy. *Free Radical Research*, ***30***, 419-427; (b) Bednarek I, Sypniewski D, Klama-Baryła A, Gałka S, Machnik G. (**2006**) Single-cell gel electrophoresis (comet assay) as a tool for apoptosis determination in tumor cell lines HL-60 and Jurkat cultures treated with anisomycin. *Annals Academy of Medicine*, ***60***, 278-284; (c) Godard T, Deslandes E, Lebailly P, Vigreux C, Sichel F, Poul JM, Gauduchon P. (**1999**) Early detection of staurosporine-induced apoptosis by comet and annexin V assays, *Histochemistry and Cell Biology*, ***112***, 155-161; (d) Steiner M, Priel I, Giat J, Levy J, Sharoni Y, Danilenko M. (**2001**) Carnosic acid inhibits proliferation and augments differentiation of human leukemic cells induced by 1,25-dihydroxyvitamin D3 and retinoic acid. *Nutrition and Cancer*, ***41***, 135-144; (e) Visanji JM, Thompson, DG, Padfield PJ. (**2006**) Induction of G2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels. *Cancer Letters*, ***237***, 130-136.

[7] (a) Wang R, Li H, Guo G, Li X, Yu X, Li H, Wang J, Liu F,Chen X. (**2008**) Apoptosis in human leukaemia cells induced by arsenic trioxide via upregulation of the tumour suppressor PTEN. *The Journal of International Medical Research*, ***36***, 682-690; (b) Russo A, Piovano M, Lombardo L, Garbarino J, Cardile V. (**2008**) Lichen metabolites prevent UV light and nitric oxide-mediated plasmid DNA damage and induce apoptosis in human melanoma cells. *Life Sciences*, ***83***, 468-474.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1711 - 1716

Antiproliferative Activity of Brown Cuban Propolis Extract on Human Breast Cancer Cells

Ada Popolo[*], Lisa Anna Piccinelli, Silvana Morello[b], Osmany Cuesta-Rubio, Rosalinda Sorrentino, Luca Rastrelli and Aldo Pinto

Department of Pharmaceutical Sciences Via Ponte Don Melillo 84135 Fisciano, Salerno, Italy

[b]Instituto de Farmacia y Alimentos (IFAL), Universidad de La Habana, La Habana, Cuba

apopolo@unisa.it

Received: September 17th, 2009; Accepted: November 4th, 2009

Brown Cuban propolis (BCP) is the major type of propolis in Cuba; its chemical composition is exclusive and the principal component is nemorosone. In this study we investigated the antiproliferative activity of the ethanol extract of BCP on human breast cancer cell lines. The MTT assay showed a significant antiproliferative activity ($P<0.005$) of BCP on MCF-7 (estrogen receptor positive ER+) rather than MDA-MB 23 1 (ER-). This effect was concentration- (1-25 μg/mL) and time- (24-48 h) dependent, but it is only partially attributable to apoptosis. Indeed, our data showed that BCP administration to MCF-7 caused a significant ($P>0.01$) inhibition of cell growth in the G_1 phase of cell cycle, which was mechanism dose- and time-dependent. 17-β Estradiol (10 nM) administration to MCF-7 caused a significant ($P<0.001$), but not total reduction of BCP antiproliferative activity at concentrations of 1, 5 and 10 μg/mL, but not at the highest concentration (25 μg/mL). The co-administration of ICI 182,780 (100nM), an antagonist of ER, on MCF-7 totally reduced the effect of BCP at 24 h, and showed a significant ($P<0.001$) reduction of BCP antiproliferative activity at 48 h.

Thus it was hypothesized that BCP possesses an estrogen-like activity. It is to be noted, however, that BCP application to MDA-MB 23 1 at 48 h also induced increased cell mortality. Thus, it cannot be ruled out that BCP could not only interact with the ER, but also have an ER-independent activity.

Keywords: Brown Cuban propolis, breast cancer, MCF-7, cytotoxicity.

Propolis is a resinous substance with a protective activity against micro-organisms and insects that invade the hives of bees. Propolis has a broad spectrum of biological properties, such as antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and antitumor activities [1-2]. Hence, propolis has attracted researchers' interest in the last decades [3].

The chemical composition of propolis can vary both qualitatively and quantitatively depending on the vegetation where it is produced [4]. Propolis from Europe and China contains many flavonoids and phenolic esters, whereas Chilean propolis is rich in phenols, able to scavenge free radical species and inhibit tumor cell growth [5]. Propolis from Brazil is mainly composed of artepillin C, a flavonoid that exhibits anti-proliferative effects by inhibiting angiogenesis in human lung, stomach and colon cancers and melanoma [6].

This study was focused on Cuban propolis that has recently attracted attention [7]. Brown Cuban propolis (BCP) is the principal type of Cuban propolis and is derived from *Clusia rosea*, the principal source of resins in the production of this material [8-11]. The major constituents are polyisoprenylated benzophenones, the principal one being nemorosone, that has been described as cytotoxic and anti-oxidant [7-9]. Interestingly, this compound showed cytotoxic activity *in vitro* against different tumor cell lines, including human cervix carcinoma (HeLa), human larynx carcinoma (Hep-2), prostate carcinoma (PC-3) and central nervous system carcinoma (U251) cells [12].

The purpose of our study was to evaluate the effect of BCP extract on human breast cancer cells. It is well known that propolis has a great impact in traditional Oriental folk medicine. Indeed, Asian women consuming propolis and traditional isoflavone-containing diets were more protected from breast cancer [13]. In particular, the ethanolic extract of propolis was found to be more effective than the aqueous extract in inhibiting mammary carcinogenesis [14]. Recently, Song and co-workers [15] demonstrated that the ethanolic extract of propolis from Korea may modulate estrogen receptor activation and more importantly estrogen-dependent breast tumors. Thus, the potential of BCP to protect against breast cancer still remains to be elucidated, as well as the determination of the active components. In this study we observed that BCP can inhibit breast tumor cell growth by arresting the cell cycle in the G0/G1 phase.

Chemical analysis revealed that nemorosone was the main component of the ethanolic extract of BCP, as demonstrated by HPLC-PDA assay. Our experimental procedure was carried out differently from the standard method, at a lower temperature, in order to decrease the co-extraction of waxes, which are inert components of propolis. The concentration of nemorosone in the BCP extract was determined to be 37.8 ± 2 mg/mL.

In order to evaluate whether BCP could exert an anti-tumor effect on breast cancer, breast cancer cell lines were used, such as MCF-7 andMDA-MB23 1, estrogen receptor positive (ER+) and negative (ER-), respectively. Cells were treated with BCP in a concentration-dependent manner (1-5-10-25 μg/mL). The cytotoxic potential of BCP was tested by means of MTT assay, considered as an index of decreased cell viability and cell growth [16]. The addition of BCP to MCF-7 increased the cell mortality in a concentration- and time-dependent manner (Figure 1a and b).

In contrast, administration of BCP on MDA-MB 23 1, ER- cells had no effect at 24 hours. However, at 48 hours the effect was similar to that on MCF-7 cells at 48 hours, implying a cytotoxic effect of the extract on both cells at this experimental time. These results suggested that the potential difference of the BCP effects on MCF-7 and MDA MB231 could be the presence of the estrogen receptor.

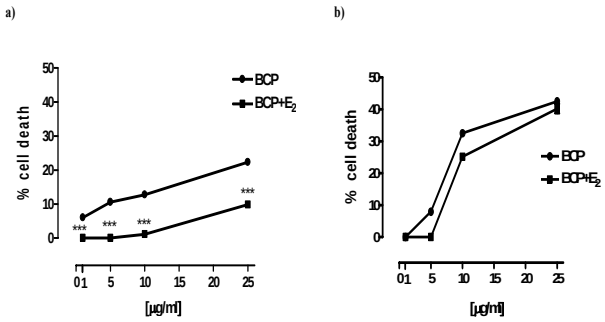


Figure 1: The cytotoxic activity of the ethanolic extract from Brown Cuban Propolis (BCP) on MCF-7. MCF-7 cells were treated with either BCP (1-5-10-25 μg/mL) or BCP plus 17β-estradiol (E2 10 nM) for 24 h (a) or 48 h (b). Cell viability was assessed by means of MTT assay. The experiments were performed in triplicate. Data were presented as mean±SEM of 3 independent experiments. Statistical analysis was performed by using One Way ANOVA, followed by Bonferroni's post test. *** represents P<0.001 of BCP alone vs BCP+E2.

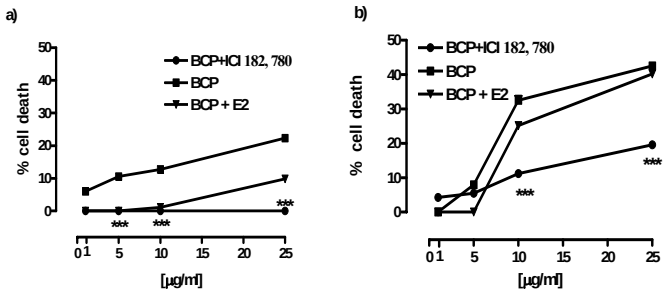


Figure 2: Effect of ICI 182,780, a selective ER antagonist, on BCP-induced cytotoxicity on MCF-7 cells. Cells were treated with BCP (1-5-10-25 μm/mL) alone, or in combination with ICI 182,780 (100 nM) or 17β-estradiol (E2 10 nM) for 24 h (a) or 48 h (b). Cell viability was assessed by means of MTT assay. The experiments were performed in triplicate. Data are presented as mean±SEM of three independent experiments. *** represent P<0.001 of BCP+ICI vs BCP, as determined by the statistical One Way ANOVA, followed by Bonferroni's post test.

In order to confirm this hypothesis, we used either the endogenous agonist, 17β-estradiol (E2; 10nM) or the ER antagonist, ICI 182,780 (100nM). As expected, these drugs had no effect on MDA-MB 23 1 (data not shown). Instead, the addition of E2 reduced the capability of BCP to reduce cell viability at 24 hours, but not at 48 hours. ICI 182,780 completely abolished BCP-induced cell mortality on MCF-7 at 24 hours (Figure 2a), in the same manner as at 48 hours (Figure 2b). The BCP vehicle, ethanol and DMSO mixture did not affect either MCF-7 or MDA-MB231 viability (data not shown).

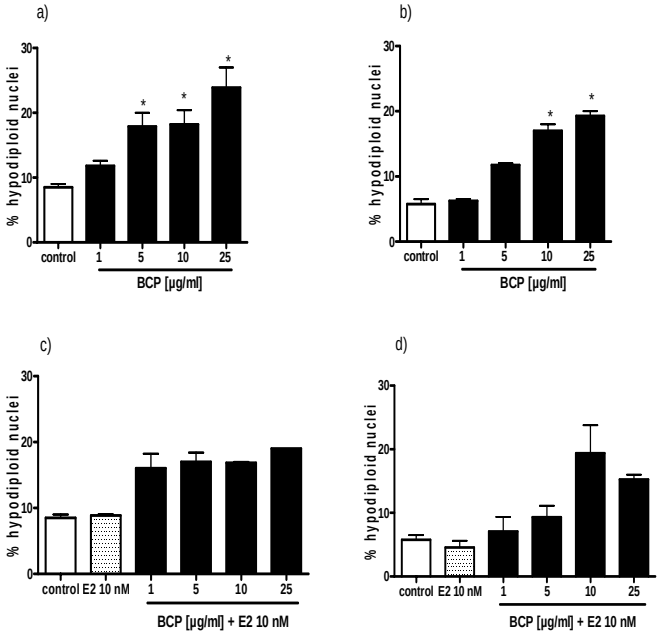


Figure 3: Apoptosis detection by propidium iodide (PI) staining of hypodiploid nuclei. Apoptosis rate was evaluated after the treatment of MCF-7 with BCP (1-5-10-25 µg/mL) alone or in combination with 17β-estradiol (E2 10 nM) for 24 h (a, c) or 48 h (b, d). PI positive cells identified hypodiploid nuclei by means of flow cytometry. Each experiment was performed three times. Data are presented as mean±SEM and the statistical analysis (One Way ANOVA, followed by Bonferroni's post test) determined * as P<0.05 (compared with the medium control).

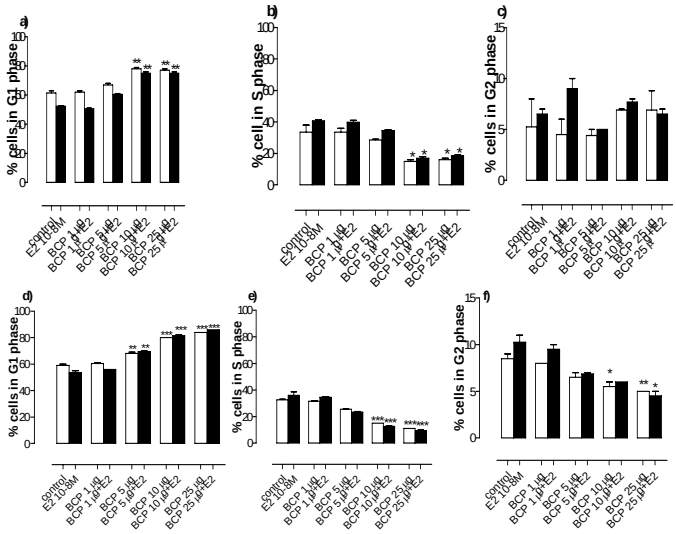


Figure 4: Quantitative analysis of MCF-7 cells in G0/G1, S, and G2/M phases. BCP (1-5-10-25 µm/mL) alone or in addition to 17β-estradiol (E2 10 nM) revealed a cell cycle arrest in the G0/G1 phase at 24 (a, b, c) and 48 h (d, e, f). The experiments were performed in triplicate. Data are expressed as mean ± SEM percentage of cell cycle distribution. Statistical analysis (One Way ANOVA, followed by Bonferroni's post test) determined * as P<0.05, ** as P<0.01, and *** as P<0.001 (compared with the medium control).

To evaluate whether apoptosis may be induced by BCP addition to MCF-7, flow cytometric analysis was performed by means of PI positive staining to distinguish hypodiploid nuclei. Again, a concentration-dependent curve was assessed in a time-dependent manner. BCP induced slight apoptosis at 24 hours in MCF-7 (Figure 3a). The mean values were 11.8 ± 0.8% for 1 µg/mL; 17.9 ± 2.1% for 5 µg/mL; 18.2 ± 2.2% for 10 µg/mL and 24 ± 3.1% for 25 µg/mL of administered BCP vs 8.5 ± 0.5% for the control medium (Figure 3a). The same effect was observed at 48 hours (Figure 3b). The addition of E2 slightly decreased the apoptosis rate, especially at the highest concentration (Figure 3c, 3d), confirming our previous results.

Chemotherapeutics are usually able to arrest the cell cycle of cancerous cells [17]. Thus, we determined whether our extract was able to exert this type of activity. The addition of BCP to MCF-7 was able to induce arrest in the G0/G1 cell cycle phase in a concentration-dependent manner after 24 hours of treatment (Figure 4a). This effect was also observed for the cells at 48 hours (Figure 4d). The G2/M cell cycle phase was not affected (Figure 4c,f), in contrast to the S phase that was significantly reduced at 24 (Figure 4b) and 48 hours (Figure 4e).

Propolis has been used for thousands of years in traditional medicine all over the world. The composition of propolis depends on the vegetation of the area from where it was collected and on the bee species [18]. In fact, the chemical composition of propolis from tropical zones is very different from that of temperate zones, as the different vegetation promotes more terpenoids and prenylated derivatives of *p*-coumaric acids in Brazilian propolis [19], lignans in Chilean propolis [5] and polyisoprenylated benzophenones in Venezuelan, Brazilian, and Cuban propolis [7]. Brown Cuban propolis (BCP) has a simple and constant chemical composition, of which the major component is nemorosone, a member of the polyisoprenylated benzophenone family. Our study utilized a BCP extract that contained nemorosone as its principal component; the extraction procedures excluded other compounds [7]. Our study revealed that BCP was able to exert an anti-proliferative activity on breast cell line cells. The interesting effect of BCP was its capability to induce cell cycle arrest in the G0/G1 phase. This kind of effect is typical of several anti-tumor chemotherapeutic agents [17]. In a previous report, it was demonstrated that nemorosone may exert a cytotoxic and antioxidant activity on several cancer cell lines [9]. This cytotoxic effect of BCP was observed using the MTT assay. To date, the diminished cell viability had not been ascribed to

apoptosis. Indeed, our experimental conditions revealed a very low percentage of hypodiploid nuclei. Instead, cell mortality was more likely defined as increased G1 phase.

Of interest was the observation that the cytotoxic effect of BCP was only evident on MCF-7 cells rather than MDA-MB 23 1, which are ER negative compared with the other ER positive cells. Indeed, the addition of 17β-estradiol reduced the percentage of dead cells compared with BCP administration alone. This surprising effect was attributable to the proliferative effect exerted by estrogen on these cells [20]. Indeed, the addition of ICI 182,780, an ER antagonist, completely abolished the BCP-induced cell mortality on MCF-7. This pharmacological activity was also demonstrated by the use of ER negative cells, such as MDA-MB 23 1, that as expected, were not affected by BCP treatment at 24 hours. Another explanation of our result could be that the receptor activation by the ligand at maximal or sub-maximal concentration may produce a proliferative activity. However, the addition of BCP and E2 did not promote the S and/or G2/M cell cycle phases. Thus, we attributed BCP to an estrogen-like activity. However, further studies need to be conducted to elucidate the real interaction of BCP on ER. Nevertheless, it has been demonstrated that many natural compounds, so called phytoestrogens, have weak *in vivo* estrogenic activities [21] and their mixed estrogen agonist/antagonist properties are analogous to those of the clinically useful anti-estrogen tamoxifen [15,22-23]. However, BCP also induced increased cell mortality of MDA-MB 23 1 and MCF-7 at 48 hours. Thus, it cannot be ruled out that BCP could not only interact with ER, but also have an ER-independent activity, as shown on the MDA-MB 231 cell line, which was ER negative, at 48 h.

In fact, it has been demonstrated that nemorosone, the main component of BCP, showed a good cytotoxic activity on a large number of cell lines [9]. So, we can hypothesize that the effect of BCP at 24 h on the estrogen receptor is an early event that follows the activation of other pathways, such as have been reported in the literature [24].

In conclusion, BCP could prove of potential therapeutic value as an anti-cancer agent. Its anti-proliferative and anti-estrogenic activity could shed light on new chemotherapeutic compounds, even though further studies are required to prove its activity in an *in vivo* breast cancer model.

Experimental

Standard and sample: Brown Cuban propolis (BCP) was provided by "La Estación Experimental Apicola" in Cuba and characterized by spectroscopic techniques, as reported previously [7]. Propolis was kept at 0-5°C and protected from light.

Extraction procedure: The propolis sample was ground prior to extraction. BCP propolis (20 g) was extracted with 80% ethanol (100 mL) by sonication-assisted extraction for 2 h at 20°C.

Quantitative analysis: A HPLC system including a Surveyor Autosampler, Surveyor LC pump and Surveyor PDA detector (ThermoFinningan, San Jose, CA, USA) and equipped with Xcalibur 3.1 software, was used for quantitative analysis. HPLC separations were performed using a Luna C8 (150 mm x 20 mm i.d., 5 μm) column protected by a guard cartridge (4 mm x 2.0 mm i.d.) and a binary gradient composed of water, solvent A, and acetonitrile, solvent B (linear gradient of B from 55% to 95% for 40 min, isocratic elution of 95% B for 10 min) at a flow rate of 0.2 mL/min. The chromatograms were recorded at 280 nm for quantification. Nemorosone was used as a standard for calibration and an external standard method was utilized for its quantification. The quantitative determination was performed using a four-point regression curve in the range 1-30 μg/mL, and triplicate injections were made for each level. UV peak areas of external standard were plotted against the corresponding standard concentration using weighed linear regression to generate standard curves. BCP hydroalcoholic extract was diluted 100 times and 20 μL of this solution was injected for analysis. The amounts of nemorosone were finally expressed as mg/mL of hydroalcoholic extract. Data are reported as mean±standard deviation (SD) of triplicate determinations.

Chemicals: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (1x) and antibiotic solution (penicillin and streptomycin), and phosphate-buffered saline (PBS) were purchased from Cambrex Biosciences (Microtech, Naples, Italy). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton x-100, propidium iodide, 17 β-estradiol and ICI 182,780 were purchased from Sigma (Milan, Italy).

Cell culture: MCF-7 and MDA-MB 23-1 human breast cancer carcinoma cell lines were used. Cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 U/mL streptomycin and 2 mmol/L glutamine in 5% CO_2 at 37°C. At the beginning of each experiment, in order to avoid interference of the phenol red with the estrogen receptor, MCF-7 cells were supplemented with phenol red-deprived DMEM, as described by So *et al*. [25]. To avoid any type of biological differences, MDA-MB 231 cells were grown under the same conditions as MCF-7, although they are estrogen receptor negative [26]. BCP was added to the cells in a concentration- (1-5-10-25 µg/mL) and time-dependent (24-48 h) manner.

MTT assay: MCF-7 and MDA-MB 23 1 cells (4 x 10^3 cells/mL in a 96-well culture plates) were incubated for 24 or 48 h with BCP (1-5-10-25 µg/mL) and/or 17 β-estradiol (10 nM) and ICI 182,780 (100 nM). After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT; 5 mg/mL in PBS) was added to each well in order to evaluate cell viability. After 3 h of incubation a lysis buffer (200 g/L SDS, 50% formamide, pH = 4.7) was added to each well to dissolve formazan. The absorbance was measured at 620 nm in a microplate reader. Data were expressed as % cell death = 100-[(OD control – OD treatment) / OD control] x 100.

Flow cytometric analysis: MCF-7 cells (2 x 10^5 cells/mL in 12-well culture plates) were incubated for 24 or 48 h with BCP in the presence or absence of 17 β-estradiol. The cells were then washed with PBS and suspended by trypsinization. Cells were spun at 2000 x *g* for 10 min, and incubated with a staining solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/mL propidium iodide at 4°C for 30 min in the dark. Samples were analyzed by a Becton Dickinson FACScan flow cytometer. The cell cycle distribution, expressed as percentage of cells in the G_0/G_1, S, and G_2/M phases, was calculated by using a ModFit LT 3.0 program. The apoptotic cells were expressed as a percentage of hypodiploid nuclei.

Statistical analysis: Results are expressed as mean ± SEM. The one-way ANOVA test was used for statistical analysis, followed by Bonferroni's Multiple Comparison test. A value of $P< 0.05$ was considered as statistically significant.

References

[1] Burdock GA. (**1998**) Review of the biological properties and toxicity of bee propolis (propolis). *Food and Chemical Toxicology*, ***36***, 347-363.

[2] Banskota AH, Tezuka Y, Kadota S. (**2001**) Recent progress in pharmacological research of propolis. *Phytotherapy Research*, ***15***, 561-571.

[3] Bankova VS, Popova M, Trusheva B. (**2006**) Plant sources of propolis: an update from a chemist's point of view. *Natural Product Communications*, ***1***, 1023-1028.

[4] Kumazawa S, Hamasaka T, Nakayama T. (**2004**) Antioxidant activity of propolis of various geographic origins. *Food Chemistry*, ***84***, 329-339.

[5] Russo A, Cardile V, Sanchez F, Troncoso N, Vanella A, Garbarino JA. (**2004**) Chilean propolis: antioxidant action in human tumor cell lines. *Life Sciences*, ***76***, 545-558.

[6] Ahn MR, Kunimasa K, Ohta T, Kumazawa S, Kamihira M, Kaji K, Uto Y, Hori H, Nagasawa H, Nakayama T. (**2007**) Suppression of tumor-induced angiogenesis by Brazilian propolis: Major component artepillin C inhibits *in vitro* tube formation and endothelial cell proliferation. *Cancer Letters*, ***252***, 235-243.

[7] Cuesta-Rubio O, Piccinelli AL, Fernandez MC, Hernández IM, Rosado A, Rastrelli L. (**2007**) Chemical characterization of Cuban propolis by HPLC-PDA, HPLC-MS, and NMR: the brown, red, and yellow Cuban varieties of propolis. *Journal of Agricultural and Food Chemistry*, ***55***, 7502-7509.

[8] Cuesta-Rubio O, Cuellar Cuellar A, Rojas N, Velez Castro H, Rastrelli L, Aquino R. (**1999**) A polyisoprenylated benzophenone from Cuban propolis. *Journal of Natural Products*, ***62***, 1013-1015.

[9] Cuesta-Rubio O, Frontana-Uribe BA, Ramirez-Apan T, Cárdenas J. (**2002**) Polyisoprenylated benzophenones in Cuban propolis; biological activity of nemorosone. *Zeitschrift fuer Naturforschung*, ***57C***, 372-378.

[10] Marquez Hernandez I, Campo Fernandez M, Cuesta-Rubio O, Piccinelli AL, Rastrelli L. (**2005**) Polyprenylated benzophenone derivatives from Cuban propolis. *Journal of Natural Products*, ***68***, 931-934.

[11] Piccinelli AL, Campo Fernandez M, Cuesta-Rubio O, Marquez Hernandez I, De Simone F, Rastrelli L. (**2005**) Isoflavonoids isolated from Cuban propolis. *Journal of Agricultural and Food Chemistry*, ***53***, 9010-9016.

[12] Cuesta-Rubio O, Velez-Castro H, Frontana-Uribe BA, Cardenas J. (**2001**) Nemorosone, the major constituent of floral resins of *Clusia rosea*. *Phytochemistry*, ***57***, 279-283.

[13] Barnes S. (**1998**) Phytoestrogens and breast cancer. *Bailliere's Clinical Endocrinology and Metabolism*, ***12***, 559-579.

[14] Padmavathi R, Senthilnathan P, Chodon D, Sakthisekaran D. (**2006**) Therapeutic effect of paclitaxel and propolis on lipid peroxidation and antioxidant system in 7,12 dimethyl benz(a)anthracene-induced breast cancer in female Sprague Dawley rats. *Life Sciences*, ***78***, 2820-2825.

[15] Song YS, Jin C, Jung KJ, Park EH. (**2002**) Estrogenic effects of ethanol and ether extract of propolis. *Journal of Ethnopharmacology*, ***82***, 89-95.

[16] Green LM, Reade JL, Ware CFR. (**1984**) Rapid colormetric assay for cell viability: Application to the quantitation of cytotoxic and growth inhibitory lymphokines. *Journal of Immunological Methods*, ***70***, 257-268.

[17] Renoir JM, Bouclier C, Seguin A, Marsaud FV, Sola B. (**2008**) Antiestrogen-mediated cell cycle arrest and apoptosis induction in breast cancer and multiple myeloma cells. *Journal of Molecular Endocrinology*, ***40***, 101-112.

[18] Bankova V, Popova M, Bogdanov S, Sabatini AG. (**2002**) Chemical composition of European propolis: expected and unexpected results. *Zeitschrift fuer Naturforschung*, ***57C***, 530-533.

[19] Park YK, Alencar SM, Aguiar CL. (**2002**) Botanical origin and chemical composition of Brazilian propolis. *Journal of Agricultural and Food Chemistry*, ***50***, 2502-2509.

[20] Jamerson MH, Johnson MD, Dickson RB. (**2004**) Of mice and Myc: c-Myc and mammary tumorigenesis. *Journal of Mammary Gland Biology and Neoplasia*, ***9***, 27-37.

[21] Diel P, Smolnikar K, Michna H. (**1999**) *In vitro* test systems for the evaluation of the estrogenic activity of natural products. *Planta Medica*, ***65***, 197-203.

[22] Jordan VC. (**1997**) Tamoxifen: the herald of a new era of preventive therapeutics. *Journal of the National Cancer Institute*, ***89***, 747-749.

[23] Santell RC, Chang YC, Nair MG, Halferich WG. (**1997**) Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and hypothalamic/pituitary axis in rats. *Journal of Nutrition*, ***127***, 263-269.

[24] Díaz-Carballo D, Malak S, Bardenheuer W, Freistuehler M, Reusch HP. (**2008**) Cytotoxic activity of nemorosone in neuroblastoma cells. *International Journal of Clinical Pharmacology Therapy*, ***46***, 428-439.

[25] So FV, Guthrie N, Chambers AF, Carroll KK. (**1997**) Inhibition of proliferation of estrogen receptor-positive MCF-7 human breast cancer cells by flavonoids in the presence and absence of excess estrogen. *Cancer Letters*, ***112***, 127-133.

[26] Das JR, Fryar-Tita EB, Zhou Y, Green S, Southerland WM, Bowen D. (**2007**) Sequence-dependent administration of 5-fluorouracil maintains methotrexate antineoplastic activity in human estrogen-negative breast cancer and protects against methotrexate cytotoxicity in human bone marrow. *Anticancer Research*, ***27***, 3791-3799.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1717 - 1728

Cancer Chemopreventive and Anticancer Evaluation of Extracts and Fractions from Marine Macro- and Micro-organisms Collected from Twilight Zone Waters Around Guam[1]

Peter J. Schupp[a,c,*], Claudia Kohlert-Schupp[a], Susanna Whitefield[a], Anna Engemann[a], Sven Rohde[a], Thomas Hemscheidt[c], John M. Pezzuto[b,c,*], Tamara P. Kondratyuk[b], Eun-Jung Park[b], Laura Marler[b], Bahman Rostama[b] and Anthony D. Wright[b,c,*]

[a]*Marine Laboratory*, *University of Guam*, *Mangilao*, *Guam 96923*

[b]*College of Pharmacy*, *University of Hawaii at Hilo*, *34 Rainbow Drive*, *Hilo*, *Hawaii 96720*, *USA*

[c]*Cancer Research Center of Hawaii*, *Honolulu*, *Hawaii 96813*, *USA*

peter.schupp@gmail.com, pezzuto@hawaii.edu and adwright@hawaii.edu

Received: July 30th, 2009; Accepted: September 25th, 2009

The cancer chemopreventive and cytotoxic properties of 50 extracts derived from Twilight Zone (50–150 m) sponges, gorgonians and associated bacteria, together with 15 extracts from shallow water hard corals, as well as 16 fractions derived from the methanol solubles of the Twilight Zone sponge *Suberea* sp, were assessed in a series of bioassays. These assays included: Induction of quinone reductase (QR), inhibition of TNF-α activated nuclear factor kappa B (NFκB), inhibition of aromatase, interaction with retinoid X receptor (RXR), inhibition of nitric oxide (NO) synthase, inhibition 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), and inhibition of HL-60 and MCF-7 cell proliferation. The results of these assays showed that at least 10 extracts and five fractions inhibited NFκB by greater than 60%, two extracts and two fractions inhibited DPPH by more than 50%, nine extracts and two fractions affected the survival of HL-60 cells, no extracts or fractions affected RXR, three extracts and six fractions affected quinone reductase (QR), three extracts and 12 fractions significantly inhibited aromatase, four extracts and five fractions inhibited nitric oxide synthase, and one extract and no fractions inhibited the growth of MCF-7 cells by more than 95%. These data revealed the tested samples to have many and varied activities, making them, as shown with the extract of the *Suberea* species, useful starting points for further fractionation and purification. Moreover, the large number of samples demonstrating activity in only one or sometimes two assays accentuates the potential of the Twilight Zone, as a largely unexplored habitat, for the discovery of selectively bioactive compounds. The overall high hit rate in many of the employed assays is considered to be a significant finding in terms of "normal" hit rates associated with similar samples from shallower depths.

Keywords: Chemoprevention, anticancer, NFκB, aromatase, quinone reductase, nitric oxide synthase, DPPH, RXR, MCF-7 and HL-60 proliferation, extracts, fractions, Twilight Zone, Guam, micro-organisms, marine organisms.

The marine environment is a proven rich source of natural products that have a wide variety of biological activities. During the last three decades, more than 15,000 natural products have been isolated from marine organisms [2]. Sessile organisms, such as sponges, ascidians and gorgonians, often lack any physical protection, and are of special interest in this respect. Sponges, with more than 600 million years of evolution, have evolved a vast number of bioactive secondary metabolites to protect themselves against predation, bacterial and fungal infection, and overgrowth by other organisms [3,4]. The ecological bioactivity of sponge, ascidian and gorgonian secondary metabolites often correlates remarkably well with bioactivity in pharmaceutical assays [5,6]. Many of these more than 5000 metabolites [2] revealed strong activity in pharmaceutical bioassays and thus became promising targets for further preclinical studies as agents against a series of human diseases (for example, cancer, AIDS, malaria, and inflammation) [7-9].

One major reason for the slow progress of a newly discovered biologically active natural compound is the issue of 'supply' [10]. Often, the bioactive compounds are found in only minute quantities in the source organism, making it necessary to collect and extract large amounts of material to supply appropriate amounts for preclinical assays. This cannot be justified from an ecological point of view nor is it congruent with the laws of countries in which the invertebrates occur [11]. Thus, alternatives to harvesting specimens from the wild are often required to take a compound beyond phase I clinical trials. Unfortunately, the supply problem has largely been ignored by marine natural products researchers [10].

For the commercial production of an active compound discovered from nature the pharmaceutical industry would almost certainly select synthesis when practicable over all other possible methods of compound generation. However, many bioactive natural products are structurally extremely complex and require multi-step syntheses that typically are not economically viable for drug development [10,12], even with the advent of approaches used by companies such as Eisai. Mariculture or aquaculture, the farming of marine organisms, is sometimes considered as an alternative to collecting specimens from the wild [13]. A promising example in this respect was *Bugula neritina*, a bryozoan that is the source of bryostatins, a group of anticancer compounds already in clinical use [14]. Although *B. neritina* is now grown in mariculture [10], Lopanik and colleagues [15] discovered that bryostatins are actually produced by a microbial symbiont (*Endobugula sertula*) that protects *Bugula* larvae from predators by its production of bryostatins. This example clearly shows that culturing marine invertebrates can only be an economically relevant alternative if the organisms lend themselves to a viable cost-effective cultivation, and if they produce the metabolites of interest in large and constant quantities.

Another interesting and promising approach to the "supply problem" is the possibility that in many cases, as mentioned above, it is not the marine invertebrates themselves, but their associated microbes that are the true producers of the pharmaceutically interesting compounds [10,16-19]. In this respect, the micro-organisms, assuming they can be cultured, would represent a more attractive source of marine natural products since fermentation is more feasible than synthesis or massive collections [10]. Again, sponges are of special interest in this respect, as they often harbor significant amounts of bacteria in their tissues. In some cases bacteria make up more than 40% of sponge biomass [20,21]. So far, only few studies have identified the actual producers of secondary metabolites of interest, indicating either the sponge itself [22] or the associated bacteria [23-25]. In many cases it is only an assumption that "sponge" metabolites are actually produced by bacterial symbionts, based on the structural characteristics of the metabolites that are typical of prokaryotic rather than eukaryotic biosynthetic processes [17,26].

Determining the true origin of secondary metabolites in marine invertebrates is a difficult task. Bacterial communities in sponges and gorgonians are often complex, making interactions between the macro-organism and bacterial symbionts highly intricate [27-29]. This relationship complicates the process of defining culture conditions for many of the invertebrate (e.g., sponge) associated bacteria and it is currently accepted that only a small percentage of the total associated bacterial community in a given sponge can be cultured. Hence, the goal of the current study was to establish the feasibility of collecting Twilight Zone macro- and micro-organisms in waters around Guam, and to assess biological activity relevant to cancer prevention and treatment. Based on these data, more advanced studies can be designed for the isolation and testing of active chemical constituents. By targeting bacteria from unusual sources and relatively untouched locations (i.e., sponges, ascidians and gorgonians from Twilight Zone habitats around Guam) and by tapping into Guam's enormous, unexploited resources, we are confident we have been able to identify numerous extracts with interesting biological activity from the macro-organisms as well as from bacterial strains isolated from these sources. The Key to our approach was the use of experienced technical divers who are comfortable working at depths typically not approached by the average scientific diver (50-150 m).

The project involved the screening of 65 extracts from unusual sources; 25 represented sponges and gorgonians from the Twilight Zone (50-150 m depth), 25 were bacterial isolates obtained from the Twilight zone sponges, and 15 were extracts from hard (Scleractinian) corals, obtained through access to a NAVY-dredging project. By tapping this

diverse, yet largely untapped biodiversity, we were able to obtain a normally unthinkable hit rate of 42% active samples in the various screens employed. A closer look at the results (Table 1), reveals that although the coral samples and bacterial isolates from the sponges generated 27% and 20% hits, respectively, extracts from the Twilight Zone sponges and gorgonians resulted in an astonishing 72% hit rate. Based on these screening results we are now in the process of fractionating the most promising lead extracts to ensure that these activities are "real" and not caused by so called nuisance compounds, these being usually too toxic or generalist for further development.

When these results are compared with reports in the literature, the true value of this unexplored biodiversity becomes even more apparent. Laird and Kate [30] estimated that screening 5 million compounds/extracts would roughly translate to 1000 hits; 1/5000 or a 0.02% hit rate. Following fractionation, purification, dereplication and structure elucidation only an estimated 10 out of these 1000 hits would become a lead.

Cragg and Newman [31] point to a similar bleak reality referring to the commonly used quote that, "only 1 in 10,000 biologically active leads will result in a commercial drug". They also reiterate that for natural products the ratio might be even worse, as it does not take into account the initial phase of fractionation and purification of compounds from the respective extracts.

In a more detailed analysis, Cragg and colleagues [32] evaluated the number of marine specimens with antileukemic activity as a percent of the total tested specimens for different phyla. When comparing their results with our outcomes, it becomes apparent that extracts of invertebrates from the Twilight Zone have a much higher hit rate than their shallow water counterparts in comparable assays with comparable cut-offs. For example, Cnidaria samples produced a hit rate of 4.4% active samples of the total tested, while our samples demonstrated a hit rate of 8%. The higher hit rates were most pronounced for Porifera, with a hit rate of 8.7% in their analysis; our Twilight Zone specimens exhibited an unprecedented 47% hit rate. The bacterial strains obtained from the Twilight Zone sponges also showed considerable promise with a hit rate of 20%. However, it should be noted here that isolated bacteria were first sequenced to obtain information regarding their taxonomy, and only those bacteria from known producing genera (genera reported in the literature to produce active metabolites) were selected for grow-out and subsequent extract screening. This approach was chosen because limited resources necessitated prioritizing samples, and focusing on bacterial genera already known to be producers of novel, pharmacologically active secondary metabolites was deemed to be the most likely approach to yield positive outcomes.

In order to investigate the potential of extracts from Twilight Zone organisms to mediate chemopreventive and anticancer effects, a series of bioassays were employed. The assays were selected to monitor a broad spectrum of activity. First, the potential to inhibit tumor necrosis factor-α induced NFκB was determined. NFκB is a transcription factor that plays roles associated with apoptosis, cell differentiation, and cell migration. Upon activation, it may promote cell proliferation and further prevent cell death through apoptosis. Inhibition of NFκB signaling has potential application for the treatment or prevention of cancer. The greatest activity was observed in the NF-κB assay, with 15 extracts having activity above 65% at the 20 μg/mL level, with at least 50% cell survival (Table 1).

Next, antioxidant activity was assessed. Free radicals, due to their high chemical reactivity, can react with other macromolecules, including DNA, proteins, carbohydrates and lipids, leading to destructive oxidative damage. Oxidative damage has been implicated in the cause of various diseases including cancer, and also has an impact on the body's aging process. In this assay, stable purple chromogen 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) is reduced by antioxidants or free radical scavengers to the corresponding pale yellow hydrazine and the scavenging capacity is evaluated by monitoring this process. Only three extracts were active in the DPPH assay with inhibition levels of 50% or higher.

Retinoids are derivatives of vitamin A that influence cellular proliferation, differentiation, and apoptosis in a retinoid-specific and cell-type specific manner. Retinoids have shown efficacy as anti-cancer drugs that intervene in the carcinogenic process by regulating proliferation and differentiation at several stages [33]. Retinoid receptors belong to the family of nuclear hormone receptor proteins. There are two major classes of retinoid nuclear receptors: retinoic

Table 1: Results of cancer chemopreventative and anticancer assays performed with extracts from 65 samples of marine macro- and micro-organisms.

Ext. No.	Taxonomy (a, b)	NFκB (c) % Inhib	NFκB (d) % Survival	DPPH (e) % Inhib	DPPH (f) IC_{50} (ug/mL)	MTT (HL-60) (g) % Survival	MTT (h) IC_{50} (ug/mL)	RXR (i) Fold induction	QR (j) IR	QR (k) % Survival	Aroma-tase (l) % Inhib	Aroma-tase (m) IC_{50} (ug/mL)	[NO] (n) % Inhib	[NO] (o) % Survival	SRB (MCF-7) (p) % Survival
1	Thorectidae	41.4	65.2	12.8		53.8		1.8	1.6	89.9	0.00		38.2	131.9	91.2
2	Callyspongiidae	67.5	80.8	5.6		-6.4	13.3	2.4	1.1	75.9	0.00		26.0	115.4	96.8
3	Halichondriidae	63.8	100.9	76.9	97	83.4		2.1	1.3	93.8	0.00		34.0	113.7	96.7
4	Callyspongiidae	73.8	93.1	4.5		51.5		1.8	1.3	89.3	7.38		21.6	103.6	96.9
5	*Suberea* sp.	54.4	111.6	41.5		-4.9	7.7	0.9	1.8	75.7	94.93	0.6	54.9	102.1	53.8
6	Halichondriidae	64.4	84.0	2.6		19.7	1.0	0.9	2.4	74.6	89.14	3.14	31.7	94.5	3.4
7	Thorectidae	72.3	69.1	11.5		-13.2	11.9	0.6	1.5	67.7	11.47		54.9	81.3	67.6
8	Callyspongiidae	74.0	132.5	5.6		66.9		0.9	1.1	108.2	5.57		28.7	111.1	95.2
9	Mycalidae	78.1	111.4	5.6		-2.2	10.5	1.2	1.3	53.4	9.47		23.2	105.0	91.7
10	Porifera	93.0	99.0	0.2		100.6		1.5	0.9	55.8	17.43		20.9	119.8	97.9
11	Nephtheidae	77.2	111.8	0.2		102.7		1.2	1.2	95.7	0.00		27.4	130.7	92.7
12	Callyspongiidae	51.8	109.6	6.1		-2.6	11.0	1.3	1.1	48.1	3.54		34.0	96.0	88.6
13	Porifera	47.6	113.6	24.1		74.6		1.3	1.4	113.3	93.20	0.29	40.9	137.1	94.0
14	Thorectidae	48.2	101.3	2.1		-0.3	8.4	0.9	1.9	60.3	17.14		25.3	102.6	92.5
15	Octocorallia	66.3	112.5	16.3		81.6		1.2	1.2	110.7	12.91		29.4	115.6	100.5
16	Octocorallia	62.8	110.7	55.7	164.1	66.4		1.4	1.4	87.4	15.90		31.3	121.8	95.8
17	Octocorallia	48.5	122.4	1.3		79.0		1.1	1.1	102.8	3.84		29.7	115.1	98.9
18	Octocorallia	38.1	112.1	8.5		78.0		1.0	1.9	74.4	10.75		38.2	101.9	94.2
19	Octocorallia	69.5	92.5	2.9		86.4		1.0	1.3	104.6	4.43		29.5	111.1	99.1
20	Octocorallia	51.0	102.6	11.7		65.5		0.7	2.1	66.0	16.84		52.9	117.7	84.6
21	Octocorallia	59.8	120.0	28.9		76.1		1.3	1.6	78.6	9.07		29.9	116.6	89.7
22	Octocorallia	56.7	99.2	2.6		94.7		1.0	1.2	102.2	5.56		22.3	132.6	96.6
23	Petrosiidae	55.3	65.6	0.2		104.0		1.1	1.0	126.4	49.2		48.8	83.4	78.0
24	Agelasidae	28.4	95.4	76.7	97.4	118.4		1.4	0.6	132.8	15.1		31.5	108.7	106.6
25	Octocorallia	31.5	119.5	0.2		82.3		1.2	0.8	127.8	16.8		29.0	102.6	99.5
26	*Kytococcus* sp.	59.5	116.4	-10.3		98.1		0.9	1.0	110.4	10.36		23.9	139.9	90.1
27	*Gordonia* sp.1	61.7	98.1	-6.0		84.3		1.3	0.8	130.5	18.43		32.0	91.2	90.1
28	*Gordonia terrae*	54.9	137.4	-5.7		100.8		1.3	1.0	115.3	13.43		33.6	138.5	95.1
29	*Streptomyces* sp.1	56.6	102.8	-10.0		104.2		1.2	1.1	106.0	13.38		22.5	114.2	98.7
30	*Ruegia* sp.	64.0	119.2	-13.2		94.6		1.5	1.0	116.4	12.56		29.5	112.0	94.6
31	*Gordonia* sp.2	64.5	124.4	-8.9		81.6		1.6	1.6	78.3	11.87		27.1	106.1	97.7
32	*Streptomyces* sp.2	38.8	101.9	-11.1		97.2		1.0	1.2	103.4	8.29		22.5	118.0	95.6
33	Not identified	64.5	114.2	1.5		97.7		1.3	1.8	75.6	16.70		25.1	100.3	100.2
34	*Pseudoalteromonas* sp.7	77.4	109.2	4.2		85.0		0.8	1.4	103.0	0.00		20.5	115.6	89.7
34	*Gordonia* sp.3	79.7	112.1	-5.2		74.8		1.7	2.0	74.8	14.06		22.1	119.6	95.8
35	*Staphylococcus* sp.	59.0	112.6	-7.6		107.7		1.3	1.4	86.7	21.29		20.9	119.2	94.3
36	*Micrococcus* sp.	59.9	85.9	2.6		110.6		1.4	1.1	111.0	14.39		20.2	134.7	93.5
37	*Gordonia* sp.4	54.1	105.9	-8.7		118.5		1.0	1.0	109.9	19.82		26.2	143.2	91.0
38	*Streptomyces* sp.3	59.7	105.7	-11.6		104.7		1.9	1.0	115.3	21.21		39.3	78.9	92.8
39	Rhodobacteraceae	32.4	132.4	6.6		103.5		0.9	1.0	109.5	4.23		33.4	128.8	96.0
40	*Gordonia* sp.5	39.3	126.9	-11.9		91.4		0.9	1.4	86.6	7.54		22.5	109.5	97.7
42	*Pseudoalteromonas* sp.	42.2	125.9	2.6		88.3		1.1	1.3	90.5	16.25		21.6	104.5	93.8
43	*Pseudoalteromonas citrea*	41.3	125.4	-6.0		111.0		1.6	1.2	93.5	8.76		22.5	101.7	93.7
44	*Streptomyces bellus*	7.2	125.3	-8.4		93.9		0.8	1.2	95.1	0.00		23.7	106.9	96.8
45	*Pseudoalteromonas* sp.1	24.7	112.5	-0.1		88.6		1.2	1.1	102.7	0.00		20.2	112.0	99.7
46	*Pseudoalteromonas* sp.2	49.5	116.7	-22.3		90.3		1.1	1.2	103.3	13.42		25.1	118.0	97.8

47	*Pseudoalteromonas* sp.3	48.2	93.3	-0.9	107.3		1.2	1.6	83.1	15.78	25.6	125.1	94.6
48	*Pseudoalteromonas* sp.4	76.1	112.2	1.3	108.3		1.1	1.2	101.0	15.58	21.2	112.8	93.6
49	*Pseudoalteromonas* sp.5	74.3	116.7	1.8	100.8		1.2	1.3	95.8	16.88	18.9	125.1	95.8
50	*Pseudoalteromonas* sp.6	72.9	127.4	5.3	111.8		1.0	1.2	107.1	2.85	25.1	134.7	92.5
51	Poritidae	73.1	52.7	14.7	101.0		1.2	0.9	119.5	56.2	41.2	130.8	91.8
52	Siderastreidae	49.8	95.7	2.9	110.0		0.9	1.0	128.1	55.4	29.4	121.5	99.0
53	Poritidae	34.1	80.4	7.4	98.7		1.2	1.2	129.6	45.7	26.9	101.2	97.1
54	Pocilloporidae	38.9	96.7	-5.4	104.8		1.1	1.0	111.0	44.9	33.1	113.7	101.2
55	Poritidae	46.3	121.3	3.7	86.9		1.1	0.9	122.2	42.5	31.1	100.0	97.9
56	Pocilloporidae	45.0	92.8	-0.9	99.9		1.0	1.3	123.9	30.8	28.8	109.9	97.0
57	Faviidae	46.8	101.6	-3.3	99.1		1.1	1.1	128.1	32.3	29.9	121.1	101.7
58	Faviidae	23.6	96.4	1.8	99.4		0.8	1.1	116.8	35.2	28.3	125.5	96.8
59	Acroporidae	46.6	93.5	-4.6	74.6		1.8	1.0	128.1	33.3	32.4	126.9	94.5
60	Acroporidae	57.7	104.4	-7.3	99.7		0.9	1.1	122.9	28.1	29.4	133.1	96.7
61	Acroporidae	55.5	87.8	-2.8	49.2	19.5	0.8	1.0	124.3	27.9	47.1	150.8	88.1
62	Merulinidae	58.9	74.5	1.8	94.1		1.3	1.2	134.9	23.9	39.8	98.8	93.0
63	Faviidae	61.4	69.2	8.8	84.2		0.9	0.8	121.8	3.5	39.8	116.5	94.1
64	Faviidae	49.7	82.1	0.2	86.5		1.1	1.1	121.8	25.1	32.4	105.7	99.0
65	Acroporidae	33.8	87.1	1.8	33.6	3.4	1.8	1.4	90.6	29.6	55.4	92.9	94.3

Test Concentrations in each of the assays: NF□B: 20 ug/mL; DPPH: 200 ug/mL; MTT with HL-60 leukemia cells: 20 ug/mL; RXR: 20 ug/mL; QR: 20 ug/mL; Aromatase: 20 ug/mL; NO: 20 ug/mL; Cytotoxicity SRB assay: 20 ug/mL

(a) Samples 1-25 are either sponges or gorgonians, samples 26-50 are bacteria, samples 51-65 are hard corals
(b) Taxonomic identifications of samples 26-50, the bacteria, were based on comparisons of sequence data with closest taxonomic matches in the NIH BLAST data base
(c) 100 % activity is with TNF as activator
(d) To avoid false positives and to determine cytotoxic effect on NFkB 293/Luc cells, 100% is with TNF and DMSO
(e) 100% activity is with scavenging DPPH free radical
(f) Sample concentration which caused 50% inhibition of DPPH
(g) HL-60 leukemia cells. 100% survival is with DMSO
(h) Sample concentration which caused 50% inhibition in HL-60 cell survival
(i) One (1.0) fold is with control induction level of RXR transcriptional activity
(j) QR the induction ratio (IR) of QR represents the specific enzyme activity of agent-treated cells compared with DMSO-treated control
(k) 100% survival is with DMSO
(l) 100% activity is with DMSO
(m) Sample concentration which caused 50% inhibition of aromatase
(n) 100% activity is with LPS as activator
(o) To avoid false positives and to determine cytotoxic effect on RAW 264.7 cells, 100% is with LPS and DMSO
(p) 100% survival is with DMSO

acid receptors (RARs) and retinoid X receptors (RXRs). Each class contains three subtypes (α, β, γ). We utilized a cell line-based retinoid X receptor responsive element (RXRE)-luciferase reporter gene assay; no extracts were deemed to be active.

Quinone reductase 1 (QR1) is considered a detoxifying enzyme. Induction correlates with induction of related phase 2 detoxification enzymes, such as glutathione *S*-transfrease [34]. Of the extracts tested, three mediated an induction ratio of ≥2.0.

Aromatase is an enzyme that catalyzes the conversion of androgen to estrogen. Therefore, aromatase inhibition blocks the production of estrogen, which in turn can reduce the growth of breast cancer cells. Aromatase inhibitors have been used in anticancer therapy to treat breast cancer in postmenopausal women, and animal studies have shown their potential as chemopreventive agents [35,36]. As shown in Table 1, three of the extracts derived from Twilight Zone organisms were effective inhibitors of aromatase.

Nitric oxide (NO) is an ubiquitous signaling molecule that impacts many physiological and pathological processes. It has been shown to be associated with the development of cancers in the early stages with *in vivo* studies [37]. The blocked production of NO is a potential mechanism for chemoprevention; four extracts inhibited production by 50% or more.

Finally, the cytotoxic potential of the extracts was determined employing HL-60 and MCF-7 cells in culture. Inhibition of proliferation or induction of apoptosis in cancer cells is one characteristic of many anti-cancer drugs. Seven of the extracts were active

in the HL-60 cell assay, one of which was active against MCF-7 cells as well.

The assays utilized for evaluation of these samples have been devised to monitor potential inhibition of multiple stages of carcinogenesis [38]. Based on past experience, as described in the literature references for each assay, threshold levels of test material concentrations have been established to yield an active hit rate in the range of 3%. Known active compounds are used in each case to assure the accuracy of the test. Although activity in a single test system may be deemed desirable, it is becoming increasingly clear that pleiotropic mechanisms are facilitated by many promising chemopreventive agents [39]. Accordingly, it is appropriate to analyze the overall response mediated by the unique collection of extracts reported herein.

In sum, of the 65 extracts, 24 showed a positive result in at least one of the applied tests. Of these, five were active in two of the assay systems, three in three tests, and only one in four assays. Of the extracts showing positive results in two assays, two of them (PS 430, PS 432) were positive in both the NFκB (67.5 and 78.1% inhibition, respectively, with cell survival >80% at 20 μg/mL) and the HL-60 (100% cell mortality at 20 μg/mL, and IC_{50} values of 13.3 and 10.5 μg/mL, respectively) test systems, the remaining three being active in QR (IR 2.1 at 20 μg/mL) and NO (52.9% inhibition with 100% cell survival at 20 μg/mL) (PS 454), NFκB (79.7% inhibition with 100% cell survival at 20 μg/mL) and QR (IR 2.0 at 20 μg/mL) (GUDS 477), and HL-60 (50.8% cell mortality at 20 μg/mL, and an IC_{50} value of 19.5 μg/mL) and NO (55.4% inhibition at 20 μg/mL, with 92.9% cell survival). Extract PS 341 gave a high cytotoxic response towards HL-60 cells (100% cell mortality at 20 μg/mL, and an IC_{50} of 7.7 μg/mL), aromatase (94.9% inhibition at 20 μg/mL and an IC_{50} of 0.6 μg/mL) and NO assay (54.9% inhibition with 100% cell survival), while extract PS 431 was active in NFκB (72.3% inhibition at 20 μg/mL with 69.1% cell survival), HL-60 (100% cell mortality at 20 μg/mL, and an IC_{50} of 11.9 μg/mL) and NO (54.9% inhibition with 81.3% cell survival) assays. Extract PS 383 showed activity in four assays; activation of QR I (IR 2.4, with a 74.6% survival at 20 μg/mL), inhibition of aromatase (89.1% inhibition at 20 μg/mL, and an IC_{50} of 3.14 μg/mL) and cytotoxicity towards HL-60 and MCF-7 cells (80.3% cell mortality at 20 μg/mL, IC_{50} of 1.0 μg/mL, and 96.6% mortality at 20 μg/mL, respectively).

Aside from these, all other extracts were active in either only one assay system or apparently devoid of any activity in the applied assays. Eight extracts were active in the HL-60 assay with survival less than 50%. In both the aromatase (greater than 70% inhibition) and NO (greater than 50% inhibition and 80% survival) assays, three extracts were found to have activity. Finally, only one extract (PS 383) was found to have significant cytotoxicity towards MCF-7 cells (greater than 50% cell mortality).

On the basis of these findings, fractionation was initiated with one of the active extracts, a sample of the sponge *Suberea* sp (Table 1, Extract No. 5) that demonstrated significant activity in three assays; aromatase, NO and HL-60. As outlined in the experimental section, this extract was fractionated several times employing various chromatographic methods to yield 16 further sub-fractions for testing. The sub-fractions fall into two groups: 1-11 and 12-16, based on the original fractions from which they were derived. As shown in Table 2, the weak NFκB activity observed with the original extract was enhanced in sub-fractions 1, 2, 4 and 7, as was the original weak DPPH activity of the extract, which was enhanced in sub-fractions 5 and 6. The original significant cytotoxicity towards HL-60 cells of the extract was borne out by similar levels of activity being seen for sub-fractions 6 (IC_{50} 7.1 μg/mL) and 13 (IC_{50} 19.3 μg/mL), which is probably attributable to at least two different chemical entities based on the fractionation scheme. The significant QRI activity of sub-fractions 8-11 and 15 was somewhat unexpected based on the activity found for the original extract, and may also be attributable to two chemical entities, different from the one(s) responsible for the observed HL-60 activity. The aromatase activity observed for sub-fractions 3, 7-11, and 13-15 is consistent with the activity of the original extract and certainly translates into at least two chemical entities that may have similar structures to those responsible for the observed QR activity. Finally, the NO activity demonstrated by sub-fractions 9-11 and 13-14 is entirely consistent with the similar level of activity seen for the extract, if not better. Interestingly, it appears that the QR, aromatase and NO activity is localized in these groups of sub-fractions, indicating that the same or similar compounds are responsible for these activities.

Table 2: Results of cancer chemopreventative and anticancer assays performed with fractions from the extract of the sponge sponge *Suberea* sp.

Fract. No.	NFκB % Inhib (a)	NFκB % Survival (b)	NFκB IC_{50} (c)	DPPH % Inhib (d)	DPPH IC_{50} (ug/mL) (e)	MTT (HL-60 leukemia cells) % Survival (f)	MTT IC_{50} (ug/mL) (g)	RXR Fold induction (h)	QR IR (i)	QR % Survival (j)	Aroma-tase % Inhib (k)	Aroma-tase IC_{50} (ug/mL) (l)	[NO] % Inhib (m)	[NO] % Survival (n)	SRB (MCF-7) % Survival (o)	SRB (KB) % Survival (p)
1	81.7	55.0	13.8	21.9		102.9		1.1	0.9	100.0	19.90		12.25	155.25	89.2	75.0
2	78.9	77.6	16.0	26.1		82.3		1.1	1.0	100.0	46.73		15.80	143.05	93.5	72.1
3	31.7	67.3	8.9	17.2		136.3		1.3	1.5	100.0	68.75		64.45	155.95	72.7	70.0
4	68.7	62.1	2.6	19.4		103.9		1.2	0.8	100.0	23.85		15.90	134.00	91.3	75.5
5	43.1	76.9	4.2	74.7	155.2	98.4		1.3	2.2	36.4	71.77	13.32	37.10	149.15	62.8	71.9
6	31.3	47.6		61.0	181.3	-31.5	7.1	1.2	1.8	42.0	67.52		48.00	137.85	70.6	72.0
7	60.4	90.2		37.7		53.7		1.2	1.8	100.0	70.54	13.8	60.60	148.65	72.3	79.6
8	-7.8	12.0		36.3		76.3		1.0	2.0	100.0	75.71	6.16	46.10	143.85	62.0	71.2
9	-17.5	28.2		18.4		64.4		1.2	2.0	100.0	83.87	1.89	78.20	145.95	85.6	73.7
10	9.0	3.5		10.4		54.9		1.2	2.3	100.0	89.89	0.56	89.70	134.60	70.2	65.5
11	55.3	13.7		4.8		124.4		1.0	3.5	69.7	87.23	2.89	82.65	131.05	75.4	56.9
12	66.4	16.8		5.8		91.9		1.1	1.0	100.0	25.06		15.95	125.10	84.5	73.8
13	5.8	39.0		3.7		-20.4	19.3	1.2	1.1	100.0	82.76	7.45	40.10	130.15	87.8	64.0
14	14.6	56.8		5.0		93.8		1.3	1.1	100.0	89.03	0.74	89.25	129.75	68.1	71.1
15	5.3	33.6		11.2		78.0		1.3	2.2	100.0	77.13	1.73	81.95	129.60	50.6	60.8
16	-18.7	43.6		7.6		91.9		1.2	1.0	100.0	66.41		27.60	121.80	72.4	74.4

Test concentrations in each of the assays: NFkB: 20 ug/mL; DPPH: 200 ug/mL; MTT with HL-60 leukemia cells: 20 ug/mL; RXR: 20 ug/mL; QR: 20 ug/mL; Aromatase: 20 ug/mL; NO: 20 ug/mL; Cytotoxicity SRB assays: 20 ug/mL

(a) 100 % activity is with TNF as activator
(b) To avoid false positives and to determine cytotoxic effect on NFkB 293/Luc cells, 100% is with TNF and DMSO
(c) Sample concentration which caused 50% inhibition of NFkB
(d) 100% activity is with scavenging DPPH free radical
(e) Sample concentration which caused 50% inhibition of DPPH
(f) 100% survival is with DMSO
(g) Sample concentration which caused 50% inhibition in HL-60 cell survival
(h) One (1.0) fold is with control induction level of RXR transcriptional activity
(i) QR the induction ratio (IR) of QR represents the specific enzyme activity of agent-treated cells compared with DMSO-treated control
(j) 100% survival is with DMSO
(k) 100% activity is with DMSO
(l) Sample concentration which caused 50% inhibition of aromatase
(m) 100% activity is with LPS as activator
(n) To avoid false positives and to determine cytotoxic effect on RAW 264.7 cells, 100% is with LPS and DMSO
(o), 100% survival is with DMSO
(p) 100% survival is with DMSO

Although preliminary in nature, the current body of work illustrates the vast biological potential of Twilight Zone macro- and micro-organisms. Based on this prototype study, with only 50 Twilight Zone organisms and 15 shallow water hard corals out of a myriad of organisms sampled, it is clear that larger collections will yield additional leads of significant interest. Further, as judged by sub-fractionation of the sponge *Suberea* species, the rich bioactivity profile shown by this sponge's extract, and fractions, is indicative of the presence of a series of active natural products each having a specific type of activity, not of one or two compounds with a broad spectrum of activity. As this work continues, we are confident that unique and unpredictable chemical entities will emerge from the investigation of Twilight Zone and hard coral samples that will have inspiring spectra of biological activities, some of which will be relevant to human health.

Conclusion

This study is one of the first to document that tapping into largely unexplored marine diversity has great potential to yield active extracts and at hit rates much larger then previously reported. The Twilight Zone and hard corals represent a relatively unexplored habitat and organism group, due to the fact that the Twilight Zone is typically too deep for regular

SCUBA diving and only a small group of professionals have undertaken the effort to become trained to dive to these depths, and the fact that hard corals are often difficult to collect due to permit issues. Additionally, for the Twilight Zone samples, submarines are rarely deployed at these depths, because of their high operating costs. It is more usual that such equipment is employed for deep-sea exploration; depths beyond 500 m. Access to these unique samples in terms of location and organism class, combined with a large array of chemopreventive and anticancer assays, enabled us to identify a higher than usual number of leads to follow up and clearly validated our approach to finding new sources of biologically active extracts.

Experimental

Chemicals: Methanol (HPLC grade), ethyl acetate (HPLC grade), marine broth, actinomycetes agar, cyclohexamide, agar, dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine, sulforhodamine B (SRB), tetradecanoylphorbol 13-acetate (TPA), sodium-diethyldithiocarbamate trihydrate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), non-essential amino acids, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM), Antibiotic-Antimycotic, Hygromycin B, MEM, sodium pyruvate, Roswell Park Memorial Institute (RPMI) 1640 medium, and Lipofctamine™ 2000 transfection reagent were purchased from Invitrogen Co. (Carlsbad, CA). Reporter Lysis Buffer, Luciferase Assay System, *Renilla reniformis* luciferase vector (pRL-SV40), and dual luciferase assay system were purchased from Promega (Madison, WI). Tumor necrosis factor-α (TNF-α) was purchased from Calbiochem (Gibbstown, NJ). Retinoid X receptor responsive element encoding firefly luciferase reporter vector (pRXRE) was purchased from Panomics Inc. (Fremont, CA). Human RXRα protein expressing vector (phRXRα) was bought from Addgene (Cambridge, MA). All other chemicals were obtained from commercial sources and were of the highest grade and purity.

Sample Collection: Sponges and gorgonians were collected by a commercial diver from various sites around Guam. Locations were marked with GPS coordinates to allow possible recollection of active samples. Samples were collected in the Twilight Zone from depths of 50 – 150 m, and bagged underwater in Ziploc bags, and stored in a cooler at ambient seawater temperature until return to the laboratory for processing. Upon return to the laboratory, samples were photographed, DNA samples were taken and a voucher for taxonomic identification made. Vouchers were taken of all collected specimens, preserved in 70% ethanol. Subsamples taken for DNA analysis were preserved in 95% ethanol. Voucher specimens have been deposited at the National Museum of Natural History, Leiden, the Netherlands. After processing, samples were immediately frozen at -20°C and freeze-dried prior to extraction.

Macro-organisms-Extract Preparation: Freeze-dried samples were exhaustively extracted with a 1:1 mixture of methanol and ethyl acetate. Solvent was removed under reduced pressure and the resulting extract evaporated to dryness in a speed vac (Labconco). Extracts were weighed to calculate yields and a portion of each prepared for pharmacological screening.

Bacterial Isolation: Bacterial isolation has sofar focused on sponges, since they are known to harbor large and diverse microbial consortia (up to 40% biomass). Small sub-samples (3 cm^2) of collected sponges were cut in half and one freeze-dried and the other ground wet and kept cool. The dried and finely ground samples were "stamped" with a sterile cotton swab over different agar plates. Dried specimens were used for actinomycetes isolation, since these groups produce spores. Wet processing and plating of the ground extract targeted unicellular bacteria. Approximately 1 g of sponge tissue was homogenized in 1 mL of sterile seawater and serially diluted from 1:10 to 1:10,000. One hundred µL of each dilution was used to inoculate five media with varying nutrient levels: MA (Difco marine agar), M1 (1.8% agar, 1% starch, 0.4% yeast extract, 0.2% peptone, cyclohexamide (100 µg/mL), filtered (80 µm) seawater, M5 1.8% noble agar, 0.05% mannitol, 0.01% casamino acids, cyclohexamide (100 µg/mL), filtered (80 µm) seawater), M8 (1.8% agar), cyclohexamide (100 µg/ml), filtered (80 µm) seawater), AC (Actinomycete Isolation Agar). Bacteria were purified by repeatedly transferring single colonies onto Petri plates. Cryo-stocks of isolates were created by growing the purified strains in 10 mL liquid cultures of the respective isolation media and once the culture reached high densities, 1 mL of the culture was diluted with sterile glycerol, vortexed and frozen.

Bacterial Characterisation: Purified bacteria, either "fresh" or from cryo-stocks, were cultivated and single colonies used as templates for Hot Start PCR. 16S rDNA fragments were amplified with the primer set 27f and 1492r and were sequenced. The NIH BLAST database was used to search for nearest sequence matches.

Bacteria Extract Preparation: Bacterial isolates were grown in 250 mL of Difco Marine Broth until turbid. Liquid cultures were exhaustively extracted by liquid:liquid partition with ethyl acetate. Solvent was removed under reduced pressure and extracts transferred to vials for final evaporation in a speed vac (Labconco). A portion of each extract was used for pharmacological screening. Extracts exhibiting pharmacological activity were grown up in 10 x 1 L cultures to obtain sufficient material for subsequent fractionation and purification of the active principles. This process is ongoing.

Fractionation of Suberea sp. Extract: Initial tests revealed that the extract (Table 1, Extract No. 5) of the Guamenian sponge *Suberea* sp., was extremely active in several of the employed bioassays (inhibition of NFκB, Aromatase inhibition, IC_{50} of 7.7 μg/mL in MTT assays with HL-60 leukemia cells). Based on these results it was selected for further fractionation. The freeze dried sponge was extracted exhaustively with a 1:1 mixture of methanol and ethyl acetate. This extract was screened on a normal phase silica gel TLC plate for its chemical composition, such as number of possible compounds and their polarity range. Based on the results of this TLC analysis, the extract was partitioned with *n*-hexane to remove the more lipophilic components. The remaining methanol layer was concentrated under reduced pressure and fractionated using flash column chromatography employing reversed phase silica gel (RP-18) with solvent mixtures of methanol:water (40:60; 80:20; 100:0), to yield 10 fractions. Resultant fractions were pooled, according to results of an HPLC analysis, into 2 major fractions. The more polar fraction was further fractionated using flash chromatography over a RP18 sep-pack column (methanol:water; 8 solvent mixtures from 5:95 to 100:0), while the fractions containing compounds of medium polarity were fractionated using column chromatography with Sephadex LH20 and methanol. The flash chromatography with sep-pack columns yielded 8 fractions, which were again screened for activity in the various assays (7 fractions, Fractions 5-11, Table 2). One of the active fractions was further purified by column chromatography over RP18 (methanol:water; 90:10) to yield a further 4 fractions. These (Fractions 1-4, Table 2) were again screened. The Sephadex LH20 column gave 5 fractions, all of which were further screened for their pharmacological activity (Fractions 12-16, Table 2).

Biological Testing: Biological assays were performed to evaluate the potential chemopreventative and anticancer activity of extracts and fractions generated from 65 organisms. A brief description of each assay method follows:

NFκB Luciferase Assay: Studies were performed with NFκB reporter stably-transfected human embryonic kidney cells 293 from Panomics (Fremont, CA). This cell line contains chromosomal integration of a luciferase reporter construct regulated by NFκB response element. The gene product, luciferase enzyme, reacts with luciferase substrate, emitting light, which is detected with a luminometer. Data were expressed as % inhibition or IC_{50} values (i.e., concentration of test sample required to inhibit TNF-α activated NFκB activity by 50%). After incubating treated cells, they were lysed in Reporter Lysis buffer. The luciferase assay was performed using the Luc assay system from Promega, following the manufacturer's instructions [40]. In this assay Nα-tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as a positive control; IC_{50} = 5.09 μM.

DPPH Radical Scavenging Assay: In this assay, stable purple chromogen 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was reduced by antioxidants or free radical scavengers to the corresponding pale yellow hydrazine, and the scavenging capacity was evaluated by monitoring the absorbance decrease at 515 nm [41]. DPPH radical scavenging capacity of samples was performed according to Lee *et al*. [42]. DPPH radical solution (316 μM) was added to 96-well plates containing samples dissolved in DMSO. The absorbance of each well was measured at 515 nm using a microplate reader. The DPPH radical scavenging activity of each sample was evaluated by calculating % of inhibition: $\%\ \text{of Inhibition} = (1 - \frac{A_{sample}}{A_{control}}) \times 100$

In this assay, ascorbic acid was used as a positive control; IC_{50} = 36.30 μM.

Retinoid X Receptor Responsive Element (RXRE)-Luciferase Reporter Gene Assay: COS-1 cells were plated and incubated for 24 h and then transiently transfected with RXR responsive element encoding vector (pRXRE), human RXRα protein expressing vector (phRXRα), and *Renilla reniformis* luciferase vector (pRL-SV40) by using Lipofctamine™ 2000 transfection reagent. After 24 h of transfection, cells were treated with samples and further incubated for 24 h. Then cells were lysed and a dual luciferase assay was performed. Results were presented as a relative value calculated by fold increase over control after normalizing ratios of firefly luciferase/Renilla luciferase [43]. In this assay, the cut-off was set at 4-fold induction and 9-*cis*-retinoic acid was used as a positive control; 23.7-fold induction at 100 nM.

Quinone Reductase (QRI) Assay: Hepa 1c1c7 (mouse hepatoma) cells were used in the assay. Cells were incubated in a 96-well plate with test compounds at a maximum concentration of 20 μg/mL, digitonin was used to permeabaize cell membranes, and enzyme activity was measured by the reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan. Production was measured by absorption at 595 nm. A total protein assay using crystal violet staining was run in parallel [44]. 4'-Bromoflavone (CD = 0.01 μM) was used as a positive control.

Aromatase Assay: Aromatase inhibition was determined by measuring the fluorescent intensity of fluorescein, the hydrolysis product of dibenzylfluorescein by aromatase. The test substance was preincubated with a NADPH regenerating system before the enzyme and substrate mixture were added. The reaction mixture was then incubated for 30 min to allow for generation of product, and then quenched with 2 N NaOH. Fluorescence was measured at 485 nm (excitation) and 530 nm (emission) [45]. Naringenin (IC_{50} = 0.23 μM) was used as a positive control.

Nitric Oxide (NO) Synthase Assay: RAW 264.7 cells were incubated in a 96-well culture plate for 24 h. The cells were treated with various concentrations of compounds dissolved in phenol red- free DMEM for 30 min followed by 1 μg/mL of LPS treatment for 24 h. NO was oxidized to stable end product, nitrite, by the addition of Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4], and absorbance was measured at 540 nm. A standard curve was created by using known concentrations of sodium nitrite [46]. The positive control in this assay was Na-L monomethyl arginine (L-NMMA); IC_{50} = 19.7 μM.

Evaluation of Proliferation Inhibitory Potential MTT Assay: HL-60 cells (2 x 10^5 cells/well in 96-well plates) were treated with various concentrations of samples for 72 h. After treatment, MTT solution was added to the incubated plate at a concentration of 500 μg/mL and incubated for an additional 4 h. The purple formazan crystal was dissolved in DMSO and the absorbance was measured at 540 nm. The effect of samples on cell proliferation was calculated as a percentage, relative to vehicle-treated control [47].

SRB Assay: The cytotoxic potential of test substances towards MCF-7 cancer cells was determined as described previously [48]. Briefly, various concentrations of test compounds in DMSO were transferred to 96-well plates and incubated for 72 h at 37°C in a CO_2 incubator. The incubation was stopped with trichloroacetic acid. The cells were washed, air-dried, stained with SRB solution, and optical densities were determined at 515 nm using a microplate reader. In each case, a zero-day control was performed by adding an equivalent number of cells to several wells, incubating at 37°C for 30 min, and processed as described above. Percent of cell survival was calculated using the formula: $(OD_{cells+tested\ compound} - OD_{day\ 0})/OD_{cells+10\%DMSO} - OD_{day\ 0}) \times 100$.

Acknowledgments - This research was supported in part by P01 CA48112 and 5U56CA096278 grants awarded by the National Cancer Institute and NIH MBRS SCORE grant S06-GM-44796. We are indebted to J. Pinson for his assistance with sample collection. This is contribution 641 of the University of Guam Marine Laboratory.

References

[1] Schupp P, Kohlert-Schupp C, Pezzuto JM, Kondratyuk TP, Park E-J, Laura Marler L, Wright AD. (**2009**) Chemoprotective and anticancer screening of extracts from marine macro- and micro-organisms sampled from waters around Guam. Presented in part at the 50th Annual Meeting of the American Society of Pharmacology Meeting, June 27 – July 1, Honolulu, Hawaii, *Congress Abstract*, P-416.

[2] MarinLit, Version 2008. A marine literature database produced and maintained by the Department of Chemistry, University of Canterbury, New Zealand.

[3] McClintock JB, Baker BJ (**2001**) *Marine Chemical Ecology*. CRC Press, Boca Raton, USA.

[4] Proksch P, Ebel R, Edrada RA, Wray V, Steube K. (**2003**) Bioactive natural products from marine invertebrates and associated fungi. In: *Sponges (Porifera)*. Müller WEG (Ed.). Springer-Verlag, Berlin, 117-142.

[5] Faulkner DJ. (**2000**) Marine pharmacology. *Antonie Van Leeuwenhoek*, ***77***, 135-145.

[6] Wright AD, McCluskey A, Macgregor K, Guenther J. (**2008**) Potential ecological role, chemistry and molecular modeling studies of secondary metabolites isolated from the tropical marine sponge *Cymbastela hooperi*. *Planta Medica*, ***74***, 931.

[7] McKee TC, Cardellina JH, Riccio R, Dauria MV, Iorizzi M, Minale L, Moran RA, Gulakowski RJ, Buckheit RW, Snader KM, Boyd MR. (**1994**) HIV-inhibitory natural products. 11. Comparative studies of sulfated sterols from marine invertebrates. *Journal of Medicinal Chemistry*, ***37***, 793-797.

[8] Coulson FR, O'Donnell SR. (**2000**) The effects of contignasterol (IZP-94,005) on allergen-induced plasma protein exudation in the tracheobronchial airways of sensitized guinea-pigs *in vivo*. *Inflammation Research*, ***49***, 123–127.

[9] Kikuchi A, Nieda M, Schmidt C, Koezuka Y, Ishihara S, Ishikawa Y, Tadokoro K, Durrant S, Boyd A, Juji T, Nicol A. (**2001**) *In vitro* anti-tumour activity of α-galactosylceramidestimulated human invariant Vα24+NKT cells against melanoma. *British Journal of Cancer*, ***85***, 741–746.

[10] Faulkner DJ, Harper MK, Haygood MG, Salomon CE, Schmidt EW. (**2000**) Symbiotic bacteria in sponges: sources of bioactive substances. In: *Drugs from the Sea.* Fusetani N. (Ed.). Karger, Basel. 107-119.

[11] Munro MHG, Blunt JW, Lake RJ, Litaudon M, Battershill CN, Page MJ. (**1994**) From seabed to sickbed: What are the prospects? In: *Sponges in time and space.* Van Soest RWM, van Kempen TMG, Braekman JC. (Eds). AA Balkema, Rotterdam, Netherlands, 473-484.

[12] Pomponi SA, Willoughby R (**1994**) Sponge cell culture for production of bioactive metabolites. In: *Sponges in time and space.* Van Soest RWM, van Kempen TMG, Braekman, JC. (Eds.). AA Balkema, Rotterdam, Netherlands. 395-400.

[13] Osinga R, Tramper J, Wijffels RH. (**1998**) Cultivation of marine sponges for metabolite production: applications for biotechnology? *Trends in Biotechnology*, ***16***, 130-134.

[14] Blackhall FH, Ranson M, Radford JA, Hancock BW, Soukop M, McGown AT, Robbins A, Halbert G, Jayson GC. (**2001**) A phase II trial of bryostatin 1 in patients with non-Hodgkin's lymphoma. *British Journal of Cancer*, ***84***, 465–469.

[15] Lopanik N, Lindquist N, Targett N. (**2004**) Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia*, ***139***, 131-139.

[16] Haygood MG, Davidson SK. (**1997**) Small-subunit rRNA genes and *in-situ* hybridization with oligonucleotides specific for bacterial symbionts in larvae of the bryozoan *Bugula neritina* and proposal of "*Candidatus Endobugula sertula*". *Applied Environmental Microbiology*, ***63***, 4612–4616.

[17] Proksch P, Edrada RA, Ebel R. (**2002**) Drugs from the seas: Current status and microbiological implications. *Applied Microbiology and Biotechnology*, ***59***, 125-134.

[18] Thoms C, Schupp PJ. (**2005**) Biotechnological potential of marine sponges and their associated bacteria as producers of new pharmaceuticals (Part II). *Journal of International Biotechnology Law*, ***2***, 257-264.

[19] Thoms C, Schupp PJ. (**2005**) Biotechnological potential of marine sponges and their associated bacteria as producers of new pharmaceuticals (Part I). *Journal of International Biotechnology Law*, ***2***, 217-220.

[20] Vacelet J. (**1975**) Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *Journal of Microscopy and Biology of the Cell*, ***23***, 271-288.

[21] Bewley CA, Faulkner DJ. (**1998**) Lithistid sponges: Star performers or hosts to the stars? *Angewandte Chemie International Edition*, ***37***, 2162–2178.

[22] Salomon CE, Deerinck T, Ellisman MH, Faulkner DJ. (**2001**) The cellular localization of dercitamide in the Palauan sponge *Oceanapia sagittaria*. *Marine Biology*, ***139***, 313-319.

[23] Bewley CA, Holland ND, Faulkner DJ. (**1996**) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia*, ***52***, 716-722.

[24] Schmidt EW, Obraztsova AY, Davidson SK, Faulkner DJ, Haygood MG. (**2000**) Identification of the antifungal peptide-containing symbiont of the marine sponge *T. swinhoei* as a novel deltaproteobacterium, "*Candidatus Entotheonella palauensis*". *Marine Biology*, ***136***, 969-977.

[25] Burja AM, Hill RT. (**2001**) Microbial symbionts of the Australian Great Barrier Reef sponge, *Candidaspongia flabellata*. *Hydrobiologia*, ***461***, 41-47.

[26] Kerr RG. (**2000**) Biosynthesis of bioactive marine natural products. In: *Studies in Natural Products Chemistry.* Vol. ***21***. Atta-ur-Rahman (Ed.). Elsevier, Amsterdam. 293-328.

[27] Steinert M, Hentschel U, Hacker J. (**2000**) Symbiosis and pathogenesis: Evolution of the microbe-host interaction. *Naturwissenschaften*, ***87***, 1-11.

[28] Hentschel U, Fieseler L, Wehrl M, Gernert C, Steinert M, Hacker J, Horn M. (**2003**) Microbial diversity of marine sponges. In: *Sponges (Porifera).* Müller WEG. (Ed.). Springer-Verlag. 59-88.

[29] Santiago-Vazquez LZ, Brueck T, Brueck WM, Duque-Alarcon AP, McCarthy PJ, Kerr RG. (**2007**) The diversity of the bacterial communities associated with the azooxanthellate hexacoral *Cirrhipathes lutkeni. The ISME Journal*, ***1***, 654–659.

[30] Laird SA, ten Kate K. (**1999**) In: *The Commercial Use of Biodiversity. Access to Genetic Resources and Benefit-Sharing*, ten Kate K, Laird SA. (Eds.). Earthscan Publications, London. 34–77.

[31] Cragg GM, Newman DJ. (**2005**) International collaboration in drug discovery and development from natural sources. *Pure and Applied Chemistry*, ***77***, 1923–1942.

[32] Cragg GM, Newman DJ, Yang SS. (**2006**) Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience. *Journal of Natural Products*, ***69***, 488-498.

[33] Wu K, Kim HT, Rodriquez JL, Hilsenbeck SG, Mohsin SK, Xu XC, Lamph WW, Kuhn JG, Green JE, Brown PH. (**2002**) Suppression of mammary tumorigenesis in transgenic mice by the RXR-selective retinoid, LGD1069. *Cancer Epidemiology Biomarkers and Prevention*, ***11***, 467-474.

[34] Kang Y-H, Pezzuto, JM. (**2004**) Induction of quinone reductase as a primary screen for natural product anticarcinogens. In: *Quinones and Quinone Enzymes (A Volume of Methods in Enzymology)*, Sies H, Packer L (Eds.), Elsevier Science, San Diego, California, USA. 380-414.

[35] Lubet RA, Steele VE, Casebolt TL, Eto I, Kelloff GJ, Grubbs CJ. (**1994**) Chemopreventive effects of the aromatase inhibitors vorozole (R-83842) and 4-hydroxyandrostendione in the methylnitrosourea (MNU)-induced mammary tumor model in Spague-Dawley rats. *Carcinogenesis*, ***15***, 2775-2780.

[36] Gubson DE, Steele RE, Chau RY. (**1995**) Prevention of spontaneous tumours in female rats by fadrozole hydrochloride, an aromatase inhibitor. *British Journal of Cancer*, ***72***, 72-75.

[37] Crowell JA, Steele VE, Sigman CC, Fay JR. (**2003**) Is inducible nitric oxide synthase a target for chemoprevention? *Molecular Cancer Therapy*, ***2***, 815-823.

[38] Pezzuto, JM, Kosmeder II, JW, Park, EJ, Lee, SK, Cuendet, M, Gills, J, Bhat, K, Grubjesic, S, Park, H-S, Mata-Greenwood, E, Tan, YM, Yu, R, Lantvit, DD, Kinghorn, AD. (**2005**) Characterization of Natural Product Chemopreventive Agents. In: *Cancer Chemoprevention, Volume **2**: Strategies for Cancer Chemoprevention*, Kelloff GJ, Hawk ET, Sigman CC. (Eds.), Humana Press Inc., Totowa, New Jersey, USA. 3-37.

[39] Francy-Guilford, J, Pezzuto, JM. (2008) Mechanisms of cancer chemopreventive agents: A perspective. *Planta Medica*, ***74***, 1644-1650.

[40] Homhual S, Bunyapraphatsara N, Kondratyuk TP, Herunsalee A, Chaukul W, Pezzuto JM, Fong HS, Zhang HJ. (**2006**) Bioactive dammarane triterpenes from the mangrove plant *Bruguiera gymnorrhiza. Journal of Natural Products*, ***69***, 421-424.

[41] Magalhães LM, Segundo MA, Reis S, Lima JL. (**2008**) Methodological aspects about *in vitro* evaluation of antioxidant properties. *Analytica Chimica Acta*, ***613***, 1-19.

[42] Lee SK, Mbwambo ZH, Chung H, Luyengi L, Gamez EJ, Mehta RG, Kinghorn AD, Pezzuto JM.(**1998**) Evaluation of the antioxidant potential of natural products. *Combinatorial Chemistry and High Throughput Screening*, ***1***, 35-46.

[43] Tanaka T, Suh KS, Lo AM, De Luca LM. (**2007**) p21WAF1/CIP1 is a common transcriptional target of retinoid receptors: pleiotropic regulatory mechanism through retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimer and RXR/RXR homodimer. *Journal of Biological Chemistry*, ***282***, 29987-29997.

[44] Su BN, Gu JQ, Kang YH, Park EJ, Pezzuto JM, Kinghorn AD. (**2004**) Induction of the phase II enzyme, quinone reductase, by withanolides and norwithanolides from Solanaceous species. *Mini-Reviews in Organic Chemistry*, ***1***, 115-123.

[45] Maiti A, Cuendet M, Croy VL, Endringer DC, Pezzuto JM, Cushman M. (**2007**) Synthesis and biological evaluation of (+/-)-abyssinone II and its analogues as aromatase inhibitors for chemoprevention of breast cancer. *Journal of Medicinal Chemistry*, ***50***, 2799-2806.

[46] Kim Y, Min HY, Park HJ, Lee EJ, Park EJ, Hwang HJ, Jin C, Lee YS, Lee SK. (**2004**) Suppressive effects of nitric oxide production and inducible nitric oxide synthase (iNOS) gene expression by *Calystegia soldanella* methanol extract on lipopolysaccharide-activated RAW 264.7 cells. *European Journal of Cancer Prevention*, ***13***, 419-424.

[47] Lee EJ, Min HY, Chung HJ, Park EJ, Shin DH, Jeong LS, Lee SK. (**2005**) A novel adenosine analog, thio-Cl-IB-MECA, induces G0/G1 cell cycle arrest and apoptosis in human promyelocytic leukemia HL-60 cells. *Biochemistry and Pharmacology*, ***70***, 918-924.

[48] You M, Wickramaratne DB, Silva GL, Chai H, Chagwedera TE, Farnsworth NR, Cordell GA, Kinghorn AD, Pezzuto JM. (**1995**) Roemerine, an aporphine alkaloid from *Annona senegalensis* that reverses the multidrug-resistance phenotype with cultured cells. *Journal of Natural Products*, ***58***, 598-604.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1729 - 1732

Chemical Composition of the Essential Oil from *Jasminum pubescens* Leaves and Flowers

Abeer Temraz[a], Pier Luigi Cioni[b], Guido Flamini[b] and Alessandra Braca[b]

[a]*Faculty of Pharmacy, Al Azhar University, Nasr-City, 11371 Cairo, Egypt*

[b]*Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno 33, 56126 Pisa, Italy*

braca@farm.unipi.it

Received: July 23rd, 2009; Accepted: October 8th, 2009

The essential oil obtained from the leaves and flowers of *Jasminum pubescens* (Retz.) Willd. (Oleaceae) has been analyzed by GC/MS. Sixty-three and sixty-four components of the essential oils, representing 95.0% of the total oil for the leaves and 91.9% for the flowers, were identified, respectively. Both the oils were mainly constituted by non-terpene derivatives (58.2% and 50.8%, respectively), among which aldehydes (44.7%) characterized the essential oil from the leaves. Besides aldehydes (14.3%) and other carbonylic compounds (acids, esters, and ketones, 38.1%) were the main non-terpene compounds of the oil from the flowers.

Keywords: *Jasminum pubescens*, Oleaceae, essential oil, leaves and flowers, GC, GC/MS.

Jasminum (Oleaceae family) is a genus of about 200 shrubs and vines species, native to tropical and warm temperate regions [1]. *J. pubescens* (Retz.) Willd., (syn. *J. multiflorum* Burm. f.) is a freely spreading shrub with downy cordate leaves and clusters of intensely sweet scented white starlike flowers at tips and along the stems [2]. The stems and leaves are covered with a downy pubescence that gives the plant an overall grayish-green appearance. Traditionally the dried leaves are used for the treatment of ulcers including the one of the mouth in the form of poultice, and as remedy for skin diseases and otorrhea. The flowers are used for the treatment of intracranial disease, metrorrhagia, oedema, biliary infection, diarrhea, heartburn, and as antidote for poisons. The flowers cataplasm is also used to prevent the milk flow. People from Philippines use the water in which the flowers were macerated the night before as eyewash [3-5]. Phytochemical investigation of the plant aerial parts revealed the presence of secoiridoid lactones and glycosides [6-9]. Within a project related to the study of aromatic plants cultivated at El-Zohria Research Garden, Cairo, Egypt, we reported herein the essential oil composition of leaves and flowers of *J. pubescens*.

Table 1 shows the chemical composition of *J. pubescens* leaves and flowers essential oil. The oil yields were 0.04% and 0.02% (w/w) for flowers and leaves, respectively. GC and GC-MS analyses have resulted in the characterization of 63 and 64 compounds, representing 95.0% of the total oil for the leaves and 91.9% for the flowers, respectively (Table 1).

These two oils resulted quite different, both from a qualitative and, mostly, from a quantitative point of view. Both the oils were mainly constituted by non-terpene derivatives (58.2% and 50.8%, respectively), among which aldehydes (44.7%) characterized the essential oil from the leaves. Besides aldehydes (14.3%), other carbonylic compounds (acids, esters, and ketones, 38.1%) were the main non-terpene compounds of the oil from the flowers.

Among non-terpene derivatives nonanal (21.2%), phenylacetaldehyde (6.8%), and (*E*,*Z*)-2,6-nonadienal (5.8%) were the main constituents for the leaves, while (*Z*)-jasmone (8.4%), phenylacetaldehyde (5.7%), and *n*-heneicosane (5.3%) for the flowers, respectively.

Table 1: Chemical composition of *J. pubescens* leaves and flowers essential oil.

Constituents			Content (%)	
	LRI[a]	LRI[b]	Leaves	Flowers
(*E*)-2-Hexen-1-ol	862	1380	0.4	-
1-Hexanol	871	1350	1.0	0.3
Heptanal	901	1173	tr	tr
α-Pinene	941	1028	0.9	0.6
Benzaldehyde	964	1495	1.0	0.4
1-Heptanol	971	1455	-	tr
β-Pinene	982	1108	0.7	-
2-Pentyl furan	993	1241	-	0.3
6-Methyl-5-hepten-2-one	994	1340	0.9	0.4
Octanal	1003	1277	0.8	0.2
δ-3-Carene	1012	1144	2.8	-
(*E*,*E*)-2,4-Heptadienal	1017	1369	0.6	0.1
p-Cymene	1028	1243	tr	-
Phenylacetaldehyde	1045	1618	6.8	5.7
1-Octanol	1068	1563	1.5	0.2
Linalool	1100	1560	0.5	0.6
Nonanal	1104	1382	21.2	2.9
α-Campholenal	1127	1505	tr	-
cis-Limonene oxide	1136	-	tr	-
cis-Verbenol	1142	-	0.5	-
Benzyl nitrile	1145	-	-	0.2
(E,Z)-2,6-Nonadienal	1158	1573	5.8	0.7
(*E*)-2-Nonenal	1165	1445	1.7	0.3
p-Mentha-1,5-dien-8-ol	1168	-	0.7	-
Phenyl ethyl formate	1176	-	-	0.2
4-Terpineol	1179	1607	0.9	-
p-Cymen-8-ol	1185	1833	tr	-
Methyl salicylate	1192	1741	0.4	0.4
α-Terpineol	1194	1684	tr	tr
Decanal	1206	1481	0.6	0.2
Geraniol	1255	1855	-	0.3
(*E*)-2-Decenal	1263	1592	4.3	0.4
Nonanoic acid	1271	2188	-	tr
Isobornyl acetate	1285	1582	0.9	-
Undecanal	1308	1649	tr	tr
(*E*,*E*)-2,4-Decadienal	1316	1706	tr	0.2
α-Terpinyl acetate	1350	1699	1.6	-
Eugenol	1356	2159	tr	0.6
(*E*)-2-Undecenal	1368	1760	1.9	0.3
α-Copaene	1377	1477	tr	-
(*E*)-β-Damascenone	1380	1790	0.7	0.2
(*E*)-Jasmone	1391	-	-	tr
(*Z*)-Jasmone	1394	1940	1.3	8.4
Dodecanal	1409	1726	-	tr
β-Caryophyllene	1419	1598	10.1	-
(*E*)-α-Ionone	1430	-	1.2	-
Aromadendrene	1439	-	-	tr
(*E*)-Geranyl acetone	1453	1842	3.8	0.8
α-Humulene	1456	1665	1.9	-
(*E*)-β-Farnesene	1458	1661	-	tr
(*E*)−β-Ionone	1485	1960	2.7	0.4
Phenyl ethyl–3-methyl butanoate	1489	-	tr	-
(*Z*,*E*)−α-Farnesene	1491	-	-	0.4
Benzyl tiglate	1498	-	tr	1.4
(*E*,*E*)−α-Farnesene	1508	1727	0.6	8.8
Tridecanal	1511	1822	-	0.3
trans-Nerolidol	1564	2006	5.5	27.6
(*Z*)-3-Hexenyl benzoate	1572	2089	tr	1.6
Hexyl benzoate	1580	-	-	0.8
Caryophyllene oxide	1583	1966	3.9	-
(E)-2-Phenyl ethyl tiglate	1585	-	-	0.6
n-Hexadecane	1600	1600	tr	-
β-Atlantol	1608	-	-	1.0
Humulene epoxide II	1610	-	tr	-
Tetradecanal	1613	1937	-	0.2
α-Bisabolol	1685	2237	-	0.3
epi−α-Bisabolol	1688	2253	-	0.2
n-Heptadecane	1700	1700	-	0.2
Pentadecanal	1717	2060	-	2.2
Benzyl benzoate	1764	2655	tr	3.5
Octadecane	1800	1800	tr	0.2
Hexadecanal	1819	2203	-	0.2
(*E*,*E*)-α-Farnesyl acetate	1843	2280	tr	0.5
n-Nonadecane	1900	1900	tr	0.5
Methyl hexadecanoate	1927	2205	-	1.2
n-Hexadecanoic acid	1940	2938	-	2.4
n-Peicosane	2000	2000	0.5	0.6
Kaurene	2036	2427	tr	-
Abietatriene	2055	2533	tr	-
Abietadiene	2080	2569	1.0	-
Methyl linoleate	2096	-	-	0.9
n-Heneicosane	2100	2100	0.9	5.3
Methyl octadecanoate	2125	2407	-	1.4
Abieta-8(14),13(15)-diene	2150	-	tr	-
n-Docosane	2200	2200	0.4	0.5
n-Tricosane	2300	2300	0.8	2.1
Abieta-7,13-dien-3-one	2313	-	0.5	-
n-Tetracosane	2400	2400	-	0.3
n-Pentacosane	2500	2500	0.8	1.4
Monoterpene hydrocarbons			4.4	0.6
Oxygenated monoterpenes			8.9	1.7
Sesquiterpene hydrocarbons			12.6	9.2
Oxygenated sesquiterpenes			9.4	29.6
Diterpenes			1.5	-
Non-terpene derivatives			58.2	50.8
Total identified			95.0%	91.9%

[a] LRI = relative retention indices on DB-5 column.
[b] LRI = relative retention indices on DB-WAX column.
tr = trace (< 0.1%).

Among terpene derivatives, sesquiterpene hydrocarbons were the most represented (12.6% and 9.2%, respectively), with β-caryophyllene (10.1%) and *trans*-nerolidol (5.5%) as the main constituents of the essential oil of the leaves and *trans*-nerolidol (27.6%) and *(E,E)*-α-farnesene (8.8%) as the main ones of the flowers oil.

Monoterpenes were less represented, principally in the oil of the flowers (2.3% vs. 13.3%). The essential oil of the leaves also contained five diterpenes (1.5%) that were completely absent in the flowers one.

To the best of our knowledge, this is the first report of the composition of the essential oil obtained by hydrodistillation of *J. pubescens* leaves and flowers. Previously, only the chemical composition of flower absolute obtained through solvent extraction methods was reported [10-12], as well as absolute from other *Jasminum* species [13,14]. Comparing our results for flowers essential oil composition of *J. pubescens* with flower absolute of other *Jasminum* species, (including species reported in this study), we found that benzyl benzoate, linalool, methyl linoleate, eugenol, (*Z*)-jasmone, and (*E*)-jasmone are common derivatives, while benzyl acetate, benzyl alcohol, phytol, isophytol, and methyljasmonate are completely absent in *J. pubescens* flowers essential oil. Interestingly, (*Z*)-jasmone amount in *J. pubescens* flowers essential oil is higher than in all the absolutes of other *Jasminum* species [12-14]. Since

(*Z*)-jasmone is one of the component that contribute to the typical smell of jasmine, *J. pubescens* flowers essential oil could be used in perfume industry, in spite of its low yields.

Experimental

Plant materials: The leaves and flowers of *J. pubescens* were collected in June 2007 at El-Zohria Research Garden, Cairo, Egypt and identified by Dr. Mamdouh Shokry from El-Zohria Research Garden. The voucher specimen (voucher number, No. 6440 *Jasminum pubescens*/1) was deposited in Nuove Acquisizioni at Herbarium Horti Botanici Pisani, Pisa, Italy.

Essential oil isolation: The leaves and the flowers of the plant were air dried at room temperature and the essential oils were obtained by hydro-distillation using a Clevenger-like apparatus for 2 h. The oil was then aspirated from the surface by mean of a syringe.

GC and GC-MS analyses: The GC analyses were accomplished with a HP-5890 Series II instrument equipped with DB-WAX and DB-5 capillary columns (30 m × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60ºC for 10 min, ramp of 5ºC/min up to 220ºC; injector and detector temperatures 250ºC; carrier gas nitrogen (2 mL/min); detector dual FID; injection of 0.5 μL of a 10% *n*-hexane solution; split ratio 1:30. The identification of the components was performed, for both the columns, by comparison of their retention times with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of *n*-alkanes. The relative proportions of the essential oil constituents were percentages obtained by FID peak-area normalization, all relative response factors being taken as one.

GC/EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m × 0.25 mm, 0.25 μm coating thickness) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240ºC, respectively; oven temperature programmed from 60-240ºC at 3ºC/min; carrier gas helium at 1 mL/min; injection of 0.2 μL (10% *n*-hexane solution); split ratio 1:30; scan time 1s; mass range *m/z* 35-400. Identification of the constituents was based by comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-alkanes, and on computer matching against commercial (NIST 98 and ADAMS) and home made library mass spectra built up from pure substances and components of known oils and MS literature data [15]. Moreover, the molecular weights of all the identified substances were confirmed by GC/CIMS, using MeOH as CI ionizing gas (the same apparatus and analytical conditions as above).

References

[1] Dirr MA. (**1990**) *Manual of woody landscape plants: their identification, ornamental characteristics, culture, propagation and uses*, Fourth Ed., Stipes Publishing Company, Champaign, IL. 425-426.

[2] Wilder LB. (**1974**) *The Fragrant Garden. A book about sweet scented flowers and leaves*, Dover Publications, New York.

[3] Dymock W, Warden CJH, Hooper D. (**1893**) *Pharmacographia Indica*, Trubner & Co., London.

[4] Burkill HM. (**1985**) The useful plants of West tropical Africa, vol.***4***, Royal Botanic Gardens, Kew, London.

[5] Simon JE, Chadwick AF, Craker LE. (**1984**) *Herbs: an indexed bibliography. 1971-1980. The scientific literature on selected herbs, and aromatic and medicinal plants of the temperate zone*. Archon Books, Hamden, CT. 770.

[6] Shen YC, Chen CH (**1994**) Muliflorin, a new secoiridoid lactone from *Jasminum multiflorum*. *Journal of the Chinese Chemical Society*, ***41***, 473-476.

[7] Chen HY, Shen YC, Chen CH. (**1991**) Jasmultiside, a new secoiridoid glucoside from *Jasminum multiflorum*. *Journal of Natural Products*, ***54***, 1087-1091.

[8] Shen YC, Lin CY, Chen CH. (**1990**) Secoiridoid glycosides from *Jasminum multiflorum*. *Phytochemistry*, ***29***, 2905-2912.

[9] Shen YC, Chen CH. (**1989**) Novel secoiridoid lactones from *Jasminum multiflorum*. *Journal of Natural Products*, ***52***, 1060-1070.

[10] Raja MI, Devarajan L, Kanakabhushani K. (**1976**) Studies on the extraction of essential oils from flowers. *Madras Agricultural Journal*, ***63***, 253-254.

[11] Malek SN, Zakaria Z. (**1995**) The chemical composition of Malaysian jasmine flower absolute *Jasminum multiflorum* L. *ACGC Chemical Research Communications*, ***3***, 15-20.

[12] Ahmad SH, Malek AK, Gan HC, Abdullah TL, Rahman AA. (**1998**) The effect of harvest time on the quantity and chemical composition of jasmine (*Jasminum multiflorum* L.) essential oil. *Acta Horticulturae*, ***454***, 355-363.

[13] Jirovetz L, Buchbauer G, Schweiger T, Denkova Z, Slavchev A, Stoyanova A, Schmidt E, Geissler M. (**2007**) Chemical composition, olfactory evaluation and antimicrobial activities of *Jasminum grandiflorum* L. absolute from India. *Natural Product Communications*, ***2***, 407-412.

[14] Braun NA, Kohlenberg B, Sherina Sim S, Meier M, Hammerschmidt FJ. (**2009**) *Jasminum flexile* Flower Absolute from India – a Detailed Comparison with Three Other Jasmine Absolutes. *Natural Product Communications*, ***3***, 1239-1250.

[15] (a) Adams RP. (**1995**) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry.* Allured: Carol Stream, IL, USA; (b) Davies NW. (**1990**) Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. *Journal of Chromatography*, ***50***, 1-24; (c) Jennings W, Shibamoto T. (**1980**) *Qualitative Analysis of Flavour and Fragrance Volatiles by Glass Capillary Chromatography.* Academic Press, New York, USA; (d) Massada Y. (**1976**) *Analysis of Essential Oils by Gas Chromatography and Mass Spectrometry.* J. Wiley & Sons, New York, USA; (e) Stenhagen E, Abrahamsson S, McLafferty FW. (**1974**) *Registry of Mass Spectral Data.* J. Wiley & Sons, New York, USA; (f) Swigar AA, Silverstein RM. (**1981**) *Monoterpenes.* Aldrich Chem. Comp., Milwaukee, USA.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1733 - 1736

Essential Oils from two *Lantana* species with Antimycobacterial Activity

Lisieux de Santana Julião[a], Humberto R. Bizzo[b], Andressa M. Souza[b], Maria Cristina S. Lourenço[c], Pedro Eduardo A. Silva[d], Eliana S. Tavares[a], Luca Rastrelli[e] and Suzana Guimarães Leitão[f,*]

[a]*Programa de Biotecnologia Vegetal, Universidade Federal do Rio de Janeiro, CCS, Bl. K. 2º andar, Rio de Janeiro, RJ, Brazil*

[b]*Embrapa Agroindústria de Alimentos, Avenida das Américas 29501, 23020-470 Rio de Janeiro-RJ, Brazil*

[c]*Instituto de Pesquisa Clínica Evandro Chagas, Fiocruz Laboratório de Bacteriologia eBioensaios em Micobactérias, Plataforma de Bioensaios II, FIOCRUZ, 21045-900, Rio de Janeiro, Brazil*

[d]*Universidade Federal do Rio Grande, FURG, Laboratório de Micobactérias, Rio Grande, RS, Brazil*

[e]*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Fisciano (SA), Italy*

[f]*Universidade Federal do Rio de Janeiro, Faculdade de Farmácia, Departamento de Produtos Naturais e Alimentos, CCS, Bl. A, 2º andar, Ilha do Fundão, 21.941-590, Rio de Janeiro, RJ, Brazil*

sgleitao@pharma.ufrj.br

Received: August 2nd, 2009; Accepted: October 15th, 2009

Lantana trifolia L. and *L. fucata* Lindl. are two Brazilian species used in folk medicine for the treatment of respiratory disorders. The composition of the essential oils from the leaves was investigated, as well as their *in vitro* activity against *Mycobacterium tuberculosis*. *L. trifolia* yielded an oil (0.2%) rich in sesquiterpenes. The major substances found were germacrene D (45.1%), (*E*)-caryophyllene (12.8%), bicyclogermacrene (12.7%) and α-humulene (4.4%). Sesquiterpenes were also the main components of the oil of *L. fucata* (0.3% yield), the principal ones being β-elemene (27.1%), germacrene D (11.6%), (*E*)-caryophyllene (7.7%), valencene (5.7%) and germacrene A (4.6%). Both oils exhibited *in vitro* antimycobacterial activity by the MABA assay with MICs of 80 μg/mL for *L. trifolia* and 100 μg/mL for *L. fucata*.

Keywords: *Lantana trifolia* L., *Lantana fucata* Lindl., essential oil, MABA, *Mycobacterium tuberculosis*, tuberculosis, Verbenaceae.

Lantana, family Verbenaceae, has approximately 150 species distributed in the tropics and subtropics of America, Africa and Asia [1]. *L. camara* L. is the most widely known species of the genus, occurring in tropical, sub-tropical and temperate regions. Many works concerning the chemical composition and pharmacological activity of this species are described, including the essential oil composition of plants growing in different regions of the globe [1-6]. For *L. trifolia* L. and *L. fucata* Lindl., however, there are only a few reports on essential oil composition.

L. trifolia (syn. *L. celtidifolia* H.B. & K.) is a small shrub that occurs in all regions of Brazil and is extensively used in folk medicine in the form of infusions and syrups for the treatment of respiratory disorders and as a calming agent [7,8]. *L. fucata* (syn. *L. lilacina* Desf.) is also a small shrub, with pink or purple flowers, found in tropical and subtropical temperate regions of the Americas. The leaves have been used in the traditional medicine of Brazil as an anti-inflammatory, for stomach affections, and as infusions to treat cold and bronchitis [7-9]. The essential oil of both species has been analysed. Germacrene D and caryophyllene were described as the major components of the essential oil of *L. trifolia* from Rwanda [10], while caryophyllene oxide and gossonorol were the most abundant substances in the oil of *L. fucata* from Pernambuco-Brazil, where it is used as an antiseptic for wounds [9].

Table 1: Chemical composition (relative % peak area) of the essential oils from *Lantana trifolia* and *L. fucata* (leaves) obtained by GC and GC-MS on a HP-5 column.

	Compound	RI*	RI $_{lit}$**	Leaf oil (%)	
				Lantana trifolia	*Lantana fucata*
1	(*Z*)-3-Hexenol	888	859	0.5	0.1
2	Sabinene	975	975	0.2	0.3
3	1-Octen-3-ol	994	979	-	0.1
4	Limonene	1031	1029	0.1	-
5	Linalool	1100	1097	0.2	-
6	δ-Elemene	1338	1338	-	0.6
7	β-Bourborene	1384	1388	1.7	-
8	β-Cubebene	1390	1388	0.5	-
9	β-Elemene	1391	1391	2.4	27.1
10	(*E*)- Caryophyllene	1418	1419	12.8	7.6
11	β-Gurjunene	1428	1434	0.3	0.2
12	γ-Elemene	1433	1437	-	0.7
13	Aromadendrene	1448	1441	-	0.2
14	α-Humulene	1453	1454	4.4	2.3
15	*allo*-Aromadendrene	1460	1460	2.1	0.7
16	α-Amorphene	1483	1485	-	0.4
17	Germacrene D	1485	1485	45.1	11.6
18	Valencene	1496	1496	-	5.7
19	α-Muurolene	1498	1500	0.8	-
20	Bicyclogermacrene	1500	1500	12.7	0.6
21	Germacrene A	1505	1509	1.2	4.5
22	γ-Cadinene	1514	1514	1.3	-
23	Cubebol	1522	1515	1.2	-
24	δ-Cadinene	1564	1523	1.3	-
25	10-*epi*-Cubebol	1523	1535	-	0.3
26	Sesquisabinene-hydrate	1527	1544	-	0.6
27	Elemol	1550	1550	-	0.7
28	Germacrene-B	1557	1561	-	2.8
29	(*E*)-Nerolidol	1561	1563	-	0.1
30	Longicamphenylone	1565	1564	-	0.8
31	Spathulenol	1577	1578	-	0.8
32	Caryophyllene oxide	1581	1583	0.3	1.0
33	β-Atlantol	1598	1608	-	0.4
34	Humulene epoxide II	1605	1608	2.0	0.4
35	β-Oplopenona	1606	1608	0.4	-
36	*epi*-α-Cubebol	1619	1640	0.5	0.5
37	Cadinol	1641	1640	1.1	-
38	α-Muurolol	1653	1646	0.9	-
39	Cubenol	1645	1647	0.4	-
40	Himachalol	1639	1654	-	1.5
41	α-Eudesmol	1642	1654	-	0.4
42	α-Cadinol	1684	1654	1.2	0.9
43	Khusinol	1682	1680	-	0.6
44	α-*trans*-Bujanol	1705	1690	-	0.3
45	Curcumenol	1734	1734	-	0.2
46	Phytol	2113	1943	2.3	2.6
	Sesquiterpenes			94.6	74.5
	Monoterpenes			0.5	0.3
	Total Identified Compounds (%)			97.9	77.6

*RI= Retention index values are calculated from retention times in relation to *n*-alkanes. **RI from reference [27].

In the course of our continuous search for Brazilian plant extracts active against *Mycobacterium tuberculosis*, we have investigated the essential oils from *Lantana trifolia* and *L. fucata* due to their traditional uses against respiratory disorders. In this work we describe for the first time the antimycobacterial activity of both essential oils, as well as their chemical composition.

The essential oils from the leaves of *L. trifolia* and *L. fucata* were obtained in yields of 0.2% (light yellow) and 0.3% (light green-yellow), respectively. The main identified compounds are listed in Table 1. The essential oils of both species presented a high content of sesquiterpenes: 94.6% for *L. trifolia* and 74.5% for *L. fucata*, and a very low content of monoterpenes (0.5% and 0.3%, respectively). This has already been observed for the oils of other *Lantana* species [4,6,9-11], as well as their small yields [11].

Twenty-seven compounds were identified in the essential oil of *L. trifolia*, where the major components were germacrene D (45.1%), (*E*)-caryophyllene (12.8%), bicyclogermacrene (12.7%) and α-humulene (4.4%). In the essential oil of *L. fucata*, 37 compounds were detected. Among them, β-elemene (27.1%), germacrene D (11.6%), (*E*)-caryophyllene (7.7%), valencene (5.7%) and germacrene A (4.6%) were the major components. Caryophyllene is an important component of the essential oils of most *Lantana* species with high contents of sesquiterpenes, being frequently cited as their major sesquiterpene [2-6, 9,10]. Germacrene D, one of the main components in the samples analyzed in this work, was not detected in the oil of *L. fucata* samples from Pernambuco, Brazil [9].

There are few reports in the literature on the antimicrobial activity of essential oils against mycobacteria [12-19]. Among them, essential oils from *Canella winterana*, *Trachyspermum ammi* and *Heliotropium indicum* presented activity against some mycobacteria, including *M. tuberculosis* in a range that varied between 12.5 mg/L to 100 μg/mL [12-14]. These essential oils presented myrcene, thymol and phytol as the major compounds, respectively. One very interesting report in the literature is the successful inhalational use of the essential oil of *Eucalyptus globulus* to treat pulmonary tuberculosis [15]. Ten days post-inhalation of the oil, the patient was tuberculosis negative (via sputum culture), with no clinical symptoms. The traditional uses of *L. trifolia* and *L. fucata* against respiratory disorders prompted us to evaluate their essential oils against *M. tuberculosis*.

Both oils, when assayed against *M. tuberculosis* by the MABA assay [20], presented MIC values of

80 μg/mL and 100 μg/mL, respectively. Germacrene D, (*E*)-caryophyllene, valencene and β-elemene have been identified in essential oils from *Salvia tomentosa*, *Origanum minutiflorum*, *O. syriacum* and *Thymus revolutus*, which displayed *in vitro* antimycobacterial activity [16-19]. However, the microorganism assayed [16-19] was *M. smegmatis,* which is a rapidly growing environmental species not considered to be a human pathogen [21]. α-Humulene and phytol have been assayed as isolated compounds and presented MIC values of 6.3 μg/mL and of 2 μg/mL, respectively, against *M. tuberculosis* by the MABA assay [22,23]. Phytol is not a very common component of essential oils and has also been described in other works of bioassay-guided isolation of antimycobacterial active principles from plant extracts [24].

Terpene derivatives present moderate to high lipophilicity, which would aid their penetration into the mycobacterial cell wall [25]. It has been demonstrated that for each series of terpenes the activity improves with lipophilicity of a given substance when compared with their more polar analogues [23]. In the case of the studied species, some terpenoids from active antimycobacterial essential oils have been detected, including α-humulene, germacrene D and (*E*)-caryophyllene. However, the contribution of minor components to the antimycobacterial activity of theses oils cannot be ruled out.

This is the first report of the *in vitro* activity of the essential oils from *L. trifolia* and *L. fucata* against *M. tuberculosis*. These results corroborate, at least in part, the traditional use of these plants in Brazil.

Experimental

Plant material: Fresh leaves of *L. trifolia* and *L. fucata,* were collected at Mendes-RJ (22º 32' 00'' S, 43º 42' 00''W) and at the campus of the Federal University of Juiz de Fora, Juiz de Fora, Brazil, (22°46'48.6"S, 43°22'24.5"W), in January 2005 and April 2007, respectively. Plants were authenticated by Dr Fatima Regina Gonçalves Salimena, and voucher specimens were deposited at the Herbarium of the Botanical Department, Federal University of Juiz de Fora (CESJ 30801 for *L. trifolia* and CESJ 48653 for *L. fucata*).

Essential oil extraction: The essential oils from fresh leaves of *L. trifolia* and *L. fucata* were obtained by hydrodistillation in a Clevenger-type apparatus for 4 h.

GC and GC-MS analyses: Gas chromatographic analyses were performed using a HP 5890 series II gas chromatograph (Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a HP-5 (5% phenyl/95% dimethylpolysiloxane) fused silica capillary column (30 m x 0.25 mm x 0.25 μm). Hydrogen was the carrier gas (1.0 mL min^{-1}). The injector temperature was kept at 250°C and the oven temperature program was from 60° to 240°C at a rate of 3°C min^{-1}. The detector (FID) was operated at 280°C. Pure oils (0.03 μL) were injected in split mode (100:1). The GC-MS analyses were performed in an Agilent 5973N mass selective detector coupled to an Agilent 6890 gas chromatograph (Palo Alto, CA), equipped with a HP5-MS capillary column (30 m X 0.25 mm X 0.25μm), operating in electronic ionization mode at 70 eV, with the transfer line maintained at 260°C, while mass analyzer and ion source temperatures were held at 150°C and 230°C, respectively. Helium (1.0 mL min^{-1}) was used as carrier gas. Oven temperature program, injector temperature and split rate were the same as stated for GC analyses. A standard solution of *n*-alkanes (C_7-C_{26}) was used to obtain the retention indices [26]. Individual volatile components were identified by comparison of their mass spectra (MS) and retention indices (RI) with those reported in literature [27] and also in the Wiley Registry of Mass Spectral Data, 6th Edition (Wiley Interscience, New York).

Antimycobacterial tests: MABA (Microplate Alamar Blue Assay) susceptibility testing was performed at FIOCRUZ according to the method described by Franzblau [16]. Final concentration of essential oils was 100 μg/mL. Media plus bacteria with and without rifampicin were used as controls. The H37Rv (ATCC - 27294) strain was used. The minimal inhibitory concentration (MIC) was determined (starting from 100 μg/mL in 1:2 serial dilutions).

Acknowledgments - This work was supported by CNPq (MCT- CNPq/MS-SCTIE-DECIT. 410475/ 2006-8, and fellowship) and FAPERJ (E-26/ 111.614/2008). We are indebted to Dr Fatima R. G. Salimena, from Universidade Federal de Juiz de Fora, Minas Gerais, Brazil, for plant identification. The authors would also like to thank Professor Lyderson F. Viccini and his undergraduate students (Laboratory of Genetic, Department of Biology/ICB), University of Juiz de Fora (MG, Brazil) for collecting the *L. fucata*. Collaborative work was performed under the auspices of the Iberoamerican Program for Science and Technology (CYTED), Project X.11:PIBATUB.

References

[1] Ghisalberti EL. (**2000**) *Lantana camara* L. (Verbenaceae). *Fitoterapia*, ***71***, 467-486.

[2] Khan M, Srivastava SK, Syamasundar KV, Singh M, Naqvi AA. (**2002**) Chemical composition of leaf and flower essential oil of *Lantana camara* from India. *Flavour and Fragance Journal*, ***17***, 75-77.

[3] Sefidkon F. (**2002**) Essential oil of *Lantana camara* L. occurring in Iran. *Flavour and Fragance Journal*, ***17***, 78-80.

[4] Ngassoum MB, Yonkeu S, Jirovetz L, Buchbauer G, Schmaus G, Hammerschmidt FJ. (**1999**) Chemical composition of essential oils of *Lantana camara* leaves and flowers from Cameroon and Madagascar. *Flavour and Fragance Journal*, ***14***, 245-250.

[5] da Silva MHL, Andrade EHA, Zoghbi MDB, Luz AIR, da Silva JD, Maia JGS. (**1999**) The essential oils of *Lantana camara* L. occurring in north Brazil. *Flavour and Fragance Journal*, ***14***, 208-210.

[6] Walden AB, Haber WA, Setzer WN. (**2009**) Essential oil compositions of three *Lantana* species from Monteverde, Costa Rica. *Natural Product Communications*, ***4***, 105-108.

[7] Correa MP. (**1984**) *Dicionário das Plantas Úteis do Brasil e das Exóticas Cultivadas.* (Reimpressão sem alterações edição 1926). Ministério da Agricultura / IBDF, Brasília, DF.

[8] Lorenzi H. (**2000**) *Plantas Daninhas do Brasil*, 3rd Ed., Instituto Plantarum de Estudos da Flora, Nova Odessa, SP.

[9] de Oliveira JCS, Neves IA, da Camara CAG, Schwartz MOE. (**2008**) Essential oil composition of two *Lantana* species from mountain forests of Pernambuco (northeast of Brazil). *Journal of Essential Oil Research*, ***20***, 530-532.

[10] Muhayimana A, Chalchat JC, Garry RP. (**1998**) Chemical composition of essential oils of *Lantana trifolia* L. from Rwanda. *Journal of Essential Oil Research*, ***10***, 547-549.

[11] Deena MJ, Thoppil JE. (**2000**) Antimicrobial activity of the essential oil of *Lantana camara*. *Fitoterapia*, ***71***, 453-455.

[12] Rastogi N, Abaul J, Goh KS, Devallois A, Philogeéne E, Bourgeois P. (**1998**) Antimycobacterial activity of chemically defined natural substances from the Caribbean flora in Guadeloupe. *Immunology and Medical Microbiology*, ***20***, 267-273.

[13] Sinha P, Santha Kumar TR, Darokar MP, Khanuja SPS. (**2006**) Isolation of oxidative stress response mutants in *Escherichia coli* for their use as a genetic screen to identify new anti-mycobacterials as functional analogues of isoniazid. *World Journal of Microbiology & Biotechnology*, ***22***, 791–798.

[14] Machan T, Korth J, Liawruangrath B, Liawruangrath S, Pyne SG. (**2006**) Composition and antituberculosis activity of the volatile oil of *Heliotropium indicum* Linn. growing in Phitsanulok, Thailand. *Flavour and Fragrance Journal*, ***21***, 265–267.

[15] Sherry E, Warnke PH. (**2004**) Successful use of an inhalational phytochemical to treat pulmonary tuberculosis: A case report. *Phytomedicine*, ***11***, 95-97.

[16] Tepe B, Daferera D, Sokmen A, Sokmen M, Polissiou M. (**2005**) Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia tomentosa* Miller (Lamiaceae). *Food Chemistry*, ***90***, 333–340.

[17] Vardar-Ünlü G, Ünlü M, Dönmez E, Vural N. (**2007**) Chemical composition and *in vitro* antimicrobial activity of the essential oil of *Origanum minutiflorum* O Schwarz & PH Davis. *Journal of the Science of Food and Agriculture*, ***87***, 255–259.

[18] Alma MH, Mavi A, Yildirim A, Digrak M, Hirata T. (**2003**) Screening chemical composition and *in vitro* antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. growing in Turkey. *Biological and Pharmaceutical Bulletin*, ***26***, 1725-1729.

[19] Karaman S, Digrak M, Ravid U, Ilcim A. (**2001**) Antibacterial and antifungal activity of the essential oils of *Thymus revolutus* Celak from Turkey. *Journal of Ethnopharmacology*, ***76***, 183–186.

[20] Franzblau SG, Witzig RS, Mclaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH. (**1998**) Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue Assay. *Journal of Clinical Microbiology*, ***36***, 362-366.

[21] Wallace RJJr, Nash DR, Tsukamura M, Blacklock ZM, Silcox VA. (**1988**) Human disease due to *Mycobacterium smegmatis*. *Journal of Infectious Disease*, ***158***, 52-59.

[22] Kanokmedhakul S, Kanokmedhakul K, Lekphrom R. (**2007**) Bioactive constituents of the roots of *Polyalthia cerasoides*. *Journal of Natural Products*, ***70***, 1536–1538.

[23] Cantrell CL, Franzblau SG, Fischer NH. (**2001**) Antimycobacterial plant terpenoids. *Planta Medica*, ***67***, 685-694.

[24] Rajab MS, Cantrell CL, Franzblau SG, Fischer NH. (**1998**) Antimycobacterial activity of (*E*)-phytol and derivatives. A preliminary structure-activity study. *Planta Medica*, ***64***, 2-4.

[25] Copp BR. (**2003**) Antimycobacterial natural products. *Natural Products Report*, ***20***, 535-557.

[26] Van Den Dool H, Kratz PD. **(1963**) A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. *Journal of Chromatography A*, ***11***, 463-471.

[27] Adams RP. (**2001**) *Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectrometry.* Allured Publishing Corp, Carol Stream, IL.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1737 - 1739

Volatile Organic Components from Fresh Non-edible Basidiomycetes Fungi

Marisa Piovano[a], Juan A. Garbarino[a], Elizabeth Sánchez[b]and Manuel E. Young[b]

[a]*Universidad Técnica Federico Santa María*, *Departamento de Química*, *Avenida España 1680*, *Valparaíso*, *Chile*

[b]*Universidad Técnica Federico Santa María*, *Centro de Biotecnología "Dr. Daniel Alkalay L."*, *Avenida España 1680*, *Valparaíso*, *Chile*

Marisa.piovano@usm.cl

Received: October 22nd, 2009; Accepted: November 3rd, 2009

The compounds responsible for the characteristic odor of eight fresh non-edible Basidiomycetes fungi were evaluated. The volatile organic compounds from the fresh samples present in the headspace of a sealed vial were determined by solid-phase microextraction gas chromatography-mass spectrometry, using a PDMS/DVB fiber. A total of twenty-eight components were identified, the most frequent being 1-octen-3-ol and 3-octanone.

Keywords: fresh non edible fungi, Basidiomycetes, volatile organic compounds, headspace, SPME.

Mushrooms have been widely consumed since ancient times as a food source. Their characteristic flavor/odor producing substances can be cataloged into non-volatile and volatile components [1]. Among the diverse volatile metabolites, a series of aliphatic C-8 oxygenated constituents are the main compounds found in the edible species and have been reported to be the major contributors to the characteristic fungal odor [2]. They are the product of the oxidation and enzymatic cleavage of either linoleic or linolenic acids and are classified as oxylipins, molecules which take part in a wide range of biological processes [3,4].The objective of this study was to determine the odor producing compounds present in eight field collected fungal species not used as food and comparing these with those found in accepted edible species [2].

The volatile organic compounds analyzed belong to the following species: *Mycena chlorinella*, *Rhizopogon roseolus*, *Pholiota spumosa*, *Telephora terrestris*, *Hygrocybe coccinea*, *Geastrum triplex*, *Ramaria flaccida* and *Laccaria laccata* [5]. Chemical antecedents of non-volatile compounds in six of the eight studied species already exist [6-14].

Twenty-eight volatile constituents, distributed in the eight species under study, were identified using National Institute of Standards and Technology (NIST) MS SPECTRAL Library (2000) (Table 1). Although 1-octen-3-ol is one of the most abundant and organoleptically important odor components in all the species, other important flavor/odor compounds were 3-octanone, 3-octanol, and 2-octen-1-ol, among others [18], confirming the importance in aroma of the double bond and the position of the hydroxy group, as reported previously [19]. 1-Octen-3-ol has been shown to be a potent slug antifeedant [20]. All of these compounds were present in seven of the eight species under evaluation.

Only small amounts of monoterpenes have been detected in the volatile constituents of wild basidiocarps as compared with those reported for cultured species [2]. In this work, limonene was identified in five of the wild species, but only in very low concentrations (< 1%); only in *H. coccinea* was the concentration of limonene significant (3.4%).

Determination of volatile organic compounds by SPME directly in the head space of a vial containing only the fungal sample truly represents the components as they are perceived by the nose, without interference due to the soluble compounds extracted by the solvent when liquid extraction is applied, followed by traditional GC technique.

Table 1: Percentage* of volatile compounds identified in fresh wild fungi.

	Fresh Fungal Species**							
Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Pentanal	-	-	0.8	-	-	-	-	-
1-Octene	0.5	1.3	6.6	15.5	-	10.0	1.7	8.2
3-Cyclohepten-1-one	42.5	-	-	1.3	-	-	4.0	-
Hexanal	-	-	3.4	-	-	-	-	-
1,3-Octadiene	3.9	40.8	0.5	-	-	-	-	-
2-Heptanone	-	0.4	-	-	-	-	-	-
1,3-*Trans*-5-*cis*-octatriene	6.1	-	-	-	-	-	-	-
2,4,6-Octatriene	1.1	-	-	-	-	-	-	-
2-Hexenal	1.1	-	-	-	-	-	-	-
2,6-Cyclooctadien-1-ol	4.0	-	-	-	-	-	-	-
α-Pinene	-	-	-	-	2.3	-	-	0.7
1-Octen-3-ol	18.4	25.5	58.5	-	19.0	3.3	6.3	34.3
3-Octanol	-	7.3	-	16.1	-	4.2	11.5	6.3
2-Octen-1-ol	-	0.4	-	-	-	-	-	-
3-Octanone	3.2	15.2	-	55.6	2.3	50.4	67.3	34.3
4-Octen-3-one	-	-	-	1.2	-	-	-	-
2,4-Heptadienal	0.1	-	-	-	-	-	-	-
δ-Limonene	0.5	-	0.2	-	3.4	-	0.4	0.9
Octenal	-	-	4.7	-	-	-	-	-
Octanal	-	-	-	-	1,6	-	-	-
2-Octenal	0.4	-	7.5	-	24.2	8.9	-	-
1-Methyl-1-ethyl-cyclopentane	-	-	-	-	3.3	-	-	-
2-Nonen-1-ol	-	-	8.1	-	-	-	-	-
2,4-Octadienal	0.2	-	-	-	-	-	-	-
Nerodiol	-	0.3	-	-	-	-	-	-
Exo-Norbornyl alcohol	0.5	-	-	-	-	-	-	-
2,4-Hexadien-1-ol	0.4	-	-	-	-	-	-	-
Pentadecane	-	-	-	-	-	1.2	-	-

*Relative percentage of the identified volatile base on the GC-MS chromatographic area.

** **1** = *Mycena chorinella*; **2** = *Rhyzopogon roseolus*; **3** = *Pholiota spumosa*; **4** = *Thelephora terrestris*; **5** = *Hygrocybe coccinea*; **6** = *Geastrum triplex*; **7** = *Ramaria flaccida*; **8** = *Laccaria laccata.*

This is the first report on the profile of the odor producing volatile organic compounds of the wild species investigated. There was not a great difference in the content of the volatiles between the edible and non-edible fungi regarding the more abundant compounds that are defined as providing the "mushroom-like flavor" and "raw mushroom" odor [1].

Experimental

Plant materials: Fresh, whole, mature specimens, in good condition, were collected in the Reserva Nacional Forestal Lago Peñuelas (Biosphere World Reserve, UNESCO 1984), Valparaíso (V Region), Chile from August to September 2007, and transported in paper bags for immediate analysis. From each species, 2-3 g was used for the assay.

Fungal samples were identified by Dr M. Clericuzio (U. de Alessandria, Alessandria, Italy), and specimens where stored in the Herbarium of the Natural Products Laboratory, Universidad Técnica Federico Santa María.

Chromatographic conditions: Volatiles were collected from the head-space of each sample placed in a 20 mL vial by solid-phase microextraction using a polydimethylsiloxane/divinylbenzene PDMS/DVB fiber (Supelco) during 30 min. at room temperature [15-17]. The fiber was then placed for 5 min in the injection port of the GC. Analyses were performed on a Hewlett-Packard GC/MS 6890 system. The chromatograph was fitted with a 30 m x 0.25 μm x 0.25 mm BPX-5 column with helium as carrier gas at a flow rate of 1 mL/min. The injector temperature was 250°C. The oven temperature was programmed as follows: initial temp 40° C (2 min), then heated at 10° C/min until 270° (4 min).

The MS were recorded in mode SCAN, using a potential of 70 eV for ionization by electron impact.

Acknowledgments - This work was supported by a Grant (# 13.05.21.) from DGIP (Universidad Técnica Federico Santa María).

References

[1] Cho IH, Kim SY, Choi H-K, Kim Y-S. (**2006**) Characterisation of aroma-active compounds in raw and cooked pine-mushrooms (*Tricholoma matsutake* Sing.). *Journal of Agricultural and Food Chemistry*, ***54***, 6332-6335.

[2] Breheret S, Talou T, Rapior S, Bessiere J-M. (**1997**) Monoterpenes in the aromas of fresh wild mushrooms (Basidiomycetes). *Journal of Agricultural and Food Chemistry*, ***45***, 831-836.

[3] Combet E, Henderson J, Eastwood DC, Burton KS. (**2006**) Eight-carbon volatiles in mushrooms and fungi: properties, analysis, and biosynthesis. *Mycoscience*, ***47***, 317-326.

[4] Cho IH, Choi H-K, Kim Y-S. (**2006**) Difference in the volatile composition of pine-mushrooms (*Tricholoma matsutake* Sing.) according to their grades. *Journal of Agricultural and Food Chemistry*, ***54***, 4820-4825.

[5] Lazo W. (**2001**) *Hongos de Chile*, Atlas Micológico, Facultad de Ciencias, Universidad de Chile, Santiago, Chile, 1-231.

[6] Piovano M, Clericuzio M, Tabasso S, Chamy MC, Garbarino JA, Vidari G, Vita Finzi P. (**2005**) Studies on Chilean fungi III. Free and bound sterols from *Mycena* chlorinella (Basidiomycetes). *Journal of the Chilean Chemical Society*, ***50***, 389-390.

[7] Laloue M, Hall RH. (**1973**) Cytokinins in *Rhizopogon roseolus.* Secretion of *N*-[9-(β-D-ribofuranosyl-9H)purin-6-ylcarbamoyl] threonine into the culture medium. *Plant Physiology*, ***51***, 559-562.

[8] Liu JK. (**2006**) Natural terphenyls: Developments since 1877. *Chemical Review*, ***106***, 2209-2223.

[9] Clericuzio M, Piovano M, Chamy MC, Garbarino JA, Milanesio M, Viterbo D, Vidari G, Vita Finzi P. (**2004**) Structural characterisation of metabolites from *Pholiota spumosa* (Basidiomycetes). *Croatica Chemica Acta*, ***77***, 605-611.

[10] Clericuzio M, Tabasso S, Garbarino JA, Piovano M, Cardile V, Russo A, Vidari G. (**2007**) Non-phenolic dicinnamamides from *Pholiota spumosa*: isolation synthesis and antitumour activity. *European Journal of Organic Chemistry*, ***33***, 5551-5559.

[11] Radulovic N, Quang DN, Hashimoto T, Nukada M, Tanaka M, Asakawa Y. (**2005**) Pregnane-type steroids from the inedible mushroom *Telephora terrestris. Chemical & Pharmaceutical Bulletin*, ***53***, 309-312.

[12] Radulovic, N, Quang DN, Hashimoto T, Nukada M, Asakawa Y. (**2005**) Terrestrins A-G: p-terphenyl derivatives from the inedible mushroom *Telephora terrestris*. *Phytochemistry*, ***66***, 1052-1059.

[13] Torpoco V, Garbarino JA. (**1998**) Studies of Chilean fungi I. Metabolites from *Geastrum triplex* Jungh**.** *Boletín de la Sociedad Chilena de Química*, ***43***, 227-229.

[14] Torpoco V. **(1997)** Estudio químico de *Ramaria flaccida* (Fr.) Ricken var. *chilensis* Lazo y *Geastrum triplex* Jungh (Hongos). Tesis de Magister en Ciencias mención Química, Universidad Técnica Federico Santa María, Valparaíso, Chile.

[15] Demyttenaere JCR, Moriña RM, De Kimpe N, Sandra P. (**2004**) Used of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. *Journal of Chromatography A*, ***1027***, 147-154.

[16] Baroni MV, Nores ML, Díaz MDP, Chiabrando GA, Fassano JP, Costa C, Wunderlin DA. (**2006**) Determination of volatile organic compound patterns characteristic of five unifloral honey by solid-phase microextraction-gas chromatography-mass spectrometry coupled to chemometrics. *Journal of Agricultural and Food Chemistry*, ***54***, 7235-7241.

[17] La Guerche S, Dauphin B, Pons M, Blancard D, Darriet P. (**2006**) Characterisation of some mushroom and earthy off odors microbially induced by the development of rot on grapes. *Journal of Agricultural and Food Chemistry*, ***54***, 9193-9200.

[18] Venkateshwarlu G, Chandravadana MV, Tewari RP. (**1999**) Volatile flavour components of some edible mushrooms (Basidiomycetes). *Flavour and Fragrance Journal*, ***14***, 191-194.

[19] Maga JA. (**1981**) Mushrom flavour. *Journal of Agricultural and Food Chemistry*, ***29***, 4-7.

[20] Wood WF, Archer CL, Largent DL. (**2001**) 1-Octen-3-ol, a banana slug antifeedant from mushroom. *Biochemical Systematics and Ecology,* ***29***, 531-533.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1741- 1750

Chemistry, Antioxidant, Antibacterial and Antifungal Activities of Volatile Oils and their Components

Laura De Martino[a], Vincenzo De Feo[a,*], Florinda Fratianni[b] and Filomena Nazzaro[b]

[a]*Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, via Ponte don Melillo, 84084 Fisciano (Salerno), Italy*

[b]*Istituto di Scienze dell'Alimentazione, CNR, via Roma 64, 83100 Avellino, Italy*

defeo@unisa.it

Received: August 1st, 2009; Accepted: November 6th, 2009

The present paper reports the chemical composition, antioxidant and antibacterial activities of several essential oils and their components. Analysis showed that three oils (*Carum carvi* L., *Verbena officinalis* L. and *Majorana hortensis* L.) contained predominantly oxygenated monoterpenes, while others studied (*Pimpinella anisum* L., *Foeniculum vulgare* Mill.) mainly contained anethole. *C. carvi*, *V. officinalis* and *M. hortensis* oils exhibited the most potent antioxidant activity, due their contents of carvacrol, anethole and estragol. Antibacterial action was assessed against a range of pathogenic and useful bacteria and fungi of agro-food interest. *V. officinalis* and *C. carvi* oils proved the most effective, in particular against *Bacillus cereus* and *Pseudomonas aeruginosa*. Carvacrol proved most active against *Escherichia coli*, and completely inhibited the growth of *Penicillium citrinum*. The oils proved inactive towards some Lactobacilli strains, whereas single components showed an appreciable activity. These results may be important for use of the essential oils as natural preservatives for food products.

Keywords: Antimicrobial activity, chemical composition, food preservation, monoterpenes, natural antioxidants.

Essential oils arise from plant secondary metabolism, [1] and they are widely used in cosmetics as scent components, and in the food industry to improve the flavor and organoleptic properties of different foods [2]. Essential oils have interesting biological properties [3] and several investigations have demonstrated their effectiveness as natural antioxidants, prompting experimental work aimed at identifying the most bioactive compounds. Generally, in order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. However, side-effects of some synthetic antioxidants used in food processing have been documented [4].

Literature reports have described natural antioxidants with radical-scavenging activity from fruits, vegetables, herbs and cereal extracts. Due to the versatile content of essential oils they should be considered as natural agents for food preservation due to their antimicrobial and potential antioxidant activity [5].

The antimicrobial activity of the essential oils is often attributed to the presence of terpenoid and phenolic components [6]. The available literature reports carvacrol, citral, 1,8-cineole, limonene, α- and β-pinene and linalool as active compounds [7] that exhibit significant antimicrobial activities when tested separately [8]. In a previous work, we reported that some essential oils from the family Labiatae exhibited a good antimicrobial activity against different pathogenic bacteria and fungi [9].

In this paper, we report the results of a study aimed to evaluate the chemical composition of the essential oils of *Pimpinella anisum* L. (anise), *Carum carvi* L. (caraway), *Foeniculum vulgare* Miller (fennel) (Apiaceae), *Majorana hortensis* L. (marjoram) (Lamiaceae), *Verbena officinalis* L. (vervain) (Verbenaceae), and to evaluate their antioxidant and antimicrobial activities, as well as those of their main components.

Table 1 outlays the chemical composition of the investigated oils. The main constituent of *P. anisum* and *F. vulgare* (Apiaceae) essential oils was *cis*-anethole, which represented 97.1% and 76.3% of the whole composition, respectively.

Table 1: Chemical composition of essential oils of *Pimpinella anisum* (anise), *Carum carvi* (caraway), *Foeniculum vulgare* (fennel), *Majorana hortensis* (marjoram), and *Verbena officinalis* (vervain).

Compound	Ki[a]	Ki[b]	Anise	Caraway	Fennel	Marjoram	Vervain	Identification [d]
			%[c]	%	%	%	%	
α-Thujene	930	1035	---	0.2±0.0	T	0.1±0.0	---	RI, MS
α-Pinene	938	1032	0.3±0.0	0.5±0.2	1.8±0.1	9.0±0.1	0.2±0.0	RI, MS, Co-GC
(-)-Camphene	953	1076	---	---	---	0.3±0.0	---	RI, MS, Co-GC
Sabinene	973	1132	T	1.0±0.1	T	1.1±0.1	0.5±0.0	RI, MS, Co-GC
Hepten-3-one	975		---	---	---	T	0.2±0.1	RI, MS
β-Pinene	978	1118	---	7.4±0.4	0.5±0.1	3.8±0.9	T	RI, MS, Co-GC
cis-Pinane	980		---	0.1±0.0	---	---	---	RI, MS
Verbenene	982		---	T	T	T	---	RI, MS
Myrcene	993	1174	---	0.7±0.1	0.2±0.1	0.7±0.3	---	RI, MS, Co-GC
α-Phellandrene	995	1176	0.1±0.0	T	0.3±0.0	0.2±0.0	---	RI, MS, Co-GC
Δ3-Carene	997	1153	0.1±0.0	---	0.3±0.1	0.3±0.0	---	RI, MS, Co-GC
α-Terpinene	1012	1188	---	T	T	0.1±0.0	T	RI, MS, Co-GC
o-Cymene	1020	1187	0.1±0.0	0.2±0.0	0.7±0.1	2.6±0.9	0.1±0.0	RI, MS, Co-GC
p-Cymene	1024	1280	---	0.1±0.1	0.3±0.0	0.4±0.1	---	RI, MS, Co-GC
β- Phellandrene	1029	1218	T	0.6±0.2	0.4±0.1	9.1±0.5	0.7±0.2	RI, MS, Co-GC
Limonene	1030	1203	---	14.3±0.5	1.5±0.5	6.4±0.5	2.3±0.9	RI, MS, Co-GC
1,8-Cineole	1034	1213	---	0.1±0.0	T	33.5±0.3	0.4±0.1	RI, MS
(*Z*)-β-Ocimene	1038	1246	T	0.1±0.0	T	0.1±0.0	T	RI, MS, Co-GC
(*E*)-β-Ocimene	1049	1280	---	0.3±0.1	T	0.2±0.1	0.3±0.1	RI, MS, Co-GC
γ-Terpinene	1057	1255	T	T	0.1±0.0	0.8±0.3	0.1±0.0	RI, MS, Co-GC
Fenchone	1067	1392	0.2±0.0	---	14.2±0.4	---	---	RI, MS
Terpinolene	1086	1265	T	T	T	0.2±0.1	T	RI, MS
Linalol	1097	1553	0.4±0.1	0.5±0.1	T	9.8±0.7	0.1±0.0	RI, MS, Co-GC
trans-Thujone	1115	1449	---	0.1±0.0	T	T	---	RI, MS, Co-GC
trans-Pinocarveol	1138	1654	---	T	T	0.1±0.0	T	RI, MS
iso-Borneol	1144	1633	---	---	---	0.1±0.0	---	RI, MS, Co-GC
Camphor	1145	1532	---	T	T	0.2±0.0	---	RI, MS, Co-GC
iso- Pinocamphone	1153	1566	---	T	T	0.2±0.0	0.2±0.0	RI, MS
trans-Pinocamphone	1159		---	4.3±0.9	---	T	T	RI, MS
Pinocarvone	1165	1587	---	---	---	T	T	RI, MS
Borneol	1167	1719	---	---	---	2.0±0.5	0.1±0.0	RI, MS, Co-GC
Terpinen-4-ol	1176	1611	---	T	T	0.4±0.1	0.2±0.0	RI, MS, Co-GC
dihydro-Carveol	1177		---	---	0.3±0.1	0.8±0.1	---	RI, MS
p-Cymen-8-ol	1185	1864	---	---	T	0.1±0.0	T	RI, MS
α-Terpineol	1189	1706	T	T	---	0.7±0.1	0.3±0.1	RI, MS, Co-GC
Myrtenal	1193	1648	---	0.1±0.0	0.1±0.0	0.7±0.1	---	RI, MS
Estragole	1195	1670	---	65.0±0.9	0.8±0.1	0.1±0.0	---	RI, MS, Co-GC
Myrtenol	1196	1804	---	---	---	0.2±0.1	---	RI, MS
Isobornyl formate	1228		---	---	---	---	45.4±0.9	RI, MS
Linalyl acetate	1248	1565	---	---	---	3.3±0.6	---	RI, MS, Co-GC
Geraniol	1255	1857	---	---	---	0.6±0.1	---	RI, MS
cis-Anethole	1262		97.1±0.4	T	76.3±0.9	---	0.2±0.0	RI, MS
Bornyl acetate	1264	1591	---	0.1±0.0	---	1.2±0.5	T	RI, MS
(*E*)-Citral	1270		---	---	---	---	44.5±0.9	RI, MS, Co-GC
Isobornyl acetate	1277		---	0.1±0.0	---	0.6±0.1	T	RI, MS
Thymol	1290	2198	---	---	---	0.7±0.1	---	RI, MS, Co-GC
Carvacrol	1297	2239	---	---	T	4.1±0.90	---	RI, MS, Co-GC
Myrtenyl acetate	1313		---	T	---	T	---	RI, MS
Terpinyl acetate	1333		---	---	---	0.5±0.0	---	RI, MS
Methyl Eugenol	1369	2023	---	0.6±0.1	T	---	T	RI, MS
α-Copaene	1377	1497	---	T	T	0.1±0.0	0.2±0.1	RI, MS
Isoledene	1382		---	T	T	T	0.1±0.0	RI, MS
β-Elemene	1387	1600	---	0.2±0.0	T	T	0.2±0.1	RI, MS
Longifolene	1411	1576	---	---	T	0.1±0.0	T	RI, MS
β-Caryophyllene	1418	1612	T	0.1±0.0	T	0.3±0.1	0.1±0.1	RI, MS
β-Cedrene	1424	1638	---	---	---	0.5±0.1	0.4±0.1	RI, MS
Aromadendrene	1437	1628	T	0.2±0.0	T	T	---	RI, MS
α-Humulene	1455	1689	---	T	T	0.3±0.1	0.2±0.0	RI, MS
allo-Aromadendrene	1463	1661	---	T	T	T	0.1±0.0	RI, MS
γ-Gurjunene	1473	1687	---	---	T	0.1±0.0	T	RI, MS
cis-*β*-Guaiene	1490	1694	---	0.4±0.2	---	---	---	RI, MS
Bicyclogermacrene	1491	1756	---	T	---	0.1±0.0	0.1±0.0	RI, MS
cis-Muurola-4(14),5-diene	1510	1675	---	0.1±0.0	T	0.1±0.0	0.2±0.1	RI, MS
α-7-*epi*-Selinene	1518	1740	---	T	T	0.1±0.0	0.2±0.1	RI, MS
α-Cadinol	1652	2255	---	0.6±0.1	---	---	---	RI, MS
Total compounds			**98.3**	**98**	**97.8**	**97.0**	**97.6**	
Monoterpenes hydrocarbons			0.6	25.5	6.1	35.4	4.2	
Oxigenated monoterpenes			0.6	70.9	15.4	59.9	91.2	
Total Monoterpenes			1.2	96.4	21.5	95.3	95.4	
Sesquiterpenes hydrocarbons			0	1	0	1.7	1.8	
Oxigenated sesquiterpenes			0	0.6	0	0	0	
Total Sesquiterpenes			0	1.6	0	1.7	1.8	
Non terpenes			97.1	0	76.3	0	0.4	
Oxigenated compounds			0.6	71.5	15.4	59.9	91.2	

[a] = Ki = Retention Index on a HP-5 column, [b] = Ki = Retention Index on a HP Innowax column, [c] = --- = absent, T = traces, less than 0.05%, [d] = RI= Retention index identical to bibliography, MS = identification based on comparison of mass spectra, Co-GC = retention time identical to authentic compound.

The dominant components in *C. carvi* oil were estragole (65.0%), limonene (14.3%), β-pinene (7.4%) and *trans*-pinocamphone (4.3%). In the Labiatae family, marjoram essential oil was mainly constituted by 1,8-cineole (33.5%), α-pinene (9.0%) and limonene (6.4%). The vervain (Verbenaceae) essential oil was mainly represented by citral and isobornyl formate, in approximately equal proportions.

Monoterpenes were the most abundant components of the oils analysed, representing a percentage ranging between 95.4%, in vervain oil, and 96.4% in caraway oil. They were constituted mainly of oxygenated monoterpenes, present in amounts ranging between 59.9% (marjoram oil) and 91.2% (vervain). On the other hand, the oils of anise and fennel were mainly constituted of non terpenes ranging between 97.1%, in the anise oil, and 76.3%, in fennel. Our data on anise oil composition agrees with the available literature. Tabanca *et al.* [10] reported that anise oil was constituted predominantly of *E*-anethole (94.2%). Fennel oil contains mainly anethole [11], and limonene and carvone have been reported [12] as the main components of caraway oil; our study confirmed limonene as one of the most abundant components of this oil. However, for marjoram oil, our results disagree with those reported [13], in which terpinene-4-ol, *trans*-sabinene hydrate, and *cis*-sabinene hydrate acetate were the main components with limonene only a minor component. A previous study reported a different composition for vervain oil: Ardakani *et al.* [14] identified 3-hexen-1-ol, 1-octen-3-ol, linalool, verbenone and geranial as its major components.

Anti-radical scavenging activity was tested by the DPPH model system and expressed as absolute percentage of DPPH inhibition (I_{DPPH}, Table 2 and Figure 1, respectively) [15].

All the essential oils showed antioxidant activity, with marjoram and caraway exhibiting the highest activity, with values for I_{DPPH} of 84.9% and 54%, respectively. Conversely, the essential oil of anise (in which the percentage of monoterpenes was as low as 1.2%) was the least effective antioxidant (I_{DPPH}= 19%). Vervain, although containing a very high percentage of monoterpenes, exhibited an intermediate level of antioxidant activity, similar to that of fennel essential oil (I_{DPPH}= 32.3%). This latter containing 21.5% of monoterpenes, showed almost double the radical scavenging potency to anise. Vervain oil showed the same antioxidant activity as fennel oil.

Table 2: The antioxidant activity, expressed as absolute percentage of DPPH inhibition, of *Pimpinella anisum* (anise), *Carum carvi* (caraway), *Foeniculum vulgare* (fennel), *Majorana hortensis* (marjoram), and *Verbena officinalis* (vervain).

	6 min	30 min	60 min
Anise	3.7±0.6	13.0±1.7	19.0±1.8
Caraway	10.6±0.9	34.9±1.8	54.0±2.5
Fennel	7.2±1.1	23.4±1.1	32.3±1.8
Marjoram	46.4±6.3	76.1±4.7	84.9±5.2
Vervain	8.0±1.0	21.0±1.4	32.7±2.2
Control	1.2±1.2	2.4±2.1	2.8±2.5

Our results are in agreement with a previous study [16], which demonstrated for 98 pure essential oils, strong correlation between the chemical composition and antioxidant activity. The authors indicated that antioxidant activity seems directly related to the presence of monoterpenes. In our samples of marjoram and caraway, such compounds reached percentages of 95.3% and 96.4%, respectively. The appreciable antioxidant activity found in the marjoram oil is probably ascribable to carvacrol, a well known antioxidant component [17] with positive synergism with other components. The radical scavenging activity of caraway oil agrees with the literature [18] and it is possible that the strong antioxidant activity is due to estragol (a major component at 65.0%).

The essential oils and their main constituents were tested also for their antimicrobial activity against some food-borne pathogenic bacterial strains, both Gram-positive and -negative. In addition, they were tested against different useful Lactobacilli strains. The antimicrobial activity of the essential oils is reported in Table 3.

The oils appeared more effective against the Gram-positive bacteria (both *B. cereus* strains, *Ent. faecalis* and *S. aureus*) than against the Gram-negative *Ps. aeruginosa* and *E. coli* strains. The most sensitive microorganisms were the two *B. cereus* strains and *Ent. faecalis* and, to a lesser extent, *P. aeruginosa*. On the other hand, *S. aureus* and, in particular, *E. coli* were the least sensitive ones. Among the essential oils, vervain exhibited the strongest antimicrobial activity against almost all the strains tested, in particular against *B. cereus* 4384 and *P. aeruginosa* (with inhibition zones of 18.7 and 15.3 mm, respectively). A strong activity was also exhibited against *Ent. faecalis*, where a zone of about 10 mm was observed in the presence of a 445 µg/paper disc of the essential oil. Caraway oil displayed, at the highest concentration assessed, an antibacterial

Table 3: Inhibition of bacterial growth provoked by essential oils of *Pimpinella anisum* (anise), *Carum carvi* (caraway), *Foeniculum vulgare* (fennel), *Majorana hortensis* (marjoram), and *Verbena officinalis* (vervain). Data are expressed in mm and do not include the diameter of paper disc. Results are shown as mean±standard deviation (SD) of the inhibition zone (n=3).

Essential oil	*Bacillus cereus* 4313 IZ(±SD)	*Bacillus cereus* 4384 IZ(±SD)	*Pseudomonas aeruginosa* IZ(±SD)	*Escherichia coli* IZ(±SD)	*Enterococcus faecalis* IZ(±SD)	*Staphylococcus aureus* IZ(±SD)
Anise 98µg	0	0	0	0	0	0
Anise 196µg	0	0	0	0	0	0
Anise 490µg	5.7(±0.3)	6.0(±0.0)	0	0	6.8(±0.8)	0
Caraway 91µg	0	0	0	0	0	0
Caraway 182µg	5.5(±0.0)	8.8(±0.3)	7.5(±0.9)	0	8.7(±1.1)	0
Caraway 455µg	6.7(±0.6)	9.8(±0.3)	9.3(±1.1)	0	11.7(±2.9)	7.8(±0.3)
Fennel 96 µg	0	0	0	0	0	0
Fennel 193 µg	5.7(±0.3)	0	0	0	0	0
Fennel 482 µg	6.5(±0.7)	5.7(±0.3)	0	0	0	0
Marjoram 90µg	0	0	0	0	0	0
Marjoram 180µg	0	0	7.0(±0.0)	0	6.8(±0.3)	0
Marjoram 450µg	6.0(±0.0)	6.3(±0.1)	0	0	9.5(±0.9)	7.0(0.0)
Vervain 89µg	0	9.7(±0.6)	6.7(±0.6)	0	0	0
Vervain 178µg	0	12.0(±2.6)	10.3(±1.1)	0	7.3(±0.6)	7.3(±0.6)
Vervain 445µg	7.0(±0.0)	18.7(±1.5)	15.3(±1.5)	0	10.3(±0.6)	8.7(±1.1)
Gentamycin 8 µg	22.7(±1.1)	20.7(±1.1)	20.3(±0.6)	20.7(±1.1)	24.7(±0.6)	10.7(±1.1)
Chloramphenicol 66 µg	16.3(±0.6)	18.7(±0.6)	11.7(±0.6)	15.3(±0.6)	26.3(±1.1)	13.3(±2.9)
Tetracycline 7 µg	15.3(±0.6)	13.3(±0.6)	14.7(±0.6)	17.7(±1.1)	18.7(±1.1)	9.3(±0.6)

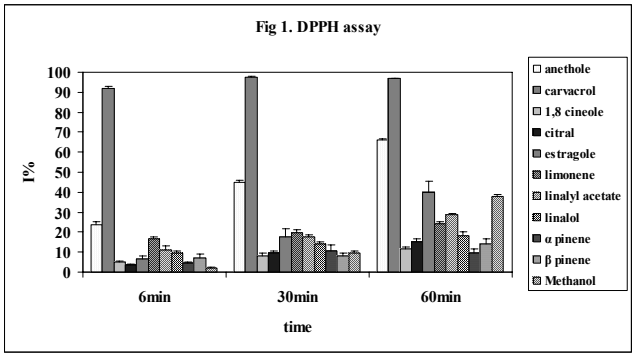


Figure 1: The antioxidant activity (DPPH assay) of main components of essential oils.

activity against almost all the strains tested, in particular *Ent. faecalis* (inhibition zone 11.7 mm), *B. cereus* 4384 and *P. aeruginosa* (inhibition zones 9.8 and 9.3 mm, respectively). An intermediate level of antimicrobial activity was reported for the marjoram essential oil, which displayed antimicrobial activity against almost all the pathogen strains, although only at the highest concentration (450 µg/paper disc). This oil appeared particularly effective against *Ent. faecalis*, with an inhibition zone of about 9.5 mm. Fennel essential oil only seemed to be selectively effective against two strains of *B. cereus* at the highest concentration tested (482 µg/paper disc).

Table 4 summarizes the antimicrobial activity of the individual oil components. Carvacrol had the widest spectrum of activity, followed by citral, linalool, estragole, limonene and linalyl acetate. Anethole, β-pinene and α-pinene were the least effective. In our experiment, carvacrol exhibited the strongest antimicrobial activity, with inhibition zones ranging from 7.3 mm (at 97.6 µg/paper disc, *versus B. cereus* 4313) to 29.7 mm (at 488 µg/paper disc, *versus E. coli*). Estragole displayed an intermediate antimicrobial activity, mainly against *S. aureus* (12.3 mm at a dose of 473 µg/paper disc). Linalyl acetate showed a weak activity only against *B. cereus* 4313 and 4384, and *Ent. faecalis*.

The essential oils appear not to inhibit significantly the Lactobacilli growth (data not reported). However, in contrast, the isolated components, with the exception of 1,8-cineole, citral, and α-pinene, were found to possess effective antimicrobial activity (Table 5) both against starters (*L. sakei*, *L. casei*) and pro-biotic microorganisms (*L. rhamnosus, L. bulgaricus* and *L. acidophilus*). Our results confirm the antimicrobial performance exhibited by vervain oil. The loss of activity exhibited by caraway oil against *E. coli* disagrees with other studies, in which a good antimicrobial action was reported [19]. The divergent results might be due to a different chemical composition of the oil, as reported by Suppakul *et al.* [20].

Hammer *et al.* [19] demonstrated, for fennel oil, an activity, at concentrations above 1%, only against *P. aeruginosa*, while *E. coli* and *S. aureus* were more sensitive. A weak activity was also observed for anise

Table 4: Inhibition of bacterial growth provoked by main components of essential oils. Data are expressed in mm and do not include the diameter of paper disc. Results are shown as mean±standard deviation (SD) of the inhibition zone (n=3).

	***Bacillus cereus* 4313**	***Bacillus cereus* 4384**	***Pseudomonas aeruginosa***	***Escherichia coli***	***Enterococcus faecalis***	***Staphylococcus aureus***
	IZ(±SD)	**IZ(±SD)**	**IZ(±SD)**	**IZ(±SD)**	**IZ(±SD)**	**IZ(±SD)**
anethole 99.8 µg	6.7(±1.1)	0	0	0	0	9.3(±0.6)
anethole 199.6 µg	7.3(±0.6)	0	0	0	0	9.0(±0.0)
anethole 499 µg	6.7(±0.6)	0	5.3(±0.6)	0	0	10.7(±1.1)
carvacrol 97.6 µg	7.3(±0.6)	7.7(±1.1)	8.3(±1.1)	8.0(±1.7)	12.0(±2.0)	20.0(±0.0)
carvacrol 195.2 µg	13.0(±1.7)	15.7(±2.9)	9.3(±0.6)	12.3(±0.6)	17.0(±1.7)	20.7(±1.1)
carvacrol 488 µg	21.7(±2.9)	23.0(±3.6)	16.7(±2.1)	29.7(±0.6)	21.7(±2.9)	23.7(±1.5)
citral 88µg	6.8(±3.2)	9.3(±0.6)	6.7(±0.6)	0	6.7(±3.2)	6.0(±0.0)
citral 176µg	6.0(±0.0)	11.3(±3.2)	12.0(±1.7)	0	6.7(±0.6)	6.3(±0.6)
citral 440µg	10.7(±1.1)	15.7(±4.0)	22.3(±4.6)	9.7(±0.6)	9.7(±0.6)	9.7(±0.6)
1,8-cineole 92.2 µg	0	6.7(±1.1)	0	0	6.3(±0.6)	8.7(±0.6)
1,8-cineole 184.4 µg	6.3(±0.6)	7.3(±1.1)	0	0	6.0(±0.0)	10.3(±0.6)
1,8-cineole 461 µg	7.0(±0.0)	7.3(±2.3)	0	0	8.3(±0.6)	11.0(±1.7)
estragole 94.6 µg	7.0(±0.0)	6.7(±1.1)	0	6.3(±1.1)	6.7(±0.6)	10.3(±0.6)
estragole 189.2 µg	7.0(±0.0)	6.7(±0.6)	7.0(±0.0)	6.7(±1.5)	5.3(±0.6)	11.3(±0.6)
estragole 473 µg	6.3(±0.6)	6.3(±0.6)	6.7(±0.6)	6.3(±1.1)	7.3(±1.5)	12.3(±0.6)
limonene 84 µg	0	0	0	0	0	9.7(±0.6)
limonene 168 µg	0	6.7(±1.1)	6.3(±1.1)	0	0	11.7(±1.5)
limonene 420 µg	6.3(±0.6)	6.7(±0.5)	6.7(±2.5)	0	6.7(±0.6)	12.0(±2.6)
linalyl acetate 89.5 µg	2.0(±0.0)	1.3(±2.3)	0	0	2.3(±0.6)	0
linalyl acetate 179 µg	3.0(±0.0)	1.3(±2.3)	0	0	2.7(±0.6)	0
linalyl acetate 447.5 µg	3.0(±0.0)	4.3(±0.6)	0	0	3.7(±0.6)	0
linalol 85.8 µg	4.0(±0.0)	2.0(±0.0)	2.3(±0.6)	2.0(±0.0)	4.3(±0.6)	9.3(±0.6)
linalol 171.6 µg	6.0(±1.0)	3.3(±0.6)	3.7(±1.5)	4.0(±0.0)	6.3(±0.6)	10.0(±0.0)
linalol 429 µg	9.3(±0.6)	4.7(±0.6)	8.7(±0.6)	5.7(±1.1)	9.3(±0.6)	14.7(±0.6)
α pinene 86 µg	0	0	0	0	0	8.7(±0.6)
α pinene 172 µg	0	0	0	0	0	10.7(±1.1)
α pinene 430 µg	0	0	0	0	0	13.0(±1.7)
β pinene 86 µg	0	0.7(±1.1)	0	0	0	11.0(±1.7)
β pinene 172 µg	0.7(±1.1)	1.3(±1.1)	0	0	0	12.7(±2.5)
β pinene 430 µg	1.3(±1.1)	2.0(±1.7)	5.7(±0.6)	0	5.7(±0.6)	13.0(±1.7)
Gentamycin 8 µg	22.7(±1.1)	20.7(±1.1)	20.3(±0.6)	20.7(±1.1)	24.7(±0.6)	10.7(±1.1)
Chloramphenicol 66 µg	16.3(±0.6)	18.7(±0.6)	11.7(±0.6)	15.3(±0.6)	26.3(±1.1)	13.3(±2.9)
Tetracycline 7 µg	15.3(±0.6)	13.3(±0.6)	14.7(±0.6)	17.7(±1.1)	18.7(±1.1)	9.3(±0.6)

essential oil, with inhibition zones not exceeding 6.8 mm (against *Ent. faecalis*), in agreement with Hammer *et al*. [19].

Phenols, like carvacrol, are well-known active substances, acting both against Gram-negative and Gram-positive microorganisms. The phenolic hydroxyl group of carvacrol seems essential also for the antimicrobial activity against the food-borne pathogen *B. cereus*, and slightly less against the other pathogens tested. In all cases, as demonstrated for *B. cereus*, it could cause the destabilization of the membrane and a depletion of the microbial ATP pools that lead to impairment of essential processes and finally to cell death [21]. The activity of carvacrol against *B. cereus* could let us hypothesise its use as a natural food preservative against this strain, which is strictly linked to food-borne illnesses and which contaminates several food products.

The strong antimicrobial activity exhibited by citral agrees with literature data [22]. However, its antimicrobial effects on lactic acid bacteria could prove problematic when they are required for a fermentative process.

Estragole was the main component in caraway oil. This showed lower activity against *S. aureus* and a stronger effect against *Ent. faecalis*.

The results, presented in Table 6, show that the tested essential oils exhibited variable degrees of antifungal activity. Marjoram and caraway oils were active against all fungal strains, with inhibition zones ranging from 9.3 mm (exhibited by caraway essential oil against *P. citrinum*) to 13.7 mm (marjoram essential oil against *D. hansenii*), at the highest concentration used in our experiments.

Table 5: Inhibition of bacterial lactic growth provoked by main components of essential oils (Data are expressed in mm and do not include the diameter of paper disc. Results are shown as mean±standard deviation (SD) of the inhibition zone (n=3).

	L. sakei IZ(±SD)	*L. rhamnosus* IZ(±SD)	*L. casei* IZ(±SD)	*L. bulgaricus* IZ(±SD)	*L. acidophilus* IZ(±SD)
anethole 99.8 µg	0	2.0(±0.0)	7.3(±0.6)	7.7(±0.6)	4.0(±0.0)
anethole 199.6 µg	1.3(±2.3)	3.7(±0.6)	11.3(±0.6)	15.3(±0.6)	6.3(±0.6)
anethole 499 µg	10.0(±0.0)	5.0(±0.0)	14.3(±2.1)	19.7(±0.6)	10.3(±0.6)
carvacrol 97.6 µg	10.0(±0.0)	9.0(±1.0)	7.3(±0.6)	8.7(±1.1)	6.3(±0.6)
carvacrol 195.2 µg	13.3(±1.1)	11.0(±0.0)	12.7(±0.6)	10.7(±1.1)	9.7(±0.6)
carvacrol 488 µg	20.3(±1.5)	15.0(±1.0)	17.3(±0.6)	17.3(±1.1)	14.0(±1.7)
citral 88 µg	0	0	0	0	0
citral 176µg	0	0	0	0	0
citral 440µg	0	0	0	0	0
1,8-cineole 92.2 µg	0	0	0	0	5.3(±0.6)
1,8-cineole 184.4 µg	0	0	10.0(±0.0)	6.7(±0.6)	5.7(±1.1)
1,8-cineole 461 µg	0	0	12.3(±0.6)	11.3(±1.1)	10.0(±0.0)
estragole 94.6 µg	6.3(±0.6)	4.3(±0.6)	8.7(±1.1)	7.7(±0.6)	5.0(±0.0)
estragole 189.2 µg	8.7(±1.1)	5.0(±0.0)	13.3(±2.9)	9.7(±0.6)	5.7(±0.6)
estragole 473 µg	10.0(±0.0)	9.3(±1.1)	14.0(±1.7)	14.7(±0.6)	10.3(±0.6)
limonene 84 µg	0	2.0(±0.0)	7.3(±2.1)	4.3(±3.8)	3.7(±0.6)
limonene 168 µg	0	4.0(±0.0)	11.3(±1.1)	9.7(±0.6)	5.0(±0.0)
limonene 420 µg	1.3(±2.3)	5.3(±2.1)	13.7(±1.5)	14.7(±0.6)	6.7(±0.6)
linalyl acetate 89.5 µg	0	2.8(±1.0)	5.3(±0.6)	0	4.7(±0.6)
linalyl acetate 179 µg	0	3.7(±0.6)	8.7(±1.1)	4.3(±3.8)	5.0(±0.0)
linalyl acetate 447.5 µg	1.3(±2.3)	5.7(±0.6)	12.3(±0.6)	13.3(±2.1)	8.0(±0.0)
linalol 85.8 µg	0	8.7(±0.6)	8.3(±1.5)	10.0(±0.0)	22.0(±0.0)
linalol 171.6 µg	0	10.7(±1.1)	10.3(±0.6)	14.3(±0.6)	22.0(±0.0)
linalol 429 µg	10.0(±0.0)	13.7(±1.5)	13.3(±1.5)	18.7(±1.1)	22.0(±0.0)
α pinene 86 µg	0	0	6.3(±2.1)	0	0
α pinene 172 µg	0	0	9.3(±0.6)	0	0
α pinene 430 µg	0	0	13.7(±1.5)	8.7(±1.1)	0
β pinene 86 µg	0	2.3(±0.6)	9.7(±0.6)	0	4.0(±0.0)
β pinene 172 µg	0	3.3(±0.6)	13.7(±1.5)	0	6.7(±0.6)
β pinene 430 µg	0	4.0(±0.0)	19.3(±1.1)	10.7(±0.6)	9.7(±0.6)

Table 6: Inhibition of fungal growth provoked by essential oils of *Pimpinella anisum* (anise), *Carum carvi* (caraway), *Foeniculum vulgare* (fennel), *Majorana hortensis* (marjoram), and *Verbena officinalis* (vervain). Data are expressed in mm and do not include the diameter of paper disc. Results are shown as mean±standard deviation (SD) of the inhibition zone (n=3).

	Penicillium simplicissimum IZ(±SD)	*Aureobasidium pullulans* IZ(±SD)	*Penicillium citrinum* IZ(±SD)	*Penicillium expansum* IZ(±SD)	*Debaryomyces hansenii* IZ(±SD)	*Penicillium aurantiogriseum* IZ(±SD)
Anise 98µg	5.0(±0.0)	2.7(±2.3)	2.3(±2.1)	0	0	0
Anise 196µg	6.0(±1.0)	6.3(±0.6)	4.7(±0.6)	0	0	1.7(±2.9)
Anise 490µg	9.7(±0.6)	7.0(±0.0)	6.7(±0.6)	0	0	6.3(±0.6)
Caraway 91µg	4.7(±0.6)	4.7(±4.0)	0	5.0(±0.0)	0	2.3(±4.0)
Caraway 182µg	7.3(±1.4)	7.0(±0.0)	7.0(±0.0)	7.0(±0.0)	10.0(±1.0)	5.7(±1.1)
Caraway 455µg	10.8(±1.0)	10.3(±1.5)	9.3(±0.6)	10.3(±1.5)	11.0(±1.7)	10.0(±0.0)
Fennel 96 µg	3.7(±0.6)	2.7(±2.3)	0	0	0	0
Fennel 193 µg	4.0(±0.0)	3.3(±2.9)	0	0	0	1.3(±2.3)
Fennel 482 µg	5.7(±0.6)	5.3(±0.6)	4.0(±3.5)	0	1.7(±2.9)	6.0(±0.0)
Marjoram 90µg	7.3(±2.1)	7.7(±1.1)	6.7(±2.9)	6.3(±0.6)	9.0(±1.0)	7.2(±0.3)
Marjoram 180µg	9.8(±0.3)	8.7(±1.5)	9.3(±2.1)	8.5(±1.3)	9.0(±1.7)	10.0(±2.0)
Marjoram 450µg	11.3(±1.1)	12.7(±1.1)	11.0(±1.0)	11.7(±2.9)	13.7(±2.3)	11.7(±2.9)
Vervain 89µg	5.3(±0.6)	3.7(±3.2)	3.7(±0.6)	0	5.2(±1.3)	0
Vervain 178µg	6.7(±0.6)	9.0(±1.7)	6.3(±0.6)	7.0(±0.0)	8.7(±1.5)	0
Vervain 445µg	11.3(±1.1)	15.0(±0.0)	11.8(±0.3)	14.0(±1.7)	12.7(±0.6)	0

Fennel and vervain essential oils exhibited different activity against the fungi tested; in particular, fennel showed a weaker activity (about 50%) than vervain and in addition, the two oils were ineffective against some strains. Fennel oil did not show activity against *P. expansum*, while vervain oil was ineffective against *P. aurantiogriseum*. On the other hand, vervain oil exhibited the highest activity against

Table 7: Inhibition of fungal growth provoked by main components of essential oils Data are expressed in mm and do not include the diameter of paper disc. Results are shown as mean±standard deviation (SD) of the inhibition zone (n=3).

	Penicillium simplicissimum **IZ(±SD)**	***Aureobasidium pullulans*** **IZ(±SD)**	***Penicillium citrinum*** **IZ(±SD)**	***Penicillium expansum*** **IZ(±SD)**	***Debaryomyces hansenii*** **IZ(±SD)**	***Penicillium aurantiogriseum*** **IZ(±SD)**
anethole 99.8 µg	4.8(±0.3)	4.8(±0.3)	4.3 (± 0.6)	0	0	0
anethole 199.6 µg	8.2(±1.0)	5.8(±0.3)	5.0(±0.0)	0	0	0
anethole 499 µg	9.3(±0.6)	10.0(±0.0)	8.3(±1.5)	0	7.3(±1.5)	0
carvacrol 97.6 µg	no growth	10.3(±0.6)	no growth	no growth	7.7(±0.6)	5.7(±0.6)
carvacrol 195.2 µg	no growth	14.3 (±0.6)	no growth	no growth	12.0(±0.0)	9.7(±0.6)
carvacrol 488 µg	no growth	16.3(±3.2)	no growth	no growth	15.7(±1.1)	15.0(±0.0)
citral 88µg	8.7(±0.6)	5.2(±1.26)	6.8(±2.0)	5.8(±0.3)	0	5.7(±0.6)
citral 176µg	10.0(±3.6)	10.0(±0.0)	8.8(±1.1)	8.3(±1.5)	8.3(±2.9)	8.3(±1.1)
citral 440µg	12.7(±3.06)	14.0(±1.7)	12.0(±1.3)	13.0(±1.7)	11.7(±2.9)	13.3(±1.1)
1,8-cineole 92.2 µg	4.0(±0.0)	7.7(±0.6)	5.2(±0.3)	0	0	4.7(±0.6)
1,8-cineole 184.4 µg	4.7(±0.6)	7.3(±1.5)	8.5(±1.3)	0	6.7(±1.1)	5.7(±1.1)
1,8-cineole 461 µg	10.0(±0.0)	14.3 (±0.6)	10.3(±0.6)	0	9.7(±0.6)	9.3(±0.6)
estragole 94.6 µg	5.0(±0.0)	5.0(±0.0)	5.0(±0.0)	9.7(±0.6)	4.3 (±0.6)	0
estragole 189.2 µg	8.5(±0.5)	10.7(±1.1)	7.7(±1.1)	10.3(±0.6)	6.3(±1.1)	0
estragole 473 µg	14.0(±1.7)	15.3 (±0.6)	15.7(±1.1)	12.3(±0.6)	12.7(±2.1)	0
limonene 84 µg	4.0(±0.0)	4.0(±0.0)	3.3(±0.6)	0	5.3(±1.1)	6.3(±2.3)
limonene 168 µg	6.8(±0.8)	6.3(±0.6)	4.0(±0.0)	0	8.0(±1.7)	9.5(±0.9)
limonene 420 µg	8.7(±0.4)	8.0(±1.7)	4.3(±0.6)	0	10.7(±1.1)	13.3(±2.9)
linalyl acetate 89.5 µg	4.7(±0.6)	4.7(±0.6)	2.0(±0.0)	0	3.3(±2.9)	2.3 (± 4.0)
linalyl acetate 179 µg	8.3(±1.1)	9.8(±1.3)	2.0(±0.0)	0	7.3(±0.6)	4.7 (± 4.0)
linalyl acetate 447.5 µg	11.0(±1.0)	12.7(±2.1)	2.0(±0.0)	0	11.3(±1.1)	4.7 (± 4.5)
linalol 85.8 µg	8.7(±3.2)	9.7(±2.5)	6.3(±0.6)	0	7.7(±0.6)	9.3(±0.6)
linalol 171.6 µg	12.3(±1.5)	13.7(±1.5)	7.3(±2.1)	10.7(±1.1)	10.7(±1.1)	15.7(±3.2)
linalol 429 µg	14.3(±2.1)	16.7(±2.9)	13.3(±1.5)	11.7(±2.9)	16.3(±1.1)	18.7(±1.5)
α pinene 86 µg	0	3.3(±0.6)	0	0	0	0
α pinene 172 µg	0	4.3(±0.6)	0	0	0	0
α pinene 430 µg	0	5.7(±1.1)	0	0	8.7(±2.3)	0
β pinene 86 µg	4.3(±0.6)	5.3(±0.6)	0	0	3.7(±0.6)	5.3(±0.6)
β pinene 172 µg	5.7(±0.6)	8.3(±1.1)	0	0	7.3(±2.3)	8.3(±1.1)
β pinene 430 µg	8.8(±0.8)	13.3(±1.5)	3.3(±2.9)	0	9.7(±0.6)	13.3(±1.5)

A. pullulans (inhibition zone of 15 mm). The growth of *P. citrinum* was appreciably reduced by the essential oils tested, with inhibition zones ranging from 4.0 mm (fennel oil), to about 12 mm (vervain oil).

Table 7 shows the antifungal activity of the components. The compounds with the strongest spectrum of activity appeared to be citral and linalool, which were effective against all fungi assayed. 1,8-Cineole, estragole, limonene and linalyl acetate acted against almost all the microorganisms. The weakest activity was exhibited by α-pinene, the best result for which was recorded against *D. hansenii* (8.7 mm inhibition zone). This compound showed activity against all the fungi assayed, producing inhibition zones always above 11 mm. Linalyl acetate, present only in the marjoram essential oil, was more active on fungi than on bacteria. However, its action was less effective than the marjoram essential oil against *P. citrinum* and *P.aurantiogriseum*. *cis*-Anethole, the main component of anise and fennel essential oils, was differently effective against the strains used in the test. It displayed an antifungal effect against *P. simplicissimum* and *P. citrinum*, as well as against *A. pullulans* and, at the highest concentration used, against *D. hansenii*. The different percent composition of anise and fennel oils, in which anethole represents 97.1% and 76.3% of the total oil, respectively could help to explain the different biological activity. 1,8-Cineole was effective against all fungal strains, except *P. expansum*. The maximum activity of anethole was recorded against *A. pullulans* (inhibition zone 10 mm). Carvacrol was the most active compound tested. It was highly effective against *A. pullulans* and *D. hansenii*, and, tested at the same concentration used in the antimicrobial assay, it did not permit any growth of almost all *Penicillium* strains tested, in particular against *P. expansum*, the agent of the blue mould which causes one of the principal postharvest diseases in agriculture, and against *P. citrinum*. The genus *Penicillium* is an

important contaminant of foods and agricultural commodities. Many *Penicillium* species are also known producers of a number of very dangerous mycotoxins. *Aureobasidium pullulans* is a saprophyte species distributed widely throughout the environment. Clinically, it has been reported to cause a variety of localized infections, including peritonitis, cutaneous infection, pneumonia, meningitis, corneal and scleral infection, as well as abscesses in the spleen and jaw. *Debaryomyces hansenii* is a hemiascomycetous yeast, often associated with the food and drink processing industries. This strain can be commonly found in freshwater and seawater or as a parasitic, opportunistic organism in humans, fish and vegetable matter [23]. The antifungal activity of caraway oil has also been reported in previous studies, particularly against several *Aspergillus* strains [24]. The activity exhibited by the essential oils against *P. citrinum* is notable due to the well known capability of this fungus to produce the toxic metabolite citrinin, a hepatonephrotoxic mycotoxin involved in different diseases in animals and human [25]. Generally, essential oils can exert their toxic effect against fungi through the disruption of the fungal membrane integrity [26], and, thereby, inhibit respiration and ion transport processes. Citral has been recently used as an ingredient for the production of edible films capable of improving shelf life and food quality by serving as selective barriers against different pathogenic bacteria [27]. The generally high antifungal activity exhibited by the essential oils could indicate, as for the antimicrobial activity, a synergistic interaction among their chemical components.

Data obtained clearly showed the inhibitory activity of the essential oils tested against pathogenic bacterial and fungal strains. On the other hand, these oils showed no inhibitory activity against lactic acid bacteria. These findings, considered together, suggest the future use of these essential oils as natural preservatives for food products, due to their positive effect on their safety and shelf life.

Experimental

Essential oils: Essential oils of *Pimpinella anisum* L., *Carum carvi* L., *Foeniculum vulgare* Miller, *Majorana hortensis* L., and *Verbena officinalis* L. were purchased from the Azienda Chimica E Farmaceutica (A.C.E.F.) Spa (Fiorenzuola d´Arda, Italy). The densities of the oils were: *P. anisum* (0.981g/mL), *C. carvi* (0.913 g/mL), *F. vulgare* (0.964 g/mL), *M. hortensis* (0.903 g/mL), and *V. officinalis* (0.889 g/mL). Anethole, carvacrol, citral, 1,8-cineole, estragole, limonene, linalyl acetate, linalol, α-pinene and β-pinene were purchased from Sigma Aldrich, Co (Milan, Italy). All samples were kept at -20°C until analysis.

Gas chromatography (GC): GC analyses were carried out using a Perkin-Elmer Sigma-115 gas chromatograph with a data handling system and a flame ionization detector (FID). Separation was achieved by a fused-silica capillary column HP-5 MS, 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. The operating conditions were as follows: injector and detector temperatures, 250°C and 280°C, respectively; oven temperature programme: 5 min isothermal at 40°C, subsequently at 2°C/min up to 250°C and finally raised to 270° at 10°C/min. Analysis was also run by using a fused silica HP Innowax polyethylene glycol capillary column (50 m x 0.20 mm i.d., 0.20 μm film thickness). In both cases, helium was used as the carrier gas (1 mL/min). Diluted samples (1/100 v/v, in *n*-hexane) of 1 μL were manually injected at 250°C, and in the splitless mode. The percentage composition of the oils was determined by normalization of the GC peak areas, calculated as mean values of 3 injections from each oil, without using correction factors.

Gas chromatography–mass spectrometry (GC-MS): GC–MS analysis was performed using an Agilent 6850 Ser. A apparatus, equipped with a fused silica HP-1 capillary column (30 m x 0.25 mm i.d.; film thickness 0.33 μm), linked on line with an Agilent Mass Selective Detector MSD 5973; ionization voltage 70 electrons, multiplier energy 2000 V. Gas chromatographic conditions were as given above, transfer line was kept at 295°C. The oil components were identified from their GC retention indices by comparison with either literature values [28] or with those of authentic compounds available in our laboratories. The identity of the components was assigned by comparing their retention indices, relative to C_8–C_{24} *n*-alkanes under the same operating conditions. Further identification was made by comparison of their MS on both columns with those stored in NIST 02 and Wiley 275 libraries, those from the literature [29], and from an 'in house' library.

Free-radical scavenging method: The free-radical scavenging activity of the essential oils and their main components was measured by using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) [30]. The analysis was performed in microplates, by

adding 7.5 µL of sample (previously diluted 1:1 in DMSO) to 303 µL of a methanol solution of DPPH (153 mM). Then, the absorbance was measured in a UV-Vis spectrophotometer (Varian Cary 50 MPR, USA). The absorbance of DPPH radical without antioxidant, i.e., the control, was measured as basis. All determinations were in triplicate. Inhibition of free radical by DPPH in percent (I%) was calculated in following way: I% $[(A_{blank} - A_{sample}/A_{blank})] \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound read at 517 nm until 60 min. Tests were carried out in triplicate.

Antimicrobial assay: The inhibition zone test on agar plates was employed to investigate the antimicrobial activity. Samples were tested against the following bacteria: non-pathogenic strains (*Lactobacillus acidophilus* DSM 20079; *L. casei* DSM 9595; *L. bulgaricus* DSM 20081; *L. sakei* DSM 20494; and *L. rhamnosus* DSM 20711); pathogenic Gram-positive strains *Bacillus cereus* (DSM 4313 and DSM 4384), *Staphylococcus aureus* DSM 25923 and *Enterococcus faecalis* DSM 2352; Gram-negative strains *Escherichia coli* DSM 8579 and *Pseudomonas aeruginosa* ATCC 50071. All strains were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ Germany). Each strain was incubated at 37°C for 18 h in its own specific growth medium. Lactic acid bacteria were grown in Man de Rogosa Sharpe (MRS) broth (Oxoid, UK), and *E. coli*, *Ent. faecalis*, *S. aureus*, *P. aeruginosa* and *B. cereus* in Nutrient Broth (Oxoid, UK). The microbial suspensions (1x 10^8 Colony Forming Units-CFU/mL) were uniformly spread onto the specific solid media plates (Ø=90 mm dishes). Sterile Whatman N° 1 paper filter discs (Ø=5 mm) were individually placed on the inoculated plates and impregnated with different doses of either essential oils or of their main compounds, previously diluted 1:10 (v/v) in dimethylsulfoxide (DMSO) (final amount ranging from 84 to 499 µg/paper disc). After 30 min under sterile conditions at room temperature, plates were incubated at 37°C for 24-48 h, depending on the strain. The diameter of the clear zone shown on plates was accurately measured and the antimicrobial activity expressed in mm (not including disc diameter of 5 mm). Sterile deionised water and pure DMSO (10 µL/paper disc) were used as negative control. Gentamycin (8 µg/paper disc), chloramphenicol (66 µg/paper disc) and tetracycline (7 µg/paper disc), in physiological solution, served as positive controls. Samples were tested in triplicate and results are expressed as mean ± standard deviation.

Antifungal activity: The inhibition zone test on agar plates was employed to investigate the antifungal activity of the essential oils and their main compounds. Six fungal strains of agro-food interest, *Penicillium citrinum* DSM 1997, *P. simplicissimum* DSM 1097, *Aureobasidium pullulans* DSM 62074, *P. expansum* DSM 1994, *P. aurantiogriseum* DSM 2429, and *Debaryomyces hansenii* DSM 70238 were used. All strains were purchased from DSMZ. Different amounts of essential oils and their components, previously diluted 1:10 (v/v) in DMSO (final doses ranging from 84 to 499 µg/paper disc), were used. A cell suspension of fungi was prepared in sterile distilled water and plated onto Potato Dextrose Agar (PDA) (Oxoid). Sterile Whatman N° 1 paper filter discs (Ø=5 mm) were individually placed on the inoculated plates and impregnated with different doses of either essential oils or of their main compounds, previously diluted 1:10 (v/v) in dimethylsulfoxide (DMSO) (final amount ranging from 84 to 499 µg/paper disc). After 20 min under sterile conditions at room temperature, plates were incubated at 28°C until the mycelium of fungi reached the edges of the control plate (negative control without the sample added extracts); the resulting clear zones of inhibition were measured in mm, expressing the antifungal activity. DMSO (10 µL) was used as negative control. Samples were tested in triplicate and the results are expressed as mean ± standard deviation.

References

[1] Di Pasqua R, De Feo V, Villani F, Mauriello G. (**2005**) *In vitro* antimicrobial activity of essential oils from Mediterranean Apiaceae, Verbenaceae and Lamiaceae against foodborne pathogens and spoilage bacteria. *Annals of Microbiology*, ***55***, 139-142.

[2] Madsen HL, Bertelsen G. (**1995**) Spices as antioxidants. *Trends in Food Science and Technology*, ***6***, 271–277.

[3] Evandri MG, Battinelli L, Daniele C, Mastrangelo S, Bolle P, Mazzanti G. (**2005**) The antimutagenic activity of *Lavandula angustifolia* (lavender) essential oil in the bacterial reverse mutation assay. *Food and Chemical Toxicology*, ***43***, 1381–1387.

[4] Ames BM. (**1983**) Dietary carcinogens and anticarcinogens: Oxygen radical and degenerative diseases. *Science*, ***221***, 1256–1263.

[5] Burt S. (**2004**) Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology*, ***94***, 223–253.

[6] Conner DE. (**1993**) Naturally occuring compounds. In *Antimicrobials in foods.* Davidson P, Branen AL (Eds) Marcel Dekker, New York, NY, 441–468.

[7] Demetzos C, Perdetzoglou DK. (**2001**) Composition and antimicrobial studies of the oils of *Origanum calcaratum* Juss. and *O. scabrum* Boiss. et Heldr. from Greece. *Journal of Essential Oil Research*, ***13***, 460–462.

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

[9] De Martino L, De Feo V, Nazzaro F. (**2008**) Chemical composition and biological activity of some Labiatae essential oils. In: *Proceedings of the 21st International ICFMH Symposium "Food Micro 2008"*, p 488.

[10] Tabanca N, Demirci B, Ozek T, Kirimer N, Can Baser KH, Bedir E, Khand IA, Wedge DE. (**2006**) Gas chromatographic–mass spectrometric analysis of essential oils from *Pimpinella* species gathered from Central and Northern Turkey. *Journal of Chromatography A*, ***1117***, 194-205.

[11] Singh G, Maurya S, de Lampasona MP, Catalan C. (**2006**) Chemical constituents, antifungal and antioxidative potential of *Foeniculum vulgare* volatile oil and its acetone extract. *Food Control*, ***17***, 745-752.

[12] Bailer J, Aichinger T, Hackl G, de Hueber K, Dachler M. (**2001**) Essential oil content and composition in commercially available dill cultivars in comparison to caraway. *Industrial Crops and Products*, ***14***, 229–239.

[13] Omidbaigi R, Bastan MR. (**2005**) Essential oil composition of marjoram cultivated in north of Iran. *Journal of Essential Oil-Bearing Plants*, ***8***, 56-60.

[14] Ardakani MS, Mosaddegh M, Shafaati A. (**2003**) Volatile constituents from the aerial parts of *Verbena officinalis* L. (vervain). *Iranian Journal of Pharmaceutical Research*, ***2***, 39-42.

[15] Gulçin I, Sat IG, Beydemir S, Elmastas M, Kufrevioglu OI. (**2004**) Comparison of antioxidant activity of clove (*Eugenia caryophylata* Thunb) buds and lavender (*Lavandula stoechas* L.). *Food Chemistry*, ***87***, 393–400.

[16] Ruberto G, Baratta MT. (**2000**) Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chemistry*, ***69***, 167-174.

[17] Aeschbach R, Loliger J, Scott BC, Murcia A, Butler J, Halliwell B, Aruoma OI. (**1994**) Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food and Chemical Toxicology*, ***32***, 31–36.

[18] Yu LL, Zhou KK, Parry J. (**2005**) Antioxidant properties of cold-pressed black caraway, carrot, cranberry, and hemp seed oils. *Food Chemistry*, ***91***, 723-729.

[19] Hammer KA, Carson CF, Riley TV. (**1999**) Antimicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology*, ***86***, 985-990.

[20] Suppakul P, Miltz J, Sonneveld K, Bigger SW. (**2003**) Antimicrobial properties of basil and its possible application in food packaging. *Journal of Agricultural and Food Chemistry*, ***51***, 3197-3207.

[21] Ultee A, Bennik MHJ, Moezelaar R. (**2002**) The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology*, ***68***, 1561-1568.

[22] Dorman HJD, Deans SG. (**2000**) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, ***88***, 308-316.

[23] Maggi RG, Govind NS. (**2004**) Regulated expression of green fluorescent protein in *Debaryomyces hansenii*. *Journal of Industrial Microbiology and Biotechnology*, ***31***, 301-310.

[24] Pawar VC, Thaker VS. (**2006**) *In vitro* efficacy of 75 essential oils against *Aspergillus niger*. *Mycoses*, ***49***, 316–323.

[25] Xu B-J, Jia X-Q, Gu L-J, Sung C-K. (**2006**) Review on the qualitative and quantitative analysis of the mycotoxin citrinin. *Food Control*, ***17***, 271–285.

[26] Knoblock K, Pauli A, Iberl B, Weis N, Weigand H. (**1988**) Antibacterial activity and antifungal properties of essential oil components. *Journal of Essential Oil Research*, ***1***, 119-128.

[27] Rojas-Graü MA, Avena-Bustillos RJ, Olsen C, Friedman M, Henika PR, Martin-Belloso O, Pan Z, McHugh TH. (**2007**) Effects of plant essential oils and oil compounds on mechanical, barrier and antimicrobial properties of alginate-apple puree edible films. *Journal of Food Engineering*, ***81***, 634-641.

[28] Davies NW. (**1990**) Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and Carbowax 20M phases. *Journal of Chromatography*, ***503***, 1-24.

[29] Adams RP. (**2001**) *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. Allured Publishing, Carol Stream, IL.

[30] Brand-Williams W, Cuvelier ME, Berset C. (**1995**) Use of free radical method to evaluate antioxidant activity. *Food Science and Technology*, ***28***, 25-30.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1751 - 1754

Chemical Composition of the Essential Oil of *Commiphora erythraea*

Maria Carla Marcotullio,* Claudio Santi, Gildas Norbert Oball-Mond Mwankie and Massimo Curini

Dipartimento di Chimica e Tecnologia del Farmaco, *Sezione Chimica Organica*, *via del Liceo 1*, *06123 Perugia*, *Italy*

marcotu@unipg.it

Received: May 27th, 2009; Accepted: September 14th, 2009

The essential oil composition of *Commiphora erythraea* (Ehrenb) Engl. is reported for the first time. The oil is rich in sesquiterpenes, particularly furanosesquiterpenes (50.3%). GC-MS analysis of the oil permitted differentiation between *C. erythraea* and *C. kataf*, two often confused species.

Keywords: *Commiphora erythraea*, *C. kataf*, *C. guidottii*, Burseraceae, GC-MS, furanosesquiterpenes, opopanax, retention indices, essential oil.

The genus *Commiphora* (Burseraceae) comprises more than 150 species of small trees and large shrubs, native particularly to Arabia, Somalia, eastern Ethiopia and Kenya. All the species produce resinous exudates commonly called myrrh containing water-soluble gum, alcohol-soluble resin and volatile oil. The most characteristic components of myrrh are furanosesquiterpenes that are responsible for the odor of the resin. *C. myrrha* (Nees) Engl. is the principal source of commercial myrrh, but *C. erythraea* (Ehrenb) Engl. was the principal source of ancient and classical times [1]. The resins of *Commiphora* spp have been used since ancient time for the production of essential oils. The chemical composition of the extracts and the essential oils of different *Commiphora* spp. have been recently reviewed [1].

The commercial importance of *Commiphora* spp resides in the use by perfumery industries of the essential oils prepared from the resins. The essential oil of *C. erythraea* var. *glabrescens,* known as "opopanax", is used in perfume of the Oriental type. The term "opopanax" is a source of confusion, since it is often used for the oil of different Commiphorae and for the gum latex oil of some Umbelliferae, for example *Opopanax chironium*. As far as we know, all the studies devoted to the analysis of opopanax were performed on commercial oils. Ikeda studied the composition of the monoterpene fraction, and found that it was constituted mostly of ocimene [2]. Nigam and coworkers reported the occurrence of isomeric bisabolenes [3], and of α-santalene [4]. Wennigen and Yates studied the composition of the sesquiterpene hydrocarbon fraction of commercial opopanax oil [5]. Besides the reported bisabolenes, they showed the presence of α-cubebene, α-copaene, caryophyllene, β-elemene, *epi*-β-santalene, δ- and γ-cadinene, α- and β-santalene, δ- and γ-elemene, *cis*- and *trans*-α-bergamotene. Later, Tucker [6] stated that opopanax is the essential oil prepared from *C. erythraea,* whilst other authors described opopanax not only deriving from *C. erythraea*, but also from other species, such as *C. kataf* (Forsk.) Engl. [7] and *C. guidottii* (Chiov) [8,9]. Wood noted also the close morphological similarity of Yemeni shrubs of *C. kataf* to *C. erythraea* var. *glabrescens* [7].

Only recently it has been stated that commercial opopanax is *C. guidottii* oil [5,10]. Several non-profit associations [e.g. IPO (Increasing People Opportunities)] [11] are committed to seeing a sustainable and better livelihood for the pastoral community of Ethiopia, and the further development of strong cooperation with communities in neighboring countries with a similar mode of life. One of the most important projects of this association is the creation of ten cooperatives for the harvesting and processing of gum resins.

At the request of IPO association we studied the composition of the essential oil of the resin of *C. erythrea* (Agarsu) commercialized by Agarsu Liben Cooperative and imported into Italy by IPO association. The aim was to define the composition of the resin oil, as the use *C. erythraea* in local African traditional medicines is common for use on livestock against ticks, for eye infections, malaria and the elimination of snake venom toxins.

Maradufu [12], from the *n*-hexane extract of *C. erythraea*, isolated furanodienone **1**, which was moderately toxic to the larvae of *Riphicephalus appendiculatus*. It was later demonstrated that this extract is repellent and toxic to several ticks [13].

The chemical composition of the resin essential oil of *C. erythraea* obtained by steam distillation has been determined by GC-MS and NMR spectroscopy (Table 1). Two dominant constituents (entry 32, 21.5% and entry 38, 14.3%) could be detected. All the monoterpenes and sesquiterpene hydrocarbons were identified by comparison of their MS with those in the NIST98 library, while entries 32-34 and 38-42 were not present in our library and we attempted to isolate them by column chromatography and identify them by NMR spectroscopy.

The oil was first divided into apolar (**I**) and polar fractions (**II**) by silica gel column chromatography and elution with *n*-hexane and Et_2O, respectively. Fraction **I**, by GC-MS analysis, was constituted of monoterpenes and sesquiterpene hydrocarbons (entries 1-10, 12-26 and 28-30), while fraction **II** contained some sesquiterpene hydrocarbons, sesquiterpene alcohols and all the unknown compounds. Column chromatographic purifications of fraction **II** led to the isolation of compounds **1-6** (entries 32, 33, 38, 39, 40 and 42, respectively), whose structures were determined by comparing their NMR data with those reported in the literature [14-17]. All these compounds have been previously reported in different *Commiphora* spp, but only **1** has been described for *C. erythraea*.

It has been reported that furanodiene and furanodienone (**1**) are thermally sensitive and during GC-MS analysis can decompose to give curzerene and curzerenone (**2**), respectively, through Cope rearrangement [18-21]. The isolation of **2** from the oil of *C. erythraea* proves that curzerenone is actually one of the components of the oil. In our oil, we could not identify either ocimene, although this was the most abundant monoterpene of the opopanax oil studied by Ikeda [2], or α- and β-bisabolenes [3,4]. It is interesting to note that the gas chromatographic

Table 1: Chemical composition of *Commiphora erythraea* essential oil.

	Component[a]	I[b]	I[c]	%[d]	Identification[e]
1	α-Thujene	921	928	1.4	MS, RI
2	α-Pinene	933	934	1.8	MS, RI, Ref
3	Camphene	942	948	0.3	MS, RI,
4	Sabinene	968	974	0.2	MS, RI, Ref
5	β-Pinene	976	976	0.9	MS, RI, Ref
6	Myrcene	987	991	0.1	MS, RI, Ref
7	3-Carene	1010	1009	0.7	MS, RI, Ref
8	*p*-Cymene	1025	1024	0.2	MS, RI, Ref
9	Limonene	1028	1030	0.5	MS, RI, Ref
10	Camphor	1145	1145	0.2	MS, RI, Ref
11	4-Terpineol	1177	1174	0.2	MS, RI, Ref
12	δ-Elemene	1339	1337	1.7	MS, RI, Ref
13	α-Cubebene	1352	1350	1.3	MS, RI, Ref
14	α-Copaene	1380	1374	3.4	MS, RI, Ref
15	β-Bourbonene	1388	1381	1.6	MS, RI, Ref
16	β-Elemene	1393	1388	1.8	MS, RI, Ref
17	α-Gurjunene	1417	1424	3.8	MS, RI, Ref
18	β-Caryophyllene	1419	1414	0.5	MS, RI, Ref
19	α-Guaiene	1425	1426	1.5	MS, RI
20	γ-Elemene	1433	1434	0.8	MS, RI, Ref
21	Aromadendrene	1441	1443	4.4	MS, RI, Ref
22	α-Humulene	1453	1450	1.0	MS, RI, Ref
23	Alloaromadendrene	1465	1458	0.7	MS, RI, Ref
24	Germacrene D	1487	1480	3.3	MS, RI, Ref
25	β-Selinene	1493	1481	2.3	MS, RI, Ref
26	α-Selinene	1501	1485	1.8	MS, RI, Ref
27	Curzerene	1504	1506	0.9	MS, RI
28	γ-Cadinene	1511	1511	2.0	MS, RI, Ref
29	δ-Cadinene	1528	1515	1.2	MS, RI, Ref
30	α-Cadinene	1541	1531	0.5	MS, RI
31	Germacrene B	1557	1552	3.0	MS, RI, Ref
32	1(10),4-Furanodien-6-one (**1**)	1628		21.5	NMR
33	Curzerenone (**2**)	1632	1601	1.5	NMR, Ref
34	NI[f]	1639		0.6	
35	β-Eudesmol	1646	1647	0.2	MS, RI, Ref
36	α-Eudesmol	1656	1650	2.2	MS, RI, Ref
37	Germacrone	1703	1693	0.9	MS, RI, Ref
38	1, 10(15)-Furanogermacra-dien-6-one (**3**)	1762		14.3	NMR
39	Dihydropyrocurzerenone (**4**)	1855		0.8	NMR
40	rel-3*R*-Methoxy-4*S*-furanogermacra-1*E*,10(15)-dien-6-one (**5**)	1890		7.4	NMR
41	NI	1894		2.2	=
42	rel-2*R*-Methoxy-4*R*-furanogermacra-1(10)*E*-en-6-one (**6**)	1905		3.9	NMR
	Total (%)			99.7	
	Total identified (%)			96.9	
	Monoterpene hydrocarbons	6.1	Monoterpene alcohols	0.4	
	Furanosesquiterpenes	50.3	Sesquiterpene hydrocarbons	36.6	
	Sesquiterpene alcohols	2.5	Sesquiterpene ketones	0.9	

[a] Components are listed in order of their elution from a HP-5MS column.
[b] RI, linear retention indices were determined relative to the retention times on HP-5MS column of homologous series of C_5-C_{20} *n*-alkanes, using the equation of Van den Dool and Krantz [23].
[c] RI of literature on the apolar column, [24]: entry 2, 4-18, 22-26, 28, 29, 31. [25]: 1, 3. [26]: (RTX column) 21, 23. [27]: 19, 20, 30, 35, 36. [28]: (DB5 column) 27. [29]: (DB5 column) 33, 37.
[d] Percentage obtained by FID peak-area normalization. Values represent an average of three determinations.
[e] Identification: MS, by comparison of the MS with those of the NIST98 library (99% matching); RI, by comparison of RI with those reported from NIST databank; Ref, by comparison with literature data; NMR, isolated compounds identified by NMR spectra; [f] NI= not identified.

analysis of the essential oil of *C. erythraea* shows many differences from the analysis reported for *C. kataf* [14]. The main differences reside in the absence of a monoterpene fraction in *C. kataf* oil and the very small percentages of furanodienone and curzerenone (together 1.0%), while in our case the two compounds amounted to approximately 23%.

As far as we know, this is the first phytochemical study of the essential oil of *C. erythraea*, although the *n*-hexane extract has been evaluated [11]. The essential oil is mostly constituted by furanosesquiterpenes, some of which have been isolated and identified by spectroscopic analysis. All the furanosesquiterpenes we isolated have been already reported in other *Commiphora* species. Our work showed that commercial opopanax oil is effectively prepared from *C. guidottii*, as the analyses reported in the 1960s [2-4] are close to the one reported by Başer [22]. Furthermore, we can affirm that gas chromatographic analysis is able to distinguish between *C. erythraea* and *C. kataf*, two often confused species.

Experimental

GC analysis: GC analyses were performed on a Hewlett Packard HP 6890, combined with HP ChemStation Software, equipped with a flame ionization detector (FID) and a fused silica capillary column (HP-5MS; 30 m x 0.25 mm i.d., 0.25 μm film thickness). The oven temperature was programmed from 60°C for 5 min, then ramped at 4°C/min to 270°C, and held for 20 min. Injector and detector temperatures were 250 and 270°C, respectively. Samples were injected in the splitless mode using helium as carrier gas (1mL/min); the samples were dissolved in CH_2Cl_2 to give 1% w/v solutions; the injection volume was 1μL. Percentage compositions of the oil components were obtained from electronic integration using FID at 270°C, dividing the area of each component by the total area of all components. The percentage values were the mean of 3 injections of the sample.

GC/MS analysis: GC/MS analyses were carried out on a Hewlett Packard HP 6890 chromatograph combined with HP ChemStation Software, equipped with a HP 5973 Mass Selective Detector and fitted with a capillary column (HP-5MS; 30 m x 0.25 mm, i.d.; 0.25μm film thickness). The oven temperature was programmed from 60°C for 5 min, then ramped at 4°C/min to 270°C, and held for 20 min. Injector and detector temperatures were 250 and 270°C, respectively. Samples were injected in the splitless mode using helium as carrier gas (1mL/min); the samples were dissolved in CH_2Cl_2 to give 1% w/v solutions; the injection volume was 1 μL. The ionization energy was 70eV.

Identification of compounds: Peak identification was accomplished by computer matching with a commercial MS library (NIST98) and comparison of MS with those of our library of pure standard authentic compounds. The identification of the oil components was also possible by comparison of their linear RI [23] with those from the literature [24-29], and comparison of ^{1}H and ^{13}C NMR spectra obtained from the oil with those from the literature [14-17].

Plant material: A voucher specimen (# MCM-1) of the resin (Agarsu grade I) is deposited at the Dipartimento di Chimica e Tecnologia del Farmaco-Sez. Chimica Organica, University of Perugia.

Preparation and purification of the essential oil*:* The essential oil (3.9 g) was obtained by steam distillation (3h) of the powdered resin (59.7 g). Methylene chloride was used as the collector solvent. The oil was dried over anhydrous sodium sulfate and the solvent removed under N_2. The oil (3 g) was purified by SiO_2 gel column chromatography. Elution with *n*-hexane gave fraction **I** (0.90 g). Further elution with Et_2O gave fraction **II** (1.98 g). Fraction **II** was purified on SiO_2 and elution with CH_2Cl_2 gave three subfractions: **II.1** (0.12 g), **II.2** (1.00 g) and **II.3** (0.44 g). Column chromatography of **II.1** (SiO_2, hexane) gave **4** (10 mg) in a pure form [14]. Purification of **II.2** on SiO_2 (*n*-hexane-Et_2O; 1-4%) gave, in order, **3** (40 mg) [14], **2** (10 mg) [14] and **1** (52 mg) [12,14,16]. Purification of **II.3** on SiO_2 (CH_2Cl_2-EtOAc, 2%) gave, in a pure form, compounds **5** (20 mg) [14,15] and **6** (15 mg) [14,15]

Acknowledgments - The authors wish to thank Ipoassociazione (www.ipoassociazione.org) and Agarsu Liben Coperative for providing Agarsu (*Commiphora erythraea* resin), Infarmazone onlus (www.infarmazone.org) and Mr Bazzucchi and Ms Messina for their work in the project.

References

[1] Hanuš LO, Řezanka T, Dembitsky VM, Moussaieff A. (**2005**) Myrrh – *Commiphora* chemistry. *Biomedical Papers*, ***149***, 3-28.

[2] Ikeda RM, Stanley WL, Vannier SH, Spitler EM. (**1962**) The monoterpene hydrocarbons composition of some essential oils. *Journal of Food Science*, ***27***, 455-458.

[3] Nigam IC, Levi L. (**1966**) Essential oils and their constituents. XXXII. Gas chromatography of sesquiterpenes hydrocarbons. *Journal of Chromatography*, ***23***, 217-226.

[4] Nigam IC, Neville GA. (**1968**) Essential oils and their constituents. Part XLI. Identification of sesquiterpene hydrocarbons in oil of opopanax. *Journal of Chromatography*, ***34***, 85-88.

[5] Wenniger JA, Yates RL. (**1969**) Constituents of opopanax oil: sesquiterpene hydrocarbons. *Journal of the Association of Official Analytical Chemistry*, ***52***, 1155-1161.

[6] Tucker AO. (**1986**) Frankicense and myrrh. *Economic Botany*, ***40***, 425-433.

[7] Wood JRI. (**1997**) *Handobook of Yemen flora*, Royal Botanic Garden Kew.

[8] Mabberley DJ. (**1998**) *The Plant Book*, 2nd rev. edn., Cambridge University Press.

[9] http://www.cropwatch.org/cropwatch11.htm

[10] Thulin M, Claeson P. (**1991**) The botanical origin of scented myrrh (bissabol or Habak Hadi). *Economic Botany*, ***45***, 487-494.

[11] www.ipoassociazione.org

[12] Maradufu A. (**1982**) Furanosesquiterpenoids of *Commiphora erythraea* and *C. myrrh. Phytochemistry*, ***21***, 677-680.

[13] Carroll JF, Maradufu A, Warthen JD Jr. (**1989**) An extract of *Commiphora erythraea*: a repellent and toxicant against ticks. *Entomologia Experimentalis et Applicata*, ***53***, 111-116.

[14] Dekebo A, Dagne E, Sterner O. (**2002**) Furanosesquiterpenes from *Commiphora sphaerocarpa* and related adulterants of true myrrh. *Fitoterapia*, ***73***, 48-55.

[15] Zhu N, Kikuzaki H, Shen S, Sang S, Rafi MM, Wang M, Nakatani N, Di Paola RS, Rosen RT, Ho C-T. (**2001**) Furanosesquiterpenoids of *Commiphora myrrha*. *Journal of Natural Products*, ***64***, 1460-1462.

[16] Hikino H, Konno C, Takemoto T. (**1969**) Structure and conformation of the sesquiterpenoids furanodienone and isofuranodienone. *Chemical Communications*, 662-663.

[17] Dekebo A, Dagne E, Hansen LK, Gautun OR, Aasen AJ. (**2000**) Crystal structures of two furanosesquiterpenes from *Commiphora spherocarpa*. *Tetrahedron Letters*, ***41***, 9875-9878.

[18] Hikino H, Agatsuma K, Konno C, Takemoto T. (**1970**) Structure of furanodiene and isofuranogermacrene (curzerene). *Chemical and Pharmaceutical Bulletin*, ***18***, 752-755.

[19] Nakagawa Y. (**1983**) Mass spectra of furanosesquiterpenes. *Journal of Mass Spectrometry Society of Japan*, ***31***, 129-135.

[20] Nagano N, Kuroda C, Moriyama Y, Tsuyuki T, Takahashi T. (**1982**) Mass spectra of furanoeremophilane derivatives. *Bulletin of the Chemical Society of Japan*, ***55***, 1221-1227.

[21] Yang FQ, Li SP, Zhao J, Lao CS, Wang FQ. (**2007**) Optimization of GC-MS condition based on resolution and stability of analytes for simultaneous determination of nine sesquiterpenoids in three species of *Curcuma* rhizomes. *Journal of Pharmaceutical and Biomedical Analysis*, ***43***, 73-82.

[22] Başer KHC, Demirci B, Dekebo A, Dagne E. (**2003**) Essential oil of some *Boswellia* spp., myrrh and opopanax. *Flavour and Fragrance Journal*, ***18***, 153-156.

[23] Van Den Dool H, Kratz PD. (**1963**) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography*, ***45***, 463-471.

[24] Saroglou V, Dorizas N, Kypriotakis Z, Skaltsa HD. (**2006**) Analysis of the essential oil composition of eight *Anthemis* species from Greece. *Journal of Chromatography A*, ***1104***, 313-322.

[25] Maggi F, Cecchini C, Cresci A, Coman MM, Tirillini B, Sagratini G, Papa F. (**2009**) Chemical composition and antimicrobial activity of the essential oil from *Ferula glauca* L. (*F. communis* L. subsp *glauca*) growing in Marche (central Italy). *Fitoterapia*, ***80***, 68-72.

[26] Paolini J, Nasica E, Desjobert J-M, Muselli A, Bernardini A-F, Costa J. (**2008**) Analysis of volatile constituents isolated by hydrodistillation and headspace solid-phase microextraction from *Adenostyles briquetii* Gamisan. *Phytochemical Analysis*, ***19***, 266-276.

[27] Karioti A, Hadjipavlou-Litina D, Mensah MLK, Fleischer TL, Skaltsa H. (**2004**) Composition and antioxidant activity of the essential oil of *Xylopia aethiopica* (Dun) A. Rich. (Annonaceae) leaves, stem bark, root bark, and fresh and dried fruits, growing in Ghana. *Journal of Agricultural Food Chemistry*, ***52***, 8094-8098.

[28] Hamm S, Bleton J, Tchapla A. (**2004**) Headspace solid phase microextraction for screening for the presence of resins in Egyptian archaeological samples. *Journal of Separation Science*, ***27***, 235-243.

[29] Adams JP. (**1995**) *Identification of essential oil components by gas chromatography/mass spectrometry.* Allured Publishing Corporation, Carol Stream, IL.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1755 - 1760

Insights on Novel Biologically Active Natural Products: 7-Isopentenyloxycoumarin

Francesco Epifano[a*], Caroline Pelucchini[b], Massimo Curini[b] and Salvatore Genovese[a]

[a]*Dipartimento di Scienze del Farmaco, Università "G. D'Annunzio" di Chieti-Pescara, Via dei Vestini 31, Chieti Scalo, Italy, 66013*

[b]*Dipartimento di Chimica e Tecnologia del Farmaco, Sezione di Chimica Organica, Università degli Studi di Perugia, Via del Liceo, Perugia, Italy 06123*

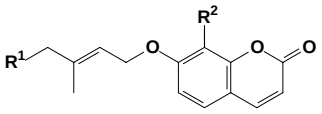
fepifano@unich.it

Received: May 27th, 2009; Accepted: September 15th, 2009

7-Isopentenyloxycoumarin is a prenyloxyphenylpropanoid derivative found in low concentration in a restricted number of plant families (Apiaceae, Asteraceae, and Rutaceae). Synthetic schemes were recently developed enabling sufficient quantities of the title coumarin to be obtained in order to evidence its valuable biological effects, mainly as an anti-cancer agent. The aim of this review is to examine the phytochemical and pharmacological properties of this compound.

Keywords: Anti-cancer activity, Apiaceae, Asteraceae, coumarins, prenyloxyphenylpropanoids, Rutaceae.

Coumarins represent a large class of natural compounds, mainly found in the families Rutaceae and Apiaceae. Although more than 1300 natural coumarins have been identified to date [1], most chemical and pharmacological studies were carried out on either coumarin itself or structurally simple derivatives. Coumarins can be divided into 3 groups: a) substituted coumarins, b) ring-fused coumarins and c) *C*- and *O*-prenylcoumarins. In particular, this third group comprises compounds in which a terpenyl side chain is attached to the benzopyrone ring either directly or through one or more phenoxy group, *via* an ether bond. While *C*-prenylcoumarins have been well studied both from a chemical and a pharmacological point of view, prenyloxycoumarins, considered for decades merely as biosynthetic intermediates of linear-, furano- and pyranocoumarins, have only in the last decade been characterized as secondary metabolites exerting valuable biological activities [1]. In this context, we have already reported the features of selected prenyloxycoumarins, like auraptene (**1**) [2], and collinin (**2**) [3]. The aim of this short review is to examine in detail from a phytochemical and pharmacological point of view, the properties of an additional coumarin derivative, 7-isopentenyloxycoumarin (**3**). This secondary metabolite was recently seen to exert promising and valuable anti-cancer, anti-inflammatory, anti-fungal, anti-microbial, and allelochemical effects.



1: R^1 = isopentenyl, R^2 = H
2: R^1 = isopentenyl, R^2 = OCH_3
3: R^1 = R^2 = H

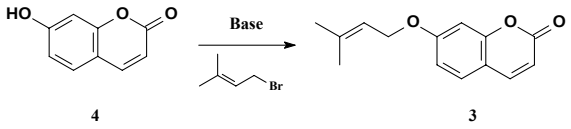
Natural and synthetic sources

Like many other coumarin derivatives, 7-isopentenyloxycoumarin has been found in a restricted number of families. In fact, to date, 7-isopentenyloxycoumarin has been obtained only from plants belonging to the Apiaceae, Asteraceae, and Rutaceae families. 7-Isopentenyloxycoumarin was first isolated in 1966 by Prokopenko from the fruits of *Libanotis intermedia* Rupr. (Apiaceae) [4]. This author provided chemical evidence for the structure of this novel secondary metabolite. A few years later, the structure of 7-isopentenyloxy-coumarin was unambiguously determined by UV, 1D and 2D NMR spectroscopy [5,6]. Compound **3** was isolated (Table 1) from several other species [7-39].

Table 1: Natural sources of 7-isopentenyloxycoumarin (**3**).

Family	Plant	Ref.
Apiaceae	*Angelica ursina* Regel	[7]
	Seseli libanotis W.D.J. Koch	[7]
	Heracleum dissectum Ledeb.	[8]
	Peucedanum stenocarpum Boiss & Reut ex Boiss	[9]
	Scandix pectens-veneris L.	[10]
	Heracleum lanatum Michx.	[10, 11]
	Ammi majus L.	[12]
	Tordylium apulum L.	[13]
	Lomatium nevadense (Watson) J. Coulter et Rose	[14]
Asteraceae	*Tagetes florida* Sweet	[15]
	Ophryosporus angustifolius B.L. Rob.	[16]
	Haplopappus tenuisectus (Greene) S.F. Blake in L.D. Benson	[17]
	Melampodium divaricatum DC.	[18]
	Haplopappus deserticola Phil.	[19]
	Baccharis pedunculata (Mill.) Cabrera	[20]
	Trichocline reptans Wedd.	[21]
	Heterotheca inuloides Cass.	[22]
	Haplopappus multifolius Phil. Ex Reiche	[23]
Rutaceae	*Euodia vitiflora* F. Muell.	[24]
	Ruta graveolens L.	[25]
	Coleonema album E. Mey	[26]
	Diosma acmaeophylla Eckl. & Zeyh.	[27]
	Coleonema aspalathoides A. Juss.	[28]
	Boenninghausenia albiflora (Hook) Meisn.	[29]
	Citrus limon (L.) Burm.f.	[30]
	Asterolasia phebalioides F. Muell.	[31]
	Ammi huntii H.C. Watson	[32]
	Haplophyllum patavinum (L.) Don. Fil.	[33]
	Melicope hayesii T.G. Hartley	[34]
	Melicope semecarpifolia (Merr.) T.G. Hartley	[35, 36]
	Phebalium brachycalyx Paul G. Wilson	[37]
	Zanthoxylum tingoassuiba A. St. Hil	[38]
	Melicope vitiflora (F. Muell.) T.G. Hartley	[39]

7-Isopentenyloxycoumarin is biosynthesized from umbelliferone (**4**) and dimethylallyl diphosphate, in turn deriving from the 1-deoxy-xylulose-5-phosphate (DOXP) pathway [40]. The coupling step is catalyzed by the enzyme dimethylallyl diphosphate umbelliferone transferase (DDU-7 transferase, E.C. 2.5.1), [41]. This enzyme is located in the membrane of the endoplasmic reticulum and is able to catalyze both the *C*-6 and the *O*-prenylation of umbelliferone. Hamerski and coworkers demonstrated that the *O*-prenylation occurred more favourably in the presence of Mn^{+2} ions as an enzymatic cofactor [41]. However, until now, 7-isopentenyloxycoumarin was obtained from natural sources in very small amounts. So, valuable, high yield, and environmentally friendly synthetic schemes were developed in order to handle this natural compound in sufficient quantities to perform tests aimed at depicting its pharmacological profile. 7-Isopentenyloxycoumarin was obtained by a Williamson etherification reaction starting from umbelliferone (**4**) and 3,3-dimethylallyl bromide as alkylating agent, and either tetrabutyl ammonium



4 **3**

Scheme 1

hydroxide [42] or dry K_2CO_3 as the base [40] (Scheme 1). In the last case, 7-isopentenyloxy-coumarin was obtained in very good yield simply by crystallization from either *n*-hexane or aqueous methanol, without the need of any chromatographic procedure [5,43].

Nowadays several efficient analytical methods (e.g HPLC, HPTLC, high speed countercurrent chromatography) for the selective separation, detection and quantification of 7-isopentenyloxy-coumarin in plant extracts are also available [30,39, 44-48].

Biological activity

The first set of data about biological activity of 7-isopenetenyloxycoumarin was reported in 1971 by Jurd and coworkers [49]. They screened 7-isopentenyloxycoumarin, at a concentration of 500 ppm, against selected bacterial (*Bacillus cereus*, *Sarcina lutea*, *Staphylococcus aureus*, *Streptococcus lactis*, *Alcaligenes faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella tiphymurium*, *Serratia marcescens*), and fungal strains (*Zygosaccharomyces japoni*, *Candida tropicalis*, *Pichia codati* var. *fermentans*, *Hansenula anomala*, *Saccharomyces cerevisiae* var. *ellipsoideus*, *Torula utilis*, *Aspergillus flavus*, *Aspergillus niger*, *Byssochlamis fluva*, *Penicillium chrysogenum*, *Rhizopus senti*), showing no activity in any case. Further investigations of the anti-fungal properties of 7-isopentenyloxycoumarin were reported in 1995 by Rahalison and coworkers. First, they tested 7-isopentenyloxycoumarin, by the agar dilution method, as an inhibitory agent of the growth of the phytopathogenic fungus *Cladosporium cucumerinum* [50]. 7-Isopentenyloxycoumarin revealed a complete inhibitory effect at a concentration of 100 μg/mL. On the basis of this result, Rahalison and coworkers tested 7-isopentenyloxycoumarin against a panel of human pathogenic fungi, such as *Candida albicans*, *Phytosporum ovale*, *Aspergillus fumigatus*, *Epidermophyton floccosum*, *Microsporum canis*, *Microsporum gypseum*, and *Trycophyton mentagrophytes*. The compound showed activity only against *E. floccosum* at a concentration of 10 μg/mL, the same value recorded for myconazole, used as the

reference drug. Considering that this fungus is among the most common causes of dermatomycoses, this effect was interesting when compared with the cytotoxic effect of 7-isopentenyloxycoumarin on human epidermal keratinocytes (LD_{50} = 15 μg/mL) [50]. Finally, 7-isopentenyloxycoumarin was not active against the phytopathogenic fungus *Botrytis cinerea* [51].

When tested as a myorelaxant, at high dosage (100 μM), 7-isopentenyloxycoumarin showed a good inhibitory effect (80 %) on the KCl (60 mM)-induced contraction of the rat thoracic aorta [29].

Only one report took into consideration 7-isopentenyloxycoumarin as an anti-inflammatory agent. However, the compound exerted a slight effect on the inhibition of LPS/IFN-γ induced NO generation in RAW 264.7 cells (30% inhibition at a concentration of 50 μM) [52].

When assayed for its capacity for anti-mutagenicity and anti-carcinogenicity against mutations induced by benzo(*a*)pyrene and hydrogen peroxide in *S. typhimurium* strains TA100 and TA102 (modified Ames test), 7-isopentenyloxycoumarin revealed only a moderate activity. At concentrations of 2, 4, and 8 μg/plate, 19, 29, and 36% protection was achieved against benzo(*a*)pyrene-induced mutations, respectively. In the case of hydrogen peroxide mutagenicity, the percentages of protection were far less (9%, 14%, and 17% at 1, 2, and 4 μg/plate, respectively) [53].

In the last decade, several studies describing the anti-cancer effects of 7-isopentenyloxycoumarin were reported. In 1998, Kofinas and coworkers assayed 7-isopentenyloxycoumarin on KB (human rhinopharinx cancer) and NSCLC-N6 (human bronchial epidermoid carcinoma) cell lines, recording ID_{50} values of 10.6 and 9.9 μg/mL, respectively [13]. In 2001, Kawaii and coworkers determined the activity of 7-isopentenyloxycoumarin on four other cancer cell lines, but virtually no activity was revealed. In fact, 7-isopentenyloxycoumarin showed IC_{50} values of 94 μM, 84 μM, 73 μM, and 57 μM against A549 (human lung carcinoma), B16 melanoma 4A5 (melanin pigment producing mouse melanoma), CCRF-HSB2 (human T-cell leukemia), and TGBC11TKB (metastasized lymphoma) cell lines, respectively [54]. The best results for 7-isopentenyloxycoumarin as an anti-cancer agent were obtained in 2002 by Baba and coworkers [55]. These authors first examined the inhibitory activity of 7-isopentenyloxycoumarin on 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen expression (EBV-EA). At a dose of 32 μM 7-isopentenyloxycoumarin exerted a 100% inhibition. It was also able to prevent the TPA-induced-$^{32}P_i$-incorporation into phospolipids in HeLa cells (89% inhibition at 1.62 mM). Encouraged by these results, Baba and coworkers studied also the effect of 7-isopentenyloxycoumarin against TPA-induced skin tumor formation in 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated mice, after topical administration. It was found that 7-isopentenyloxycoumarin strongly suppressed tumor formation in a dose dependent manner, with an inhibition degree of about 75% at a dose of 3.24 μM. Moreover, no toxicity for animals was recorded at the doses used to perform these *in vivo* tests. Finally, they examined the effects on TPA-induced murine epidermal ornithine decarboxylase (ODC) activity. Over-induction of ODC is known to be strongly associated with tumor progression. 7-Isopentenyloxy-coumarin exhibited an inhibitory effect also in this test, although not in a so evident manner as that shown in the previous assays. Taken together, the data reported by Baba and coworkers may indicate that 7-isopentenyloxycoumarin is a potent chemopreventive agent of skin tumors. The last report on anti-cancer properties of 7-isopentenyloxy-coumarin was given in 2005 by Chou and coworkers. These authors showed that 7-isopentenyloxy-coumarin exerted a mild inhibitory effect on HT-29 cell line (human colon adenocarcinoma, ED_{50} = 4.85 μg/mL), while it was virtually inactive on P-388 cells (murine leukemia, ED_{50} > 50.0 μg/mL) [36].

Finally, Epifano and coworkers found that 7-isopentenyloxycoumarin could be regarded as a novel neuroprotective agent [56]. In a preliminary *in vitro* screening, these authors showed that 7-isopentenyloxycoumarin is able to protect neuronal cells (astrocytes and neurons) from death induced by *N*-methyl-D-aspartate (50.5 % protection at 100 μM).

Conclusions

From the data reported in this short review, it can be seen that 7-isopentenyloxycoumarin may be regarded nowadays as a potential drug for the chemoprevention of some types of cancer, in particular those affecting skin. This coumarin has now become widely and easily available in huge

amounts by chemical synthesis, which is accomplished by isopentenylation of commercially available umbelliferone with 3,3-dimethylallyl bromide. The title prenyloxycoumarin and its structurally related natural and semi-synthetic compounds have become a topic of current and growing interest and it is hoped that in the near future other studies aimed at the further characterization of their pharmacological properties, not only in cancer therapy, but also in other fields, will be reported.

References

[1] Curini M, Cravotto G, Epifano F, Giannone G. (**2006**) Chemistry and biological activity of natural and synthetic prenyloxycoumarins. *Current Medicinal Chemistry*, ***13***, 199-222.

[2] Epifano F, Genovese S, Curini M. (**2008**) Auraptene: phytochemical and pharmacological properties. In *Phytochemistry Research Progress*. Matsumoto T. (Ed.). Nova Science Publishers Inc., Hauppauge, NY, USA, 145-162.

[3] Epifano F, Genovese S, Marcotullio, MC, Curini, M. (**2008**) Chemistry and pharmacology of collinin, active principle of *Zanthoxylum* spp. *Mini Reviews in Medicinal Chemistry*, ***8***, 1203-1208.

[4] Prokopenko AP (**1966**) Coumarins of *Libanotis intermedia*. *Rastitel'Nye Resursy*, ***2***, 201-203.

[5] Steck W, Bailey BK. (**1969**) Characterization of plant coumarins by combined gas-chromatography, ultraviolet absorption spectroscopy, and nuclear magnetic resonance. *Canadian Journal of Chemistry*, ***47***, 3577-3583.

[6] Chang CJ, Floss HG, Steck W. (**1977**) Carbon 13 magnetic resonance spectroscopy of coumarins. Carbon 13-proton long range couplings. *Journal of Organic Chemistry*, ***42***, 1337-1340.

[7] Bohlmann F, Rao VS, Bhaskar GM. (**1968**) Naturally occurring coumarins. III. Coumarins from *Angelica ursina* and *Seseli libanotis*. *Tetrahedron Letters*, ***36***, 3947-3950.

[8] Belenovskaya LM, Sinitskii VS, Tumbaa H. (**1977**) 7-Isopentenyloxycoumarin from *Heracleum dissectum***.** *Khimiya Prirodnykh Soedinenii,* ***4***, 574-575.

[9] Gonzalez AG, Cardona RJ, Diaz Chico E, Medina JM, Rodriguez Luis F. (**1978**) Umbelliferae components. XIX. Coumarins of *Peucedanum stenocarpum* Boiss and Revter and of *Scandix pectens-veneris* L. *Anales de Quimica*, ***74***, 637-640.

[10] Nakata H, Sashida Y, Shimomura H. (**1982**) A new phenolic compound from *Heracleum lanatum* Michx. var. *nipponicum* Hara. *Chemical & Pharmaceutical Bulletin*, ***30***, 4554-4556.

[11] Baba M, Jin Y, Mizuno A., Suzuki H, Okada Y, Takasuka N, Tokuda H, Nishino H, Okuyama T. (**2002**) Studies on cancer chemoprevention by traditional folk medicines XXIV. Inhibitory effect of a coumarin derivative, 7-isopentenyloxycoumarin, against tumor-promotion. *Biological & Pharmaceutical Bulletin*, ***25***, 244-246.

[12] Hamerski D, Schmitt D, Matern U. (**1990**) Induction of two prenyltransferases for the accumulation of coumarin phytoalexins in elicitor-treated *Ammi majus* cell suspension cultures. *Phytochemistry*, ***29***, 1131-1135.

[13] Kofinas C, Chinou I, Loukis A, Harvala C, Roussakis C, Maillard M, Hostettmann K. (**1998**) Cytotoxic coumarins from the aerial parts of *Tordylium apulum* and their effects on a non-small-cell bronchial carcinoma line. *Planta Medica*, ***64***, 174-176.

[14] Beauchamp PS, Dev BC, Dev V, Midland SL, Sims JS. (**2007**) California Lomatiums, Part VII. Analysis of the essential oils of *Lomatium nevadense* (Watson) J. Coulter et Rose var. *parishii* (J. Coulter et Rose) Jepson. Isolation of *trans*-dauc-8-en-11-ol, a new sesquiterpene alcohol and naturally occurring 2',3',3'-trimethyl-2',3'-dihydroangelicin. *Journal of Essential Oil Research*, ***19***, 117-124.

[15] Rios T, Flores RM. (**1976**) Chemical study of *Tagetes* I. *Revista Latinoamericana de Quimica*, ***7***, 33-36.

[16] Bohlmann F, Zdero C. (**1979**) New anol derivatives from *Ophryosporus angustifolius*. *Phytochemistry*, ***18***, 145-147.

[17] Bohlmann F, Fritz U, Robinson H, King RM. (**1979**) Naturally occurring terpene derivatives. Part 223. Isosesquicarene from *Haplopappus tenuisectus*. *Phytochemistry*, ***18***, 1749-1750.

[18] Borges-del-Castillo J, Martinez-Martir AI, Rodriguez-Luis F, Rodriguez-Ubis JC, Vazquez-Bueno, P. (**1984**) Salvadorian Compositae. Part 6. Isolation and synthesis of two coumarins from *Melampodium divaricatum*. *Phytochemistry*, ***23***, 859-861.

[19] Zdero C, Bohlmann F, Niemeyer HM. (**1990**) Diterpenes and umbelliferone derivatives from *Haplopappus deserticola*. *Phytochemistry*, ***29***, 326-329.

[20] Rahalison L, Benathan, Messod M, Michel FE, Gupta MP, Solis PN, Fuzzati N, Hostettmann K. (**1995**) Antifungal principles of *Baccharis pedunculata*. *Planta Medica*, ***61***, 360-362.

[21] Alarcon SR, De La Fuente JR, Novara L, Sosa VE. (**1998**) Benzofuran and coumarins in *Trichocline reptans* Wedd. *Anales de la Asociacion Quimica Argentina*, ***86***, 248-251.

[22] Delgado G, Olivares MS, Chavez MI, Ramirez-Apan T, Linares E, Bye R, Espinosa-Garcia FJ. (**2001**) Antiinflammatory constituents from *Heterotheca inuloides*. *Journal of Natural Products*, ***64***, 861-864.

[23] Torres R, Faini F, Modak B, Urbina F, Labbè C, Guerrero J. (**2006**) Antioxidant activity of coumarins and flavonoids from the resinous exudate of *Haplopappus multifolius*. *Phytochemistry*, ***67***, 984-987.

[24] Lassak EV, Southwell IA. (**1972**) Coumarins from the resin of *Euodia vitiflora*. *Australian Journal of Chemistry*, ***25***, 2491-2496.

[25] Austin DJ, Brown SA. (**1973**) Furanocoumarin biosynthesis in *Ruta graveolens* cell cultures. *Phytochemistry*, ***12***, 1657-1667.

[26] Gray AI. (**1981**) New coumarins from *Coleonema album*. *Phytochemistry*, ***20***, 1711-1713.

[27] Campbell WE, Provan GJ, Waterman PG. (**1982**) Chemosystematics in the Rutaceae. Part 14. Simple coumarins from two populations of *Diosma acmaeophylla*. *Phytochemistry*, ***21***, 1457-1458.

[28] Gray AI, Meegan CJ, O'Callaghan NB. (**1986**) Coumarins from two *Coleonema* species. *Phytochemistry*, ***26***, 257-260.

[29] Yamahara J, Miki S, Murakami H, Matsuda H, Fujimura H. (**1987**) Screening for calcium antagonists in natural products. The active principles of *Boenninghausenia albiflora var. japonica*. *Yakugaku Zasshi*, ***107***, 823-826.

[30] Ziegler H, Spiteller G. (**1992**) Coumarins and psoralens from Sicilian lemon oil [*Citrus limon* (L.) Burm. f.]. *Flavour and Fragrance Journal*, ***7***, 129-139.

[31] Sarker SD, Armstrong JA, Gray AI, Waterman PG. (**1994**) Coumarins from *Asterolasia phebalioides* (Rutaceae). *Biochemical Systematics and Ecology*, ***22***, 433-434.

[32] Antunes AM, Dos Santos SP, Brito PF. (**1995**) Coumarins from *Ammi huntii*. *Polyphenols*, ***94***, 305-306.

[33] Filippini R, Piovan A, Innocenti G, Caniato R, Cappelletti EM. (**1998**) Production of coumarin compounds by *Haplophyllum patavinum in vivo* and *in vitro*. *Phytochemistry*, ***49***, 2337-2340.

[34] Latip J, Hartley TG, Waterman PG. (**1999**) Lignans and coumarins metabolites from *Melicope hayesii*. *Phytochemistry*, ***51***, 107-110.

[35] Chen IS, Chen HF, Cheng MJ, Chang YL, Teng ML, Tsutomu I, Chen JJ, Tsai IL. (**2001**) Quinoline alkaloids and other constituents of *Melicope semecarpifolia* with antiplatelet aggregation activity. *Journal of Natural Products*, ***64***, 1143-1147.

[36] Chou HC, Chen JJ, Duh CY, Huang TF, Chen IS. (**2005**) Cytotoxic and anti-platelet aggregation constituents from the root wood of *Melicope semecarpifolia*. *Planta Medica*, ***71***, 1078-1081.

[37] Girard C, Muyard F, Colombain M, Tillequin F, Waterman PG, Bevalot F. (**2005**) Coumarins from *Phebalium brachycalyx*. *Biochemical Systematics and Ecology*, ***33***, 1195-1201.

[38] Vasconcelos da Silva C, Detoni CB, Velozo ES, Guedes MLS. (**2008**) Alkaloids and other metabolites from stems and fruits of *Zanthoxylum tingoassuiba* A. St. Hil. *Quimica Nova*, ***31***, 2052-2055.

[39] O'Donnell F, Ramachandran VN, Smyth TJP, Brooks P. (**2009**) An investigation of bioactive phytochemicals in the leaves of *Melicope vitiflora* by electrospray ionization ion trap mass spectrometry. *Analytica Chimica Acta*, ***634***, 115-120.

[40] Stanjek V, Piel V, Boland W. (**1999**) Biosynthesis of furanocoumarins: mevalonate-independent prenylation of umbelliferone in *Apium graveolens* (Apiaceae). *Phytochemistry*, ***50***, 1141-1148.

[41] Hamerski D, Schmitt D, Matern U. (**1990**) Induction of two prenyltransferases for the accumulation of coumarin phytoalexins in elicitor-treated *Ammi majus* cell suspension cultures. *Phytochemistry*, ***29***, 1131-1135.

[42] Raad I, Terreux R, Richomme P, Matera EL, Dumontet C, Raynaud J, Guilet D. (**2006**) Structure activity relationship of natural and synthetic coumarins inhibiting the multidrug transporter P-glycoprotein. *Bioorganic & Medicinal Chemistry*, ***14***, 6979-6987.

[43] Epifano F, Curini M, Genovese S, Blaskovich M, Hamilton A, Sebti SM. (**2007**) Prenyloxyphenylpropanoids as novel lead compounds for the selective inhibition of geranylgeranyl transferase I. *Bioorganic & Medicinal Chemistry Letters*, ***17***, 2639-2642.

[44] Thompson HJ, Brown SA. (**1984**) Separations of some coumarins of higher plants by liquid chromatography. *Journal of Chromatography*, ***314***, 323-336.

[45] Glowniak K, Bieganowska ML. (**1986**) Effects of modifier and molecular structure of some coumarins on retention in reversed-phase high-performance thin-layer and column chromatography**.** *Journal of Chromatography*, ***370***, 281-292

[46] Bieganowska ML, Glowniak K. (**1988**) Retention behavior of some coumarins in normal-phase high-performance thin-layer and column chromatography. *Chromatographia*, ***25***, 111-116.

[47] Akhtar S, Ahmad M, Ali E, Yamin M, Bhatty MK. (**1988**) Separation of natural coumarins in different solvent systems by thin-layer chromatography on silica gel. *Journal of Scientific and Industrial Research*, ***31***, 307-309.

[48] Feger W, Branduaer H, Gabris P, Ziegler H. (**2006**) Nonvolatiles of commercial lime and grapefruit oils separated by high-speed countercurrent chromatography. *Journal of Agricultural and Food Chemistry*, ***54***, 2242-2252.

[49] Jurd L, King AD, Miharav K. (**1971**) Antimicrobial properties of natural phenols. III. Antimicrobial properties of umbelliferone derivatives. *Phytochemistry*, ***10***, 2965-2970.

[50] Rahalison L, Benathan M, Monod M, Frenk E, Gupta MP, Solis PN, Fuzzati N, Hostettmann K. (**1995**) Antifungal principles of *Baccharis pedunculata***.** *Planta Medica*, ***61***, 360-362.

[51] Mourad Daoubi R, Duran-Patron MH, Rosario Hernandez-Galan R, Benharref A, Collado IG. (**2004**) Screening study for potential lead compounds for natural product-based fungicides: I. Synthesis and *in vitro* evaluation of coumarins against *Botrytis cinerea*. *Plant Management Science*, ***60***, 927-932.

[52] Murakami A, Gao G, Omura M, Yano M, Ito C, Furukawa H, Takahashi D, Koshimizu K, Ohigashi H. (**2000**) 1,1-Dimethylallylcoumarins potently suppress both lipopolysaccharide- and interferon-γ-induced nitric oxide generation in mouse macrophage RAW 264.7 cells. *Bioorganic and Medicinal Chemistry Letters*, ***10***, 59-62.

[53] Pillai SP, Menon SR, Mitscher LA, Pillai CA, Shankel DM. (**1999**) Umbelliferone analogues and their potential to inhibit benzo(*a*)pyrene- and hydrogen peroxide-induced mutations. *Journal of Natural Products*, ***62***, 1358-1362.

[54] Kawaii S, Tomono Y, Ogawa K, Sugiura M, Yano M, Yoshizawa Y, Ito C, Furukawa H. (**2001**) Antiproliferative effect of isopentenylated coumarins on several cancer cell lines. *Anticancer Research*, ***21***, 1905-1911.

[55] Baba M, Jin Y, Mizuno A, Suzuki H, Okada Y, Takasuka N, Tokuda H, Nishino H, Okuyama T. (**2002**) Studies on cancer chemoprevention by traditional folk medicines XXIV. Inhibitory effect of a coumarin derivative, 7-isopentenyloxycoumarin, against tumor-promotion. *Biological & Pharmaceutical Bulletin*, ***25***, 244-246.

[56] Epifano F, Molinaro G, Genovese S, Teke Ngomba R, Nicoletti F, Curini M. (**2008**) Neuroprotective effect of prenyloxycoumarins from edible vegetables, *Neuroscience Letters*, ***443***, 57-60.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1761 - 1776

Stability of Active Ingredients of Traditional Chinese Medicine (TCM)

Wang Meng[ab], Ren Xiaoliang[a], Gao Xiumei[a], Franco Francesco Vincieri[b] and Anna Rita Bilia[b*]

[a]*Tianjin Key Laboratory of Phytochemistry and Pharmaceutical Analysis*, *Tianjin University of Traditional Chinese Medicine*, *Yuquan Road*, *88, 300193*, *Tianjin*, *People's Republic of China*

[b]*Department of Pharmaceutical Sciences*, *University of Florence*, *Via Ugo Schiff*, *6*, *50019*, *Sesto Fiorentino (FI), Italy*

ar.bilia@unifi.it

Received: September 21st, 2009; Accepted: November 9th, 2009

Studies on stability of active ingredients are fundamental and critical for the rational development of Traditional Chinese Medicine (TCM) in view of its modernization and worldwide use. The stability of both active and marker constituents of plants used in TCM is reviewed for the first time. More than 100 papers, mostly written in Chinese, have been reviewed. Studies concerning plant constituents were analyzed according to their chemical classification of active ingredients. In addition, several crude drugs of animal origin are also reported. Stability of active ingredients is summarized during extraction and/or storage of the herbal drug preparations, and under stress conditions (pH, temperature, solvents, light, and humidity) and in the presence of preservatives, antioxidants, and metals.

Keywords: Traditional Chinese Medicine (TCM), active ingredients, stability, alkaloids, phenols, terpenoids.

Traditional Chinese Medicine (TCM), as one of the most representative traditional medicine systems, has contributed significantly to the health of Chinese and East Asian people for over two thousand years [1-7]. Now it still plays an indispensable role in local health care system [8-15]. In recent years, the advantages of TCM for certain diseases have been recognized and studied all around the world [16-19].

In the last two decades active principles and/or marker constituents from plant and animal TCMs have been investigated using an interdisciplinary approach, which includes phytochemistry, analytical chemistry, pharmacology, and molecular biology. In some selected cases, isolated constituents, such as polyphenols, alkaloids, terpenoids, polysaccharides, amino acids, polypeptides and enzymes, have also been developed and marketed as either new drugs or perspective pre-drugs [20-24].

Quality, and consequently safety and efficacy profiles, of Herbal Drugs (HDs) and Herbal Drug Preparations (HDPs) of TCM is strictly related to the presence of the active ingredients in defined amounts. However the integrity of these structures can be susceptible to stress conditions during collection, storage and extraction leading to irreversible chemical modifications, which can cause a dramatic decrease of the activity of the HDs or HDPs or even create toxic constituents. For these reasons, the stability of these active ingredients is considered as the basis of safety and efficacy, as is also stated by the Chinese Pharmacopeia.

In China the current methods to evaluate stability of the constituents of TCM are similar to those reported for synthetic drugs through the guidelines of SFDA (State Food and Drug Administration). These are also similar to those issued by the FDA and ICH and include stress tests, accelerating tests, and long-term stability tests [25-27]. Factors tests (stress tests) observe the changes using various kinetic models under certain stress conditions, such as acidic or alkaline pHs, oxidation, light, and humidity [28].

On the basis of accelerated and long-term stability tests, the expiration of drug substance in the plant material (Herbal Drug, HD) and in the preparations (HDPs) will be set. Furthermore, some characteristic properties such as color, flavor and odor, can also be evaluated, even if these are not reported in the present review.

A variety of well known analytical methods, such as TLC, HPLC-DAD, HPLC-MS, NMR, IR, NIR, and UV-Vis, are employed in the evaluation of the stability of the characteristic ingredients of TCM and track the possible degradation products, to deduce degradation pathways [29-32].

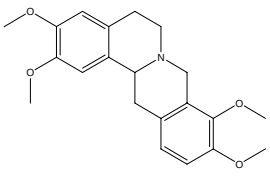
In this paper, for the first time, to the best of our knowledge, the studies concerning the stability of active ingredients represented by the characteristic constituents of herbal drugs and their preparations from TCM are reported and reviewed according to their origin (plant or animal drugs) and chemical classification.

1. Alkaloids

Alkaloids are naturally occurring constituents containing nitrogen [33,34], produced by a large variety of organisms, including bacteria, fungi, plants, and animals and many of them represent the characteristic constituents of TCM possessing diverse physiological activities.

The degradation of alkaloids is mainly caused by oxidation of nitrogen atoms. These reactions may be triggered by environmental conditions, such as light, temperature and moisture.

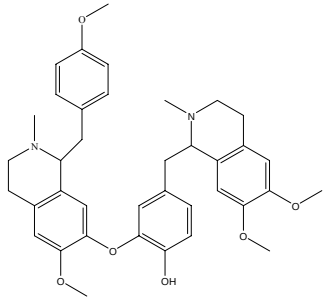
Isoquinoline alkaloids: Tetrahydropalmatine (**1**) is the characteristic constituent of *Rhizoma corydalis* (Yanhusuo), which is the dried tuber of *Corydalis yanhusuo* W. T. Wang. The drug is reported to alleviate pain and as having hypnotizing effects. Xu and coworkers found that tetrahydropalmatine in the dry extract of *Rhizoma corydalis* (Yanhusuo) can degrade at high temperature in the presence of air [35,36].



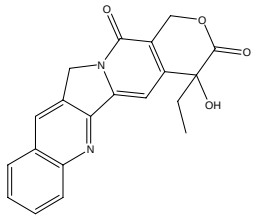
Tetrahydropalmatine (**1**)

The degradation mechanism was oxidative. The reaction rate was extremely slow below 30°C, but increased rapidly above 60°C. The long-term stability tests evidenced that tetrahydropalmatine was stable in the range 10-30°C for 12 months. *Plumula Nelumbinis* (Lianzixin) is a traditional Chinese medicine, which is the dried young cotyledons and ripe seed of *Nelumbo nucifera* Gaertn. This HD has good therapeutic effects on heat-shock, dysentery, diarrhea, and against vomiting. Liensinine, isoliensinine and neferine (**2**) are the main alkaloids from the extract of *Plumula Nelumbinis* (neferine>40%). Neferine is a bisbenzylisoquinoline alkaloid with a phenolic hydroxyl group which is susceptible to oxidation. As the most abundant compound, its stability in liquid extracts was studied by Lu and coworkers [37]. Neferine is unstable at acid and alkaline pHs, and to irradiation, moisture, andFe^{3+}, but is not affected by temperature. Oxidant and acidic media, Fe^{3+} and irradiation could catalyze conspicuously the decomposition reaction. High humidity causes moisture absorption by the dry powder of neferine, which leads to degradation, as occurs in the liquid state. As a consequence, the product should be stored only in cool and dry conditions.

Camptothecin (CPT, **3**) is a well-known anti-cancer constituent isolated from *Fructus Camptothecae Acuminatae* (Xishuguo), which is the fruit of *Camptotheca acuminata* Decne. Thermal and light stability of CPT in phosphate buffers was studied using HPLC and first order derivative spectrophotometry. CPT degradation followed first order kinetics and was catalyzed by alkali (calculated energy was 87.6l KJ/mol). Decomposition by irradiation followed zero order kinetics [38].

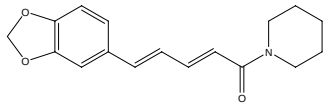


Neferine (**2**)



Camptothecin (**3**)

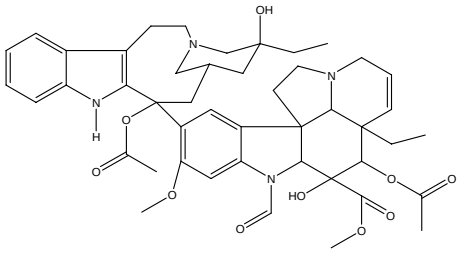
Piperidine alkaloids: Piperine (**4**), a pyrrolidine alkaloid, is the major active ingredient of *Fructus Piperis* (Hujiao), which is the dried fruit of *Piper nigrum* L. It possesses several activities, such as anti-inflammatory, anti-convulsivant, and anti-cancer [39]. High-speed counter current chromatography and atmospheric-vacuum distillation have been applied to extract and purify piperine. The stability studies revealed that piperine was stable in an acidic medium and for a short period of heating (60ºC applied for 2 h) [40]. Packaging was essential



Piperine (**4**)

to preserve stability, due to the significant decrease of piperine content by ambient irradiation [40].

Indole alkaloids: Vincristine (**5**) is very efficacious on various types of acute leukemia and malignant lymphomas, and represents the active constituent of *Herba Catharanthi Rosei* (Changchunhua), which is the whole herb of *Catharanthus roseus* (L.) Don [41]. Zuo and coworkers studied the stability of vincristine sulfate in 0.9% NaCl solution used for injections [42].



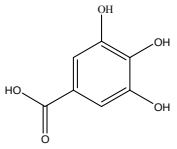
Vincristine (**5**)

The studies indicated that the degradation of vincristine sulfate was a first order reaction ($T_{0.9}$ at 25ºC and 32ºC was 12.51 h and 8.82 h, respectively) concluding that the injectable preparation of vincristine sulfate in 0.9% NaCl solution is stable for 8 hours [42].

2. Polyphenols

Polyphenols are a wide group of secondary constituents, characterized by the presence of one or more phenol units. Polyphenols, mostly derived from the shikimate pathway, are generally classified according to their basic skeletons [43]. It is well known that polyphenols have antioxidant characteristics and potential health benefits, such as anti-cancer, antibacterial, anti-inflammatory, and cholesterol-lowering [44-47].

C6-C1 derivatives: Gallic acid (**6**) is one of the most simple polyphenols, having anti-hepatitis B virus activity. The compound is widespread in food and HDs, such as the Chinese medicine *Radix et Rhizoma Rhei* (Dahuang), which is the dried rhizome and root of *Rheum palmatum* L., *R. tanguticum* Maxim, ex Balf., and *R. officinale* Baill., and from the Chinese medicine *Fructus Corni* (Shanzhuyu), which is the dried flesh of *Cornus officinalis* Sieb. et Zucc. Studies on the stability of gallic acid in aqueous solutions have been carried out by the classical isothermal accelerated test using RP-HPLC [48]. It was found that gallic acid is unstable in basic and neutral aqueous media and under strong oxidizing conditions, but stable in acidic aqueous media, after strong irradiation and high temperature. Protection by antioxidants could strongly affect the stability of gallic acid.

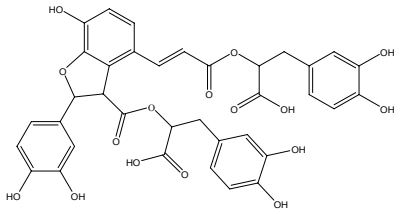


Gallic acid (**6**)

C6-C3 derivatives and their glucosides: C6-C3 derivatives are widely distributed in medicinal plants of TCM, such as *Flos Lonicerae Japonicae* (Jinyinhua) from the family Caprifoliaceae, *Herba Erigerontis* (Dengzhanxixin) and *Herba Taraxaci* (Pugongying) from the family Asteraceae, *Radix et Rhizoma Salviae Miltiorrhizae* (Danshen) from the family Lamiaceae, *Fructus Canarli* (Qingguo) from the family Burseraceae, *Radix Angelicae Sinensis* (Danggui) and *Rhizoma Chuanxiong* (Chuanxiong) from the family Apiaceae [1-5]. In addition, many foods like wine, coffee and tea represent other important sources of these constituents. A variety of biological activities are reported for these constituents such as radical scavenging, anti-inflammatory, antiviral, immunomodulatory, anti-clotting and anti-tumor.

Most of the compounds contain a carboxyl ester bond, which can undergo hydrolysis in alkaline or strong acidic conditions, while they are stable in weak acidic media. Some of them are sensitive to light and heat, probably due to the oxidation of the phenolic hydroxyl groups.

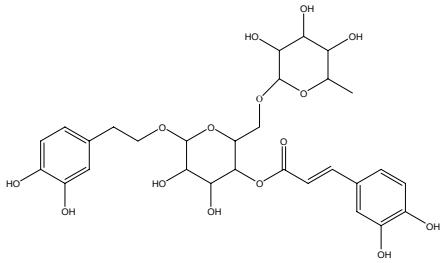
Salvianolic acid B (lithospermic acid B, **7**) is the most representative constituent of this class of constituents, having a good clinical effect on heart, brain, liver and kidney [49].



Salvianolic acid B (lithospermic acid B, **7**)

Chromatography on macroporous resin and semi-preparative HPLC were successfully adopted to isolate salvianolic acid B from *Rhizoma Salviae Miltiorrhizae* (Danshen), which is the dried rhizome and root of *Salvia miltiorrhiza* Bge. The compound, the structure of which consists of four units of caffeic

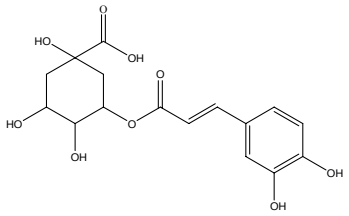
acid, is one of the most important active ingredients of the drug, and many researchers have investigated its stability using various methods [50-56]. Using 1mg/mL aqueous solutions at 20ºC, it has been found that salvianolic acid B is stable under acidic conditions (pH=2), but degraded significantly when the pH value rises to pH> 6. The degradation of salvianolic acid B was related to the pH and temperature of the solutions and followed first order kinetics. Hydrolysis of the ester moiety and ring cleavage of the benzofuranyl unit represented the two main pathways, as found by the degradation products indentified by HPLC-MS and NMR. After hydrolysis, salvianolic acid B can be converted to danshensu and alkannic acid, while protocatechualdehyde represents the product obtained by oxidative degradation. Salvianolic acid E was obtained by opening the ring of benzofurane. Salvianolic acid B had a faster degradation rate in alkaline intestinal juice rather than in simulated acidic gastric juice [50-56].



Forsythoside (**8**)

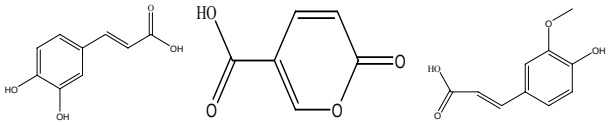
Forsythoside (**8**) is the bioactive constituent of *Fructus Forsythiae* (Lianqiao) from *Forsythia suspensa* (Thunb.) Vahl. It has strong inhibitory effect against various pathogenic bacteria, fungi and viruses [57]. Being a derivative of caffeic acid, forsythoside can be successfully separated with macroporous resins. Both the ester and glucosidic bonds are susceptible to strong acidic and alkaline media, and to high temperature (>80ºC) [58]. If the pH is above 9.40, ester hydrolysis can occur and the degradation products are caffeic acid, D-glucose, and L-isodulcitol. The stability of forsythoside was good at pH=4.03 and lower pHs.

Chlorogenic acid (**9**) is a widespread caffeoylquinic acid ester, well known for many activities, including anti-viral and antihypertensive properties [59]. The stability of chlorogenic acid in the extract of *Flos Lonicerae Japonicae* (Jinyinhua), the dried flower and bud of *Lonicera japonica* Thunb., was investigated using the classical accelerated test. The hydrolysis of chlorogenic acid can be catalyzed by both acidic and basic media [60]. The most stable aqueous solution was found at pH=3.0. After comparison of different organic solvents, ethanol was elected as the best for chlorogenic acid, having a good profile of stability [59].



Chlorogenic acid (**9**)

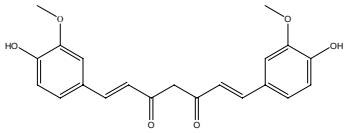
Caffeic acid (**10**) and *p*-coumaric acid (**11**) are two further common active ingredients in a variety of medicinal plants and animals used in TCM. Lu and coworkers studied their stability in a 70% v/v methanol extract of *Spora Lygodii* (Haijinsha), the mature sporophytes of *Lygodium japonicum* (Thunb.) Sw. [61]. Decomposition and structural transformations of these constituents occurred under irradiation, so light was considered a determinant factor concerning their stability. The content of caffeic acid decreased rapidly with increasing temperatures, while minor changes were found for *p*-coumaric acid. Furthermore, in acidic conditions, other phenolic derivatives present in the extract of *Spora Lygodii* could hydrolyze to caffeic acid, which resulted in significant increases in the content of caffeic acid, while the content of *p*-coumaric acid remained unchanged.



Caffeic acid (**10**) *p*-Coumaric acid (**11**) Ferulic acid (**12**)

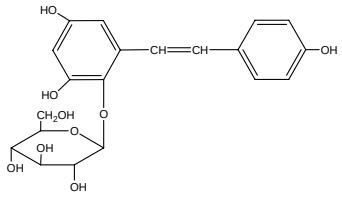
Ferulic acid (**12**) is the active ingredient of a variety of commonly used TCMs, such as *Radix Angelica Sinensis* (Danggui), which is the dried root of *Angelica sinensis* (Oliv.) Diels, and *Rhizoma Chuanxiong* (Chuanxiong), which is the dried rhizome of *Ligusticum chuanxiong* Hort. The compound has several therapeutic effects, including the inhibition of the release of 5-HT from platelets [62]. Ferulic acid is very sensitive to light and decomposes rapidly in 80% v/v ethanol solution after 2 hours decoction. The crude drug of *Rhizoma Chuanxiong* is stable in dry conditions [63-64]. 70% v/v methanol is the best solvent medium for ferulic acid if compared with other organic solvents. In addition, small amounts of glacial acetic acid can significantly stabilize ferulic acid in solution [63-64].

Curcuminoids represent a group of rare dione pigments with a C6-C3 backbone structure, present in *Rhizoma Curcumae Longae* (Jianghuang), the dried rhizome of *Curcuma longa* L. They have anti-inflammatory, antioxidant, anti-lipidemic and anti-cancer activities, widely used in medicine and health food products [65]. Different methodologies have been applied for the isolation of such molecules, including activated charcoal and acid-base extraction. Different studies on the stability of curcumin (**13**) and related molecules, such as demethoxycurcumin and bis-demethoxycurcumin, have been reported under different stress conditions (temperature, pH, metal ions, with different surfactants, oxidizing and reducing agents) [66-68]. Curcumin is photosensitive, and is unstable at high temperature, and in strong acidic or alkaline media. Sucrose, maltose, Zn^{2+}, Fe^{2+} and Fe^{3+} have an hyperchromic effect on its UV-vis absorptions, while discoloration is caused by sodium benzoate, citric acid, tartaric acid and Cu^{2+}. Vitamin C, Na^{+}, K^{+}, and Mg^{2+} have slight effects on curcumin. PEG400 and DMSO could degrade it, and furthermore sodium bisulfite, sodium sulphite, and sodium thiosulfate can significantly accelerate its degradation rate. Glutathione and SDS can stabilise curcumin. Under the same conditions, the stability of curcuminoid derivatives follow the order: bis-demethoxycurcumin>demethoxycurcumin>curcumin. However, bis-demethoxycurcumin and demethoxycurcumin are less active, probably due to their weaker electrophilic reactivity when compared with curcumin, and demethoxycurcumin was found as the natural stabilizer for curcumin [66-68].



Curcumin (**13**)

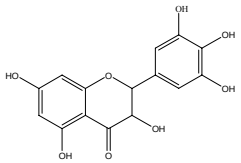
C6-C2-C6 derivatives: Stilbenes are simple phenolic derivatives present in diverse mosses and plants. 2,3,5,4'-Tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG) (**14**) is the active principle of *Radix Polygoni Multiflori* (Heshouwu), which is the dried root of *Polygonum multiflorum* Thunb. [69]. THSG is well known for its anti-aging and hypolipidemic activities and can be isolated pure (99.9%) by liquid-liquid extraction, silica gel column chromatography and repeated crystallizations. THSG in solution (0.1 mg/mL) undergoes rapid degradation in either acidic or strong alkaline conditions, after irradiation and in methanol solutions [70]. Strong acidic conditions result in the hydrolysis of THSG to form glucose and the aglycone, which can further degrade into small molecules. In the presence of strong alkaline media, THSG can be easily oxidized into quinones, whereas in neutral or weak basic conditions it is quite stable.



2,3,5,4'-Tetrahydroxystilbene-2-*O*-β-D-glucoside (THSG) (**14**)

C6-C3-C6 derivatives: Flavonoids have a characteristic C6-C3-C6 backbone structure, and represent an important group of active ingredients of TCM. Flavonoids are commonly known for their antioxidant and radical scavenger activities. However, they are known for many other activities, such as anti-cancer and heart disease [71-72]. The general structure of flavonoids is represented by a 2-phenyl-chromone constituted of three rings (A-C rings) [73].

Dihydromyricetin (**15**) is the characteristic constituent of *Ampelopsis Grossedentata* (Tengcha), the leaf and stem of *Ampelopsis grossedentata* (Hand-Mazz) W.T.Wang, a widely used TCM, traditionally employed to cure dysentery and diarrhea [74].

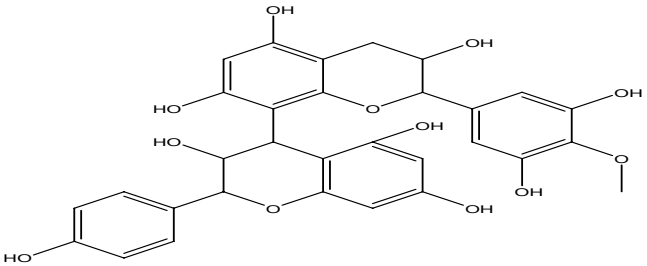


Dihydromyricetin (**15**)

Dihydromyricetin possesses several common activities typical of flavonoids, for example, free radical scavenging, antioxidant, anti-thrombosis, anti-tumor, and anti-inflammatory, and in addition, has excellent liver protecting effects. It can be isolated (99% pure) using high-speed counter current chromatography. HPLC analysis in different aqueous media determined the maximum stability of the compound at pH=4, while oxidative degradation increased with increasing pH values and oxidation was immediate from pH 9 upward.. In addition, strong irradiation and high temperature accelerated the oxidation of dihydromyricetin [75].

Oligomeric proanthocyanidins (**16**) are widely recognized as powerful natural antioxidants, and they are widely spread in various TCMs, for example,

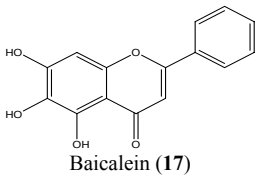
Fructus Crataegi (Shanzha), the dried fruit of *Crataegus pinnatifida* Bge. and *C. pinnatifida* Bge. var. *major* N. E. Br.; *Semen Ginkgo* (Baiguo), the dried seed of *Ginkgo biloba* L.; *Radix Puerariae Lobatae* (Gegen), the dried root of *Pueraria lobata* (Willd.) Ohwi; *Rhizoma Drynariae* (Gusuibu), the dried rhizome of *Drynaria fortune* (Kunze) J. Sm.; *Radix et Rhizoma Glycyrrhizae* (Gancao), the dried root or rhizome of *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., and *G. glabra* L.; *Herba Epimedii* (Yinyanghuo), the dried aerial parts of *Epimedium brevicornum* Maxim., *E. sagiuarum* (Sieb. et Zucc.) Maxim., *E. pubescens* Maxim., *E. wushanense* T. S.Ying, and *E. koreanum* Nakai [76]. Studies carried out on fractions of oligomeric proanthocyanidins (purity 95.0%) extracted from *Semen Vitis Viniferae* (Putaozi), the seed of *Vitis vinifera* evidenced a stability in acidic conditions, but degradation occurred at increasing pH values up to alkaline conditions [77].



A typical oligomeric proanthocyanidin (**16**) of *Semen Vitis Viniferae*

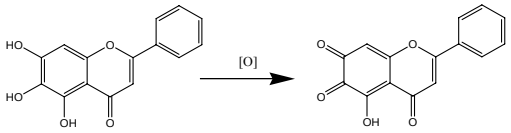
Temperatures above 60°C can also affect the stability of oligomeric proanthocyanidins, while their stability can be increased in the presence of some antioxidants, such as ascorbic acid and Na_2SO_3 [77].

Baicalein (**17**) is another very common flavonoid in TCMs and represents the anti-inflammatory bioactive ingredient of *Radix Scutellariae* (Huangqin), the dried root of *Scutellaria baicalensis* Georgi [78]. Studies with isolated baicalein (purity 97.2%) in different pH media evidenced its stability in acidic conditions, the optimum being pH 4.28 [79-81].



Baicalein (**17**)

In addition, baicalein and flavonoids containing *o*-dihydroxy, *o*-trihydroxy, and a 3,4'-dihydroxy moiety are easily oxidized to colored quinones in alkaline media (Figure 2) [82-83]. Other studies showed that the decomposition of baicalein and its 7-glucuronide



(Yellow) (Green)

Figure 2: Structural changes of baicalein in alkaline media (Ph = 10)

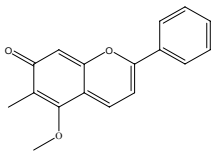
can be accelerated by increasing temperature, especially near to their melting points [84]. Baicalin also has poor stability in the presence of light. The complex between baicalin and zinc showed similar results to those reported for the stress conditions [85].

Similar results have been found by Jing and co-workers for the stability of the total flavonoid fraction of *Fructus Hippophae* (Shaji), the dried fruit of *Hippophae rhamnoides* L. [83]. Different results were found according to reaction time. When the extract was treated with an alkaline solution for less than five hours and then neutralized, the stability did not change significantly when compared with the TFH solution at pH=7, probably due to a reversible reaction of the phenolic moieties to quinone structures. However, an irreversible reaction was observed by UV spectrophotometry when the time of the alkaline treatment was more than 5 hours.

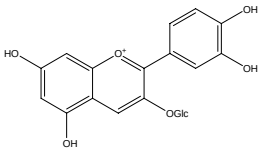
Other studies of the flavonoid fraction of *Cacumen Platycladi* (Cebaiye), the leaf of *Platycladus orientalis* (L.) Franco confirmed the above results. Good stability was found in weakly acidic conditions (pH 5-6) [86-87]. Similar results were also found for flavonoids isolated from *Flos Paulowniae* (Maopaohua) [88-89], whilst the flavonoids of *Herba Tagetis Patulae* (Kongquecao), the whole herb of *Tagetes patula,* were unstable after either irradiation or increased temperature (>60 °C) [90].

Sanguis Draconis (Xuejie), the resin of *Daemonorops draco* Bl., is a valuable Chinese medicine for surgery [91]. Its major bioactive ingredient, dracorhodin (**18**), is structurally related to flavonoids and is unstable if submitted to dry heat [92]. The results of classic thermal accelerated tests indicated that the kinetic constants increased conspicuously with increasing temperatures. A first order kinetic relationship was found by the Arrhenius equation; expiration was set at 2.23 years at 25ºC.

The extract of *Herba Emiliae* (Yidianhong), the stem and leaf of *Emilia sonchifolia* DC., is largely employed for its many pharmacological effects, such as its anti-inflammatory, analgesic and immunological properties [93]. Anthocyanin **19** represents the characteristic constituent of this TCM



Dracorhodin (**18**)



Anthocyanin (**19**)

and can be isolated using microwave extraction. The compound is stable in 80% ethanol solution below 80°C, but very sensitive to acids under boiling water bath conditions, which caused hydrolysis to the aglycone [94]. In addition, the aqueous solution of the anthocyanin is not stable in the presence of sucrose, citric acid, sodium benzoate and potassium sorbate, because of degradation of the chromophore moiety.

Another TCM containing anthocyanins is *Caulis Parthenocissi Tricuspidatae* (Pashanhu), which is the dried stem and root of *Parthenocissus tricuspidata* (S. et Z.) Planch., used for its effects in arthromyodynia and chronic bronchitis [95]. The constituents are very stable in the presence of sodium sulphite, citric acid, and glucose, but unstable in the presence of sodium benzoate and sodium chloride [95].

The anthocyanins from the fresh crude drug *Radix Rehmanniae* (Dihuang), the fresh root of *Rehmannia glutinosa* Libosch., were evaluated for their stability at different pH values and temperatures, under ultraviolet irradiation, and in the presence of metal ions, oxidizing and reducing agents [96]. The complex was stable in mild alkaline conditions, but degraded slightly in the present of ultraviolet light and reducing agents. Heat, oxidants, and several ions, Sn^{3+}, Fe^{2+}, Si^{2+}, Pb^{2+}, Cu^{2+}, also had a great impact on stability, and generated colored sediments [96].

Several studies were carried out to evaluate the stability of anthocyanins of *Gladiolus Gandavensis* (Tangchangpu), the corm of *Gladiotus huhridus* Bort, to sunlight, heat, ultraviolet light (365 nm), acidic and basic solutions, metal ions, reducing agents, oxidizing agents, sugars and preservatives [97]. It was found that these anthocyanins are stable in strong acidic conditions (pH=1) and to heat, while reducing agents, oxidizing compounds, sucrose, and sodium benzoate led to their degradation. In addition, anthocyanins and many other flavonoids can easily form colored complexes with Pb^{2+}, Al^{3+}, Fe^{3+}, Mg^{2+}, and other cations, that oxidized the compounds to quinones, changing the colour of the aqueous

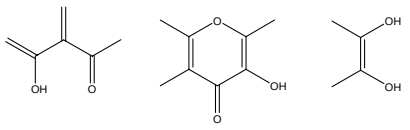
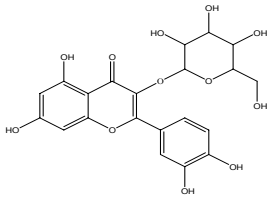


Figure 3: Structural moieties of flavonoids capable of interacting with cations

solutions [97]. The structural units can interact with such cations to give complexes with the OH in the 5 position of ring A, the carbonyl at C-4 of ring C, by the 3-OH and the carbonyl of ring and the catecol moiety of ring B, as shown in Figure 3.

Hyperoside (**20**), the 3-*O*-galactoside of quercetin, represents the main and characteristic constituent of a series of widely used TCMs, including *Herba Hyperici Perforate* (Guanyelianqiao), the whole herb of *Hypericum perforatum* L., *Herba Polygoni Avicularis* (Bianxu), the dried aerial parts of *Polygonum aviculare* L., *Fructus Evodiae* (Wuzhuyu), the dried fruit of *Evodia rutaecarpa* (Juss.) Benth., *Semen Cuscutae* (Tusizi), the dried seed of *Cuscuta chinensis* Lam. *Folium Crataegi* (shanzhaye), the dried leaf of *Crataegus pinnatifida* Bge. var. *major* N. E. Br. and *C. pinnatifida* Bge. These TCMs have numerous therapeutic effects, including decreasing blood lipids, cardiovascular and cerebrovascular protection and immunological properties [98]. Wang and coworkers found that strong acidic conditions (1 M HCl) cause the degradation of hyperoside in the extract of *Herba Hyperici Perforate* [99]. A certain level of degradation of this compound was also detected in the presence of strong oxidants, high temperature and moisture.



Hyperoside (**20**)

Formononetin-7-*O*-β-D-glucoside-6"-*O*-malonate is one of the major flavonoids of *Radix Hedysari* (Hongqi), the dry roots of *Hedysarum polybotrys* Hand.-Mazz., a health food, but also a very famous TCM. The most suitable conditions to optimize the stability of the compound were represented by an acidic aqueous methanol solution at pH=3 [100]. In conclusion, the studies on flavonoids suggested that the optimum conditions for maintaining their stability are represented by low temperature, weak acidity, and protection from light and oxidants.

3. Terpenoids

Terpenoids are widespread constituents in nature, including higher plants, fungi, micro-organisms, insects and marine organisms. Terpenoids represent very important bioactive ingredients of TCM and play an indispensable role in the cosmetic and food industries, due to the aromatic properties of those having smaller molecular weight.

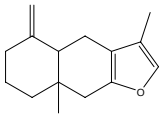
Volatile terpenoids: Volatile terpenoids (mainly mono- and sesquiterpenes) represent the constituents of essential oils, which are generally extracted by steam distillation. Essential oils are very effective TCM ingredients, such as *Herba Menthae* (Bohe), *Herba Ocimi Basilici* (Luole), *Herba Pogostemonis* (Guanghuoxiang) from Lamiaceae; *Fructus Foenicull* (Xiaohuixiang), *Radix Angelicae Sinensis* (Danggui), *Radix Angelicae Dahuricae* (Baizhi), *Rhizoma* Chuanxiong (Chuanxiong) from Apiaceae; *Folium Artemisiae Argyi* (Aiye), *Herba Artemisiae Scopariae* (Yinchen), *Rhizoma Atractylodis* (Cangzhu), *Rhizoma Atractylodis Macrocephalae* (Baizhu) from Asteraceae; *Pericarpium Citri Reticulatae* (Chenpi), *Exocarpium Citri Rubrum* (Juhong), *Semen Citri Reticulatae* (Juhe), *Fructus Aurantii* (Zhiqia), *Fructus Aurantii Immaturus* (Zhishi) from Rutaceae; *Borneolum* (Bingpian), *Cortex Cinnamoni* (Rougui) from Lauraceae; and *Rhizoma Zingiberis Recens* (Shengjiang), *Rhizoma Curcumae Longae* (Jianghuang), and *Rhizoma Curcumae* (Ezhu) from Zingiberaceae.

Various medical applications have been ascribed to the essential oils, such as accelerating blood and lymph circulation, and antihypetensive, antibacterial, antifungal, anti-inflammatory, insecticide, and anti-viral properties.

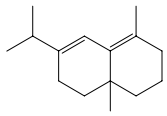
In general, essential oils are unstable towards air and light, and they can resinify, changing in color and viscosity.

The essential oil of *Fructus Evodia* (Wuzhuyu), the dried fruit of *Evodia rutaecarpa* (Juss.) Benth., has good anti-bacterial and analgesic effects [101]. Zhen and coworkers established the relationship between refractive index and degree of oxidation of this essential oil [102]. The degradation follows first order kinetics, as observed by the classic isothermal test. At 293K, the experimental values $t_{0.9}$, $t_{0.7}$, and $t_{0.8}$ were approximately 5 months, 10 months, and 16 months, respectively.

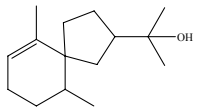
Atractylone (**21**) is the bioactive constituent of *Rhizoma Atractylodis Macrocephalae* (Baizhu), the dried rhizome of *Atractylodes macrocephala* Koidz. and *Rhizoma Atractylodis* (Cangzhu), the dried rhizome of *A. lancea* (Thunb.) DC. and *A. chinensis* (DC.) Koidz. Atractylone represents the marker of several Chinese patented medicines. Pure atractylone (99%) is unstable at room temperature and can be self-oxidized rapidly to butenolide I and butenolide III. Its residual content in the essential oil stored at room temperature is 31% and 15% of the initial value after 0.5 h and 2 h, respectively [103].



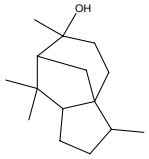
Atractylone (**21**)



β-Selinene (**22**)

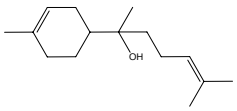


Hinesol (**23**)



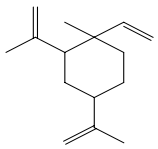
β-Eudesmol (**24**)

Deng and coworkers investigated the instability of the constituents of the essential oil obtained from *Rhizome Atractylodis Macrocephalae*. In the study 17 main constituents were identified by GC/MS. After a long-term storage period (about 6 months), the content of the four main components (total content more than 4%), β-selinene (**22**), hinesol (**23**), β-eudesmol (**24**), α-bisabolol (**25**), and one of the unknown compounds decreased dramatically. Isomerization of some compounds was also observed [104].

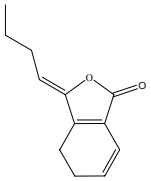


α-Bisabolol (**25**)

β-Elemene (**26**) is a characteristic sesquiterpene of the essential oil extracted from *Rhizoma Curcumae* (Ezhu), the dried rhizome of *Curcuma phaeocaulis* Val., *C. kwangsiensis* S. G. Lee et C. F. Liang and *C. wenyujing* Y. H. Chen et C. Ling [105]. Supercritical carbon dioxide extraction was carried out to obtain elemene mixture, which consists of three isomers, β-, γ- and δ- elemene having similar physico-chemical properties. Sun and coworkers reported that β-elemene is more stable than γ-elemene, which can be converted to β-elemene and other compounds. The stability of each isomer was also related to the different percentages of the three isomers [106].



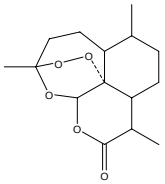
β-Elemene (**26**)



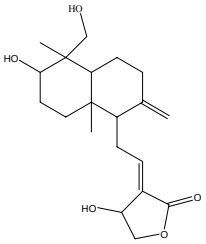
Ligustilide (**27**)

Ligustilide (**27**) is the active ingredient, commonly extracted by steam distillation of many TCMs, including *Rhizoma Chuanxiong* (Chuanxiong), which is the dried rhizome of *Ligusticum chuanxiong* Hort., *Radix Angelicae Sinensis* (Danggui), which is the dried root of *Angelica sinensis* (Oliv.) Diels, and *Rhizoma et Radix Ligustici Sinensis* (Gaoben), the dried rhizome or root of *Ligusticum sinense* Oliv.,and *L. jeholense* Nakai et Kitag,. Ligustilide has spasmolytic and vasodilator activities [107]. In the essential oil of *Rhizoma Chuanxiong*, more than 45 components have been identified by GC-MS analysis, which represent 93.2% of the total constituents [108]. The stability of ligustilide in the volatile oil of *Rhizoma Chuanxiong*, as well as during the extraction process, has been extensively studied. The major constituents, ligustilide (27.4%) and 2-propylene-1-hexanoic acid-3-ene (41.8%), are both unstable during storage (3 months at 37ºC). This was reflected by a decrease of ligustilide and an increase in 2-propylene-1-hexanoic acid-3-ene, this probably being the degradation product of ligustilide. Several isomerization products were also observed [109]. Chloroform and cyclohexane can stabilise ligustilide. Ligustilide (purity>98%) was stable in chloroform at -20°C, if protected from light. Strong acidic/basic solutions can irreversibly modify the constituents during extraction and the best conditions were low-temperatures, dark conditions, and a pH=5 [110].

Iridoids, non volatile sesquiterpenoids and diterpenoids: Iridoids contain a hemi-acetal moiety, which can easily be hydrolyzed and/or oxidized [111]. Iridoids are major constituents of *Herba Hedyotis Diffusae* (Baihuasheshecao), the whole herb of *Hedyotis diffusa* Willd. and *Oldenlandia diffusa* (Willd.) Roxb. Aqueous solutions of iridoids can turn blue in plastic containers and studies have suggested that to preserve the structure of iridoids they need to be stored at temperatures lower than 60°C for not more than 6 hours. Iridoids with carboxyl groups at C-4 were found to be more stable than those having carboxymethyl groups, which can be easily hydrolyzed in acidic media [111].



Artemisinin (**28**)



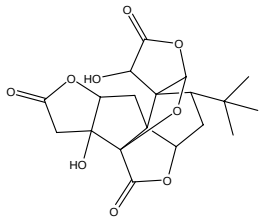
Andrographolide (**29**)

Artemisinin (**28**) is a unique sesquiterpene endoperoxide lactone. It is considered to be the best and most well-known clinically effective drug from a Chinese medicine, *Herba Artemisiae Annuae* (Qinghao), obtained from the dried aerial parts of *Artemisia annua* L. Artemisinin has been successfully used all around the world to cure malaria since the middle of the last century [112]. The highly unusual endoperoxide moiety present in artemisinin, which is essential for the activity, has been focused as the possible degradation target, in addition to the lactone moiety, which is not stable in acidic media. Artemisinin is surprisingly stable in neutral solvents heated to 150°C. Dihydroartemisinin represents the natural metabolite of artemisinin and it is chemically more vulnerable than artemisinin. Artemether, arteether, artesunate and artelinic acid, which are acetal-type prodrugs, are susceptable to moisture and acidic conditions [113].

Several researchers have studied the stability of andrographolide (**29**) and its succinate salt (DAS), respectively [114-116]. Andrographolide is the characteristic constituent of *Herba Andrographis* (Chuanxinlian), the dried aerial parts of *Andrographis paniculata* (Burm.f.) Nees., which can be obtained by supercritical fluid extraction. Both andrographolide and DAS are not stable to either heat or to acidic and alkaline conditions, but are not sensitive to light and oxygen, as reported by HPLC-MS analyses, which led to the characterization of two products of degradation. DAS, in aqueous methanol solution, degraded by 1% at 25°C in one month. The $t_{0.9}$ of andrographolide at 25°C was about 4 months at pH=3, which was found as the most stable condition. Andrographolide was degraded rapidly in protic solvents, but was stable in organic solvents, such as chloroform. In biological media, it was found that the compound was more stable in bovine serum than in mice homogenate [114-116].

Ginkolides are the characteristic and unique trilactone diterpene constituents of *Folium Ginkgo* (Yinxingye), obtained from the leaves of *Ginkgo*

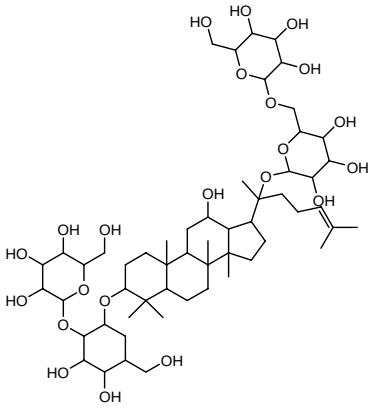
biloba L. Liquid-liquid extraction and supercritical fluid extraction can be adopted successfully to extract these constituents. This herbal drug represent one of the most known and used TCMs, mentioned in the book of Liu Wen-Tai in 1505 AD and has gained wide interest for its biological activities, especially for the treatment of memory related impairments [117].



Ginkgolide B (**30**)

In particular, ginkgolide B (**30**) is the most effective natural inducer of platelet aggregation [117]. Evaluation of stability performed on pure ginkgolide B in an injection solution evidenced that the molecule is tolerant to acidic media, oxidation and light exposure, but can be degraded in alkaline conditions and high temperature [118]. Two degradation products were found in the samples submitted to a long-term stability test (two years) at room temperature; one was recognised as ginkgolide A, and the other an unknown compound with a molecular ion [M-H-H_2O] at m/z 405 in its MS [118].

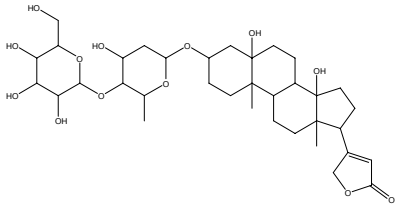
Triterpene and steroidal saponins: Saponins are a large class of secondary constituents found in both vegetable and animal sources. Specifically, they are amphiphilic molecules having a polar part, represented by sugars (one or more moieties), combined with a lipophilic triterpene or steroidal part [119-121]. Steroidal saponins are widely distributed in plants of the Liliaceae and animal sources, while saponins having a triterpenoid skeleton are commonly extracted from plants, for example, species of Araliaceae, Apiaceae, and Fabaceae. Saponins have been shown to have antibacterial, antipyretic, sedative, anti-cancer, cardioactive and corticosteroid effects. Most saponins from TCMs can be separated and isolated with macroporous resins, silica gel and C18 preparative columns. Saponins are stable in mild conditions (pH near neutral and/or temperatures below 50°C), but can be hydrolyzed in strong acidic and alkaline media. Under mild conditions, the stability is quite good and most saponins are stable, such as those of *Herba Gynostemmae Pentaphylli* (Jiaogulan), the whole herb of *Gynostemma pentaphyllum* (Thunb.) Mak.,



Ginsenoside Rb1(**31**)

and momordicosides in *Fructus Momordicae Charantiae* (Kugua), obtained from the fruit of *Momordica charantia* L., and *Radix Panacis Quinquefolii* (Xiyangshen), from the dried root of *Panax quinquefolium* L. [122-126].

In particular, ginsenoside Rb1(**31**), the characteristic and active constituent of *Radix et Rhizoma Ginseng* (Renshen), the dried root or rhizome of *Panax ginseng* C. A. Mry., *Radix Panacis Quinquefolii* (Xiyangshen) and *Radix et Rhizoma Notoginseng* (Sanqi), the dried root or rhizome of *Panax notoginseng* (Burk.) F. H. Chen, isolated by macroporous resins and silica gel chromatography has been extensively studied. Song and coworkers investigated the stability of ginsenoside Rb1 in different pH media, including gastric and intestinal juices [127]. The results showed that ginsenoside Rb1 was relatively stable at intestinal pH, but degraded rapidly at the pH of the stomach.



Periplocin (**32**)

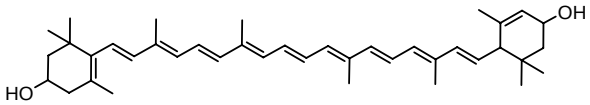
Periplocin (**32**) is a cardiac glycosides form *Cortex Periplocae* (Xiangjiapi), the dried root bark of *Periploca sepium* Bge., a traditional Chinese herb medicine used to treat chronic congestive heart failure [128]. Periplocin (99.5%) was isolated using macroporous resin and silica gel and its stability in mimetic gastrointestinal fluids was investigated by TLC and HPLC-MS analyses [129]. The degradation was pH-dependent, but was not influenced by the presence of pepsin and trypsin. Periplocin hydrolyzed

rapidly to its aglycone, periplogenin, in mimetic gastric fluid in the fasting state, but no decomposition in mimetic gastric fluid, small intestinal fluid and large intestinal fluid in the feeding state was found [129].

Tetraterpenoids (Carotenoids): Carotenoids are characteristic polyisoprenes having conjugated double bonds, well known for their antioxidant proprieties and as pigments. Due to their peculiar structure, carotenoids are very unstable in the presence of light, oxidants or very polar solutions, mainly attributed to photocatalytic oxidation of the chromophore moiety of these molecules [130].

Lutein (**33**) and lutein esters are widely used for their anti-oxidant properties, but also for their therapeutic action on cataract and age-related macular degenerations [131]. Lutein esters can be isolated by supercritical CO_2 fluid extraction from *Flos Calendulae* (Jinzhanju), the dried flower of *Calendula officinalis* L.

Li and coworkers studied the stability of lutein and lutein esters after irradiation, heat treatment, in acidic and alkaline media, and in the presence of metal ions, oxidizing and reducing agents [132]. Generally lutein was less stable than its ester under the same thermal and photostability conditions. The studies showed that irradiation had a great influence on the stability of these molecules and only residual (0.35%) luteolin was present after exposing to sunlight for 0.5 h. Lutein and its esters were stable to heat, even if temperature could accelerate the degradation reaction in the presence of light. It was found that strong acidic media and certain metal ions, like Fe^{3+}, Fe^{2+} and Cu^{2+} could modify these structures. Degradation can also occur, even if slightly, in the presence of H_2O_2; reducing agents (such as Na_2SO_3) have a minor impact [132].



Lutein (**33**)

4. Other constituents of animal origin

Polysaccharides: *Stichopus Japonicus Selenka* (Cishen) is a marine animal medicine used in TCM since the Ming Dynasty for its tonic and replenishing effects. The bioactive ingredient is an acidic mucopolysaccharide (SJAMP) having good anti-tumor, anti-coagulation and immunological enhancing properties [133,134]. The stability of SJAMP at high temperature, humidity and after irradiation was investigated and humidity was ascribed as the main cause of its instability [135].

Polypeptides: Hirudin (Shuizhi) is an antithromboticpeptide composed of 64-66 amino acid residues derived originally from the salivary gland of the medicinal leech, *Hirudo medicinalis* [136]. These acidic, low molecular weight peptides were adopted in Chinese medicine about one thousand years ago to relieve congestion. It was one of the most active specific thrombin inhibitors, with strong anticoagulant, antithrombotic and anti-inflammatory activities. Today, as recombinant hirudin (rHV3), it is used as an inhibitor to prevent phlebothrombosis, and is available on the USA and German markets. Stability of hirudin, based on accelerating and long-term tests was evaluated by its bioactivity. Major determinative factors in its stability were humidity and temperature, while light had a minor effect [136]. Samples under accelerated and long-term experiments maintained their bioactivity.

Ahalysantinfarctasum is a polypetptide consisting of several arginines, characteristic of *Agkistrodon Halys* (Fushe), a TCM widely used for rheumatism. Its chemical stability was assessed by enzyme activity under different temperatures and at different pH values. The inactivation of ahalysantinfarctasum was caused by an acid-base catalytic reaction under first order kinetics. The maximum value of activation energy was found at 55°C and 60°C at pH 6 and 7.6, respectively [137].

5. Conclusions

TCMs have more than 5000 years of history and during these millennia have played and still play an important and indispensable role in treating diseases in the health care for Chinese and East Asian people. This increasing recognition, especially in Europe, is shown by the acceptance of TCM by the European Pharmacopeia and the decision by this institution to organise an ad hoc working group on TCM to develop monographs (more than 70 are expected to be published in the next years). This increasing interest is also reflected by the increasing modernization of TCM and, in particular, the increasing development of stability studies of active ingredients in order to have a rational development of TCMs in view of its worldwide acceptance and use. Stability of active or marker constituents of TCMs from animal or plant sources is reviewed for the first time.. More than 100 papers, mostly of them written

in Chinese, have been reviewed and commented upon. Studies concerning plant constituents were analyzed according to their phytochemical classification of active ingredients. In addition several studies of constituents from animal origin, such as polysaccharides and polypeptides are also reported. Stability of active ingredients as pure drug substances or in the herbal drug or in extracts is summarized during extraction and/or storage of the herbal drug preparations. In addition, the performance of these constituents under stress conditions, including different pHs, storage temperatures, solvents, light and humidity, is reported and, in some cases, the kinetics of degradation are also described. For some active principles used for oral administration, their stability in the presence of simulated gastric or intestinal fluids are reported, while for injectable preparations, the effects of serum proteins, physiological solutions and glucose are described. Finally, the presence of some preservatives, antioxidants, antimicrobials and metals is reviewed, in order to evidence the effects on the drug substances.

Acknowledgments – The work is supported by the Fund of The Ministry of Science and Technology of the People's Republic of China (No. 2007DFG31670).

References

[1] Jiang WY. (**2005**) Therapeutic wisdom in traditional Chinese medicine: a perspective from modern science. *Trends in Pharmacological Science*, ***26***, 558-563.

[2] Li WF, Jiang JG, Chen J. (**2008**) Chinese Medicine and Its Modernization Demands. *Archives of Medical Research*, ***39***, 246-251

[3] Dong XH, Dai RW. (**2003**) Traditional Chinese medicine from the point of view of system science. *Clinical Acupuncture and Oriental Medicine*, ***4***, 34-37.

[4] Feng LJ. (**2002**) Exchange of traditional Chinese medicine between China and Southeast Asian in ancient times. *Southeast Asian Affairs*, ***3***, 8-19.

[5] Zhao PY. (**1997**) Status quo of traditional Chinese medicine and prospect of its development. *Management of Chinese Medicine*, ***7***, 64.

[6] Chen YJ, Jia J. (**2004**) Strategic reconstruction of Chinese medicine for the health benefit of 1.3 billion people-Current development of traditional Chinese medicine and its management reform. *Medicine World*, ***5***, 14-23.

[7] Shi XM. (**2002**) Prospect of the constant development of TCM in the new century. *Tianjin Journal of Traditional Chinese Medicine*, ***19***, 3-4.

[8] Wang ZY, Liu H. (**2000**) Advances in the studies on ventricular premature strike-treated by TCM. *Henan Traditional Chinese Medicine*, ***20***, 71-72.

[9] Zhao LP, Jia HP, Liu JY, Zhao BH. (**1996**) Application of Chinese medicine in ophthalmology. *Nei Mongol Journal of Traditional Chinese Medicine*, ***s1***, 78.

[10] Luo XX, Weng SH, Zhao YH. (**2004**) Application of Chinese medicine in ICU. *Chinese Archives of Traditional Chinese Medicine*, ***22***, 449-451.

[11] Cui KM, Jiang JQ, Wei XM, Wang SJ. (**2001**) The influence of traditional Chinese medicine and herbs on oncogene and antioncogene expression. *Shandong Journal of Biomedical Engineering*, ***20***, 52-55.

[12] Wu XQ, Qin J. (**2007**) Advance in research of the anti aging mechanism of TCM. *Traditional Chinese Medicine Research*, ***20***, 59-61.

[13] Cao Y. (**1994**) Mechanism of TCM with hypoglycemic and immunoregulatory effect. *Acta Chinese Medicine and Pharmacology*, ***1***, 9-12.

[14] Gao JF, Li JS. (**2005**) The present situation in studies on interfering inflammatory cascade reaction caused by cerebral ischemia. *Journal of Henan University of Chinese Medicine*, ***20***, 81-84.

[15] Zhang JD. (**2004**) Overview of the treatment of epilepsy with Chinese medicine. *Chinese Journal of Traditional Medical Traumatology & Orthopeics*, ***s1***, 81-84.

[16] Cao HJ. (**1996**) The international development of TCM treatment to treat cancer. *Journal of Huaihai medicine*, ***14***, 46-47.

[17] Zhou CF. (**2000**) The international superiority of TCM. *Chinese Journal of Information on Traditional Chinese Medicine*, ***7***, 4-5.

[18] Zhou ZK. (**1994**) Opportunities and current status of traditional Chinese medicine on the international arena. *Chinese Journal of Information on Traditional Chinese Medicine*, ***1***, 16-18.

[19] Peng X, Wang YL. (**2004**) The study of symbiosis of traditional Chinese medicine and western modern medicine. *Forecasting*, ***23***, 76-79.

[20] Zhou XZ, Liu BY, Wu ZH, Feng Y. (**2007**) Integrative mining of traditional Chinese medicine literature and MEDLINE for functional gene networks. *Artificial Intelligence in Medicine*, ***41***, 87-104.

[21] Yang M, Sun JH, Lu ZQ, Chen GT, Guan SH, Liu X, Jiang BH, Ye M, Guo DA. (**2009**) Phytochemical analysis of traditional Chinese medicine using liquid chromatography coupled with mass spectrometry. *Chinese Journal of Chromatography A*, ***1216***, 2045-2064.

[22] Shen ML (**1983**) The contribution of traditional Chinese medicine to modern pharmacology. *Trends in Pharmacological Science*, ***4***, 496-500.

[23] Liu CX. (**1987**) Development of Chinese medicine based on pharmacology and therapeutics. *Journal of Ethnopharmacology*, ***19***, 119-123.

[24] Li P, Yang LP, Gong YW. (**2009**) Application of systems biology technology in research of traditional Chinese medicine. *Journal of Traditional Chinese Medicine*, ***29***, 153-157.

[25] ICH, Stability Testing of New Drug Substances and Products (Q1AR), International Conference on Harmonization, IFPMA, Geneva, 2000.

[26] FDA, Guidance for Industry Q1A (R2) Stability Testing of New Drug Substances and Products, Department of Health and Human Services Food and Drug Administration, US, 2003.

[27] SFDA, Guidance for Stability Testing of Chinese Medicine and Nature Drug Substances, State Food and Drug Administration, China, 2006.

[28] Waterman KC, Adami RC. (**2005**) Accelerated aging: Prediction of chemical stability of pharmaceuticals. *International Journal of Pharmaceutics*, ***293***, 101–125.

[29] Gao Y, Lv QJ, Liu YX, Chen P, Tan HL. (**2000**) The study of Chinese prescription on analysis methods. *Chinese New Drug Journal,* ***9***, 307-308.

[30] Liu YM, Xue XY, Xu Q, Zhang FF, Liang XM. (**2008**) Development and comparison of three different methods for the analysis of traditional Chinese medicine. *Journal of Biotechnology*, ***136S***, S75–S98.

[31] Zhou JL, Qi LW, Li P. (**2009**) Herbal medicine analysis by liquid chromatography/time-of-flight mass spectrometry. *Journal of Chromatography*, Available online.

[32] Deng CH, Liu N, Gao MX, Zhang XM. (**2007**) Recent developments in sample preparation techniques for chromatography analysis of traditional Chinese medicines. *Journal of Chromatography A*, ***1153***, 90-96.

[33] Wang FP. (**2008**) *Collection of Natural Product Chemistry-Alkaloid Chemistry.* Chemical Industry Press, Beijing, China.

[34] International Union of Pure and Applied Chemistry. (1995) Alkaloids. *Compendium of Chemical Terminology Internet edition.*

[35] Lv QW, Cong YQ. (**2005**) The pharmacological effect and clinical application of *Rhizoma corydalis. Internal Mongolian Journal of Traditional Chinese Medicine*, ***24***, 889-891.

[36] Xu M, Fu XT, Zhu ZY, Nie J. (**2007**) Thermal stability of tetrahydropalmatine in *Rhizoma corydalis* extract. *Chinese Traditional and Herbal Drugs*, ***29***, 746-747.

[37] Lu DG, Li Y, Liu S, Leng P, Zhu ST, Li XZ. (**2008**) Experimental analysis of stability factors on neferine from total alkaloids extract portion of *Lotus plumule*. *China Journal of Chinese Materia Medica*, ***33***, 2418-2420.

[38] Zhang QM, Gu XQ, Zhang YH. (**1988**) Studies on the chemical stability of S-CPT in phosphate buffer (pH =7.6). *Journal of Shengyang College of Pharmacy*, ***5***, 243-246.

[39] Ma CJ, Bo GRLT. (**2007**) Research progress of pharmacological effect of piperine and its derivates. *Chinese Journal of Traditional Medical Science and Technology*, ***16***, 381-382.

[40] Mo ZR, Zhang Q. (**2006**) Study of the antioxidant effect and stability of piperine. *Journal of Hainan Normal University (Natural Science)*, ***19***, 52-54.

[41] Lu Y, Hou SX, ChenT. (**2007**) Advances in the study of vincristine, an anticancer ingredient from *Catharanthus roseus. China Journal of Chinese Materia Medica*, ***28***, 1006-1009.

[42] Zhou Y, Chi JP. (**1995**) Study on the stability of vincristine sulfate in 0.9% sodium chloride injection. *Chinese Journal of Hospital Pharmacy*, ***15***, 165-167.

[43] Dewick PM. (**1995**) The biosynthesis of shikimate metabolites, *Natural Product Reports*, ***12***, 579-607.

[44] Arts, IC., Hollman PC. (**2005**) Polyphenols and disease risk in epidemiologic studies. *American Journal of Clinical Nutrition*, ***81***, 317S-325S.

[45] Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. (**2004**) Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition*, ***79***, 727-747.

[46] Matuschek E., Svanberg U. (**2002**) Oxidation of polyphenols and the effect on *in vitro* iron accessibility in a model food system, *Journal of Food Science*, ***67***, 420-424.

[47] Zhao YF, Zheng BD. (**2006**) The research progress of polyphenols and their functions in plants. *The Light & Textile Industries of Fujian*, ***11***, 107-110.

[48] Wu XC, Yu BT, Hou AL, Hu TT, Lu GQ. (2006) Study on stability of gallic acid. *Medical Journal of National Defending Forces in Southwest China*, ***16***, 484-485.

[49] Zhan YE, Xu JP. (**2007**) Pharmacological research progress on salvianolic acid B. *Military Medical Journal of South China*, ***21***, 37-39.

[50] Zhang CG, Cui HM, Zhang QY, Wang J. (**2009**) Stability studies of salvianolic acid B in the extract of *Salvia miltiorrhiza* Bge. at different pHs. *Chinese Journal of Experimental Traditional Medical Formulae*, ***15***, 3-5.

[51] Guo YX. (**2007**) Studies on degradation mechanism and purification technique of lithospermic acid B. Ph.D. Thesis, Dalian University of Technology, China.

[52] Lin Q, Huang L, Xiao XL, Chen W, Li CY. (**2008**) Kinetic study of the degradation of salvianolic acid B in extract of *Salvia miltiorrhiza* Bge. *Modern Chinese Medicine*, ***10***, 29-31.

[53] Li ZX, Zou HY, Gong MX. (**2007**) Studies on critical relative humidity and stability of salvianolic acids. *Lishizhen Medicine and Materia Medica Research*, ***18***, 2214-2215.

[54] Chai SW, Pan GX, Zhang YB. (**2006**) Stability investigation of salvianolic acid B in the minosis fluid of digestive tract. *Tianjin Journal of Traditional Chinese Medicine*, ***23***, 332-333.

[55] Lu ZZ, Zhu JB, Liu TC. (**2008**) Influence of pH and additives on stability of salvianolic acid B. *Journal of Dalian Polytechnic University*, ***27***, 209-211.

[56] Zhang J, Wang JY, Zhan LL, Lai XP. (**2005**) Investigation of factors influencing chemical stability of salvianolic acid B in liquid extract of *Salvia miltiorrhiza* Bge. *China Journal of Chinese Materia Medica*, ***30***, 789-790.

[57] Feng SY, Li XR, Sun JN. (**2006**) Study of the effects of forsythoside in infections and decreasing fever. *Progress in Modern Biomedicine*, ***6***, 73-75.

[58] Zhang LW, Yang P. (**2003**) Study on the stability of forsythiaside. *Chinese Traditional Patent Medicine*, ***25***, 353-356.

[59] Wu WH, Kang Z, Ouyang DS, Zhou HH. (**2006**) Progresses in the pharmacology of chlorogenic acid. *Nature Product Research and Development*, ***18***, 691-694.

[60] Chen G, Hou SX, Hu P, He N, Zhu YN, Cao L. (**2003**) Studies on stability of chlorogenic acid in extract of Flos lonicerae. *China Journal of Chinese Materia Medica*, ***28***, 223-226.

[61] Lv HT, Zhao YJ, Deng R, Chen WP. (**2008**) Study on the determination and stability of caffeic acid and coumaric acid extracted from *Lygodium japonicum*. *Qilu Pharmaceutical Affairs*, ***27***, 305-307.

[62] Zhang J, Jin QZ, Wang XG. (**2007**) Research progresses on synthesis and pharmacological activities of ferulic acid and its derivatives. *Cereals & Oils*, ***1***, 43-45.

[63] Xu ZS, Cai BC, Zhang X. (**2004**) Research of the stability of ferulic acid in Rhizoma Chuanxiong. *Research and Practice of Chinese Medicines*, ***18***, 25-27.

[64] He SL. (**2008**) Stability studies of ferulic acid in the extract liquid of *Radix Angelica Sinensis*. *Chinese Traditional Patent Medicine*, ***30***, 1555-1557.

[65] Xu DH Wang S, Jin J, Mei XT, Xu SB. (**2005**) Advances in study pharmacological effects of curcumin. *China Traditional and Herbal Drugs*, ***36***, 1737-1740.

[66] Han G, Cui JJ, Bi R, Zhao LL, Zhang WG. (**2008**) Study on stability of curcumin, demethoxycurcumin and bisdemethoxycurcumin. *China Journal of Chinese Materia Medica*, ***33***, 2611-2614.

[67] Qi LL, Wang JB. (**2007**) Studies on the stability of curcumin. *Science and Technology of Food Industry*, ***28***, 81-82.

[68] Han G, Huo W, Li QY, Sun GL, Duan LT. (**2007**) Studies on the stability of curcumin. *Chinese Traditional Patent Medicine*, ***29***, 291-293.

[69] Wang CY, Zhang LT. (**2008**) The research progress of tetrahydroxyldiphenyl in *Polygonum multiflorum*. *Journal of Hebei Medical University*, ***29***, 157-160.

[70] Ban Y, Liu QL, Jin Y, Wang JL. (2004) Determination of stilbene-glucoside and investigation of its stability. *Chinese Traditional and Herbal Drugs*, ***35***, 1235-1237.

[71] Stauth D. (**2007**) Studies force new view on biology of flavonoids. *EurekAlert!* Adapted from a news release issued by Oregon State University. URL accessed.

[72] Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N. (**2007**) Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: Chemical diversity, impacts on plant biology and human health. *Biotechnology Journal*, ***2***, 1214-1234.

[73] Xiao ZH. (**2001**) Chinese medicine chemistry. Shanghai Science and Technology Publication, Shanghai, China.

[74] Zhong ZX, Tan JP, Zhou GF. (**1998**) Pharmacological study on dihydromyricetin from *Guangxi ampelopsis*. *Journal of Medicine & Pharmacy of Chinese Minorities*, ***4***, 42-44.

[75] He GX, Pei Gang, Li Bin, Ouyang W. (**2007**) Studies on stability of dihydromyricetin. *Chinese Journal of New Drugs*, ***16***, 1888-1890.

[76] Bagchi D, Garga A, Krohn RL, Bagchi M, Tran MX, Stohs SJ. (**1997**) Oxygen free radical scavenging abilities of vitamins C and E, and a grape seed proanthocyanidin extract in vitro. *Research Communications in Molecular Pathology and Pharmacology*, ***95***, 179-189.

[77] Zheng YS, Mo Q, Liao ZD. (**2008**) Study on the stability of proanthocyandin in the seeds of wild *Vitis vinifera*. *Journal of Anhui Agricultural Science*, ***36***, 5259-5260.

[78] Zhang XP, Li ZF, Liu GX. (**2001**) Review of pharmacological studies of baicalein. *Shanxi Journal of Traditional Chinese Medicine*, ***23***, 1127-1129.

[79] Yao YH, Zhang LW. (**2006**) Studies of stability of baicalein. *Chinese Journal of Spectroscopy Laboratory*, ***23***, 346-348.

[80] Tan SX. (**2000**) Stability study of baicalein aqueous solution. *Guangxi Medical Journal*, ***22***, 920-922.

[81] Qiu F, Tang X, He ZG, Li HZ. (**2004**) Stability of baicalein aqueous solution by validated RP-HPLC. *Journal of Pharmaceutical Sciences*, ***13***, 134-137.

[82] Wan CX, Lin YQ. (**2007**) Study on pH stability of scutellaria preparations. *Heilongjiang Medicine Journal*, ***20***, 239-240.

[83] Jin LY, Ling CS, Wang W, Zhang D, Su DL. (**2008**) Study on the stability of total flavonones of *Hippophae rhamnoides* in alkaline solution. *Journal of Henan Univ*ersity (Medical Science), ***27***, 29-31.

[84] Zhu ZH, Yan HM, Wen YJ, Yang Y. (**2006**) Influence of heat in air tight condition on thermostability of baicalin and baicalein. *Henan Science*, ***24***, 353-355.

[85] Feng L, Liu X, Yang H. (**2008**) A study of influencing factors on baicalin- zinc（HBZn） stability. *Journal of Kunming Medical University*, ***5***, 26-28.

[86] Cao YD, Zeng XL, Shan MQ, Ding AW. (**2008**) Progresses in the research of *Cacumen Platycladi. Jiangsu Journal of Traditional Chinese Medicine*, ***40***, 86-88.

[87] Zhao Y, Zhang JP, Zhao YG, Zhou YG, Song AJ, Cao XW. (**2008**) Stability of flavonoids extracted from *Platycladus orientalis* leaves. *Journal of Hebei Normal University of Science & Technology*, ***22***, 12-15.

[88] Meng ZF, Guo XF, Kui W. (**2008**) Preliminary study on the antioxidant activity of flavonoids from *Paulownia tomentosa* Steud flower. *Chinese Journal of Spectroscopy Laboratory*, ***25***, 914-917.

[89] Meng ZF, Guo XF, Jiang Y. (**2008**) Study on the stability of flavonoids from *Paulownia tomentosa* Steud flower. *Chinese Journal of Spectroscopy Laboratory*, ***25***, 655-659.

[90] Zhang R, Xing J. (**2008**) Study of the optimisation of extraction and stability under light or heating of the flavonoids of *Tagetes patula* L. *Food Research and Development*, ***29***, 38-41.

[91] He SP. (**2008**) Pharmacological research on *Resina Draconis. China Pharmacy,* ***19***, 1912-1913.

[92] Xu SQ, Hao Y, Zhao Q. (**2006**) Stability studies of *Resina Draconi*. *Chinese Traditional Patent Medicine*, ***28***, 586-587.

[93] Zheng YX. (2008) Progresses in the pharmacological research of anthocyanin. *Shanxi Medical Journal*, ***37***, 255-257.

[94] Han M, Zhang YH. (**2008**) Study on the ultrasonic extraction of anthocynin from *Emilia sonchifoli* and its stability. *Journal of Henan University of Technology (Natural Science Edition),* ***29***, 29-33.

[95] Diao HP, Sun TJ, Gong H, Ji XL, Yan X, Zhao Y. (**2008**) Stability of red pigment from leafage of *Parthenocissus tricuspidata* Planch. *Journal of Shanxi Medical University*, ***39***, 1009-1011.

[96] Zhang ZL, Zhang QL, Yang HL. (**2007**) Study on pigment extraction from *Rehmannia glutinosa* with ultrasound and its stability. *Food Science*, ***28***, 162-165.

[97] Wei L, Li CX, Gao HN, Jiao Y, Zhang Y. (**2008**) Study on the stability of red pigment in *Gladiolus gandavensis*. *Journal of Qinghai Normal University (Natural Science Edition)*, ***3***, 75-79.

[98] Li MF, Li H, Wang XM. (**2008**) Progresses in the research of pharmacological action of hyperin. *Chinese Journal of Information on TCM*, ***15***, 102-104.

[99] Wang HC, Fan GR. (**2007**) Research on the distinction and stability of hyperinin *Hypericum perforatum* L. extracts. *Lishizhen Medicine and Materia Medica Research*, ***17***, 2544-2545.

[100] Liu, Y, Chen HB. (**2006**) Quantification and stability studies of the flavonoids of *Radix Hedysari. Journal of Agricultural and Food Chemistry*, ***54***, 6634-6639.

[101] Wang SY. (**2008**) Studies on extraction, purification, identification, of chemical constituents, antioxidant and antimicrobial activities of the essential oil from *Fructus Evodia*. Ph.D. Thesis. Huazhong Agricultural University, Wuhan, China.

[102] Zheng P, Liu XH, Liang HH. (**2005**) Extraction of in *Evodia rutaecarpa* (Juss.) Benth volatile oil and investigation of its stablity. *Lishizhen Medicine and Materia Medica Research*, ***16***, 513-514.

[103] Hao YJ, Sang YL, Li BL, Jia TZ. (**2007**) Evaluation of stability of atractylone at ambient temperature. *Chinese Traditional Patent Medicine*, ***29***, 895-896.

[104] Deng SG, Hu XJ, Li WY. (**2008**) Preliminary studies on the instability of volatile oil in *Rhizoma Atractrylodis* with different anti-inflammation effects. *Liaoning Journal of Traditional Chinese Medicine,* ***35***, 3371.

[105] Zhu SL, Huang PF, Wang YF. (**2007**) Progresses in the research of pharmacological effects of *Rhizoma Atractrylodis. Strait Pharmaceutical Journal*, ***19***, 9-11.

[106] Sun H, Lian XH. (**1999**) Study on the stability of β-elemene and γ-elemene. *Shenyang Chemical Industry*, ***28***, 11-13.

[107] Wang CS, Du JR, Qian ZM. (**2006**)) Progresses in the research of ligustilide. *Chinese Pharmaceutical Journal*, ***41***, 889-891.

[108] Shi LF, Deng YZ, Wu BS. (**1995**) Studies on the constituents and their stability of the essential oil from dry *Rhizome Ligusticum chuanxiong* Hort. *Chinese Journal of Pharmaceutical Analysis*, ***15***, 26-30.

[109] Zhan Aijun. (**2008**) The extraction and stability of ligustilide. *Journal of Liaoning University of TCM*, ***10***, 162-163.

[110] Li GS, Ma CJ, Li XY, Liu K. (**2000**) Studies on the stability of ligustilide and the analysis of its isomerized products by GC-MS. *Chinese Traditional and Herbal Drugs,* ***31***, 405-407.

[111] Li CM, Xu Q, Xue XY, Zhang FF, Liang XM. (**2007**) Stability of iridoid glycosides in *Hedyotis Diffusa*. *World Science and Technology/ Modernization of Traditional Chinese Medicine and Materia Medica*, ***9***, 51-55.

[112] Xu XX, Zhu SH, Lin QS. (**1986**) Pharmacological action of arteannuin. *Guangdong Medical Journal*, ***7***, 25-28.

[113] Jung M, Lee S. (**1998**) Stability of acetal and non-acetal-type analogs of artemisinin in simulated gastric acid. *Bioorganic & Medicinal Chemistry Letters*, ***8***, 1003-1006.

[114] Yu BT, Zhang ZR, LiuWS, Yang T, Wang P (**2008**) Study on the stability of the compatibility of kalii dehydrographolidi succinas injection with other drugs. *China Foreign Medical Treatment,* ***33***, 74-75.

[115] Hou QY, Dai Z, Lin RC. (**2008**) Study on stability of dehydroandrographolide succinate. *Chinese Journal of Pharmaceutical Analysis*, ***28***, 752-754.

[116] Lv HT, Wang YL, Wang YQ. (**2002**) Study on stability *in vitro* of andrographolide. *Chinese Traditional Patent Medicine,* ***24***, 331-333.

[117] Xia SH, Fang DC. (**2007**) Pharmacology and mechanism of action of ginkgolide B. *China Medical Journal,* ***120***, 922-928.

[118] Song YQ, Liao J, Tao HH, Li SF, Mei SC. (**2008**) Study on stability of ginkgolide B in sodium chloride injection. *Modern Instruments,* ***1***, 31-33.

[119] Hostettmann K, Marston A. (**1995**) *Saponins,* ISBN 0-521-32970-1. Cambridge University Press, UK.

[120] Suzuki H, Achnine L, Xu R, Matsuda SPT, Dixon RA. (**2002**) A genomics approach to the early stages of triterpene saponin biosynthesis in *Medicago truncatula. Plant Journal,* ***32***, 1033-1048.

[121] Waller GR, Yamasaki K . (**1996**) Biological effects of feed and forage saponins and their impacts on animal production. *Advances in Experimental Medicine and Biology*, ***405***, 337-385.

[122] Xu P, Li Y. (**2008**) Saponins used in food and agriculture. *Chinese Journal of Modern Drug Application*, ***2***, 58-59.

[123] Wang QZ, Ding QL, Li Y. (**2008**) Stability study of saponins of *Herba Gynostemmae Pentaphylli* in jiaogulan capsule. *Chinese Journal of Modern Drug Application*, ***2***, 84-85.

[124] Li J, Huang Y, Liu N, Chen SJ, Liu X. (**2008**) Study of stability of momordicosides. *Food Science*, ***29***, 109-111.

[125] Ning N, Yang XC, Chen J. (**2005**) The study of the stability of β-aesine sodium under heat. *Nanfang Journal of Nursing,* ***12***, 10-11.

[126] Stability studies of saponins from the stems and leaves of *Panax quinquefolium* L.. *Northwest Pharmaceutical Journal*, ***10***, 30-32.

[127] Song L, Zhang CN, Guo SR, Xu QF. (**2004**) Quantification and stability of ginsenoside Rb1. *Chinese Journal of Clinical Pharmacy*, ***13***, 211-213.

[128] Li YH, Gao XM, Zhang BL, Xu Q, Liu H, Pan GX. (**2005**) Effect of the medicine xiangjiapi on cardiac function of isolated hearts. *Journal of Liaoning College of TCM*, ***7***, 396-397.

[129] Wang Y, Ren XL, Pan GX, Qi AD, Gao XM. (**2007**) Stability of periplocin in the mimetic digestive fluid. *Journal of Tianjin University of Traditional Chinese Medicine*, ***26***, 74-76.

[130] Zepka LQ, Mercadante AZ. (**2009**) Degradation compounds of carotenoids formed during heating of a simulated cashew apple juice. *Food Chemistry*, ***117***, 28-34.

[131] Wang MF, Lin XM. (**2008**) Protective role of lutein on light-damage of retina. *Journal of Hygiene Research*, ***37***, 115-117.

[132] Li DJ, Fang GZ, Liu CQ, Yu JP. (**2007**) Stability of lutein and its esters. *Chemistry and Industry of Forest Products*, ***27***, 112-116.

[133] Hu RJ, Yu SP, Jiang H. (**1997**) The inhibitory effect of SJAMP combined with cortisone on murine solid tumors.. *Cancer,* ***16***, 422-424.

[134] Wang JF, Pang L, Xue Y, Dong P, Sheng WJ, Xue CH. (**2008**) Protective effects of polysaccharides, triterpene glycosides and collagen polypeptides from *Apostichopus japonicus* on vascular endothelial cells. *Chinese Pharmacological Bulletin,* ***24***, 227-232.

[135] Liu GM, Zhao XM, Chen JD, Hu RJ. (**2004**) Study of the determination method and stability of acidic mucopolysaccharide in Stichopus Japonicus Sekenka. *Hebei Medical Journal,* ***26***, 398-399.

[136] Teng FF, Hang CB, Jiang WQ, Ping QN. (**2008**) Stability of recombinant hirudin for injection. *Chinese Journal of Biochemical Pharmaceutics*, ***29***, 334-336.

[137] Hu LC, Li B, Chen CP. (**1991**) A study of chemical stability of ahalysantinfarctasum. *Journal of Shenyang College of Pharmacy,* ***8***, 32-35.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1777 - 1784

Pharmaco-Toxicological Aspects of Herbal Drugs Used in Domestic Animals

Rosario Russo[a], Giuseppina Autore[b] and Lorella Severino[a,*]

[a]*Department of Pathology and Animal Health*, *University of Naples Federico II*, *via Delpino 1*, *80137*, *Naples*, *Italy*

[b]*Department of Pharmaceutical Sciences*, *University of Salerno*, *via Ponte Don Melillo*, *84084*, *Fisciano (SA)*, *Italy*

lorella.severino@unina.it

Received: July 31st, 2009; Accepted: November 4th, 2009

Herbal drugs are more and more used both in human and veterinary medicine to mitigate and prevent minor diseases and to support conventional medicine using allopathic drugs. Nevertheless, 'natural product' does not mean lack of adverse effects, and many people and veterinarians do not know enough about the adverse reactions that can occur following the administration of such drugs in domestic animals. Moreover, herbal products can interact with each other when administered concomitantly or can agonize or antagonize the effects of synthetic drugs administered as primary therapy. The use of non-conventional medicines (NCM) should be considered as a veterinary practise. In this paper, the herbal drugs most utilized in domestic animals, both pets and large animals, are reviewed, as their use is increasing, despite the prejudices of the academic world and some of the adverse effects and interactions that can occur in domestic animals.

Keywords: phytotherapy, adverse reactions, domestic animals, dog, cat.

Introduction

In recent years, the use of non-conventional medicines (NCMs) in humans has been increasing all over Europe, particularly in the United Kingdom, France, Germany and Switzerland. It is estimated that from 70 to 80 per cent of the world's population uses NCM, such as phytotherapy, homeopathy, acupuncture and homotoxicology, to treat several diseases [1]. The number of both doctors and veterinarians using NCM is increasing in Italy too; moreover, the number of people who request for either themselves or their pets such practices is higher and higher.

Phytotherapy represents one of the most used NCMs in both human and veterinary medicine. It utilizes plants, parts of them such as flowers, leaves, roots, and seeds, and substances extracted from them to treat many different minor diseases. Phytotherapy is also often utilized to support traditional treatment with synthetic drugs.

Phytotherapy is a very ancient practice; medical plants or extracts of vegetable origin have been utilized by Asian populations for many centuries as natural remedies for the treatment of several illnesses, while native Americans used *Echinacea* spp. to treat cold and flu symptoms, wounds and snake bites, and to reduce inflammation because of its anti-inflammatory, antibiotic and healing properties [2].

Medicinal plants used in veterinary practice

Medicinal properties of the plants used in phytotherapy are due to the large amount of active compounds that can be found in the vegetable kingdom. Often, active principles extracted from plants are equivalent to synthetic drugs according to their therapeutic efficacy; for this reason they are utilized in veterinary medicine, mainly as antibacterial, antimycotic, antiparasitic, disinfectants and immunostimulants (Table 1).

According to Viegi *et al*. [3], who carried out a veterinary ethno-botanical study in Italy, large animals (cattle, horses, sheep, goats and pigs) represent 70.5% of domestic animals treated with herbal remedies in Italy, followed by poultry (9.1%), dogs (5.3%) and rabbits (4.3%).

Table 1: Main plants used in veterinary phytotherapy.

Scientific name	Used parts	Use	Species	Reference
Allium sativum	Cloves	Endoparasites	P; LA	6
Aloe vera	Latex	Skin diseases; Gastrointestinal diseases	LA	43
Arctostaphylos uva-ursi	Leaves	Urinary diseases	P	44
Artemisia spp.	Aerial parts	Gastrointestinal diseases; Endoparasites	P; LA	6 ; 23
Avena sativa	Aerial parts	Mastitis	LA	3
Calendula officinalis	Leaves; Flowers	Wounds; Gingivitis	P; LA	45
Capsella bursa pastoris	Aerial parts	Hemorrhages, Reproductive disorders	LA	3
Carica papaya	Seeds; Latex	Endoparasites	P; LA	25; 46
Cichorium intybus	Aerial parts	Endoparasites	LA	26
Crataegus oxyacantha	Leaves; Flowers	Cardiotonic	P; LA	47
Cucurbita pepo	Seeds	Endoparasites	P	6
Cynara scolymus	Leaves	Hepatic diseases; Gastrointestinal spasms	P	48
Dorycnium spp.	Aerial parts	Endoparasites	LA	20
Echinacea spp.	Root; Aerial parts	Immunostimulant; Wounds	P; LA	9
Eucalyptus globulus	Leaves; Essential oil	Ectoparasites; Respiratory diseases	P	49
Euphrasia officinalis	Aerial parts	Eye affection	P	6
Genziana lutea	Root	Gastrointestinal diseases	P	6
Glycyrrhiza glabra	Root	Gastrointestinal diseases; Otitis	P	10
Gossypium spp.	Leaves	Endoparasites	P	46
Hedera helix	Leaves	Placental retention	LA	3
Hypericum perforatum	Flowers	Wounds	LA	3
Juniperus communis	Aerial parts; Oil	Skin diseases; Ectoparasites	P	10
Lavandula officinalis	Essential oil; Stems	Ectoparasites; Wounds	LA	10
Lotus corniculatus	Aerial parts	Endoparasites	LA	26
Malva sylvestris	Aerial parts	Immunomodulation; Respiratory diseases	P; LA	50
Matricaria chamomilla	Flowers	Eye Inflammation; Ear problems	P	12
Melissa officinalis	Leaves	Anxiety; Stress	P; LA	14
Mentha piperita and *M. cardifolia*	Leaves; Essential oil	Ectoparasites and Endoparasites	P; LA	51
Onobrychis viciifolia	Aerial parts	Endoparasites	LA	19
Passiflora incarnata	Aerial parts	Anxiety; Hormonal imbalance	P ; LA	52
Plantago major	Leaves	Gastrointestinal diseases; Wounds	P; LA	53
Rosa canina	Hip	Inflammation	P	54
Ruta graveolens	Leaves	Ectoparasites	LA	43
Salvia officinalis	Leaves; Flowers	Endoparasites; Dehydration	P; LA	6
Silybum marianum	Fruits	Hepatic diseases	P	55
Taraxacum officinalis	Root; Leaves	Gastrointestinal diseases	P; LA	6
Thymus vulgaris	Flowers	Respiratory and gastrointestinal diseases	P	46
Tilia cordata	Flowers	Respiratory diseases	P	46
Urtica dioica	Seeds	Endoparasites; Diarrhea	LA	43
Valeriana officinalis	Root	Analgesic; Stress	P; LA	14
Zingiber officinale	Rhizomes	Vomiting	P	7

Table legend: P, pets; LA, large animals.

Table 2: Adverse reactions of some medicinal plants and plant products in domestic animals.

Scientific name	Adverse effects	Reference
Allium sativum	Antiplatelet effect; Hematologic disorders	35; 36
Artemisia absinthum	Convulsions	38
Echinacea spp.	Hepatotoxicity	29
Ephedra spp.	Hyperactivity, tremors, seizures, behaviour changes, vomiting, tachycardia, hyperthermia	41
Juniperus sabina	Gastrointestinal and respiratory disorders; haemorrhages	11
Mentha piperita	Hepatotoxicity	39
Rubus idaeus	Reproductive disorders	42

Regarding the large animals, phytotherapy is mainly utilized in the organic farms to reduce more and more the use of allopathic drugs. In the organic farms, in fact, not only herbal drugs, such as plant extracts and essential oils, but also homeopathic products, nutraceuticals and oligoelements, such as sodium, calcium, phosphorus, magnesium, and sulphur are considered the main drugs to administer to animals for the treatment of different diseases. Nevertheless, it is possible to use synthetic allopathic drugs only when the previous products are ineffective; in such eventuality, it is necessary to prefer drugs that are metabolized rapidly, with a low environmental impact and less adverse effects in the animal organism [4].

Herbal drugs usually used in human practice are often utilized in pets, in particular by owners that used such remedies for themselves. They are given to companion animals to treat respiratory, skin, urinary, digestive, and cardiovascular affections, and to reduce stress (Table 1); moreover, they are also used to treat some chronic diseases instead of conventional drugs in order to avoid adverse effects that sometimes

could occur as a consequence of a prolonged administration of synthetic drugs. Finally, phytotherapy could represent a useful support to conventional therapies in the case of severe illness [5].

Although the utilizing of phytotherapeutic products is increasing in companion animals, there are few studies and clinical trials reported in the literature regarding the therapeutic use of phytomedicine in pets. We collected some information by local veterinarians and reported some examples of plants and plant products used in the clinical practice for the treatment of different minor diseases (unpublished data). Mother tincture obtained from *Calendula officinalis, Centella asiatica* and *Commiphora myrrha* can be useful in the case of gingivitis; *Euphrasia officinalis* is commonly used in the treatment of conjunctivitis. Camomile infusion, carrot juice and a 10% rice decoction are often used to treat diarrhea in pets, while thyme (*Thymus vulgaris*) essential oil (3 drops body weight until remission) or syrup (2 teaspoons 3 times per day until remission) are prescribed for the treatment of cough in both dogs and cats. Finally, lemon-balm (*Melissa officinalis*), valeriana (*Valeriana officinalis*) and hawthorn (*Crataegus oxyacantha*) mother tincture (1 drop/kg body weight 2-3 times per day) are often utilized for the treatment of anxiety, to reduce stress and control behavioral or psychological problems in pets.

Lans and colleagues [6] claim that an infusion of peppermint (*Mentha piperita*) and lemon-balm (*Melissa officinalis*) administered orally to dogs for 2 days is useful to treat stomach and intestinal illness; while *Aloe vera* juice (3 mL orally), made by pulverizing the leaf gel in water, represents an effective remedy for vomiting and irritation. An *in vivo* study revealed that the acetone and ethanolic extracts of ginger (*Zingiber officinale*) exerted a significant antiemetic effect in dogs treated with doses of 100 and 200 mg/kg orally, respectively [7]. These results have been recently confirmed by Lans *et al*. [8], who showed that ginger infusion can be administered to pregnant dogs to treat nausea and vomiting.

Significant reductions in the severity and resolution of clinical symptoms, such as clear nasal secretions, enlargement of lymph nodes, dry cough, dyspnea and dry lung sounds, are evident after 4 weeks of treatment with *Echinacea* powder, suggesting this preparation as an alternative remedy for canine upper respiratory tract infections [9].

Many plants are used in domestic animals for their anti-parasitic activity. A spray made with one to five drops (0.25 mL) of essential oil of juniper (*Juniperus communis*) and water represents an effective flea repellent to be used for topical treatment of these parasites in dogs and cats [10], although this plant could be dangerous for domestic animals, if ingested [11]. An *in vitro* study revealed that a 10% decoction of dried flower heads of *Matricaria chamomilla* showed 100% acaricidal activity when tested against the mite *Psoroptes cuniculi*, one of the agents responsible for otoacariasis in domestic animals. For this reason, chamomile is suggested for ear problems in pets [12].

While few studies have been carried out to evaluate the therapeutic efficacy of herbal remedies in companion animals, many studies have been found in the literature relating to the use of plants and plant materials in farm animals. A lot of plants, such as *Brassica oleracea*, *Avena sativa*, *Anagallis arvensis*, *Linum usitatissimum*, *Scrophularia canina* and *Buxus sempervirens* are often used by farmers in many regions of Italy to either treat or prevent mastitis in cattle because of their anti-inflammatory and emollient properties [3]. An *in vivo* study showed that Echinacea extracts administered to horses for 42 days not only enhanced the immune system (increased phagocytic ability of isolated neutrophils, boosted peripheral lymphocyte counts and stimulated neutrophil migration from peripheral circulation into the tissues), but also improved the quality of blood by increasing hemoglobin levels and the number of erythrocytes. Therefore, considering the following effects on oxygen transport cells, *Echinacea* spp. could be considered to improve parameters of exercise physiology and performance [13].

Peeters *et al.* [14] suggested sedative and antianxiety effects of a commercial herbal product containing *Valeriana officinalis* and *Passiflora incarnata* in pigs during transport stimulation. In particular, it was found that the administration of 2.5 g/L drinking water of the herbal product for two days resulted in significantly smaller increases in some heart parameters (minimum heart rate; ventricular ectopic beats; ST elevation) during and after stress evocation compared with the control group.

Many plants are used to alleviate anxiety in domestic animals, especially in horses, such as leaves of magnolia (*Magnolia acuminata*) and flowers of passion flower (*Passiflora incarnata*). Alternatively,

lavender (*Lavandula officinalis*) infusion or tincture was given in water or placed on the feed. For nervousness and restlessness, flowers of chamomile (*Matricaria recutita*, *M. chamomilla*) were added to the feedstuffs. One or two teaspoons of valerian (*Valeriana officinalis*) ground root was given to horses that froze in stressful situations. In addition, one teaspoon of powdered valerian, hops (*Humulus lupulus*) and skullcap (*Scutellaria lateriflora*) was put into the feed, twice a day [15].

Most of the studies found in the literature focus on the anti-parasitic effects of different plants, such as *Allium sativum*, *Anethum graveolens*, *Eucalyptus globulus*, *Mentha piperita*, *Lavandula officinalis* and forages in livestock, especially in ruminants [16-21]. Such effects seem to be due to essential oil, secondary metabolites and other active substances such as terpenes, alkaloids, glycosides and tannins contained in the medicinal plants [22].

A number of medicinal plants, plant extracts and other products of vegetable origin used for the treatment of parasitism in domestic animals have been studied and reviewed. *Artemisia absinthium* extracts (2 g/kg body weight orally) represent an effective natural anthelmintic agent for the treatment of gastrointestinal nematodes of sheep causing a significant reduction in fecal egg counts [23]. The active principle is santonin, a sesquiterpene lactone, which has a selective toxic action, particularly against nematodes [24]. The oral administration of 8 g/kg body weight of papaya latex (*Carica papaya*) to pigs naturally infested with *Ascaris suum* caused a reduction of parasitic burden by up to 100% seven days after the treatment [25]. Finally, some forages such as sulla (*Hedysarum coronarium*), chicory (*Chicorium intybus*), alfalfa (*Medicago sativa*), and lotus major (*Lotus pedunculatus*) exert anti-parasitic properties, which are likely to be related to the content of tannins and other active secondary metabolites [21, 22]. In particular, Marley *et al.* [26] showed that lambs naturally infected with helminth parasites had a lower parasitic burden after grazing birdsfoot trefoil (*Lotus corniculatus*) and chicory for 5 weeks than sheep grazing other forages (*Loluim perenne/Trifolium repens*).

Nevertheless, some studies showed no significant differences in parasite levels between animals fed with forages supposed to have anti-parasite properties and the control group [27; 28]. However, it is very important not to exceed a tolerable daily intake because many of the active principles contained in some medicinal plants could be toxic to domestic animals, in particular for grazing herbivores that can ingest large amount of vegetables. Moreover, some substances contained in plants and forages can act as antinutritional factors and reduce the absorption of nutrients with consequent loss of body weight and reduction of food intake and performances [11].

Toxicological aspects of medicinal plants

Adverse reactions, often completely un-known to people that use herbal drugs, have been reported for many medicinal plants, although these are well known for their efficacy (Table 2) [29, 30]. In fact, the opinion that medicinal plants are mostly harmless ("*natural = safe*") is widespread, not only in the normal population using phytotherapy, but also in practitioners working in this field. As a result, medicinal plants are often used in self-medication without consulting a doctor. Sometimes, many people that use herbal drugs for themselves also administer such products to their pets without the prescription of a veterinarian. These habits increase the risk of adverse reactions, such as allergy. Drug interactions, although infrequent, can also occur between herbal drugs and synthetic ones or with concomitantly used herbal therapies. Other specific contraindications could be represented by pre-existing pathologies (for example, peptic ulcer, kidney and hepatic failure) or surgery that could increase the risk of adverse effects by modification of the kinetics of the active principles. Finally, the quality is very important for the safety of herbal drugs; in fact, adverse effects could occur because of the presence of residues of environmental pollutants (heavy metals, mycotoxins, radionuclides) in the phytotherapeutic product [31].

In 1997, the American Herbal Products Association (AHPA) proposed a classification of the medicinal plants into 4 classes: the first class includes plants with a large margin of safety, such as calendula, hawthorn, euphrasy, lavender, taraxacum, nettle, valerian, camomile, echinacea, peppermint, lemon-balm, and thistle; the second class, further subdivided into four subclasses, includes herbs for which some limitation exists, such as artemisia, St. John's wort, sage, liquorice; the third class includes herbs for which scientific evidence exists to make necessary the supervision of a specialized practitioner; finally, the fourth class includes all herbs not yet classified in the previous classes [32].

Many studies can be found in the literature regarding the possible interactions (in relation to botanical species, dose, treatment) between the active principles of a specific phytocomplex and synthetic drugs [29, 33]. Garlic, reducing the production of thromboxane B2, could increase the fibrinolytic activity and induce an antiplatelet effect [34]. For this reason, the contemporary administration of garlic and anticoagulants is discouraged. Oxidative damage in the erythrocytes with formation of Heinz bodies and eccentrocytes appeared in dogs after the administration of garlic extract (1.25 ml/kg body weight) for 7 days [35]. Additionally, horses fed freeze dried garlic at 0.4 g/kg per day showed Heinz bodies anemia [36]. Liquorice induced an increase of serum levels of corticosteroids [37] and caused a reduction of circulating concentrations of salicylates. It has also been reported in the literature that the effects of immunosuppressant drugs could be antagonized by plants with immunostimulant activities, such as echinacea, astragalus and liquorice [38]. Some species of *Echinacea* contain pyrrole alkaloids that increase the risk of hepatic toxicity inducing the depletion of glutathione, particularly in patients treated with paracetamol [29]. Therefore, in cases of co-administration, it is necessary to monitor possible signs of hepatotoxicity in the patients [33]. The American Society for the Prevention of Cruelty to Animals (ASPCA) recorded 45 calls from 1992 to 2000 for accidental ingestion of drugs containing *Echinacea* spp. in pets: a few animals showed symptoms, including vomiting, sialorrhea, and erythema.

The oil obtained from *Mentha piperita* is currently used in veterinary medicine as a flea repellent. Peppermint oil is composed primarily of menthol and menthone; other minor constituents include pulegone, menthofuran and limonene. *In vivo* studies showed that pulegone is hepatotoxic for rabbits and is able to induce lesions in the cerebellum of rats at a dose of 200 mg/kg body weight [39]. Moreover, Sudekun *et al*. [40] found that pennyroyal, an oil derived from *Mentha pulegium* and *Hedeoma pulegoides* that contains pulegone, was associated with toxic effects (vomiting, diarrhea, hemoptysis and hepistaxis) in a dog treated for fleas. The dog died within 48 hours of treatment.

Ooms *et al*. [41] described the clinical signs following the ingestion of a herbal supplement containing guarana and ma huang (*Ephedra* spp.) in 47 dogs. Most dogs (83%) developed signs of toxicosis (hyperactivity, tremors, seizures, behavior changes, vomiting, tachycardia, hyperthermia) and 17% of intoxicated dogs died. Estimated doses of guarana and ma huang ranged from 4.4 to 296.2 mg/kg body weight and 1.3 to 88.9 mg/kg body weight, respectively.

Wormwood (*Artemisia absinthium*) could be dangerous for domestic animals, particularly in ruminants where it is used for the treatment of gastrointestinal nematodes [23]. An *in vivo* study showed that the intravenous injection of thujone, a toxic compound found in wormwood, induces convulsion (40 mg/kg body weight) and death (120 mg/kg body weight) in rats [38].

Infusion of raspberry (*Rubus idaeus*) leaf is sometimes used for pregnancy support, postpartum supplement and to tone uterine muscles in companion animals [8]. An *in vivo* study carried out by Johnson *et al*. [42] to evaluate the consequences of the administration of raspberry leaf to Wistar rats (10 mg/kg per day until parturition) revealed that this remedy was associated with altered reproductive development and functions (increased gestation length, accelerated reproductive development, time to vaginal opening and lower birth weight) in the offspring.

Conclusions

In recent years, many ethnobotanical studies have been carried out and there is evidence that some plants used in folk veterinary medicine contain active compounds that may explain their popular use. Nevertheless, despite many anecdotal reports of the efficacy of herbal remedies, most of them have never been proven effective in domestic animals. It is necessary to undertake scientific studies and clinical trials to achieve a validation and standardization.

People should be informed that the use of herbal drugs in domestic animals does not imply the absence of risks, particularly if they are administered simultaneously with synthetic drugs or when plants for which scientific evidence able to justify their therapeutic use does not exist or in case of utilizing unsafe herbs. Also, the consumption of marked herbal products could represent either a risk or a lack of therapeutic efficacy because the content could be uncertain. Scientific validation of therapeutic effects and the evaluation of the possible side effects of plant products and drug interactions in domestic animals

are necessary prior to the adoption of such remedies as alternative therapeutic methods in clinical practice.

Herbal drugs like allopathic drugs should be prescribed by a specialized practitioner; the National Federation of the Orders of Italian Veterinarians (F.N.O.V.I.) asserted in 2005 that that the use of NCM has to be considered a veterinary practise.

References

[1] Hahn I, Zitterl-Eglseer K, Franz C. (**2005**) Phytomedicine in dogs and cats: web-based survey among veterinarians in Austria, Germany and Switzerland. *Schweizer Archiv für Tierheilkunde*, ***147***, 135-141.

[2] Foster S. (**1995**) Echinacea. The cold and flu remedy. *Alternative and Complementary Therapies*, 254-257.

[3] Viegi L, Pieroni A, Guarrera PM, Vangelisti R. (**2003**) A review of plants used in folk veterinary medicine in Italy as basis for a databank. *Journal of Ethnopharmacology*, ***89***, 221-244.

[4] Severino L, Russo R. (**2007**) Use of non conventional medicine in farm animals. www.zootec.it.

[5] Severino L, Russo R, Autore G, Marzocco S, De Tommasi N. (**2008**) Use of phytotherapics in dogs and cats. *Pharmacologyonline*, ***2***, 12-21.

[6] Lans C, Turner N, Khan T, Brauer G. (**2007**) Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia, Canada. *Veterinary Parasitology*, ***148***, 325-340.

[7] Sharma SS, Kochupillai V, Gupta SK, Seth SD, Gupta YK. (**1997**) Antiemetic efficacy of ginger (*Zingiber officinale*) against cisplatin-induced emesis in dogs. *Journal of Ethnopharmacology*, ***57***, 93-96.

[8] Lans C, Turner N, Brauer G, Khan T. (**2009**) Medicinal plants used in British Columbia, Canada for reproductive health in pets. *Preventive Veterinary Medicine*, ***90***, 268-273.

[9] Reichling J, Fitzi J, Furst-Jucker J, Bucher S, Saller R. (**2003**) Echinacea powder: treatment for canine chronic and seasonal upper respiratory tract infections. *Schweizer Archiv fur Tierheilkunde*, ***145***, 223-231.

[10] Lans C, Turner N, Khan T. (**2008**) Medicinal plant treatments for fleas and ear problems of cats and dogs in British Columbia, Canada. *Parasitologcal Research*, ***103***, 889-898.

[11] Severino L. (**2009**) Toxic plants and domestic animals. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, ***4***, 1-6.

[12] Macchioni F, Perrucci S, Cecchi F, Cioni PL, Morelli I, Pampiglione S. (**2004**) Acaricidal activity of aqeous extracts of camomile flowers, *Matricaria chamomilla*, against the mite *Psoroptes cuniculi*, *Medical and Veterinary Entomology*, ***18***, 205-207.

[13] O'Neill W, McKee S, Clarke AF. (**2002**) Immunological and haematinic consequences of feeding a standardized Echinacea (*Echinacea angustifolia*) extracts to healthy horses. *Equine Veterinary Journal*, ***34***, 222-227.

[14] Peeters E, Driessen B, Steegmans R, Henot D, Geers R. (**2004**) Effect of supplemental tryptophan, vitamin E, and a herbal product on responses by pigs to vibration. *Journal of Animal Science*, ***82***, 2410-2420.

[15] Lans C, Turner N, Brauer G, Lourenco G, Georges K. (**2006**) Ethnoveterinary medicines used for horses in Trinidad and in British Columbia, Canada. *Journal of Ethnobiology and Ethnomedicine*, ***2***, 31-51.

[16] Scharenberg A, Heckendorn F, Arrigo Y, Hertzberg H, Gutzwiller A, Hess HD, Kreuzer M, Dohme F. (**2008**) Nitrogen and mineral balance of lambs artificially infected with *Haemonchus contortus* and fed tanniferous sainfoin (*Onobrychis viciifolia*). *Journal of Animal Science*, ***86***, 1879-1890.

[17] Heckendorn F, Haring DA, Maurer V, Senn M, Hertzberg H. (**2007**) Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemoncus contortus* and *Cooperia curticei*. *Veterinary Parasitology*, ***146***, 123-134.

[18] Heckendorn F, Haring DA, Maurer V, Zinsstag J, Langhans W, Hertzberg H. (**2006**) Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemoncus contortus* and *Cooperia curticei*. *Veterinary Parasitology*, ***142***, 293-300.

[19] Paolini V, De La Farge F, Prevot F, Dorchies P, Hoste H. (**2005**) Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. *Veterinary Parasitology*, ***127***, 277-283.

[20] Niezen JH, Waghorn GC, Graham T, Carter JL, Leathwick DM. (**2002**) The effect of diet fed to lambs on subsequent development of *Trichostrongylus colubriformis* larvae *in vitro* and on pasture. *Veterinary Parasitology*, ***105***, 269-283.

[21] Waller PJ, Bernes G, Thamsborg SM, Sukura A, Richter SH, Ingebrigtsen K, Hoglund J. (**2001**) Plants as de-worming agents of livestock in the Nordic Countries: historical perspective, popular beliefs and prospects for the future. *Acta Veterinaria Scandinavica*, ***42***, 31-44.

[22] Githiori JB, Athanasiadou S, Thamsborg SM. (**2006**) Use of plants in novel approaches for control of gastrointestinal helminths in livestock with emphasis on small ruminants. *Veterinary Parasitology*, ***139***, 308-320.

[23] Tariq KA, Chishti MZ, Ahmad F, Shawl AS. (**2009**) Anthelmintic activity of extracts of *Artemisia absithium* against ovine nematodes. *Veterinary Parasitology*, ***160***, 83-88.

[24] Githiori JB, Hoglund J, Waller PJ, Leyden Baker R. (**2003**) Evaluation of anthelmintic properties of extracts from some plants used as livestock dewormers by pastoralist and smallholder farmers in Kenya against *Heligmosomoides polygyrus* infections in mice. *Veterinary Parasitology*, ***118***, 215-226.

[25] Satrija F, Nansen P, Bjorn H, Murtini S, He S. (**1994**) Effect of papaya latex against Ascaris suum in naturally infected pigs. *Journal of Helminthology*, ***68***, 343-346.

[26] Marley CL, Cook R, Keatinge R, Barrett J, Lampkin NH. (**2003**) The effect of birdsfoot trefoil (*Lotus corniculatus*) and chicory (*Cichorium intybus*) on parasite intensities and performance of lambs naturally infected with helminth parasites. *Veterinary parasitology*, ***112***, 147-155.

[27] Pomroy WE, Adlington BA. (**2006**) Efficacy of short-term feeding of sulla (*Hedysarum coronarium*) to young goats against a mixed burden of gastrointestinal nematodes. *Veterinary Parasitology*, ***136***, 363-366

[28] Athanasiadou S, Tzamaloukas O, Kyriazakis I, Jackson F, Coop RL. (**2005**) Testing for direct anthelmintic effects of bioactive forages against *Trichostrongylus colubriformis* in grazing sheep. *Veterinary Parasitology*, ***127***, 233-243.

[29] Abebe W. (**2002**) Herbal medication: potential for adverse interactions with analgesic drugs. *Journal of Clinical Pharmacy and Therapeutics*, ***27***, 391-401.

[30] Means C. (**2002**) Selected herbal hazards. *The Veteterinary Clinics Small Animal Practice*, ***32***, 367-382.

[31] Severino L. (**2005**) Residues of xenobiotici in medicinal plants. www.b2bfarmacia.it.

[32] Guffin M, Hobbs C, Upton R. (**1997**) *Botanical safety handbook*. Boca Raton, CRC Press.

[33] Miller LG. (**1988**) Herbal medicinals: selected clinical considerations focusing on known or potential drug-herb interactions. *Archives of Internal Medicine*, ***158***, 2200-2211.

[34] Harenberg J, Giese C, Zimmermann R. (**1988**) Effect of dried garlic on blood coagulation fibrinolysis, platelet aggregation and serum cholesterol levels in patients with hyperlipoproteinemia. *Atherosclerosis*, ***74***, 247-249.

[35] Lee KW, Yamato O, Tajima M, Kuraoka M, Omae S, Maede Y. (**2000**) Hematologic changes associated with the appearance of eccentrocytes after intragastric administration of garlic extract to dogs. *American Journal of Veterinary Research*, ***61***, 1446-1450.

[36] Pearson W, Boermans HJ, Bettger WJ, McBride BW, Lindinger MI. (**2005**) Association of maximum voluntary dietary intake of freeze-dried garlic with Heinz body anemia in horses. *American Journal of Veterinary Research*, ***66***, 457-465.

[37] Tamura Y. (**1979**) Effects of glycyrrhetinic acid and its derivates on delta-4-5-alfa and 5-beta-reductase in rat liver. *Arzneimittelforschung*, ***29***, 647-649.

[38] Poppenga RH. (**2001**) Risks associated with the use of herbs and other dietary supplements. *Veterinary Clinics of North America: Equine Practice*, ***17***, 455-477.

[39] Nair B. (**2001**) Final report on the safety assessment of *Mentha piperita* (peppermint) oil, *Mentha piperita* (peppermint) leaf extract, *Mentha piperita* (peppermint) leaf, and *Mentha piperita* (peppermint) leaf water, *International Journal of Toxicology*, ***20***, 61-73.

[40] Sudekun M, Poppenga RH, Raju N, Braselton WE. (**1992**) Pennyroyal oil toxicosis in a dog. *Journal of the American Veterinary Medical Association*, ***200***, 817-818.

[41] Ooms TG, Khan SA, Means C. (**2001**) Suspected caffeine and ephedrine toxicosis resulting from ingestion of an herbal supplement containing guarana and ma huang in dogs: 47 cases (1997-1999). *Journal of the American Veterinary Medical Association*, ***218***, 225-229.

[42] Johnson JR, Makaji E, Ho S, Boya X, Crankshaw DJ, Holloway AC. (**2009**) Effects of maternal raspberry leaf consumption in rats on pregnancy outcome and the fertility of the female offspring. *Reproductive Science*, ***16***, 605-609.

[43] Lans C, Turner N, Khan T, Brauer G, Boepple B. (**2007**) Ethnoveterinary medicines used for ruminants in British Columbia, Canada. *Journal of Ethnobiology and Ethnomedicine*, ***26***, 3-11.

[44] Beaux D, Fleurentin J, Mortier F. (**1999**) Effect of extracts of *Orthosiphon stamineus* Benth, *Hieracium pilosella* L., *Sambucus nigra* L. and *Arctostaphylos uva-ursi* (L.) Spreng. in rats. *Phytotherapy Research*, ***13***, 222-225.

[45] Preethi KC, Kuttan R (**2009**) Wound healing activity of flower extract of *Calendula officinalis*. *Journal of Basic and Clinical Physiology and Pharmacology*, ***20***, 73-79.

[46] Lans C, Harper T, Georges K, Bridgewater E. (**2000**) Medicinal plants used for dogs in Trinidad and Tobago, *Preventive Veterinary Medicine*. ***45***, 201-220.

[47] Gildor A. (**1998**) *Crataegus oxyacantha* and heart failure. *Circulation*, ***98***, 2098-2102.

[48] Emendorfer F, Emendorfer F, Bellato F, Noldin VF, Cechinel-Filho V, Yunes RA, Delle Monache F, Cordozo AM. (**2005**) Antispasmodic activity of fractions and cynaropicrin from *Cynara scolymus* on Guinea-pig ileum. *Biological Pharmaceutical Bulletin*, ***28***, 902-904.

[49] Cermelli C, Fabio A, Fabio G, Quaglio P. (**2008**) Effect of eucalyptus essential oil on respiratory bacteria and viruses. *Current Microbiology*, ***56***, 89-92.

[50] El Ghaoui WB, Ghanem EB, Chedid LA, Abdelnoor AM. (**2008**) The effects of *Alcea rosea* L., *Malva sylvestris* L. and *Salvia libanotica* L. water extracts on the production of anti-egg albumin antibodies, interleukin-4, gamma interferon and interleukin-12 in BALB/c mice. *Phytotherapy Research*, ***22***, 1599-1604.

[51] Villasenor IM, Angelada J, Canlas AP, Echegoyen D. (**2002**) Bioactivity studies on beta-sitosterol and its glucosides. *Phytotherapy Research*, ***16***, 417-421.

[52] Nassiri-Asl M, Shariati-Rad S, Zamansoltani F. (**2007**) Anticonvulsant effects of aerial parts of *Passiflora incarnata* extract in mice: involvement of benzodiazepine and opioid receptors. *BMC Complementary and Alternative Medicine*, ***7***, 26-31.

[53] Samuelsen AB. (**2000**) The traditional uses, chemical constituents and biological activities of *Plantago major* L. A review. *Journal of Ethnopharmacology*, ***71***, 1-21.

[54] Deliorman Orhan D, Hartevioglu A, Kupeli E, Yesilada E. (**2007**) *In vivo* anti-inflammatory and antinociceptive activity of the crude extract and fractions from *Rosa canina* L. fruits. *Journal of Ethnopharmacology,* ***112***, 394-400.

[55] Pradhan SC, Girish C. (**2006**) Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. *The Indian Journal of Medical Research*, ***124***, 491-504.

NPC Natural Product Communications

2009
Vol. 4
No. 12
1785 - 1796

Garlic: Empiricism or Science?

Gabriella Aviello[1], Ludovico Abenavoli[2], Francesca Borrelli[1], Raffaele Capasso[1], Angelo Antonio Izzo[1], Francesca Lembo[1], Barbara Romano[1] and Francesco Capasso[1,*]

[1]*Department of Experimental Pharmacology, University of Naples Federico II, Naples, Italy*

[2]*Department of Experimental and Clinical Medicine, University "Magna Graecia", Catanzaro, Italy*

fcapasso@unina.it

Received: July 17th, 2009; Accepted: October 2nd, 2009

Garlic (*Allium sativum* L. fam. Alliaceae) is one of the best-researched, best-selling herbal remedies and is also commonly used as a food and a spice. Garlic constituents include enzymes (for example, alliinase) and sulfur-containing compounds, including alliin, and compounds produced enzymatically from alliin (for example, allicin). Traditionally, it has been employed to treat infections, wounds, diarrhea, rheumatism, heart disease, diabetes, and many other disorders. Experimentally, it has been shown to exert antilipidemic, antihypertensive, antineoplastic, antibacterial, immunostimulant and hypoglycemic actions. Clinically, garlic has been evaluated for a number of conditions, including hypertension, hypercholesterolemia, intermittent claudication, diabetes, rheumatoid arthritis, common cold, as an insect repellent, and for the prevention of arteriosclerosis and cancer. Systematic reviews are available for the possible antilipidemic, antihypertensive, antithrombotic and chemopreventive effects. However, the clinical evidence is far from compelling. Garlic appears to be generally safe although allergic reactions may occur.

Keywords: *Allium sativum*, garlic, Alliaceae, hypercholesterolemia, hypertension, cancer.

HISTORY

The historic role played by garlic in human health is fascinating (Table 1). Nicholas Culpeper in the Complete Herbal, dated 1653, wrote "… *a remedy for all diseases and hurt. It provokes urine, and women courses, helps the biting of mad dogs and other venomous creatures, kills worms in children, cuts and voids tough phlegm, purges the head, helps the lethargy, it is a good preservative against and a remedy for any plague, sore or foul ulcers*…". Garlic was already in use by the ancient Egyptians for both medicine and alimentary purposes. Several garlic bulbs have been found in the grave of Tutankhamun, probably to protect the boy-king on his journey to the afterlife. The image of garlic was found in ancient Egyptian burial grounds and on Sumerian clay tablets dating from 2600-2100 BC [1,2]. It is said that large supplies of garlic were given to the construction workers of the great pyramid of *Cheops*, which would give them strength to continue their work and protect them from illness. The application of crushed garlic to the teeth protected ancient Egyptians from mouth abscesses. The Bible cites garlic repeatedly. Cultivation and use were also described in the oldest Chinese texts. Longevity seems to be one of the main reasons why the ancient Chinese people used garlic. They was strongly convinced that regular consumption of garlic would lengthen their lives, and, according to an ancient writer "*if when a person reached the age of fifty, he ate garlic for fifty days, he would live a further fifty years*".

Garlic was introduced into Mediterranean regions by the Phoenicians and was used by the Greeks and Romans [3]. Homer, in the Iliad, ascribes to garlic magical properties. The Greeks regarded it as an aphrodisiac and, moreover, they believed that it might lend strength and physical resistance. For this reason garlic was considered by athletes as an essential part of their diet just before competitions. The Greek physician Hippocrates (around 430 BC) treated gangrene, bronchitis, pneumonia and other ailments of the respiratory tract with garlic. Galen and Dioscorides, both Greek physicians, used garlic for its antibacterial effects and to obtain some protection against stomach upsets. During the Middle Ages the use of garlic as a medicine and food become

Table 1: Garlic as medicine in the course of time.

Ancient Egypt	In an ancient Egyptian manuscript know as the *Codex Ebers*, garlic is cited as being useful in the treatment of heart disease, tumors, worms, bites, month abscesses and many other ailments.
Bible	In the *Book of Numbers* (chapter 11, verse 5), which describes the Israelites flight from Egypt, it is reported that Jewish slaves, accustomed to taking garlic to stay strong, were unhappy to be deprived of it throughout their escape.
Ancient India	The Indian ancient medical text, *Charaka-Samhita*, recommends garlic for the treatment of heart disease, and arthritis. In another ancient medicinal textbook, *Bower Manuscript,* garlic is recommended for parasites, fatigue and digestive problems.
Ancient China	Garlic was used for respiratory and digestion problems and to enhance male potency.
Ancient Greece	The Greek physicians prescribed garlic extensively for several ailments (just before Olympic Games). Soldiers and athletes ate garlic to increase their stamina.
Ancient Rome	In Roman times garlic was used for conditions such as gastrointestinal diseases, cardiovascular and musculoskeletal problems, epilepsy, abscesses and boils.
Middle Ages	In medieval times and later, garlic became a popular remedy against the plague and other epidemics. Garlic was stored at home, because the strong aroma could give protection against infections. There was also the practice of drinking wine vinegar containing crushed garlic. A German herbal (*Nenc Kreuterbuch*) of 1626 recommended garlic for chills, flatulence, colic and worms.
Our own times	During the First Word War garlic was the main source of antibacterial treatment and Albert Schweitzer, medical missionary in Africa between 1913 and 1965, had success in treating long-standing dysentery. In the last few years garlic has been used to treat infection, wounds, heart disease, diabetes, abnormal cholesterol levels and blood pressure.

important in Europe. Paracelsus and Lonicerus reputed garlic as an antibiotic agent. At that time, its effectiveness against intestinal disturbances was also been noted. Later, it was forgotten in Europe, but during the First World War, garlic was used by the Russians to treat gangrene. During the Second World War, garlic was reputed to be an excellent antibacterial agent and, therefore, was called "Russian penicillin".

BOTANY

The botanical name for garlic is *Allium sativum* L. (Fam. Alliaceae). A taxonomic classification considers the following varieties: *A. sativum* var. *sativum* (common garlic), *A. sativum* var. *ophioscordum* (Link) Doll. (rocambole) and *A. sativum* var. *pekinense* (Prokh.) Maekawa (Peking garlic). Allium is a perennial herb, up to 90 cm tall, with mostly composite bulbs (cloves), oblong-ovoid (var. *sativum*) or roundish ovoid (var. *ophioscordum*), enclosed in a thin whitish skin, which holds them in a sac. Leaves are green to bluish gray, flat, broadly linear, acuminate, with a rough margin (smooth margin in var. *ophioscordum*) up to 2 cm wide (more than 2 cm wide in var. *pekinense*). Flowers, placed at the end of a stalk rising directly from the bulb, are whitish, grouped together in a globular head, or an umbel, with an enclosing kind of leaf. Flowering period is July-August. The whole bulb, diameter about 4 cm, consists of a hard, flattened base with root fibers on the lower end, and a longish main bulb on top, surrounded by 8-12 angular secondary bulbs. Each bulb is enclosed by a white to reddish paper-like membrane. Bulbs are harvested when the leaves wither, between July and August; if harvested later the bulbs begin to disintegrate in the cloves. Once dried, bulbs can be stored in a dry location at between 0 and 2°C. Bulbs, cut or crushed, present a characteristic odor; the taste is pungent, burning, and characteristic. Microscopic examination of powdered garlic reveals (i) numerous parenchyma fragments, (ii) groups of spiral and annular vessels, (iii) numerous oxalate crystals in the form of prisms.

A. sativum is reported to be native of central Asia (SW Siberia), where wild garlic still grows. Cultivation of *A. sativum* probably began in this area and from here it spread to southern Europe, where it became naturalized. It is widely cultivated in Hungary, Czech Republic, Russia and in the countries bordering the Mediterranean. Due to its self-incompatibility, *Allium* species should not be planted in the some location for at least 5 years. The culture requires loamy-humus, deep and nutrient-rich soil in sunny locations.

CHEMISTRY

Since the beginning of the 20th century up until now, garlic has been the subject of many chemical studies that highlighted some differences in the chemical composition of the drug examined (fresh or stored garlic). The most important chemical ingredients contained in the garlic are divided into two groups: sulfur- and non sulfur-containing compounds. Most of the pharmacological effects of garlic are due to sulfur compounds, in particular allicin [4-6]. The sulfur compounds derive from the amino acid cysteine or its derivatives S-allylcysteine sulfoxide and γ-glutamyl-(S)-allycysteine. Fresh garlic contains 0.35-1.15 % of cysteine sulfoxides, mostly alliin

(about 0.5%) and not less than 0.2% γ-glutamyl-(S)-ally-L-cysteine, calculated on a dried basis. Another fresh garlic constituent is the acid-sensitive enzyme, alliinase. Other constituents include glutamyl peptides (proteins), saponins (proto-eruboside B, eruboside B, sativoside), lectins, flavonoids, polysaccharides, oligosaccharides and mono-saccharides (particularly fructans, about 30%), minerals (selenium, tellurium), vitamins (C and E), prostaglandins (A_2, D_2, E_2, $F_{1\alpha}$, F_2), aminoacids (arginine, glutamic acid, methionine, threonine), several enzymes (peroxidase, myrosinase, catalase, superoxide dismutase, arginase, lipase) and terpenes (for example, citral, geraniol) [7,8]. Alliin is separated from alliinase (stored in vacuoles) in intact clove. However, when the bulb is chopped or crushed, alliinase transforms alliin into allicin.

Allicin, once produced, rapidly degrades, but the speed of this reaction depends on temperature. A few days after it has been produced, allicin can still be detected if the garlic is kept refrigerated, but at room temperature, within a few hours, it breaks down into strongly smelling volatile sulfur compounds like diallyl di- and tri-sulphides, ajoene, and vinyldithiins. It has been assumed that the sulfur compounds are responsible for the medical effects of garlic. As a consequence, all commercial garlic products are standardized on their content of alliin and/or allicin (1 mg alliin is considered equivalent to 0.45 mg allicin). However, some studies indicate that sulfur compounds are not completely essential for efficacy and, on the contrary, steroids and terpenes are the active compounds. Garlic powder that has been immediately dried at 60°C may contain up to twice the concentration of allicin (0.5-2.5%).

PHARMACOKINETICS

The health benefits of garlic likely arise from a wide variety of components, possibly working synergistically. The pharmacokinetics of garlic's constituents have been poorly investigated. Highly unstable thiosulfinates, such as allicin (diallyl thiosulfinate), disappear during processing and are quickly transformed into a variety of organosulfur components. Allicin and allicin-derived compounds are rapidly metabolized to allyl methyl sulfide [9]. Most garlic supplements are enteric-coated to prevent gastric acid inactivation by the enzyme alliinase.

Sulfur-containing compounds, such as diallyl-disulphide, diallyl-sulphide, dimethyl-sulphide and mercapturic acids have been isolated and identified in human urine following the ingestion of garlic [10,11]. It has also been reported that the flavor of human breast milk is altered when lactating women consume garlic [12].

PHARMACOLOGY

Among the components that have been identified in garlic sulfur compounds are the most interesting constituents from a pharmacological point of view. Allicin has potent antibacterial activity and ajoene is reported to have antithrombotic properties. Furthermore, sulfur-containing compounds are reported to lower serum lipids, decrease platelet aggregation and show vasodilator, anticancer and antioxidant effects. The pharmacological properties of garlic (Table 2) and its constituents have been summarized in many reviews [6,13-21]

Effects on atherogenesis and lipid metabolism: Cholesterol helps to maintain cell integrity and initiates the production of bile acids that process fats in the intestine so that they can be absorbed into the bloodstream. Cholesterol is also essential for the production of the sex hormones and, therefore, plays a key role in reproduction. However, several studies have demonstrated a strong relationship between excessive blood cholesterol levels and heart disease. The effect of garlic and its constituents on cholesterol and fatty acid biosynthesis was first documented by Quereshi and colleagues [22] in cultured chicken hepatocytes and monkey livers. In other *in vitro* studies with rat hepatocytes, Gebhardt and colleagues [23,24] identified the steps in cholesterol biosynthesis that are modified by garlic and its constituents. These effects are based on the inactivation of enzymes involved in lipid synthesis, such as HMG-CoA reductase, lanosterol-14-demethylase and squalene monooxygenase [25,10]. Moreover, it has also been reported that compounds containing an allyl-disulfide or allyl-sulfhydril group inhibit cholesterol biosynthesis acting on sterol 4α-methyl oxidase [26]. Other mechanisms proposed include increased excretion of bile acids [18,27] and reduced *de novo* fatty acid synthesis via inhibition of fatty acid synthase [28]. Some studies have also reported a reduction by garlic of aortic lipid deposition and atheromatous lesions in rabbits fed a high-fat diet. It has been also observed that administration of aged garlic extract or allicin exerts an anti-atherogenic effect via inhibition of smooth muscle proliferation and lipid accumulation in the artery wall [29,30]. These results have been supplemented by more recent studies [31].

Table 2: Summary of garlic biological effects.

BIOLOGICAL EFFECT	MAIN MECHANISM	CLINICAL EVIDENCE
Antilipidemic	Inactivation of enzymes involved in lipid synthesis (HMG-CoA reductase, lanosterol-14-demethylase, squalene monooxygenase).	The available evidence from randomized controlled trial does not demonstrate any beneficial effects of garlic on serum cholesterol [71].
Antihypertensive	Direct vasodilatative effect by acting on potassium channels; modulation of relaxing and contracting factors of endothelium cells.	Garlic preparations are superior to placebo in reducing blood pressure in individuals with hypertension [73].
Antiatherosclerotic	Inhibition of smooth muscle proliferation and lipid accumulation in the artery wall; antioxidant effects.	Preliminary clinical evidence demonstrates preventive and curative effect of garlic on the development of artherosclerosis [70].
Antineoplastic	Blockage of cell proliferation; induction of apoptosis; inhibition of COX-2 expression and carcinogenic nitrosoamines synthesis; stimulation of glutathione-*S*-transferase activity; antioxidant activity.	There is no credible evidence to support a relation between garlic intake and a reduced risk of gastric, breast, lung, or endometrial cancer. Very limited evidence supported a relation between garlic consumption and reduced risk of colon, prostate, esophageal, larynx, oral, ovary, or renal cell cancers [79].
Antibacterial	Interaction with important thiol-containing enzymes (alcohol dehydrogenases, thioredoxin reductases, cysteine proteinases, alcohol dehydrogenases, thioredoxin reductases).	No reliable evidence.
Immunostimulant	Enhances cellular immunity by direct and/or indirect modification of the functions of mast cells, basophils and activation of T lymphocytes.	No reliable evidence.
Antiplatelet, antitrombotic and fibrinolytic	Inhibition of thromboxane synthesis, membrane phospholipase activity and calcium uptake into platelet adhesion.	Clinical trials have shown a modest, but significant decrease in platelet aggregation; controversy over results on fibrinolytic activity and plasma viscosity were reported [80].
Hypoglycemic activity	Antioxidant activity.	It is premature to actively recommend use of garlic to treat hyperglycemia [68].

Effects on fibrinolysis, platelet aggregation and vascular resistance: The effects of garlic and its derivatives on fibrinolysis, platelet aggregation and blood pressure are well documented in both *in vitro* and *in vivo* studies. The ability of garlic to lower blood pressure in animals was described by Chandorkar and Jain [32] and successively by Malik and Siddiqui [33], Al-Qattan and Alnaqeeb [34], and Ali and colleagues [35]. It was also observed that the effect of garlic on blood pressure was accompanied by a reduction in secondary myocardial injury [36]. The antihypertensive effect of garlic is partly due to direct vasodilatative action of its sulfur compounds. These substances act as a potassium channel openers [37] and modulate the production and the function of relaxing and contracting factors of endothelium cells [38,39]. The inhibitory effect of garlic on platelet aggregation and its activating effect on fibrinolysis are well documented in several animal studies. Most of these show that garlic preparations, allicin and ajoene, inhibit human platelet aggregation caused by several inducers (ADP, collagen, arachidonic acid, adrenaline and calcium ionophore A23187) [40-43]. The mechanisms proposed include (i) inhibition of thromboxane synthesis, (ii) inhibition of membrane phospholipase activity and incorporation of aminoacids into platelet membrane phospholipid [44] and (iii) inhibition of calcium uptake into platelet adhesion [45]. There is a current opinion that all these effects, together with antilipidemic, antihypertensive and anti-atherosclerotic actions of garlic contribute to lowering the risk of infarction.

Cardioprotective and antioxidantive effects: Several experimental studies suggest that a high consumption of fruits and vegetables, especially garlic, is effective in the prevention and treatment of atherosclerosis and other risk factors for cardiovascular diseases. As regards garlic, it has been shown that treatment for 11 days protected rats by isoproterenol-induced myocardial damage [46]. In another series of experiments, rats were fed a standard chow enriched with 1% garlic for 10 weeks, and then Langerdorff heart preparations were performed under conditions of cardiac ischemia; the size of the ischemic zone was significantly reduced in the garlic treated animals [47].

Oxygen free radicals are recognized as having an important role in several pathological processes, such as ischemic heart disease and atherosclerosis. Therefore, the antioxidant effects of garlic could be of interest in relation to anti-atherosclerotic properties. These effects have been documented in human granulocytes *in vitro* [48], in isolated hepatic microsomes [49], in pulmonary artery endothelial cells [50], in isolated LDL particles [51] and using radical generating systems [52,53]. *In vivo* studies have also documented a reduction in liver lipid peroxidation and inhibition of ethanol-induced mitochondrial lipid peroxidation in animals fed garlic oil [10]. It has been suggested that allicin is the constituent of garlic responsible for these positive effects.

Effects on carcinogenesis: It has been documented that tumors induced by carcinogenic agents (for example, benzopyrine, 7,12-dimethyl benzanthracine, *N*-methyl-*N*-nitrosourea, dimethylhydrazine, nitroso-methyl benzylamine, diethylnitrosamine) can be inhibited (as determined by evidence of either a significant reduction in the incidence and growth of experimental tumors or a significant reduction in the number of aberrant crypt *foci*, which are pre-neoplastic lesions) by garlic preparations and some of its constituent (for example, S-allylcysteine, diallyl disulfide, diallyl-trisulfide, diallyl-sulfide) [54]. The antineoplastic activity of garlic and its constituents may be due to multidirectional mechanisms, such as blockage of cell proliferation, induction of apoptosis, inhibition of carcinogen-induced DNA adduct formation, enhancement of carcinogen-metabolizing enzymes, inhibition of cyclooxygenase-2 expression, inhibition of *N*-nitroso compounds synthesis, and enhancement of glutathione-*S*-transferase activity. Other possible mechanisms are scavenging carcinogen-induced free radicals and inhibition of lipid peroxidation. Sulfur compounds are considered to be the major ones responsible for the protective effect of garlic against cancer [6]. However, anticancer properties are also attributed to other garlic constituents, including kaempferol, selenium, vitamin A and C, arginine and fructooligosaccharides [55-59].

Other actions: Studies carried out *in vitro* have shown that garlic and its active compounds inhibit the growth of both Gram-negative and Gram-positive bacteria [60-64]. The antibacterial effects have been demonstrated against, among other species, *Helicobacter pylori, Staphylococcus aureus, Escherichia coli, Listeria monocytogenes, Staphylococcus epidermidis, Salmonella thyphi* and *Cryptococcus neoformans.* Garlic also stimulates production of cytokines (IL-2, TNF, γ-interferon) and the proliferation of macrophages, NK-cells and T-cells [65,66], and so the immune system. Several experimental models have then demonstrated the antitoxic [67] and antidiabetic [68] effects of garlic. All these effects seem to have little practical importance.

CLINICAL STUDIES

Garlic has been clinically evaluated for a number of conditions, including hypercholesterolemia, hypertension, intermittent claudication, common cold, for the prevention of arteriosclerosis and cancer, and as a topical treatment for infections of skin and mucosa.

Serum cholesterol: Numerous randomized controlled trials (RCTs) published in the late 1980s and early 1990s demonstrated that garlic was effective in reducing total cholesterol and low-density lipoprotein cholesterol. Several systematic reviews of these data therefore arrived at positive conclusions, although the effect was believed to be modest [69]. Subsequently, however, several negative RCTs have emerged [70]. A recent systematic review has critically summarized the evidence on the effect of garlic on serum cholesterol levels. Thirteen randomized controlled trials ranging from 11 to 24 weeks in duration and including a total of 1056 subjects were used for the meta-analysis. Overall, administration of garlic did not show any significant difference in effects on all outcome measures examined when compared with placebo. Garlic therapy did not produce any statistically significant reduction in serum total cholesterol level, LDL-cholesterol level, triglycerides level or apolipoprotein B level. There was no difference between garlic and placebo on HDL-cholesterol level. It was concluded that the available evidence from randomized controlled trials does not demonstrate any beneficial effects of garlic on serum cholesterol [71].

Blood pressure reduction: Reinhart and colleagues have recently examined the effect of garlic on blood pressure in patients with and without elevated systolic blood pressure (SBP) through meta-analyses of randomized controlled trials [72]. To be included in the analysis, studies must have reported endpoints of either SBP or diastolic blood pressure (DBP). Studies whose population had a mean baseline SBP greater than 140 mmHg were evaluated separately from those with lower baseline blood pressures. Ten trials were included in the analysis; 3 of these had patients with elevated SBP. Garlic reduced SBP by 16.3 mmHg and DBP by 9.3 mmHg compared with placebo in patients with elevated SBP. However, the use of garlic did not reduce SBP or DBP in patients without elevated SBP. This meta-analysis suggests that garlic is associated with blood pressure reductions in patients with an elevated SBP, but not in those without elevated SBP [72]. These results are in agreement with a further recent meta-analysis which showed a mean decrease of 8.4 mmHg in SBP and 7.3 mmHg for DBP compared with placebo in hypertensive subjects [73]. Collectively, these results

suggest that garlic preparations may be effective in subjects with mild hypertension.

Preeclampsya: Preeclampsya is a hypertensive disorder which occurs during the second half of pregnancy. A Cochrane review identified one single-blind RCT (n=100) that did not show a clear difference between garlic and placebo in the risk of developing gestational hypertension or preeclampsya. The participants were women at moderate risk of preeclampsya, as determined by a positive roll-over test. The review concluded that the evidence is insufficient to recommend garlic for the prevention of preeclampsya and its complications [74].

Prevention of atherosclerosis: Some interesting, although not compelling data suggest that, due to its broad-ranging effect on cardiovascular risk factors, the regular intake of garlic might prevent or delay the development of atherosclerosis [70]. Garlic intake was shown to reduce (5-18% compared with placebo) the volume of existing atherosclerotic plaques in 152 patients after 4-18-months of treatment [75]. Another study demonstrated that chronic garlic powder intake (900 mg/daily of garlic powder for 4 years) delayed age-related increases in aortic stiffness in the elderly (101 healthy adults) [76]. More recently, a placebo-controlled, double-blind, randomized pilot study (19 patients with coronary high-risk subjects) evaluating coronary artery calcification and the effect of 1-year garlic extract therapy in a group of patients who were also on statin therapy, suggested incremental benefits [77].

Chemoprevention: Numerous animal and *in vitro* studies provided evidence for a relation between garlic intake and cancer risk reduction. Accumulating evidence from epidemiological studies, as well as clinical data, also support the anticancer properties of garlic widely used as a medicinal herb and food [5,15,70]. Some organosulfur compounds, such as diallyl-sulphide, diallyl-disulphide and diallyl-trisulphide present in garlic have been shown to possess pronounced chemoprotective and chemopreventive properties [78]. Certain garlic-derived organosulfur compounds can inhibit metabolic activation of carcinogens, thereby blocking initiation of carcinogenesis [79]. In addition, the induction of antioxidant and phase II detoxifying enzymes by organosulfur compounds has been reported [80]. Since allylsulfides undergo metabolic conversion to form sulfone derivatives [81] that may act as electrophiles, these organosulfur compounds are thought to target cysteine sulfhydryl of Keap1, thereby activating Nrf2-regulated gene transcription and ARE activity. In support of this hypothesis, Chen *et al*. [82] reported that treatment of human hepatoma HepG2 cells with various organosulfur compounds resulted in Nrf2 activation, leading to the induction of HO-1. Nrf2-mediated HO-1 induction by diallyl sulfide in HepG2 cells has also been reported by Gong *et al*. [83]. According to this work, diallyl sulfide-induced heme oxygenase-1 (HO-1) gene expression was accompanied by a transient increase in reactive oxygen species (ROS) production. Treatment of HepG2 cells with *N*-acetyl-L-cysteine, a ROS scavenger, blocked diallyl sulfide-induced ROS production, ERK activation, nuclear translocation of Nrf2, and subsequently HO-1 expression. However, the same study revealed that diallyl trisulfide-induced ARE activity was mediated via Ca^{2+}- dependent signalling, but not that of either MAPKs or PKC [82]. Therefore, the differential effects of garlic-derived organosulfur compounds on the MAPK-mediated activation of Nrf2 and HO-1 induction may be due to structural differences in terms of the number of sulfur moieties and the length of the alkyl side chain, which may confer varying degrees of electrophilicity to parent compounds, as well as their active metabolites [84]. However, several clinical studies report an inverse association in humans, especially for colon cancer [70]. For example, a published meta-analysis (level III) of 7 case/cohort studies confirmed this inverse association, with a 30% reduction in the colon cancer related risk [85]. However, a recent analysis re-evaluated the possible effect of garlic intake on different types of cancer using the US Food and Drug Administration's evidence-based review system for the scientific evaluation of health claims. Nineteen human studies were identified and reviewed to evaluate the strength of the evidence that supports a relation between garlic intake and reduced risk of different cancers. It was concluded that there is no credible evidence to support a relationship between garlic intake and a reduced risk of gastric, breast, lung, and endometrial cancer. Moreover, very limited evidence supported a relation between garlic consumption and reduced risk of colon, prostate, oesophageal, larynx, oral, ovary, and renal cell cancers [84].

Other relevant clinical trials: Ackerman and colleagues retrieved 10 trials assessing the effectiveness of garlic on potential prothrombotic risk factor. Of 6 trials measuring effects on spontaneous platelet aggregation, 5 provided significant inhibitory

Table 3: Summary of garlic adverse effects (modified from Borrelli *et al*. [90]).

Adverse effect	Source of evidence
Garlic odor on breath and skin	Randomized controlled trials, observational studies
Allergic reactions (allergic contact dermatitis, urticaria/angioedema, pemphigus anaphylaxis)	Multiple case reports for allergic contact dermatitis and urticaria. Single case reports for anaphylaxis and pemphigus
Photoallergy	A case report and a case series
Cutaneous manifestations (garlic burns)	Multiple case reports
Coagulation alterations (spinal epidural hematoma, increased clotting time, post-operative bleeding, retrobulbar hemorrhage)	Single case reports
Gastrointestinal adverse effects (mild: nausea, bloating, flatulence	Mild adverse events: randomized clinical trials, observational studies
severe: small intestinal obstruction, epigastric and esophageal pain, hematemesis, hematochezia)	Severe adverse events: single case reports
Others (hypotensive effects, myocardial infarction, Meniere's disease)	Observational study for hypotensive effects; single case reports for myocardial infarction and Meniere's disease
Herb drug interactions (chlorzoxazone, fluindione, paracetamol, ritonavir, saquinavir, warfarin)	Two clinical trials for chlorzoxazone, one case report for fluindione, one clinical trial for paracetamol, one case report for ritonavir (although a clinical trial showed no effect of garlic on ritonavir pharmacokinetics), one clinical trial for saquinavir, one case report for warfarin (although two clinical trials showed no effect of garlic on warfarin pharmacokinetics)

effects compared with placebo in platelet aggregation [86]. Garlic had a positive response in the inhibition of platelet aggregation in both healthy subjects and subjects with cardiovascular disease. Mixed effects on fibrinolytic activity and plasma viscosity were reported, while no trial assessing serum fibrinogen levels, or serum homocysteine levels reported significant results [86].

A recent analysis identified one double-blind RCT assessing 146 patients over a 12-week treatment period [87]. Patients in the treatment group had significantly fewer colds than patients in the placebo group who had also a longer duration of symptoms. Finally, topical application of garlic (as a cream or a paste) has been found to be effective for superficial *Tinea* infections and oral candidiasis [88].

SIDE EFFECTS

Garlic odor is the most frequent complaint associated with drug ingestion: this odor is perceived on the breath after a single consumption of the drug and on skin after several days of garlic intake. A regular use of garlic as either a food or herbal remedy does not cause acute or chronic toxicity to healthy people (Table 3). In sensitive subjects, therapeutic doses or high doses of raw garlic may cause mild gastrointestinal complaints (stomach disturbances, nausea, vomiting, bloating) and sometime micturition, cystitis and fever [69,89-90]. As a consequence, raw garlic is contra-indicated for patients with stomach and duodenal ulcers. Experimentally, it is worthy of note that, among a number of garlic preparations (namely raw garlic powder, dehydrated boiled garlic powder and aged garlic extract), administrated directly into the dog's stomach, only aged garlic extract caused no side effects [91]. Garlic active components pass into breast milk causing bloating in babies, and, therefore, nursing mothers should avoid garlic. It may also cause allergic reactions [92,93], which are due to its constituents diallyl-disulphide, allyl-propyl-sulphide and allicin [89,94]. Notably, alliin lyase was found to be the major garlic allergen in a garlic-allergic group of patients in Taiwan [95]. Case reports of allergic reactions associated with garlic intake include allergic contact dermatitis, generalized urticaria, angioedema, pemphigus, anaphylaxis and ulceration of the buccal vestibule [96-103].

Drug interactions: Using well-established probe drugs (for example, alprazolam and midazolam for CYP3A4, caffeine for CYP1A2, chlorzoxazone for CYP2E1, dextromethorphan and debrisoquine for CYP2D6) clinical trials have shown that garlic oil may selectively inhibit CYP2E1, but not other CYP isoforms such as CYP1A2, CYP3A4 or CYP2D6 [104,105].

Case reports have suggested that garlic might influence platelet function and blood coagulation leading to a risk of bleeding [90]. However, two trials

have reported that garlic did not alter either the pharmacokinetics or pharmacodynamics of warfarin and does not induce serious hemorrhagic risk in adequately monitored patients under warfarin treatment [106,107].

Garlic might cause interactions in patients on antiretroviral therapy [90]. A significant decline in the plasma concentrations of the protease inhibitor saquinavir was observed in healthy volunteers after administration of garlic for three weeks [108]. However, another trial showed that garlic did not significantly alter the single-dose pharmacokinetics of the protease inhibitor ritonavir [109]. The reason for the discrepancies is presently unclear.

One report described the case of two HIV-positive individuals who developed severe gastrointestinal toxicity from ritonavir after ingesting garlic supplements [110]. Symptoms recurred after re-challenge with low-dose ritonavir in the absence of garlic intake, suggesting that the elevated ritonavir concentration was not the cause.

In summary, caution is advised if garlic is taken concomitantly with either CYPE1 substrates or with antiretroviral drugs such as ritonavir and saquinavir. The possibility that garlic may cause over-anticoagulation if co-administered with anticoagulant drugs has not been confirmed by clinical trials [106,107].

PREPARATIONS

There are various preparations, such as garlic powder, aged garlic extract (AGE), garlic oil, and garlic homogenase. Due to the odoriferous components, coated tablets are the form mostly used, followed by tablets and capsules. Since *S*-allyl cysteine and *S*-allyl mercaptocysteine are major organosulfur compounds found in AGE, both substances must be used to standardize AGE preparations. Garlic powder is generally standardized to contain 1.3% alliin and 0.6% allicin [111]. Actually, monopreparations and combination products containing garlic as the active ingredient are available. Examples of monopreparations: Allioson ® film coated tablets (300 mg dry extract); Kwai ® coated tablets (100 mg garlic powder); and Valvarde ® garlic capsules (300 mg garlic powder). Examples of combination products: Klasterfrom-Aktiv capsules (garlic oily macerate, hypericum oily macerate, vitamin A, α-tocopherol acetate); and Kneipp ® (coated tablets containing garlic, hawthorn and mistletoe).

CONCLUSIONS

Garlic has been extensively studied and animal data suggest its potential use for either the treatment or prevention of a wide range of diseases, including hypertension, hypercholesterolemia, atherosclerosis, cancer, infections and many others [112]. However, the real efficacy of garlic has been doubted by recently-published systematic reviews and meta-analyses. Garlic products do not lower cholesterol, but it has been shown to be effective in patients with mild hypertension. The possible chemopreventive effect of garlic against colon cancer requires further investigations.

Garlic is considered as safe, although it may cause allergic reactions. Garlic may interact with anti-AIDS medicines or with drugs which are substrates of CYPE1. The possibility that garlic may cause over-anticoagulation if co-administered with anticoagulant/antiplatelet drugs has not been confirmed by controlled clinical trials.

References

[1] Rivlin RS. (**2001**) Historical perspective on the use of garlic. *Journal of Nutrition*, ***131***, 9515-9545.

[2] Banerjee SK, Maulik SK. (**2002**) Effect of garlic on cardiovascular disorder: a review. *Journal of Nutrition*, ***136***, 1-14.

[3] Rahman K, Lowe GM. (**2006**) Garlic and cardiovascular disease: a critical review. *Journal of Nutrition*, ***136***, 736S-740S.

[4] Amagase H, Petesch BL, Matsuura H, Kasuga S, Itakura Y. (**2001**) Intake of garlic and its bioactive components. *Journal of Nutrition*, ***131***, 9555-9625.

[5] Nagini S. (**2008**) Cancer chemoprevention by garlic and its organosulfur compounds-panacea or promise? *Anticancer Agents in Medicinal Chemistry*, ***8***, 313-321.

[6] Seki T, Hosono T, Hosono-Fukao T, Inada K, Tanaka R, Ogihara J, Ariga T. (**2008**) Anticancer effects of diallyl trisulfide derived from garlic. *Asia Pacific Journal of Clinical Nutrition*, ***17***, 249-252.

[7] Münchberg U, Anwar A, Mecklenburg S, Jacob C. (**2007**) Polysulfides as biologically active ingredients of garlic. *Organic & Biomolecular Chemistry*, ***5***, 1505-1518.

[8] Block E. (**1985**) The chemistry of garlic and onions. *Scientific American*, ***252***, 114-119.

[9] Lawson LD, Wang ZJ. (**2005**) Allicin and allicin-derived garlic compounds increase breath acetone through allyl methyl sulfide: use in measuring allicin bioavailability. *Journal of Agriculture and Food Chemistry*, ***53***, 1974-1983.

[10] Koch HP, Hahn G, Lawson L, Reuter HD, Siegers CP. (**1996**) Garlic. The science and the therapeutic applications of *Allium sativum* L. and related species. 2nd Ed, Williams and Wilkins.

[11] Jandkle J, Spiteller G. (**1987**) Unusual conjugates in biological profiles originating from consumption of onions and garlic. *Journal of Chromatography*, ***421***, 1-8.

[12] Mennella JA, Beauchamp GK. (**1991**) Maternal diet alters the sensory qualities of human milk and the nursling's behaviour. *Pediatrics*, ***88***, 737-744.

[13] Banerjea SK, Mukherjee PK, Mauli KSK. (**2003**) Garlic as an antioxidant: the good, the bad and the ugly. *Phytotherapy Research*, ***17***, 97-106.

[14] Rahman K. (**2007**) Effects of garlic on platelet biochemistry and physiology. *Molecular Nutrition & Food Research*, ***51***, 1335-1344.

[15] Devrim E, Durak I. (**2007**) Is garlic a promising food for benign prostatic hyperplasia and prostate cancer? *Molecular Nutrition & Food Research*, ***51***, 1319-1323.

[16] Milner JA. (**1996**) Garlic: its anticarcinogenic and antitumorigenic properties. *Nutrition Reviews*, ***54***, 582-586.

[17] Adetumbi M, Lau BHS. (**1983**) *Allium sativum* (garlic) a natural antibiotic. *Medical Hypotheses*, ***12***, 227-237.

[18] Fulder S. (**1989**) Garlic and the prevention of cardiovascular disease. *Cardiology Practice*, ***7***, 30-35.

[19] Borek C. (**2001**) Antioxidant health effects of aged garlic extract. *Journal of Nutrition*, ***131***, 51010-51015.

[20] Yhe Y-Y, Liu L. (**2001**) Cholesterol-lowering effect of garlic extract and organosulfur compounds: human and animal studies. *Journal of Nutrition*, ***131***, 5989-5993.

[21] Le Bon A-M, Siness M-H. (**2001**) Organosulfur compounds from *Allium* and the chemoprevention of cancer. *Drug Metabolism and Drug Interaction*, ***17***, 51-79.

[22] Qureshi AA, Abiurmeileh N, Din ZZ, Elson CE, Burger WC. (**1983**) Inhibition of cholesterol and fatty acid biosynthesis in liver enzymes and chicken hepatocytes by polar fractions of garlic. *Lipids*, ***18***, 343-348.

[23] Gebhardt R. (**1993**) Multiple inhibitory effects of garlic extracts on cholesterol biosynthesis in hepatocytes. *Lipids*, ***28***, 613-619.

[24] Gebhardt R. (**1995**) Amplification of palmitate-induced inhibition of cholesterol biosynthesis in cultured rat hepatocytes by garlic-derived organosulfur compounds. *Phytomedicine*, ***2***, 29-34.

[25] Gupta N, Porter TD. (**2001**) Garlic and garlic-derived compounds inhibit human squalene monooxygenase. *Journal of Nutrition*, ***131***, 1662-1667.

[26] Singh DK, Porter TD. (**2006**) Inhibition of sterol 4α-methyl oxidase is the principal mechanism by which garlic decreases cholesterol synthesis. *Journal of Nutrition*, ***136***, 759S-764S.

[27] Sheela CG, Augusti KT. (**1995**) Effects of S-allyl cysteine sulfoxide isolated from *Allium sativum* Linn and gugulipid on some enzymes and faecal excretions of bile acids and sterols in cholesterol fed rats. *Indian Journal of Experimental Biology*, ***33***, 749-751.

[28] Liu L, Yhe Y-Y. (**2001**) Water-soluble organosulfur compounds of garlic inhibit fatty acid and triglyceride synthesis in cultured rat hepatocytes. *Lipids*, ***36***, 395-400.

[29] Campbell JH, Efendy JL, Smith NJ, Campbell GR. (**2001**) Molecular basis by which garlic suppresses atherosclerosis. *Journal of Nutrition*, ***131***, 1006-1009.

[30] Abramowitz D. (**1999**) Allicin-induced decrease in formation of fatty steaks (atherosclerosis) in mice fed a cholesterol-rich diet. *Coronary Artery Disease*, ***10***, 515-519.

[31] Gorinstein S, Jastrzebski Z, Namiesnik J, Leontowicz H, Leontowicz M, Trakhtenberg S. (**2007**) The atherosclerotic heart disease and protecting properties of garlic: contemporary data. *Molecular Nutrition & Food Research*, ***51***, 1365-1381.

[32] Chandorkar AG, Jain PK. (**1973**) Analysis of hypertensive action of *Allium sativum* (garlic). *Indian Journal of Physiology and Pharmacology*, ***17***, 132-133.

[33] Malik ZA, Siddiqui S. (**1981**) Hypotensive effect of freeze-dried garlic (*Allium sativum*) SAP in dog. *Journal of the Pakistan Medical Association*, ***31***, 12-13.

[34] Al-Qattan KK, Alnaqeeb MA, Ali M. (**1999**) The antihypertensive effect of garlic (*Allium sativum*) in the rat two-kidney-one-clip Goldblatt model. *Journal of Ethnopharmacology*, ***66***, 217-222.

[35] Ali M, Al-Qattan KK, Al-Enezi F, Khanafer RM, Mustafa T. (**2000**) Effect of allicin from garlic powder on serum lipids and blood pressure in rats fed with a high cholesterol diet. *Prostaglandins, Leukotrienes & Essential Fatty Acids*, ***62***, 253-259.

[36] Jacob R, Rhrsam M, Ohkubo T, Rupp H. (**1991**) Antihypertensive and kardioprotektive effekt von knoblauchpulver (*Allium sativum*). *Medizinische Welt*, ***7***, 39-41.

[37] Siegel G, Emden J, Wenzel K, Mironneau J, Stock G. (**1992**) Potassium channel activation in vascular smooth muscle. In: Frank GB. (Ed.). *Excitation-contracting coupling in skeletal, cardiac and smooth muscle*. Plenum Press, New York.

[38] Das I, Khan NS, Sooranna SR. (**1995**) Potent activation of nitric oxide synthase by garlic: a base for its therapeutic applications. *Current Medical Research and Opinion*, ***13***, 257-263.

[39] Kim-Park S, Ku DD. (**2000**) Garlic elicits a nitric oxide-dependent relaxation and inhibits hypoxic pulmonary vasoconstriction in rats. *Clinical and Experimental Pharmacology and Physiology*, ***27***, 780-786.

[40] Boullin DJ. (**1981**) Garlic as a platelet inhibitor. *Lancet*, ***1***, 776-777.

[41] Gaffen JD, Tavares IA, Bennett A. (**1984**) The effect of garlic extracts on contractions of rat gastric fundus and human platelet aggregation. *Journal of Pharmacy and Pharmacology*, ***36***, 272-274.

[42] Srivastava KC. (**1986**) Evidence for the mechanism by which garlic inhibits platelet aggregation. *Prostaglandins,Leukotrienes and Medicine*, ***22***, 313-321.

[43] Apitz-Castro R, Cabrera S, Cruz MR, Ledezma E, Jain MK. (**1983**) Effects of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ultrastructure. *Thrombosis Research*, ***32***, 155-169.

[44] Wagner H, Wierer M, Fessler B. (**1987**) Effects of garlic constituents on arachidonate metabolism. *Planta Medica*, ***53***, 305-306.

[45] Sharma CP, Nirmala NV. (**1985**) Effects of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ultrastructure--comments. *Thrombosis Research*, ***37***, 489-490.

[46] Ciplea AG, Richter KD. (**1988**) The protective effect of *Allium sativum* and *Crataegus* on isoprenaline-induced tissue necrosis in rats. *Arzneimittel-Forschung*, ***38***, 1583-1592.

[47] Isansee H, Riatz B, Jacob R. (**1993**) Cardioprotective actions of garlic (*Allium sativum*). *Arzneimittel-Forschung*, ***43***, 94-98.

[48] Siegers CP, Röbke A, Pentz R. (**1999**) Effects of garlic preparations on superoxide production by phorbol ester activated granulocytes. *Phytomedicine*, ***6***, 13-16.

[49] Kourounakis PN, Rekka EA. (**1991**) Effect of active oxygen species of alliin and *Allium sativum* (garlic) powder. *Research Communications in Chemical Pathology & Pharmacology*, ***74***, 249-252.

[50] Ide N, Lau BH. (**1997**) Garlic compounds protect vascular endothelial cells from oxidized low density lipoprotein-induced injury. *Journal of Pharmacy and Pharmacology*, ***49***, 908-911.

[51] Lewin G, Popov I. (**1994**) Antioxidant effects of aqueous garlic extract. 2nd communication: Inhibition of the Cu(2+)-initiated oxidation of low density lipoproteins. *Arzneimittel-Forschung*, ***44***, 604-607.

[52] Popov I, Blumstein A, Lewin G. (**1994**) Antioxidant effects of aqueous garlic extract. 1st communication: Direct detection using the photochemiluminescence. *Arzneimittel-Forschung*, ***44***, 602-604.

[53] Török B, Belágyi J, Rietz B, Jacob R. (**1994**) Effectiveness of garlic on the radical activity in radical generating systems. *Arzneimittel-Forschung*, ***44***, 608-611.

[54] Herman-Antosiewicz A, Powolny AA, Singh SV. (**2007**) Molecular targets of cancer chemoprevention by garlic-derived organosulfides. *Acta Pharmacologica Sinica*, ***28***, 1355-1364.

[55] El-Bayoumy K, Sinha R, Pinto JT, Rivlin RS. (**2006**) Cancer chemoprevention by garlic and garlic-containing sulfur and selenium compounds. *Journal of Nutrition*, ***136***, 864S-869S.

[56] Milner JA. (**2006**) Preclinical perspectives on garlic and cancer. *Journal of Nutrition*, ***136***, 827S-831S.

[57] Khanum F, Anilakumar KR, Viswanathan KR. (**2004**) Anticarcinogenic properties of garlic: a review. *Critical Reviews in Food Science and Nutrition*, ***44***, 479-488.

[58] Pinto JT, Lapsia S, Shah A, Santiago H, Kim G. (**2001**) Antiproliferative effects of garlic-derived and other *Allium* related compounds. *Advances in Experimental Medicine and Biology*, ***492***, 83-106.

[59] Bianchini F, Vainio H. (**2001**) Allium vegetables and organosulfur compounds: do they help prevent cancer? *Environmental Health Perspectives*, ***109***, 893-902.

[60] Sharma VD, Sethi MS, Kumar A, Rarotra JR. (**1977**) Antibacterial property of *Allium sativum* Linn.: in vivo & in vitro studies. *Indian Journal of Experimental Biology*, ***15***, 466-468.

[61] Kumar M, Berwal JS. (**1998**) Sensitivity of food pathogens to garlic (*Allium sativum*). *Journal of Applied Microbiology*, ***84***, 213-215.

[62] Davis LE, Shen J, Royer RE. (**1994**) *In vitro* synergism of concentrated *Allium sativum* extract and amphotericin B against *Cryptococcus neoformans*. *Planta Medica*, ***60***, 546-549.

[63] Ross ZM, O'Gara EA, Hill DJ, Sleightholme HV, Maslin DJ. (**2001**) Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Applied and Environmental Microbiology*, ***67***, 475-480.

[64] Shashikanth KN, Basappa SC, Sreenivasa Murthy V. (**1984**) A comparative study of raw garlic extract and tetracycline on caecal microflora and serum proteins of albino rats. *Folia Microbiologica*, ***29***, 348-352.

[65] Lamm DL, Riggs DR. (**2000**) The potential application of *Allium sativum* (garlic) for the treatment of bladder cancer. *Urologic Clinics of North America*, ***27***, 157-162.

[66] Lau BH, Yamasaki T, Gridley DS. (**1991**) Garlic compounds modulate macrophage and T-lymphocyte functions. *Molecular Biotherapy*, ***3***, 103-107.

[67] Reuter HD, Sendl A. (**1994**) *Alium sativum* and *Allium ursinum*: *Chemistry, Pharmacology and Medicinal Applications*. In Wagner H. (Ed.), Economic and medicinal plant research, 6. Academic Press, London, 55-113.

[68] Liu C-T, Sheen L-Y, Lii C-K. (**2007**) Does garlic have a role as an antidiabetic agent? *Molecular Nutrition & Food Research*, ***51***, 1353-1364.

[69] Stevinson C, Pittler MH, Ernst E. (**2000**) Garlic for treating hypercholesterolemia. A meta-analysis of randomized clinical trials. *Annals of Internal Medicine*, ***133***, 420-429.

[70] Ernst E, Pittler MH, Wider B, Boddy K. (**2008**) The Oxford Handbook of Complementary Medicine, Oxford University Press, Oxford.

[71] Khoo YSK, Aziz Z. (**2009**) Garlic supplementation and serum cholesterol: a meta-analysis. *Journal of Clinical Pharmacy and Therapeutics*, ***34***, 133-145.

[72] Reinhart KM, Coleman CI, Teevan C, Vachhani P, White CM. (**2008**) Effects of garlic on blood pressure in patients with and without systolic hypertension: a meta-analysis. *Annals of Pharmacotherapy*, ***42***, 1766-1771.

[73] Ried K, Frank OR, Stocks NP, Fakler P, Sullivan T. (**2008**) Effect of garlic on blood pressure: a systematic review and meta-analysis. *BMC Cardiovascular Disorders*, ***8***, 13-24.

[74] Meher S, Duley L. (**2006**) Garlic for preventing pre-eclampsia and its complications. *Cochrane Database Systematic Reviews*, ***3***, CD006065.

[75] Koscielny J, Klussendorf D, Latza R, Schmitt R, Radtke H, Siegel G, Kiesewetter H. (**1999**) The antiatherosclerotic effect of *Allium sativum*. *Atherosclerosis*, ***144***, 237-249.

[76] Breithaupt-Grogler K, Ling M, Boudoulas H, Belz GG. (**1997**) Protective effect of chronic garlic intake on elastic properties of aorta in the elderly. *Circulation*, ***96***, 2649-2655.

[77] Budoff M. (**2006**) Aged garlic extract retards progression of coronary artery calcification. *Journal of Nutrition*, ***136***, 741S-744S.

[78] Herman-Antosiewicz A, Singh SV. (**2004**) Signal transduction pathways leading to cell cycle arrest and apoptosis induction in cancer cells by *Allium* vegetable-derived organosulfur compounds: a review. *Mutation Research*, ***555***, 121-131.

[79] Milner JA. (**2001**) Mechanisms by which garlic and allyl sulphur compounds suppress carcinogen bioactivation. Garlic and carcinogenesis. *Advances in Experimental Medicine and Biology*, ***492***, 69-81.

[80] Sheen LY, Chen HW, Kung YL, Liu CT and Lii CK. (**1999**) Effects of garlic oil and its organosulfur compounds on the activities of hepatic drug-metabolizing and antioxidant enzymes in rats fed high- and low-fat diets. *Nutrition and Cancer*, ***35***, 160-166.

[81] Germain E, Chevalier J, Siess MH, Teyssier C. (**2003**) Hepatic metabolism of diallyl disulphide in rat and man. *Xenobiotica* ***33***, 1185-1199.

[82] Chen C, Pung D, Leong V, Hebbar V, Shen G, Nair S, Li W and Kong AN. (**2004**) Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. *Free Radical Biology and Medicine*, ***37***, 1578-1590.

[83] Gong P, Hu B, Cederbaum AI. (**2004**) Diallyl sulphide induces heme oxygenase-1 through MAPK pathway. *Archives of Biochemistry and Biophysics*, ***432***, 252-260.

[84] Kim JY, Kwon O. (**2009**) Garlic intake and cancer risk: an analysis using the Food and Drug Administration's evidence-based review system for the scientific evaluation of health claims. *American Journal of Clinical Nutrition*, ***89***, 257-264.

[85] Ngo SNI, Williams DB, Cobiac L, Head RJ. (**2007**) Does garlic reduced risk of colorectal cancer? A systematic review. *Journal of Nutrition*, ***137***, 2264-2269.

[86] Ackermann RT, Mulrow CD, Ramirez G, Gardner CD, Morbidoni L, Lawrence VA. (**2001**) Garlic shows promise for improving some cardiovascular risk factors. *Archives of Internal Medicine*, ***161***, 813-824.

[87] Pittler MH, Ernst E. (**2007**) Clinical effectiveness of garlic (*Allium sativum*). *Molecular Nutrition & Food Research,* ***51***, 1382-1385.

[88] Izzo AA. (**2007**) Efficacy and safety of *Allium sativum* (garlic). *Focus on Alternative and Complementary Therapies*, ***12***, 162–166.

[89] Alvarez MS, Jacobs S, Jiang SB, Brancaccio RR, Soter NA, Cohen DE. (**2003**) Photocontact allergy to diallyl disulfide. *American Journal of Contact Dermatitis*, ***14***, 161-165.

[90] Borrelli F, Capasso R, Izzo AA. (**2007**) Garlic (*Allium sativum* L.): adverse effects and drug interactions in humans. *Molecular Nutrition & Food Research*, ***51***, 1386-1397.

[91] Blumenthal M, Busse WR, Goldberg A, Gruenwald J, Hall T, Riggins CW, Rister RS. (Eds). (**1998**) *The complete German Commission E monographs: therapeutic guide to herbal medicines*. American Botanical Council, Austin, TX.

[92] Hoshino T, Kashimoto N, Kasuga S. (**2001**) Effects of garlic preparations on the gastrointestinal mucosa. *Journal of Nutrition*, ***131***, 1109S-1113S.

[93] Beck E, Grunwald J. (**1993**) *Allium sativum* in der Stufentherapie der Hyperlipidaemie. *Medizinische Welt*, ***44***, 516-520.

[94] Papageorgiou C, Corbet JP, Menezes-Brandao F, Pecegueiro M, Benezra C. (**1983**) Allergic contact dermatitis to garlic (*Allium sativum* L.). Identification of the allergens: the role of mono-, di-, and trisulfides present in garlic. A comparative study in man and animal (guinea-pig). *Archives of Dermatology Research*, ***275***, 229-234.

[95] Kao SH, Hsu CH, Su SN, Hor WT, Chang T WH, Chow LP. (**2004**) Identification and immunologic characterization of an allergen, alliin lyase, from garlic (*Allium sativum*). *Journal of Allergy Clinical Immunology*, ***113***, 161-188.

[96] Bagge S, Thomas BS, Bhat M. (**2008**) Garlic burn as self-inflicted mucosal injury- a case report and review of the literature. *Quintessence International*, ***39***, 491-494.

[97] Bojs G, Svensson A. (**1988**) Contact allergy to garlic used for wound healing. *Contact Dermatitis*, ***18***, 179–181.

[98] Burden AD, Wilkinson SM, Beck MH, Chalmers RJ. (**1994**) Garlic-induced systemic contact dermatitis. *Contact Dermatitis,* ***30*****,** 299–300.

[99] Pires G, Pargana E, Loureiro V, Almeida MM, Pinto JR. (**2002**) Allergy to garlic. *Allergy*, ***57***, 957-958.

[100] Asero R, Mistrello G, Roncarolo D, Antoniotti PL, Falagiani P. (**1998**) A case of garlic allergy. *Journal of Allergy and Clinical Immunology*, ***101***, 427-428.

[101] Sanchez-Hernandez MC, Hernandez M, Delgado J, Guardia P, Monteseirin J, Bartolomé B, Palacios R, Martinez J, Conde J. (**2000**) Allergenic cross-reactivity in the Liliaceae family. *Allergy*, ***55***, 297-299.

[102] Brenner S. (**1999**) Pemphigus and diet. Have we solved the mystery of fogo selvagem? *Advances in Experimental Medicine and Biology*, ***455***, 267-269.

[103] Ruocco V, Brenner S, Lombardi ML. (**1996**) A case of diet-related pemphigus. *Dermatology*, ***192***, 373-374.

[104] Gurley BJ, Gardner SF, Hubbard MA, Williams DK, Gentry WB, Cui Y, Ang CY. (**2005**) Clinical assessment of effects of botanical supplementation on cytochrome P450 phenotypes in the elderly: St John's wort, garlic oil, *Panax ginseng* and *Ginkgo biloba. Drugs Aging*, ***22***, 525-539.

[105] Gurley BJ, Gardner SF, Hubbard MA, Williams DK, Gentry WB, Cui Y, Ang CY. (**2002**) Cytochrome P450 phenotypic ratios for predicting herb-drug interactions in humans. *Clinical Pharmacology & Therapeutics*, ***72***, 276-287.

[106] Macan H, Uykimpang R, Alconcel M, Takasu J, Razon R, Amagase H, Niihara Y. (**2006**) Aged garlic extract may be safe for patients on warfarin therapy. *Journal of Nutrition*, ***136***, 793S-795S.

[107] Mohammed Abdul MI, Jiang X, Williams KM, Day RO, Roufogalis BD, Liauw WS, Xu H, McLachlan AJ. (**2008**) Pharmacodynamic interaction of warfarin with cranberry but not with garlic in healthy subjects. *British Journal of Pharmacology*, ***154***, 1691-1700.

[108] Piscitelli SC, Burstein AH, Welden N, Gallicano KD, Falloon J. (**2002**) The effect of garlic supplements on the pharmacokinetics of saquinavir. *Clinical Infectious Diseases*, ***34***, 234-238.

[109] Gallicano K, Foster B, Choudhri S. (**2003**) Effect of short-term administration of garlic supplements on single-dose ritonavir pharmacokinetics in healthy volunteers. *British Journal of Clinical Pharmacology*, ***55***, 199-202.

[110] Laroche M, Choudhuri S, Gallicano K, Foster B. (**1998**) Severe gastrointestinal toxicity with concomitant ingestion of ritonavir and garlic. *The Canadian Journal of Infectious Diseases*, ***9***, 471.

[111] Barnes J, Anderson LA, Phillipson JD. (**2002**) *Herbal Medicine*, 2nd Ed., Pharmaceutical Press, London.

[112] Capasso F, Gaginella TS, Grandolini G, Izzo AA. (**2003**) *Phytotherapy. A Quick Reference to Herbal Medicine*, Springer-Verlag, Berlin, Heidelberg.

Natural Product Communications

Manuscripts in Press
Volume 4, Number 12 (2009)

Natural Product Communications

2009 Volume 4

Natural Product Communications 4 (1-12) 1-1798 (2009)

ISSN 1934-578X (print)
ISSN 1555-9475 (online)

NPC Natural Product Communications

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Natural Product Communications

Volume **4** (1-12)
2009
Contents

Number 1

Number 2

Number 3

Number 4

Number 5

Number 6

Number 7

Number 8

Number 9

Number 10

Number 11

Chemical Biodiversity of Secondary Metabolites from Endophytic Fungi

Original Paper

Letter to Editor

Number 12

Natural Product Communications

Volume **4** (1-12)
2009
Author Index

Natural Product Communications

Volume **4** (1-12)
2009
Keyword Index

Review/Account

Natural Product Communications
2009
Volume 4, Number 12

Contents

Continued inside backcover