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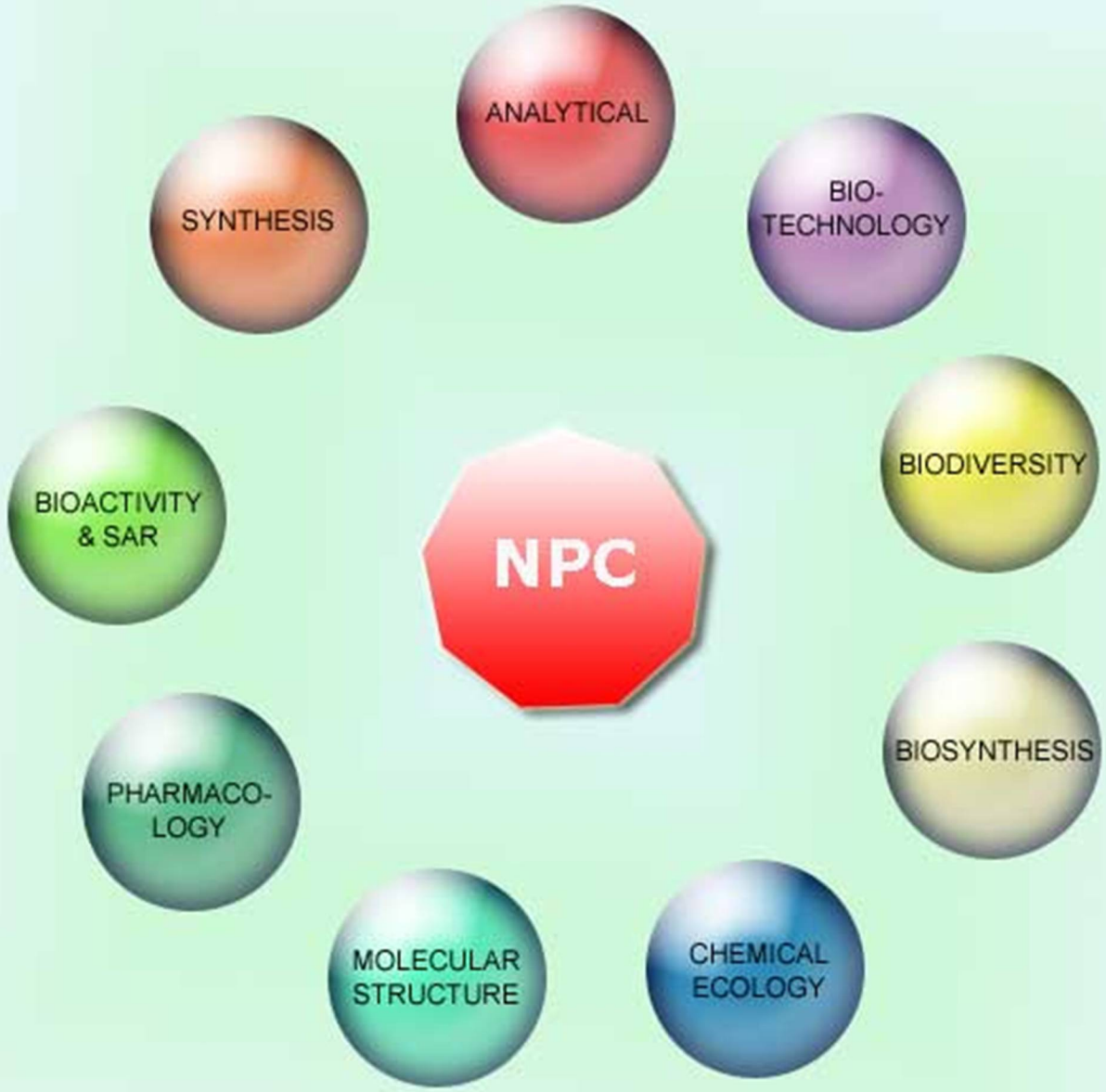

Volume 6. Issue 12. Pages 1799-1968. 2011
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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Natural Product Communications
2011
Volume 6, Number 12

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Published online (www.naturalproduct.us)

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No. 12
1799 - 1800

Sibiralactone: A New Monoterpene from *Sibiraea angustata*

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Received: August 23rd, 2011; Accepted: October 26th, 2011

Sibiralactone (**1**), a new monoterpene derivative, was isolated from the leaves of *Sibiraea angustata*. The structure was determined by the analysis of its NMR data and the absolute configuration was established by TDDFT ECD calculation of the solution conformers.

Keywords: *Sibiraea angustata*, sibiralactone, monoterpene, TDDFT ECD calculation.

Sibiraea angustata (Rehd.) Hand.-Mazz. is indigenous of the bush flora and gravel areas found in Qinghai, Gansu, Sichuan and Yunnan provinces of China at an altitude of 3000-4000 m [1]. When it is fed to domestic animals, loss of weight was observed [2a]. Previous phytochemical investigations of the plant revealed the presence of several triterpenoids, phenolic acids, fatty alcohols [2b-2e] and a monoterpene lactone, 1-*O*-*β*-D-glucopyranosylgeraniol-10,5-olide exhibiting lipid metabolism-ameliorating and hypoglycemic activity [3]. Herein we report the structure elucidation of sibiralactone (**1**), the second monoterpene lactone from *S. angustata* leaves.

Sibiralactone (**1**) was determined as $C_{10}H_{16}O_4$ on the basis of a HRESI-MS peak of $[M + Na]^+$ at 223.0942 (Calcd. 223.0946), indicating three degrees of unsaturation. The IR spectrum showed absorption bands characteristic of hydroxyl groups (3307 cm^{-1}) and a γ-lactone moiety (1780 cm^{-1}). The ^1H NMR spectrum showed proton signals for two isolated methyl groups [δ_H 1.80 (3H, s), 1.81 (3H, s)], two oxymethine groups [δ_H 3.88 (1H, t, J = 8.4 Hz), 4.85 (1H, t, J = 8.4 Hz)], one methine group [2.60 (1H, dt, J = 2.8, 9.6 Hz)], one olefinic H-atom [δ_H 5.21 (1H, br. d, J = 9.2 Hz)], one oxymethylene group [δ_H 3.72 (1H, dt, J = 2.4, 10.8 Hz), 4.00 (1H, dt, J = 4.0, 10.8 Hz)] and one methylene group [δ_H 1.73 (1H, m), 2.25 (1H, dq, J = 3.2, 6.4, 14.8 Hz)]. The proton spin system of (O)-CH_2-CH_2-CH-CH(O)-CH(O)-CH=$C(CH_3)_2$ could be deduced by an ^1H-^1H COSY measurement. The ^{13}C NMR spectroscopic data, including the DEPT spectrum, showed two methyls (δ_C 18.6 and 25.9), two oxymethines (δ_C 78.6 and 79.7), one methine (δ_C 49.0), one oxymethylene (δ_C 61.8), one methylene (δ_C 31.2), one olefinic methine (δ_C 120.1), one quaternary olefinic carbon (δ_C 143.0), and a carbonyl (δ_C 176.0). The ester carbonyl at δ_C 176.0 showed HMBC correlation with the C-7 methylene

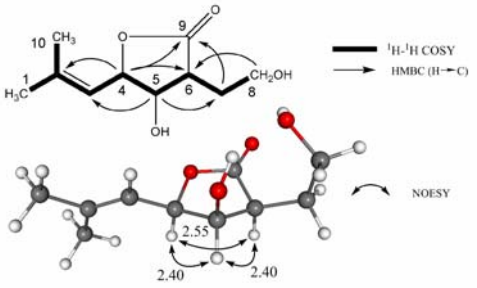

Figure 1: ^1H-^1H COSY, key HMBC and selected NOE correlations of **1**.

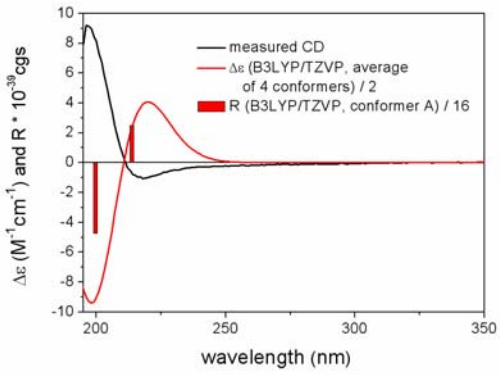

Figure 2: Experimental and B3LYP/TZVP ECD spectra of (4*S*, 5*S*, 6*R*)-**1** obtained as the Boltzmann-weighted average of the computed four solution conformers.

protons at δ_H 1.73 and 2.25, and the oxymethine at δ_H 4.85, which together with other correlations confirmed the γ-lactone moiety and the planar structure of **1** (Figure 1). The all *cis* relative configuration was deduced on the ground of NOE effects between H-4 (δ_H 4.85), H-5 (δ_H 3.88) and H-6 (δ_H 2.60) (Figure 1), implying (4*R**, 5*R**, 6*S**) relative configuration. The all *cis* relative configuration also corroborated well the observed $^3J_{H,H}$ coupling constants of 4-H (J = 8.4 Hz), 5-H (J = 8.4 Hz), and 6-H (J = 2.8, 9.6 Hz).

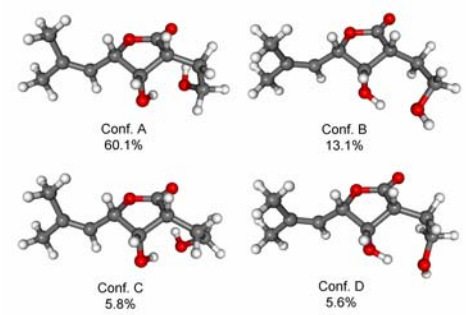

Figure 3: DFT optimized geometries of the four lowest-energy conformers of (4*S*, 5*S*, 6*R*)-**1**.

For the determination of absolute configuration, the electronic circular dichroism (ECD) spectra were calculated with three functionals (B3LYP, BH&HLYP, PBE0) for the computed four lowest-energy conformers (with populations larger than 5%) of (4*S*, 5*S*, 6*R*)-**1** and the Boltzmann-weighted ECD spectrum was compared with the experimental ECD curve. Since the calculated and experimental ECD curves were mirror images (Figure 2), the absolute configuration of sibiralactone (**1**) was determined as (+)-(4*R*, 5*R*, 6*S*).

Experimental

General: Optical rotation, Perkin-Elmer Model 341 polarimeter; IR, Nicolet NEXUS 670 FT-IR spectrometer; ECD, Applied Photophysics Ltd. Chirascan spectropolarimeter; HRESI-MS, Waters Q-TOF Premier; NMR (1D and 2D) on a Varian Unity 400/54 instrument.

Plant material: The leaves of *Sibiraea angustata* were collected at Songpan County, Sichuan Province in August, 2008. The plant was identified by Prof. Tiznzhi Wang. A voucher specimen (No. SA0808) was deposited at the Herbarium of the Sichuan University, Chengdu, China.

Extraction and isolation: Powdered leaves of *S. angustata* (5 kg) were extracted with 80% aq. ethanol under reflux. The extracts were evaporated *in vacuo*, then partitioned between H_2O and EtOAc. The EtOAc extract (185 g) was chromatographed over a silica gel column (2000 g, 100-200 mesh), eluted with a gradient solvent system [$CHCl_3$-MeOH (90:1-2:1)] to give 12 fractions (Fr.1-12). Fr. 4 (2.9 g) was chromatographed over a silica gel column (200-300 mesh, 60 g) eluted with solvent systems of light petroleum-acetone (5:1) and cyclohexane-EtOAc (4:1) to afford compound **1** (24 mg).

Sibiralactone (**1**)
colorless gum
$[\alpha]_D$: +32.7 (*c* 1.15, $CHCl_3$).
CD (MeOH, λ [nm] (Δε), c = 0.028): 217 (-1.41), 196 (9.12).
IR ν_{max} (ethanol): 3307, 2848, 1780, 1454, 1378, 1045, 879 cm^{-1}.
^{1}H NMR (400 MHz, $CDCl_3$): 1.73 (1H, m, H-7a), 1.80 (3H, s, H-10), 1.81 (3H, s, H-1), 2.25 (1H, dq, *J* = 14.8, 6.4, 3.2 Hz, H-7b), 2.60 (1H, dt, *J* = 9.6, 2.8 Hz, H-6), 3.72 (1H, dt, *J* = 10.8, 2.4 Hz, H-8a), 3.88 (1H, t, *J* = 8.4 Hz, H-5), 4.00 (1H, dt, *J* = 10.8, 4.0 Hz, H-8b), 4.85 (1H, t, *J* = 8.4 Hz, H-4), 5.21 (1H, br.d, *J* = 9.2 Hz, H-3).
^{13}C NMR (100 MHz $CDCl_3$): 18.6 (CH_3, C-10), 25.9 (CH_3, C-1), 31.2 (CH_2, C-7), 49.0 (CH, C-6), 61.8 (CH_2, C-8), 78.6 (CH, C-5), 79.7 (CH, C-4), 120.1 (CH, C-3), 143.0 (C, C-2), 176.0 (C, C-9).
HRESI-MS: *m/z* 223.0942 [M + Na^+] (calcd. 223.0946 for $C_{10}H_{16}N_4NaO_4$.

Computational section: Conformational searches were carried out by means of the Macromodel 9.7.211 [4] software using Merck Molecular Force Field (MMFF) with implicit solvent model for chloroform. Geometry reoptimizations at B3LYP/6-31 G(d) level of theory followed by TDDFT calculations using various functionals (B3LYP, BH&HLYP, PBE0) and TZVP basis set were performed by the Gaussian 03 [5] package. Boltzmann distributions were estimated from the ZPVE corrected B3LYP/6-31 G(d) energies. ECD spectra were generated as the sum of Gaussians [6] with 3000 cm^{-1} half-height width (corresponding to ca. 12 nm at 200 nm), using dipole-velocity computed rotational strengths for conformers above 5%. The MOLEKEL [7] software package was used for visualization of the results.

Acknowledgments - The authors are grateful to Shepo Shi (Institute of Biological Chemistry, Washington State University) for discussion on the structure elucidation. This work was supported financially by the Fundamental Research Funds for the Central Universities (ZYGX2010J102). T. K. thanks the National Office for Research and Technology (NKTH, K-68429), TÁMOP 4.2.1/B-09/1/KONV-2010-0007 and János Bolyai Foundation.

References

[1] Yu DJ, Gu CZ. (**1974**) *Flora Republicae Popularis Sinicae*. Volume ***36***. Science Publishing House, Beijing. 69-70.
[2] (a) Wang XL, Guo JM, Yang BX. (**1995**) Studies on narrowleaf Sibiraea (*Sibiraea angustata*) in regulating lipoid metabolism. *Chinese Traditional and Herbal Drugs*, ***26***, 473-475, 504; (b) Zhang CZ, Feng SL, Li C, Zhao CX, Shang XY, Ma HP. (**1992**) Triterpenoid constituents from liucha (*Sibiraea angustata*). *China Journal of Chinese Materia Medica*, ***17***, 360, 373; (c) Zhang CZ, Li C, Feng SL, Zhao CL, Wang WH. (**1993**) Isolation and structure of sibirate from *Sibiraea angustata*. *Acta Pharmaceutica Sinica*, ***28***, 798-800; (d) Liu JX, Wu SJ, Wei XY, Yang RZ. (**1999**) Studies on the chemical constituents of *Sibiraea angustata*. *Journal of Tropical and Subtropical Botany*, ***7***, 248-251; (e) Tao TT, Sei Y, Wang TZ, Bai C, Chang YB. (**2006**) Chemical constituents of *Sibiraea angustata*. *Chinese Journal Natural Medicines*, ***4***, 257-259.
[3] Sei Y, Kano Y, Takabori R. (**2007**) 1-*O*-beta-D-glucopyranosyleraniol-10,5-olide and use thereof, US 2007/0059339.
[4] MacroModel, Schrödinger LLC, **2009**. http://www.schrodinger.com/Products/macromodel.html
[5] Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JR, Montgomery Jr JA, Vreven T, Kudin KN, Burant JC, *et al*. Gaussian 03, Revision C.02. **2004**, Gaussian Inc., Wallingford CT.
[6] Stephens PJ, Harada N. (**2010**) ECD cotton effect approximated by the Gaussian curve and other methods. *Chirality*, ***22***, 229-233.
[7] Varetto U. MOLEKEL 5.4., **2009**, Swiss National Supercomputing Centre: Manno, Switzerland.

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2011
Vol. 6
No. 12
1801 - 1804

Bioconversion of Proposed Precursors into Theobroxide and Related Compounds

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Received: June 14th, 2011; Accepted: September 2nd, 2011

We have previously reported a tetraketide origin for theobroxide and its related compound. In the present study, bioconversion of natural and deuterium-labeled precursors of this proposed biosynthetic pathway by *Lasiodipoldia theobromae* was investigated. Theobroxide was quantified after bioconversion from each proposed precursor. The transformation of the isotopically labeled precursor to products was tracked by ^{2}H NMR measurement.

Keywords: *Lasiodiplodia theobromae*, bioconversion, fungal biosynthesis, theobroxide, ^{2}H NMR.

Fungi are lower eukaryotic microbes having important relationships with human beings not only as plant and animal pathogens, but also as major producers of enzymes, amino acids, and biologically active secondary metabolites. *Lasiodiplodia theobromae* (synonym *Botryodiplodia theobromae*) is a common pathogenic fungus that has been found throughout the tropics and the subtropics [1]. The culture filtrate of *L. theobromae* showed significant plant growth inhibitory activity [2]. For several decades, a lot of attention has been paid to this fungus. A variety of bioactive components have been isolated from it and been characterized, such as jasmonic acid (JA), a plant hormone widely distributed in plants [3] and micro-organisms [2]; mellein, a metabolite of *Aspergillus melleus* [4] and *A. ochraceus* [5], as a hair pencil component of the male oriental fruit moth *Graphholitha molesta* [6]; and theobroxide, a potato-tuber and flower bud inducing substance (1*S*,2*R*,5*S*,6*R*)-3-methyl-7-oxa-bicyclohept-3-en-2,5-diol (**1**), with its related compound (1*S*,4*R*,5*S*,6*R*)-7,9-dioxa-3-methyl-8-oxobicyclo[4.3.0]-2-nonene-4,5-diol (**2**), both showing inducing activity for different physiological phenomena. Research on some plant growth regulators from *L. theobromae* and their biosynthesis, translocation, functions, and modes of action has also been intensively undertaken by the authors' group [7–12].

In our previous study, we reported a tetraketide origin for biosynthesis of **1** and **2** by using ^{13}C and ^{2}H labeled

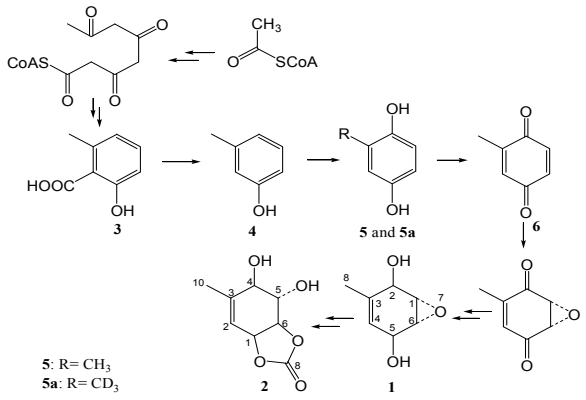

Figure 1: The proposed biosynthetic pathway of theobroxide **1** and compound **2**.

acetates [7]. In the proposed biosynthetic pathway (Figure 1), we supposed that four acetyl-CoA are linked 'head-to-tail' to generate a triketo-CoA, and then one of the resulting keto groups is reduced to a hydroxyl group. Generation of a carbanion at the β-keto residue would allow an aldol condensation to form a six-membered carbocycle. Then a sequence of plausible dehydration and enolisation reactions would produce the 6-methylsalicylic acid (**3**, 6-MSA) as a natural precursor [13,14]. Decarboxylation of compound **3** gives 3-methylphenol (**4**), which is *para*-hydroxylated to 2-methylbenzene-1,4-diol (**5**). Precursor **5** is then oxidized to 2-methyl-[1,4]benzoquinone (**6**). Finally, theobroxide (**1**) is

produced through epoxidization and reduction of both hydroxyl groups, and is converted to its carbonyldioxy derivative **2** by an unidentified mechanism.

As a continuing study, we performed bioconversion experiments to confirm this proposed biosynthetic pathway by incorporating both natural and isotopic proposed precursors into these two target compounds.

In this study, the appropriate conditions and process for feeding of four proposed precursors to *L. theobromae* were investigated first. The mycelia grew well when the fungal culture was fed with these chemicals at a concentration of under 10^{-4} M. However, they did not grow when fed with compound **6** at 10^{-3} M, implying that a higher concentration of **6** may kill the fungus. No obvious difference in mycelial growth was observed when fed with the other three compounds at 10^{-3} M compared with the control group, which was fed only with 0.5 mL of DMSO (average weight: 3.98–4.35 g/200 mL culture). The average mycelial weight of the positive control group was 4.57 g/200 mL culture. The results indicated that these four compounds did not significantly suppress growth of *L. theobromae* OCS 71 at a concentration of 10^{-4} M. TLC monitoring also showed that theobroxide can be produced well in the presence of 10^{-4} M of **6**. Therefore, feeding experiments with *L. theobromae* using the proposed precursors **3**–**6** were carried out at a concentration of 10^{-4} M.

The results are presented in Figure 2. As can be seen, feeding of compounds **3** or **4** increased the amount of theobroxide in the fungal culture by two times compared with the control. In the case of fungal culture fed with compounds **5** or **6**, theobroxide was produced in almost three times higher amount compared with the control group. The higher content of theobroxide might be because compounds **5** and **6** are closer to theobroxide in the biosynthetic pathway. The above results provide evidence for compounds **3**, **4**, **5**, and **6** as intermediates in the biosynthetic pathway of theobroxide.

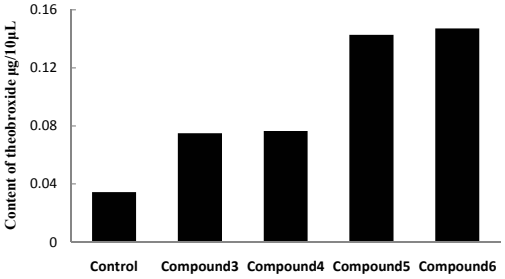

Figure 2: Content of theobroxide after bioconversion from proposed precursors. Values represent the means of three independent experiments with ±SE which indicate significant differences ($p < 0.05$) with respect to the control.

The biosynthesis of theobroxide **1** and compound **2** from the proposed precursor of compound **5** in *L. theobromae* was further examined by means of ^{2}H NMR based isotopic tracing. As shown in Table 1, ^{2}H NMR spectroscopic data of the resulting compounds **1** and **2** revealed that the chemical shifts of the incorporated deuterium were δ 1.79 for **1** and δ 1.84 for **2**, respectively. These chemical shifts are consistent with those of the methyl proton of natural theobroxide **1** and compound **2**. These results indicated that retention of deuterium of the methyl group occurred during bioconversion from **5a** to **1** and **2** in *L. theobromae.* Accordingly, compound **5** was further proved to be an intermediate in the biosynthetic pathway of theobroxide.

Table 1: Incorporation of deuterium-labelled **5a** into compounds **1** and **2**.

Position	Compound **1**		Compound **2**	
	^{1}H[a]	^{2}H[a]	^{1}H[b]	^{2}H[b]
1	3.31 (1H, br s)	-	5.10 (1H, m)	-
2	4.20 (1H, br s)	-	5.56 (1H, m)	-
3	-	-	-	-
4	5.46 (1H, br d, *J* = 5.4 Hz)	-	3.88 (1H, br d, *J* = 8.1 Hz)	-
5	4.41 (1H, br s)	-	3.55 (1H, br t, *J* = 8.1 Hz)	
6	3.25 (1H, br s)	-	4.59 (1H, br t, *J* = 8.1 Hz)	-
8	1.79 (3H, s)	1.79 (3D, s)	-	-
10	-	-	1.84 (3H, s)	1.84 (3D, s)

[a]Measured in $CDCl_3$ for ^{1}H NMR and in $CHCl_3$ for ^{2}H NMR.
[b]Measured in CD_3OD for ^{1}H NMR and in CH_3OH for ^{2}H NMR.

In conclusion, in this study, some evidence was provided for the previously proposed biosynthetic pathway of theobroxide (**1**) and compound **2** as depicted in Figure 1. For future work, the biotransformation mechanism of theobroxide to its carbonyldioxy derivative **2** should be clarified experimentally.

Experimental

General experimental procedures: Spores of *L. theobromae* were maintained on agar (1.5%) in a 2%-potato D-glucose medium (Nissui Seiyaku) at 25°C and transferred at intervals of 6 months. All chemicals were purchased from either Wako or Aldrich Chemical Company. Compounds **3** and **5a** were prepared by published methods [15, 16]. Preparative and analytical TLC were performed on Merck Kieselgel $60F_{254}$ precoated glass plates (0.5 mm and 0.25 mm, respectively). Compounds were visualized by either exposure to UV light or by staining with 5% molybdophosphoric acid *n*-hydrate in EtOH followed by heating on a hot plate. Open column chromatography was performed with silica gel (60N spherical neutral 63–210 μm, Kanto Chemical). ^{1}H NMR (270 MHz and 500 MHz) and ^{2}H NMR (76.5 MHz) spectra were recorded on a Bruker AMX-500 FT-NMR.

Determination of feeding conditions: Two different concentrations (10^{-3} M and 10^{-4} M) of each proposed precursor were tested for fungal growth. For one group, 3 500 mL Erlenmeyer flasks containing 200 mL of a potato D-glucose medium (1% glucose) were inoculated with a

piece (1 cm^2) of agar bearing the slantwise culture of *L. theobromae* OCS 71, individually. Chemicals were first dissolved in 0.5 mL DMSO (dimethyl sulfoxide) and then fed into the culture medium. As a control group, 3 flasks of fungal culture were fed with 0.5 mL of DMSO without chemicals. As a positive control group, 3 flasks only contained fungal culture. All groups were statically cultured at 25°C in the dark for 7 days, and then the mycelia were harvested and dried upon filter paper under reduced pressure for 12 h. The fresh weight of mycelia was measured. The presence of theobroxide in the culture medium of each group was monitored by TLC with MeOH–$CHCl_3$ (5:95, v/v).

Quantification of theobroxide: After 7 days, the fungal culture in each flask was filtered using 3 layers of gauze. One mL of culture filtrate from each flask was added into 100 µL of internal standard, 2-methoxy-4-methylphenol (100 µg in EtOAc), and then extracted with 1 mL of EtOAc, 3 times. The EtOAc layer was again filtered using a cellulose acetate 0.45 µm filter, dried over Na_2SO_4 and then concentrated to dryness under vacuum. The concentrated sample was re-dissolved in 1 mL of acetonitrile (HPLC grade). Ten µL of sample solution was analyzed by HPLC (Waters model 600 system) equipped with an RP ODS column (GL Science, 4.6 mm ID × 250 mm) and a photodiode array detector (Waters 996). Acetonitrile-water (1:1, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The detection wavelength was set at 210 nm and the column temperature was 25°C. The retention time of theobroxide was 2.7 min and that of the internal standard 6.1 min. The content of theobroxide was estimated using the following equation: ($C_t = f \times A_t / A_s \times C_s$), where C_t, A_t, A_s, and C_s are the content of target compound, peak area of target compound, peak area of standard substance, and content of injected standard substance, respectively. The correction factor f is the average value (R.S.D. < 2.0%) of 4 tests, which is calculated as $f = (M_t / A_t) / (M_s / A_s)$ using the mass (M) and peak area (A) of the target compound (t) and standard substance (s). Statistical analysis of data was performed by ANOVA analysis and the Bonferoni test ($p < 0.05$ and $p < 0.01$) in order to compare different data in the experiments.

Feeding experiments: *L. theobromae* was grown by static culture on liquid potato-1%-D-glucose medium supplemented with 10^{-3} M [7,7-$^2H_3$]-2-methyl-benzene-1,4-diol (**5a**). The culture broth from each feeding experiment of either 7-day-old or 14-day-old cultures was combined individually, filtered, and concentrated to 100 mL. The filtrate was extracted 3 times with an equal volume of EtOAc. The EtOAc extract was concentrated to dryness under reduced pressure and then chromatographed using a silica gel column (24 g silica gel for 7-day-old culture and 45 g for 14 day-old culture) eluting with MeOH–$CHCl_3$ (5:95, v/v). Compound **1** (0.9 mg/100 mL from 7-day-old culture) and compound **2** (0.36 mg/100mL from 14-day-old culture) were purified by recrystallization from EtOAc: *n*-hexane.

Acknowledgments - The authors thank Dr Eri Fukushi (GC-MS and NMR Laboratory, Hokkaido University, Japan) for NMR measurements.

References

[1] Hirai T. (**1938**) Disease of the banana in transport from Formosa (Japanese). *Annual Meeting of the Phytopathological Society of Japan*, ***8***, 145-166.

[2] Aldridge DC, Galt S, Giles D, Turner WB. (**1971**) Metabolites of *Lasiodiplodia theobromae*. *Journal of Chemical Society*, ***9***, 1623-1627.

[3] Meyer A, Miersch O, Buttner C, Dathe W, Sembdner G. (**1984**) Occurrence of the plant growth regulator jasmonic acid in plants. *Journal of Plant Growth Regulation*, ***3***, 1-8.

[4] Nishikawa H, Kaishi NN. (**1993**) Metabolite from *Aspergillus melleus*. *Nippon Nogeikagaku Kaishi*, *Journal of the Agricultural Chimerical Society of Japan*, ***9***, 772-774.

[5] Yabuta T, Sumiki Y, (**1993**) Metabolite from *Aspergillus ochraceus*. *Nippon Nogeikagaku Kaishi*, *Journal of the Agricultural Chimerical Society of Japan*, ***9***, 1264-1275.

[6] Mori K, Gupta AK, (**1985**) Chiral synthesis of (*R*)-(-)-mellein and (3*R*, 4a*S*)-(+)-ramulosin. *Tetrahedron*, ***41***, 5295-5299.

[7] Peng L, Ryo T, Kosaku T, Kensuke N. (**2006**) Biosynthesis of theobroxide and its related compounds, metabolites of *Lasiodiplodia theobromae*. *Phytochemistry*, ***68***, 819-823.

[8] Peng L, Kosaku T, Hideyuki M, Yoshihara T. (**2005**) Novel potato micro-tuber-inducing compound, (3*R*,6*S*)-6-Hydroxylasiodiplodin, from a strain of *Lasiodiplodia theobromae*. *Bioscience, Biotechnology, and Biochemistry*, ***69***, 1610-1612.

[9] Nakamori K, Matsuura H, Yoshihara T, Ichihara A, Koda Y. (**1994**) Potato micro-tuber inducing substances from *Lasidiplodia theobromae*. *Phytochemistry*, ***35***, 835-839.

[10] Yoshihara T, Amanuma M, Tsutsumi T, Okumura Y, Matsuura H, Ichihara A. (**1996**) Metabolism and transport of [2-^{14}C]jasmonic acid in potato plant. *Plant Cell Physiology*, ***37***, 586-590.

[11] Kashima T, Takahashi K, Matsuura H, Nabeta K. (**2009**) Biosynthesis of resorcylic acid lactone lasiodiplodin in *Lasiodiplodia theobromae*. *Bioscience, Biotechnology, and Biochemistry*, ***73***, 1118-1122.

[12] Takei R, Takahashi K, Matsuura H, Nabeta K. (**2009**) Biosynthesis of resorcylic acid lactone. (5*S*)-5-Hydroxylasiodiplodin in *Lasiodiplodia theobromae*. *Bioscience, Biotechnology, and Biochemistry*, ***72***, 2522-2524.

[13] Birch AJ, Donovan FW. (**1953**) Studies in relation to biosynthesis. Some possible routes to derivatives of orcinol and phloroglucinol. *Australian Journal of Chemistry*, ***6***, 360-368.

[14] Birch AJ, Massy-Westropp RA, Moye CJ. (**1955**) Studies in relation to biosynthesis. 2-Hydroxy-6-methylbenzoic acid in *Penicillium griseofulvum* Dierckx. *Australian Journal of Chemistry*, ***8***, 539-544.
[15] Eliel EL, Rivard DE, Bergstahler AW. (**1953**) The synthesis of 6-formylsalicylic acid, esters of 6-bromomethylsalicylic acid, and related substances. *Journal of Organic Chemistry*, ***18***, 1979-1988.
[16] Francesco M, Salvadori P. (**2005**) An easy two-step reduction of salicylic acids and alcohols to 2-methylphenols. *Synthesis*, ***15***, 2479-2481.

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2011
Vol. 6
No. 12
1805 - 1806

Microbial Hydroxylation of *S*-(-)-Perillyl Alcohol by *Fusarium heterosporium*

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Received: March 23rd, 2011; Accepted: October 4th, 2011

S-(-)-Perillyl alcohol (*p*-mentha-1, 8-diene-7-ol) (**1**) (500 mg) was converted by *Fusarium heterosporium* ATCC 15625 over 10 days at 25°C to a new metabolite, 1,2-dihydroxyperillyl alcohol (*p*-mentha-8-en-1,2,7-triol) (**3**) in a yield of 13% (70 mg). The structure of **3** was established by IR and NMR spectroscopic, specific rotation, and mass spectral studies.

Keywords: Biotransformation, *Fusarium heterosporium*, *S*-(-)-perillyl alcohol.

Perillyl alcohol (POH) is a dietary monoterpene found in a variety of foods such as mints, cranberries and cherries [1,2]. It is a hydroxy derivative of limonene and possesses chemopreventive properties [3]. At present it is claimed that POH and limonene inhibit, in a dose-dependent manner, the development of various kinds of carcinoma [4-6]. They may act during the initiation phase of carcinogenesis, preventing interaction of carcinogens with DNA, or during the promotion phase, inhibiting cancer cell development and migration. These led to induction of cancer cell apoptosis, re-differentiation of tumor cells, and influence the molecular mechanisms which regulate their functions. It is believed that post-translational isoprenylation of proteins controlling the growth of cells is the most important mechanism that perillyl alcohol and limonene influence [3,7]. Therefore, the biological derivatization of perillyl alcohol and its structural analog limonene with the aim of producing new metabolites with a variety of biological properties, besides their use as flavoring agents, is an important field of xenobiochemistry, pharmacology and toxicology [8]. The aim of this study was to obtain derivatives of perillyl alcohol through microbial biotransformation.

A few studies are available for the biotransformation of *S*-(-)-perillyl alcohol by microorganisms, plant cell cultures and mammals. These include epoxidation by *Streptomyces ikutamanensis* Ya-2-1 giving 8,9-epoxy perillyl alcohol and glycosylation by *Eucalyptus perriniana* suspension cells, which produced the monoglucoside [9,10]. In the present study, the microbiological transformation of *S*-(-)-perillyl alcohol (**1**) by the plant pathogenic fungus *Fusarium heterosporium* ATCC 15625 was examined. The microbial transformation was carried out on an orbital shaker for 10 days at 25°C. This resulted in the production

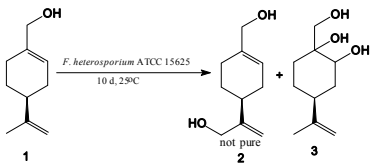

Figure 1: Biotransformation of *S*-(-)-perillyl alcohol by *F. heterosporium*.

of a complex mixture of products as detected by TLC. The pure metabolite, dihydroxyperillyl alcohol (**3**), was obtained from the column by eluting with 90%, v/v, ethyl acetate in light petroleum (60-80°C) (see Figure 1). The MS of the metabolite showed a molecular ion at *m/z* 186 corresponding to the molecular formula of $C_{10}H_{18}O_3$. The ^{13}C NMR spectrum indicated a new lowfield hydroxyl-bearing carbon and methine signals at δ_C 69.8 and 72.2 ppm, respectively, whereas two carbon signals resonating at δ_C 137.2 and 122.1 disappeared. This indicated that the endocyclic double bond of *S*-(-)-perillyl alcohol (**1**) had been dihydroxylated.

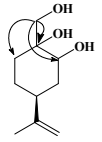

Figure 2: HMBC correlations of 7-CH_2OH signals for p-mentha-8-en-1,2,7-triol.

The position of the newly introduced hydroxyl groups were established by the use of Heteronuclear Multi Bond Coherence (HMBC) spectra and comparing the ^{13}C NMR and the DEPT spectra of the metabolite with that of **1** and its known metabolites. The DEPT spectrum displayed the presence of a methyl, a hydroxyl bearing methylene, four methylenes, one methine, one hydroxyl bearing methine and a hydroxyl bearing quaternary carbon signals,

indicating that hydroxylations had taken place on C-2 and C-3. The HMBC spectrum confirmed this and showed the key bond connectivity of proton signals of the hydroxy-bearing carbon atoms at 3.45 (1H, d, *J* 11.4 MHz, 7-C*H*$_A$H$_B$OH) and 3.82 (1H, d, *J* 11.4 MHz, 7-CH$_A$*H*$_B$OH) to the carbon signals at δ_C 72.2 (C-1), 69.8 (C-2) and 25.4 (C-6).

In summary, *F. heterosporium* ATCC 15625 efficiently dihydroxylated perillyl alcohol at the C–2 and C-3 positions, producing a dihydroxylated metabolite, which is recorded in the literature for the first time. The pure metabolite was found to be optically active.

Experimental

General experimental procedures: IR spectra were recorded in KBr pellets. Optical rotation was measured on a Krüss Optronic P8000-T series polarimeter. GC-MS and LCMS spectra were recorded on a 6890 N network GC system, a 5975 inert mass and Applied Biosystems API 3200 instruments, respectively. ^{1}H NMR and ^{13}C NMR spectra were determined on a Bruker DPX 500 spectrometer, while DEPT spectra were measured at 125 MHz in deuteriochloroform with tetramethylsilane as an internal standard reference. *S*-(-)-perillyl alcohol (*p*-mentha-1,8-diene-7-ol) (**1**) was purchased from Aldrich (218391). The solvents used for purification were distilled prior to use. Silica gel type 60 (Merck, 230–400 mesh) was used for CC. TLC was carried out on a 0.25 mm thick silica gel plate (Merck silica gel 60 GF_{254}) in *n*-hexane/ethyl acetate (1:1, v,v). Compounds were detected either under UV light (at 254 nm) or by spraying with vanillin/sulfuric acid (1:1).

Microorganism: *Fusarium heterosporium* ATCC 15625 was provided by Prof. Dr Fatih Demirci, Faculty of Pharmacy at Anadolu University, Eskişehir, Turkey.

***General culture conditions*:** The cultures were maintained and precultured on potato dextrose agar (PDA) slants at 5^{o}C and 25^{o}C, respectively [11]. A medium for growing *F. heterosporium* was prepared by mixing glucose (20 g), yeast extract (5 g), polypeptone (5 g), NaCl (5 g) and Na_2HPO_4 (5 g) in distilled water (1 L). *S*-(-)-perillyl alcohol (**1**) (500 mg) was added into 20 Erlenmeyer flasks (250 mL) containing 100 mL of freshly prepared and autoclaved medium after 2 days' growth. The fermentations were continued for a further 10 days. The mycelium was filtered off and the broth was extracted with ethyl acetate. The extract was dried over Na_2SO_4 and the solvent evaporated to give a residue which was purified by means of CC (3 cm wide, 60 cm long) on silica eluting with increasing concentrations of ethyl acetate in light petroleum. A control experiment was run simultaneously.

Biotransformation by F. heterosporium: The product, 1,2-dihydroxyperillyl alcohol (**3**) (70 mg; 13 % yield), was eluted with 90%, v/v, ethyl acetate in light petroleum (60-80^{o}C) as an oily compound.

$[\alpha]^{20}_D$: 96.0 (2.5 mg in 1 mL acetone, 20°C).

FT-IR ν_{max} cm^{-1}: 3404 (OH), 1641 (C=C);

^{1}H NMR (500 MHz, $CDCl_3$): 1.75 (3H, s, 10-C*H*$_3$), 3.45 (1H, d, *J* = 11.4 MHz, 7-C*H*$_A$H$_B$OH), 3.82 (1H, d, *J* = 11.4 MHz, 7-CH$_A$*H*$_B$OH), 3.91 (1H, s, 2-CH), and 4.75 (2H, br. s, 9-C*H*$_2$OH).

^{13}C NMR (125 MHz, $CDCl_3$): C-8 (149.3), C-9 (108.9), C-1 (72.2), C-2 (69.8), C-7 (68.0), C-4 (37.8), C-3 (33.9), C-5 (28.7), C-6 (25.4), C-10 (20.8).

LCMS m/z: [M+1] 187.066, (calcd for $C_{10}H_{18}O_3$, [M+1] 187.241).

Acknowledgements - Dr Hakan DAL and Dr Fatih Göger from Anadolu University, Eskişehir-TURKEY are kindly acknowledged for providing the NMR and LCMS analyses, respectively.

References

[1] Belanger JT. **(1998)** Perillyl alcohol: applications in oncology. *Alternative Medicine Review*, ***3***, 448–457.
[2] Paduch R, Kandefer−Szerszeń M, Trytek M, Fiedurek J. (**2007**) Terpenes: substances useful in human healthcare. *Archivum Immunologiae et Therapia Experimentalis*, ***55***, 315–327.
[3] Chan NLS, Wang H, Wang Y, Leung HY, Leung LK. (**2006**) Polycyclic aromatic hydrocarbon-induced CYP1B1 activity is suppressed by perillyl alcohol in MCF–7 cells. *Toxicology and Applied Pharmacology*, ***213***, 98–104.
[4] Crowell PL. (**1999**) Prevention and therapy of cancer by dietary monoterpenes. *Journal of Nutrition*, ***129***, 775S–778S.
[5] Bardon S, Foussard V, Fournel S, Loubat A. (**2002**) Monoterpenes inhibit proliferation of human colon cancer cells by modulating cell cycle-related protein expression. *Cancer Letters*, ***181***, 187–194.
[6] Gupta A, Myrdal PB. (**2004**) Development of a perillyl alcohol topical cream formulation. *International Journal of Pharmacology*, ***269***, 373–383.
[7] Crowell PL. (**1997**) Monoterpenes in breast cancer chemoprevention. *Breast Cancer Research Treatment*, ***46***, 191–197.
[8] Bhatti HN, Zubair M, Rasool N, Hassan Z, Ahmad VU. (**2009**) Transformation of sesquiterpenoids. *Natural Product Communications*, ***4***, 1155-1168.
[9] Noma Y, Asakawa Y. (**2010**) Biotransformation of monoterpenoids by microorganisms, insects and mammals. In *Handbook of essential oils*, Baser KHC, Buchbauer G. (Eds), CRC Press, New York, USA, 645–646.
[10] Cardillo R, Nasini G, De Pava OV. (**1992**) Isolation of illudin M, illudinine and shisool from the mushroom *Clitocybe phosphorea*; biotransformation of perillyl alcohol and aldehyde. *Phytochemistry*, ***31***, 2013–2014.
[11] Kiran I, Yildirim HN, Hanson JR, Hitchcock PB. (**2004**) The antifungal activity and biotransformation of diisophorone by the fungus *Aspergillus niger*. *Journal of Chemical Technology and Biotechnology*, ***79***, 1366–1370.

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2011
Vol. 6
No. 12
1807 - 1808

A Phytochemical Investigation of *Zanthoxylum setulosum*

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Received: September 19th, 2011; Accepted: September 28th, 2011

The crude bark extract of *Zanthoxylum setulosum* from Monteverde, Costa Rica was notably cytotoxic (100% kill at 100 μg/mL) to MCF-7, MDA-MB-231, and MDA-MB-468 cells *in vitro*. Phytochemical studies of the bark extract revealed the triterpenoid lupeol, the lignan sesamin, the sesquiterpene sesquichamaenol, and the xanthone lichexanthone. This is the first report of the isolation of sesquichamaenol and lichexanthone from the bark extract of *Z. setulosum*. All structures were determined using NMR spectroscopic techniques (^{1}H NMR and ^{13}C NMR) and GC-MS and by comparison with literature data. Lupeol proved to be the cytotoxic component of *Z. setulosum* bark.

Keywords: *Zanthoxylum setulosum*, lupeol, sesamin, sesquichamaenol, lichexanthone, cytotoxicity.

Tropical rainforest plants have evolved chemical defensive mechanisms to avoid herbivory and resist pathogens [1]. Monteverde, Costa Rica, is home to numerous species of *Zanthoxylum* that contain common bioactive constituents and have various traditional uses [2a]. *Zanthoxylum*, a genus of about 250 species in Rutaceae, is a rich source of lignans, alkaloids, coumarins, amides, flavonoids, terpenes and other metabolites with pharmacological properties [2b,2c].

Z. setulosum P. Wilson is a medium to large tree [3]. Lupeol and sesamin have been previously identified in the leaves of *Z. setulosum* [4]. *Z. setulosum* bark extract had been previously shown to be cytotoxic [5] and in this work, the crude chloroform bark extract was cytotoxic to MCF-7, MDA-MB-231, and MDA-MB-468 breast tumor cells (100% kill at 100 μg/mL).

The crude chloroform bark extract of *Z. setulosum* was subjected to flash chromatography leading to the isolation of lupeol, sesamin, lichexanthone, and sesquichamaenol. The structures of the isolated components were determined using NMR and MS spectral data and by comparison of these with the literature [6-8].

Previous phytochemical investigation of *Z. setulosum* has shown the isolation from the leaf extract of sesamin, syringaresinol, the isopentyl ether of pluviatol, savinin, kusunokinin, lupeol, aurantiamide, and skimmianine [4]. In this work we report the isolation of sesquichamaenol and lichexanthone that have not been previously reported from *Z. setulosum*. In addition, lupeol and sesamin have been isolated in larger quantities from the bark than previously isolated from the leaves of this tree [4].

Sesquichamaenol is a sesquiterpene that is reported to be a source of various metabolites with pharmacological activities [9]. Lichexanthone is recorded as having antibacterial, antimalarial, antioxidant, and antitumor activity [10]. Lichexanthone has been isolated from a variety of *Zanthoxylum* species, but has not been reported in *Z. setulosum* [11]. Cytotoxicity testing of isolated and purified compounds indicated lupeol to be the cytotoxic material in the crude bark extract (100% kill at 100 μg/mL). Lupeol had been previously shown to be cytotoxic to Hep-G2, A-431 and H-4IIE cells [12]. However, neither sesamin, sesquichamaenol, nor lichexanthone showed cytotoxic activity.

Experimental

Collection and extraction of plant material: *Zanthoxylum setulosum* bark was collected in June, 2004, in Provincia Puntarenas, Costa Rica, 6 km SSW of Santa Elena on the main road from Monteverde. *Z. setulosum* was identified and authenticated by W.A. Haber and a voucher specimen (Haber 9982) has been deposited in Missouri Botanical Garden Herbarium. The chopped, dried bark (952 g) was extracted with refluxing chloroform for 6 h. Evaporation of the solvent gave 31.9 g of crude chloroform extract.

Chromatographic separation of bark extract: The crude chloroform bark extract (25.02 g) was subjected to flash chromatography using a silica gel (200-400 mesh) column, 70 cm in length × 5 cm in diameter. Elution was carried out using a solvent gradient (*n*-hexane with increasing proportions of ethyl acetate, followed by ethyl acetate with increasing proportions of ethanol). TLC was performed on silica gel precoated aluminum plates with a fluorescent indicator 254 nm for analysis of isolated fractions, and spots were detected by illumination with UV light. Lupeol was isolated from fraction 14 (yield 1.28 g). Sesamin crystallized from fraction 29 and 30, as colorless prisms (yield 2.05 g).

Fractions were checked by TLC and HPLC analysis and fractions having comparable TLC features were combined to form "superfractions." Combined fractions 5-11 (22 mg) and 20-23 (69 mg) were subjected to chromatographic separation on a silica gel (200-400 mesh) column, eluting with a *n*-hexane/EtOAc step gradient. Superfraction 5-11 was separated to give 10 mg of sesquichamaenol, and superfraction 20-23 to give 10 mg of lichexanthone.

Gas chromatography-mass spectrometry: Superfractions of *Z. setulosum* were subjected to gas chromatographic-mass spectral analysis on an Agilent system consisting of a 6890 Gas Chromatograph, a 5973 Mass selective detector (MSD), and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary consisting of a (5% phenyl)-methylpolysiloxane stationary phase, a film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The GC carrier gas was helium and the column had a head pressure of 7.07 psi and a flow rate of 1.0 mL/min. The inlet temperature was 200°C and the MSD temperature 280°C. The GC oven temperature was programmed as follows: 40°C initial temperature held for 10 min; increased at 3°/min to 200°C; increased 2°/min to 220°C. The extracts were dissolved in dichloromethane and 1 μL of the sample was injected using a split injection method. The MS of constituents were compared with those in the literature [7-9].

Cytotoxicity screening: *In-vitro* cytotoxic activity against MCF-7, MDA-MB-231, and MDA-MB-468 cells was determined using the MTT method for cell viability, as previously described [13].

Acknowledgments – We are grateful to the Monteverde Cloud Forest Preserve and the Tropical Science Center for granting permission to collect plant materials under a cooperative rights agreement and to the Commission for the Development of Biodiversity of Costa Rica's Ministry of the Environment, Energy, and Telecommunications for Research Permit R-001-2006-OT-CONAGEBIO.

References

[1] Howe HF, Westley LC. (**1990**) *Ecological Relationships of Plants and Animals*, Oxford University Press, Oxford, UK.

[2] (a) Haber WA, Zuchowski W, Bello E. (**2000**) *An Introduction to Cloud Forest Trees, Monteverde, Costa Rica*, Mountain Gem Publications, Monteverde de Puntarenas, Costa Rica; (b) Gentry AH. (**1993**) *A Field Guide to the Families and Genera of Woody Plants of Northwest South America*, University of Chicago Press, Chicago, Illinois; (c) Stermitz FR, Sharifi IA. (**1977**) Alkaloids of *Zanthoxylum monophyllum* and *Zanthoxylum punctatum*. *Phytochemistry*, ***16***, 2003-2006.

[3] Boehme AK, Noletto JA, Haber WA, Setzer WN. (**2008**) Bioactivity and chemical composition of the leaf essential oils of *Zanthoxylum rhoifolium* and *Zanthoxylum setulosum* from Monteverde, Costa Rica. *Natural Product Research*, ***22***, 31-36.

[4] Mora A, Castro V, Poveda L, Chavarria M, Murillo R. (**2011**) Chemical constituents from *Zanthoxylum setulosum* (Rutaceae). *The Latin American and Carribbean Bulletin of Medicinal and Aromatic Plants*, ***10***, 155-158.

[5] Setzer MC, Moriarity DM, Lawton RO, Setzer WN, Gentry GA, Haber WA. (**2003**) Phytomedicinal potential of tropical cloudforest plants from Monteverde, Costa Rica. *Revista de Biologia Tropical*, ***51***, 647-674.

[6] (a) Baures PW, Miski M, Eggleston DS. (**1992**) Structure of sesamin. *Acta Crystallographica Section C Crystal Structure Comminucations*, ***48***, 574-576; (b) Laggoune S, Brouard I, Leon F, Calliste CA, Duroux J, Bermejo J, Kabouche Z, Kabouche A. (**2011**) Lignans and an abundant flavone glycoside with free-radical scavenging activity from the roots of the endemic species *Stachys mialhesi* de Noe. *Records of Natural Products*, ***5***, 238-241.

[7] (a) Letcher RM. (**1968**) Chemistry of lichen constituents-VI: Mass spectra of usnic ascid, lichexanthone and their derivatives. *Organic Mass Spectrometry*, ***1***, 551-561; (b) Díaz AB, Vera JR, Cote V, Bruno-Colmenarez J, Diaz de Delgado G. (**2010**) NMR elucidation and crystal structure analysis of 1-hydroxy-3,6-dimethoxy-8-methyl-9h-xanthen-9-one (lichexanthone) isolated from *Vismia baccifera* (Guttiferae). *Latin American and Caribbean Bulletin of Medicinal and Aromatic Plants*, ***9***, 470-474.

[8] Koorbanally NA, Randrianarivelojosia M, Mulholland DA, van Ufford LQ, van den Berg AJ. (**2002**) Bioactive constituents of *Cedrelopsis microfoliata*. *Journal of Natural Products*, ***65***, 1349-1352.

[9] Warrior G, Lima EJS, Fernandes JB, da Silva MFGF, Vieira PC, Magellan CMI, Pagnocca CF, Bueno OC, Hebling MJA, Pirani JR. (**2005**) Sesquiterpenes from the stem of *Pilocarpus riedelianus* and activities of microorganisms. *Quimica Nova*, ***40***, 986-990.

[10] Pinto MM, Sousa ME, Nascimento MS. (**2005**) Xanthone derivatives: New insights in biological activity. *Current Medicinal Chemistry*, ***12***, 2517-2538.

[11] Jimenez C, Marcos M, Villaverde MC, Riguera R, Castedo L, Stermitz F. (**1989**) A chromone from *Zanthoxylum* species. *Phytochemistry*, ***28***, 1992-1993.

[12] Moriarity DM, Huang J, Yancey CA, Zhang P, Setzer WN, Lawton RO, Bates RB, Caldera S. (**1998**) Lupeol is the cytotoxic principle in the leaf extract of *Dendropanax* cf. *querceti*. *Planta Medica*, ***64***, 370-372.

[13] Moriarity DM, Bansal A, Cole RA, Takaku S, Haber WA, Setzer WN. (**2007**) Selective cytotoxic activities of leaf essential oils from Monteverde, Costa Rica. *Natural Product Communications*, ***2***, 1263-1268.

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2011
Vol. 6
No. 12
1809 - 1812

Cytotoxic Cembranoids from the Red Sea Soft Coral *Sarcophyton glaucum*

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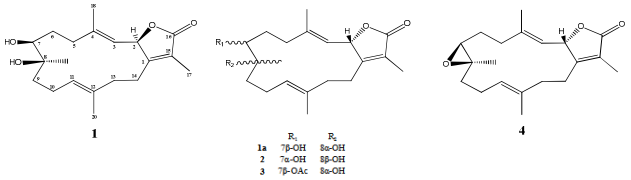
Received: June 21st, 2011; Accepted: August 3rd, 2011

One new cembrane diterpene, 2*R*,7*R*,8*R*-dihydroxydeepoxysarcophine (**1**), together with three known compounds, 7α,8β-dihydroxy-deepoxysarcophine (**2**), 7β-acetoxy-8α-hydroxydeepoxysarcophine (**3**), and sarcophine (**4**), have been isolated from the Red Sea soft coral *Sarcophyton glaucum*. Their structures were determined using 1D and 2D NMR spectroscopy. 7β-Acetoxy-8α-hydroxydeepoxysarcophine (**3**) exhibits cytotoxic activity against HepG2, HCT-116, and HeLa cells with IC_{50} values of 3.6, 2.3, and 6.7 μg/mL, respectively.

Keywords: *Sarcophyton glaucum*, cembrane diterpenes, dihydroxydeepoxysarcophine, sarcophine.

Marine organisms, comprising more than half of the total global diversity, offer an enormous source of novel and biologically active compounds. The soft corals (phylum Cnidaria, class Anthoza, subclass Octocorallia, order Alycyonacea) have proven to be a biochemical warehouse for terepenoids. Nearly 30 species of soft corals of the genus *Sarcophyton*, from different geographical areas, have been chemically examined. They contain antiproliferative sesquiterpenes [1] and an impressive series of cembranoid diterpenes [2]. Many cembrane-type compounds have been shown to exhibit cytotoxicity and anti-inflammatory activity [3-5]. Herein, we report the isolation of the new compound 2*R*,7*R*,8*R*-dihydroxydeepoxysarcophine (**1**), together with three known cembranolides, 7α,8β-dihydroxydeepoxysarcophine (**2**), 7β-acetoxy-8α-hydroxy-deepoxysarcophine (**3**), and sarcophine (**4**), from the Red Sea soft coral *S. glaucum* and cytotoxic activity against HepG2, HCT-116, HeLa, and MCF-7 cells. Structures of these isolated metabolites were elucidated through extensive 1D and 2D spectroscopic analyses.



Compound **1** was obtained as colorless crystals, $[\alpha]_D^{25}$ –21.2 (*c* 1.86, MeOH). The HR-CI-MS exhibited a $[M+Na]^+$ ion at m/z 357.20371 (calc. 357.20363), suggesting a molecular formula $C_{20}H_{29}O_4Na$ and six degrees of unsaturation that was supported by NMR data. The IR spectrum indicated the presence of an α,β-unsaturated-γ-lactone group (1750 and 1686 cm^{-1}), a ketone group (1707 cm^{-1}), an olefinic group (1669 cm^{-1}), and broad absorption for OH stretching (3000–3353 cm^{-1}) functionalities. A literature survey and careful comparison of the ^1H and ^{13}C NMR spectroscopic data of **1** (Table 1) revealed that it was in part close to the known compounds, 7β,8α-dihydroxydeepoxysarcophine (**1a**) and 7α,8β-dihydroxydeepoxysarcophine (**2**) [6a]. The ^{13}C NMR and

Table 1: NMR spectroscopic data of compound **1**.

Position	δ_H (*J* in Hz)	δ_C	HMBC
1	--	163.1 C	
2	5.43 (dd, 10.0, 1.5)	80.1 CH	C-1, C-3, C-4
3	4.95 (dd, 10.0, 1.0)	118.7 CH	C-5, C-18
4	--	146.2 C	
5	2.24 (m)	36.5 CH_2	C-3, C-4, C-6, C-7, C-18
6	1.60 (m)	29.1 CH_2	C-4, C-5, C-7
	1.79 (m)		C-4, C-5, C-7, C-8
7	3.33 (dd, 10.0, 1.5)	73.8 CH	C-5, C-6, C-8, C-19
8	--	75.3 C	
9	1.68 (m)	38.4 CH_2	C-7, C-8, C-10, C-11, C-19
10	2.10 (m)	22.4 CH_2	C-12
11	5.24 (td, 7.5, 1.0)	126.6 CH	C-9, C-10, C-13, C-20
12	--	135.3 C	
13	2.37 (m)	36.7 CH_2	C-1, C-11, C-12, C-14, C-20
14	2.10 (m)	26.1 CH_2	C-1, C-2, C-12, C-13, C-15
	2.62 (m)		C-1, C-2, C-12, C-13, C-15
15	--	123.1 C	
16	--	175.1 C	
17	1.88 (brt, 1.5)	9.3 CH_3	C-1, C-15, C-16
18	1.90 (d, 1.0)	17.7 CH_3	C-3, C-4, C-5
19	1.20 (s)	24.3 CH_3	C-7, C-8, C-9
20	1.64 (s)	16.1 CH_3	C-11, C-12, C-13

Recorded in $CDCl_3$ and obtained at 500 and 125 MHz for ^1H and ^{13}C NMR, respectively.

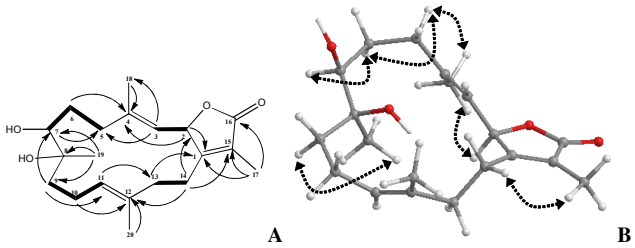


Figure 1: (A) ^1H-^1H COSY (bold lines) and key HMBC (arrows) correlations of **1**; (B) Selected NOESY correlations in compound **1**.

DEPT spectra (Table 1) revealed the presence of four olefinic functionalities at C-3 (δ 118.7), C-11 (δ 126.6), C-12 (δ 135.3), and C-4 (δ 146.2), two oxymethine carbons at C-7 (δ 73.8) and C-2 (δ 80.1), and one oxygenated quaternary carbon at C-8 (δ 75.3). The carbon signal resonances at C-15 (δ 123.1), C-1 (δ 163.1), and C-16 (δ 175.1) were assigned to the *α,β*-unsaturated-*γ*-lactone functionality that was confirmed by comparison of NMR data for **1** with **4**.

From the ^1H NMR and ^1H-^1H COSY spectra, the following proton correlations were assigned H-2/H-3, H_2-5/H_2-6/H-7, H_2-9/H_2-10/H-11, and H_2-13/H_2-14. The HMBC correlations H-2/C-1, H-2/C-4, H-3/C-5, H_2-9/C-7, H_2-10/C-12, H-11/C-13, H_2-13/C-1, and H_2-14/C-2 confirmed the connectivities of the 14-membered ring from C1–C14. The position of the methyl groups H_3-18 (δ 1.90), H_3-19 (δ 1.20), and H_3-20 (δ 1.64) was deduced from the HMBC correlations H-3/C-18, H_3-18/C-4, H_3-19/C-8, H_3-19/C-7, H_3-19/C-9, and H_3-20/C-12. The last methyl group, H_3-17 (δ 1.88), was connected to the α,β-unsaturated-γ-lactone ring from the HMBC correlations with the olefinic carbons C-1 (δ 163.1) and C-15 (δ 123.1) and the carbonyl carbon C-16 (δ 175.1). The most significant differences observed

Table 2: Comparison of ^1H and ^{13}C NMR spectroscopic data of compounds **1**, **2**, and **1a**.

	1	**2**	**1a**		**1**	**2**	**1a**
H-2	5.43, dd (10.0, 1.5)	5.57, dq (13.6, 2.4)	5.55, dq (10.1, 1.3)	C-2	80.1	79.3	79.1
H-7	3.33, dd (10.0, 1.5)	3.47, d (13.5)	3.66, dd (9.6, 9.5)	C-7	73.8	72.5	72.3
H-11	5.24, td (7.5, 1.0)	4.98 (m)	5.10, dd (6.7, 6.6)	C-11	126.6	125.2	124.0
H_3-19	1.20 (s)	1.18 (s)	1.54 (s)	C-19	24.3	24.2	26.4

in the ^1H NMR spectrum of **1** are the downfield chemical shift of H-11, which resonates at δ 5.24 (td, *J* = 7.5, 1.0), and the upfield chemical shift of H-7 [δ 3.33 (dd, *J* = 10.0, 1.5)] rather than the values reported for compounds **2** and **1a** [6b] (Table 2). These data indicate differences in the stereochemistry of the three chiral carbons C-2, C-7, and C-8 compared with those of **1a** and **2**.

The relative configuration of **1** was determined on the basis of the study of the coupling constants and NOESY experiments. The H-2 and H-3 vicinal coupling constant (10.0 Hz), as well as a NOESY correlation of H-2 with H_3-18, establish a *trans* configuration between the *γ*-lactone (H-2) and the olefinic protons (H-3); the proton signal H-5 (2.24, m) showed clear correlations with the H_3-18 methyl protons (1.90, d, *J*= 1.0); the oxygenated proton at C-7 (δ_H 3.33, dd, *J* =10.0, 1.5) showed a clear correlation with the H-6 (1.60, m) and H_3-19 (1.20, s) protons. From this information, we noted that H-2, H-7, and H_3-19 all faced in the same direction (Figure 1b).

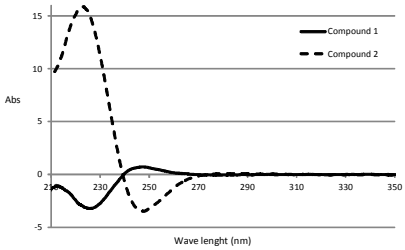


Figure 2: Circular dichroism spectra of compounds**1** and **2** in MeOH.

The absolute stereochemistry of **1** at C-2 was determined via circular dichroism (CD) analysis (Figure 2). The positive and negative Cotton effect ($[\theta]_{248}$ +1.12; $[\theta]_{225}$ –2.19) observed in the CD spectrum for the electronic transitions of the 2(5*H*)-furanone moiety indicate a left-handed (*M*) helix for the five-membered *α,β*-unsaturated-*γ*-lactone ring [6c]. Consistent with this interpretation, the similar optical rotation for **1** {$[\alpha]_D^{25}$ –21.2 (*c* 1.86, MeOH)} and *ent*-sarcophine {$[\alpha]_D^{25}$ = –16.0 (*c* 1.19, MeOH)} [7], confirm the same *R* absolute configuration at C-2, C-7, and C-8 [6-8].These data establish the isolation and identification of 7*β*,8*β*-dihydroxydeepoxysarcophine, a newly identified natural product.

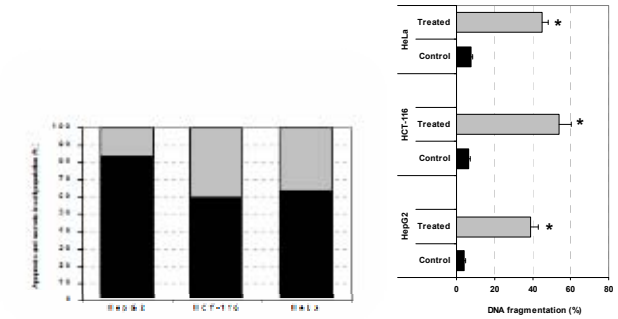
Compounds **2**–**4** were previously reported from the soft coral *S. glaucum* [6b,9] with no report of cytotoxicity. The

Table 3: Cytotoxic effect of different samples against different human cancer cell lines, using MTT assay (n=4); data expressed as the mean value of IC_{50} (μg/mL) ± S.E. Sample results are compared with paclitaxel.

	Cell line		
Compound	Hep-G2	HCT-116	HeLa
1	38.8 ± 1.0	26.3 ± 2.7	> 50
2	23.5 ± 2.4	15.6 ± 1.9	44.1 ± 5.4
3	3.6 ± 1.0	2.3 ± 1.5	6.7 ± 0.8
Paclitaxel	0.49 ± 0.1	0.4 ± 0.1	0.6 ± 0.08

cyototoxic activity of compounds **1**–**3** was tested against HepG2, HCT-116, HeLa, and MCF-7 cells. 7β-Acetoxy-8α-hydroxydeepoxy-sarcophine (**3**) showed strong cytotoxic activity against HepG2, HCT-116, and HeLa cells with IC_{50} values of 3.6, 2.3, and 6.7 μg/mL, respectively, while no activity against MCF-7 cells was observed for compounds **1** and **2**.

Using MTT assay, the effect of isolated compounds on the growth of various cell lines was studied after 48 h of incubation. As shown in Table 3, compound **3** showed a potential cytotoxic activity (low IC_{50} values <10 μg/mL) against HepG2, HCT-116, and HeLa cells, but not against MCF-7 cells, while the other tested compounds were not cytotoxic (IC_{50} values >10 μg/mL). Paclitaxel, a known anti-cancer drug, was used as a positive control.



(**A**) (**B**)

Figure 3: (A) Type of cell death was investigated in HepG2, HCT-116, and HeLa cells after treatment with compound **3**, using acridine orange/ethidium bromide staining to compare between the percentage of necrotic cells (grey segment) and apoptotic cells (black segment). (B) Effect of compound **3** (grey bars) on the percentage of DNA fragmentation in different cancer cell types compared with untreated cells (black bars). The fragmented DNA was determined with the diphenylamine reaction. Values are means of three measurements (mean ± SD), where (*) represent the p<0.05 in comparison with control.

To detect the type of cell death induced by compound **3** in different cancer cell lines, cells were treated with the appropriate IC_{50} value of compound **3** for 12 h and the apoptosis and necrosis percentages were recorded using acridine orange/ethidium bromide staining and analysis under a fluorescence microscope. As shown in Figure 3A, compound **3** led to an apoptosis-dependant cell death in all of the tested cell lines (59.7–83.2% of the total number of dead cell population), while the percentage of necrotic cells was 16.8–40.3% of the total number of the dead cell population. Comparing the untreated cells with paclitaxel-treated cells indicated that the latter significantly induced DNA fragmentation up to 57% (p< 0.001) in different cell lines, as shown in Figure 3B. Similarly, treatment of different cells with compound **3** resulted in high, to variable extents, DNA fragmentation levels that were significantly different from the control (p< 0.01–0.001), in the following order: HCT-116 >HeLa> HepG2.

Experimental

General experimental procedures: 1D and 2D NMR, Varian Mercury Plus 300 MHz and Varian Unity INOVA 500 spectrometer; CI-MS, Finnigan LCQ Ion Trap mass spectrometer; HR-CI-MS, Fourier transform ion cyclotron mass spectrometer (Ion Spec, Varian); Optical rotations, Perkin–Elmer-341 MC digital polarimeter; CD, OLIS DSM-10UV/Vis CD; CC, Silica gel 60 (Merck, 230–400 mesh) and Sephadex LH-20 (Sigma); TLC, Pre-coated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm).

Animal material: The *S. glaucum* was collected by SCUBA in June 2009 from Hurghada, on the Egyptian Red Sea coast. A voucher specimen (03RS24) was deposited in the Marine Biological Station, Hurghada, Red Sea, Egypt.

Extraction and isolation: The frozen soft coral was chopped into small pieces (4 kg, wet wt) and extracted with EtOAc at room temperature (4L × 5). The combined EtOAc extracts were concentrated to a brown gum (20.0 g), which was subjected to CC on a silica gel column (6 × 120 cm) using *n*-hexane–EtOAc (gradient separation) to yield 8 fractions. Fraction 2, eluted with *n*-hexane–EtOAc (3:1), was separated by Sephadex LH-20 using *n*-hexane–$CHCl_3$–MeOH (7:4:0.5) to give **1** (10 mg) and **3** (15 mg). Fraction 6 eluted with *n*-hexane–EtOAc (1:1) was separated on silica gel using *n*-hexane–EtOAc, followed by Sephadex LH-20 using *n*-hexane–$CHCl_3$–MeOH (7:4:0.5), to give **2** (12 mg).

2*R*,7*R*,8*R*-Dihydroxydeepoxysarcophine (1)
Colorless crystals.
$[\alpha]_D^{25}$: –21.2 (c 1.86, MeOH).
IR (film): 1750, 1686, 1707, 1669 cm^{-1}.
^1H and ^{13}C NMR: Table 1.
HRCIMS: m/z $[M + Na]^+$calcd for $C_{20}H_{29}O_4Na$: 357.20363; found: 357.20371.

Cell culture: Human breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), cervical carcinoma (HeLa), and colon carcinoma cells (HCT-116) were purchased from ATCC, USA, and used to evaluate the cytotoxic effect of the tested extracts. Cells were routinely cultured in Dulbeco's modified Eagle's medium (DMEM), while HCT-116 cells were grown in McCoy's medium. Media were supplemented with 10% fetal bovine serum (FBS), 2 mL glutamine, containing 100 units/mL penicillin G sodium, 100 units/mL streptomycin sulfate, and 250 ng/mL amphotericin B. Cells were maintained at sub-confluency at 37°C in humidified air containing 5% CO_2. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%. Test samples were dissolved in DMSO, and then diluted 1000 times for the assay. All experiments were repeated 3 times, unless mentioned.

Cytotoxicity assay: The cytotoxic effect of the compounds on the growth of different human cancer cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [10]. Cells (5×10^4 cells/well) were incubated for 48 h with various concentrations of the compounds at 37°C in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with a microplate reader (BioRad, München, Germany) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared with untreated cells. The relative cell viability was expressed as the mean percentage of viable cells compared with the respective untreated cells (control). The half maximal growth inhibitory concentration (IC_{50}) value was calculated from the line equation of the dose-dependent curve of each compound. The results were compared with paclitaxel.

Apoptosis and necrosis staining: The type of cell death was investigated in compound-treated and -untreated cells using acridine orange/ethidium bromide staining [11,12]. In brief, a mixture of 100 μg/mL acridine orange and 100 μg/mL ethidium bromide was prepared in PBS. The cell uptake of the stain was monitored under a fluorescence microscope, and the apoptotic, necrotic, and viable cells were counted. The early apoptotic cells had yellow chromatin in the nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner.

DNA fragmentation: DNA fragmentation was essentially assayed as reported previously [13]. Briefly, compound-treated and -untreated cell pellets were re-suspended in 250 μL 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation. Pellets were re-suspended in TE-buffer and samples were precipitated by 10% trichloroacetic acid at 48°C. The sample pellets were added to 5% trichloroacetic acid and boiled. DNA contents were quantified using the diphenylamine reagent [14]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Acknowledgements - Financial assistance was provided in part by a grant from the Robert Welch Foundation (D-1478).

References

[1] Sawant SS, Youssef DTA, Sylvester PW, Wali V, El Sayed KA. (**2007**) Antiproliferative sesquiterpenes from the Red Sea soft coral *Sarcophyton glaucum*. *Natural Product Communications*, ***2***, 117-119.

[2] Yan XH, Gavagnin M, Cimino G, Guo YW. (**2007**) Two new biscembranes with unprecedented carbon skeleton and their probable biogenetic precursor from the Hainan soft coral *Sarchophyton latum*. *Tetrahedron Letters*, ***48***, 5313–5316.

[3] Gross H, Wright AD, Beil W, Konig G. (**2004**) Two new bicyclic cembranoides from a new *Sarcophyton* species and determination of the absolute configuration of sarcoglaucol-16-one. *Organic & Biomolecular Chemistry*, ***2***, 1133–1138.

[4] Sawant S, Youssef D, Mayer A, Sylvester P, Wali V, Arant M, Sayed KE. (**2006**) Anticancer and anti-inflammatory sulfur-containing semisynthetic derivatives of sarcophine. *Chemical & Pharmaceutical Bulletin*, ***54***, 1119–1123.

[5] Sawant S, Youssef D, Reiland J, Ferniz M, Marchtti D, Sayed KE. (**2006**) Biocatalytic and antimetastatic studies of the marine cembranoidssarcophine and 2-*epi*-16-deoxysarcophine. *Journal of Natural Products*, ***69***, 1010–1013.

[6] (a) Yao LG, Liu HL, Guo YW, Mollo E. (**2009**) New cemranoids from the Hainan soft coral *Sarcophyton glaucum*. *Helvetica Chimica Acta*, ***92***, 1085–1091; (b) El Sayed KA, Hamann MT, Waddling CA, Jensen C, Lee SK, Dunstan CA, Pezzuto JM. (**1998**) Structurally novel bioconversion products of the marine natural product sarcophin effectively inhibit JB6 cell transformation. *Journal of Organic Chemistry*, ***63***, 7449–7455; (c) Kamel HN, Ferreira D, Garcia-Fernandez LF, Slattery M. (**2007**) Cytotoxic Diterpenoids from the hybrid soft coral *Sinularia maxima* × *Sinularia polydactyla*. *Journal of Natural Products*, ***70***, 1223–1227.

[7] Kashman Y, Zadock E, Neeman I. (**1974**) Some new cembrane derivatives of marine origin. *Tetrahedron*, ***30***, 3615–3620.

[8] Yin SW, Shi YP, Li XM, Wang BG. (**2005**) A novel hydroperoxyl substituted cemranolidediterpene from marine soft coral *Lobophytum crissum*. *Chinese Chemical Letters*, ***16***, 1489–1491.

[9] Grote D, Shaker KH, Soliman HSM, Hegazi MM, Seifert K. (**2008**) Cembranoid diterpenes from the soft corals *Sarcophyton* sp. and *Sarcophyton glaucum*. *Natural Product Communications*, ***3***, 1473–1478.

[10] Hansen MB, Nielsen SE, Berg K. (**1989**) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of Immunological Methods*, ***119***, 203–210.

[11] Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. (**1995**) Current protocols in immunology. In *Related isolation procedures and functional assays*. Vol.**1**, Coico R. (Ed.). John Wiley & Sons, Inc., New York, NY, p 3.17.1.

[12] Ribble D, Goldstein NB, Norris DA, Shellman YG. (**2005**) A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnology*, ***5***, 12, doi:10.1186/1472-6750-5-12 (http://www.biomedcentral.com/1472-6750/5/12).

[13] Messmer UK, Reed UK, Brüne B. (**1996**) Bcl-2 protects macrophage from nitric oxide-induced apoptosis. *Journal of Biological Chemistry*, ***271***, 20192–20197.

[14] Burton KA. (**1956**) A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochemical Journal*, ***62***, 315–323.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1813 - 1816

C-Lactam Derivatives of Oleanolic Acid. The synthesis of C-lactam by Beckmann rearrangement of C-oxime

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Received: May 18th, 2011; Accepted: October 11th, 2011

Oleanolic acid, one of the most known triterpenes, was subjected to different chemical transformations within C-3 β-hydroxyl group, a double bond between C-12 and C-13, and a carboxyl function at C-17 in order to obtain new derivatives. The key compound consists of four six-membered rings (A, B, D, E) and one enlarged ring (C ring) containing a nitrogen atom and a carbonyl function – lactam. This type of derivative can be obtained by Beckmann rearrangement of the appropriate oxime. The lactam can be transformed into thiolactam with the use of Lavesson's reagent. The method is also presented for new derivatives synthesis, as well as their structure elucidation by spectroscopic means.

Keywords: triterpenes, oleanolic acid, azaderivatives of oleanolic acid, lactams, Beckmann rearrangement.

Oleanolic acid is a very-well tested compound that is broadly distributed in the plant kingdom. This acid is present, often together with other triterpenes, in a great number of plant sources [1]. The presence of a β-hydroxyl group at C-3, a double bond between carbons 12 and 13, and a carboxyl function at C-17 allows the possibility of performing a set of transformations that lead to many new derivatives.

For many years oleanolic acid has been subjected to numerous tests to determine its biological activity [eg. 2 and works cited within]. These efforts were focused on obtaining new types of derivatives with special pharmacological activity [eg. 3–7 and works cited therein].

Lactam derivatives are a special type of new compound within oleanolates. Lactams are internal amides. The nitrogen atom constitutes an element of a ring. The lactam system appears in numerous compounds with pharmacological activity, for example, psychotropic drugs, antiviral medicines and antibiotics.

There are only a few known derivatives of oleanolic acid with a lactam system. The first sythesis of this type of compound was published in 1971 [8]. The scientists developed a method of oleanolic acid azaderivatives synthesis with an expanded A-ring, pyridine as solvent, and tosyl chloride as a rearrangement activator. The reaction was carried out at 100°C for 3 hours. Two main products were separated, one with a nitrile function and cleaved A-ring (about 24%), and the second with a seven-membered lactam system (32%). Sundararamaiah *et al*. [9] performed some experiments with the usage of phosphoryl chloride as a rearrangement agent in pyridine at either slightly elevated temperature (30 – 35°C) or at 130°C. Better results were obtained in the latter case and the yield of nitrile and lactam were 12% and 40%, respectively.

The next azaderivative of oleanolic acid was obtained in 1974 [10]. The new compounds contained two nitrogen atoms, one within a morpholide function, and the second within a lactam or nitrile system. The synthesis was performed in pyridine with the use of tosyl chloride as a rearrangement activator at room temperature. The nitrile was obtained in a yield of 74%, but the yield of lactam is unknown.

The synthesis of a six-membered lactam system within an oleanolic acid molecule is also known, but this compound is deprived of its C-23 and C-24 angular groups [11]. As a result of phosphorus pentachloride action on methyl oleanolate at -15°C, dehydratation took place and a new bond between C-3 and C-5 was formed. Further transformation led to a product with a five-membered A-ring of the nor-type and with a C-3 carbonyl group. This compound, when treated with hydroxylamine hydrochloride, formed an oxime that by the action of phosphorus pentachloride was transformed into a lactam.

Oleanolic acid (**1**) used for transformations was isolated by us from a by-product residue obtained during production of mistletoe extract. The triterpenic acid was transformed into its methyl ester (**2**). Physico-chemical and spectral data of both compounds were in agreement with literature data [12].

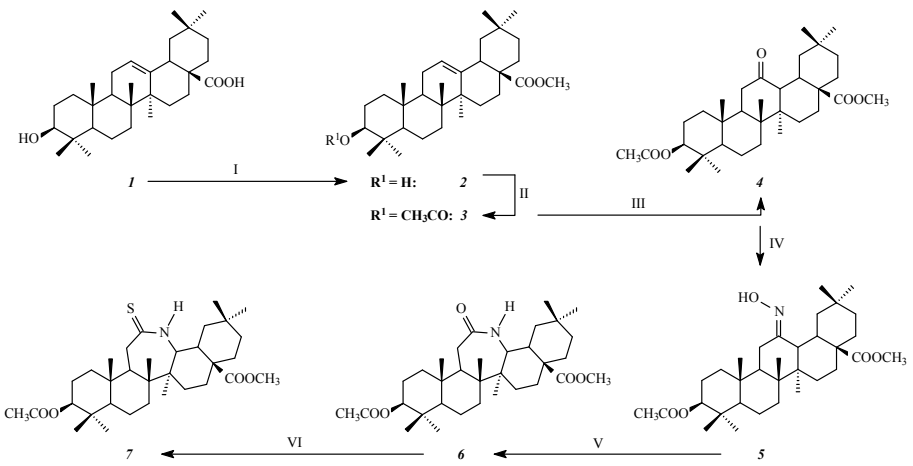


Figure 1: Synthesis of C-lactam derivatives of oleanolic acid.

Reagents and conditions: I: $(CH_3)_2SO_4$, EtOH, NaOH, heating; II: $(CH_3CO)_2O$, pyridine, rt.; III: *m*-CPBA, CH_2Cl_2, rt; IV: NH_2OH x HCl, CH_3COONa, EtOH, heating; V: $POCl_3$, pyridine, rt; VI: Lavesson's reagent, C_6H_6, heating.

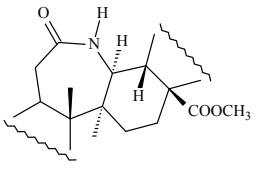
Next, methyl oleanolate (**2**) was acetylated with 10 equiv. of acetic anhydride in anhydrous pyridine to give methyl 3-acetoxyoleanolate (**3**), the spectral data of which were in accordance with those in the literature [12]. On oxidation of **2** with 2 equiv. of 3-chloroperbenzoic acid in dry $CHC1_3$ at room temperature overnight, methyl 3-acetoxy-12,13-epoxyoleanolate was formed. The crude product was purified by column chromatography. Under acidic conditions, the 12,13-epoxide underwent hydrolysis and furher oxidation to form the 12-oxo compound. Pure product (**4**) was obtained as oil that solidified after cooling. The crystallization from ethanol led to white needles, the physico-chemical and spectral data of which were in agreement with those in the literature [13].

The product of the above reaction was converted into the appropriate hydroxyimine compound by the action of hydroxylamine hydrochloride in ethanol. The total transformation of ketone **4** into oxime **5** finished after 3 hours of heating; the yield was about 80%. After crystallization from ethanol, 3β-acetoxy-12-hydroxyiminoolean-28-oic acid methyl ester (***5***) formed white crystals.

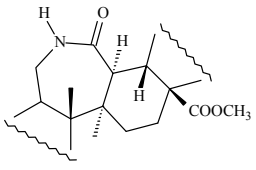
The obtained 12-oxime (**5**) was subjected to Beckmann rearrangement in pyridine at room temperature with phosphoryl chloride as Lewis acid (rearrangement agent). As a result, a mixture of at least three compounds was obtained with one of them, the most polar, as the main product. The mixture was subjected to column chromatography and derivative **6** was isolated in about 70% yield.

In the ^{1}H NMR spectrum of the above compound the presence of a characteristic N-H signal, located at δ 5.53, was observed. The signal, a doublet, J = 3.4 Hz, indicated the coupling of the lactam proton with a single atom of hydrogen, which can be only that at C-13. It is stated on the basis of this observation that the product of this Beckmann rearrangement of oxime **5** has the structure **6a**. If the product had the structure **6b**, the N-H proton would be coupled with two hydrogen atoms at C-11, and the signal for this lactam proton would be observed as a triplet. On the basis of the above evidence it can be stated that the starting oxime **5** was the *E* isomer. The structures of the less polar products was not established and so regiostructural tests were performed in order to establish them.

The lactam system in 3β-acetoxy-12-oxo-12a-aza-C-homoolean-28-oic acid methyl ester (**6**) is not a reactive function. It forms the appropriate thioxoderivative, named thiolactam (**7**). The reaction is carried out in boiling benzene with Lavesson's reagent. After purification by column chromatography, the main product was obtained in a yield of about 88%.



6.a



6.b

In the IR spectrum of the newly obtained triterpene the alteration of the N-H signal was noticed. This was caused by the presence of a neighbouring thiocarbonyl group instead of the carbonyl one. The signal was observed at about 3395 cm^{-1} for thiolactam (for lactam: 3410 cm^{-1}). The signal indicating the presence of a thiocarbonyl function was found at 1445 cm^{-1}. In the ^{1}H NMR spectrum of product **7**, the thiolactam signal, observed at δ 7.77, had a higher value in comparison with the signal for a lactam system (δ 5.53). As in the case of the mother lactam, the signal for **7** had the form of a doublet. Comparison of the ^{13}C NMR spectra of thioazaderivative **7** and azaderivative

6 showed the change of the C-12 signal from δ 176.6 (lactam) to δ 208.2 (thiolactam). The molecular ion in the MS of the thiolactam was at m/z = 559.3, with an intensity of 100.00%, which confirmed the structure of the obtained product **7**.

Experimental

General: The solvents used for experiments (pure or pure for analysis) were purchased from either Chempur or POCh; the other reagents were purchased from Sigma-Aldrich. TLC analysis (reactions progress and level of compounds purity) was conducted on HPTLC aluminum sheets (Merck, HPLC Alufolien Kieselgel 60 F245, Merck Art. 5547) with benzene: ethyl acetate mixtures in different ratios used as eluents. The chromatograms were vizualised by spraying them with 10% ethanolic sulphuric acid solution and then heating at about 110°C for several minutes. Column chromatography was performed using Kieselgel 60; 0.063 – 0.200 mm (70 – 230 mesh), Merck. Melting points were determined using an open capillary method in a Kopfler apparatus and are uncorrected. IR spectra were recorded using a Specord IR-75 spectrophotometer, for 0.5% mixtures of tested compounds and KBr. The position of absortion bands is given in cm^{-1}, exact to ± $5cm^{-1}$. NMR spectra for hydrogen (^1H) and carbon atoms (^{13}C) were recorded in $CDCl_3$ solutions using a Varian Gemini 300 VT apparatus, for frequencies of 300 MHz and 75 MHz, respectively, with Me_4Si as the internal standard. The values for chemical shifts are given in δ, exact to ± 0.01 ppm and ± 0.1 ppm, respectively; *J* values are given exact to ± 0.1 Hz. The multiplicity of signals in ^1H NMR spectra are marked as follows: **s** – singlet, **s/br**/ – broad singlet, **d** – doublet, **t** – triplet, **dd** – doublet of doublets, **dt** – doublet of triplets. MS were recorded using an AMD 402 spectrometer with electroionisation. The m/z values are given exact to ± 0.1. HREIMS were also recorded using an AMD 402 spectrometer; m/z values are given exact to ± 0.0001. Elemental analyses (C, H, N) were performed with Perkin-Elmer 2400 CHN analyzer. The elucidation of the chemical structures was based on IR, ^1H NMR, ^{13}C NMR and MS analysis.

3β-Acetoxy-12-oxoolean-28-oic acid methyl ester (4): To a solution of 3β-acetoxyolean-12-en-28-oic acid methyl ester (**3**, 5.13 g, 10 mmol) in dry chloroform (150 cm^3), a dry solution of *m*-CPBA (3.45 g, 20 mmol) in chloroform (50 cm^3) was added and the resulting solution was left at room temperature in darkness for 2 days. The mixture was washed with 5% solutions of $FeSO_4$, Na_2CO_3, HCl and with water. The organic solution was dried with $MgSO_4$, the solvent removed and the obtained solid (5.04 g) was subjected to column chromatography.
Yield: 3.84 g, 72.6%.
White needles (ethanol).
MP: 198 – 199°C.
R_f : 0.82 (C_6H_6 – AcOEt, 4:1), dark-yellow spot.
Spectral data and melting point agreed with literature data [14].

3β-Acetoxy-12-hydroxyiminoolean-28-oic acid methyl ester (5): To a hot solution of 3β-acetoxy-12-oxoolean-28-oic acid methyl ester (**4**, 5.28 g, 10 mmol) in ethanol (105 cm^3), hydroxylamine hydrochloride (3.47 g, 50 mmol) and anhydrous sodium acetate (6.56 g, 80 mmol) were added. The obtained mixture was refluxed for 3 h, cooled and poured into water (525 cm^3) slightly acidified with HCl, The resulted precipitate was filtered, washed with water and dried.
Yield: 4.24 g, 78.0%.
White needles (ethanol).
MP: 196 – 198.5°C.
R_f : 0.80 (C_6H_6 – AcOEt, 4:1), yellow-brown spot.
IR (KBr): 3430 (OH, N-OH), 1730 and 1710 (C=O, CH_3COO and $COOCH_3$), 905 (N-O, N-OH).
^1H NMR (300 MHz, $CDCl_3$): 8.05 (1H, s/br/, N-OH), 4.47 (1H, dt, *J* = 5.7 and 10.5 Hz, C_3-H_α), 3.67 (3H, s, $COOCH_3$), 2.85 (1H, dt, *J* = 3.3 and 11.0 Hz; C_{18}-H_β), 2.52 (1H, d, *J* = 3.7 Hz; C_{13}-H), 2.04 (3H, s, CH_3COO), 0.91, 0.91, 0.88, 0.87, 0.85, 0.85, 0.81 (7 x s, 7 x C*H*$_3$).
^{13}C NMR (75 MHz, $CDCl_3$): 178.5 (C-28), 170.8 (CH_3*C*OO), 159.8 (C-12), 80.7 (C-3), 51.8 (COO*C*H_3), 48.1 (C-17), 43.3 (C-13), 21.3 (*C*H_3COO).
EIMS, *m/z* (%): 543.4 (49.6) $M^{+\bullet}$.
Anal. calcd for $C_{33}H_{53}NO_5$ (%): C = 72.89; H = 9.82; N = 2.58; found: C = 72.53; H = 9.86; N = 2.85.

3β-Acetoxy-12-oxo-12a-aza-C-homoolean-28-oic acid methyl ester (6): To a solution of 3β-acetoxy-12-hydroxyiminoolean-28-oic acid methyl ester (**5**, 5.44 g, 10 mmol) in dried pyridine (125 cm^3), phosphoryl chloride (2.8 cm^3, 4.60 g, 30 mmol) was added dropwise with cooling. The obtained mixture was left at room temperature for one day and then poured into water (625 cm^3), slightly acidified with HCl. The resulting precipitate was filtered, washed with water, dried and subjected to column chromatography.
Yield: 3.94 g, 72.5%.
Long, colorless needles (ethanol).
MP: 289 – 290°C.
$[\alpha]_D$: –14.0 (*c* 0.58, $CHCl_3$).
R_f : 0.10 (C_6H_6 – AcOEt, 4:1), dark-pink spot.
IR (KBr): 3410 (N-H, lactam), 1730 and 1710 (C=O, CH_3*C*OO and *C*$OOCH_3$), 1670 (C=O, lactam).
^1H NMR (300 MHz, $CDCl_3$): 5.53 (1H, d, *J* = 3.4 Hz; N-H, lactam), 4.49 (1H, dd, *J* = 5.1 and 11.3 Hz; C_3-H_α), 4.01 (1H, t, *J* = 6.0 Hz; C_{13}-H), 3.73 (3H, s, COOC*H*$_3$), 2.04 (3H, s, C*H*$_3$COO), 1.00, 0.98, 0.96, 0.87, 0.86, 0.84, 0.82 (21H, 7 x s, 7 x C*H*$_3$).
^{13}C NMR (75 MHz, $CDCl_3$): 177.3 (*C*$OOCH_3$), 176.6 (C-12), 170.6 (CH_3*C*OO), 80.2 (C-3), 52.0 (COO*C*H_3), 50.4 (C-13), 47.3 (C-17), 21.3 (*C*H_3COO).
EIMS, *m/z* (%): 543.0 (27.7) $M^{+\bullet}$.
HREIMS: for $M^{+\bullet}$ of $C_{33}H_{53}NO_5$ calcd.: 543.3924, found: 543.3939.
Anal. calcd. for $C_{33}H_{53}NO_5$ (%): C = 72.89; H = 9.82; N = 2.58, found: C = 73.14; H = 10.01; N = 2.72.

3β-Acetoxy-12-thioxo-12a-aza-C-homoolean-28-oic acid methyl ester (7): To a hot solution of 3β-acetoxy-12-oxo-12a-aza-C-homoolean-28-oic acid methyl ester (**6**, 2.72 g, 5 mmol) in dry benzene (130 cm^3), Lavesson's reagent (2.02 g, 5 mmol) was added and the resulting solution was refluxed for 90 minutes. Next it was cooled, washed with a 5% solution of K_2CO_3 and water. The organic solution was dried with $MgSO_4$, the solvent removed and the obtained solid (2.63 g) subjected to column chromatography.
Yield: 2.47 g, 88.3%.
White, thick needles (ethanol).
MP: 235 – 237°C.
$[\alpha]_D$: –53.6 (*c* 0.50, $CHCl_3$).
R_f : 0.80 (C_6H_6 – AcOEt, 4:1), yellow-brown spot.
IR (KBr): 3395 (N-H, thiolactam), 1720 and 1710 (C=O, CH_3COO and $COOCH_3$), 1495 (C=S, thiolactam).
^{1}H NMR (300 MHz, $CDCl_3$): 7.77 (1H, d, J = 5.7 Hz; N-H, thiolactam), 4.49 (1H, dd, J = 4.7 and 11.7 Hz; C_3-H_α), 4.22 (1H, t, J = 6.3 Hz; C_{13}-H), 3.73 (3H, s, $COOCH_3$), 2.04 (3H, s, CH_3COO), 1.01, 0.99, 0.98, 0.90, 0.85, 0.84, 0.82 (21H, 7 x s, 7 x CH_3).
^{13}C NMR (75 MHz, $CDCl_3$): 208.2 (C-12), 177.2 (C-28), 170.7 (CH_3COO), 80.2 (C-3), 54.4 (C-13), 52.0 ($COOCH_3$), 47.1 (C-17), 21.2 (CH_3COO).
EIMS, *m/z* (%): 559.3 (100.0) $M^{+\bullet}$.
HREIMS: for $M^{+\bullet}$ of $C_{33}H_{53}NO_4S$ calcd.: 559.3695, found: 559.3696.

References

[1] Yeung MF, Che ChT. (**2009**) A review on presence of oleanolic acid in natural products. *Natura Proda Medic*a, ***2***, 77–290.
[2] Liu J. (**2005**) Pharmacology of oleanolic acid and ursolic acid. *Journal of Ethnopharmacology*, ***100***, 92–94.
[3] Couch RD, Browning RG, Honda T, Gribble GW, Wright DL, Sporn MB, Anderson AC. (**2005**) Studies on the reactivity of CDDO, a promising new chemopreventive and chemotherapeutic agent: Implications for a molecular mechanism of action. *Bioorganic and Medicinal Chemistry Letters*, ***15***, 2215–2219
[4] García – Granados A, López PE, Melguizo E, Parra A, Simeo Y. (**2004**) Partial synthesis of C-ring derivatives from oleanolic and maslinic acids. Formation of several triene systems by chemical and photochemical isomerization processes. *Tetrahedron*, ***60***, 1491–1503.
[5] Hichri F, Jannet HB, Cheriaa J, Jegham S, Mighri Z. (**2003**) Antibacterial activities of a few prepared derivatives of oleanolic acid and of other natural triterpenic compounds. *Comptes Rendus Chimie*, ***6***, 473–483.
[6] Finlay HJ, Honda T, Gribble GW. (**2002**) Synthesis of novel [3,2-b]Indole fused oleanolic acids as potential inhibitors of cell proliferation. *ARKIVOC*, ***12***, 38–46.
[7] Honda T, Honda Y, Favaloro FGJr, Gribble GW, Suh N, Place EA, Rendi MH, Sporn MB. (**2002**) A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooloana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. *Bioorganic and Medicinal Chemistry Letters*, ***12***, 1027–1030.
[8] Wrzeciono U, Turowska W, Kusnierkiewicz G. (**1971**) Azotowe pochodne trójterpenów. IV. 3,4-Seko- i A-homo-pochodne oleananu. *Roczniki Chemii* (*Polish Journal of Chemistry*), ***45***, 1233–1241.
[9] Sundararamaiah T, Ramraj SK, Lakshman Rao K, Vimala Bai V. (**1976**) Synthesis of A-azatriterpenes-I: A-aza triterpenes from methyl oleanonate, methyl betulonate and lupenone. *Journal of Indian Chemical Society*, ***53***, 664–665.
[10] Yasue M, Sakakibara J, Kaiya T. (**1974**) Syntheses of nitrogen-containing triterpenes. III. Derivatives of betulinic acid and oleanolic acid. *Yakugaku Zasshi*, ***94***, 1468–1474.
[11] García – Granados A, López PE, Melguizo E, Moliz JN, Parra A, Simeó Y, (**2003**) Epoxides, cyclic sulfites, and sulfate from natural pentacyclic triterpenoids: theoretical calculations and chemical transformations. *Journal of Organic Chemistry*, ***68***, 4833–4844.
[12] Ahmad VU, Atta-ur-Rahman. (**1994**) *Handbook of Natural Products Data*. Volume 2. Pentacyclic Triterpenoids, Elsevier, Amsterdam – London – New York – Tokyo, 1–509.
[13] Zhu YM, Shen JK, Wang HK, Cosentino LM, Lee KH. (**2001**) Synthesis and anti-HIV activity of oleanolic acid derivatives. *Bioorganic and Medicinal Chemistry Letters*, ***11***, 3115–3118.
[14] Okamoto I., Takeya T., Kagawa Y., Kotani E. (**2000**) Iron(III)picolinate-induced oxygenation and subsequent rearrangement of triterpenoid derivatives with hydrogen peroxide. *Chemical & Pharmaceutical Bulletin*, ***48***, 120–125.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1817 - 1820

Analysis of Native Carotenoid Composition of Sweet Bell Peppers by Serially Coupled C_{30} Columns

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Received: June 29th, 2011; Accepted: September 26th, 2011

Serial coupled columns reversed-phase separations in high-performance liquid chromatography can be a useful tool for the analysis of complex real samples. The great difficulties found when analyzing complex carotenoid samples, due to the high natural variability of these compounds, as well as to the presence of carotenoid esters, are well documented. In the present contribution, the applicability of connecting two C_{30} columns to increase significantly the separation power, resolution and peak capacity for the analysis of carotenoids in a complex carotenoid sample, like sweet bell peppers, has been shown for the first time. By using LC coupled to PDA/APCI-MS detectors, 56 different carotenoids have been detected in red sweet bell peppers. By using two serial coupled C_{30} columns a peak capacity of 95.4 was obtained, compared with 73 achieved using a single column. Moreover, resolution greatly improved between different critical peaks when using two serial coupled C_{30} columns, compared with a single column. Interestingly, free carotenoids, mono-esters and di-esters were quantitatively equally represented (around 33% for each different class) in red sweet bell pepper, showing, therefore, a value for the ratio of mono-esters/di-esters of around 1, which could be considered a parameter of typicality. Free β-carotene (12.6%), capsanthin-C14:0 (8.4%), and capsanthin-C12:0-C14:0 (8.9%) were the most abundant carotenoids in the three different classes of red sweet bell pepper. No carotenoid esters were detected in either yellow or green sweet bell peppers. The application of such methodology in the analysis of other complex carotenoid matrices could be a future objective of research.

Keywords: carotenoids, sweet bell peppers, liquid chromatography-DAD-APCI-MS.

Carotenoids are based on a C_{40} tetraterpenoid skeleton which can undergo a high diversity of modifications, such as cyclization in one or both ends, hydrogenation, dehydrogenation, and addition of lateral groups, among others, resulting in an extremely wide group of compounds. Usually, these compounds are divided into two groups: hydrocarbons (commonly known as carotenes) and oxygenated compounds (generally named xanthophylls). To further increase the natural variability of these compounds, it has to be considered that the carotenoids can be present in nature as either free carotenoids or in a more stable form esterified with fatty acids, in the case of the oxygenated compounds. To simplify to some extent their analysis, a saponification procedure has been traditionally employed to release all the carotenoid esters and to analyze all these compounds in their free form. Although this saponification step acts also as a clean-up procedure, some drawbacks are found, mainly related to the formation of artefacts, as well as to the production of carotenoid degradation. Moreover, as a result of the saponification step, information on the native carotenoid composition of the studied samples is lost. Thus a better approach to carotenoid content is through classifying plant materials depending on either a free or esterified xanthophylls profile. In fact, the carotenoid esters could be used as a marker of authenticity of food products and could be useful as a ripeness degree index; moreover, esters may enhance food product oxidative stability and may improve carotenoid bioavailability. Although esterification does not change the chromophore properties of the carotenoid molecules, it does modify the immediate molecular environment; thus, chemical activities may be altered depending on the kind of fatty acid bound to the xanthophylls. Carotenoids are an important kind of natural pigment that can be widely found in plant-derived food and products. Although these compounds have been traditionally used in the food industry as colorants, nowadays, they attract a great deal of attention since they have been described to possess several important functional properties, mainly antioxidant activity [1], as well as prevention of cardio vascular diseases [2,3], cancer [4] and macular degeneration [5]. These properties make these compounds ideal for the always increasing functional food industry, as well as promoting the

required and recommended amounts of carotenoids in the human diet. *Capsicum annuum* is a carotenogenic fruit: during ripening, the transformation of the chloroplast into chromoplast occurs. Chlorophylls disappear and more and new carotenoids are formed. *Capsicum* species uniquely have capsanthin-capsorubin synthase that synthesizes two red pigments, capsanthin and capsorubin. Moreover, esterification greatly increases during the fruit ripening process. *Capsicum* is one of the oldest and most popular vegetables and spices in the world. Considering the carotenoid profile among various *C. annuum* cultivars [6-16], the spicy ones have received greater attention than the non spicy cultivars; the native carotenoid profile in sweet bell peppers had not been previously investigated. Therefore, the aim of this study was to investigate the native carotenoid composition in sweet bell peppers by developing an analytical LC method which allowed the direct identification of the carotenoids in the samples, based on the use of serially connected C_{30} columns coupled with DAD and APCI-MS detectors. High performance liquid chromatography has been selected as the analytical tool for a large number of applications, including carotenoid analysis. However, due to the great complexity of some natural samples containing this kind of compound, conventional LC could not have enough separation power. In this work, serial connection of two columns is proposed as an alternative to conventional LC. The applicability of connecting two C_{30} columns to increase significantly the separation power, resolution and peak capacity for the analysis of carotenoids in sweet bell peppers has been demonstrated. Moreover, here we report the first investigation of the native carotenoid profile in sweet bell peppers at three different ripening stages: green, yellow and red (Figures 1, 2 and 3). As shown in Figure 3B, 56 different carotenoids have been detected in red sweet bell peppers, including many esters (for peak identification see Table 1). No carotenoid esters were detected in either yellow or green sweet bell peppers. The identification of these compounds was carried out combining the information provided by the two detectors employed (i.e., DAD and APCI-MS detectors) and the commercial standards available. As can be observed in Figure 3, the order of elution of the different compounds is highly dependent on the polarity and hydrophobicity of the molecules. Therefore, free xanthophylls elute before mono-esterified ones, and the di-esterified xanthophylls have longer retention times. By considering the different fragmentations in the APCI-MS producing different regioisomers, various xanthophyll di-esters were detected in red bell peppers.

As can be seen in Table 2, by using two serially coupled C_{30} columns a peak capacity of 95.4 was obtained, compared with 73 using a single column. Moreover, the resolution values of some critical pairs (12-13, 29-30 and 32-33) were significantly improved by the coupling of the two C_{30}columns.

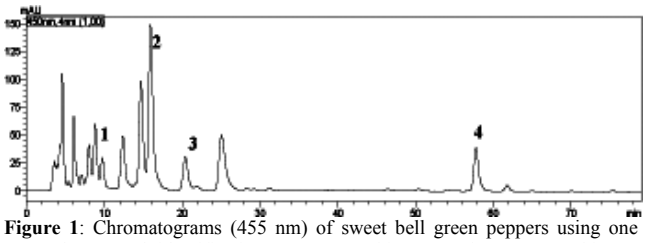

Figure 1: Chromatograms (455 nm) of sweet bell green peppers using one C30 column. Peak identification: 1. Luteoxanthin; 2. Lutein; 3. Zeaxanthin ; 4. β-Carotene.

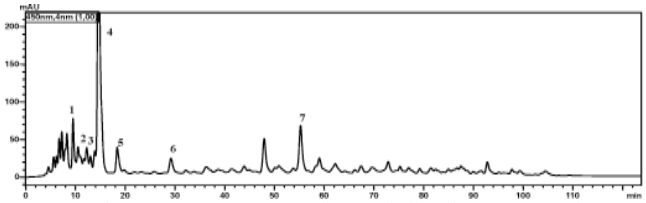

Figure 2: Chromatograms (455 nm) of sweet bell yellow peppers using one C30 column. Peak identification: 1. Luteoxanthin; 2. Auroxanthin; 3. Antheraxanthin ; 4. Lutein; 5. Zeaxanthin; 6. β-cryptoxanthin; 7. β-carotene

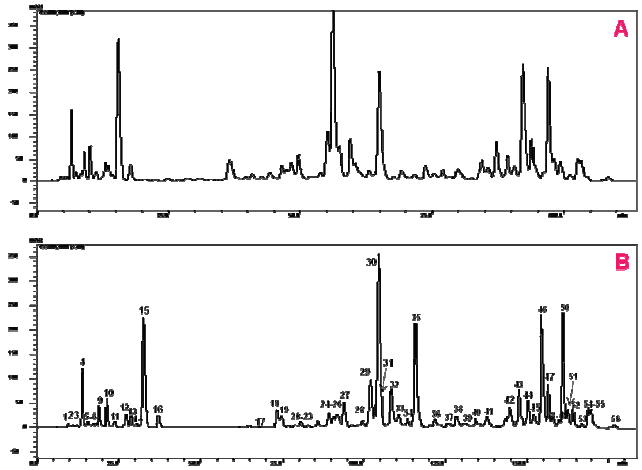

Figure 3: Chromatograms (455 nm) of sweet bell red peppers using one C_{30} column (A), and two C_{30} columns (B). For peak identification see Table 1.

Interestingly, free carotenoids, mono-esters and di-esters were quantitatively equally represented (around 33% for each different class) in red sweet bell pepper, and the ratio of di-esters/mono-esters was around 1, which could be considered a typicality parameter of red peppers. β-Carotene (6%), lutein (20-30%) and zeaxanthin (2-5%) were the most abundant carotenoids present in yellow and green sweet bell peppers, whereas violaxanthin (2.2%) capsanthin (8.1%) and β-carotene (12.6%) characterized the free carotenoid fraction of sweet red bell peppers. Myristic acid (C14:0) was the most abundant fatty acid present as mono-ester with capsanthin (8.4%), capsorubin (3.1%) and antheraxanthin (2.7%) in red bell peppers. Lauric and myristic acids were mainly present in the di-esters of capsanthin and capsorubin in red bell peppers. In particular, the most abundant di-esters in red bell peppers were capsanthin-C12:0, C14:0 (8.9%), capsanthin-C14:0, C14:0 (7.1%) and capsorubin-C14:0, C14:0 (3.3%).

No carotenoid esters were detected in either green or yellow sweet bell peppers. Interestingly, Minguez-Mosquera [6]

Table 1: UV-Vis, MS data and identification of the different carotenoids separated in sweet bell red pepper using two serially connected C_{30} columns.

No.	Peak identification	λ_{max} (nm)	$[M + H]^+$
1	Neoxanthin	416,440,469	601,583
2	n.i.	419,446,467	601,583
3	β-Apo-10-carotenal	436	376,361
4	Violaxanthin	418,441,470	601,583
5	Luteoxanthin	400,422,448	601,583
6	n.i.	418,440,467	613,595
7	n.i.	400,424,448	601,583
8	n.i.	400,423,448	615,597
9	Capsanthin-5,6-epoxide	469,486	601,583,565
10	Anteraxanthin	424s,445,474	585,567
11	n.i.	357,448,469	585,567
12	Mutatoxanthin A	406,429,451	585,567,549
13	n.i.	457s,479,507	601,583,567,491
14	Mutatoxanthin B	406,429,452	585,567,549
15	Capsanthin	474	585,567,479
16	Zeaxanthin	427,451,477	569,551,476
17	Phytofluene	332,348,367	543
18	β-Cryptoxanthin	426,451,477	553,535
19	Anteraxanthin-laureate	422,447,474	767,749,567
20	n.i.	400,424,448	875,597
21	n.i.	424,443,469	875,597,565
22	n.i.	451,467	823,583
23	n.i.	425s,447,467	877,823,597
24	n.i.	466	879,599,583
25	n.i.	400,424,448	879,793,597
26	Anteraxanthin-myristate	424,446,474	795,777,567
27	n.i.	425,446,474	876,777
28	Mutatoxanthin-laureate	408,425,452	767,749,567
29	Capsorubin-myristate	479	811,583,565
30	β-carotene	452,478	537
31	Capsanthin-laureate	474	767,567,549
32	Anteraxanthin-myristate	425,447,474	795,777,567
33	n.i.	424s,448,471	853,795,567
34	Mutatoxanthin-myristate	406,429,452	795,777,567
35	Capsanthin-myristate	474	795,777,567
36	n.i.	460	795,567,549
37	n.i.	426,451,474	879,684,533
38	n.i.	424,443,469	961,821,547
39	n.i.	466,472	823,567,549
40	β-Cryptoxanthin-laureate	426,451,477	735,535
41	n.i.	469	765,549
42	β-Cryptoxanthin-miristate	425,452,479	765,535
43	Capsanthin-di-laureate (C12:0,C12:0)	473	949,749,549
44	Capsorubin-laureate-myristate (C12:0,C14:0)	479	993,793,765,565
45	n.i.	428,451,470	1021,793,565
46	Capsanthin-laureate-myristate (C12:0,C14:0)	474	977,777,749,549
47	Capsorubin-di-myristate (C14:0,C14:0)	481	1021,793,565
48	Mutatoxanthin-laureate-myristate (C12:0,C14:0)	406,429,454	977,793,765,565
49	Zeaxanthin-laureate-myristate (C12:0,C14:0)	425,451,478	961,761,733,533
50	Capsanthin-di-myristate (C14:0,C14:0)	474	1005,777,549
51	n.i.	469	1005,777,749,549
52	Capsorubin-myristate-palmitate (C14:0-C16:0)	479	1049,821,793,565
53	Zeaxanthin-di-myristate (C14:0,C14:0)	427,452,477	989,761,533
54	Capsanthin-myristate-palmitate (C14:0,C16:0)	474	1033,805,777,549
55	n.i.	469	805,777,549
56	n.i.	469	805,713,549,551

Table 2: Values of Peak capacity (Pc) and resolution (Rs) between selected peaks for the analysis of sweet bell red peppers using two different set ups.

Set Up	Pc	Rs (12-13)	Rs (29-30)	Rs (32-33)
1 x C30	73.0	0.23	0.66	0.33
2 x C30	95.4	0.85	1.28	0.87

reported that in the yellow spicy *C. annuum* cultivar Bola, 50% of the carotenoids were esterified, and that at the fully ripened red stage the percentages of the free carotenoid pigments and the partially and totally esterified forms of these were 21.3%, 35.6%, and 43.1%, respectively, and, therefore, different from the percentages reported in this work for red sweet bell peppers, where the three fractions were equally represented. The increase in xanthophyll esterification during ripening reported in this work is in agreement with the report by Hornero-Mendez [8] for various cultivars of spicy *C. annuum* cultivars. This process has been related to a phenomenon intimately linked with and inherent to the degeneration of chloroplast and formation of chromoplast . Such a phenomenon might be the result of hydrophobicity requirements on the part of the carotenoid, so that, with all its hydroxyl groups esterified, it will be included more readily in the lipid matrix of chromoplast membranes and organelles (plastoglobules) [8]. Schweiggert *et al*. [9] reported that capsanthin and β-carotene were the main free carotenoids in spicy red peppers, in agreement with the results reported in this work for sweet red bell peppers, and they reported also a similar esters profile for spicy red pepper pods, although the sweet bell red peppers analyzed in this work showed a higher degree of esterification with capsanthin rather than with capsorubin. Apart from a very early report by Gregory *et al*. [11] on the carotenoid esters in sweet red bell peppers carried out by gas chromatographic analysis of the transesterified fatty acids obtained after saponification and which reported some generic tentative identifications, the work here reported is the first direct study of the native carotenoid composition of sweet bell peppers using a liquid chromatographic methodology. In fact, previous works on the carotenoid composition of sweet bell peppers [12-16] were carried out after a saponification step.

Although the application of the methodology reported in this work is instrumentally quite simple, it has been demonstrated to be a valid way to further improve resolution and efficiency in LC.

Experimental

Samples and chemicals: Fresh samples of green, yellow and red sweet bell peppers were supplied by a local producer. Carotenoid standards, namely, β-carotene, lutein, β-cryptoxanthin, zeaxanthin, capsanthin and lutein-di-palmitate ($C_{16:0}$, $C_{16:0}$) were purchased from Extrasynthese (Genay, France). All the carotenoid standards were stored protected from light at -18°C. All the solvents used, namely, methanol, methyl tert-butyl ether (MTBE) and water, were HPLC grade and purchased from Sigma-Aldrich (Milan, Italy). BHT (butylatedhydroxy

toluene) and potassium hydroxide were obtained from Sigma-Aldrich (Milan, Italy).

Carotenoid extraction: The sweet bell pepper samples (200 g) were homogenized, and the carotenoids extracted 4 times with methanol/ethyl acetate/light petroleum (1:1:1). The upper phase was kept and ca. 2 mg of BHT was added prior to evaporation under vacuum until dryness. The dry residue was then resuspended in a given volume of MTBE/methanol (1:1) and stored protected from light at -18°C until use.

LC-DAD/APCI-MS analyses: To carry out the analyses a Shimadzu HPLC instrument (Shimadzu, Milan, Italy) was employed including a SCL-10A-*VP* system controller, two LC-10AD-*VP* pumps and a SPD-10A*vp* diode array detector. Besides a Shimadzu mass spectrometer LCMS-2010 equipped with an APCI interface in the positive ion mode was installed in parallel. The APCI parameters were set as follows: probe voltage (kV), 4.5; probe temperature, 400°C; block temperature, 200°C; CDL temperature, 250°C; Q array voltage, 20 and 80 V; gas flow, 2.5 L/min. Data acquisition and system control was performed by the LCMSsolutionver 3.30 software (Shimadzu). The injections were made manually through a Rheodyne injection valve (injection volume 20 μL). The C_{30} columns employed consisted of two YMC 30 analytical columns (YMC Europe, Schermbeck, Germany) with 5 μm C_{30} reversed-phase material (250 × 4.6 mm I.D.), including a pre-column YMC 30 (S-5 μm, 10 × 4.0 mm I.D.). The HPLC solvent systems were (A) methanol/MTBE/water (83:15:2, v/v/v) and (B) methanol/MTBE/water (8:90:2, v/v/v) used following a linear gradient depending on the use of one or two serial coupled columns. Gradient for the separation with 1 x C_{30} column: 0-20 min, 0% B; 20-160 min 100% B; then reconditioning. Gradient for the separation with 2 x C_{30} columns: gradient times were doubled keeping unchanged the B%. The flow rate employed was 1 mL/min and the chromatograms were recorded at 450 nm and the UV-Vis spectra were recorded in the range from 250 to 600 nm (sampling rate: 12.5 Hz; time constant: 0.64 s). The column oven temperature was 40°C.

References

[1] Beutner S, Bloedorn B, Frixel S, Hernandez Blanco I, Hoffman T, Martin HD, Mayer B, Noack P, Ruck C, Schmidt M, Schulke I, Sell S, Ernst H, Haremza S, Seybold G, Sies H, Stahl W, Walsh R. (**2001**) Quantitative assessment of antioxidant properties of natural colorants and phytochemicals: carotenoids, flavonoids, phenols and indigoids. The role of β-carotene in antioxidant functions. *Journal of Science Food & Agriculture*, ***81***, 559-568.

[2] Arab L, Steck S. (**2000**) Lycopene and cardiovascular disease. *American Journal of Clinical Nutrition*, ***71***, 1691S–1695S.

[3] Rao AV, Rao RG. (**2007**) Carotenoids and human health. *Pharmaceutical Research*, ***55***, 207-216.

[4] Nishino H, Tokuda H, Satomi Y, Masuda M, Bu P, Onozuka M, Yamaguchi S, Okuda Y, Takayasu J, Tsuruta J, Okuda M, Ichiishi E, Murakoshi M, Kato T, Misawa N, Narisawa T, Takasuka N, Yano M. (**1999**) Cancer prevention by carotenoids. *Pure & Applied Research*, ***12***, 2273-2278.

[5] Snodderly M D. (**1995**) Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. *American Journal of Clinical Nutrition*, ***62***, S1448-S1461.

[6] Minguez-Mosquera MI, Hornero-Mendez D. (**1994**) Changes in carotenoid esterification during the fruit ripening of *Capsicum annuum* Cv. Bola. *Journal of Agricultural and Food Chemistry*, ***42***, 640-644.

[7] Markus F, Daood HG, Kapitany J, Biacs PA. (**1999**) Changes in the carotenoid and antioxidant content of spice red pepper (paprika) as a function of ripening and some technological factors. *Journal of Agricultural and Food Chemistry*, ***47***, 100-107.

[8] Hornero-Mendez D, Minguez-Mosquera MI. (**2000**) Xanthophyll esterification accompanying carotenoid overaccumulation in chromoplast of *Capsicum annuum* ripening fruits is a constitutive process and useful for ripeness index. *Journal of Agricultural and Food Chemistry*, ***48***, 1617-1622.

[9] Schweiggert U, Kammerer DR, Carle R, Schieber A. (**2005**) Characterization of carotenoids and carotenoid esters in red pepper pods (*Capsicum annuum* L.) by high-performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*, ***19***, 2617-2628.

[10] Hernandez-Hervert D, Sayago-Ayerdi SG, Goni I. (**2010**) Bioactive compounds of four hot pepper varieties (*Capsicum annuum* L.), antioxidant capacity, and intestinal bioaccessibility. *Journal of Agricultural and Food Chemistry*, ***58***, 3399-3406.

[11] Gregory GK, Chen TS, Philip T. (**1987**) Quantitative analysis of carotenoids and carotenoid esters in fruits by HPLC: red bell peppers. *Journal of Food Science*, ***52***, 1071-1073.

[12] Sun T, Xu Z, Wu CT, Janes M, Prinyawtwatkul W, No HK. (**2007**) Antioxidant activities of different colored sweet bell peppers (*Capsicum annuum* L.). *Journal of Food Science*, ***72***, S98-S102.

[13] de Azevedo-Meleiro CH, Rodriguez-Amaya DB. (**2009**) Qualitative and quantitative differences in the carotenoid composition of yellow and red peppers determined by HPLC-DAD-MS. *Journal of Separation Science*, ***32***, 3652-3658.

[14] Matsufuji H, Ishikawa K, Nunomura O, Chino M, Takeda M. (**2007**) Anti-oxidant content of different coloured sweet peppers, white, green, orange and red (*Capsicum annuum* L.). *International Journal of Food Science and Technology*, ***42***, 1482-1488.

[15] Guil-Guerrero JL, Martinez-Guirado C, Rebelloso-Fuentes M, Carrique-Perez A. (**2006**) Nutrient composition and antioxidant activity of 10 pepper (*Capsicum annuum*) varieties. *European Food Research and Technology*, ***224***, 1-9.

[16] Marin A, Ferreres F, Tomas-Barberan FA, Gil MI. (**2004**) Characterization and quantification of antioxidant constituents of sweet pepper (*Capsicum annuum* L.). *Journal of Agricultural and Food Chemistry*, ***52***, 3861-3869.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1821 - 1824

New Antifungal Cholestane and Aldehyde Derivatives from the Red Alga *Laurencia papillosa*

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Received: July 2nd, 2011; Accepted: October 20th, 2011

The chloroform/methanol extract of the red alga, *Laurencia papillosa*, collected from the Red Sea in Saudi Arabia, was found to contain two cholestane derivatives: 3α, 6α-dihydroxy-5β-cholestan-12-one (**1**) and the known, 6β-hydroxycholest-4-en-3-one (**2**), which was isolated separately in a pure form for the first time. In addition to these compounds, a new aldehyde derivative, (*E*)-2-{(*E*) tridec-2-en-2-yl} heptadec-2-enal (**3**), was isolated. The structures of all compounds were established based on extensive spectroscopic (1D and 2D NMR, UV, IR) and mass spectrometric studies. All compounds, except **2**, were tested for their antifungal activity. Significant activities were associated with **1** and **3** against *Candida albicans*, *Aspergillus fumigatus*, and *A. flavus.*

Keywords: Red alga, *Laurencia papillosa*, cholestane derivatives, aldehyde, antifungal activity.

Marine organisms have historically provided a rich source of structurally diverse, biologically active secondary metabolites [1a-1d]. The genus *Laurencia* (family Rhodomelaceae, order Ceramiales) possesses nearly 140 species worldwide are considered as a rich source of bioactive metabolites [2a-2c]. *L. papillosa* C. Agardh is a source of different substances such as steroids [3a-3c] and an unsaturated fatty acid aldehyde [4a]. A number of long chain unsaturated fatty acid aldehydes with antibiotic activity were reported from the fungus *Cladosporium* sp., obtained from intertidal marine sediment [4b]. It was clear from the literature that steroidal and aldehydic compounds have antimicrobial activities. On this basis, *L. papillosa*, collected from the water around El-Shuaiba, Saudi Arabia, was extracted with chloroform and methanol (1:1) and the extract fractionated by NP-silica gel column chromatography, followed by preparative thin-layer chromatography to yield a new cholestane derivative, 3α,6α-dihydroxy-5β-cholestan-12- one (**1**), and a known steroid, 6β-hydroxycholest-4-en-3-one (**2**). In addition, a new aldehydic derivative {(2E)-2-(tridec-2-en-2-yl) heptadec-2-enal} (**3**) was also isolated.

The molecular formula of **1** was established as $C_{27}H_{46}O_3$ by EI-MS {*m/z*: 418 $[C_{27}H_{46}O_3]^+$ and 400 $[M- H_2O]^+$}. This result was validated by HRFABMS (*m/z* 441.3357 $[M+Na]^+$). The ^{13}C NMR spectra (^1H decoupled and DEPT) of **1** showed 27 resonances attributable to 5 X CH_3, 10 X CH_2, 9 X CH, and 3 X C (Table 1). One of the five elements of unsaturation, as indicated by the molecular formula of **1**, deduced to be a carbonyl group, appeared at δ_c 212.8 with the absence of any olefinic proton or any double bond signal. The IR spectrum showed a $\nu(C-H)_{Aliphatic}$ vibration band at 2930 cm^{-1}. The spectrum also displayed two strong bands at 3450 and 1706 cm^{-1} assigned to ν(OH) and a six membered ring ν(C=O) [4c], respectively; the molecule thus has four rings. As the ^1H and ^{13}C NMR spectroscopic data enabled all but two of the hydrogen atoms within **1** to be accounted for, it was evident that the remaining two protons were present as part of a hydroxyl function, After association of all the protons with directly bonded carbons via 2D NMR (HMQC)

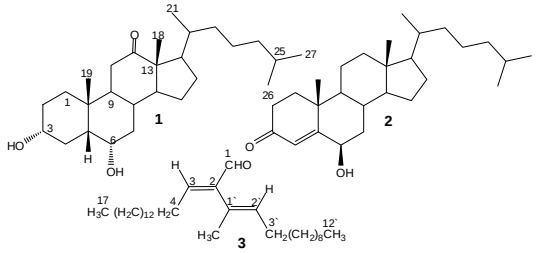

Figure 1: Structures of isolated compounds **1**-**3**.

Table 1: ^{1}H ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) spectral data of compound **1**[a].

P.	^{13}C	H (m, *J* Hz)	P	^{13}C	H (m, *J* Hz)
1a	41.3	1.67 (1H, m) [b, c]	13	53.8	
1b		1.32 (1H, m)	14	56.4	1.26 (1H, m)
2a	31.7	1.77 (1H, m)	15	27.2	1.66 (2H, m)
2b		1.36 (1H, m)	16	27.6	1.76 (2H, m)
3	65.4	4.18 (1H, m)	17	53.1	1.22 (1H, m)
4a	36.1	2.02 (1H, m)	18	12.0	0.68 (3H, s)
4b		1.67 (1H, m)	19	12.3	0.74 (3H, s)
5	42.0	1.64 (1H, brs)	20	38.0	1.36 (1H, m)
6	73.9	3.61 (1H, dd, 12, 1.8)	21	21.1	0.93(3H,d, 6.6)
7a	39.5	1.71 (1H, dd, 12, 3.0)	22a	40.0	1.32 (1H, m)
7b		1.44 (1H, m)	22b		1.10 (1H, m)
8	38.0	1.51 (1H, m)	23a	24.0	1.33 (1H, m)
9	51.7	1.98 (1H, ddd, 13.3, 4.5, 1.8)	23b		1.13(1H, m)
10	35.9		24	29.7	1.12 (1H, m)
11a	46.9	2.31(1H,dd,13.0, 4.5)	25	28.2	1.39 (1H, m)
11b		2.03(1H, d, 13.0)	26	23.0	0.90(3H, d, 6.6)
12	212.8		27	22.5	0.91(3H, d, 6.6)

All assignments are based on 1D and 2D NMR measurements (HMBC, HMQC, COSY). [b] Implied multiplicities as determined by DEPT (C= s, CH = d, CH_2 = t, CH_4= q). [c] *J* in Hz.

spectral measurements, it was possible to deduce the structure of **1** by interpretation of the ^{1}H–^{1}H COSY and ^{1}H –^{13}C HMBC spectra. From the ^{1}H–^{1}H COSY spectrum of **1**, ^{1}H–^{1}H spin system between H_2-1 and H_2-2, H_2-2 and H-3, H-3 and H_2-4, H_2-4 and H-5, H-5 and H-6, H-6 and H_2-7, H_2-7 and H-8, and H-8 and H-9 were observed. Long-range C-H correlations observed between the resonances of H-9 and those of C-1, C-5, C-7, C-8 and C-10; between H_2-1 and C-2, C-3, C-5, C-9 and C-19; between H-3 and C-1 and C-5; between H-6 and C-8 and C-10 established that rings A and B were fused together. In the ^{1}H–^{1}H COSY spectrum of **1**, a ^{1}H–^{1}H spin system between H_2-11 and H-9 indicated that C-11 was attached to C-9. A CH long-range coupling between H-9 and C-8, C-10, C-11, C-14 and C-19 were observed. HMBC correlations, this time between H-14 and C-8, C-13, C-15, and C-18, between H_3-18 and C-12, C-13, C-14, and C-17 were observed. Long-range C-H correlations observed between the resonances of C-12 and H_2-11, H-14 were observed. Ring C was established by deduction through the previous data. From the ^{1}H–^{1}H COSY spectrum of **1**, a ^{1}H–^{1}H spin system between H_2-15 and H-14 and H_2-16, and between H_2-16 and H-17 were observed indicating a C-C bond between C-14 to C-15, C-15 to C-16, and C-16 to C-17. Ring D was established based on^{1}H–^{1}H COSY and ^{1}H–^{13}C HMBC spectra. The main skeleton of **1** was established as a steroidal derivative. From the ^{1}H–^{1}H COSY spectrum of **1**, a ^{1}H–^{1}H spin system between H_3-26 and H-25 and between H-25 and H_3-27 indicated a forked tail isopropane derivative [4d]. The positions of the two hydroxyl groups were determined by examination of the ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HSQC and HMBC spectra. From the ^{1}H–^{1}H COSY spectrum, correlations were observed between H-25 and H_2-24, between H_2-24 and H_2-23, between H_2-23 and H_2-22, and between H_2-22, H_2-20 and H_2-21, which led to establishing the side chain as an iso-octane derivative. This side chain is attached to the steroidal nucleus between C-17 and C-20 based on HMBC correlations between H_3-21 and C-17, C-20 and C-22. A proton at δ 4.18 appears as a multiplet at position 3, with equatorial orientation (hydroxyl group occupies α-position). The proton at δ 3.61, appearing as a double doublet, with *J* values of 12 and 1.8 Hz, was located at position 6 with axial orientation (OH-6 occupies α-orientation). The weak or zero chemical shift effect of the hydroxyl group located at C-6 on Me-19 at δ 0.74 is further evidence for the α-position of OH-6. The proton at position 5 was found to have a β- orientation, owing to the signal at δ 1.64 (brs) which implies an equatorial orientation for H-5. The configuration of the OH at C-6 is equatorial downward, a deduction which led to the axial orientation of H-5. The *cis*- junction between rings A and B is responsible for the strain on both rings. On this basis, the chemical shifts of C-1 and C-11 appeared more downfield than the normal values. The configuration of 3-OH is alpha based on the ^{13}C chemical shift (δ 65.4) [5a]. The ^{13}C chemical shifts of C-1 and C-11 are downfield from the normal ones, based on the strain that appears to be due to the *cis*-junction between rings A and B. Also the α-OH located at C-6 was confirmed by the correlation between H-8 and C-6, which is in good agreement with the ^{13}C chemical shift of C-6 (δ 73.9) [5a,5b]. A literature survey indicated that **1** is a new cholestanone derivative and was named as 6α-dihydroxy-5β-cholestan-12-one.

The structure of **2** was based on the molecular formula of $C_{27}H_{44}O_2$, which was abstracted from the EI-MS: *m/z* 400 $[C_{27}H_{44}O_2]^+$ and 382 $[M\text{-}H_2O]^+$. After extensive study of the ^{1}H and ^{13}C NMR spectral data of **2** (see experimental part), it was clear that these matched those published for the steroidal derivative; 6β-hydroxycholest-4-en-3-one, isolated from the red alga *Hypnea musciformis* as non solvable mixture with 6β-hydroxycholest-4, 22-dien-3-one [6a]. A computer survey, including Science Finder, indicated that **2** is published here for the first time in a pure form, and the complete physical properties are presented.

Compound **3** had the molecular formula $C_{30}H_{56}O$, derived from the high-resolution mass measurement of the parent ion, which implied three degrees of unsaturation. The IR bands at 2730, 1680 and 1640 ν_{max} were characteristic of an α, β-unsaturated aldehyde. This conjugation was supported by the ^{1}H NMR spectrum, which showed an aldehyde proton at δ9.32 and one vinyl proton at δ 6.44 (H-3, *t, J*= 7 Hz). ^{1}H NMR spectral data also revealed the presence of an olefinic proton at δ 5.33 (H-2', m), two primary methyls at δ 0.88 (6 H, t, *J*= 6.6 Hz), four methylene protons at δ 2.20-2.35 (4 H, m, 4, 3'), four methylene protons at δ 1.50 (4H, m, 5, 4'), olefinic methyl protons at δ 1.61 (3 H, m), and a huge methylene envelope at δ 1.27 (cf Experimental). The ^{13}C NMR and DEPT spectral data of **3** were supportive of the above analysis, showing an aldehyde group at δ_c 195.4 (C-1, s), two double bonds [δ_c 143.7 (C-2, s), 155.5 (C-3, d), 129.6 (C-1', s), 130.1 (C-2', d)], aliphatic methylene carbons at δ_c 23.0-31.0, two methyls at δ_c 14.2, and an olefinic methyl at δ_c 22.0. Thus, three degrees of unsaturation were accounted for as an aldehydic group and two double bonds, which are *trans* oriented owing to the values of the

chemical shifts of the allylic methylene at $\delta_c > 30$, and the *J* values, which are similar to those published [6b]. Hence **1**, had an acyclic α, β-unsaturated aldehyde with two fatty acid chains terminating at methyl groups, in which the length of both chains was determined by MS, and ^{1}H and ^{13}C NMR spectra. The structure connectivity of **3** was concluded by interpretation of its ^{1}H-^{1}H COSY cross peaks, which showed that H-3 at δ 6.44 correlated with H_2-4 at δ 2.35; H-2' at δ 5.33 correlated with H_2-3' at δ 2.22; there was no observed correlation between H-3 and H-2'. The structure was unambiguously indicated as **3** by studying the HMBC spectrum in which H-1, resonating at δ 9.32, correlates with C-2 at δ_c 143.7, C-3 at δ_c 155.5 and C-1' at δ_c 129.6; also the olefinic proton H_1-3, resonating at δ_c 6.44, correlates with C-2 and C-4 at δ_c 31.0; the CH_3 protons resonating at δ_H1.60 correlate with both C-1', C-2 and C-2' at δ_c 130.1. The above correlations denoted that the CHO group is connected to the quaternary carbon C-2, (δ_c 143.7, which in turn is connected to both C-3 and C-1', and the olefinic methyl is attached to C-1'; other correlations are shown in figure 2. From the above, compound **3** was assigned the structure (*E*)-2-((*E*) tridec-2-en-2-yl) heptadec-2-enal.

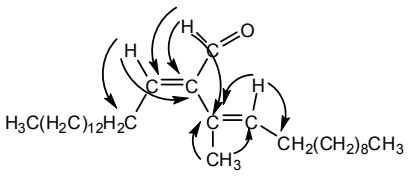

Figure 2: Selected HMBC correlations of **3**

The antifungal activity of all compounds, except **2**, was assessed using the microtiter broth dilution method for yeast susceptibility testing. Compound **1** had significant activity against *Candida albicans* with a MIC of 2000 µg/mL. Although **1** showed no activity against *A. fumigatus* and *A. flavus*, **3** had significant activity against these with MIC of 200 and 1000 µg/mL, respectively. The positive control, ketoconazole, showed potent activity against *C. albicans* and *A. fumigatus* with a MIC of 40 µg/mL, and 625µg/mL towards *A. flavus*.

Experimental

General: Optical rotations, ATAGO POLAX-L 2 polarimeter; GC/MS, Shimadzu-QP 2010 spectrometer; TLC was performed on silica gel (Kieselgel 60, F_{254}) of 0.25 mm layer thickness. Compounds were detected by using ethanol /sulfuric acid as spray reagent.

Algal sample: *Laurencia papillosa* was collected in June 2009, off the Saudi Arabian Red Sea Coast at Jeddah. A voucher sample (JAD 03050) is deposited at the Marine Chemistry Department, King Abdulaziz University, Jeddah, Saudi Arabia.

Extraction and isolation: The air dried algal material (200 g) was extracted with equal volumes of a mixture of light petroleum, chloroform and methanol (2 X 6 L, 24 h for each batch) at room temperature. The extract was concentrated under reduced pressure to obtain 6 g residue. The residue (6 g) was homogenized with a small amount of silica gel (50 g) and poured onto the top of the column that was packed in light petroleum (40-60°C). The fractions were collected (50 mL each) employing a gradient elution from light petroleum to diethyl ether and from diethyl ether to ethyl acetate. The similar fractions were pooled together according to TLC pattern into three pools (P-A, P-B and P-C), employing 50%-sulfuric acid in methanol as spraying reagent. P-A was purified by PTLC-Silica gel, using light petroleum-diethyl ether (9:1), yielded **3**. P-B was purified by PTLC-Silica gel, using benzene: ethyl acetate (4:1) to give **2**. Finally, P-C was purified by PTLC-Silica gel, using benzene-ethyl acetate (5: 2) and re-chromatographed on Sephadex LH-20 using a mixture of MeOH-$CHCl_3$ (8:2), and finally purified by preparative TLC-silica gel employing benzene-ethyl acetate (6: 4), yielded **1**.

3*α*, 6*α*-Dihydroxy-5β- cholestan-12-one (**1**)
White solid (7 mg, 0.0028 %).
MP: 170°C.
$[\alpha]_D^{18}$: +93 (*c* 0.1, $CHCl_3$).
R_f: 0.48 (benzene/EtOAc , 6:4).
IR (neat) ν_{max}: 3450, 2930, 1706 cm^{-1}.
^{1}H and ^{13}C NMR ($CDCl_3$): Table 1.
HRFAB MS: (*m/z* 441.3357 $[M+Na]^+$). Calcd. 441.3345, EI-MS *m/z*: 418 $[M]^+$, 400 $[M- H_2O]^+$, 385 $[M-CH_3-H_2O]^+$, 318 (100) $[M-C_7H_{16}]^+$.

6β-Hydroxycholest-4-en-3-one (**2**)
White solid (2 mg, 0.0008 %).
MP: 119-120°C.
$[\alpha]_D^{18}$: +29 (*c* 0.03, $CHCl_3$).
R_f: 0.35 (*n*-hexane/EtOAc, 7:3).
IR (KBr): 3635, 3520, 1685 cm^{-1}.
UV (MeOH): strong absorption at λ_{max} 237 nm.
^{1}H NMR ($CDCl_3$), δ (ppm): 0.85 (d, *J*=6.6 Hz, 3H, CH_3-26), 0.87 (d, *J*=6.6 Hz, 3H, CH_3-27) 0.92 (d, *J*=6.6 Hz, 3H, CH_3-21), 0.74 (s, 3H, CH_3-18), 1.38 (s, 3H, CH_3-19), 1.52 (m, 1H, H-25), 1.28 (m, 2H, H-24), 1.16 (m, 2H , H-23), 1.24 (m, 2H , H-22), 1.35 (m, 1H, H-20), 1.12 (m, 1H, H-17), 1.28 (m, 1H, H-16), 1.24 (m, 2H, H-15), 1.03 (m, 1H, H-14), 1.12 (m, 2H, H-12), 1.51 (m, 2H, H-11), 1.33 (m, 1H, H-9), 1.97 (m, 1H, H-8), 2.54-2.00 (m, 2H, H-7), 4.35 (t, *J*= 2.8 Hz, 1H, H-6), 5.82 (s, 1H, H-4), 2.40 (m,2H, H-2), 2.05-1.60 (m, 2H, H-1).
^{13}C NMR ($CDCl_3$), δ (ppm): 37.0 (C-1), 34.4 (C-2), 200.5 (C-3), 126.3 (C-4), 168.5 (C-5), 73.3 (C-6), 38.5 (C-7), 29.7 (C-8), 53.6 (C-9), 37.9 (C-10), 21.0 (C-11), 39.5 (C-12), 42.2 (C-13), 55.8 (C-14), 24.1 (C-15), 56.1 (C-17), 12.1 (C-18), 19.5 (C-19), 32.8 (C-20), 18.7 (C-21), 38.0 (C-22), 24.1(C-23), 39.5 (C-24), 28.1 (C-25), 22.5 (C-26), 22.5 (C-27).
EIMS *m/z*: 400 $[M, C_{27}H_{44}O_2]^+$, 385 $[M-CH_3]^+$, 370 $[M-CH_3-H_2O]^+$, 339 $[M-C_3H_7-H_2O]^+$, 269 $[M-C_8H_{17}-H_2O]^+$, 199 $[M-C_{13}H_{27}-H_2O]^+$.

(*E*)-2-((E) Tridec-2-en-2-yl) heptadec-2-enal (**3**)
White residue (10 mg, 0.005%).
R_f: 0.70 (light petroleum/diethylether, 9:1).
IR (neat): ν_{max} 2930, 1680, 1640 cm^{-1}.
UV (Et_2O): 259 nm.
^1H (600 MHz, $CDCl_3$) δ: 9.32 (1H, s, H-1), 6.44 (1H, t, *J* = 7 Hz, H-3), 2.20-2.35 (4H, m, H-4, 3'), δ 5.33 (1H, m, H-2'), 1.60 (3H, s, CH_3-1'), 1.50 (4H, m, H-5, 4'), 1.27 (36H) and 0.88 (6H, t, *J*= 6.6 Hz H-17 and H-13').
^{13}C NMR (150 MHz, $CDCl_3$): 195.4 (C-1), 143.7 (C-2), 155.5 (C-3), 129.6 (C-1'), 130.1 (C-2'), 31 (C-4 and C-3'), 23-29 ($19CH_2$), 22.0 (CH_3-1') and 14.2 (C-17 and C-12').
GCMS *m/z*: 432 [M, $C_{30}H_{56}O]^+$, 431 [M- H]$^+$, 305 [M-$C_9H_{19}]^+$, 249 [M-$C_{13}H_{27}]^+$, 183, 127, 43 (100).
GCMS *m/z*: 432 [M, $C_{30}H_{56}O]^+$, 431 [M- H]$^+$, 305 [M-$C_9H_{19}]^+$, 249 [M-$C_{13}H_{27}]^+$, 183, 127, 43 (100).
HR-EIMS: 432.4319 requires $C_{30}H_{56}O$, calcd. 432.4331;

Microtiter broth dilution method for yeast susceptibility testing: Sterile microtiter assay trays containing 96 round-bottom wells (Dynatech Laboratories, Inc., Alexandria, Va.) were employed. The stock solutions of antifungal agents were appropriately diluted in the various assay media to give the following working solution of ketoconazole (Janssen Pharmaceuticals, Piscataway, N.J.), starting from 20 to 1000 μg/mL. Two fold serial dilutions were performed with respective assay broths and dispensed into appropriate wells. Each vertical column of wells contained a single antifungal agent in progressive dilutions and was inoculated with a single clinical isolate. The first horizontal row of wells (A1-A12) contained no antifungal agent and served both as a growth and sterility control. The inoculation of 0.1 mL of yeast suspension created a final range of antifungal concentrations identical to that of the agar dilution plates. The final volume in each well was 0.2 mL.

Inoculum: *Aspergillus fumigatus* WT (Af293), *A. flavus* WT (NRRL3357), and *Candida albicans* were grown in YPD (1% yeast, 2% peptone and 2% dextrose) medium. After 24 h of incubation at 37°C in a 5% CO_2 atmosphere, the isolates were diluted 1:100 in normal saline, followed by a 1:100 dilution in yeast peptone dextrose medium. Prepared in this manner, a volume of 0.1 mL of the final broth dilution contained approximately 1000 CFU of the previously mentioned fungi. Microtiter broth dilution plates were incubated at 37°C in 5% CO_2 for 48 h. MIC was defined as that concentration of the antifungal agent contained in the microtiter well or in the agar dilution plate in which the absence of visual turbidity (colonies) was first observed.

Acknowledgment - This work was supported by grants from the Deanship of Scientific Research (DSR) No. 429/003-8, King Abdul-Aziz University (KAU). Dr Nessim, I. Rady, Assistant Prof. of algal flora, Faculty of Science, Umm Al-Qura University, is acknowledged for algal identification, and Dr Shaymaa Hassan, Faculty of Pharmacy, Mansoura University, for her help in measuring the antifungal activity.

References

[1] (a) Kladi M, Xenaki H, Vagias C, Papazafiri P, Roussis V. (**2006**) New cytotoxic sesquiterpenes from the red algae *Laurencia obtusa* and *Laurencia microcladia*. *Tetrahedron*, ***62***, 182-189; (b) Kaur K, Jain M, Kaur T, Jain R. (**2009**) Antimalarials from nature. *Bioorganic and Medicinal Chemistry*, ***17***, 3229-3256; (c) Li H-Y, Matsunaga S, Fusetani N. (**1998**) Antifungal metabolites from marine sponges. *Current Organic Chemistry*, ***2***, 2649–682; (d) Vairappan CS. (**2003**) Potent antibacterial activity of halogenated metabolites from Malaysian red alga, *Laurencia maiuscula* (Rhodomelaceae, Ceramiales). *Biomolecular Engineer*, ***20***, 255–259.

[2] (a) Bruno LA, Beatriz GF, Mutue TF, Valéria LT. (**2008**) Sesquiterpenes of the Brazilian marine red alga *Laurencia filiformis* (Rhodophyta, Ceramiales). *Natural Product Communications*, ***3***, 1653-1654; (b) Dias DA, White JM, Urban S. (**2009**) *Laurencia filiformis*: Phytochemical profiling by conventional and HPLC-NMR approaches. *Natural Product Communications*, ***4***, 157-172; (c) Kladi M, Vagias C, Papazafiri P, Furnari G, Serio D, Roussis V. (**2007**). New sesquiterpenes from the red alga *Laurencia microcladia. Tetrahedron*, ***63***, 7606-7611.

[3] (a) Al-lihaibi SS, Ayyad SN, Al-wessabi EO, Alarif WM. (**2010**) 3β, 7β-Dihydroxy-5α-cholestan-11-one: A new oxidation pattern of cholestane skeleton from *Laurencia papillosa. Biochemical Systematics and Ecology*, ***38***, 861-863; (b) Lisboa BP, Ganshow LI, Halket JM, Pinheiro-Joventio F.(**1982**) The trace sterols of the red alga *Laurencia papillosa* investigated by open tubular capillary column gas chromatography. *Compound Biochemical Physiology*, ***73B***, 257-264; (c) Zornista K, Kamen S, Rosalina S, Stefka D-K, Simon P. (**2006**) Comparative investigation on sterols from some Black Sea red algae. *Natural Products Research*, ***20***, 113-118.

[4] (a) Suzuki M, Kurosawa E, Kurata K. (**1987**) (*E*)-2-Tridecyl-2-heptadecenal, an unusual metabolite from the red alga *Laurencia* species. *Bulletin of the Chemical Society of Japan*. ***60***, 3793–3794; (b) Gallo ML, Seldes AM, Cabrera GM. (**2004**) Antibiotic long-chain and α,β-unsaturated aldehydes from the culture of the marine fungus *Cladosporium* sp., *Biochemical Systematics and Ecology*, ***32***, 545–551; (c) Anderson IG, Haslewood AD. (**1967**) New evidence for the structure of myxinol. *Biochemistry Journal*, ***104***, 1061-1063; (d) Goad JL, Akihisa T. (**1977**) Analysis of sterols 1st. ed. Blackie Academic & Professional, 197-217.

[5] (a) Zhao K, Wang Y, Han L. (**2007**) 4,5-Epoxy cholestane-3,6- diols: templates for generating the full set of eight cholestane -3, 5, 6- triol stereoisomerisms in multigram scales, but not for cholestane- 3, 4, 6- triol. *Steroids*, ***72***, 95-104; (b) Jiang B, Shi H-P, Xu M, Wang W-J, Zhao W-S. (**2008**) Stereoselective synthesis of certonardolsterol D3. *Tetrahedron*, ***64***, 9738-9744.

[6] (a) Bultel-Ponce V, Etahriri S, Guyot M. (**2002**) New ketosteroid from the red alga *Hypnea musciformis*. *Bioorganic Medicinal Chemistry Letter*, ***12***, 1715-1718; (b) Su B-N, Takaishi Y, Morinins H-K. (**1999**) Four novel phenylpropanol ester lipid metabolites from *Morina chinesis*, *Journal of Natural Products*, ***62***, 1325-1327.

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2011
Vol. 6
No. 12
1825 - 1826

Steroidal Saponins from the Fruits of *Cestrum ruizteranianum*

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Received: September 23rd, 2011; Accepted: September 29th, 2011

Seven spirostane and furostane-type glycosides were isolated from the aqueous methanolic extract of the fruits of *Cestrum ruizteranianum* and characterized mainly by 2D NMR spectroscopy and mass spectrometry. These known saponins belong to the Δ^5-spirostene and Δ^5-furostene series and are reported in this species for the first time.

Keywords: Solanaceae, *Cestrum ruizteranianum*, steroidal saponins.

Cestrum ruizteranianum Benítez & D'Arcy is a medium height shrub endemic of the Venezuelan Andes [1]. The genus *Cestrum* (Solanaceae) comprises more than 300 species, which are native to warm temperate to tropical regions, mainly distributed in South America, but known as ornamental plants in the whole world [2]. The species of this genus are rich in steroidal saponins and exhibit many potential medicinal uses as insecticide, herbicide, molluscicide, antimicrobial agent, and anticancer agents for instance [2]. We chose to study *C. ruizteranianum* for chemotaxonomic reasons, and because no work on its saponin composition had been reported.

Seven saponins were obtained by several chromatographic methods from the dried fruits of *C. ruizteranianum*. The isolated compounds (**1**-**7**) were unambiguously identified by 2D NMR spectroscopy and mass spectrometry (FAB) as: pennogenin 3-*O*-β-chacotrioside (**1**) [3], (25*R*,26*R*)-spirost-5-ene-3β,17α,26-triol 3-*O*-β-chacotrioside (**2**) [4], methyl protodioscin (**3**) [5], protodioscin (**4**) [5], 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,17α,22,26-tetraol 3-*O*-β-chacotrioside (**5**) [4], 26-*O*-β-D-glucopyranosyl-22-methoxy-(25*R*)-furost-5-ene-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (Methyl proto Pb) (**6**) [6], and 26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**7**) [7]. All spectroscopic data were in agreement with those previously reported.

Chemotaxonomic significance: Steroidal saponins with spirostane and furostane-type skeletons are very common in *Cestrum* species [2], like in *C. parqui* [8], *C. diurnum* [9], *C. nocturnum* [10], *C. sendtenerianum* [11], *C. axillare* [12], and *C. laevigatum* [13]. From a chemotaxonomic point of view, it is not surprising to find steroidal saponins in the fruits of *C. ruizteranianum*, but the isolated compounds (**1**-**7**) are found for the first time in this species.

Experimental

Plant material: Plant material was collected in Mucuchies-Gavidia (2950 m), Municipio Rangel, Mérida-Venezuela, in April 2010 and identified by Ing. Juan Carmona Arzola, Universidad de Los Andes (Mérida-Venezuela). A voucher specimen (J. M. Amaro *et al*., No. 1862) was deposited in the MERF Herbarium, Faculty of Pharmacy, ULA.

Extraction and isolation: Eight g of the MeOH/H_2O (7:3) extract of the dried fruits was fractionated by isocratic vacuum liquid chromatography (VLC) on silica gel (40-63 μm) ($CHCl_3$/MeOH/H_2O, 13:7:2, lower phase, 200 mL, each) yielding 3 fractions (A-C). Fraction A (360 mg) was then separated by medium pressure liquid chromatography (MPLC) on silica gel (15-40 μm) ($CHCl_3$/MeOH/H_2O,

13:7:2, lower phase) to obtain compounds **1** (11 mg) and **2** (15 mg). Fraction B (4.4 g) was chromatographed on a Sephadex LH-20 column (MeOH/H_2O, 1:1) yielding 5 subfractions (I-V). Subfraction I (1.1 g) was then submitted to successive MPLC on normal and RP-18 silica gel to afford compounds **3** (9.8 mg), **4** (10.0 mg), **5** (11.1 mg), **6** (11.6 mg) and **7** (10.8 mg). All saponins were isolated as white amorphous powders.

References

[1] Jauregui D, de Rios NR, Benitez de Rojas CE. (**2001**) Leaf anatomic studies in Solanaceae of Venezuela. VI. Leaf anatomy of 10 species of *Cestrum* L. *Acta Científica Venezolana*, ***52***, 248-260.

[2] Begum AS, Goyal M. (**2007**) Research and medicinal potential of genus *Cestrum* (Solanaceae)-a review. *Pharmacognosy Reviews,* ***1,*** 320-332.

[3] Nohara T, Miyahara K, Kawasaki T. (**1975**) Steroid saponins and sapogenins of underground parts of *Trillium kamtschaticum* Pall. II. Pennogenin- and kryptogenin 3-*O*-glycosides and related compounds. *Chemical & Pharmaceutical Bulletin*, ***23***, 872-885.

[4] Ando J, Miyazono A, Zhu XH, Ikeda T, Nohara T. (**1999**) Studies on the constituents of Solanaceous plants, steroidal glycosides from *Solanum nodiflorum*. *Chemical & Pharmaceutical Bulletin*, ***47***, 1794-1796.

[5] Kawasaki T, Komori T, Miyahara K, Nohara T. (**1974**) Furostanol bisglycosides corresponding to dioscin and gracillin. *Chemical & Pharmaceutical Bulletin*, ***9***, 2164-2175.

[6] Miyamura M, Nakano K, Nohara T, Tomimatsu T, Kawasaki T. (**1982**) Steroid saponins from *Paris polyphylla* Sm.-Supplement. *Chemical & Pharmaceutical Bulletin*, ***30***, 712-718.

[7] Kawabe N, Togami Y, Uenishi N, Rii H, Chen S. (**1996**) Extraction of anticancer saponins from *Ypsilandra thibetica.* Jpn. *Kokai Tokkyo Koho*, JP 08040914 A.

[8] Chaieb I, Tayeb W. (**2009**) Comparison of the molluscicidal activity of *Cestrum parqui* (Solanaceae) and *Quillaja saponaria* (Quillajaceae) saponins*. Tunisian Journal of Medicinal Plants and Natural Products*, ***2***, 31-35.

[9] Fouad MA, Mohamed KM, Kamel MS, Matsunami K, Otsuka H. (**2008**) Cesdiurins I-III, steroidal saponins from *Cestrum diurnum*. *Journal of Natural Medicines*, ***62***, 168-173.

[10] Mimaki Y, Watanabe K, Sakagami H, Sashida Y. (**2002**) Steroidal glycosides from the leaves of *Cestrum nocturnum*. *Journal of Natural Products*, ***65***, 1863-1868.

[11] Haraguchi M, Mimaki Y, Motidome M, Morita H, Takeya K, Itokawa H, Yokosuka A, Sashida Y. (**2000**) Steroidal saponins from the leaves of *Cestrum sendtenerianum. Phytochemistry*, ***55***, 715-720.

[12] De Amorim A, Borba HR, Pereira NA, Matsunami K, Otsuka H. (**1992**) Effect of the toxic fraction of *Cestrum axillare* on mitochondria. *Revista Brasileira de Farmacia*, ***73***, 2-4.

[13] Canham PAS, Warren FL (**1950**) Saponins. I. Isolation of gitogenin and digitogenin from *Cestrum laevigatum*. *Journal of the South African Chemical Institute*, ***3***, 9-12.

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2011
Vol. 6
No. 12
1827 - 1830

Isolation and Cholinesterase Activity of Amaryllidaceae Alkaloids from *Nerine bowdenii*

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Received: September 26th, 2011; Accepted: October 20th, 2011

Amaryllidaceae species are known as ornamental plants. Some contain galanthamine, an acetylcholinesterase inhibitor. The chemical composition of the alkaloid extract of bulbs of *Nerine bowdenii* Watson has been analyzed by means of GC/MS. Twenty-two compounds were detected and nineteen of them identified, one of which was belladine. The alkaloid extract showed promising cholinesterase inhibitory activities against human blood acetylcholinesterase (HuAChE; IC_{50}= 87.9±3.5 μg/mL) and human plasma butyrylcholinesterase (HuBuChE; IC_{50} = 14.8±1.1 μg/mL). Belladine inhibited HuAChE and HuBuChE in a dose-dependent manner with IC_{50} values of 781±12.5 μM and 284.8±4.2 μM, respectively.

Keywords: *Nerine bowdenii*, GC/MS, belladine, acetylcholinesterase, butyrylcholinesterase.

Alzheimer´s disease (AD) is the most common form of dementia. Epidemiological data indicate a considerable potential increase in the prevalence of the disease over the next two decades [1]. Although the pathogenesis of AD is complicated and not fully established, two major hypotheses (amyloid cascade and cholinergic) are currently under consideration regarding the molecular mechanism.

In AD patients, deficit of cholinergic functions, which results in decreased levels of the neurotransmitter acetylcholine (ACh) in the cortex, is responsible for the memory impairments [2a]. The principal role of acetylcholinesterase (AChE) is the termination of nerve impulse transmission at the cholinergic synapses by rapid hydrolysis of ACh. Inhibition of AChE serves as a strategy for the treatment of AD, senile dementia, ataxia, myasthenia gravis, and Parkinson´s disease [2b]. In the healthy brain, AChE is the most important enzyme regulating the level of ACh, while another enzyme, butyrylcholinesterase (BuChE) plays only a minor role. Moreover, in late AD stages, levels of AChE have declined by up to 85% and BuChE represents the predominant cholinesterase in the brain. BuChE, primarily associated with glial cells, but also with specific neuronal pathways, cleaves ACh in a manner similar to that of AChE to terminate its physiological action [2c]. Galanthamine, which is used for the treatment of AD, has been shown to be much less potent against BuChE than AChE [2d]. This fact has targeted BuChE as a new approach to intercede in the progression of AD and requires research into new inhibitors with dual enzymatic activity. Currently, cholinesterase inhibition is the most used therapeutic treatment for the symptoms of AD [2e, f].

Extracts from plants belonging to the Amaryllidaceae family have long been used as herbal remedies. These plants are known to synthesize a particular type of bioactive compound known as Amaryllidaceae alkaloids [3]. Some species of this family contain galanthamine, a long-acting, selective, reversible and competitive acetylcholinesterase inhibitor, as well as other alkaloids with interesting pharmacological activities, such as anticancer, antiviral, antimalarial, anti-inflammatory and antiparasitic [4a-e]. Among the bulbs of Amaryllidaceae plants screened previously for AChE inhibitory activity, *Nerine bowdenii* showed strong inhibition [5a,b].

The genus *Nerine*, the second largest within Amaryllidaceae with ca 30 species, is an autumn-flowering perennial bulbous plant group, whose species inhabit areas with summer rainfall and cool, dry winters [6]. Previous investigations led to the isolation of more than 30 Amaryllidaceae alkaloids. Some of these have been tested for their acetylcholinesterase inhibitory activity, but only ungeremine and undulatine showed interesting inhibition

activity with IC_{50} values of 0.35 μM [7a] and 37 μM [7b], respectively. In both studies acetylcholinesterase from electric eel was used.

The ethanolic extract of *N. bowdenii* bulbs exhibited significant HuAChE and HuBuChE inhibition activity (61.5% ± 4.0% for HuAChE and 89.8% ± 5.2% for HuBuChE, respectively) at a concentration of 500 μg/mL, thus indicating the presence of cholinesterase inhibitors in this plant taxon. The crude extract from the bulbs was concentrated into an alkaloid fraction and, as expected, this possessed a promising activity with an IC_{50} value of 87.9 ± 3.5 μg/mL for HuAChE and 14.8 ± 1.1 μg/mL for HuBuChE (values for reference compounds: belladine 781 ± 12, galanthamine 6.9 ± 0.3, huperzine A 0.25 ± 0.01 μg/mL for HuAChE, and 285 ± 5, 156 ± 2.9, > 500 μg/mL, respectively, for HuBuChE).

In order to identify the compounds in the complex alkaloid fraction of *N. bowdenii* bulbs, capillary GC/MS was employed. In the bulb extract, twenty-two alkaloids of the crinine, lycorine, belladine and haemanthamine types were detected by GC (Fig. 1); nineteen of them were identified based on their retention times (RT) and mass spectra as buphanisine (**1**,**2**), crinine (**3**), belladine (**4**), caranine (**5**), *N*-demethylbelladine (**7**), acetylfalcatine (**8**), buphanidrine (**9**), buphanamine (**10**), crinamine (**11**), powelline (**12**), undulatine (**15**), 11-*O*-acetylambelline (**16**), ambelline (**17**), 3-acetylnerbowdine (**18**), crinamidine (**19**), bowdensine (**20**), and 11-*O*-acetyl-1,2-epoxyambelline (**21**) (Table 1). Compound **2** showed a longer retention time than buphanisine (**1**) by GC-MS, but its MS pattern was the same, indicating that it is an isomer of **1**. Compounds **13**, **14** and **22** remained unidentified. Considering the low concentration of **13** and **14** (< 1% of TIC) and the complexity of the alkaloid mixture, their isolation and structural elucidation could be problematic. The mass spectrum of compound **22** was not found either in the databases or in the literature. For the identification of this compound, one of the major components of the alkaloid extract (6.8% of TIC), isolation in larger amount and structure elucidation is needed. The haemanthamine and crinine series of Amaryllidaceae alkaloids only differ in the position of the 5,10*b*-ethano bridge [8a]. The alkaloids of belladine type, which are biosynthetic precursors of Amaryllidaceae alkaloids [8b], have been previously reported only in the genera *Nerine* (*N. filifolia*) and *Crinum* [8c,d]. For *N. bowdenii*, belladine (**4**) and *N*-demethylbelladine (**7**) are reported here for the first time, which is quite surprising when considering their domination of the alkaloid mixture and the number of previous phytochemical investigations of the species. Alkaloids of the lycorine type (**5**, **6** and **8**) were identified only as minor components in the mixture. The relative proportion of each alkaloid was determined as a percentage of the total ion current. The alkaloid pattern of bulbs of *N. bowdenii* was dominated by belladine (**4**; 49.9 % of TIC), ambelline (**17**; 9.1 % of TIC) and *N*-demethylbelladine (**7**; 8.5 % of TIC).

A wide range of Amaryllidaceae alkaloids belonging to various ring types has been evaluated in recent studies for their *in vitro* AChE inhibitory activity with either galanthamine or physostigmine as a positive control [7b, 9a-c]. Results of these studies showed that the AChE inhibitory activity is associated mainly with galanthamine- and lycorine-type alkaloids. The lycorine-type compounds are less active inhibitors than the galanthamine type and their activity is associated with a substitution at positions C-1 and C-2 [9d]. Crinine-type alkaloids have only a weak activity against AChE and it appears that the stereochemistry of the 5,10b-ethanobridge has no effect on the inhibition of AChE activity [9c]. In comparison with our previous studies on other Amaryllidaceae plants, such as *Zephyranthes robusta* [9e], *Z. grandiflora* [9f] and *Chlidanthus fragrans* [9g], the alkaloid extract from *N. bowdenii* showed the weakest AChE inhibition activity, which is in agreement with the above mentioned conclusions, because galanthamine-type alkaloids were not detected and the lycorine-type alkaloids represented only a minor fraction (2.9 % of TIC). The alkaloid extracts of *Z. robusta*, *Z. grandiflora* and *Ch. fragrans* were dominated by galanthamine- and lycorine-type alkaloids, and consequently the HuAChE inhibition activity of these extracts was markedly stronger. On the other hand, the extract of *N. bowdenii* showed promising inhibition activity against HuBuChE with an IC_{50} value of 14.8 ± 1.1 μg/mL. As mentioned, BuChE plays an important role in the late AD stages, but only a limited number of alkaloids have been tested for their BuChE inhibitory activity so far. There are no data on BuChE inhibition activity of pure Amaryllidaceae alkaloids.

Relatively surprising was the weak cholinesterase activity of the isolated alkaloid belladine (**4**), previously isolated only from *N. filifolia* [8c], in comparison with the alkaloid extract due to its dominant amount in the mixture (49.9% of TIC) of *N. bowdenii*, and the very good HuBuChE inhibition activity of this extract. It is clear that the BuChE inhibition activity of *N. bowdenii* must be connected to some compound/compounds present in a smaller amount. The isolation and identification of this/these alkaloids will be the next step in searching for new bioactive natural compounds with BuChE inhibition activity.

Experimental

Plant materials: The fresh bulbs of *Nerine bowdenii* were obtained from Lukon Glads (Sadská, Czech Republic). The botanical identification was performed by Prof. Lubomír Opletal, CSc. A voucher specimen is deposited in the Herbarium of the Faculty of Pharmacy at Hradec Králové.

Extraction of alkaloids: Fresh bulbs (150 g) were extracted 3 times with EtOH (150 mL) at room temperature for 24 h. The solvent was evaporated under reduced pressure and the residue dissolved in 50 mL 2% HCl. After removing the neutral compounds with diethyl ether (3 x 50 mL), the extract was basified with 25% ammonia solution and the alkaloids extracted with EtOAc

Table 1: Alkaloids identified by GC/MS in bulbs of *Nerine bowdenii*.

Compound	Retention time,[a] (min)	[M^+] and characteristic ions *m/z* (% relative intensity)	%[b]	Reference for MS data
1 Buphanisine, isomer I	25.34	285 (100), 270 (23), 253 (25), 230 (28), 215 (80), 201 (22), 187 (20), 157 (15)	1.4	NIST 08, [10c]
2 Buphanisine, isomer II	25.47	285 (100), 270 (23), 253 (25), 230 (28), 215 (80), 201 (22), 187 (20), 157 (15)	0.7	NIST 08, [10c]
3 Crinine	26.45	271 (100), 254 (8), 242 (8), 228 (25), 216 (14), 199 (48), 187 (43)	1.3	NIST 08, [10c]
4 Belladine	26.65	315 (2), 194 (70), 152 (90), 151 (100), 135 (10), 121 (18), 107 (18)	49.9	NIST 08, [10d], [d]
5 Caranine	27.10	271 (86), 270 (46), 252 (52), 250 (10), 240 (12), 227 (48), 226 (100), 212 (5)	0.2	NIST 08
6 Acetylcaranine	27.24	313 (80), 270 (5), 253 (20), 252 (95), 250 (15), 240 (10), 225 (40), 226 (100)	2.6	NIST 08
7 *N*-Demethylbelladine	27.64	301 (2), 299 (3), 273 (1), 195 (6), 180 (20), 151 (100), 138 (4), 128 (2), 121 (9), 107 (6)	8.5	[8c]
8 Acetylfalcatine	28.49	343 (75), 300 (5), 283 (25), 282 (80), 266 (30), 257 (77), 256 (100), 241 (12)	0.1	NIST 08
9 Buphanidrine	28.82	315 (100), 300 (26), 287 (30), 272 (10), 260 (45), 245 (60), 231 (30), 228 (22)	2.9	NIST 08, [10c]
10 Buphanamine	28.94	301 (100), 286 (12), 284 (14), 272 (14), 256 (18), 231 (25), 218 (18), 204 (18)	1.1	NIST 08
11 Crinamine	29.34	301 (1), 269 (100), 240 (35), 225 (20), 224 (25), 211 (17), 181 (58)	1.9	NIST 08
12 Powelline	29.67	301 (100), 284 (8), 272 (7), 258 (20), 246 (12), 244 (12), 229 (80), 217 (40)	0.3	NIST 08
13 A13[c]	30.07	331 (22), 313 (10), 299 (28), 287 (100), 284 (14), 282 (10), 270 (20), 255 (45)	0.4	-
14 A14[c]	30.19	373 (90), 314 (72), 299 (38), 284 (20), 270 (20), 254 (54), 242 (50), 226 (38), 216 (20), 115 (25)	0.3	-
15 Undulatine	31.14	331 (100), 316 (6), 302 (9), 300 (6), 286 (20), 272 (5), 260 (18), 258 (40)	3.6	[10c]
16 11-*O*-Acetylambelline	31.24	373 (100), 358 (15), 342 (15), 330 (10), 314 (70), 313 (69), 298 (30), 282 (55),270 (25)	4.5	[10c]
17 Ambelline	31.49	331 (100), 316 (5), 299 (30), 287 (55), 270 (25), 260 (50), 257 (35), 255 (23),241 (27)	9.1	NIST 08
18 Acetylnerbowdine	32.10	361 (75), 318 (30), 302 (60), 273 (20), 254 (50), 244 (25), 43 (100)	0.7	NIST 08
19 Crinamidine	32.62	317 (75), 288 (100), 274 (5), 259 (22), 258 (25), 243 (28), 244 (30), 230 (25), 217 (40), 205 (40)	0.4	NIST 08
20 Bowdensine	32.75	403 (82), 344 (80), 288 (75), 284 (50), 272 (40), 230 (40), 217 (42), 189 (28), 173 (50), 43 (100)	2.9	NIST 08
21 11-*O*-Acetyl-1,2-epoxyambelline	33.52	389 (60), 330 (75), 316 (100), 274 (30), 256 (55), 231 (40), 228 (12), 205 (25), 203 (27), 190 (18)	0.4	[10e]
22 A22[c]	33.87	329 (100), 314 (18), 300 (8), 286 (12), 270 (6), 231(28), 218 (15), 204 (20), 190 (10)	6.8	-

[a] For GC conditions see the Experimental section, [b] Values are expressed as a percentage of the total ion current (TIC), [c]not identified, [d]standard.

(3 x 50 mL). The organic solvent was evaporated and 10 mg of alkaloid extract was removed for HuAChE and HuBuChE assay. 10 mg of the dry alkaloid fraction was dissolved in MeOH to a final concentration of 10 mg/mL for further analysis. The rest of the fraction (180 mg) was used for the isolation of pure alkaloids.

GC/MS analysis and identification of alkaloids: The GC/MS analysis of alkaloids from *N. bowdenii* was carried out on a gas chromatograph (Focus Thermo Scientific, USA) with a splitless injector (280°C) and a mass detector (200°C, GC-MS MD 800 Fisons, Manchester, UK). A DB-5MS column (30 m x 0.25 mm x 0.25 μm, Agilent Technologies Santa Clara, CA, USA) and helium gas (constant flow 1 mL/min) were used for separation. The temperature program was: 100 - 180°C at 15°C/min, 1 min held at 180°C, and 180 – 300°C at 5°C /min and 5 min held at 300°C, detection range *m/z* 40-600. The injector temperature was 280°C. The alkaloids were identified by comparison of their MS with those in the NIST library and with those reported in the literature [8c, 9a, 10a-e].

Isolation and identification of belladine: The alkaloid extract of *N. bowdenii* was subjected to preparative TLC (silica gel 60, F_{254}, Merck) and eluted with toluene:Et_2NH (95:5, 3 times) to give 4 subfractions A/1-A/4. Subfraction A/1 (35 mg) was further subjected to preparative TLC (cHx: Et_2NH, 95:5, 3 times), which led to the isolation of compound **4** (20 mg). Because of the small amount of other subfractions, they were not used for the isolation of other alkaloids. The structure of belladine (**4**) was determined by comparison of its spectral data with those reported in the literature [8c]. NMR spectra were recorded on a Varian VNMRS500 spectrometer, operating at 500 MHz (^{1}H) and at 125 MHz (^{13}C). ESI-MS were measured on a Thermo Finnigan LCQDuo spectrometer.

Preparation of red blood cell ghosts: Ghosts were prepared from freshly drawn blood (taken from healthy volunteers), to which 1 mL of sodium citrate per 10 mL of blood was added, according to the method of Steck and Kant [11a], with slight modification. Briefly, plasma (HuBuChE) was removed from the whole blood by centrifugation at 4000 rpm in a Boeco U-32R centrifuge with a Hettich 1611 rotor. Red blood cells were transferred to 50 mL tubes and washed 3 times with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride (12000 rpm, Avanti J-30I, rotor JA-30.50). The washed erythrocytes were stirred with 5 mM phosphate buffer (pH 7.4) for 10 mins to ensure lysis. The lysed cells were centrifuged at 20,000 rpm for 10 mins and then the ghosts (HuAChE) were washed 3 times with phosphate buffer.

Acetylcholinesterase and butyrylcholinesterase assay: HuAChE and HuBuChE activities were determined using a modified method of Ellman *et al*. [11b] at concentrations of 0.5, 2.5, 5, 12.5, 25, 50, 125, 250 and 500 μg/mL using acetylthiocholine iodide and butyrylthiocholine iodide as substrates, respectively. Galanthamine and huperzine A were used as positive standards. The % inhibition was calculated according to the formula: $\%I = 100-(\Delta A_{BL}/\Delta A_{SA})*100$, where ΔA_{BL} is increase of absorbance of blank sample and ΔA_{SA} the increase of absorbance of the measured sample.

Statistical analysis: The IC_{50} values were calculated with the use of GraphPad Prism 5.02 software.

Acknowledgments – This project was supported by grants SVV-2011-263002 and MSM0021620822.

References

[1] Johnson N, Davis T, Bosanquet N. (**2000**) The epidemic of Alzheimer´s disease; how can we manage the costs? *Pharmacoeconomics*, ***18***, 215-223.

[2] (a) Lahiri DK, Farlow MR, Greig NH, Sambamurti K. (**2002**) Current drug targets for Alzheimer´s disease treatment. *Drug Development Research*, ***56***, 267-281; (b) Rahman AU, Choudhary MI. (**2001**) Bioactive natural products as a potential source of new pharmacophores a theory of memory. *Pure and Applied Chemistry*, ***73***, 555-560; (c) Greig NH, Lahiri DK, Sambamurti K. (**2002**) Butyrylcholinesterase: an important new target in Alzheimer´s disease therapy. *International Psychogeriatrics*, ***14***, 77-91; (d) Fulton B, Benfield P. (**1996**) Galanthamine. *Drugs & Aging*, ***9***, 60-65; (e) Lleó A. (**2007**) Current therapeutic options for Alzheimer´s disease. *Current Genomics*, ***8***, 550-558; (f) Recanatini M, Valenti P. (**2004**) Acetylcholinesterase inhibitors as a starting point towards improved Alzheimer´s disease therapeutics. *Current Pharmaceutical Design*, ***10***, 3157-3166.

[3] Bastida J, Lavilla R, Viladomat F. (**2006**) Chemical and biological aspects of *Narcissus* alkaloids. In *The Alkaloids*, Vol. ***63***, Cordell GA. (Ed.). Elsevier Scientific: Amsterdam, NL, pp. 87-179.

[4] (a) Evidente A, Kornienko A. (**2009**) Anticancer evaluation of structurally diverse Amaryllidaceae alkaloids and their synthetic derivatives. *Phytochemistry Reviews*, ***8***, 449-459; (b) Toriizuka Y, Kinoshita E, Kogure N, Kitajima M, Ishiyama A, Otoguro K, Yamada H, Ōmura S, Takayama H. (**2008**) New lycorine-type alkaloid from *Lycoris traubii* and evaluation of antitropanosomal and antimalarial activities of lycorine derivatives. *Bioorganic & Medicinal Chemistry*, ***16***, 10182-10189; (c) Weniger B, Italiano L, Beck JP, Bastida J, Bergoñon S, Codina C, Lobstein A, Anton R. (**1995**) Cytotoxic activity of Amaryllidaceae alkaloids. *Planta Medica*, ***61***, 77-79; (d) Zupkó I, Réthy B, Hohmann J, Molnár J, Ocsovczki I, Falaky G. (**2009**) Antitumor activity of alkaloids derived from Amaryllidaceae species. *In Vivo*, ***23***, 41-48; (e) Lamoral-Theys D, Andolfi A, van Goietsenoven G, Cimmino A, Le Calvé B, Wauthoz N, Mégalizzi V, Gras T, Bruyère C, Dubois C, Mathieu V, Kornienko A, Kiss R, Evidente A. (**2009**) Lycorine, the main phenanthridine Amaryllidaceae alkaloid, exhibits significant antitumor activity in cancer cells that display resistance to proapoptotic stimuli: an investigation of structure-activity relationship and mechanistic insight. *Journal of Medicinal Chemistry*, ***52***, 6244-6256.

[5] (a) Rhee IK, van de Meent M, Ingkaninan K, Verpoorte R. (**2001**) Screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. *Journal of Chromatography A*, ***915***, 217-223; (b) Marston A, Kissling J, Hostettmann K. (**2002**) A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. *Phytochemical Analysis*, ***13***, 51-54.

[6] Rix M, Phillips R. (**1983**) *The bulb book: a photographic guide to over 800 hardy bulbs*, Pan Books Ltd., London, GB, pp. 171.

[7] (a) Rhee IK, Appels N, Hofte B, Karabatak B, Erkelens C, Stark LM, Flippin LA, Verpoorte R. (**2004**) Isolation of the acetylcholinesterase inhibitor ungeremine from *Nerine bowdenii* by preparative HPLC coupled on-line to a flow assay system. *Biological & Pharmaceutical Bulletin*, ***27***, 1804-1809; (b) van Rijn RM, Rhee IK, Verpoorte R. (**2010**) Isolation of acetylcholinesterase inhibitory alkaloids from *Nerine bowdenii*. *Natural Product Research,* ***24***, 222-225.

[8] (a) De Angelis G, Wildman W. (**1969**) Identification of Amaryllidaceae alkaloids using ORD and CD spectroscopy. *Tetrahedron Letters,* ***9***, 729-732; (b) Eichhorn J, Takada T, Kita Y, Zenk MH. (**1998**) Biosynthesis of the Amaryllidaceae alkaloid galanthamine. *Phytochemistry,* ***49***, 1037-1047; (c) Nair JJ, Campbell WE, Brun R, Viladomat F, Codina C, Bastida J. (**2005**) Alkaloids from *Nerine filifolia*. *Phytochemistry*, ***66***, 373-382; (d) Jin Z. (**2009)** Amaryllidaceae and *Sceletium* alkaloids. *Natural Product Reports*, ***26***, 363-381.

[9] (a) López S, Bastida J, Viladomat F, Codina C. (**2002**) Acetylcholinesterase inhibitory activity of same Amaryllidaceae alkaloids and *Narcissus* extracts. *Life Sciences*, ***71***, 2521-2529; (b) Elgorashi EE, Stafford GI, van Staden J. (**2004**) Acetylcholinesterase enzyme inhibitory effects of Amaryllidaceae alkaloids. *Planta Medica*, ***70***, 258-260; (c) Houghton PJ, Agbedahunsi JM, Adegbulugbe A. (**2004**) Choline esterase inhibitory properties of alkaloids from two Nigerian *Crinum* species. *Phytochemistry*, ***65***, 2893-2896; (d) Houghton PJ, Ren Y, Howes MJ. (**2006**) Acetylcholinesterase inhibitors from plants and fungi. *Natural Product Reports*, ***23***, 181-189; (e) Cahlíková L, Kulhánková A, Urbanová K, Valterová I, Macáková K, Kuneš J. (**2010**) Analysis of Amaryllidaceae alkaloids from *Zephyranthes robusta* by GC-MS and their cholinesterase activity. *Natural Product Communications*, ***5***, 1201-1204; (f) Cahlíková L, Valterová I, Macáková K, Opletal L. (**2011**) Analysis of Amaryllidaceae alkaloids from *Zephyranthes grandiflora* by GC-MS and their cholinesterase activity. *Revista Brasileira de Farmacognosia/Brazilian Journal of Pharmacognosy,* ***21***, 575-580; (g) Cahlíková L, Macáková K, Zavadil S, Jiroš P, Opletal L, Urbanová K, Jahodář L. (**2011**) Analysis of Amaryllidaceae alkaloids from *Chlidanthus fragrans* by GC-MS and their cholinesterase activity. *Natural Product Communications*, ***6***, 603-606.

[10] (a) Kreh M, Matusch R, Witte L. (**1995**) Capillary gas chromatography-mass spectrometry of Amaryllidaceae alkaloids. *Phytochemistry*, ***38***, 773-776; (b) Berkov S, Bastida J, Nikolova M, Viladomat F, Codina C. (**2008**) Rapid TLC/GC-MS identification of acetylcholinesterase inhibitors in alkaloid extract. *Phytochemical Analysis*, ***19***, 411-419; (c) Viladomat F, Codina C, Bastida J, Mathee S, Campbell WE. (**1995**) Further alkaloids from *Brunsvigia josephinae*. *Phytochemistry*, ***40***, 961-965; (d) Ghosal S, Shanthy A, Singh SK. (**1988**) Isocraugsodine, an *N*-arylidenephenethylamine from *Crinum asiaticum* and its *E-Z* isomerism. *Phytochemistry*, ***27***, 1849-1852; (e) Machocho A, Chhabra SC, Viladomat F, Codina C, Bastida J. (**1999**) Alkaloids from *Ammocharis tinneana*. *Phytochemistry*, ***51***, 1185-1191.

[11] (a) Steck TL, Kant JA. (**1974**) Preparation of impermeable ghosts and inside-out vesicles from human erythrocyte membranes. *Methods in Enzymology*, ***31 (Pt A)***, 172-180; (b) Ellman GL, Courtney KD, Andres V, Featherstone RM. (**1961**) A new rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, ***7***, 88-95.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1831 - 1834

HPLC Determination of Majdine in *Vinca herbacea*

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Received: May 19th, 2011; Accepted: October 26th, 2011

A reliable HPLC method coupled with DAD detection was developed and validated for determination of majdine in *Vinca herbacea*. The chromatographic separation was carried out on a Symmetry C_{18} column (250 mm x 4.6 mm, 5 µm, Waters) with an isocratic solvent system of 25 mM potassium phosphate buffer (pH=3.0)-acetonitrile. UV detection was performed at 225 nm. Good linear behavior over the investigated concentration range was observed with the value of $r^2 > 0.9978$. The method was reproducible with intra- and inter-day variations of less than 4.38%. The proposed method was linear, accurate, precise and specific. The validated method was successfully applied to quantify majdine in various parts of *V. herbacea*, which was collected during the flowering months of April and May. The results indicated that the developed HPLC method could be used for the quality control of *V. herbacea* and for the standardization of its extracts in majdine.

Keywords: HPLC-DAD, validation, *Vinca herbacea*, alkaloids, majdine.

Vinca herbacea Waldst. and Kit. (Apocynaceae) is a flowering plant native to eastern and southeastern Europe, from south Austria to Greece and east to the Crimea, and also in southwestern Asia east to the Caucasus, especially in the eastern part of Georgia. *V. herbacea* is an herbaceous perennial plant growing as a trailing vine, growing up to 10-20 cm in height.

The anticancer compounds vinblastine and vincristine are present in *V. rosea* leaves, thus investigations of *Vinca* species became very interesting. Previous phytochemical studies have reported the presence of alkaloids in various parts of *V. herbacea*: akuamicine, akuamine, herbaine, hervine, herbaline, herbaceine, lochnerine, norfluo-kurarine, 11-methoxytabersonine, tabersonine, reserpine, reserpinine, isoreserpinine, vincamine, vincaherbine, and vincaherbinine. The alkaloids belonging to the oxindole group, such as majdine, isomajdine and carapanaubine, were detected in leaves and roots of *V. herbacea.* Majdine has been shown to be the major alkaloid in the roots and leaves during the period of flower formation. [1-7].

The pharmacological activity of the total alkaloids of *V. herbacea* has been investigated. They markedly reduced blood pressure for an extended period [8]. Also, the blocking action of *V. herbacea* on neuromuscular synapses has been studied. Intravenous injection of total alkaloids in doses greater than 8 mg/kg was able to induce a curare-like action in rabbits and cats [9]. Similarly, a mixture of alkaloids named Vinherbine, has been shown to produce spasmolytic effects in Guinea pig ileum preparation.

Pharmacological studies have demonstrated that these alkaloids also possess cardio stimulating and antiarythmic activity [10-13]. The bacteriostatic effect of the alkaloids has been tested on *Staphylococcus aureus* and *Escherichia coli*. The indole alkaloids were shown to be the most active [14]. In addition, recent pharmacological experiments have revealed that majdine and isomajdine were responsible for apoptotic and antioxidant activity [15].

Majdine is one of the main alkaloids in the plant. Based on pharmacological studies, majdine has been selected as the chemical marker of *V. herbacea*. Therefore, the quantification of this alkaloid is necessary to evaluate the quality of this species. No analytical method has been reported in the literature for alkaloid quantification in *V. herbacea*. Thus, the development of an analytical method is necessary for the quality control of the plant.

HPLC methods have been reported for indole alkaloids such as vincamine. Mostly, mobile phases with buffer are used for determination of these alkaloids [16,17].

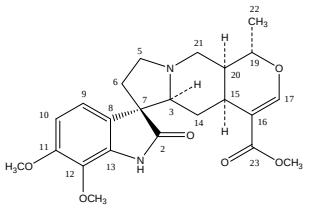

Figure 1: Chemical structure of majdine.

The aim of this study was the development and validation of a HPLC-DAD method for the quantification of majdine (Figure 1) in *V. herbacea*.

The separation conditions were optimized to achieve satisfactory resolution. Various reversed phase columns such as Symmetry C18, SunFire C18 and Atlantis C18 were tested. The optimal separation of leaf extract was obtained with a Symmetry C_{18} column (250 mm x 4.6 mm, 5 µm, Waters). The effect of mobile phase was also examined. In initial experiments, mixtures composed of methanol and water acidified with acetic acid (pH=3.0) in different ratios were tested. This mobile phase led to bad resolution and an unstable baseline. Satisfactory separation was obtained with a mobile phase consisting of 25 mM potassium phosphate buffer-acetonitrile in a ratio of 8 /2, v/v. A pH value of 3.0 was selected for the optimal separation of the compound in the extracts; the pH was adjusted with orthophosphoric acid. The retention time of majdine was observed at 15.6 min. Measurement at 225 nm displayed sufficient sensitivity and a satisfactory chromatographic baseline.

In the present study, quantification with an internal standard was used because majdine was not commercially available. Several alkaloids, such as palmatine, berberine, caffeine, papaverine, quinine and chelidonine, were tested as internal standard. Among the alkaloids, papaverine was chosen because it was stable, commercially available and displayed a suitable chromatographic retention time. The chromatogram showed complete baseline separation of papaverine (**2**) and majdine (**1**) in *V. herbacea* (Figure 2).

The method was validated according to the ICH guidelines on the validation of analytical methods. The specificity was investigated. HPLC chromatogram of the sample solution revealed no interference with majdine and other compounds in the extract of *V. herbacea*. The peak purity was verified using Agilent Chemstation software.

The calibration curves were plotted by correlating the area ratio (y = majdine/internal standard) versus the corresponding concentration ratio (x = majdine/internal standard). Assay results of majdine standard (0.006-0.019 mg/mL, n=15) were linear within the studied concentration range. The regression equation was y = 1.5812 x - 0.0221, and the correlation coefficient (r^2) 0.9978.

The intra-day (3 days, n=6) and inter-day precisions (n=18) were assessed on dried leaves of *V. herbacea*. The

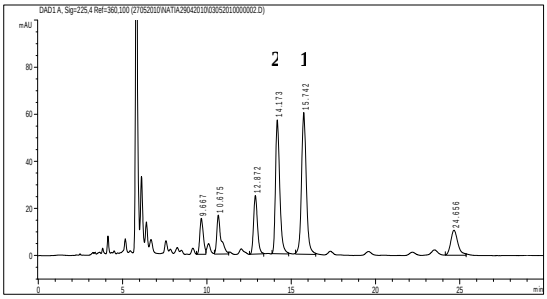

Figure 2: HPLC profile of *Vinca herbacea* leaf extract collected in May 2008. Peaks: **1**, majdine; **2**, internal standard.

Table 1: Content of majdine in extracts prepared from different parts of *V. herbacea* collected in April/May 2008.

	Plant part	Content of majdine %
April	Leaves	3.0
	Stems	0.2
	Roots	1.1
May	Leaves	11.9
	Stems	7.5
	Roots	0.7

data obtained for the repeatability were: day 1 = 12.2% (RSD % = 5.1), day 2 = 11.4% (RSD % = 3.7), day 3 = 12.0% (RSD % = 4.3). The intermediate precision was 11.9 %, RSD % = 5.3%. The mean recoveries of majdine ranged from 94.4 (± 0.1) to 105.9 (± 0.1)%. The limit of quantification and the limit of detection were 13 ng/mL and 65 ng/mL, respectively.

The validated HPLC-DAD method was applied to the determination of majdine in extracts from different parts of *V. herbacea* collected during the flowering period (Table 1). The concentration varied significantly according to the different plant parts. The highest content (11.9%) was found in the leaves, which are considered to be the best raw material.

The content of majdine was also studied during two consecutive months of the flowering period. Important variations were noted between April and May. The content of the leaves was four times higher in May than in April, and forty times higher in the stems. The best harvesting period for the leaves seems to be in May. These results could be completed by analyzing samples from different locations of Georgia and collected at different periods to confirm these first results.

The proposed HPLC-DAD method is simple, linear, accurate, precise and specific. The validation procedure confirmed that the method afforded reliable analyses of majdine. Finally, the developed method has been applied successfully to quantify majdine in different parts of the plant.

Experimental

Plant material: Different parts (leaves, roots, stems) of *Vinca herbacea* were collected in Shiraki, East Georgia in

April and May of 2008. The plant was identified by B. Grigolava (Institute of Pharmacochemistry, Tbilisi, Georgia) and a voucher specimen n° 9 (120) was deposited in the herbarium of the Institute of Pharmacochemistry. The freshly picked parts were air dried at room temperature for 3 weeks and kept in the dark until chemical analysis. The samples were ground to a fine powder before analysis.

Chemicals and reagents: HPLC grade acetonitrile and other solvents of analytical grade were purchased from Carlo Erba (Val de Reuil, France). Potassium dihydrogen phosphate was purchased from Fluka (Saint Quentin Fallavier, France). Ultrapure water (18,2MΩ) for HPLC analysis was obtained from an Elga purelab Classic purification system (Elga, France). Papaverine hydrochloride was purchased from Sigma (ref P3510, purity ≥ 98%). Majdine was isolated from *Vinca herbacea* and the purity, determined by HPLC, was 98%.

Chromatographic conditions: The HPLC system consisted of an Agilent Technologies Model 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a photodiode array detector (DAD). The system was piloted by Chemstation computer software. The chromatographic separation was achieved using a Symmetry C18 column (250 mm x 4.6 mm, 5 µm, Waters). The isocratic mobile phase was composed of 25 mM phosphate buffer (pH=3.0, adjusted with orthophosphoric acid) and acetonitrile (8/2, v/v). The mobile phase flow rate was 1mL/min. The injection volume was 20 µL. UV spectra were recorded in the range 200-400 nm for all peaks. Quantification was carried out at a single wavelength of 225nm.

Standard preparation: A stock solution containing 0.425 mg/mL of internal standard in methanol was used. Standard stock solution of majdine was prepared in methanol to give a final concentration of 0.163 mg/mL. A series of working solutions (n=5) was prepared in order to obtain various concentration levels (0.006-0.019 mg/mL). The appropriate volume of stock solution of majdine was introduced to a 25 mL volumetric flask, 1 mL of internal standard solution was added, and the volume was adjusted to 25.0 mL with the mobile phase. The final concentration of papaverine in each standard solution was 0.017 mg/mL. All prepared standard solutions were filtered through 0.45 µm membrane filter (Millipore, ref HVPL04700) before HPLC analysis.

Sample preparation: Powdered plant material (100 g) was moistened with water alkalinized with ammonia (100/5, v/v). The material was macerated for 4 h and then percolated with methylene chloride at room temperature. Liquid/liquid extraction was performed with 2N HCL. The aqueous layer was alkalinized with ammonia (28%) and extracted with methylene chloride. The organic layer was collected and evaporated to dryness to yield the total alkaloids (0.4%). Ten mg of the total alkaloids was introduced to a 100 mL volumetric flask and solubilized in the mobile phase. Afterwards, 1.0 mL of the internal standard solution was added to obtain a final concentration of 0.017 mg/mL of papaverine, and the final volume was adjusted to 100.0 mL with the mobile phase. Then, 2 ml of each solution was filtered through a syringe filter (0.45 mm Millipore) into a HPLC vial.

Validation and assay: A validation procedure was performed to check the performance of the chromatographic technique. The method was validated on a leaf extract of *V. herbacea* according to the ICH guidelines [18]. The specificity was performed by comparing the UV spectrum and retention time of majdine in the sample preparation and the reference compound solution. The linearity of the HPLC method was evaluated by analyzing 5 different concentrations of majdine standard solutions. Each concentration was assayed in triplicate. The limits of detection and quantification were considered to be the concentrations that produced signal-to-noise ratios of 3:1 and 10:1, respectively.

The precision of the method was evaluated with respect to both intra- and inter-day precision. Intra-day precision was calculated from the analysis of 6 sample solutions, which were prepared independently on the same day. Inter-day precision was evaluated by replicating the procedure on 2 consecutive days, The standard deviation and RSD (relative standard deviation) values were calculated for each day.

The accuracy of the method was evaluated using the recovery test. This involved the spiking of known quantities of majdine standard solutions into the tested samples. The standard solutions were prepared at 3 concentration levels (50,100 and 150%). At each level, samples were analyzed in triplicate according to the established chromatographic conditions.

Acknowledgments - We thank Dr M. De Meo for stylistic and grammatical corrections.

References

[1] Vachnadze VY, Malikov VM, Mudzhiri KS, Yunusov SY. (**1971**) A study of the alkaloids of *Vinca herbacea*. *Khimiya Prirodnykh Soedinenii*, ***5***, 661-662.

[2] Ognyanov I, Pyuskyulev B, Kompiš I, Sticzay T, Spiteller G, Shamma M, Shine RJ. (**1968**) Alkaloids from *Vinca herbacea* W.K. The structures and stereochemistry of majdine and isomajdine. *Tetrahedron*, ***24***, 4641-4648.

[3] Chikvadze G. (**1985**) Dynamics of the accumulation of alkaloids in *Vinca herbacea* cultivated in eastern Georgia. *Chemistry of Natural Compounds*, ***21***, 681-682.

[4] Chikvadze G. (**1978**) Isoreserpinine from *Vinca herbacea*. *Chemistry of Natural Compounds*, ***14***, 420-422.
[5] Ognyanov J, Dalev P, Dutschevska H, Mollov M. (**1964**) Neue alkaloide aus *Vinca herbacea* W. et K C.R. *Bulgarian Academy of Sciences*, ***17***,153-156.
[6] Ognyanov J, Pyuskyulev B, Bonjanov B, Hesse M. (**1967**) Alkaloide aus *Vinca herbacea* W et K, mittelung isolierung and structur von Hervin. *Helvetica Chimica Acta*, ***50***, 754-759.
[7] Pyuskyulev B, Ognyanov J, Panov P. (**1967**) Alkaloide aus *Vinca herbacea*, XI, Norfluokurarin. *Tetrahedron Letters*, ***4***, 4559-4562.
[8] Rusinov K, Zhelyazkov D, Georgiev V. (**1962**) Pharmacological investigation of the alkaloids of *Vinca herbaceae*. *Bulgaria Akademii Nauk*, ***5***, 271-306.
[9] Roussinoff K, Jelyazkoff D, Guerguieff V. (**1961**) The blocking action of the alkaloids of *Vinca herbacea* on the neuromuscular synapses. *Archivio Italiano di Scienze farmacologiche*, ***11***, 83-91.
[10] Chkhikvadze G, Vachnadze V, Gambashidze N, Gvishiani E, Mudjiri L. (**1981**) The method of receipt of compound with antiarythmic activity, USSR patent SU 834964, Russian.
[11] Chkhikvadze G, Gambashidze N, Gvishiani E, Vachnadze V, Giorgadze N. (**1982**) Chemical and pharmacological investigation of alkaloids of herb Vinca Herbaceae W. and Kit. Presented at conference *Creation, investigation and complex utilization of medicinal drugs for therapy of cardio vascular diseases*, Tbilisi, Georgia.
[12] Novikova Z, Gotsiridze I, Abuladze G. (**1984**) Comparative study of pharmacological activity of individual and combined alkaloids of *Vinca herbaceae*. *Izvestiia Akademii Nauk SSSR Seriia Biologicheskaia*, ***10***, 54-55.
[13] Gagua N, Chkikvadze G, Vachnadze N, Vachnadze V, Bakuridze A. (**2010**) Vingerbin an indolin alkaloid preparation with antiarrhythmic activity produced by *Vinca herbacea* W. and K. *Allergology and Immunology Journal*, ***11***, 160-162.
[14] Hava M. (**1973**) The pharmacology of Vinca species and their alkaloids. In *Vinca alkaloids*. Taylor WI, Farnsworth N (Eds). Marcel Dekker, New York, USA, 305-338.
[15] Gülçin İ, Gagua N, Bursal E, Bakuridze A, Beydemir Ş, Gepdiremen A, Bayram R. (**2011**) Apoptotic, antioxidant, antiradical and antiglaucoma effects of majdine and isomajdine from *Vinca herbacea* Waldst. and Kit. *Journal of Enzyme Inhibition and Medicinal Chemistry,* in press.
[16] Yongijian W, Duowel L, Minjie P, Wei L. (**2009**) Determination of vincamine by RP-HPLC. *Xibex Yoxue Zazhi*, ***24***, 23-24.
[17] El-Saharty, Yasser Shaker I. (**2008**) Simultaneous determination of piracetam and vincamine by spectrophotometric and high-performance liquid chromatographic methods. *Journal of Association of Official Analytical Chemists International*, ***91***, 311-321.
[18] *Validation of analytical procedures: Text and methodology* - International Conference on Harmonisation. ICH-Q2 (R1) (**2005**) Geneva.

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2011
Vol. 6
No. 12
1835 - 1838

Pyridine Metabolism and Trigonelline Synthesis in Leaves of the Mangrove Legume trees *Derris indica* (*Millettia pinnata*) and *Caesalpinia crista*

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Received: September 29th, 2011; Accepted: October 5th, 2011

The aim of this study was to reveal the pyridine metabolism in leaves of two mangrove legumes, *Derris indica* (=*Millettia pinnata* or *Pongamia pinnata*) and *Caesalpinia crista*. Radioactivity from [*carbonyl*-^{14}C]nicotinamide supplied exogenously to young leaf disks was recovered in nicotinic acid, nicotinic acid mononucleotide, NAD, NADP, nicotinamide mononucleotide and trigonelline. These mangrove species, especially *D. indica,* have strong ability to convert nicotinamide to trigonelline, but not to nicotinic acid glucoside. The endogenous trigonelline content in leaves of *D. indica* was more than 830 μg/g dry weight. This value is 5-12 times greater than that in leaves of *Glycine max*. There was little short-term effect of 250 and 500 mM NaCl (equivalent to *ca.* 50% and 100% sea water) on nicotinamide metabolism.

Keywords: Nicotinamide adenine dinucleotide, nicotinamide, nicotinic acid, salt stress, trigonelline.

Derris indica and *Caesalpinia crista* are mangrove plants belonging to the Leguminosae family. They are widely distributed on seashores in Southeast Asia and Pacific Islands. Different organs of these plants possess anti-mycobacterial activity [1] and have been used in folk medicine. Seeds of *D. indica* contain oils and fatty acids suitable for biodiesel production [2]. These plants grow by seashores with their roots in the sea. They therefore apparently have salt-tolerant properties. These mangrove legumes are interesting from the viewpoint of biotechnology as well as basic plant biology. There have, however, been few physiological studies of these plants.

Nicotinamide is formed as a catabolite of NAD and NADP, and is a key metabolite of pyridine metabolism. In plants, nicotinamide is not directly used for re-synthesis of NAD, as it is in animals [3], but it is readily deaminated to nicotinic acid and then used for pyridine nucleotide synthesis [4-6]. As well as the salvage pathway for NAD synthesis, nicotinic acid is used for the synthesis of secondary metabolites. In a few plants, nicotinic acid is a substrate for the synthesis of unique pyridine alkaloids, such as nicotine and ricinine [7,8], but most plants produce simple and common secondary products: trigonelline (1-*N*-methylnicotinic acid) or nicotinic acid *N*-glucoside (Figure 1) [9,10].

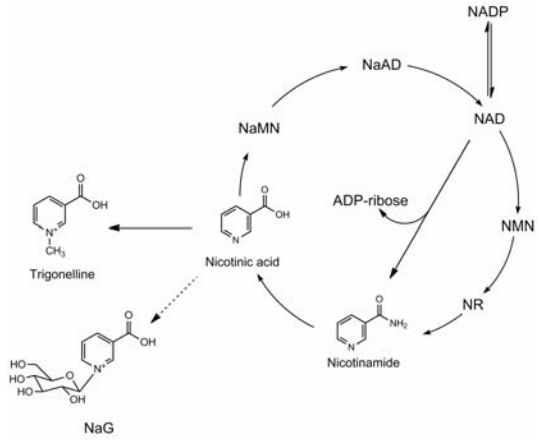

Figure 1: Possible metabolic pathways of nicotinamide in *Caesalpinia crista* and *Derris indica*.

The biosynthesis of these two compounds is an example of biochemical evolution in plant chemosystematics [11]. Diverse physiological functions of trigonelline have been proposed by several researchers [9-10,12], and one role of trigonelline is as a compatible solute to adjust the osmotic pressure when the plants are affected by salt stress [13-16].

As part of our studies of pyridine metabolism in plants, we examined the metabolic fate of [*carbonyl*-^{14}C]nicotinamide in leaves of two mangrove legumes in the presence and

absence of NaCl. The short-term effect of NaCl on endogenous trigonelline content was also examined in leaf segments of *D. indica*.

We examined the changes in ^{14}C-labelled metabolites, along with the incubation time. Figure 2 shows the results from leaf disks of *C. crista*. [^{14}C]Nicotinamide, taken up by the disks, was readily converted into nicotinic acid. Within 30 min of administration, radioactivity was found in NaMN, NAD and NADP. This implies that nicotinic acid derived from nicotinamide was salvaged to NaMN by nicotinate phosphoribosyltransferase (EC 2.4.2.11), and was then utilized for pyridine nucleotide synthesis.

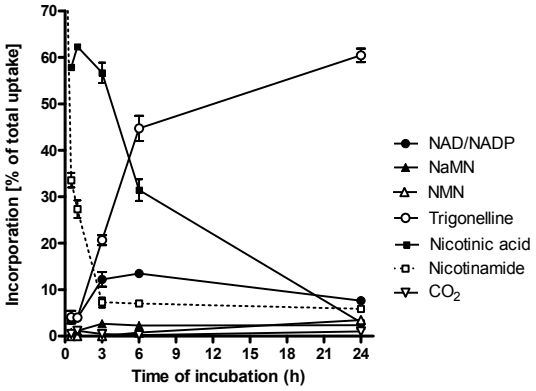

Figure 2: Time-course studies of [carbonyl-^{14}C]nicotinamide in leaf disks of *Caesalpinia crista*. Rates of incorporation ± SD are expressed as a percentage of total radioactivity. The total radioactivity (kBq per 100 mg fresh weight) taken up at 0.5, 1, 3, 6 and 24 h after administration was respectively 1.8 ± 0.1, 2.6 ± 0.1, 6.1 ± 0.1, 11.0 ± 0.7 and 20.6 ± 0.3.

Radioactivity was also found in trigonelline; the proportion gradually increased with incubation time to 60% of the total radioactivity at 24 h after initiation. In these disks, therefore, nicotinic acid appears to be converted to trigonelline by nicotinic acid *N*-methyltransferase (trigonelline synthase, EC 2.1.1.7); this pyridine alkaloid was accumulated. Release of $^{14}CO_2$ from [*carbonyl*-^{14}C]nicotinamide was low; less than 1.2% of total activity was found during the time of incubation. Small amounts of radioactivity were found in NMN at 6 h after administration of [^{14}C]nicotinamide. Although it is difficult to infer the metabolic sequence from these results alone, our data support the functioning of the pyridine nucleotide cycle shown in Figure 1, which has been reported in other plants [5,16-17].

We examined the metabolic fate of [*carbonyl*-^{14}C]nicotinamide in leaf disks from *C. crista* and *D. indica* at 3 h after administration in the presence and absence of NaCl (Figure 3A,B). In leaf disks of both species the radioactivity was recovered in nicotinamide, nicotinic acid, NAD/NADP, NaMN and trigonelline. Only a small amount of radioactivity was found in CO_2. No radioactivity was detected in nicotinic acid glucoside.

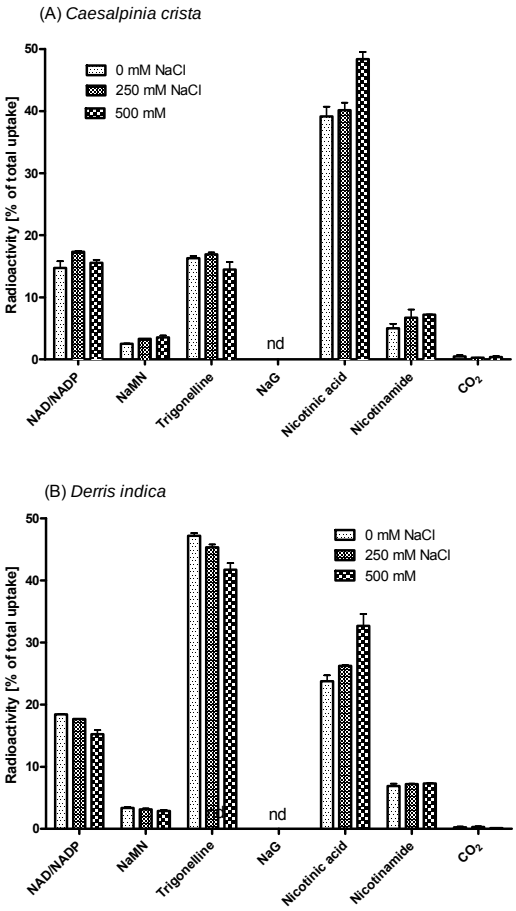

Figure 3: Metabolic fate of [*carbonyl*-^{14}C]nicotinamide in leaf disks of *Caesalpinia crista* (A) and *Derris indica* (B) in the presence of 0, 250 and 500 mM NaCl. Incubation took place for 3 h. Values ± SD (n = 3) are expressed as % of total uptake by leaf disks. Total radioactivity (kBq per 100 mg fresh weight) taken up by the leaf disks treated with 0, 250 and 500 mM NaCl was respectively 6.1 ± 0.5, 6.1 ± 0.4 and 7.8 ± 0.2 (A) and 9.0 ± 0.3, 7.5 ± 0.1 and 7.2 ± 0.4 (B).

The activity of pyridine salvage, estimated from the sum of the incorporation rate of radioactivity into NAD, NADP and NaMN, varied from 18% to 22% of the total uptake in leaf disks of both species, and no large difference was caused by NaCl. In contrast, widely differing trigonelline synthesis activity was found in the different species; the incorporation rate in *D. indica* (42-47% of total uptake) was much greater than in *C. crista* (15-17%). No increase in trigonelline synthesis due to NaCl took place, and there was even a slight reduction in activity (88% of control) in *D. indica*.

The endogenous concentration of trigonelline in leaf disks of *D. indica* at 3 h after incubation with 0, 250 and 500 mM NaCl was respectively 831.5 ± 53.3, 857.0 ± 22.0 and 893.9 ± 13.7 µg per g dry weight. Cho *et al.* [13] reported that the trigonelline concentration in leaf tissue of cultivated *Glycine max* ranged from 64 to 162 µg per g dry weight. Trigonelline concentration in *D. indica* leaves is consequently 5-12 times higher than in *G. max* leaves. High concentrations of trigonelline may act as compatible

solutes in this mangrove legume. Although we found that the concentration of trigonelline increased slightly as a result of 3 h salt stress, these differences were not statistically significant ($P > 0.05$).

Our observations suggest that trigonelline synthesis in mangrove leaves does not respond quickly to salt stress. However, it is clear that *D. indica* has a mechanism for producing trigonelline, so that its content in leaves is much greater than in *G. max*, even if the plant is grown in a salt-free environment. It is, therefore, plausible that the strong trigonelline formation in *D. indica* is an inherent property of this mangrove legume.

Experimental

Plant materials and chemicals: Seeds of *Derris indica* were collected from the basin of the Khanom River, and seeds of *Caesalpinia crista* from the Pang Yi River basin, both in Thailand. Young leaves of 6-month-old seedlings grown in a greenhouse were used as plant materials. [Carbonyl-^{14}C]nicotinamide (2.04 GBq/mmol) was purchased from Moravek Biochemicals Inc., Brea, CA, USA, and other chemicals were from either Sigma-Aldrich, St. Louis, MO, USA, or from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Radioactivity incorporation: Administration of [*carbonyl*-^{14}C]nicotinamide to the leaf disks and analysis of labeled metabolites were essentially the same as in a previous paper [18]. Leaf disks (*ca.* 100 mg fresh weight), together with 2.0 mL of 30 mM potassium phosphate buffer (pH 5.6) containing 10 mM sucrose, 50 mM sodium ascorbate, and 0, 250 or 500 mM NaCl, were placed in the main compartment of a 30 mL Erlenmeyer flask. The flask was fitted with a glass tube containing a piece of folded filter paper that had been impregnated with 0.1 mL of 20% KOH in the centre well. Each reaction was started by adding 10 µL (37 kBq) of a solution of [carbonyl-^{14}C]nicotinamide to the main compartment of the flask. The flasks were incubated in an oscillating water bath at 27ºC. After incubation, the glass tube was removed from the centre well and placed in a 50 mL Erlenmeyer flask containing 10 mL distilled water. At the same time, the leaf disks were harvested and washed with distilled water, frozen with liq. N_2, and then stored at -80ºC prior to extraction. The filter paper, containing CO_2 trapped as potassium bicarbonate, was soaked overnight in distilled water (10 mL), and aliquots of the resulting solution (usually 0.5 mL) were used for determination of radioactivity. Radioactivity was measured with a liquid scintillation counter (Beckman, type LS 6500, Fullerton, CA, USA).

Analysis of metabolites: For analysis of ^{14}C-labelled metabolites, the frozen samples were homogenized using a mortar and pestle with ice-cold 6% perchloric acid (PCA) containing 0.1 mM NAD. NAD was supplemented to prevent the degradation of labeled nucleotides during extraction and analysis, and also for action as endogenous markers in the TLC analysis. The homogenate was centrifuged at 3,000 x *g* for 7 min, the supernatant was collected, and the precipitate was re-suspended in the same extraction reagent and washed 3 times. The ^{14}C-labelled metabolites extracted with 6% PCA were combined, neutralized with 20% KOH, and centrifuged briefly (3,000 x *g*, 7 min) to remove potassium perchlorate as precipitate. The radioactivity of aliquots of supernatant was measured with a liquid scintillation counter. The neutralized fractions were freeze-dried and then redissolved in a small amount of 50%, v/v, ethanol.

The concentrated PCA-soluble metabolites (free nicotinic acid related small molecular weight compounds) were separated by using microcrystalline cellulose TLC sheets (200 × 200 mm, Merck, Darmstadt, Germany). As solvent systems, system I (*n*-butanol: acetic acid: water, 4:1:2, v/v) and system IV (isobutyric acid: ammonia: water, 660: 17: 330, v/v) as specified by Zheng and Ashihara [19] were used. The distribution of ^{14}C to individual spots on the TLC sheet was estimated using a bio-imaging analyzer (Type, FLA-2000, Fuji Photo Film Co., Ltd. Tokyo, Japan). The total radioactivity of each fraction was determined by a liquid scintillation counter (Beckman, type LS 6500). Incorporation into individual metabolites was calculated using the total radioactivity values in the PCA-soluble fraction, as measured by a liquid scintillation counter, and the % distribution of radioactivity on TLC plates was obtained from the bio-imaging analyzer.

Effects of NaCl: To determine the short-term effect of NaCl on the accumulation of trigonelline, leaf disks (ca. 300 mg fresh weight) were incubated with 2 mL of the medium which had been used for the ^{14}C-tracer experiments, supplemented with 0, 250 or 500 mM NaCl, in a 30 mL Erlenmeyer flask at 27ºC for 3 h. After incubation, the leaf disks were washed with distilled water, boiled in 10 mL of hot water (> 95ºC) for 10 min, and then homogenized using a mortar and pestle. After brief centrifugation (20,000 x *g*, for 10 min), the water-soluble supernatant fraction was collected and freeze dried. The samples were dissolved in small amounts of distilled water and analyzed by HPLC, as in our previous paper [20], except that the absorbance was monitored using a Shimadzu Diode Array Detector, type SPD-M10A (Shimadzu Corp., Kyoto, Japan).

Acknowledgements - We thank Dr Reiko Minagawa, Tokyo University of Agriculture, for her generous supply of plant materials. This research was supported by a JSPS Grant-in-Aid for Scientific Research (No. 22510226) to H.A.

References

[1] Satnami DK, Yadava RN. (**2011**) Potential phytochemical from *Caesalpinia crista* Linn. *Research Journal of Phytochemistry*, ***5***, 22-31

[2] Scott P, Pregelj L, Chen N, Hadler J, Djordjevic M, Gresshoff P. (**2008**) *Pongamia pinnata*: An untapped resource for the biofuels industry of the future. *BioEnergy Research*, ***1***, 2-11.

[3] Revollo JR, Grimm AA, Imai S. (**2004**) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates *Sir2* activity in mammalian cells. *Journal of Biological Chemistry*, ***279***, 50754-50763.

[4] Wang G, Pichersky E. (**2007**) Nicotinamidase participates in the salvage pathway of NAD biosynthesis in Arabidopsis. *Plant Journal*, ***49***, 1020-1029.

[5] Zheng XQ, Hayashibe E, Ashihara H. (**2005**) Changes in trigonelline (*N*-methylnicotinic acid) content and nicotinic acid metabolism during germination of mungbean (*Phaseolus aureus*) seeds. *Journal of Experimental Botany*, ***56***, 1615-1623.

[6] Zrenner R, Ashihara H. (**2011**) Nucleotide metabolism. In *Plant Metabolism and Biotechnology,* Ashihara H, Crozier A, Komanine A. (Eds), John Wiley & Sons, Ltd, Chichester, England, 135-162.

[7] Katoh A, Hashimoto T. (**2004**) Molecular biology of pyridine nucleotide and nicotine biosynthesis. *Frontier in Bioscience*, ***9***, 1577-1586.

[8] Waller GR, Yang KS, Gholson RK, Hadwiger LA, Chaykin S. (**1966**) The pyridine nucleotide cycle and its role in the biosynthesis of ricinine by *Ricinus communis* L. *Journal of Biological Chemistry*, ***241***, 4411-4418.

[9] Ashihara H. (**2008**) Trigonelline (*N*-methylnicotinic acid) biosynthesis and its biological role in plants. *Natural Product Communnications*, ***3***,1423-1428.

[10] Barz W. (**1985**) Metabolism and degradation of nicotinic acid in plant cell cultures, In *Primary and Secondary Metabolism of Plant Cell Cultures,* Neumann KH, Barz W, Reinhard E. (Eds) Springer-Verlag, Berlin, 186-195.

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

[12] Minorsky PV. (**2002**) Trigonelline: A diverse regulator in plants. *Plant Physiology*, ***128***, 7-8.

[13] Cho Y, Lightfoot DA, Wood AJ. (**1999**) Trigonelline concentrations in salt stressed leaves of cultivated *Glycine max*. *Phytochemistry*, ***52***, 1235-1238.

[14] Rajasekaran LR, Aspinall D, Jones GP, Paleg LG. (**2001**) Stress metabolism. IX. Effect of salt stress on trigonelline accumulation in tomato. *Canadian Journal of Plant Science*, ***81***, 487-498.

[15] Tramontano WA, Jouve D. (**1997**) Trigonelline accumulation in salt-stressed legumes and the role of other osmoregulators as cell cycle control agents. *Phytochemistry*, ***44***, 1037-1040.

[16] Wood AJ. (**1999**) Comparison of salt-induced osmotic adjustment and trigonelline accumulation in two soybean cultivars. *Biologia Plantarum*, ***42***, 389-394.

[17] Ashihara H, Stasolla C, Yin Y, Loukanina N, Thorpe TA. (**2005**) *De novo* and salvage biosynthetic pathways of pyridine nucleotides and nicotinic acid conjugates in cultured plant cells. *Plant Science*, ***169***, 107-114.

[18] Katahira R, Ashihara H. (**2009**) Profiles of the biosynthesis and metabolism of pyridine nucleotides in potatoes (*Solanum tuberosum* L.). *Planta*, ***231***, 35-42.

[19] Zheng XQ, Ashihara H. (**2004**) Distribution, biosynthesis and function of purine and pyridine alkaloids in *Coffea arabica* seedlings. *Plant Science*, ***166***, 807-813.

[20] Ashihara H, Yin Y, Deng WW, Watanabe S. (**2010**) Pyridine salvage and nicotinic acid conjugate synthesis in leaves of mangrove species. *Phytochemistry*, ***71***, 47-53.

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2011
Vol. 6
No. 12
1839 - 1841

Anti-adipogenic Activity of *Cordyceps militaris* in 3T3-L1 Cells

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Received: July 14th, 2011; Accepted: October 7th, 2011

Inhibition of adipocytes differentiation is suggested to be an important strategy for prevention and/or treatment of obesity. In our present study, *Cordyceps militaris* showed significant inhibitory activity on adipocyte differentiation in 3T3-L1 preadipocytes as assessed by measuring fat accumulation using Oil Red O staining. Activity-guided fractionation led to the isolation of cordycepin (**1**), guanosine (**2**) and tryptophan (**3**) as active compounds. All the three compounds were more effective in the prevention of early stage of adipogenesis than in lipolysis. In addition, combinational treatment of three compounds significantly increased anti-adipogenic activity.

Keywords: *Cordyceps militaris*, cordycepin, 3T3-L1 preadipocytes, Oil Red O staining, obesity.

Obesity has become a widespread issue in modern society, due to a global rise in obesity-associated problem. It is no longer considered only a cosmetic problem but associated with several pathological disorders, including diabetes, hypertension, atherosclerosis and cancer [1]. Adipose tissue is an important metabolic organ that is crucial for insulin sensitivity and energy homeostasis. Adipocyte differentiation is an important process for its function in normal condition [2]. In obesity, adipocytes undergo abnormal growth and differentiation, which result in increased numbers of fat cells storing their lipids and consequent pathological disorders [3]. Therefore, inhibition of adipocytes differentiation is suggested to be an important strategy for prevention and/or treatment of obesity [4].

Cordyceps, also known as 'winter worm summer grass', is a well-known traditional medicine in Asian countries. It has been known as a rich source of biologically active components and used for treatment of several diseases such as cancer, fatigue, hyperglycemia and hyperlipidemia [5,6]. Recently, favorable role of *Cordyceps* in the regulation of obesity has been reported [7]. However, little is known about its active components related to adipocyte differentiation.

In this study, we evaluated the anti-adipogenic activity of *Cordyceps militaris* (Hypocreaceae) employing 3T3-L1, a mouse preadipocyte cell line as an *in vitro* assay system. The *n*-BuOH fraction of *C. militaris* showed potent inhibitory activity on adipocyte differentiation at a concentration of 10 μg/mL (6.5% of differentiated control, $p<0.001$). Activity-guided fractionation of *n*-BuOH fraction resulted in the isolation of three constituents which were identified as cordycepin (**1**), guanosine (**2**) and tryptophan (**3**) by direct comparison of their physicochemical and spectroscopic data with those previously reported (Figure 1) [8].

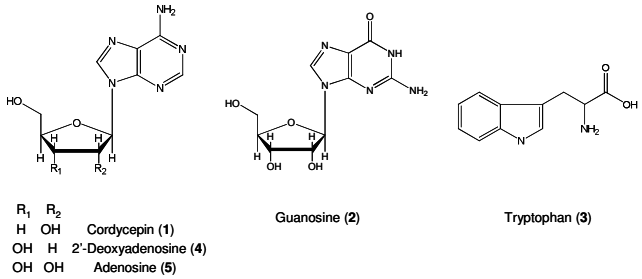

Figure 1: Structure of compounds isolated from *C. militaris* (**1**–**3**) and cordycepin derivatives (**4**, **5**).

The inhibitory activity of isolated compounds (**1**–**3**) on adipocyte differentiation was also evaluated in our assay system. Among the compounds isolated, guanosine (**2**) and tryptophan (**3**) showed the strong inhibitory activity, followed by cordycepin (**1**) (Figure 2B). At a concentration of 10 μg/mL, guanosine (**2**) and tryptophan (**3**) completely inhibited adipocyte differentiation as measured by fat accumulation, which was supported by microscopic observation (Figure 2A).

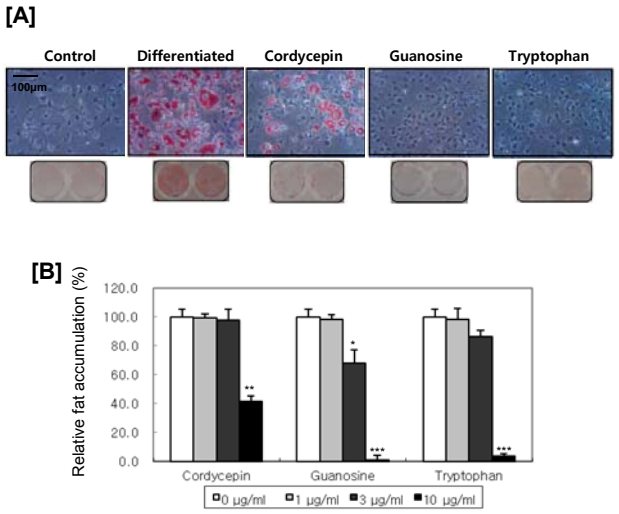

Figure 2: Effect of cordycepin, guanosine and tryptophan on fat accumulation in 3T3-L1 cells. Cultures were stained with Oil Red O, photographed (A) and quantitated (B). Results are expressed as mean ± SD of three independent experiments, each performed using triplicate wells. $^{*}p<0.05$; $^{**}p<0.01$;$^{***}p<0.001$ compared with differentiated control.

Adipogenic differentiation is a well-organized process, occurs in several stages [9]. Therefore, we further examined which stage of adipogenic differentiation was effectively inhibited by these compounds. During differentiation, 3T3-L1 cells were incubated with cordycepin, guanosine and tryptophan at different periods of differentiation, namely, during days 0–2, 2–4, 4–8, 0–4, 2–8 and 0–8.As shown in Figure 3A, cordycepin was most effect when treated during days 2–4, whereas guanosine and tryptophan significantly inhibited adipocyte differentiation when treated during days 0–2.However, all the compounds showed weak activity when treated during days 4–8. In addition, these compounds did not show significant effects when treated to differentiated adipocytes (data not shown). These results suggest that cordycepin, guanosine and tryptophan could effectively inhibit the early stage of adipocyte differentiation and might be effective in the prevention of adipogenesis but not in lipolysis.

Cordycepin is a characteristic constituent of *C. militaris* and diverse biological activity such as anticancer and neuroprotective activity [10,11]. Structurally, cordycepin has same structure with adenosine except for the absence of hydroxyl group at C-3' (Figure 1). Adipocytes express adenosine A1 receptor and activation of adenosine A1 receptor by agonists inhibits lipolysis and enhances the lipogenesis, whereas antagonists exerts opposite action [12,13]. Therefore, we compared the effect of cordycepin and other adenosine derivatives such as adenosine and 2'-deoxyadenosine. Consistent with previous report adenosine slightly increased fat accumulation at the concentration of 10 μg/mL. In addition, 2'-deoxyadenosine also failed to reduced fat accumulation. Therefore, we suppose that the absence of hydroxyl group at C-3' may play an important role in inhibitory activity of cordycepin on adipocyte differentiation, and cordycepin might act as a

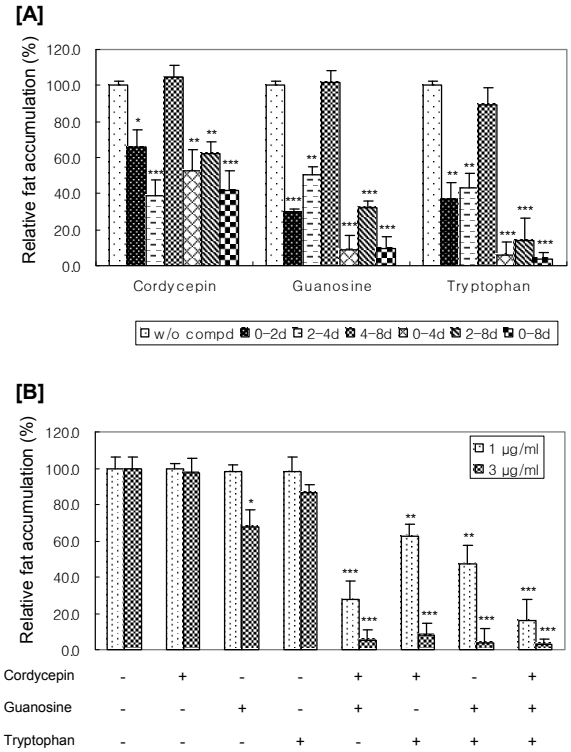

Figure 3: Effect of cordycepin, guanosine and tryptophan on adipocyte differentiation during differentiation process (A) and combinational treatment (B). Compounds were added at different periods of differentiation, namely, during days 0-2, 2-4, 4-8, 0-4, 2-8 and 0-8 (A). In other cultures, Cordycepin, guanosine and tryptophan were treated with combination as indicated (B). On day 8, cells were subjected to Oil Red O staining. Values are expressed as means ± SD of triplicate experiments. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ compared with differentiated control.

partial antagonist of adenosine A1 receptor, which needs to be clarified by further investigation.

Natural products are known to exert strong biological activity by additive effects of its constituents. Therefore, combinatorial effect of cordycepin, guanosine and tryptophan was investigated. Interestingly, combination of each compounds strongly increased anti-adipogenic activity (Figure 3B). Although each compound showed little effect at the concentration of 1 μg/mL, combination of two or three compounds showed potent inhibition on fat accumulation. These results suggest that combinational action of each compound contributes to the anti-adipogenic activity of *C. militaris*.

Experimental

Extraction and isolation: Dried *Cordyceps* samples were provided from Rural Development Administration in November 2009.A voucher specimen (CBNU-2009-CM) has been deposited in the Herbarium of College of Pharmacy, Chungbuk National University. The powdered *C. Militaris* were extracted 3 times with EtOH, which yielded the total ethanolic extract. The ethanolic extract was then suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$and *n*-BuOH. The *n*-BuOH fraction, which showed most potent activity, was subjected to XAD

column chromatography with the mixture of MeOH–H_2O (0%, 20%, 40%, 60%, 80%, 100% MeOH in H_2O) to give 6 fractions (B1-B6).B2was subjected to column chromatography over Sephadex LH-20 with MeOH to give 9 subfractions (B21–B29). Compound **1** was obtained from B24 by recrystallization using MeOH. Compounds **2** and **3** were isolated from B24 by semipreparative HPLC eluting with MeOH-water (23:77, F=2 min/mL, Rt=7.67 and 8.65 min, respectively).

Cell culture and differentiation induction: 3T3-L1 mouse embryo fibroblasts were obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) until confluence. Two days after confluence (day 0), cells were stimulated to differentiate with differentiation medium containing DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μg/mL insulin and 1 μM dexamethasone for 2 days (day 2). Cells were then maintained in DMEM supplemented with 10% FBS and 1 μg/mL for another 2 days (day 4), followed by culturing with DMEM with 10% FBS for an additional 4 days (day 8). All media contained 100 IU/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 95% air-5% CO_2 [14].

Oil Red O staining: Lipid droplets in cells were stained with Oil Red O. Eight days after differentiation induction, cells were washed three times with PBS and fixed with 10% formalin at room temperature for 1 hr. After fixation, cells were washed twice with PBS and stained with freshly diluted Oil Red O solution (3 parts of 0.6% Oil Red O in isopropyl alcohol and 2 parts of water) for 15 min. Cells were then washed twice with water and visualized. For quantitative analysis, Oil Red O staining was dissolved with isopropyl alcohol and optical density was measured at 550 nm by ELISA plate reader [14].

Statistical analysis: The evaluation of statistical significance was determined by one-way ANOVA test with a value of $p<0.05$ or less considered to be statistically significant.

Acknowledgments –This work was supported by the grant from the Korean Rural Development Administration (Agenda Program, PJ0067792010) and the grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/Chungbuk BIT research-Oriented University Consortium).

References

[1] Kopelman PG. (**2000**) Obesity as a medical problem. *Nature*, ***404***, 635-643.
[2] Rosen ED, Spiegelman BM. (**2006**) Adipocytes as regulators of energy balance and glucose homeostasis. *Nature*, ***444***, 847-853.
[3] Spielgelman BM, Enerback S. (**2006**) The adipocyte: a multifunctional cell. *Cell Metabolism*, ***4***, 425-427.
[4] Rayalam S, Cella-Fera MA, Baile CA. (**2008**) Phytochemicals and regulation of the adipocyte life cycle. *Journal of Nutrition and Biochemistry*, ***19***, 717-726.
[5] Koh JH, Kim KM, Kim JM, Song JC, Suh HJ. (**2003**) Antifatigue and antistress effect of the hot-water fraction from mycelia of *Cordyceps sinensis*. *Biological & Pharmaceutical Bulletin*, ***26***, 691-694.
[6] Lee H, Kim YJ, Kim HW, Lee DH, Sung MK, Park T. (**2006**) Induction of apoptosis by *Cordyceps militaris* through activation of caspase-3 in leukemia HL-60 cells. *Biological & Pharmaceutical Bulletin*, ***29***, 670-674.
[7] Shimada T, Hiramatsu N, Kasai A, Mukai M, Okamura M, Yao J, Huang T, Tamai M, Takahashi S, Nakamura T, Kitamura M. (**2008**) Suppression of adipocyte differentiation by *Cordyceps militaris* through activation of the aryl hydrocarbon receptor. *American Journal of Physiology*, ***295***, E859-867.
[8] Sawai H, Imai J, Lesiak K, Johnson MI, Torrence PF. (**1983**) Cordycepin analogues of 2-5A and its derivatives. *Journal of Biological Chemistry*, ***258***, 1671-1677.
[9] Lefterova MI, Lazar MA. (**2009**) New developments in adipogenesis. *Trends in Endocrinology and Metabolism*, ***20***, 107-114.
[10] Cheng Z, He W, Zhou X, Lv Q, Xu X, Yang S, Zhao C, Guo L. (**2011**) Cordycepin protects against cerebral ischemia/reperfusion injury *in vivo* and *in vitro*. *European Journal of Pharmacology*, ***664***, 20-28.
[11] Lee EJ, Kim WJ, Moon SK. (**2010**) Cordycepin suppresses TNF-alpha-induced invasion, migration and matrix metalloproteinase-9 expression in human bladder cancer cells. *Phytotherapy Research*, ***24***, 1755-1761.
[12] Liang HX, Belardinelli L, Ozeck MJ, Shryock JC. (**2002**) Tonic activity of the rat adipocyte A1-adenosine receptor. *British Journal of Pharmacology*, ***135***, 1457-1466
[13] Szkudelski T, Szkudelska K, Nogowski L. (**2009**) Effect of adenosine A1 receptor antagonism on lipogenesis and lipolysis in isolated rat adipocytes. *Physiological Research*, ***58***, 863-871.
[14] Shin EJ, Choi KM, Yoo HS, Lee CK, Hwang BY, Lee MK. (**2010**) Inhibitory effects of coumarins from the stem barks of *Fraxinus rhynchophylla* on adipocyte differentiation in 3T3-L1 cells. *Biological & Pharmaceutical Bulletin*, ***33***, 1610-1614.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1843 - 1846

Two New Cyclopeptides and One New Nonenolide from *Xylaria* sp. 101

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Received: August 1st, 2010; Accepted: October 26th, 2011

Two novel cyclopeptides, xylarotides A (**1**), and B (**2**), and one novel nonenolide, xylarolide (**3**), along with two known compounds, coriloxin (**4**), and 2-hydroxy-3-methoxy-5-methyl-*p*-benzoquinone (**5**) were isolated from the fungal strain *Xylaria* sp. 101. This strain was isolated from the fruiting body of *Xylaria* sp. collected in Gaoligong Mountain, Yunnan Province. The chemical structures were elucidated by spectroscopic analyses, including 1D- and 2D-NMR spectroscopic experiments, and on the basis of HR-Q-TOF mass spectrometry. Antibacterial assays of **1** - **3** were carried out; no effects on the growth of the tested bacteria and yeast were observed.

Keywords: *Xylaria* sp., cyclopeptides, nonenolide.

Fungi of the genus *Xylaria* are a rich source of bioactive metabolites, including terpenoids [1-5], cyclopeptides [6,7], polyketides [8,9], cytochalasins [10], xanthones [11,12], and unique unclassified xyloketals [13]. We isolated a fungal strain named 101 from Gaoligong Mountain of southwestern China, and identified it as *Xylaria* sp. (family Xylariaceace) based on its complete ITS1-5.8S-ITS2 sequences. Previously, four novel terpenoids were isolated from the still suspension cultures of *Xylaria* sp. 101 in potato-dextrose (PD) medium [5]. Here we report the isolation and structure elucidation of two novel cyclopeptides, namely xylarotide A (**1**) and xylarotide B (**2**), and one novel nonenolide, xylarolide (**3**), plus the previously known compounds, coriloxin (**4**) [14], and 2-hydroxy-3-methoxy-5-methyl-*p*-benzoquinone (**5**). Antibacterial assays of **1** – **3** were also performed.

The morphological properties of the isolate 101 were examined after incubation for 2 months at 28°C in potato-dextrose agar (PDA) medium. The strain 101 was identified as *Xylaria* sp. according to its ITS rDNA sequence (ITS1-5.8S-ITS2). The culture of 101 was concentrated and extracted successively with AcOEt. This extract was purified by repeated column chromatography (RP-18, Sephadex LH-20, and silica gel) to obtain compounds **1** – **5**.

Compound **1** was obtained as a white amorphous solid. The molecular formula $C_{29}H_{51}N_5O_5$ was determined by HR-Q-TOF MS and NMR spectroscopic data (Table 1). The IR spectrum indicated the presence of NH groups (3287cm^{-1}) and C=O groups (1640 cm^{-1}), respectively. The ^{13}C NMR spectrum of **1** (Table 1) displayed five C=O signals. By extensive analysis of the HSQC, HMBC, ^{1}H-^{1}H COSY and NOE spectra, five amino acid residues were established, and shown to be one proline, one leucine, one *N*-methyl-leucine, one isoleucine, and one valine. The seven degrees of unsaturation were deduced from the molecular formula, and attributed to five C=O groups, a cyclic moiety in proline, and the last one suggesting that **1** was a cyclopeptide. The sequence of these amino acid residues was elucidated on the basis of HMBC and NOE correlations (Figures 1 and 2). The HMBC correlations from α–H of proline to C=O of leucine, from α–H of leucine to C=O of isoleucine, from α–H of isoleucine to C=O of valine, from α–H of valine to C=O of *N*-methyl-leucine, and from α–H and *N*-methyl of *N*-methyl-leucine to C=O of proline, together with the NOE correlations between NH of leucine and α–H of isoleucine, and NH of isoleucine and α–H of valine, indicated the sequential connections of these amino acid residues. Thus, compound **1** was established to be cyclo-(Pro-Me-Leu-Val-Ile-Leu), and named as xylaropeptide A.

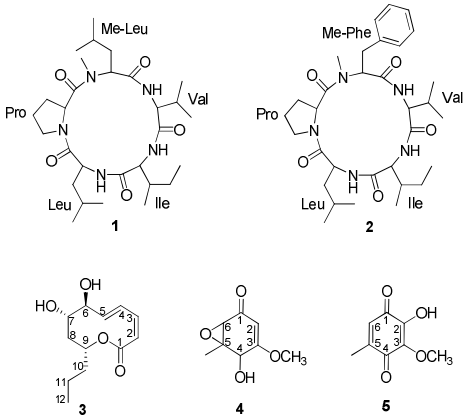
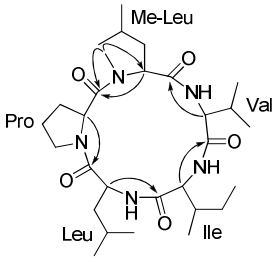


Table 1: ^{1}H- and ^{13}C-NMR spectroscopic data of **1** and **2** (600/150 MHz, resp., in $CDCl_3$; δ in ppm, *J* in Hz).

		1				**2**	
		δ_H (multiplicity, *J* in Hz)	δ_C			δ_H (multiplicity, *J* in Hz)	δ_C
Pro[1]	CO		172.3 (s)	Pro[1]	CO		172.5 (s)
	CH (α)	5.15 (d, *J* = 6.5)	59.5 (d)		CH (α)	5.05 (d, *J* = 6.5)	58.9 (d)
	CH_2 (β)	2.11-2.18 (m), 1.88-1.91 (m)	31.1 (t)		CH_2 (β)	1.94-2.01 (*m*), 1.30-1.33 (*m*)	30.5 (t)
	CH_2 (γ)	1.86-1.90 (m), 1.65-1.68 (m)	21.0 (t)		CH_2 (γ)	1.78-1.82 (*m*), 1.61-1.64 (*m*)	20.9 (t)
	CH_2 (δ)	3.88 (t, *J* = 8.5)	45.7 (t)		CH_2 (δ)	3.77 (br d, *J* = 10.5)	45.9 (t)
		3.43 (br d, *J* = 9.0)				3.45 (br d, *J* = 7.5)	
Me-Leu[2]	CO		171.7 (s)	Me-Phe[2]	CO		170.4 (s)
	N- CH_3	3.18 (s)	30.4 (q)		N- CH_3	3.15 (s)	30.6 (q)
	CH (α)	4.64 (t, *J* = 6.5)	55.3 (d)		CH (α)		57.8 (d)
	CH_2 (β)	1.62-1.64 (m)	37.4 (t)		CH_2 (β)	3.18-3.20 (*m*), 3.04-3.11 (*m*)	34.5 (t)
	CH (γ)	1.55-1.57 (m)	24.8 (d)		C (γ)		136.8 (s)
	CH_3 (δ)	0.91 (overlap)	22.1 (q)		CH (δ)×2	7.21 (*d*, *J* = 6.0)	128.8 (d)
	CH_3 (δ)	0.98 (d, *J* = 5.5)	22.8 (q)		CH (δ)×2	7.25 (*d*, *J* = 6.6)	128.5 (d)
					CH (ε)	7.20 (*d*, *J* = 6.0)	126.8 (d)
Val[3]	CO		171.4 (s)	Val[3]	CO		171.7 (s)
	NH	7.67 (br s)			NH		
	CH (α)	4.11 (t, *J* = 8.5)	58.1 (d)		CH (α)	4.06 (*t*, *J* = 8.0)	58.3 (d)
	CH (β)	2.37-2.41 (m)	26.8 (d)		CH (β)	2.17-2.20 (*m*)	27.1 (d)
	CH_3 γ)	0.94 (d, *J* = 5.0)	18.4 (q)		CH_3 (γ)	0.75 (*d*, *J* = 4.5)	18.2 (q)
	CH_3 (γ)	0.95 (d, *J* = 5.0)	19.7 (q)		CH_3 (γ)	0.88 (overlap)	19.7 (q)
Ile[4]	CO		171.2 (s)	Ile[4]	CO		171.0 (s)
	NH	6.78 (d, *J* = 7.8)			NH		
	CH (α)	4.55 (t, *J* = 7.0)	56.8 (d)		CH (α)	4.33 (*br s*)	57.0 (d)
	CH (β)	1.62-1.65 (m)	38.0 (d)		CH (β)	1.67-1.70 (*m*)	37.5 (d)
	CH_2 (γ)	1.50-1.54 (m), 1.09-1.14 (m)	25.8 (t)		CH_2 (γ)	1.40-1.47 (*m*), 1.09-1.12 (*m*)	25.9 (t)
	CH_3 (δ)	0.85 (d, *J* = 6.0)	14.4 (q)		CH_3(δ)	0.87 (overlap)	14.5 (q)
	CH_3 (δ)	0.87 (overlap)	11.3 (q)		CH_3 (δ)	0.91 (overlap)	11.3 (q)
Leu[5]	CO		169.5 (s)	Leu[5]	CO		169.5 (s)
	NH	7.67 (br s)			NH		
	CH (α)	4.91 (q, *J* = 6.5)	47.4 (d)		CH (α)	4.85 (*q*, *J* = 6.0)	47.7 (d)
	CH_2 (β)	1.62-1.65 (m)	42.1 (t)		CH_2 (β)	1.64-1.68 (*m*)	42.2 (t)
		1.56-1.59 (m)				1.50-1.54 (*m*)	
	CH (γ)	1.53-1.56 (m)	25.1 (d)		CH (γ)	1.45-1.52 (*m*)	24.8 (d)
	CH_3 (γ)	0.89 (overlap)	22.6 (q)		CH_3 (γ)	0.85 (overlap)	22.6 (q)
	CH_3 (γ)	0.88 (overlap)	22.7 (q)		CH_3 (γ)	0.89 (overlap)	22.7 (q)



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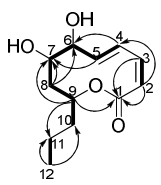

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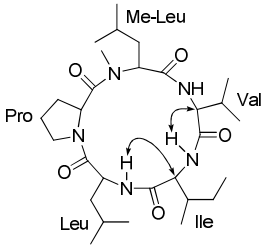
Figure 1: The structures of compounds **1** and **3**, and selected HMBC correlations (H→C) and ^{1}H,^{1}H-COSY (bold line).



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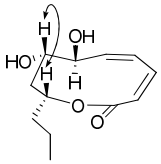

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Figure 2: The selected NOE correlations for compounds **1** and **3** (H↔H).

Compound **2** was obtained as a white amorphous powder, and established as having a molecular formula of $C_{32}H_{49}N_5O_5$ by its HR-Q-TOF MS and NMR spectroscopic data (Table 1). The IR spectrum indicated the presence of NH and C=O groups at 3264 and 1627 cm^{-1}, respectively. The ^{1}H- and ^{13}C-NMR spectral data of **2** (Table 1) were similar to those of **1**, except for the presence of a mono-substituted phenyl group instead of an isopropylidene, indicating that the *N*-methyl-leucine residue in **1** was replaced by *N*-methyl-phenylalanine in **2**. The sequence of these amino acid residues was elucidated on the basis of the same HMBC and NOE correlations as those of **1**, except for the HMBC correlations from α–H of *N*-methyl-phenylalanine to C=O of proline, and from α–H of valine to C=O of *N*-methyl-phenylalanine. Thus, the structure of compound **2** was established to be cyclo-(Pro-Me-Phe-Val-Ile-Leu), and named as xylaropeptide B.

Compound **3** was obtained as colorless oil. Its molecular formula was determined to be $C_{12}H_{18}O_4$ according to HR-Q-TOF MS and NMR spectroscopic data. The IR absorption at 3427 cm^{-1} indicated the presence of OH groups. The ^{13}C NMR and DEPT spectra of **3** (Table 2) exhibited 12 signals corresponding to one Me, three CH_2 groups, seven CH (three oxygenated and four olefinic)

groups, as well as one quaternary C-atom (C=O functional group). Inspection of the ^{1}H-^{1}H COSY, HMQC and HMBC data readily revealed a nonenolide core for **3** [15,16]. The HMBC correlations from H-C(2) to C(1) and C(4), from H-C(9) to C(1) and C(11), and from Me(12) to C(10) and C(11), along with ^{1}H-^{1}H COSY correlations H-C(2)↔H-C(3), and H-C(4)↔H-C(5)↔H-C(6)↔H-C(7)↔H_2-C(8)↔H-C(9)↔H_2-C(10), and H_2-C(11)↔H-C(12) established the structure of **3** (Figure 1). The configurations of C(2)/C(3) and C(4)/C(5) double bonds were identified to be *cis* and *trans*, respectively, according to the coupling constants of those corresponding protons.

Table 2: ^{1}H- and ^{13}C-NMR spectroscopic data of **3** (600/150 MHz, resp., in $CDCl_3$).

Position	δ_H (multiplicity, *J* in Hz)	δ_C
1		168.2 (s)
2	5.94 (d, *J* = 10.6)	126.1 (d)
3	6.66 (d, *J* =10.6)	139.2 (d)
4	6.26 (d, *J* = 15.2)	130.2 (d)
5	5.48 (dd, *J* = 10.1, 15.2)	133.4 (d)
6	3.90 (t, *J* = 9.1)	78.5 (d)
7	3.53 (t, *J* =7.7)	76.7 (d)
8	1.87-1.89 (m)	40.0 (t)
9	4.91-4.95 (m)	75.5 (d)
10	1.42-1.54 (m) 1.55-1.62 (m)	38.7 (t)
11	1.35 (m)	18.5 (t)
12	0.93 (t, *J* =7.3)	13.9 (q)

The relative stereochemistry of **3** was determined by the analysis of the ROESY spectrum. The presence of NOE correlations H-C(7)↔H-C(9) indicated that H-C(7) and H-C(9) were *β*-oriented, while H-C(6) was in an *α*-orientation (Figure 2). Thus, the structure of **3** was established as (3*Z*,5*E*)-7,8-dihydroxy-10-propyl-7,8,9,10-tetrahydro-2*H*-oxecin-2-one, and named as xylarolide.

The antibacterial activities of **1** – **3** were tested against bacteria [*Escherichia coli* (CMCC (B) 44103], *Bacillus subtilis* [CMCC (B) 63501], *B. pumilus* [CMCC (B) 63202], and *Staphylococcus aureus* (CMCC (B) 26003)), and a yeast [*Candida albicaus* (AS 2.538)] using an Oxford plate assay system. Two replicates were performed for each compound at a concentration of 0.3 mg/mL with a loading volume of 100 μL. Compounds **1**–**3** had no effects on the growth of the tested bacteria and yeast at 30 μg/plate.

Previously, two antibacterial nonenolides, phomolides A and B, were isolated from *Phomopsis* sp.[16]. Phomolides A and B are epoxide analogues of **3**, indicating that the epoxy group between C(6) and C(7) is critical for the antimicrobial activities. The biological activities of cyclopeptides **1** and **2** need to be further explored.

Experimental

General experimental procedures: For column chromatography (CC): silica gel (SiO_2, 200 - 300 and 80 - 100 mesh; Qingdao Marine Chemical Factory, Qingdao, P. R. China), SiO_2 GF_{254} (Merck), RP-18 (Merck), and Sephadex LH-20 (Amersham Biosciences) were used. TLC: precoated SiO_2 GF_{254} plates (0.20 - 0.25 mm, Qingdao Marine Chemical Factory). Optical rotations: Perkin-Elmer 341 polarimeter with either $CHCl_3$ or MeOH as solvent. UV spectra: Amersham Biosciences Ultrospec 1100 pro spectrophotometer; λ_{max} (log ε) in nm. IR spectra: Nicolet FT-IR 380 in KBr. HR-Q-TOF-MS: Bruker Daltonios BioTOF-Q mass spectrometers; in *m/z* (rel. %). ^{1}H- and ^{13}C-NMR spectra: Bruker DRX-600 spectrometer, at 600 (^{1}H), and150 (^{13}C) MHz; in $CDCl_3$ or $(CD_3)_2CO$; δ in ppm rel. to Me_4Si, *J* in Hz.

Isolation and fermentation of the fungal strain: The fungus was isolated from the fruiting body of *Xylaria* sp., collected at "Gaoligong Mountain National Natural conservation Area", Yunnan Province, P. R. China. Both a traditional morphological assessment and internal transcribed spaces (ITS) sequence analysis were performed to characterize it as *Xylaria* sp. and named 101. Fermentation was performed, and the mycelia of 101 grown on PDA plates were used to inoculate 200 mL PD medium (potato 200 g/L, glucose 20 g/L, pH-neutral) in a 1 L Erlenmeyer flask. The flask was incubated on a rotary shaker for 5 d at 28°C with shaking at 160 rpm. The culture was transferred into 4 flasks (20 L) each containing PD medium (5 L), and cultivated for 2 months at 28°C without agitation.

Extraction and isolation: The culture filtrate (20 L) was concentrated under vacuum at 45°C to a volume of 2 L, and then extracted with an equal volume of AcOEt (3×). The combined org. layer, upon evaporation, yielded a brown syrupy extract (4.8 g). This was subjected to MPLC [RP-18 (145g); gradient aq. acetone (0%, 30%, 50%, 70%, and 100% respectively, 2 L each)] to yield 14 fractions (*Fr.a* – *Fr.m*). *Fr.a* (516 mg) was subjected to CC [Sephadex LH-20 (140 g); MeOH] to obtain 2 subfractions *Fr.a.1* and *Fr.a.2*. *Fr.a.1* (286 mg) was purified by CC [Sephadex LH-20 (140 g); MeOH] again, and purified by CC (SiO_2; light petroleum (PE)/ Me_2CO 15 : 1) to obtain *Fr.a.1.a* and coriloxin (**4**, 20 mg). *Fr.a.1.a* (6 mg) was further purified by MPLC [RP-18 (30 g); Me_2CO/H_2O 3:7] to afford **3** (25 mg). *Fr.a.2* (26 mg) was purified by CC (SiO_2; PE/Me_2CO 50: 1) to obtain 2-hydroxy-3-methoxy-5-methyl-*p*-benzoquinone (**5**, 4 mg). *Fr.j* (56 mg) was subjected to MPLC [RP-18 (30g); Me_2CO/H_2O 1: 1], and further purified by CC (SiO_2; chloroform/Me_2CO 15: 1) to obtain **1** (30 mg). *Fr.k* (160 mg) was subjected to CC [Sephadex LH-20 (140 g); MeOH], and further purified by CC (SiO_2; chloroform/Me_2CO 12: 1), followed by MPLC [RP-18 (30 g); Me_2CO/H_2O 1:1] to obtain **2** (6 mg).

Xylarotide A (**1**)

Colorless powder.
$[\alpha]_D$: -24.2 (*c* 0.8, $CHCl_3$).
IR (KBr): 3287, 2959, 2872, 1640, 1528, 1451, 755 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 216 (3.09), 225 (2.98), 242 (3.22), 279 (2.85).

^{1}H NMR and ^{13}C NMR: Table 1.
HR-Q-TOF MS: 550.3921 (calcd. 550.3968 for $C_{29}H_{52}N_5O_5^+$, $[M + H]^+$).

Xylarotide B (**2**)

Colorless powder.
$[\alpha]_D$: -3.6 (*c* 0.18, $CHCl_3$).
IR (KBr): 3263, 2958, 2925, 2871, 1642, 1529, 698cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 208 (3.44), 212 (3.30), 229 (3.35), 233 (3.34), 239 (3.52), 278 (3.33).
^{1}H NMR and ^{13}C NMR: Table 1.
HR-Q-TOF MS: 584.3772 (calcd. 584.3812 for $C_{32}H_{50}N_5O_5^+$, $[M + H]^+$).

Xylarolide (**3**)

Colorless oil.
$[\alpha]_D$: -18.0 (*c* 0.54, $CHCl_3$).
IR (KBr): 3427, 2959, 2934, 2874, 1713, 1387, 1254, 1163, 1091, 1039, 952, 797 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 247 (3.05).
^{1}H NMR and ^{13}C NMR: Table 2.
HR-Q-TOF MS: 227.1256 (calcd. 227.1283 for $C_{12}H_{19}O_4^+$, $[M + H]^+$).

Acknowledgments - This work was financially supported by the National Science Fund for Distinguished Young Scholars to Y.-M. Shen (No. 30325044) and the Key Grant of Chinese Ministry of Education (No. 306010).

References

[1] Coval SJ, Puar MS, Phife DW, Terracciano JS, Patel M. (**1995**) SCH57404, an antifungal agent possessing the rare sodaricin skeleton and a tricyclic sugar moiety. *The Journal of Antibiotics*, ***48***, 1171-1172.
[2] Smith CJ, Morin NR, Bills GF, Dombrowski AW, Salituro GM, Smith SK, Zhao A, MacNeil DJ. (**2002**) Novel sesquiterpenoids from the fermentation of *Xylaria persicaria* are selective ligands for the NPY Y5 receptor. *Journal of Organic Chemistry*, ***67***, 5001-5004.
[3] Deyrup ST, Gloer JB, O'Donnell K, Wicklow DT. (**2007**) Kolokosides A-D: triterpenoid glycosides from a Hawaiian isolate of *Xylaria* sp. *Journal of Natural Products*, ***70***, 378-382.
[4] Hu ZY, Li YY, Huang YJ, Su WJ, Shen YM. (**2008**) Three new sesquiterpenoids from *Xylaria* sp. NCY2. *Helvetica Chimica Acta*, ***91***, 46-52.
[5] Li YY, Hu ZY, Lu CH, Shen YM. (**2010**) Four new terpenoids from *Xylaria* sp. 101. *Helvetica Chimica Acta*, ***93***, 796-802.
[6] Lin YC, Wu XY, Feng S, Jiang GG, Zhou SN, Vrijmoed LLP, Jones EBG. (**2001**) A novel *N*-cinnamoylcyclopeptide containing an allenic ether from the fungus *Xylaria* sp. (strain # 2508) from the South China Sea. *Tetrahedron Letters*, ***42***, 449-451.
[7] Huang H, She Z, Lin Y, Vrijmoed LL, Lin W. (**2007**) Cyclic peptides from an endophytic fungus obtained from a mangrove leaf (*Kandelia candel*). *Journal of Natural Products*, ***70***, 1696-1699.
[8] O'Hagana D, Rogers SV, Duffin GR, Edwards RL. (**1992**) Biosynthesis of the fungal polyketide, cubensic acid from *Xylaria cubensis*. *Tetrahedron Letters*, ***33***, 5585-5588.
[9] Boonphong S, Kittakoop P, Isaka M, Pittayakhajonwut D, Tanticharoen M, Thebtaranonth Y. (**2001**) Multiplolides A and B, new antifungal 10-membered lactones from *Xylaria multiplex*. *Journal of Natural Products*, ***64***, 965-967.
[10] Jayasuriya H, Herath KB, Ondeyka JG, Polishook JD, Bills GF, Dombrowski AW, Springer MS, Siciliano S, Malkowitz L, Sanchez M, Guan Z, Tiwari S, Stevenson DW, Borris RP, Singh SB. (**2004**) Isolation and structure of antagonists of chemokine receptor (CCR5). *Journal of Natural Products*, ***67***, 1036-1038.
[11] Healy PC, Hocking A, Tran-Dinh N, Pitt JI, Shivas RG, Mitchell JK, Kotiw M, Davis RA. (**2004**) Xanthones from a microfungus of the genus *Xylaria*. *Phytochemistry*, ***65***, 2373-2378.
[12] Davis RA, Pierens GK. (**2006**) ^{1}H and ^{13}C NMR assignments for two new xanthones from the endophytic fungus *Xylaria* sp. FRR 5657. *Magnetic Resonance in Chemistry*, ***44***, 966-968.
[13] Lin Y, Wu X, Feng S, Jiang G, Luo J, Zhou S, Vrijmoed LL, Jones EB, Krohn K, Steingrover K, Zsila F. (**2001**) Five unique compounds: xyloketals from mangrove fungus *Xylaria* sp. from the South China Sea coast. *Journal of Organic Chemistry*, ***66***, 6252-6256.
[14] Shiono Y, Murayama T, Takahashi K, Okada K, Katohda S, Ikeda M. (**2005**) Three oxygenated cyclohexenone derivatives produced by an endophytic fungus. *Bioscience, Biotechnology, and Biochemistry*, ***69***, 287-292.
[15] Rivero-Cruz JF, Macias M, Cerda-Garcia-Rojas CM, Mata R. (**2003**) A new phytotoxic nonenolide from *Phoma herbarum*. *Journal of Natural Products*, ***66***, 511-514.
[16] Du XP, Lu CH; Li YY, Zheng ZH, Su WJ, Shen YM. (**2008**) Three new antimicrobial metabolites of *Phomopsis* sp. *Journal of Antibiotics*, ***61***, 250-253.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1847 - 1850

A Novel Flavonoid and Furoquinoline Alkaloids from *Vepris glomerata* and their Antioxidant Activity

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Received: June 23rd, 2011; Accepted: October 4th, 2011

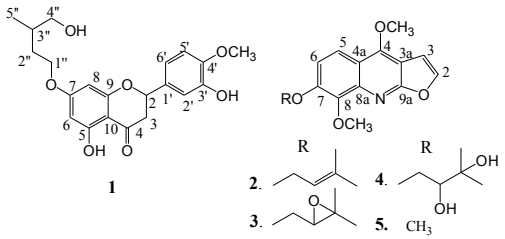
The dichloromethane extract of the aerial part of the plant *Vepris glomerata* (Rutaceae) yielded a new flavonoid, which was accorded the trivial name veprisinol (**1**), together with four known furoquinoline alkaloids: haplopine-3,3'-dimethylallyl ether (**2**), anhydroevoxine (**3**), evoxine (**4**) and skimmianine (**5**). The structures of the compounds were established by 1D and 2D NMR spectroscopy, as well as HREIMS. Compounds **1** and **2** have strong antioxidant potential, similar to and in some instances better than ascorbic acid and can be used as beneficial additives to antioxidant supplements.

Keywords: *Vepris glomerata*, veprisinol, furoquinoline alkaloids, antioxidant activity.

The African *Vepris* species have proved to be a good source of furoquinoline and acridone alkaloids that typify the genus as a whole. *V. bilocularis* has been found to have both furoquinoline as well as acridone alkaloids [1,2], while furoquinoline alkaloids alone have been found in *V. ampody* [3], *V. heterophylla* [4], *V. punctata* [5] and *V. stolzii* [6], and acridone alkaloids alone in *V. fitoravina* and *V. macrophylla* [7]. The alkaloids are reported to possess broad spectrum antimicrobial [8], antiradical [9], antioxidant [10], antiplasmodial [11], anticancer [12] and antimutagenic [13] activities. *V. glomerata* is used in African traditional medicine, where its aqueous root extract is used to treat malaria, epilepsy, psychosis and stroke, when mixed with tea [14]. Earlier pharmacological studies on this plant reported antiplasmodial activities of the ethanol extract [15].

Since the species of Rutaceae are often cited as antimalarials or febrifuges in African traditional medicine [14], and the antioxidant activity of alkaloids [10] and flavonoids [16] has previously been demonstrated, all the five compounds isolated were assessed for antioxidant activity using three methods.

Here we report on the isolation and structure elucidation of a new flavonoid, in addition to four known furoquinoline alkaloids: haplopine-3,3'-dimethylallyl ether (**2**), anhydroevoxine (**3**), evoxine (**4**) and skimmianine (**5**) from the dichloromethane extract of *V. glomerata*, together with their antioxidant activities *in vitro*. The structures of the known compounds **2**-**5** were determined by comparison



of their physical and spectroscopic data with those reported in literature; **2** and **3** [17], **4** [18] and **5** [19]. Only skimmianine was previously reported from the leaves of *V. glomerata* endemic to Ethiopia, in addition to kokusaginine [20]. It is not apparent if the different compounds found in this study are as a result of either geographical or seasonal differences.

Compound **1** was obtained as a yellow solid. Its mass was established to be 388.1573 amu, based on HREIMS data, corresponding to a molecular formula of $C_{21}H_{24}O_7$, which indicates a double bond equivalence of 10, eight being due to the aromatic rings, one being due to the carbonyl group and one to ring C of the flavanone skeleton. The IR spectrum showed a carbonyl stretching band at 1705 cm^{-1} and a hydroxyl absorption band at 3364 cm^{-1}. This compound was identified as a flavanone based on its characteristic ^{1}H NMR spectral pattern. The characteristic ABX coupling system of H-2β, H-3α and H-3β appeared at δ_H 5.29 (1H, dd, J = 12.84, 2.84 Hz, H-2β), δ_H 3.04 (1H, dd, J = 17.12, 2.84 Hz, H-3α) and δ_H 2.75 (1H, dd, J = 17.12, 12.84, Hz, H-3β). These signals also showed COSY and NOESY correlations with each other.

Another characteristic pattern was that of the trisubstituted aromatic B ring. The proton resonances of this ring occurred as a singlet at δ_H 7.00 (s, H-2') and doublets at δ_H 6.89 and 6.84 (1H each, d, J = 8.48 Hz, H-5' and H-6'). The small coupling constant of about 2 Hz for $J_{H2',H6'}$ could not be detected for the H-2' resonance. The ^{1}H NMR spectrum also showed the presence of a methoxy group at δ_H 3.88 (s), its position at C-4' being confirmed by both a 1D NOE and a NOESY correlation with the resonances at δ_H 6.89 and 6.84 (H-5' and H-6'). Five aromatic C-O resonances were seen at δ_C 164.0, 167.2, 162.8, 145.0 and 147.0 attributed to oxygenation at C-5, 7, 9, 3' and 4'.

A pair of doublets at δ_H 6.02 (1H, d, J = 1.76 Hz, H-6) and δ_H 6.00 (1H, d, J = 1.76 Hz, H-8) were attributed to the *meta* coupled, H-6 and H-8 protons on ring A. These two proton resonances showed NOESY correlations to 2H-1'' at δ_H 4.02, confirming the position of the side chain at C-7. Its corresponding carbon resonance showed HMBC correlations to two multiplets at δ_H 1.87 (overlapping resonances of H-2''a and H-3'') and δ_H 1.61 (H-2''b). The H-2'' resonances were diastereotopic and appeared as two separate resonances. COSY correlations were also observed between H-1'' and H-2''a and H-2''b and between H-2''b and H-3''. The H-3'' methine proton was coupled to the methyl proton resonance at δ_H 0.95 (d, J = 6.52 Hz) attributed to 3H-5'' and the methylene proton at δ_H 3.50 (2H-4'') in the COSY spectrum. These correlations formed a side chain which was attached to ring A by an ether linkage at C-7. Compound **1** was thus identified as 4H-1-benzopyran-4-one, 2, 3-dihydro-5-hydroxy-2-(4'-methoxy-3'-hydroxybenzyl)-7-*O*-(2-methyl butanol) ether, and given the trivial name veprisinol.

The results of the reducing potential (transformation of Fe^{3+}-Fe^{2+}) of the standard (ascorbic acid) and compounds **1**-**5** are shown in Figure 1. The activity of haplopine-3,3'-dimethylallyl ether, **2** and veprisiniol (**1**) was significantly higher than the activity of the other three alkaloids at all concentrations. However, the reducing power of compound **1** was significantly lower than that of compound **2**. The reducing power of the compounds and standard followed the order: ascorbic acid > **2** > **1** > **3** > **4** > **5**.

The DPPH radical scavenging assay results are shown in Fig. 2. The results revealed that the scavenging activity of the standard ascorbic acid was significantly higher than all other compounds tested. At concentrations of 62.5 µg mL^{-1} and above, the activity decreased in the order ascorbic acid > **1** > **2** > **4** > **3**, whereas at the lower concentrations, 31.25 and 15.625 µg mL^{-1}, evoxine (**4**) had the highest percentage antioxidant activity of 41%. The activity of compounds **1** and **2** was increased with their concentration and significantly higher than other compounds, particularly at higher concentrations (Figure 2).

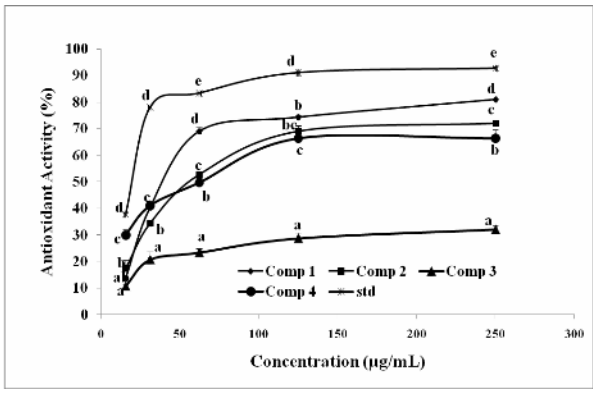


Figure 1: Free radical reducing potential of compounds **1**-**5** and standard ascorbic acid as evaluated by the spectrophotomeric detection of the Fe^{3+}-Fe^{2+} transformation (FRAP method).

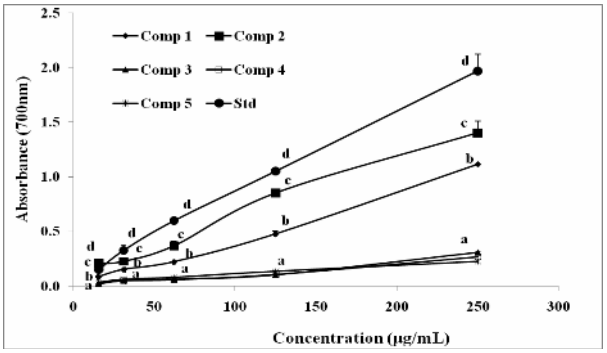


Figure 2: Antioxidant activity of compounds 1-4 and ascorbic acid standard, as measured by the DPPH method.

The hydroxyl radical scavenging activities in the deoxyribose assay are shown in Figure 3. The results revealed that compound **1** possesed significantly higher activity than all the other compounds tested, including the standard, ascorbic acid, at most concentrations. Compounds **1**, **2** and **4** had hydroxyl radical scavenging activity comparable with and in the case of **1** and **2**, better than that of ascorbic acid. Skimmianine (**5**) was not tested in either the DPPH or deoxyribose assays due to insufficent amount.

The three assays revealed that compounds **1** and **2** are good antioxidant compounds, while compound **4** shows high activity at a lower concentration in the DPPH assay. Flavonoids are known to be potent antioxidants and their activity is dependent on their molecular structure. The activity of **1** could be attributed to the hydroxyl (OH) groups in the molecule, which donate hydrogen to reduce the DPPH radical to DPPH-H. The alkaloids **2**-**5** have the same basic skeleton, the only difference being in their side chain. The reductive ability of **2** may be attributed to the double bond of the isoprenyl unit, rich in delocalized pi-electrons, which are easily donated during reduction of Fe^{3+} to Fe^{2+}. Sang *et al.* also reported that the double bond of the isoprenyl group was responsible for the antioxidant activity of garcinol [21]. The antioxidant activity of **2** in the DPPH assay, like **1**, could also be attributed to the hydroxyl groups in the molecule.

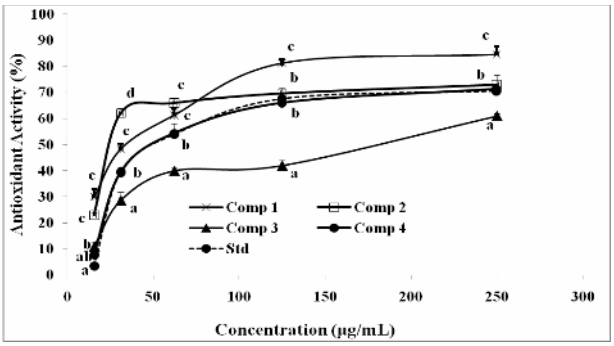


Figure 3: Hydroxyl radical scavenging activity of compounds **1-4** and standard ascorbic acid as measured by the deoxyribose method.

In conclusion, five compounds were isolated (a flavonoid and four alkaloids) from the aerial parts of *V. glomerata*. Verification of their antioxidant activities, as well as comparison with known antioxidants, will provide herbalists and traditional healers with scientific evidence for the use of the aerial parts of this plant as natural antioxidants.

Experimental

General experiment procedures: The melting points were recorded on an Ernst Leitz Wetzer micro-hot stage melting point apparatus and are uncorrected. UV spectra were obtained on a Varian Cary UV-VIS Spectrophotometer in chloroform. IR spectra were recorded on a Perkin-Elmer Universal ATR Spectrometer. The 1D and 2D NMR spectra were recorded using a Bruker Avance^{III} 400 MHz NMR spectrometer. All the spectra were recorded at room temperature using deuterated chloroform ($CDCl_3$) as solvent. The HREIMS was measured on a Bruker Micro TOF-QII instrument. Specific rotations were measured at room temperature in chloroform on a PerkinElmerTM, Model 341 Polarimeter with a 10 mm flow tube. The separation, isolation and purification of compounds were carried out by gravity CC and monitored by TLC. Merck silica gel 60 (0.040-0.063 mm) was used for CC. Merck 20 × 20 cm silica gel 60 F_{254} aluminum sheets were used for TLC. TLC plates were analyzed under UV light (254 and 366 nm) before being sprayed with anisaldehyde: concentrated sulfuric acid: methanol [1:2:97] spray reagent and then heated.

Plant material: *Vepris glomerata* was collected from the Rift Valley province of Kenya and identified by Dr S. T. Kariuki from the Department of Botany, Egerton University, Kenya. A voucher specimen (Kiplimo 01) was deposited at the University of KwaZulu-Natal Ward Herbarium, Westville Campus, Durban, South Africa.

Extraction and isolation: The air-dried aerial parts (980 g) of *V. glomerata* were sequentially extracted with *n*-hexane, followed by dichloromethane in a Soxhlet apparatus for 48 h, yielding crude extracts of 46 and 32 g, respectively. The oily residue of the dichloromethane extract obtained after evaporation under vacuum, was separated by CC on silica gel with *n*-hexane and then increasing the concentration of ethyl acetate from 10 to 80% in *n*-hexane, to give 10 fractions (fr.); fr. 8-16 (1.27 g), fr. 17-19 (0.5 g), fr. 20-26 (2.36 g), fr. 27-32 (2.35 g), fr. 33-39 (1 g), fr. 40-43 (2.1 g), fr. 44-49 (0.5 g), fr. 52-56 (3.9 g), fr. 57-62 (1.75 g) and fr. 63-67 (5.1 g).

Fraction 52-56 was separated by CC with *n*-hexane/EtOAc (7:3) as the solvent to afford sub-fractions A-C. Sub-fraction A was further purified using 100% dichloro-methane to afford compound **2**, a green solid (51 mg). Sub-fraction B yielded compound **3**, a brownish solid (43 mg), which needed no further purification. Sub-fraction C was crystallized in methanol to afford **4** (62 mg). Fraction 44-49 was purified using 100% dichloromethane to afford **5** (60 mg). Fraction 63-67 was separated with *n*-hexane/EtOAc (4:1) to yield 4 sub-fractions A-D. Sub-fraction B was crystallized in methanol to afford yellow crystals of compound **1** (18 mg).

Veprisinol (**1**)

4H-1-Benzopyran-4-one, 2, 3-dihydro-5-hydroxy-2-(4'-methoxy-3'-hydroxylbenzyl)-7-*O*-(2-methyl butanol) ether

Yellow solid.

M.p: 78-80°C.

$[\alpha]^{20}_D$: +55.30 (*c* 0.056, $CHCl_3$).

IR: 3364 (O-H), 2928, 1705 (C=O), 1636, 1512, 1162 cm^{-1}.

UV λ_{max} ($CHCl_3$) nm (log ε): 337 (4.45), 285 (5.13), 239 (5.44).

^{1}H NMR (400 MHz, $CDCl_3$): 11.97 (H, s, OH), 7.00, (H, s, H-2'), 6.89 (H, d, *J* = 8.28 Hz, H-5'), 6.84 (H, d, *J* = 8.28 Hz, H-6'), 6.02 (H, d, *J* = 1.76 Hz, H-6), 6.00 (H, d, *J* = 1.76 Hz, H-8), 5.29 (H, dd, *J* = 12.84, 2.84 Hz, H-2β), 4.02 (2H, dd, *J* = 12.88, 6.24 Hz, 2H-1''), 3.88 (3H, s, OCH_3), 3.50 (2H, d, *J* = 5.68 Hz, 2H-4''), 3.04 (H, dd, *J* = 17.12, 12.84 Hz, H-3α), 2.75 (H, dd, *J* = 17.12, 2.84, Hz, H-3β), 1.87 (2H, m, H-2''a and H-3''), 1.61 (H, m, H-2''b), 0.95 (3H, d, *J* = 6.52 Hz, H-5'').

^{13}C NMR: 195.97 (C, C-4), 167.29 (C, C-7), 164.05 (C, C-5), 162.85 (C, C-9), 147.02 (C, C-4'), 145.93 (C, C-3'), 131.52 (C, C-1'), 118.15 (CH, C-5'), 112.71 (CH, C-2'), 110.71 (CH, C-6'), 103.11 (C, C-10), 95.54 (CH, C-6), 94.60 (CH, C-8), 78.92 (CH, C-2), 67.86 (CH_2, C-4''), 66.70 (CH_2, C-1''), 56.06 (OCH_3), 43.15 (CH_2, C-3), 32.94 (CH, C-3''), 32.37 (CH_2, C-2''), 16.60 (CH_3, C-5'').

HREIMS *m/z* 388.1573 $[M]^+$ (calcd. for $C_{21}H_{24}O_7$, 388.1522)

Antioxidant activity: The total reducing power was determined according to the method described previously [22]. The free radical scavenging activity (antioxidant capacity) of the plant phytochemicals on the stable radical 2, 2-diphenyl-β-picrylhydrazyl (DPPH) was evaluated by the method established by Shirwaikar *et al*. [23], and the deoxyribose assay for hydroxyl radical scavenging activity was performed as described previously by Chung *et al*. [24].

Statistical analysis: The data in Figures 1-3 are presented as mean ± SD of triplicates. [a-d]Values with different superscript letters for a given concentration are significantly diferent from each of the other compounds. The data were statistically analyzed using a statistical software program SPSS (SPSS for Windows, version 18, SPSS Science, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's multiple range post-hoc test was employed to find the differences. The data were considered significantly different at $p < 0.05$.

Acknowledgments - The authors wish to acknowledge the financial support received from the Organization for Women in Science for the Developing World (OWSDW).

References

[1] Govindachari TR, Sundararajan VN. (**1961**) Alkaloids of *Vepris bilocularis*. *Journal of Scientific and Industrial Research*, ***20***, 298-299.
[2] Ganguly AK, Govindachari TR, Manmade A, Mohamed PA. (**1966**) Alkaloids of *Vepris bilocularis*. *Indian Journal of Chemistry*, ***4***, 334-335.
[3] Kan-Fan B, Das C, Boiteau P, Potier P. (**1970**) Alcaloïdes de *Vepris ampody* (Rutacées). *Phytochemistry*, ***9***, 1283–1291.
[4] Gomes E, Dellamonica G, Gleye J, Moulis C, Chopin MJ, Stanislas E. (**1983**) Phenolic compounds from *Vepris heterophylla*, *Phytochemistry*, ***22***, 2628–2629.
[5] Chaturvedula VSP, Schilling JK, Miller JS, Andransiferana R, Rasamison VE, Kingston DGI. (**2003**) New cytotoxic alkaloids from the wood of *Vepris punctata* from the Madagascar rainforest. *Journal of Natural Products*, ***66***, 532-534.
[6] Khalid SA, Waterman PG. (**1982**) Chemosystematics in the Rutaceae 15. Furoquinoline and pyrano-2-quinolone alkaloids of *Vepris stolzii*. *Journal of Natural Products*, ***45***, 343-346.
[7] Koffi Y, Gleye J, Moulis C, Stanislas E. (**1987**) Acridones from *Vepris fitoravina* and *Vepris macrophylla*. *Planta Medica*, ***53***, 570-571.
[8] Karou D, Savadogo A, Canini A, Yameogo S, Montesano C, Simpore J, Colizzi V, Traore AS. (**2006**) Antibacterial activity of alkaloids from *Sida acuta*, *African Journal of Biotechnology*, ***5***, 195-200.
[9] Račková L, Májeková M, Košt'álová D, Štefek M, (**2004**) Antiradical and antioxidant activities of alkaloids isolated from *Mahonia aquifolium*. Structural aspects. *Bioorganic & Medicinal Chemistry*, ***12***, 4709-4715.
[10] Chung HS, Woo SO. (**2001**) A quinolone alkaloid with antioxidant activity from the aleurone layer of anthocyanin-pigmented rice. *Journal of Natural Products*, ***64***, 1579-1580.
[11] Waffo KFA, Coombes PH, Crouch NR, Mulholland DA, Amin SMM, Smith PJ. (**2007**) Acridone and furoquinoline alkaloids from *Teclea gerrardii* (Rutaceae: Toddalioideae) of Sourthern Africa. *Phytochemistry*, ***68***, 663-667.
[12] Iwasa K, Moriyasu M, Yamori T, Turuo T, Lee DU, Eigrebe W. (**2001**) *In vitro* cytotoxicity of the protoberberine-type alkaloids. *Journal of Natural Products*, ***64***, 896-898.
[13] Fukuda K, Hibiya Y, Mutoh M, Koshiji M, Akao S, Fujiwara H. (**1999**) Inhibition by berberine of cyclooxygenase-2 transcriptional activity in human colon cancer cells. *Journal of Ethnopharmacology*, ***66***, 227-233.
[14] Moshi JM, Mbwambo ZH, Nondo RSO, Masimba PJ, Kamuhabwa A, Kapingu MC, Thomas P, Richard M. (**2006**) Evaluation of ethnomedical claims and brine shrimp toxicity of some plants used in Tanzania as Traditional Medicines. *African Journal of Traditional Complementary and Alternative Medicine*, ***3***, 48–58.
[15] Innocent E, Moshi M, Masimba P, Mbwambo Z, Kapingu M, Kamuhabwa A. (**2009**) Screening of traditionally used plants for *in vivo* antimalarial activity in mice. *African Journal of Traditional Complementary and Alternative Medicine*, ***6***, 163-167.
[16] Dimitrios B. (**2006**) Sources of natural phenolic antioxidants. *Trends in Food Science Technology*, ***17***, 505–512.
[17] Al-Rehaily AJ, Ahamad MS, Muhammad IM, Al-Thukair AA, Perzanowski HP. **(2003**) Furoquinoline alkaloids from *Teclea nobilis, Phytochemistry*, ***64***, 1405-1411.
[18] Dreyer DL. (**1970**) Chemotaxonomy of the Rutaeceae.VII. Alkaloids in *Evodia zanthoxyloides* F. Muell. *Journal of Organic Chemistry*, ***35***, 2420-2422.
[19] Latip J, Ali MNH, Yamin BM. (**2005**) 4, 6, 7-Trimethoxyfuro[2, 3-b]quinoline-water (2/3). *Acta Crystallographica*. ***E61***, 02230-02232.
[20] Dagne E, Yenesew A, Waterman PG, Gray AI. (**1988**) The chemical systematics of the Rutaceae subfamily Toddalioideae, in Africa. *Biochemical Systematics and Ecology*, ***16***, 179-188.
[21] Sang S, Liao CH, Pan MH, Rosen RT, Lin-Shiau SY, Lin JK, Ho CT. (**2002**) Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities. *Tetrahedron*, ***58***, 10095-10102.
[22] Ferreira ICFR, Baptista M, Vilas-Baos, Barros L. (**2007**) Free radical scavenging capacity and reducing capacity of wild edible mushrooms from northeast Portugal: individual cap and stipe activity. *Food Chemistry*, ***100***, 1511-1516.
[23] Shirwakar A, Shirwakar AR, Rajendran K, Punitha IRS. (**2006**) *In vitro* antioxidant studies on the benzyltetraisoquinoline alkaloid berberine. *Biological and Pharmaceutical Bulletin*, ***29***, 1906-1910.
[24] Chung SK, Osawa T, Kawakishi S. (**1997**) Hydroxyl radical-scavenging effects of species and scavengers from brown mustard (*Brassica nigra*). *Bioscience, Biotechnology and Biochemistry*, ***61***, 118-123.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1851 - 1854

Flavonoid Constituents and Free Radical Scavenging Activity of *Alchemilla mollis*

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Received: June 29th, 2011; Accepted: October 24th, 2011

Antioxidant capacity of the methanolic extract of *Alchemilla mollis* was measured by its ability to scavenge the DPPH radical. The EtOAc fraction obtained after partition of the total extract was found to be the most active radical scavenger (IC_{50} 9.8 ± 1.8 μg/mL) and was subjected to fractionation by Sephadex LH-20 CC. Further purification by RP-18 CC led to the isolation of eight flavonoid glycosides: *cis*- and *trans*-tiliroside (**1** and **2**), rhodiolgin (**3**), hyperoside (**4**), isoquercitrin (**5**), miquelianin (**6**), sinocrassoside D_2 (**7**), and gossypetin-3-*O*-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (**8**). It was found that **8** is a new compound and its antioxidant activity is also reported. Identification of the isolated compounds was carried out by spectroscopic and spectrometric analysis (1D and 2D NMR, UV and MS).

Keywords: *Alchemilla mollis,* Rosaceae, flavonoid glycosides, antioxidant activity.

Alchemilla mollis (Buser) Rothm. (Rosaceae) is a herbaceous perennial plant that grows in mountainous regions of the Balkan Peninsula (Romania, Greece and Bulgaria) [1]. The species, with its only known locality in the National Park "Central Balkan", is critically endangered in Bulgaria according to the IUCN criteria and was included in the Red Book of Bulgaria, the Red List of Bulgarian vascular plants, the Biological Diversity Act, and the Medicinal Plants Act [2a-2c]. Because of its very limited occurrence, its cultivation has been started recently [3,4].

A. mollis forms part of the commercial drug "Herba Alchemillae", an aggregate of species collectively referred to as Lady's mantle. The drug possesses astringent, diuretic and antispasmodic properties, and is commonly used in traditional medicine as a cure for excessive menstruation and wounds [5,6]. Different studies showed that the phenolic compounds (for example, tannins, and flavonoids) present in the plant are responsible for the pharmacological activity of Lady's mantle [7a-7d]. Surprisingly, the literature data concerning the chemical composition of *A. mollis* have proved to be quite limited, with only a few reports on total flavonoid and tannin contents of the plant [8a-8c]. Consequently, the aim of the present work was to study the flavonoid constituents of this species and their antioxidant capacity.

The DPPH scavenging assay is widely used for preliminary evaluation of the antioxidant potential of extracts and individual compounds [10,11]. In this work, the MeOH extract of *A. mollis* and its fractions obtained after re-extraction with light petroleum (PE), chloroform ($CHCl_3$), and ethyl acetate (EtOAc), as well as the remaining H_2O residue were studied for their general antioxidant effect, as indicated by their potential to scavenge the stable DPPH radical.

Table 1: DPPH radical scavenging activity of *A. mollis* total methanolic extract, fractions and individual compounds.

	IC_{50} (μg/mL)
total MeOH extract	31.7 ± 4.9
PE fraction	>200
$CHCl_3$ fraction	>200
EtOAc fraction	9.8 ±1.8
H_2O residue	42.5 ± 4.3
Quercetin (Reference)	3.2 ± 0.4
Hyperoside (**4**)	5.1 ± 0.4
Compound **8**	4.9 ± 0.5

The total MeOH extract exhibited a significant dose dependent inhibition of DPPH activity with 50% inhibition (IC_{50}) at a concentration of 31.7 ± 4.9 μg/mL. As shown in Table 1, the scavenging activities of the fractions on the DPPH radical increased in the order of PE<$CHCl_3$ <H_2O<EtOAc. Although the DPPH free radical scavenging ability of the EtOAc fraction (IC_{50} 9.8 ± 1.8 μg/mL) was less than that of quercetin (IC_{50} 3.2 ± 0.4 μg/mL) it was evident that the EtOAc-soluble fraction had hydrogen donating ability and could serve as either a free radical inhibitor or scavenger, possibly acting as a primary antioxidant.

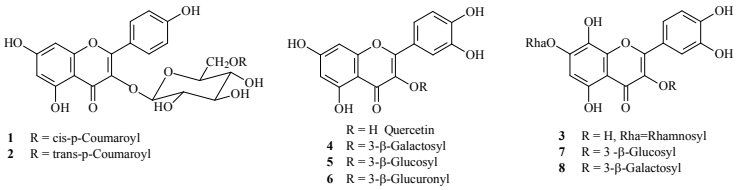

Figure 1: Structures of flavonoid glycosides **1-8**.

The most active EtOAc-soluble fraction was subjected to Sephadex LH-20 column chromatography. Further purification by RP-18 silica gel column chromatography afforded eight flavonoid glycosides. The structures (Figure 1) of the known *cis*- and *trans*-tiliroside (**1** and **2**) [11], rhodiolgin (**3**) [12], hyperoside (**4**) [13], isoquercitrin (**5**) [13], miquelianin (**6**) [13] and sinocrassoside D_2 (**7**) [14] were determined by comparison of their spectral data (UV, ^{1}H NMR, ^{13}C NMR and MS) with those published previously.

Table 2: ^{1}H and ^{13}C NMR spectroscopic data of compound **8** (CD_3OD).

Position	δ_H J (Hz)	δ_C	Position	δ_H J (Hz)	δ_C
2		159.4			
3		135.3	1”	5.20 d (7.2)	105.4
4		180.1	2”	3.85 m*	73.2
5		154.3	3”	3.65 m*	75.1
6	6.67 s	99.9	4”	3.85 m*	70.1
7		152.2	5”	3.50 m*	77.2
8		128.5	6”	3.50 m*	62.0
9		146.2			
10		107.1			
1’		123.0	1’”	5.54 brs	100.9
2’	7.94 d (2.2)	116.1	2’”	4.14 brs	71.7
3’		145.8	3’”	3.99 dd (2.8, 9.4)	72.1
4’		150.2	4’”	3.65 m*	73.8
5’	6.87 d (8.5)	118.1	5’”	3.55 m*	71.3
6’	7.72 dd (2.2,8.5)	123.5	6’”	1.26 d (6.2)	18.1

*overlapped signals

Compound **8**, isolated as yellowish amorphous powder, displayed spectral properties very similar to those of sinocrassoside D_2 (**7**) [14]. The negative HR-ESI-MS of **8** showed a $[M-H]^+$ peak at *m/z* 625.14035, which corresponded to a molecular formula $C_{27}H_{29}O_7$. The ^{1}H NMR spectrum (Table 2) contained signals assignable to a gossypetin moiety [δ_H 6.67 (1H, s, H-6), 6.87 (1H, d, J = 8.5 Hz, H-5’), 7.72 (1H, dd, J = 2.2, 8.5 Hz, H-6’), 7.94 (1H, d, J = 2.2 Hz, H-2’)], an α-L-rhamnopyranosyl unit [δ_H 1.26 (3H, d, J=6.2 Hz) and δ_H 5.54 (1H, brs)] and the doublet at δ_H 5.20 (J = 7.2 Hz) characteristic of an anomeric proton of a sugar moiety with β-configuration. This was identified as D-galactose after acid hydrolysis of **8** and confirmed by co-TLC with an authentic sample. The full NMR assignments and connectivities of **8** were determined by HMBC and NOESY data analysis (Figure 2). Thus, the observed HMBC long-range correlations between anomeric proton H-1” (δ_H 5.20) and C-3 δ_C (135.3), as well as between the second anomeric proton H-1’” (δ_H 5.54) and C-7 (δ_C 152.2) supported the location of sugar moieties at C-3 and C-7. Furthermore, NOE interactions of the aromatic singlet at δ_H 6.67 (H-6) with H-1’” and H-6’” confirmed the position of the

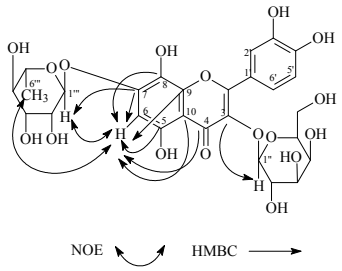

Figure 2: Significant HMBC and NOE correlations in **8**.

α-L-rhamnopyranosyl unit at C-7. Thus, the new compound (**8**) was identified as gossypetin-3-β-D-galactopyranosyl-7-α-L-rhamnopyranoside.

Finally, the antioxidant activity of the new compound **8** was evaluated by the DPPH free radical scavenging assay (Table 1) and compared with that of hyperoside (**4**) and quercetin. As can be seen, **8** exhibited antioxidant activity against the DPPH radical with an IC_{50} value (4.9 ± 0.5 μg/mL) similar to that of hyperoside (IC_{50} 5.1 ± 0.4 μg/mL) and lower than that of quercetin (IC_{50} 3.2 ± 0.4 μg/mL). This significant radical scavenging activity of **8** could be explained by the presence of *ortho*-dihydroxyl groups at C-3’ and C-4’ in ring B of the flavonoid structure [15].

The results described above showed that the EtOAc fraction obtained from the total methanolic extract of *A. mollis* was characterized by the presence of three different types of flavonoid glycosides – kaempferol (**1** and **2**), quercetin (**4**, **5** and **6**) and gossypetin (**3**, **7** and **8**). It is noteworthy, that kaempferol and quercetin glycosides have been previously found in *A. speciosa* [16], *A. xanthochlora* [17,18] and *A. vulgaris* [19], while gossypetin glycosides were detected for the first time in the genus *Alchemilla*. These differences in the chemical composition of the investigated species could be of chemotaxonomic importance.

Experimental

General: UV spectra were taken on a Helios Gamma spectrophotometer (Thermo Electron Corporation). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance II+ 600 NMR spectrometer with an operating frequency of 600.13 MHz (^{1}H) and 150.903 MHz (^{13}C), using TMS as internal standard and CD_3OD as solvent. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hertz. 1D and 2D NMR spectra were recorded using the standard Bruker pulse sequence. The HR-ESI-TOF-MS

was measured using a 6210 Time-of-flight LC/MS system (Agilent technologies) coupled with a HPLC instrument 1200 Series (Agilent Technologies). TLC analyses were carried out on silica gel 60 F_{254} precoated plates (Merck). The compounds were visualized by spraying with either H_2SO_4 or NP-reagent (Fluka). For open column chromatography, Sephadex LH-20 (Fluka) was used as stationary phase. A LiChroprep RP-18 column (Merck) was used for medium pressure liquid chromatography (MPLC).

Plant material: *A. mollis* plants from a native Bulgarian population (Stara planina mt. -1150 m a.s.l.), cultivated in the experimental field of the Institute of Biodiversity and Ecosystem Research (Sofia – 570 m a.s.l.), were used in the experiments. The aerial flowering parts of the plants were collected within phenophase – full blossoming, air-dried and kept in the dark. The voucher specimen (SOM 159980) was deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia.

Extraction and isolation: Powdered plant material (70 g) was extracted with MeOH (3 x 1 L) at room temperature in an ultrasonic bath for 30 min each time. After filtration, the solvent from the combined extracts was evaporated under vacuum to give the total methanolic extract (MeOH, 9.10 g). This was further dissolved in distilled water (200 mL) and partitioned with light petroleum (PE, 3 x 80 mL), chloroform ($CHCl_3$, 3 x 80 mL) and ethyl acetate (EtOAc, 3 x 80 mL) to yield PE (0.57 g), $CHCl_3$ (0.28 g) and EtOAc (0.44 g) fractions. The remaining aqueous phase was evaporated to dryness (7.51 g).

The EtOAc extract was dissolved in MeOH (15 mL) and filtered through celite in order to remove insoluble parts. The clear methanolic solution was concentrated to 5 mL and applied to a Sephadex LH-20 column (equilibrated with MeOH) to give 4 main fractions [TLC: Silica gel, EtOAc/MeOH/H_2O, 5:0.8:0.6, and EtOAc/HCOOH/ CH_3COOH/H_2O, 100:11:11:26]. The flavonoid containing fraction (65 mg) was further subjected to MPLC (Li Chroprep RP-18) and eluted with increasing concentrations of MeOH in H_2O (20 to 70%). Repeated MPLC (Li Chroprep RP-18, MeOH/H_2O, 50:50) of selected fractions yielded mixture of *cis*- and *trans*-tiliroside (**1** and **2**, 4.8 mg), rhodiolgin (**3**, 0.8 mg), hyperoside (**4**, 10.8 mg), isoquercetrin (**5**, 2.2 mg), miquelianin (**6**, 4.9 mg), sinocrassoside D_2 (**7**, 1.2 mg) and gossypetin-3-*O*-β-D-galactopyranosyl-7-*O*-α-L-rhamnopyranoside (**8**, 5.9 mg). Known compounds were identified by comparison of their spectral data with those reported in the literature and by comparison with authentic samples. It should be noted that the isolated amounts do not represent the real concentration of the compounds in the plant.

Gossypetin 3-*O*-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside (**8**)

Yellowish amorphous powder.
UV λ_{max} nm (MeOH): 260, 275, 303sh, 352; + NaOMe 267, 339sh, 398; + $AlCl_3$ 277, 315sh, 452; + $AlCl_3$ / HCl 249sh, 282, 312sh, 365, 429; + NaOAc 270, 336, 390.
^{1}H NMR (CD_3OD): Table 2.
^{13}C NMR (CD_3OD): Table 2.
Negative HR-ESI-TOF-MS: *m/z* 625.14035 (calcd. for $C_{27}H_{29}O_7$ 625.14102) $[M-H]^+$

Acid hydrolysis: Flavonoid **8** (2 mg) was dissolved in MeOH (5 mL) and refluxed with 8% HCl (2 mL) for 2 h. The reaction mixture was evaporated to dryness, dissolved in H_2O (2 mL) and neutralized with NaOH. The neutralized product was analyzed by TLC (silica gel, *n*-PrOH-EtOAc-H_2O, 7:2:1) in the presence of authentic samples.

Free radical scavenging activity of the DPPH radical: The DPPH radical scavenging method was used for determination of the antioxidant capacity of the extracts and individual compounds [9,10]. Different concentrations of the extracts (10, 20, 50, 100 and 200 μg/mL in MeOH) and individual compounds (5, 10, 20 and 50 μg/mL in MeOH) were added in equal volume (2.5 mL) to the MeOH solution of DPPH$^\bullet$ (0.3 mM, 1 mL). After 30 min at room temperature, the absorption values were measured at 517 nm on a spectrophotometer (Jenway 6320D) and converted into the percentage antioxidant activity using the following equation: DPPH$^\bullet$ anti-radical scavenging capacity (%) = $[1-(A_{sample} - A_{blank})/A_{control}]x100$. MeOH (1.0 mL). The solution plus plant extract solution (2.5 mL) was used as a blank, while DPPH$^\bullet$ solution plus MeOH was used as a control. The extracts were measured in triplicate on 2 different days. The results are presented as a mean ± SD. The IC_{50} values were calculated by Software Prizm 3.00.

Acknowledgements - The authors are thankful to Dr Milka Jadranin (University of Belgrade, Faculty of Chemistry, Belgrade) for preparing the HRMS. This work was supported by the National Science Fund, Ministry of Education, Youth and Science, Bulgaria, project DTK 02/38 (2009).

References

[1] Kurtto A, Fröhner SE, Lampinen R. (Eds) (**2007**) *Atlas Florae Europaeae. Distribution of Vascular Plants in Europe*. Vol. ***14***. *Rosaceae* (*Alchemilla* and *Aphanes*). The Committee for Mapping the Flora of Europe & Societas Biologica Fennica Vanamo, Helsinki. pp.200.

[2] (a) Biological Diversity Act. State Gazette, №77/02.08. 2002. (in Bulgarian); (b) Vitkova A. (**2009**) *Alchemilla mollis* (Buser) Rothm. In *Red list of Bulgarian vascular plants*. Petrova A, Vladimirov V. (Eds). *Phytologia Balcanica, **15***, 63-94; (c) Vitkova A. (**2011**) *Alchemilla mollis* (Buser) Rothm. In *Red Book of R Bulgaria*. Vol. 1. Plants, Peev D. (Ed.) In press.

[3] Vitkova A. (**1997**) Contribution to biological investigations of *Alchemilla mollis* (Buser) Rothm. *Phytologia Balcanica*, ***3***, 57-61.

[4] Vitkova A, Gavrilova A, Tashev A. (**2011**). *Alchemilla mollis* (Rosaceae) – critically endangered species in Bulgaria. *Phytologia Balcanica*, in press.

[5] Ivanov I, Landgev I, Neshev G. (**1977**) *Bilkite v Bulgaria i izpolzvaneto im* (Medicinal plants in Bulgaria and their applications), Sofia. 256-257.

[6] Bisset NG. (**1994**) Lady's Mantle In *Herbal Drugs and Phytopharmaceuticals*. Wichtl M and NG Bisset (Eds), Medpharm Scientific Publishers, Stuttgart. 52-54.

[7] (a) Lamaison JL, Carnat A, Petitjean-Freytet C. (**1990**) Tannin content and inhibiting activity of elastase in Rosaceae. *Annales Pharmaceutiques Francaises*, ***48***, 335-340; (b) Filipek J. (**1992**) Effect of *Alchemilla xanthochlora* water extracts on lipid peroxidation and superoxide anion scavenging activity. *Pharmazie*, ***47***, 717-718; (c) Jonadet M, Meunier MT, Villie F, Bastide JP, Lamaison JL. (**1986**) Flavonoids extracted from *Ribes nigrum* L. and *Alchemilla vulgaris* L.: 1. *In vitro* inhibitory activities on elastase, trypsin and chymotrypsin. 2. Angioprotective activities compared *in vivo*. *Journal of Pharmacology*, ***17***, 21-27; (d) Schimmer O, Lindenbaum M. (**1995**) Tannins with antimutagenic properties in the herb of *Alchemilla* species and *Potentilla anserine. Planta Medica*, ***61***, 141-145.

[8] (a) Tuka L, Tamas M. (**1977**) Determination of flavonoids in *Alchemilla mollis* (Buser) Rothm and *Alchemilla* L. (Lady's mantle), *Farmacia*, ***25***, 247-252; (b) Vitkova A. (**1996**) A comparative assessment and dynamics of accumulation of flavonoids and tannins in species of *Alchemilla* L. (Rosaceae). *Fitology*, ***48***, 11-18; (c) Ayaz F A, Hayirlioglu-Ayaz S. (**2001**) Total phenols and condensed tannins in the leaves of some *Alchemilla* species. *Biologia*, ***6***, 449-453.

[9] Cimpoiu C. (**2006**) Analysis of some natural antioxidants by thin–layer chromatography and high performance thin-layer chromatography. *Journal of Liquid Chromatography & Related Technologies*, ***29***, 1125-1142.

[10] Matkowski A, Piotrowska M. (**2006**) Antioxidant and free radical scavenging activities of some medicinal plants from the Lamiaceae. *Fitoterapia*, ***77***, 346-353.

[11] Matlawska I, Sikorska M, Bylka W. (**1999**) Flavonoid compounds in *Lavatera thuringiaca* L. (Malvaceae) flowers. *Acta Poloniae Pharmaceutica*, ***56***, 453-445.

[12] Zapesochnaya GG, Kurkin VA, Shchavlinskii AN. (**1985**) Flavonoids of the epigeal part of *Rhodiola rosea*. II. Structures of new glycosides of herbacetin and of gossypetin. *Chemistry of Natural Compounds*, ***21***, 464-473**.**

[13] Tatsis EC, Boeren S, Exarchou V, Troganis AN, Vervoort J, Gerothanassis IP. (**2007**) Identification of the major constituents of *Hypericum perforatum* by LC/SPE/NMR and LC/MS. *Phytochemistry*, ***68***, 383-393.

[14] Morikawa T, Xie H, Wang T, Matsuda H, Yoshikawa M. (**2008**) Bioactive constituents from Chinese natural medicines. XXXII.1) Aminopeptidase N and aldose reductase inhibitors from *Sinocrassula indica*: Structures of sinocrassosides B_4, B_5, C_1, and D_1—D_3. *Chemical & Pharmaceutical Bulletin*, ***56***, 1438-1444.

[15] Zheng CD, Li G, Li HQ, Xu XJ, Gao JM, Zhang AL. (**2010**) DPPH-scavenging activities and structure-activity relationships of phenolic compounds. *Natural Product Communications*, ***5***, 1759-1765**.**

[16] Felser C, Schimmer O. (**1999**) Flavonoid glycosides from *Alchemilla speciosa*, *Planta Medica*, ***65***, 668-670.

[17] Lamaison JL, Carnat A, Petitjean-Freytet C, Carnat AP. (**1991**) Quercetin-3-glucuronide, the main flavonoid of Lady's Mantle, *Alchemilla xanthochlora* Rothm. (Rosaceae). *Annales Pharmaceutiques Francaises*, ***49***, 186-189.

[18] Fraisse D, Heitz A, Carnat A, Carnat A-P, Lamaison J-L. (**2000**) Quercetin 3-arabinopyranoside, a major flavonoid compound from *Alchemilla xanthochlora*. *Fitoterapia*, ***71***, 463-464.

[19] D'Agostino M, Dini I, Ramundo E, Senatore F. (**1998**) Flavonoid glycosides of *Alchemilla vulgaris* L. *Phytotherapy Research*, ***12***, (Suppl. 1), S162–S163.

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2011
Vol. 6
No. 12
1855 - 1856

Ultrasound-assisted Extraction of Total Phenols and Flavonoids from Dry Tobacco (*Nicotiana tabacum*) Leaves

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Received: October 12th, 2010; Accepted: October 23rd, 2011

Yields of extracted substances, as well as total phenol and flavonoid compounds obtained by classical and ultrasonic extractions from dry leaves of two tobacco types (oriental and Virginia) by two different solvents (acetone and methanol) at two operating temperatures (25 and 40°C) were compared. The yield of extractive, as well as total phenol and flavonoid compounds depended on the type of solvent, operational temperature and the tobacco type. The importance of these factors was assessed using 2^4 full factorial experiments without replication.

Keywords: Total phenol content, flavonoids, extraction, full factorial experiments.

Tobacco (*Nicotiana tabacum* L.) leaves are the basic raw materials for cigarettes, directly influencing their quality and style, but are also highly appreciated material for perfumes, cosmetics and pharmaceuticals [1a]. Tobacco leaf is rich in polyphenols, which affect its color and quality [1a-1e]. Wang *et al*. extracted dry tobacco leaves with methanol through microwave-assisted technology and reached a total polyphenols yield of 22.4 mg/g (dry weight) [1a]. The yield of polyphenols from tobacco leaves extracted with 80% aqueous ethanol in the presence of ultrasound (40 kHz, 15 minutes, room temperature), reached 23.6 ± 2.3 mg/g dry weight [1e]. The present study deals with the effects of extraction conditions on the yields of total phenolic content and flavonoids from dry tobacco leaves. Both silent and ultrasound-assisted extractions were used to study the recovery of total extracted substances, phenols and flavonoids from oriental and Virginia tobacco leaves by methanol and acetone at 25 and 40°C. The statistical significance of extraction conditions was evaluated by 2^4 full factorial experiments without replication.

As seen in Table 1, all four factors affected the yields of total phenolic content and flavonoids obtained after a 20 minute extraction from dry tobacco leaves. Independently of the recovery technique, operating temperature and tobacco type, methanol provided much higher extractive yields than acetone. The increase of extraction temperature slightly affected the extractive yields from both tobacco types, while the ultrasound-assisted extraction provided higher extractive substance yields than the classical method, approximately 8 and 24% in the case of oriental and Virginia types of tobacco, respectively. The data given in Table 1 show also that the extracts obtained by classical extraction had higher amounts of phenolics than those obtained by ultrasound extraction. These results confirmed the previously reported benefit of ultrasound action for extraction from plant materials [2a-2c]. This was explained by oxidation and degradation of some compounds either during sonication or at a higher temperature [2c].

The major results of the analysis of variance (ANOVA), used for screening out the important factors, are given in Table 2. The experiment was not replicated, so the residual variance was determined based on the variance of three- and four-way interactions. That these interactions could be included in the residual variance, it was checked by the Bartlett's test for homogeneity of variances. Bartlett's test statistics of 0.87, 8.53 and 0.55 in the case of yield of extract, and total phenolic and flavonoid content were respectively smaller than the upper critical value of the chi-square distribution with four degrees and with a significance level of 0.05 ($\chi^2_{4;0.05}$ = 9.49). Thus, the variances of three- and four-way interactions were judged to be equal. The obtained results confirm that for the total phenolic content, the important factors, with the significance level of 0.05, are the tobacco type, the type of solvent and the operational temperature, while the effects of the extraction technique are ignorable. All four factors, as well as the interaction of factors A and D and factors B and C are important, with the significance level of 0.05 in the case of the total flavonoid content.

Experimental

Plant materials: Dry oriental and Virginia tobacco (*Nicotiana tabacum* L.) leaves harvested in Bulgaria and Brazil, respectively, were used; moisture contents of dry tobacco leaves, determined by drying at 105°C to constant weight, were 6.7 and 7.2%, respectively.

Extraction procedures: Dry leaves were crushed and sieved (0.250 mm sieve). Methanol and acetone were utilized as extraction solvents and the resulting extracts were stored

Table 1: Total phenolic compounds and flavonoids of tobacco leaves extracts*.

Tobacco type	Extraction solvent	Extraction temperature	Extraction technique	Yield of extracted substances, g/100 g dry plant material	Total phenolic content, mg GAE/g dry extract	Total flavonoids, mg RE/g dry extract
Oriental	Acetone	25°C	CE[a]	7.4	122.0±0.97	100.2±1.67
			USE[b]	8.8	121.4±1.80	96.3±2.35
		40°C	CE	7.8	115.3±0.14	94.8±2.34
			USE	9.0	111.7±1.24	87.9±0.64
	MeOH	25°C	CE	22.6	105.9±1.80	51.8±0.12
			USE	23.4	99.7±1.94	50.2±0.30
		40°C	CE	24.7	90.0±1.94	49.7±0.67
			USE	26.6	87.8±3.87	43.9±0.91
Virginia	Acetone	25°C	CE	3.6	164.4±2.77	132.8±0.66
			USE	4.0	145.6±2.49	128.6±1.76
		40°C	CE	4.8	135.9±0.00	122.2±1.64
			USE	5.2	129.7±0.14	108.3±3.59
	MeOH	25°C	CE	18.2	117.3±0.41	59.1±0.83
			USE	21.2	110.6±2.07	54.8±0.51
		40°C	CE	22.4	116.3±1.66	55.7±0.65
			USE	23.4	102.6±3.18	43.6±1.26

*Data were expressed as the mean of three replicates ± standard deviation. [a] Classical (silent) extraction [b] Ultrasound extraction.

Table 2: Results of the variance analysis.

Source of variation	Degrees of freedom	Yield of extract		Total phenolic content		Total flavonoids		$F_{1;5;0.95}$
		Mean square	Test statistic	Mean square	Test statistic	Mean square	Test statistic	
A (Tobacco type)	1	62.02	**28.66**	1612.02	**42.87**	1061.13	**108.01**	**6.61**
B (Extraction technique)	1	2.33	1.08	132.25	3.52	122.66	**12.49**	
C (Temperature)	1	7.16	3.31	457.96	**12.18**	286.46	**29.16**	
D (Type of solvent)	1	1154.30	**533.41**	2595.90	**69.04**	13357.6	**1359.69**	
AB	1	0.77	0.36	125.44	3.34	3.9	0.40	
AC	1	2.98	1.38	28.09	0.75	33.93	3.45	
AD	1	0.031	0.01	180.90	4.81	565.25	**57.54**	
BC	1	0.53	0.24	13.32	0.35	68.48	**6.97**	
BD	1	0.031	0.01	4.41	0.12	0.68	0.07	
CD	1	1.38	0.64	64.00	1.70	29.43	3.00	
ABC+ ABD+ ACD+ BCD+ ABCD	5	10.85		187.99		49.12		
Total	15							

at 4°C. The plant material (5 g) and extraction solvent (50 mL) were put in Erlenmayer flasks (100 mL) and placed in an ultrasonic cleaning bath (Sonic, Niš, Serbia; total nominal power: 3 x 50 W; and internal dimensions: 30 x 15 x 20 cm) operating at a frequency of 40 kHz. Sonication was performed in 20 mins. The bath was filled with distilled water up to 1/3 of its volume (about 2.5 L). The extraction was carried out at 25 and 40°C. The temperature was maintained (± 0.1°C) by water circulating from a thermostated bath by means of a pump. The classical extraction was carried out with the ultrasound power switched off. At the end of the extraction procedure, the liquid extract was separated from the solid residue by vacuum filtration. The solid residue was washed twice using fresh solvent (20 mL). The filtrates were collected, the solvent evaporated in a rotary vacuum evaporator at 40°C. Each experiment was carried out in duplicate.

Determination of total phenolic and total flavonoid contents: The total phenolic and flavonoid contents of the extracts was determined by the Folin-Ciocalteu method [3a] and aluminum chloride colorimetric method [3b] respectively. Results were expressed as means of 3 replicate measurements.

Statistical analysis: The comparison of means was analyzed by Student's *t-test* and differences were considered significant when $p<0.05$. The four factors' effects (tobacco type, extraction technique, solvent and temperature) on the yields of extractive substances, total phenolic content and flavonoids were analyzed by 2^4 full factorial experiments with no replication.

Acknowledgements - This work was supported by the Ministry of Science of the Republic of Serbia; Projects OI 172047.

References

[1] (a) Wang HY, Cui C, Zhao MM, Shen GL, Rao GH. (**2008**) Extraction optimization and component identification of tobacco polyphenols. *Huanan Ligong Daxue Xuebao*, ***36***, 64-68; (b) Bazinet L, DeGrandpré Y, Porter A. (**2005**) Electromigration of tobacco polyphenols. *Separation and Purification Technology*, ***4***, 101-107; (c) Bazinet L, DeGrandpré Y, Porter A. (**2005**) Enhanced tobacco polyphenol electromigration and impact on membrane integrity. *Journal of Membrane Science*, ***25***, 111-118; (d) Kierulff JV, Jorgenson OB, Hansen TT, Knox AJ, De Grandpré Y. (**2001**) Use of a phenol oxidizing enzyme in the treatment of tobacco. *US Patent* 6298859; (e) Wang H, Zhao M, Yang B, Jiang Y, Rao G. (**2008**) Identification of polyphenols in tobacco leaf and their antioxidant and antimicrobial activities. *Food Chemistry*, ***107***, 1399-1406.

[2] (a) Stojičević S, Stanisavljević I, Veličković D, Veljković V, Lazić M. (**2008**) Comparative screening of antioxidant and antimicrobial activities of *Sempervivum marmoreum* L. extracts obtained by various extraction techniques. *Journal of the Serbian Chemical Society*, ***73***, 597-607; (b) Stanisavljević IT, Lazić ML, Veljković VB. (**2007**) Ultrasonic extraction of oil from tobacco (*Nicotiana tabacum* L.) seeds. *Ultrasonic Sonochemistry*, ***14***, *646*-652; (c) Vinatoru M. (**2001**) An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonic Sonochemistry*, ***8***, 303-313.

[3] (a) Singleton VL, Rossi JA. (**1965**) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, ***16***, 144-158; (b) Chang C, Yang M, Wen H, Chern J. (**2002**) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*, ***10***, 178-182.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1857 - 1862

Characterization of Polyphenolic Compounds in Unripe Chinotto (*Citrus myrtifolia*) Fruit by HPLC/PDA/ESI/MS-MS

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Received: July 1st, 2011; Accepted: September 25th, 2011

The flavonoid and furocoumarin composition was investigated of peel and pulp tissues of unripe fruits of *Citrus myrtifolia* Rafinesque, an ingredient of the popular soft drink "chinotto". Compound separation and identification was made using an HPLC-PDA detector coupled to ESI/MS/MS in positive and negative mode. Eighteen compounds (3-hydroxy-3-methylglutaryl-, *C*- and *O*-glycosyl flavonoids, furocoumarins and polymethoxylated flavones) were identified and quantified. Data indicated that the overall amount of flavonoids and furocoumarins in peel was higher than in the pulp, even though their relative distribution did not significantly change, apart from a different distribution of flavones and a lower content of naringin in the peel.

Keywords: chinotto, citrus, flavonoids, furocoumarins, peel, pulp.

Over the past few years, increasing attention has been paid by consumers to the nutritional aspects of products containing significant amounts of biologically active components. Nowadays, many studies are carried out on the thousands of natural phytochemicals that may have important physiological effects [1]. Scientific data demonstrated that an increased consumption of fruit and vegetables may protect against degenerative pathologies such as cancer and atherosclerosis [2]. Epidemiological studies have shown an inverse relationship between dietary flavonoids intake and cardiovascular diseases [3]. Many citrus flavonoids have been shown to have antioxidative activity to inhibit angiogenesis and to slow down cancer cell migration and proliferation [4,5]. Among the phytochemicals, flavonoids are widely contained in Citrus fruits [3].

Recently our research group has studied the unripe fruits of *Citrus myrtifolia* Rafinesque in terms of bioactive compounds and antioxidant activity; results indicated that it is a good source of phytochemicals, mainly vitamin C and flavonoids [6]. *Citrus myrtifolia* is a taxon of Citrus with foliage similar to that of the common myrtle. It is a compact tree with small leaves and no thorns which grows to a height of three meters and can be found in Malta and in the Liguria, Tuscany, Sicily, and Calabria regions of Italy. The fruit of the tree resemble small oranges. They are either sour or bitter and are commonly called by their Italian name, chinotto. They are an essential flavoring agent of most Italian amari, of the popular Campari aperitif, and of several brands of carbonated soft drinks that are generically called "chinotto". Native of southern China, its origin has not been exactly ascertained; probably it is a mutation of sour orange that eventually evolved into the species known today [7]. The scientific literature about *C. myrtifolia* [8,9] is related prevalently to the juice and no information is available about bioactive compounds quantitation and distribution in the whole fruit. Interest in the Italian citrus industry for chinotto production could be increased by studies on its composition.

The aim of the present work was to elucidate by HPLC/PDA/ESI/MS-MS the identity and relative distribution of flavonoids and furocoumarins in pulp and peel tissues of unripe *C. myrtifolia* used for industrial beverage purposes.

Figure 1 shows the PDA chromatograms of unripe *C. myrtifolia* pulp and peel at different λ allowing for the discrimination of flavanone, furocoumarin and flavone derivatives. All peaks identified were numbered from 1 to 18. The UV spectra, recorded in correspondence with peaks 1, 2, 3, 7 and 9 showed absorptions at 270 and 350 nm (peaks 1, 3 and 9) and 270 and 335 nm (peaks 2 and 7), which can be attributed to bands II (due to the A ring benzoyl system) and I (associated with absorption due to

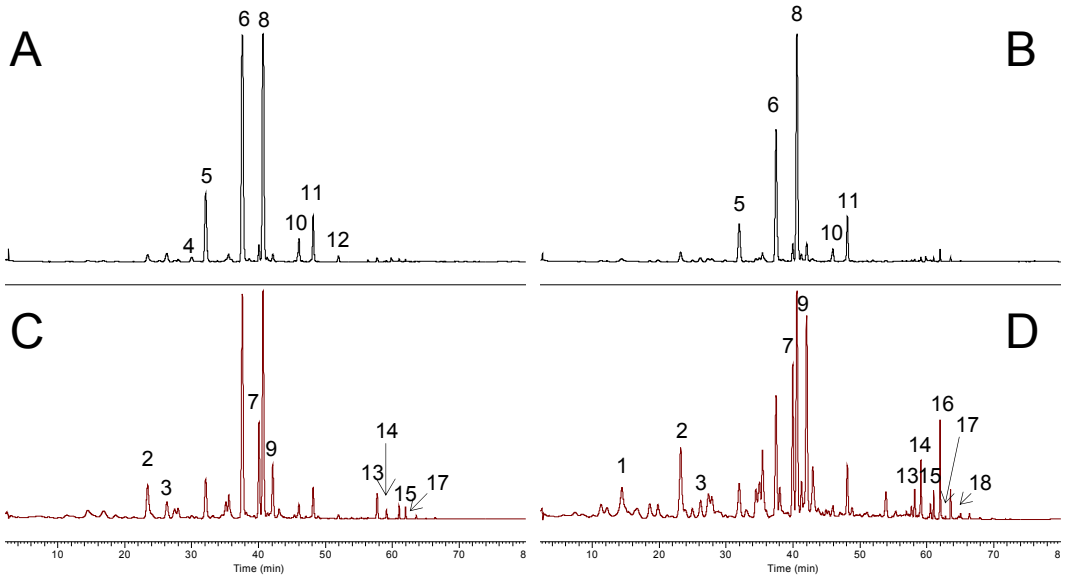


Figure 1: Typical PDA chromatograms of *C. myrtifolia* pulp and peel from immature fruits recorded at 280 (**A**, **B**) and 330 nm (**C**, **D**). Compounds **1-18** were identified as follows: **1**, lucenin-2; **2**, vicenin-2; **3**, lucenin-2 4'-methyl ether; **4**, eriocitrin; **5**, neoeriocitrin; **6**, naringin; **7** rhoifolin; **8**, neohesperidin; **9**, neodiosmin; **10** melitidin; **11**, brutieridin; **12**, poncirin; **13,** bergapten; **14**, epoxybergamottin; **15**, sinensetin; **16**, nobiletin; **17**, heptamethoxyflavone; **18**, tangeretin.

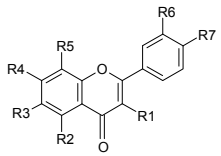
the B ring cinnamoyl system) of a flavone structure, respectively. The UV spectra of compounds 4, 5, 6, 8, 10, 11 and 12 indicated the flavanone nature of the aglycone, showing absorptions centered at 285 and 330 nm. Compounds 13 and 14 showed UV absorption maxima at 260 and 310, indicating a possible coumarin moiety. The lag-times and UV maxima (335 nm) of peaks from 15 to 18 suggested the presence of less polar polymethoxylated flavones.

The MS/MS spectra recorded in positive and negative mode in correspondence with the studied peaks and comparison with analytical standards and literature data, when available, permitted the unambiguous structural assignment for all compounds (Figures 2, 3 and 4), which were quantified separately in pulp and peel (Table 1).

Peaks 1-3 were identified as the flavone-*C*-glucosides lucenin-2, vicenin-2 and lucenin-2 4'-methyl ether. The precursor ions (m/z 611 ($[M+H]^+$) and 609 ($[M-H]^-$); m/z 595 ($[M+H]^+$) and 593 ($[M-H]^-$); 625 ($[M+H]^+$) and 623 ($[M-H]^-$) for 1, 2 and 3, respectively) were in agreement with their nominal masses. The product ions showed the characteristic loss of 120 amu, ascribable to the fragment mass of *C*-glucosyl flavonoids. These flavones were recently detected in chinotto juice [9, 10] and in taxonomically close Citrus juices, such as bergamot and sour orange [11-13]. Peak 7 was assigned to the flavone-*O*-glycoside rhoifolin, as confirmed by the molecular fragmentation pattern, which showed the precursor ion at 579 m/z and the loss of an *O*-diglycoside fragment mass at 273 m/z. Compound 9 was identified as a diosmetin aglycone *O*-linked with saccharide substituents; the MS/MS fragmentation pattern indicated the loss of a rhamnose unit ($[M+H-146]^+$) and a further loss of a glucose unit ($[M+H-308]^+$), revealing that the substituent is a rhamnose–glucose disaccharide. Coelution with a standard allowed us to assign to this peak the structure of neodiosmin (7-*O*-neohesperidosil diosmetin). Neodiosmin presence in *Citrus* was earlier reported by Gattuso *et al*. in *Citrus bergamia* Risso juice [12].

All flavanone compounds showed the characteristic loss of 308 amu, corresponding to the *O*-diglycoside breakage. Moreover, two 3-hydroxy-3-methylglutaric acid conjugates of neohesperidin and naringin, namely, brutieridin and melitidin were identified as peaks 10 and 11, respectively. The positive and negative ESI/MS showed a $[M + H]^+$ ion at m/z 725 and 755 and a $[M - H]^-$ ion at m/z 723 and 753, which corresponded to the elemental composition of melitidin and brutieridin, respectively. A detailed analysis by tandem mass spectrometry showed highly diagnostic fragment ions: the loss of 3-hydroxy-3-methylglutaryl moiety was confirmed by the positive daughter ion at m/z 273 for melitidin, and at m/z 303 for brutieridin. These data are in agreement with those reported by Barreca *et al.* [9] and Scordino *et al.* [10] for chinotto juice.

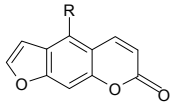
Finally, the present paper reported the occurrence of polymethoxylated flavones (PMFs) (Figure 2) in *C. myrtifolia* (peaks 15-18). PMFs are a class of minor components usually found in the essential oil fraction of citrus peels [14]. Mass spectra generated in positive mode showed $[M+H]^+$ ions at m/z 403, 373 and 433, well-matched in fragmentation, in UV-vis spectra and retention times with data reported by Scordino *et al.* [15]. The parent ions $[M+H]^+$ were further fragmented and the breakage of methyl and methoxy groups were in agreement with the loss of 15 and 30 amu. Compounds 15 to 18 were identified as sinensetin, nobiletin, heptametoxyflavone and tangeretin, respectively.



Peak	Structure assignment	R1	R2	R3	R4	R5	R6	R7
1	Luteolin 6,8-di-*C*-glucoside (Lucenin-2)	H	OH	Glu	OH	Glu	OH	OH
2	Apigenin 6,8-di-*C*-glucoside (Vicenin 2)	H	OH	Glu	OH	Glu	H	OH
3	Diosmetin 6,8-di-*C*-glucoside (Lucenin-2 4'-Me)	H	OH	Glu	OH	Glu	OH	OCH_3
7	Apigenin 7-*O*-neohesperidoside (Rhoifolin)	H	OH	H	*O*-Nh*	H	H	OH
9	Diosmetin 7-*O*-neohesperidoside (Neodiosmin)	H	OH	H	*O*-Nh*	H	OH	OCH_3
15	Sinensetin	H	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3
16	Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
17	3,5,6,7,8,3',4'-heptamethoxylflavone	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
18	Tangeretin	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3

*Neohesperidoside

Figure 2: Structure assignments of identified flavones.



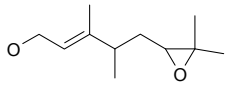
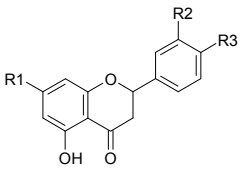
Peak	Structure assignment	R
13	5-Methoxypsoralen (Bergapten)	OCH_3
14	5-(6',7'-Epoxy)geranyloxypsoralen (Epoxybergamottin)	

Figure 3: Structure assignments of identified furocoumarins.

***Quantitative evaluation*:** Naringin and neohesperidin are the flavanone-*O*-glycosides found in the highest amounts in chinotto fruit (Table 1), ranging from about 200 to 175 mg/100 g in the pulp and from about 347 to 472 mg/100 g in the peel, respectively. Eriocitrin (6.9 mg/100 g) and poncirin (5.6 mg/100 g) were detected only in pulp, while neoericitrin was found in both analyzed matrixes in significant quantity (58.9 and 131.5 mg/100 g in pulp and peel, respectively). The two 3-hydroxy-3-methylglutaryl flavanone glycosides melitidin and brutieridin were found to be present in large amounts (14.2 and 28.6 mg/100 g in the pulp and 30.3 and 95.4 mg/100 g in the peel, respectively). The flavone *O*-glycosides rhoifolin and neodiosmin were the most abundant flavones of chinotto tissues, ranging from about 7 mg/100 g in the pulp to about 29.5 and 57.4 mg/100 g in the peel, respectively. The peel was also characterized by about 30 mg/100 g of the flavone-*C*-glucosides lucenin-2, vicenin-2 and lucenin-2 4'-methyl ether; on the contrary, only traces of these compounds were detected in the pulp. The total amount of



Peak	Structure assignment	R1	R2	R3
4	Eriodictyol 7-*O*-rutinoside (Eriocitrin)	O-Rutinoside	OH	OH
5	Eriodictyol 7-*O*-neohesperidoside (Neoeriocitrin)	*O*-Neohesperidoside	OH	OH
6	Naringenin 7-*O*-neohesperidoside (Naringin)	*O*-Neohesperidoside	H	OH
8	Hesperetin 7-*O*-neohesperidoside (Neoesperidin)	*O*-Neohesperidoside	OH	OCH_3
10	Naringenin 7-(2″-α-rhamnosyl-6″-(3‴'-hydroxy-3‴'-methylglutaryl)-β-glucoside (Melitidin)	3-Hydroxy-3-Methylglutaryl	OH	OCH_3
11	Hesperetin 7-(2″-α-rhamnosyl-6″-(3‴'-hydroxy-3‴'-methylglutaryl)-β-glucoside (Brutieridin)	3-Hydroxy-3-Methylglutaryl	H	OH
12	Isosakuranetin 7-*O*-neohesperidoside (Poncirin)	*O*-Neohesperidoside	H	OCH_3

Figure 4: Structure assignments of identified flavanones.

the two furocoumarins bergapten and epoxybergamottin in the peel (25.1 mg/100 g) was two times that of the pulp (12.0 mg/100 g). A small but distinctive amount of polymethoxylated flavones (16.4 mg/100 g), among which nobiletin predominated, was detected in the peel; the same components were detectable in traces in the peel. It is well known that polyphenolic contents are usually higher in peels [3], therefore it is not surprising that the total amount of flavonoids and furocoumarins in the peel (1235 mg/100 g) was more than two times higher than that of the pulp (519 mg/100 g). The flavonoid contents were also determined in all samples by the method indicated by the Association of the Industry of Juices and Nectars (AIJN) using the colorimetric Davis method; data obtained were about 423.7 mg/100 g and 736.4 mg/100 g for the pulp and the peel, respectively. Taking into account the relative distribution of the different classes discussed above in the two tissues (Figure 5), no major changes could be observed between the pulp and the peel contribution to the total polyphenolics apart from some differences in the relative distribution of the flavones, and in the naringin amounts, which predominated in the pulp.

In conclusion, our results elucidated the distribution of flavonoids and furocoumarins in *C. myrtifolia* peel and pulp. The HPLC/PDA/ESI/MS-MS technique allowed the identification and relative quantification of 18 compounds, among which many compounds have been detected for the first time in *C. myrtifolia*. Nowadays these aspects are considered to be highly valuable for the commercial valorization of chinotto as a citrus with high potential as a nutraceutical source.

Table 1: Polyphenolic contents of *Citrus myrtifolia* pulp and peel.

		Pulp Mean	Peel Mean
1	Lucenin-2 [b]	traces	4.0 (0.2)
2	Vicenin-2 [c]	0.03 (0.01)	21.3 (1.3)
3	Lucenin-2 4'-OMe [b]	1.9 (0.2)	4.6 (0.2)
4	Eriocitrin	6.9 (0.3)	traces
5	Neoeriocitrin	58.9 (3.3)	131.5 (6.7)
6	Naringin	200.3 (10.7)	347.0 (17.6)
7	Rhoifolin [c]	6.6 (0.4)	29.5 (1.6)
8	Neohesperidin	175.7 (7.3)	472.4 (21.9)
9	Neodiosmin	7.4 (0.3)	57.4 (1.8)
10	Melitidin [d]	14.2 (0.8)	30.3 (1.5)
11	Brutieridin [e]	28.6 (0.9)	95.4 (5.2)
12	Poncirin	5.6 (0.3)	traces
13	Bergapten [f]	7.0 (0.6)	12.0 (0.7)
14	Epoxybergamottin [f]	5.0 (0.1)	13.1 (0.6)
15	Sinensetin	0.24 (0.02)	1.8 (0.2)
16	Nobiletin [g]	0.91 (0.12)	11.2 (0.5)
17	Heptamethoxyflavone [g]	traces	0.39 (0.03)
18	Tangeretin [g]	traces	3.0 (0.1)
	Total flavonoids (HPLC)	519.1 (13.5)	1235.0 (29.5)
	Total flavonoids (according to Davis)	423.7 (22.7)	736.4 (29.7)

[a] mean value of five determinations; standard deviation in parentheses.
[b] expressed as neodiosmin; [c] expressed as apigenin; [d] expressed as naringin; [e] expressed as neohesperidin; [f] expressed as bergamottin; [g] expressed as sinensetin.

Figure 5: Relative distribution (%) of flavonoids and furocoumarins in *C. myrtifolia* pulp and peel.

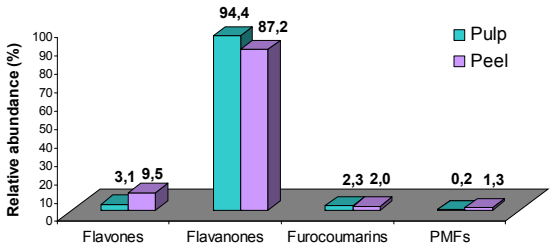


Table 2: Polyphenolic contents in *Citrus myrtifolia* pulp and peel.

Parameter	Mean Value[a]	
Hunter color parameters	*Peel*	*Pulp*
a	36.3 (6.2)	5.4 (1.5)
b	-34.4 (4.2)	-14.3 (2.7)
L	66.3 (2.2)	78.8 (2.4)
Mean diameter (cm)	2.5 (0.3)	
Mean weight (g)	27 (2)	
Total soluble solids (Brix)[b]	8.4 (0.1)	
Titrable acidity (% citric acid) [b]	0.83 (0.05)	
pH [b]	3.87 (0.21)	
Ratio (Brix/acidity) [b]	10.1	

[a] mean value of five determinations; standard deviation in parentheses.
[b] referred to the juice.

Experimental

Reagents and standards: HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA [Millipore, Vimodrone (MI), Italy] purification system. Apigenin, bergamottin, neodiosmin, neoeriocitrin, naringin, neohesperidin, poncirin and sinensetin were obtained from Extrasynthèse (Genay, France).

Plant materials: Fruits used in this study were grown in Castiglione di Sicilia (Catania, Italy). Immature *Citrus myrtifolia* (chinotto) fruits were picked in October 2010 with the degree of fruit maturity determined from the Hunter colour parameters, mean diameter, total soluble solids, titratable acidity and pH (Table 2). Samples for analysis were prepared from 25 fruits; pulp and peel tissues were separated and analyzed individually. Peels and pulps represented 55% and 38% of the studied sample, while the remaining part was constituted by seeds. The samples were stored at -20°C until needed for the study.

Extraction and analysis of flavonoids and furocoumarins: Five g of sample was extracted for 2 h with 10 mL of 50% aqueous methanol containing 0.3% formic acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000 rpm for 15 min and the supernatant decanted. The pellets were re-extracted under identical conditions. Supernatants were combined and used for the LC analyses, after filtration through 0.45µm PTFE filters (LabService Analytica, Bologna, Italy). The juice was analyzed before centrifugation and filtration through 0.45 µm PTFE filters. The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Luna C18 250 x 4.6 mm, 5 µm i.d. (Phenomenex), the flow rate was 1 mL/min, the column temperature 30°C and the injection volume 20 µL. Flow rate was split 1/10 before MS interface. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43% B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 190–700 nm, and for quantitative determinations the chromatograms were recorded at 285 nm (flavanones), 310 nm (furocoumarins), 335 nm (polymethoxylated flavones) and 340 nm (flavones). Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative and positive ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 4.0 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 12 (arbitrary units). The MS-MS spectra were obtained using collision energy of 25% of instrument maximum, operating in selected reaction monitoring (SRM). Preliminary positive and negative tunings were carried out with continuous introduction of dilute solutions of sinensetin and naringin, respectively, at a flow rate of 5 µL/min and the voltages on the lenses were optimized in TunePlus (Excalibur software).

The chinotto polyphenolics were characterized in terms of retention times, lambda max, MS and MS/MS data operating in positive and negative mode as follows:

Compound **1**: Rt, 16.9 min; UV, 270, 350 nm. MS, 611 [M + H]$^+$ (100); MS, 609 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 611 [M + H]$^+$ (20), 593 [M + H-18]$^+$ (100), 575 [M + H - 36]$^+$ (10), 545 [M + H - 66]$^+$ (5), 491 [M + H - 120]$^+$ (15), 473 [M + H - 138]$^+$ (15).
Compound **2**: Rt, 23.5 min; UV, 270, 335 nm. MS, 595 [M + H]$^+$ (100); MS, 593 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 595 [M + H]$^+$ (20), 577 [M + H-18]$^+$ (100), 559 [M + H - 36]$^+$ (10), 529 [M + H - 66]$^+$ (5), 475 [M + H - 120]$^+$ (15), 457 [M + H - 138]$^+$ (10).
Compound **3**: Rt, 26.3 min; UV, 270, 350 nm. MS, 625 [M + H]$^+$ (100); MS, 623 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 625 [M + H]$^+$ (70), 607 [M + H -18]$^+$ (100), 559 [M + H - 66]$^+$ (5), 505 [M + H - 120]$^+$ (8), 463 [M + H - 162]$^+$ (70), 445 [M + H -180]$^+$ (8).
Compound **4**: Rt, 29.9 min; UV, 285, 330 nm. MS, 597 [M + H]$^+$ (100); MS, 595 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 597 [M + H]$^+$ (100), 451 [M + H - 146]$^+$ (40), 289 [M + H - 308]$^+$ (80).
Compound **5**: Rt, 31.8 min; UV, 285, 330 nm. MS, 597 [M + H]$^+$ (100); MS, 595 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 597 [M + H]$^+$ (100), 451 [M + H - 146]$^+$ (65), 289 [M + H - 308]$^+$ (90).
Compound **6**: Rt, 37.4 min; UV, 285, 330 nm. MS, 581 [M + H]$^+$ (100); MS, 579 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 581 [M + H]$^+$ (10), 563 [M + H-18]$^+$ (30), 545 [M + H - 36]$^+$ (25), 435 [M + H - 146]$^+$ (65), 419 [M + H - 162]$^+$ (100), 315 [M + H - 266]$^+$ (25), 271 [M + H - 308]$^+$ (10).
Compound **7**: Rt, 40.0 min; UV, 265, 335 nm. MS, 579 [M + H]$^+$ (100); MS, 577 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 579 [M + H]$^+$ (50), 432 [M + H-146]$^+$ (50), 273 [M + H - 308]$^+$ (100).
Compound **8**: Rt, 40.4 min; UV, 285, 330 nm. MS, 611 [M + H]$^+$ (100); MS, 609 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 611 [M + H]$^+$ (50), 593 [M + H-18]$^+$ (30), 575 [M + H - 36]$^+$ (30), 490 [M + H - 120]$^+$ (15), 449 [M + H - 162]$^+$ (80), 345 [M + H - 266]$^+$ (20), 303 [M + H - 308]$^+$ (70).
Compound **9**: Rt, 42.2 min; UV, 255, 340 nm. MS, 609 [M + H]$^+$ (100); MS, 607 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 609 [M + H]$^+$ (50), 463 [M + H -146]$^+$ (40), 302 [M + H- 308]$^+$ (100). MS-MS focused on [M - H]$^-$, 607 [M - H]$^-$ (40), 299 [M – H - 308]$^-$ (100).
Compound **10**: Rt, 46.2 min; UV, 285, 330 nm. MS, 725 [M + H]$^+$ (100); MS, 723 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 725 [M + H]$^+$ (5), 707 [M + H -18]$^+$ (100), 461 [M + H- 264]$^+$ (90), 273 [M + H - 452]$^+$ (10). MS-MS focused on [M - H]$^-$, 723 [M - H]$^-$ (100), 661 [M – H - 62]$^-$ (10), 621 [M – H - 102]$^-$ (20), 579 [M – H - 144]$^-$ (80).
Compound **11**: Rt, 48.2 min; UV, 285, 325 nm. MS, 755 [M + H]$^+$ (100); MS-MS focused on [M + H]$^+$, 755 [M + H]$^+$ (100), 609 [M + H- 146]$^+$ (30), 301 [M + H- 452]$^+$ (50); MS-MS focused on [M - H]$^-$, 753 [M - H]$^-$ (100); 691 [M + H - 62]$^-$ (10); 651 [M + H - 102]$^-$ (15); 609 [M + H - 144]$^-$ (40).
Compound **12**: Rt, 50.7 min; UV, 285, 330 nm. MS, 595 [M + H]$^+$ (100); MS, 593 [M - H]$^-$ (100). MS-MS focused on [M + H]$^+$, 595 [M + H]$^+$ (20), 577 [M + H-18]$^+$ (30), 559 [M + H-36]$^+$ (40), 449 [M + H-146]$^+$ (70), 433 [M + H-162]$^+$ (100), 287 [M + H-308]$^+$ (25). MS-MS focused on [M - H]$^-$, 593 [M - H]$^-$ (10), 473 [M – H-120]$^-$ (30), 431 [M – H-162]$^-$ (15), 327 [M – H-266]$^-$ (30), 285 [M – H-308]$^-$ (100).
Compound **13**: Rt, 57.4 min; UV, 260, 315 nm. MS, 217 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 217 [M + H]$^+$ (10), 202 [M + H - 30]$^+$ (100).
Compound **14**: Rt, 59.3 min; UV, 260 (sh), 310 nm. MS, 355 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 355 [M + H]$^+$ (30), 337 [M + H - 18]$^+$ (100).
Compound **15**: Rt, 61.3 min; UV, 335 nm. MS, 373 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 373 [M + H]$^+$ (100), 358 [M + H - 15]$^+$ (50), 312 [M + H - 61]$^+$ (30).
Compound **16**: Rt, 62.0 min; UV, 335 nm. MS, 403 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 403 [M + H]$^+$ (60), 388 [M + H - 15]$^+$ (100), 373 [M + H - 30]$^+$ (50).
Compound **17**: Rt, 62.8 min; UV, 335 nm. MS, 433 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 434 [M + H]$^+$ (60), 419 [M + H - 15]$^+$ (100), 403 [M + H - 30]$^+$ (50).
Compound **18**: Rt, 63.6 min; UV, 335 nm. MS, 373 [M + H]$^+$ (100). MS-MS focused on [M + H]$^+$, 373 [M + H]$^+$ (40), 358 [M + H - 15]$^+$ (100), 343 [M + H - 30]$^+$ (40).

The external calibrations were obtained using methanolic standard solutions of known concentration (1-100 mg/L). Lucenin-2 and lucenin-2 4'-methyl ether were expressed as neodiosmin; vicenin-2 and rhoifolin as apigenin; meltidin and brutieridin as naringin and neohesperidin, respectively; bergapten and epoxybergamottin in terms of bergamottin relative amount; and nobiletin, heptamethoxylflavone and tangeretin in terms of sinensetin relative amount.

The total flavonoid content was also determined in all of the samples according to the colorimetric method of Davis [16] and the values were expressed as mg naringin/100 g FW (fresh weight).

Statistical analysis: To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where appropriate, the data were tested by two-way ANOVA. $P<0.05$ was adopted as statistically significant. All data are means of 5 measurements.

References

[1] Patil BS, Jayaprakasha GK, Murthy KNC, Vikram A. (**2009**) Bioactive compounds: historical perspectives, opportunities, and challenges. *Journal of Agricultural and Food Chemistry*, ***57***, 8142–8160.
[2] Shahidi F, Marian N. (**2003**) *Phenolics in Food and Nutraceuticals*, CRC Press, Boca Raton.

[3] Tripoli E, La Guardia M, Giammanco S, Di Majo D, Giammanco M. (**2007**) Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry*, ***104***, 466–479.
[4] Cao G, Sofic E, Prior R. (**1997**) Antioxidant and prooxidant behavior of flavonoids: Structure–activity relationships. *Free Radical Biology and Medicine*, ***22***, 749–760.
[5] Rice-Evans C, Miller NJ, Paganda G. (**1996**) Structure–antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical and Medicine*, ***20***, 933–956.
[6] Scordino M, Sabatino L, Belligno A, Gagliano G. (**2011**) Preliminary study on bioactive compounds of *Citrus* x *myrtifolia* Rafinesque (Chinotto) to its potential application in food industry. *Food and Nutritional Sciences*, ***2***, 685-691.
[7] Hanelt P, Büttner R, Mansfeld, R. (**2001**) *Mansfeld's encyclopedia of agricultural and horticultural crops (except ornamentals)*. Springer, Germany.
[8] Cautela D, Pirrello AG, Espositom C, Minasi P. (**2004**) Caratteristiche compositive del chinotto (*Citrus myrtifolia*). *Essenze dei Derivati Agrumari*, ***74***, 51-57.
[9] Barreca D, Bellocco E, Caristi C, Leuzzi U, Gattuso G. (**2010**) Flavonoid composition and antioxidant activity of juices from Chinotto (*Citrus* x *myrtifolia* Raf.) fruits at different ripening stages. *Journal of Agricultural and Food Chemistry*, ***58***, 3031-3036.
[10] Scordino M, Sabatino L, Belligno A, Gagliano G. (**2011**) Flavonoids and furocoumarins distribution of unripe chinotto (*Citrus* x *myrtifolia* Rafinesque) fruit: beverage processing homogenate and juice characterization. *European Food Research and Technology*, ***233***, 759-767.
[11] Barreca D, Bellocco E, Caristi C, Leuzzi U, Gattuso G. (**2011**) Distribution of C- and O-glycosyl flavonoids, (3-hydroxy-3-methylglutaryl)glycosyl flavanones and furocoumarins in *Citrus aurantium* L. juice. *Food Chemistry*, ***124***, 576−582.
[12] Gattuso G, Barreca D, Caristi C, Gargiulli C, Leuzzi U. (**2007**) Distribution of flavonoids and furocoumarins in juices from cultivars of *Citrus bergamia* Risso. *Journal of Agricultural and Food Chemistry*, ***55***, 9921−9927.
[13] Caristi C, Bellocco E, Gargiulli E, Toscano G, Lezzi E. (**2006**) Flavone-di-C-glycosides in citrus juices from Southern Italy. *Food Chemistry*, ***95***, 431–437.
[14] Miyake Y, Yamamoto K, Morimitsu Y, Osawa T. (**1997**) Isolation of C-glucosylflavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. *Journal of Agricultural and Food Chemistry*, ***45***, 4619–4623.
[15] Scordino M, Sabatino L, Traulo P, Gargano M, Panto´ V, Gagliano G. (**2011**) HPLC–PDA/ESI–MS/MS detection of polymethoxylated flavones in highly degraded citrus juice: a quality control case study. *European Food Research and Technologies*, ***232***, 275–280.
[16] Davis WB. (**1947**) Determination of flavanones in citrus fruits. *Analytical Chemistry*, ***19***, 476–477.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1863 - 1872

Bioactive Compounds, RP-HPLC Analysis of Phenolics, and Antioxidant Activity of Some Portuguese Shrub Species Extracts

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Received: August 12th, 2011; Accepted: October 24th, 2011

In the ecosystem of Serra Da Estrela, some plant species have the potential to be used as raw material for extraction of bioactive products. The goal of this work was to determine the phenolic, flavonoid, tannin and alkaloid contents of the methanolic extracts of some shrubs (*Echinospartum ibericum*, *Pterospartum tridentatum*, *Juniperus communis*, *Ruscus aculeatus*, *Rubus ulmifolius*, *Hakea sericea*, *Cytisus multiflorus*, *Crataegus monogyna*, *Erica arborea* and *Ipomoea acuminata*), and then to correlate the phenolic compounds and flavonoids with the antioxidant activity of each extract. The Folin-Ciocalteu's method was used for the determination of total phenols, and tannins were then precipitated with polyvinylpolypyrrolidone (PVPP); a colorimetric method with aluminum chloride was used for the determination of flavonoids, and a Dragendorff's reagent method was used for total alkaloid estimation. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene bleaching tests were used to assess the antioxidant activity of extracts. The identification of phenolic compounds present in extracts was performed using RP-HPLC. A positive linear correlation between antioxidant activity index and total phenolic content of methanolic extracts was observed. The RP-HPLC procedure showed that the most common compounds were ferulic and ellagic acids and quercetin. Most of the studied shrubs have significant antioxidant properties that are probably due to the existence of phenolic compounds in the extracts. It is noteworthy to emphasize that for *Echinospartum ibericum*, *Hakea sericea* and *Ipomoea acuminata*, to the best of our knowledge, no phytochemical studies have been undertaken nor their use in traditional medicine been described.

Keywords: Phenolic compounds, flavonoids, antioxidant activity, vegetal extracts, RP-HPLC.

The Portuguese forest area occupies around 3.3 million hectares, which represents about 38% of the territory. The Serra Da Estrela Mountain supports a rich plant cover constituting the crown of Portugal's interior ecological network**.** In this ecosystem grow numerous species of trees and shrubs. Examples of shrubs that grow spontaneously in Portuguese forests in general and in this region in particular are *Echinospartum ibericum* Rivas Mart., Sánchez-Mata & Sancho, *Pterospartum tridentatum* (L.) Willk subsp. *cantabricum* Spach, *Juniperus communis* L. subsp. *alpina* (Suter) Celak., *Ruscus aculeatus* L., *Rubus ulmifolius* Schott, *Hakea sericea* Schrader, *Cytisus multiflorus* (L'Hér) Sweet, *Crataegus monogyna* Jacq., *Erica arborea* L., and *Ipomoea acuminata* (Vahl) Roemer & Schultes (*I. indica* Burm.).

The genus *Echinospartum* (Spach) Fourr. (family Fabaceae), mostly endemic to the Iberian Peninsula, is composed of both silicicolous and calcicolous taxa, growing in mountain areas usually above 1000 meters above sea level [1]. The authors are not aware of any known application of these species in folk medicine.

Pterospartum tridentatum (Fabaceae; subfamily Papilionoideae) grows spontaneously in Portugal, where it is known as Carqueja or Carqueija. The flowers are used in traditional medicine for the treatment of throat irritation conditions and in herbal mixtures for diabetes [2]. No phytochemical, pharmacological and toxicological studies have been reported for this species, but alkaloids and isoflavonoids are characteristic secondary metabolites of Fabaceae species [2].

Juniperus communis or common juniper is a coniferous shrub distributed throughout the Arctic and temperate zone of the northern hemisphere. Its dried bluish-black cones, known as "juniper berries", are said to stimulate the appetite and are used as a flavoring agent for culinary purposes and in the preparation of gin spirits [3]. They have also been used for various medicinal purposes, including as an abortifacient, contraceptive, diuretic, and as a remedy for urinary tract infections, chest complaints, diabetes, rheumatism and backache. More interestingly, juniper has been reported as a traditional cure for chest troubles such as bronchitis and for tuberculosis [3].

An alcoholic infusion of the rhizomes of *Ruscus aculeatus* has been used for the treatment of some veinous ailments for decades [4].

Leaves and young shoots of *Rubus ulmifolius* are used in folk medicine for their anti-inflammatory, anti-odontalgic and gastrointestinal spasmolytic properties; crushed young shoots are applied to wounds, infected insect bites and pimples [5].

A key characteristic of *Hakea sericea* is its extreme serotinous habit: all of its seeds are retained in pairs in tough woody follicles, which accumulate along the branches throughout the life of the plant [6]. As far as we know, neither medicinal uses nor phytochemical studies are described to this shrub.

Cytisus spp. is used as a diuretic, hypnotic, sedative, anti-diabetic and also as a hepatoprotective [7].

Crataegus monogyna has been used as a folk medicine due its sedative actions, protective effects against arrhythmias and increase of coronary vessel flow [8]. Previous chemical studies on *C. monogyna* resulted in isolation of know flavonoids and phenolic acids [8].

Erica multiflora is used to treat hyperlipidaemia, and in folk medicine as a diuretic and antiseptic agent [9]. The phytochemical study of this plant showed that tannins, proanthocyanidols and flavonoids represent major compounds of its flowers [9].

Ipomoea species accumulate high levels of anthocyanin pigments in the storage root [10]. To the best of our knowledge, no medical applications are described for this plant.

Several constituents of plant extracts have been shown to have antioxidant activity, such as ascorbic acid, tocopherol, β-carotene, flavonoids, tannins, phenolics, and anthocyanins [11]. Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, reactive oxygen species (ROS) are often over-produced under pathological conditions, resulting in oxidative stress. An over-production of various forms of activated oxygen species, such as free-radical and non-free-radical species is involved in the onset of many diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, and atherosclerosis, as well as in degenerative processes associated with aging [11]. In order to reduce damage to the human body, synthetic antioxidants are used in industrial processing. However, the presence of unwanted side effects is almost unavoidable, and some have been suspected of being responsible for liver damage and carcinogenesis [11]. Thus, it is essential to develop antioxidants that can protect the human body from free radicals and retard the progress of many chronic diseases.

No single chemical component is responsible for the medicinal properties of plant-based drugs, and their synergic action or bioenhancement is due to the presence of several chemical substances in the plant material. Therefore, the determination of the total amount of different classes of components is essential for the standardization of the plants [12].

The purpose of this work was to determine the phenolic, flavonoid, tannin and total alkaloid contents of the methanolic extracts of the species above mentioned, and then to correlate phenolic compounds and flavonoids with antioxidant activity of corresponding extracts. The Folin-Ciocalteu's method was used for the determination of total phenols, and tannins were then precipitated with polyvinylpolypyrrolidone (PVPP); a colorimetric method with aluminum chloride was used for the determination of total flavonoids, and a Dragendorff's reagent method was used for alkaloid estimation. A 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and a β-carotene bleaching test were used to assess the antioxidant activity of extracts. The identification of phenolic compounds present in extracts was performed using a RP-HPLC technique.

From the results presented in Table 1 it can be verified that fruits of *Juniperus communis* had a higher extraction yield than the other shrubs. By contrast, the extract of fruits of *Hakea sericea* produced the lowest extraction yield. The extraction yields obtained in the present study are very similar to those reported for some medicinal plants reported in the literature [13].

Medicinal plants are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. They have multiple biological properties including antioxidant activity. The antioxidant activity of phenolic acids and flavonoids is due to their redox properties, ability to chelate metals and quenching of singlet oxygen [14]. In this sense, it is reasonable to determine the amount of phenolic compounds in the extracts of these plants.

The quantitative determination of phenolic compounds using Folin-Ciocalteu's method is a widespread assay. [15]. As can be observed in Table 2, in general, the extracts from stems and leaves have the highest amount of phenolic compounds. The extract of stems of *Crataegus monogyna* is the one that had the greatest concentration of total phenols. This result is similar to those obtained by other researchers [16]. Barros *et al*. studied the composition and bioactivity of *Crataegus monogyna* and concluded that phenolics were the major antioxidant components present in this shrub [17]. *Ipomoea acuminata* and *Ruscus aculeatus* are species that are poor in these compounds. Many studies indicate that the roots of sweet potato (*Ipomoea batatas*) are a source of bioactive compounds [18]. In previous studies by our group, we concluded that the ethanolic extract of *Cytisus* spp. had more phenolics than that of the aqueous extract [19]. Methanolic extracts of the different parts of *Cytisus multiflorus* have significant amounts of total phenols. *Pterospartum tridentatum* and *Erica arborea* are also species rich in these compounds. Fruits of *Rubus*

Table 1: Extraction yields of methanolic extracts.

Species	Plant part	Extraction yield (%) *
Echinospartum ibericum	Stems and Leaves	21.1 ± 0.1
	Flowers	19.7 ± 0.4
Pterospartum tridentatum	Stems and Leaves	26.1 ± 1.7
	Flowers	25.1 ± 1.4
Juniperus communis	Stems	15.6 ± 2.1
	Leaves	31.4 ± 2.0
	Fruits	45.7 ± 4.8
Ruscus aculeatus	Stems and Leaves	18.3 ± 3.5
Rubus ulmifolius	Stems	9.9 ± 1.0
	Leaves	23.8 ± 5.6
	Flowers	22.6 ± 0.7
	Fruits	27.5 ± 2.1
Hakea sericea	Stems	11.0 ± 0.1
	Leaves	16.4 ± 2.4
	Fruits	3.2 ± 0.1
Cytisus multiflorus	Stems	10.1 ± 0.6
	Leaves	15.5 ± 1.5
	Flowers	27.6 ± 2.7
	Fruits	25.0 ± 1.1
Crataegus monogyna	Stems	13.1 ± 1.7
	Leaves	23.1 ± 3.1
	Flowers	23.5 ± 9.9
	Fruits	10.2 ± 1.5
Erica arborea	Stems	12.9 ± 0.1
	Leaves	29.5 ± 1.8
	Flowers	38.6 ± 0.7
Ipomoea acuminata	Stems	9.3 ± 1.7
	Leaves	12.0 ± 0.3
	Flowers	14.4 ± 5.4

* Results in terms of mean ± standard deviation

ulmifolius are the part of this shrub that has less phenolic compounds, in contrast with stems, leaves and flowers. Other studies [20] demonstrated that this shrub had a lower concentration of phenols that the one now estimated. There are no literature data for *Echinospartum ibericum* and *Hakea sericea* phenol contents, but in this work they were shown to be rich in these compounds. Öztürk *et al.* conducted an exhaustive study of several species of Turkish juniper, and concluded that *Juniperus communis* is rich in bioactive compounds, and that methanol is one of the best solvents for their extraction [21]. Regarding the determination of tannins (Table 2), it can be concluded that the composition varies in the different extracts (it was not possible to observe a trend). Significant differences were also found in the tannin content among different parts of the same shrub, which is in agreement with the behavior described for tannins in plants.

Flavonoids are polyphenolic compounds that occur ubiquitously in plants and structurally have variations in one of the aromatic rings that characterize the different types, namely, flavonols, flavones, isoflavones, flavonones, flavanol and anthocyanins [14,22]. Flavonoids could act as antioxidants because they can readily donate an electron or a hydrogen atom to a peroxyl or alkoxy radical to terminate a lipid peroxidation chain reaction or to regenerate a phenolic/flavonoid compound, which can effectively chelate a pro-oxidant transition metal [22]. The process used in quantitative methods for flavonoid determination is performed by precipitating them with aluminum chloride in an alkalinized medium. This precipitate acquires a color and, therefore, lends itself to the colorimetric assay. The flavonoids in the presence of aluminum chloride have an intense yellow fluorescence when observed under filtered UV light [23]. As can be seen in Figure 1, extracts from flowers and fruits generally have more flavonoids than the extracts of stems and leaves of the same shrub species. However, *Ipomoea acuminata* had the highest flavonoids content of the tested species, especially the extract of leaves. The results obtained for the studied species are very similar to those of our previous studies for other Portuguese shrubs [19]. Andrade *et al*., have studied the bioactivity of *Cistus ladanifer* and *Arbutus unedo* extracts, and the flavonoids contents were very similar to those presented here [24]. In the present study, *Erica arborea*, *Cytisus multiflorus*, *Rubus ulmifolius* and *Pterospartum tridentatum* are noted for being species with large concentrations of flavonoids. There are no previous studies in the literature about *Hakea sericea* and *Echinospartum ibericum* on the characterization of flavonoids; however, large amounts of these compounds were detected in all these species. Previous research on flavonoids of various species of juniper showed that this shrub could be a source of these biologically active compounds [25]. The results now obtained showed that the leaves of this species are the richest part of the plant in these compounds. Methanol was shown to be a good solvent for flavonoids, as its high polarity favors the extraction of compounds with aromatic rings possessing hydroxyl groups.

Alkaloids are responsible for the therapeutic effect of many plant materials, but several alkaloids are toxic. Over 200 alkaloids have been identified in 300 plant species of up to 13 families. It has been estimated that up to 3% of the world's flowering plants contain toxic alkaloids [26]. The alkaloids were precipitated as BiI_3(Alk·HI) by Dragendorff's reagent ($KBiI_4$). Bismuth forms a yellow bismuth complex $\{Bi[CS(NH_2)_3]\}(NO_3)_3$ in nitric acid medium with thiourea. The bismuth from the alkaloidal complex is completely released by disodium sulfide [12].

Looking at Figure 1 it could be noted that all studied extracts have significant amounts of alkaloids. *Cytisus multiflorus* is the richest species in these compounds, specially the extracts of the stems. This species is known to possess alkaloids, namely spartein, with anti-arrhythmic properties [7]. The extract of leaves of *Ipomoea acuminata* also has a high concentration of alkaloids. By contrast, *Juniperus communis* is the poorest species in alkaloids. The extract of the leaves of that shrub is the one that has the smallest concentration of alkaloids.

One problem in assessing antioxidant activity is that this activity is variable and depends on the method used. It is known that an antioxidant mechanism in various biological matrices is very complex and several factors may intervene [27]. Given this complexity, only one method to determine the antioxidant activity of the extracts is not sufficient to draw relevant conclusions, and thus, we have applied two different methods and determined diverse antioxidant properties.

Table 2: Total phenolic compounds and tannin contents of methanolic extracts.

Species	Plant part	Total phenolic compounds (mg GAE / g dry matter) *	Tannin content (mg GAE / g dry matter)
Echinospartum ibericum	Stems and Leaves	99.1 ± 1.5	26.3
	Flowers	71.9 ± 0.8	N/D
Pterospartum tridentatum	Stems and Leaves	113.6 ± 1.5	43.4
	Flowers	171.4 ± 0.7	55.3
Juniperus communis	Stems	221.3 ± 2.5	79.3
	Leaves	155.6 ± 3.2	60.4
	Fruits	44.7 ± 2.6	N/D
Ruscus aculeatus	Stems and Leaves	32.9 ± 0.3	N/D
Rubus ulmifolius	Stems	186.8 ± 3.8	0.5
	Leaves	167.6 ± 6.1	21.9
	Flowers	291.4 ± 0.4	132.7
	Fruits	54.5 ± 0.6	6.8
Hakea sericea	Stems	267.6 ± 5.9	74.8
	Leaves	217.0 ± 2.7	103.5
	Fruits	110.1 ± 2.7	N/D
Cytisus multiflorus	Stems	142.4 ± 2.0	11.1
	Leaves	176.1 ± 3.6	61.5
	Flowers	120.4 ± 0.9	21.1
	Fruits	155.1 ± 5.6	20.6
Crataegus monogyna	Stems	377.4 ± 4.5	134.0
	Leaves	225.5 ± 2.5	81.1
	Flowers	186.2 ± 2.7	86.5
	Fruits	55.3 ± 2.7	N/D
Erica arborea	Stems	270.2 ± 4.3	133.8
	Leaves	260.2 ± 1.9	92.5
	Flowers	178.1 ± 0.2	83.0
Ipomoea acuminata	Stems	23.5 ± 5.1	N/D
	Leaves	32.2 ± 1.3	N/D
	Flowers	84.3 ± 1.7	N/D

* Results in terms of mean ± standard deviation; N/D – Not detected.

Results for the DPPH radical scavenging assay are reported as IC_{50}, values, which are defined as the amount of antioxidant required to inhibit 50% of DPPH free radicals under the experimental conditions [28]. Table 3 shows that *Echinospartum ibericum*, *Ruscus aculeatus* and *Ipomoea acuminata* had the poorest antioxidant activity in this assay, whereas that of stems of *Crataegus monogyna* had both the greatest concentration of total phenols and, simultaneously, the greatest antioxidant activity. In contrast, *C. monogyna* fruits had only moderate antioxidant activity. Other researchers demonstrated that the ethanolic extract of berries of *C. monogyna* possessed DPPH free-radical-scavenging activity [29]. All *Erica arborea* and *Hakea sericea* extracts had very strong antioxidant activity. As far as we know, no medicinal uses are described for this latter shrub and no previous phytochemical studies have been carried out on it, but it is rich in phenols with antioxidant activity. The results obtained for the parameters of antioxidant activity for the standard compounds are very similar to those determined by the same method by Faustino *et al*., and Scherer and Godoy [30,31].

The β-carotene/linoleic acid bleaching assay is one of the antioxidant assays suitable for plant samples [28,32]. All the shrub extracts tested presented some degree of antioxidant properties when compared with the synthetic antioxidant BHT. The extracts of *Echinospartum ibericum* (Figure 2) showed a high degree of inhibition of lipid oxidation (linoleic acid) and sequestration of radicals resulting from its possible oxidation, when compared with the extracts of *Pterospartum tridentatum* and *Hakea sericea* (Figure 2).

In contrast with the results of the DPPH scavenging assay, the extracts of *Echinospartum ibericum* had no significant ability to sequester free radicals such as DPPH, but displayed a high degree of inhibition of lipid oxidation, which may predict a potential use as a food antioxidant. The extract of *Pterospartum tridentatum* flowers showed the weakest inhibition of lipid oxidation. *Rubus ulmifolius* (Figure 3) showed the highest antioxidant potential measured by this method. By contrast, *Ipomoea acuminata* (Figure 3) had no capacity to promote inhibition of lipid oxidation. Stems of *Juniperus communis* (Figure 4) is the part of this shrub that provided higher percentages of inhibition, and the extracts of leaves and fruits presented antioxidant activity similar to that of the extract of *Ruscus aculeatus* (Figure 4).

Other studies [21] undertaken with juniper species allow us to conclude that in the β-carotene-linoleic acid assay, oxidation of linoleic acid was effectively inhibited by methanol extracts of various species of *Juniperus*, which is in agreement with the results now obtained. *Erica arborea*, *Cytisus multiflorus* and *Crataegus monogyna* were grouped in the same graph (Figure 5) because they have very similar antioxidant activity. It should be noted the extract of flowers of *Cytisus multiflorus* (Figure 5) presented percentages of inhibition slightly higher than those of the other extracts.

According to Figure 6, there was a positive linear correlation between antioxidant activity index and total phenolic content of methanolic extracts (R^2=0.8442). These results indicate that the phenolic compounds could be the main contributor to the antioxidant properties of these shrubs. This result is in agreement with several previous studies [19,33]. For example Fu *et al*. studied the antioxidant activity and phenolic compounds of 56 wild fruits from South China and found that the results showed a positive linear correlation between the antioxidant capacities and total phenolic content (R^2>0.86) [33]. The same analysis was conducted for the flavonoids (Figure 6) and it was verified that there was no correlation (R^2=0.0083) between these compounds and the antioxidant activity of the extracts. It is known that only flavonoids of a certain structure and particular hydroxyl position in the molecule determine the antioxidant properties; in general these properties depend on the ability to donate either hydrogen or an electron to a free radical [34].

RP-HPLC analysis was employed to identify major phenolic compounds in the methanolic shrub extracts. Satisfactory separation with good resolution could be achieved with the method now used. Such data will be helpful to compare the antioxidant activities with the

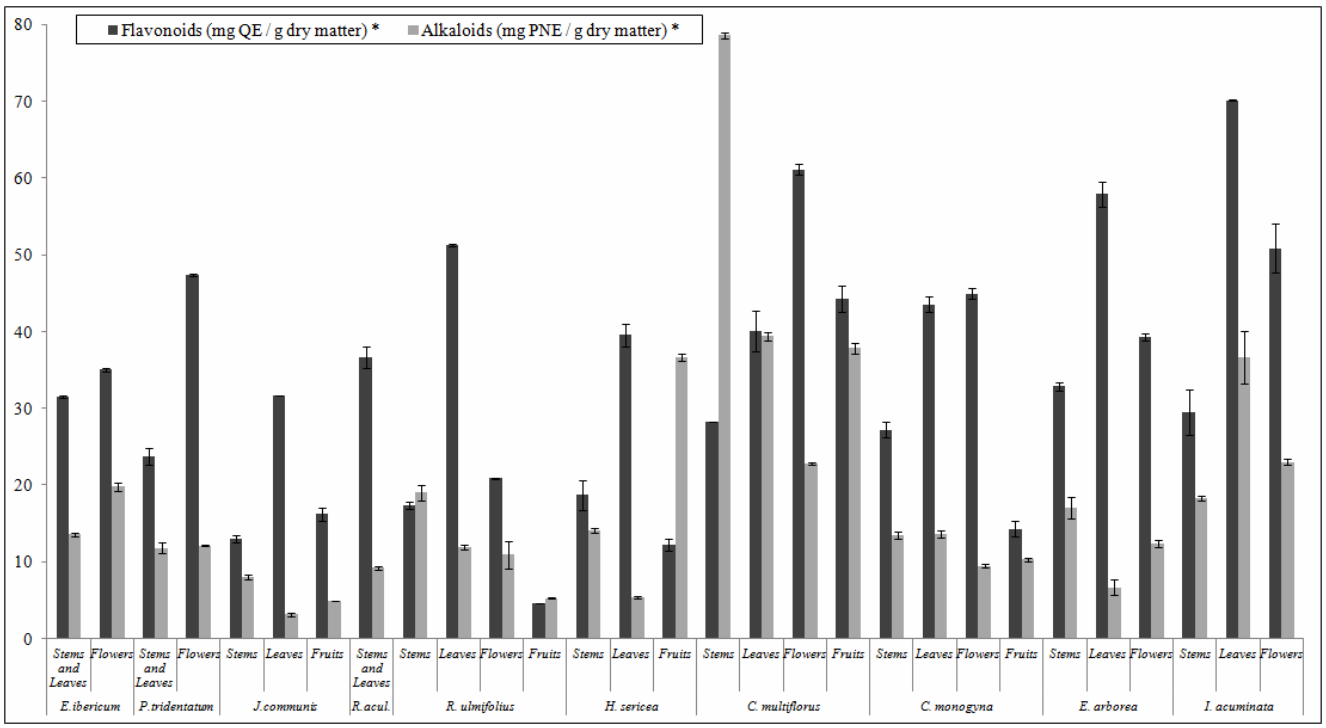

Figure 1: Concentration of flavonoids and alkaloids in studied extracts (* Results in terms of mean ± standard deviation).

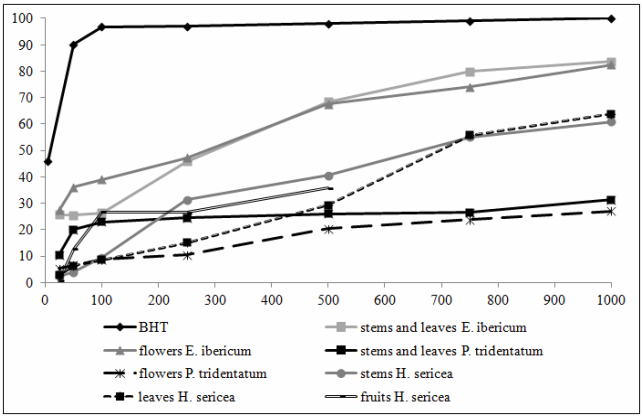

Figure 2: Antioxidant activity of the extracts of *Echinospartum ibericum*, *Pterospartum tridentatum* and *Hakea sericea* measured by β-carotene bleaching test.

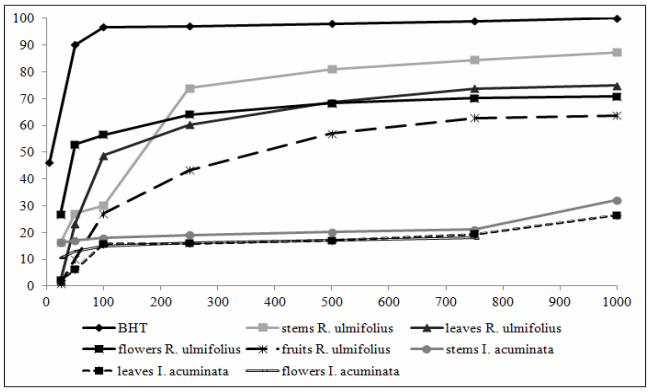

Figure 3: Antioxidant activity of the extracts of *Rubus ulmifolius* and *Ipomoea acuminata* measured by β-carotene bleaching test.

phenolic compounds of the different studied shrubs and will be also useful to understand their chemical constituents and functionality. RP-HPLC analysis is the most used method to identify plant phenolics. Because of the diversity and complexity of natural phenolic compounds in hundreds of medicinal plant extracts, it is rather difficult to characterize every compound and elucidate its structure. It is not difficult, however, to identify major categories of phenolic compounds and representative phenolics. In the present study, we identified representative phenolic compounds from selected plants. A total of nine phenolic compounds were identified and quantified in the methanolic extracts of the shrubs (Table 4), including hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. The total amount of phenolic compounds in all the extracts was 1380.8 mg/g of dry matter. Gallic acid is one of the most important hydroxybenzoic acids, but, surprisingly, in the extracts now analyzed, it was present in low amounts (1.26%). However, the hydroxycinnamic acids identified (caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid) were abundantly present. Ferulic acid was the most dominant hydroxycinnamic acid, taking a percentage of occurrences of 29.0 %. These results concerning phenolic acids are in agreement with previously published data for other biological samples [35]. Ellagic acid and quercetin are also present in large amounts, with percentages of occurrence of 17.9% and 13.7%, respectively. Vanillic acid is the phenolic compound that is present in greater quantity in the extract of the stems and leaves of *Pterospartum tridentatum*, having a concentration of 32.2 mg/g of dry matter, corresponding to a percentage of 27.6 %. *Juniperus*

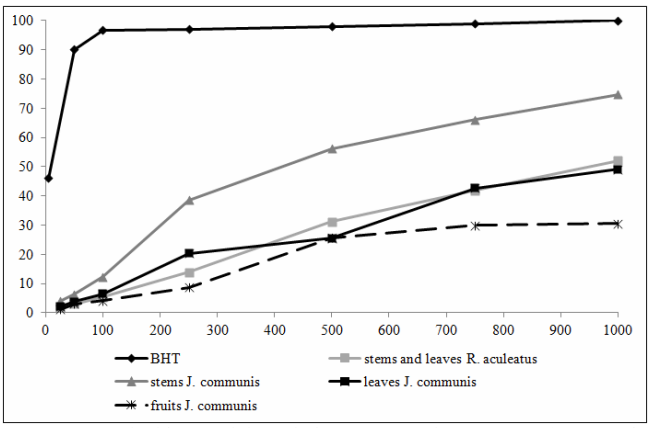

Figure 4: Antioxidant activity of the extracts of *Ruscus aculeatus* and *Juniperus communis* measured by β-carotene bleaching test.

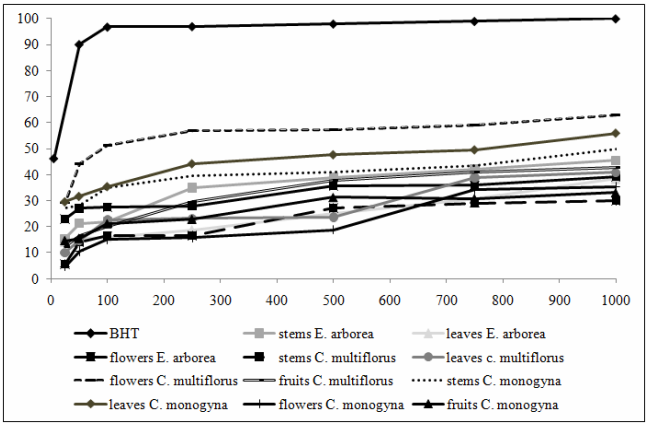

Figure 5: Antioxidant activity of the extracts of *Erica arborea*, *Cytisus multiflorus* and *Crataegus monogyna* measured by β-carotene bleaching test.

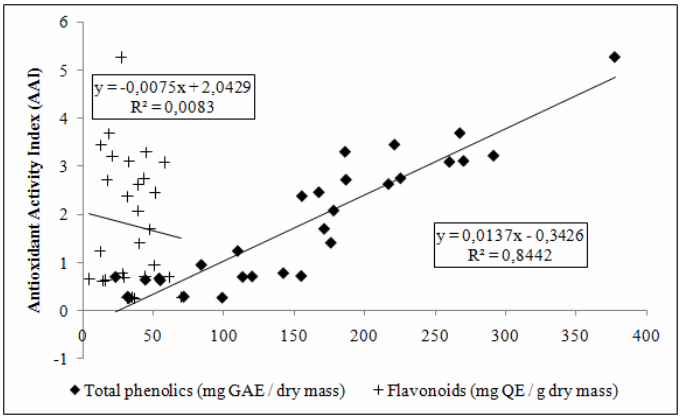

Figure 6: Correlation between antioxidant activity and total phenolics and flavonoids of extracts.

communis, *Ruscus aculeatus*, *Hakea sericea* and *Ipomoea acuminata* are species relatively poor in phenolic compounds. A total of 34.5% of the analyzed phenols of the extract of *Rubus ulmifolius* leaves correspond to ferulic acid, with a concentration of 18.5 mg/g dry matter. In the extract of fruits of the same species, chlorogenic acid is the predominant compound. *Cytisus multiflorus* is rich in ferulic acid and ellagic acid, especially its leaves, flowers and fruits. The extract of stems of *Erica arborea* presents a large amount of ferulic acid, while the extracts of flowers

Table 3: Antioxidant properties of methanolic extracts studied and standard compounds.

Species / Standard	Plant part	IC_{50} (mg/L) *	AAI *	Antioxidant Activity
Echinospartum ibericum	Stems and Leaves	162.7 ± 13.8	0.3 ± 0.02	Poor
	Flowers	125.3 ± 12.4	0.3 ± 0.02	Poor
Pterospartum tridentatum	Stems and Leaves	69.7 ± 11.9	0.7 ± 0.06	Moderate
	Flowers	26.1 ± 1.3	1.7 ± 0.06	Strong
Juniperus communis	Stems	12.4 ± 0.3	3.4 ± 0.07	Very Strong
	Leaves	16.8 ± 0.6	2.4 ± 0.07	Very Strong
	Fruits	81.1 ± 6.9	0.6 ± 0.05	Moderate
Ruscus aculeatus	Stems and Leaves	171.9 ± 21.1	0.3 ± 0.03	Poor
Rubus ulmifolius	Stems	14.7 ± 1.2	2.7 ± 0.2	Very Strong
	Leaves	17.3 ± 1.2	2.5 ± 0.4	Very Strong
	Flowers	11.0 ± 0.5	3.2 ± 0.2	Very Strong
	Fruits	78.8 ± 5.9	0.7 ± 0.04	Moderate
Hakea sericea	Stems	9.5 ± 0.1	3.7 ± 0.03	Very Strong
	Leaves	13.4 ± 0.4	2.6 ± 0.06	Very Strong
	Fruits	28.3 ± 1.8	1.2 ± 0.07	Strong
Cytisus multiflorus	Stems	64.9 ± 3.2	0.8 ± 0.05	Moderate
	Leaves	35.1 ± 1.4	1.4 ± 0.05	Strong
	Flowers	71.5 ± 8.8	0.7 ± 0.09	Moderate
	Fruits	77.0 ± 8.6	0.7 ± 0.07	Moderate
Crataegus monogyna	Stems	8.5 ± 0.2	5.3 ± 0.1	Very Strong
	Leaves	16.5 ± 0.3	2.7 ± 0.05	Very Strong
	Flowers	13.6 ± 0.2	3.3 ± 0.05	Very Strong
	Fruits	86.5 ± 12.5	0.6 ± 0.06	Moderate
Erica arborea	Stems	14.6 ± 1.5	3.1 ± 0.3	Very Strong
	Leaves	10.9 ± 0.1	3.1 ± 0.04	Very Strong
	Flowers	16.1 ± 0.7	2.1 ± 0.1	Very Strong
Ipomoea acuminata	Stems	77.3 ± 6.0	0.7 ± 0.06	Moderate
	Leaves	204.1 ± 12.8	0.3 ± 0.09	Poor
	Flowers	57.4 ± 1.4	0.9 ± 0.03	Moderate
Rutin	---	10.7 ± 0.4	4.9 ± 0.2	Very Strong
Quercetin	---	4.3 ± 0.4	12.2 ± 1.7	Very Strong
Trolox	---	8.4 ± 0.1	6.6 ± 0.1	Very Strong
Gallic Acid	---	2.2 ± 0.02	22.8 ± 0.3	Very Strong

* Results in terms of mean ± standard deviation

are rich in ellagic acid. The extract of *Crataegus monogyna* flowers has the greatest concentration of ferulic acid (116.8 mg/g, dry matter), which corresponds to a percentage of 75.7%, relatively to the total phenolics quantified in this extract.

Experimental

Plant material: Aerial parts (stems, leaves, flowers and fruits) of *Echinospartum ibericum*, *Pterospartum tridentatum*, *Juniperus communis*, *Ruscus aculeatus*, *Rubus ulmifolius*, *Hakea sericea*, *Cytisus multiflorus*, *Crataegus monogyna*, *Erica arborea* and *Ipomoea acuminata* were collected in Serra Da Estrela. Table 5 shows the GPS coordinates and altitude at which the different shrubs were collected. Plant materials were dried at 35ºC in a ventilated oven during 48 h and reduced to coarse powder (< 2 mm) using a laboratory cutting mill. Harvesting, transport and storage of plant species were authorized by *Instituto da Conservação da Natureza e da Biodiversidade*. All vegetal species were identified by a botanist and a voucher specimen of all species used has been deposited in the Herbarium of the *Instituto Superior de Agronomia* (*Jardim Botânico d'Ajuda*, *Lisboa*). Table 5 lists the full scientific name of the vegetal species and the number of the voucher specimens.

Extraction process: Methanolic extracts were obtained using a Soxhlet apparatus until the solvent became colorless, using approximately 100 g of raw material and 1 L of solvent. The extracts were filtered under vacuum

using a crucible of porosity #2 then distilled under vacuum to remove the solvents to a final volume of 100 mL. Then, 5 mL of each extract was diluted in 45 mL of methanol. Aliquots (5 mL) of the extracts were removed for subsequent evaporation to dryness for the calculation of extraction yield and extract concentration.

Determination of total phenolic compounds: The phenols were determined by Folin-Ciocalteu's colorimetric method. The methanolic solutions of each extract (50 µL) or gallic acid (standard phenolic compound) were mixed with 450 µL of distilled water, and then 2.5 mL of Folin-Ciocalteu's reagent 0.2 N (diluted with distilled water) was added. The mixtures were allowed to stand for 5 mins, and then 2 mL of aqueous Na_2CO_3 (75 g/L) was added. After incubation of these reaction mixtures (90 mins / 30ºC), the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 500, 400, 350, 325, 300, 250, 225, 200, 150, 125, 100 and 50 mg/L solutions of gallic acid in methanol ($y=0.0009x$; $R^2=0.9875$). Total phenol values were expressed as gallic acid equivalents (mg GAE / g of dry mass) [36,37]. The assays were conducted in triplicate.

Tannin content: Tannins were measured as the difference in total phenolics (measured by Folin–Ciocalteu's reagent) before and after treatment with insoluble polyvinylpolypyrrolidone (PVPP), as this polymer binds strongly to tannins [38]. About 1 mL of each methanolic extract was added to 1 mL of an aqueous solution of PVPP (70 mg/mL). After vigorous shaking, the samples remained for 15 mins at 4°C, to develop the tannin-PVPP complex [39,40]. Then the samples were centrifuged for 10 mins at 3000 rpm; the tannins were found in the residue and the free phenols/non-adsorbed phenolics in the supernatant. Total phenols in the supernatant were determined by the Folin-Ciocalteu colorimetric method, as described above, and the concentration of tannins was calculated as the difference between the total phenols and free phenols, and is expressed in their respective units [39,40]. These determinations were made in triplicate.

Flavonoids determination: The aluminum chloride colorimetric method was used for flavonoids determination according to Pourmorad *et al.* [37]. Each methanol extract (500 µL) was separately mixed with 1.5 mL of methanol, 0.1 mL 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. This solution remained at room temperature for 30 mins; the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer. The calibration curve was constructed by preparing 8 quercetin solutions at concentrations ranging from 12.5 to 200 µg/mL in methanol ($y=0.0074x$; $R^2=0.9980$). Total flavonoid values were expressed as quercetin equivalents (mg QE / g of dry mass) [36]. These determinations were made in duplicate.

Alkaloid estimation: The alkaloid estimation was performed by a spectrophotometric method, as described by Sreevidya and Mehotra [12]. Briefly, 10 mL of each crude extract was centrifuged for 10 mins (3000 rpm) to remove residual suspended particles and then 5 mL of the supernatant was mixed with 1 mL of HCl, 0.1 N. Then, 2.5 mL of Dragendorff's reagent was added to the previous mixture, for precipitation and the precipitate was centrifuged for 5 mins (3000 rpm). This precipitate was further washed with 2.5 mL of ethanol. The filtrate was discarded and the residue was then treated with 2.5 mL of disodium sulfide solution (1%, w/v). The brownish black precipitate formed was then centrifuged (5 mins, 3000 rpm). This residue was dissolved in 2 mL of concentrated nitric acid, with warming, if necessary; this solution was diluted to 10 mL in a standard flask with distilled water and 1 mL was then pipetted out and mixed with 5 mL of thiourea solution (3%, w/v). The absorbance of this solution was measured at 435 nm against a blank containing 1 mL of concentrated nitric acid and 2.5 mL of thiourea solution (3%, w/v). The standard curve was prepared using 750, 500, 400, 250, 200, 150 and 100 mg/L solutions of pilocarpine nitrate in HCl, 0.1N ($y=0.0013x-0.2750$; $R^2=0.9957$). Alkaloid contents were expressed as pilocarpine nitrate equivalents (mg PNE / g of dry mass) [12]. These tests were performed in duplicate.

Evaluation of antioxidant activity

DPPH scavenging assay: The antioxidant activity of the extracts and standards was determined by the radical scavenging activity method using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [31]. Briefly, 0.1 mL aliquots of methanolic solutions of the extracts or standards at different concentrations were added to 3.9 mL of a DPPH methanolic solution. Three DPPH solutions were tested, 0.2000, 0.1242 and 0.0800 mM that were prepared by dissolving 39.4, 24.5 and 15.8 mg in 500 mL of methanol, respectively. These concentrations were selected due to the linearity range of DPPH solutions: above 0.2 mM the concentration is very high, and below 0.5 mM the color is very weak having a limited range of absorbance reading. The control sample consisted of a solution of 0.1 mL of methanol mixed with 3.9 mL of DPPH. After a 90 mins incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: $I\ \% = [(Abs_0 - Abs_1) / Abs_0] \times 100$, where Abs_0 was the absorbance of the control and Abs_1 was the absorbance in the presence of the test sample at different concentrations. The IC_{50} was calculated graphically using a calibration curve in the linear range by plotting the extract concentration *vs*. the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows: AAI = (final concentration of DPPH in the control sample - $\mu g.mL^{-1}$) / (IC_{50} - $\mu g.mL^{-1}$) [31]. Thus, the AAI was calculated considering the mass of DPPH and the mass of the tested sample in the reaction, resulting in a constant for each sample, independent of the concentration of DPPH and sample used. In this work, it was considered that shrub

Table 4: Phenolic content of extracts determined by RP-HPLC.

		Standard Phenolic Compounds (mg/g dry matter)*								
Species	**Plant part**	**Gallic Acid**	**Vanillic Acid**	**Caffeic Acid**	**Chlorogenic Acid**	**Syringic Acid**	***p*-Coumaric Acid**	**Ferulic Acid**	**Ellagic Acid**	**Quercetin**
Echinospartum ibericum	Stems and Leaves	1.9 ± 0.07	N/D	N/D	8.1 ± 1.6	15.9 ± 0.2	5.4 ± 0.2	23.8 ± 3.2	6.2 ± 1.8	25.3 ± 0.1
	Flowers	N/D	5.0 ± 1.0	3.8 ± 0.6	8.5 ± 0.4	16.3 ± 3.1	5.8 ± 0.4	13.5 ± 3.9	40.1 ± 2.6	2.2 ± 0.7
Pterospartum tridentatum	Stems and Leaves	N/D	32.2 ± 2.6	18.2 ± 3.0	17.8 ± 0.6	8.3 ± 0.8	16.8 ± 3.2	7.4 ± 0.8	9.7 ± 1.8	6.6 ± 0.3
	Flowers	N/D	4.2 ± 0.9	3.1 ± 0.3	N/D	1.9 ± 0.4	N/D	22.2 ± 0.6	10.4 ± 0.4	9.3 ± 0.7
Juniperus communis	Stems	N/D	N/D	N/D	2.2 ± 0.8	N/D	2.1 ± 0.4	2.0 ± 0.07	2.9 ± 0.2	6.7 ± 0.1
	Leaves	N/D	N/D	1.1 ± 0.0	N/D	N/D	2.8 ± 0.6	3.7 ± 0.3	12.7 ± 0.3	9.8 ± 0.7
	Fruits	N/D	N/D	N/D	N/D	N/D	1.0 ± 0.04	0.9 ± 0.2	1.8 ± 0.1	N/D
Ruscus aculeatus	Stems and Leaves	N/D	N/D	N/D	N/D	N/D	3.0 ± 0.08	N/D	N/D	4.1 ± 0.4
Rubus ulmifolius	Stems	1.8 ± 0.3	2.5 ± 0.1	N/D	8.2 ± 0.9	3.6 ± 0.2	3.5 ± 0.1	13.1 ± 0.8	1.3 ± 0.08	N/D
	Leaves	7.0 ± 0.4	1.1 ± 0.02	2.1 ± 0.2	4.2 ± 0.8	3.6 ± 0.5	4.1 ± 0.9	18.5 ± 0.6	4.9 ± 0.5	8.2 ± 0.5
	Flowers	3.3 ± 0.3	3.3 ± 0.5	4.6 ± 0.2	6.7 ± 0.3	4.7 ± 0.2	7.6 ± 0.5	7.0 ± 0.2	15.6 ± 0.9	33.3 ± 2.2
	Fruits	N/D	1.4 ± 0.2	0.8 ± 0.09	6.1 ± 0.2	0.9 ± 0.3	1.2 ± 0.08	3.6 ± 0.2	0.7 ± 0.09	1.3 ± 0.1
Hakea sericea	Stems	N/D	N/D	N/D	N/D	N/D	2.1 ± 0.6	11.3 ± 1.1	3.5 ± 0.6	N/D
	Leaves	N/D	1.2 ± 0.08	1.2 ± 0.1	1.9 ± 0.2	2.8 ± 0.4	3.8 ± 0.4	21.5 ± 0.9	2.6 ± 0.06	6.0 ± 0.5
	Fruits	1.2 ± 0.2	0.7 ± 0.07	1.1 ± 0.2	N/D	0.7 ± 0.01	1.7 ± 0.07	1.9 ± 0.2	3.7 ± 0.6	1.6 ± 0.03
Cytisus multiflorus	Stems	N/D	1.2 ± 0.6	1.0 ± 0.2	3.4 ± 0.1	2.7 ± 0.2	6.9 ± 0.8	1.8 ± 0.2	9.2 ± 0.3	37.1 ± 2.6
	Leaves	1.6 ± 0.02	2.3 ± 0.6	2.0 ± 0.1	3.2 ± 0.02	1.0 ± 0.08	3.8 ± 0.8	5.7 ± 0.8	16.1 ± 2.0	7.9 ± 1.9
	Flowers	N/D	N/D	N/D	N/D	0.7 ± 0.04	1.3 ± 0.3	13.0 ± 1.6	5.4 ± 0.4	3.5 ± 0.5
	Fruits	N/D	4.3 ± 0.6	2.0 ± 0.2	2.2 ± 0.4	2.9 ± 0.2	4.2 ± 0.3	25.4 ± 4.2	16.0 ± 1.0	3.0 ± 0.08
Crataegus monogyna	Stems	N/D	8.2 ± 0.5	7.7 ± 1.0	20.5 ± 1.4	4.4 ± 0.6	5.1 ± 0.7	17.2 ± 1.5	9.4 ± 1.0	N/D
	Leaves	N/D	18.9 ± 2.4	4.9 ± 0.9	10.3 ± 1.2	2.0 ± 0.3	5.3 ± 0.6	11.0 ± 1.6	7.4 ± 1.1	1.7 ± 0.2
	Flowers	0.6 ± 0.1	9.7 ± 1.5	4.4 ± 0.3	1.5 ± 0.3	1.8 ± 0.2	1.3 ± 0.06	116.8 ± 8.4	8.4 ± 1.2	9.8 ± 0.5
	Fruits	N/D	0.5 ± 0.05	N/D	3.0 ± 0.3	N/D	0.6 ± 0.1	3.3 ± 0.4	1.2 ± 0.1	0.6 ± 0.03
Erica arborea	Stems	N/D	1.3 ± 0.05	3.6 ± 0.6	0.8 ± 0.1	4.4 ± 0.4	3.1 ± 0.2	7.7 ± 0.9	5.1 ± 0.8	1.2 ± 0.3
	Leaves	N/D	15.1 ± 0.7	3.9 ± 0.3	17.5 ± 1.8	16.3 ± 1.0	7.5 ± 0.6	25.6 ± 1.5	9.3 ± 1.9	N/D
	Flowers	N/D	6.4 ± 0.1	2.3 ± 0.05	1.7 ± 0.3	2.5 ± 0.4	3.5 ± 0.3	6.0 ± 0.3	23.4 ± 0.9	N/D
Ipomoea acuminata	Stems	N/D	2.2 ± 0.1	N/D	N/D	N/D	N/D	2.4 ± 0.2	N/D	N/D
	Leaves	N/D	1.4 ± 0.2	N/D	N/D	N/D	1.0 ± 0.1	1.0 ± 0.1	N/D	9.8 ± 0.4
	Flowers	N/D	4.8 ± 0.2	N/D	N/D	N/D	2.6 ± 0.4	12.8 ± 1.7	20.5 ± 2.3	N/D

* Results in terms of mean ± standard deviation; N/D – Not detected

Table 5: GPS coordinates and altitude at which the different shrubs were collected and number of the vouchers specimens.

Full Scientific Name	**GPS coordinates**	**Altitude (m)**	**Number of Voucher Specimen**
***Echinospartum ibericum* Rivas Mart., Sánchez-Mata & Sancho**	N 40º19.046' W 07º34.365'	1606	LISI 8/2011
***Pterospartum tridentatum* (L.) Willk subsp. *cantabricum* Spach**	N 40º18.825' W 07º33.630'	1541	LISI 9/2011
***Juniperus communis* L. subsp. *alpina* (Suter) Celak.**	N 40º19.046' W 07º34.365'	1606	LISI 10/2011
***Ruscus aculeatus* L.**	N 40º19.105' W 07º26.791'	567	LISI 11/2011
***Rubus ulmifolius* Schott**	N 40º19.167' W 07º27.247'	519	LISI 12/2011
***Hakea sericea* Schrader**	N 40º20.296' W 07º27.491'	730	LISI 13/2011
***Cytisus multiflorus* (L'Hér) Swett**	N 40º18.668' W 07º27.076'	504	LISI 14/2011
***Crataegus monogyna* Jacq.**	N 40º18.668' W 07º27.076'	504	LISI 15/2011
***Erica arborea* L.**	N 40º19.046' W 07º34.365'	1606	LISI 16/2011
***Ipomoea acuminata* (Vahl) Roemer & Schultes**	N 40º16.719' W 07º30.744'	649	LISI 18/2011

extracts showed poor antioxidant activity when AAI < 0.5, moderate antioxidant activity between 0.5 and 1.0, strong antioxidant activity between 1.0 and 2.0, and very strong when AAI > 2.0 [31]. Assays were carried out in duplicate and all DPPH solutions were prepared daily.

β-Carotene bleaching test: After preparation of β-carotene solution (20 mg/mL in chloroform), 20 µL was added to 40 µL of linoleic acid, 400 mg of Tween 40 and 1 mL of chloroform. This mixture was then evaporated at 45ºC for 5 mins in a rotary vacuum evaporator to remove chloroform and immediately diluted with 100 mL of oxygenated distilled water. The water was added slowly to the mixture and vigorously agitated to form an emulsion. Five mL of the emulsion was transferred into test tubes containing 300 µL of extracts in methanol at different concentrations. About 5 mL of the emulsion and 300 µL of samples in methanol were used as control. Standard butylated hydroxytoluene (BHT) in methanol, at the same concentration as samples, was used as reference. The tubes were then gently shaken and placed at 50ºC in a water bath for 2 h. The absorbances of the extracts, standard and control were measured at 470 nm, using a spectrophotometer, against a blank consisting of an emulsion without β-carotene. The measurements were carried out at initial time (t=0 h) and at final time (t=2 h). The antioxidant activity was measured in terms of percentage of inhibition of β-carotene's oxidation by:

$$\% \text{ Inhibition} = (Abs^{t=2}_{sample} - Abs^{t=2}_{control}) / (Abs^{t=0}_{control} - Abs^{t=2}_{control})$$

Where $Abs^{t=2}$ was the absorbance of the sample or control at final time of incubation and $Abs^{t=0}$ was the absorbance in the control at initial time of incubation [19].

RP-HPLC analysis of phenolics: Chromatographic analysis was performed with the use of a liquid chromatographic system, which consisted of a Perkin Elmer Binary LC Pump 250, Perkin Elmer UV-visible Spectrophotometric Detector LC 290 and Perkin Elmer LC Oven 101 with a loop of 50 µL. The chromatographic system was connected through the Data Apex U-PAD2

USB Acquisition Device to a PC computer. Software used for data acquisition and evaluation was Clarity Lite Data Apex. The separation was carried out on a 150×4.60 mm, Phenomenex Kinetex Luna 2.6µ PFP 100A reversed phase column equipped with a Phenomenex KrudKatcher Ultra HPLC In-Line Filter with 0.5 µm porosity. Standard solutions were filtered through a 0.22 µm pore membrane filter before injection and crude methanolic extracts were centrifuged and then filtered through a 0.22 µm pore membrane filter before injection. The injection volume for all samples was 50 µL. The mobile phase consisted of 2 solvents: Solvent A, water with acetic acid (pH=3) and Solvent B, acetonitrile/solvent A (6:4; v/v). The mobile phase was filtered through a 0.22 µm pore membrane filter and degasified with nitrogen. Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the column temperature was set at 35°C, isocratic conditions from 0 to 10 mins with 0% B, linear gradient conditions from 0% to 5% B in 30 mins, from 5% to 15% B in 18 mins, from 15% to 25% B in 14 mins, from 25% to 50% B in 31 mins, from 50% to 100% B in 3 mins, followed by washing and reconditioning the column. For detection, chromatograms were monitored by ultra-violet-visible spectra (280 nm), which were recorded for all peaks. Duplicate analyses were performed for each sample. The identification of phenolic compounds was obtained by using authentic standards, while quantification was performed using the external standard method. Stock solutions of standard compounds at a concentration of 1 mg/mL each were prepared in methanol, and several dilutions with methanol were made. The solutions of standards at various concentrations (1.00, 0.75, 0.50, 0.25, 0.15 mg/mL) were injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of the compound was calculated from peak area according to calibration curves. The amount of each phenolic compound was expressed as mg/g of dry mass [35,41,42].

Acknowledgments - Ângelo Luís acknowledges a fellowship from *Fundação Para a Ciência e a Tecnologia* (Reference: SFRH / BD / 65238 / 2009). Authors wish to thank Prof. Dr Dalila Espírito Santo and Dr Paula Paes, from the Herbarium of the *Instituto Superior de Agronomia* (*Jardim Botânico d'Ajuda*, *Lisboa*), for the identification and deposit of the vouchers specimens of the plant species used in this work.

References

[1] Aparicio A, Guisande R. (**1997**) Replenishment of the endangered *Echinospartum algibicum* (Genisteae, Fabaceae) from the soil seed bank. *Biological Conservation*, ***81***, 267-273.

[2] Vitor R, Mota-Filipe H, Teixeira G, Borges C, Rodrigues A, Teixeira A, Paulo A. (**2004**) Flavonoids of an extract of *Pterospartum tridentatum* showing endothelial protection against oxidative injury. *Journal of Ethnopharmacology*, ***93***, 363-370.

[3] Gordien A, Gray A, Franzblau S, Seidel V. (**2009**) Antimycobacterial terpenoids from *Juniperus communis* L. (Cupressaceae). *Journal of Ethnopharmacology*, ***126***, 500-505.

[4] Mimaki Y, Kuroda M, Yokosuka A, Sashida Y. (**1999**) A spirostanol saponin from the underground parts of *Ruscus aculeatus*. *Phytochemistry*. ***51***, 689-692.

[5] Sisti M, Santi M, Fraternale D, Ninfali P, Scoccianti V, Brandi G. (**2008**) Antifungal activity of *Rubus ulmifolius* Schott standardized in *in vitro* culture. *LWT-Food Science and Technology*. ***41***, 946-950.

[6] Le Maitre D, Krug R, Hoffmann J, Gordon A, Mgidi T. (**2008**) *Hakea sericea*: Development of a model of the impacts of biological control on population dynamics and rates of spread of an invasive species. *Ecological Modelling*, ***212***, 342-358.

[7] Raja S, Ahamed K, Kumar V, Mukherjee K, Bandyopadhyay A, Murkherjee P. (**2007**) Antioxidant effect of *Cytisus scoparius* against tetrachloride treated liver injury in rats. *Journal of Ethnopharmacology*, ***109***, 41-47.

[8] García M, Sáenz M, Ahumada M, Cert A. (**1997**) Isolation of three triterpenes and several aliphatic alcohols from *Crataegus monogyna* Jacq.. *Journal of Chromatography A*, ***767***, 340-342.

[9] Harfani H, Bouanani N, Aziz M, Caid H, Ghalim N, Amrani S. (**2007**) The hypolipidaemic activity of aqueous *Erica multiflora* flowers extract in Triton WR-1339 induced hyperlipidaemic rats: A comparison with fenofibrate. *Journal of Ethnopharmacology*, ***109***,156-160.

[10] Terahara N, Konczak I, Ono H, Yoshimoto M, Yamakawa O. (**2004**) Characterization of acylated anthocyanins in callus induced from storage root of purple-fleshed sweet potato, *Ipomoea batatas* L. *Journal of Biomedical Biotechnology*, ***5***, 279-286.

[11] Xu W, Zhang F, Luo Y, Ma L, Kou X, Huang K. (**2009**) Antioxidant activity of a water-soluble polysaccharide purified from *Pteridium aquilinum*. *Carbohydrate Research*, ***344***, 217-222.

[12] Sreevidya N, Mehotra S. (**2003**) Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials. *Journal of AOAC International*, ***86***, 1124-1127.

[13] Silva E, Souza J, Rogez H, Rees J, Larondelle Y. (**2007**) Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian regions. *Food Chemistry*, ***101***, 1012-1018.

[14] Surveswaran S, Cai, Y, Corke H, Sun M. (**2007**) Systematic evaluation of natural phenolic antioxidant from 133 Indian medicinal plants. *Food Chemistry*, ***102***, 938-953.

[15] Cicco N, Lanorte M, Paraggio M, Viggiano M. (**2009**) A reproducible, rapid and inexpensive Folin-Ciocalteu micro-method in determining phenolics of plant methanol extracts. *Microchemical Journal*, ***91***, 107-110.

[16] Bahorun T, Aumjaud E, Ramphul H, Rycha M, Luximon-Ramma A, Trotin F, Aruoma O. (**2003**) Phenolic constituents and antioxidant capacities of *Crataegus monogyna* (hawthorn) callus extracts. *Nahrung/Food*, ***43***, 191-198.

[17] Barros L, Carvalho A, Ferreira C. (**2010**) Comparing the composition and bioactivity of *Crataegus monogyna* flowers and fruits used in folk medicine. *Phytochemical Analysis DOI:10.1002/pca.1267*.

[18] Konczak-Islam I, Yoshimoto M, Hou D, Terahara N, Yamakawa O. (**2003**) Potential chemopreventive properties of anthocyanin-rich aqueous extracts from *in vitro* tissue of sweetpotato (*Ipomoea batatas* L.). *Journal of Agricultural and Food Chemistry*, ***51***, 5916-5922.

[19] Luís A, Domingues F, Gil C, Duarte AP. (**2009**) Antioxidant activity of extracts of Portuguese shrubs: *Pterospartum tridentatum*, *Cytisus scoparius* and *Erica* spp. *Journal of Medicinal Plants Research*, ***3***, 886-893.

[20] Dall'Acqua S, Cervellati R, Loi M, Innocenti G. (**2008**) Evaluation of *in vitro* antioxidant properties of some traditional Sardinian medicinal plants: Investigation of the high antioxidant capacity of *Rubus ulmifolius*. *Food Chemistry*, ***106***, 745-749.

[21] Öztürk M, Tümen İ, Ugŭr A, Aydoğmuş-Öztürk F, Topçu G. (**2011**) Evaluation of fruit extracts of six Turkish *Juniperus* species for their antioxidant, anticholinesterase and antimicrobial activities. *Journal of the Science of Food and Agriculture*, ***91***, 867-876.

[22] Arouma O. (**2003**) Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Fundamental and Molecular Mechanisms of Mutagenesis*, ***523-524***, 9-20.

[23] Alim A, Goze I, Goze H, Tepe B. (**2009**) *In vitro* antimicrobial and antiviral activities of the essential oil and various extracts of *Salvia cedronella* Boiss. *Journal of Medicinal Plants Research*, ***3***, 413-419.

[24] Andrade D, Gil C, Breitenfeld L, Domingues F, Duarte A. (**2009**) Bioactive extracts from *Cistus ladanifer* L. and *Arbutus unedo* L. *Industrial Crops and Products*, ***30***, 165-167.

[25] Orhan N, Orhan I, Ergun F. (**2011**) Insights into cholinesterase inhibitory and antioxidant activities of five *Juniperus* species. *Food and Chemical Toxicology DOI:10.1016/j.fct.2011.06.031*.

[26] Prakash A, Pereira T, Reilly P, Seawright A. (**1999**) Pyrrolizidine alkaloids in human diet. *Genetic Toxicology and Environmental Mutagenesis*, ***443***, 53-67.

[27] Çam M, Hışıl Y, Durmaz G. (**2009**) Classification of eight pomegranate juices based on antioxidant capacity measured by four methods. *Food Chemistry*, ***112***, 721 – 726.

[28] Krishnaiah D, Sarbatly R, Nithyanandam R. (**2010**) A review of the antioxidant potential of medicinal plant species. *Food Bioproducts Processsing*, *DOI:10.1016/j.fbp.2010.04.008.*

[29] Tadić V, Dobrić S, Marković G, Đorđević S, Arsić I, Menković N, Stević T. (**2008**) Anti-inflammatory, gastroprotective, free-radical-scavenging, and antimicrobial activities of hawthorn berries ethanol extract. *Journal of Agricultural and Food Chemistry*, ***56***, 7700-7709.

[30] Faustino H, Gil N, Baptista C, Duarte A. (**2010**) Antioxidant activity of lignin phenolic compounds extracted from kraft and sulphite black liquors. *Molecules DOI:10.3390/molecules15129308.*

[31] Sherer R, Godoy H. (**2009**) Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. *Food Chemistry*, ***112***, 654-658.

[32] Wang B, Zhang W, Duan X, Li X. (**2009**) *In vitro* antioxidative activities of extract and semi-purified fractions of the marine red alga, *Rhodomela confervoides* (Rhodomelaceae). *Food Chemistry*, ***113***, 1101-1105.

[33] Fu L, Xu B, Xu X, Qin X, Gan R, Li H. (**2010**) Antioxidant capacities and total phenolic contents of 56 wild fruits from South China. *Molecules. DOI:10.3390/molecules15128602.*

[34] Miliauskas G, Venskutonis P, van Beek T. (**2004**) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, ***85***, 231-237.

[35] Kelebek H, Selli S, Canbas A, Cabaroglu T. (**2009**) HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan. *Microchemical Journal*, ***91***, 187-192.

[36] Pourmorad F, Hosseinimehr S, Shahabimajd N. (**2006**) Antioxidant activity, phenol and flavonoids contents of some selected Iranian medicinal plants. *African Journal of Biotechnology*, ***5***, 1142-1145.

[37] Tawaha K, Alali F, Gharaibeh M, Mohammad M, El-Elimat T. (**2007**) Antioxidant activity and total phenolic content of selected Jordanian species. *Food Chemistry*, ***104***, 1372-1378.

[38] Vitti D, Abdalla A, Bueno I, Filho J, Costa C, Bueno M, Nozella E, Longo C, Vieira E, Filho S, Godoy P, Mueller-Harvey I. (**2005**) Do all tannins have similar nutritional effects? A comparison of three Brazilian fodder legumes. *Animal Feed Science and Technology*, ***119***, 345-361.

[39] Wannes W, Mhamdi B, Sriti J, Jemia M, Ouchick O, Hamdaoui G, Kchouk M, Marzouk B. (**2010**) Antioxidant activities of the essential oils and methanol extracts from myrtle (*Myrtus communis* var. *italica* L.) leaf, stem and flower. *Food and Chemical Toxicology*, ***48***, 1362-1370.

[40] Rubanza C, Shen M, Otsyina R, Bakengesa S, Ichinohe T, Fujihara T. (**2005**) Polyphenolics and tannins effect on *in vitro* digestibility of selected *Acacia* species leaves. *Animal Feed Science and Technology*, ***119***, 129-142.

[41] Fernández-Arroyo S, Barrajón-Catalán E, Micol V, Segura-Carretero A, Fernández-Gutiérrez A. (**2010**) High-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from a *Cistus ladanifer* aqueous extract. *Phytochemical Analysis*, ***21***, 307-313.

[42] Tarnawski M, Depta K, Grejcium D, Szelepin B. (**2006**) HPLC determination of phenolic acids and antioxidant activity in concentrated peat extract - a natural immunomodulator. *Journal of Pharmaceutical and Biomedical Analysis*, ***41***, 182-188.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1873 - 1876

HPLC/PDA/ESI-MS Evaluation of Saffron (*Crocus sativus L.*) Adulteration

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Received: July 20th, 2011; Accepted: September 28th, 2011

The present study evaluated the reliability of the ISO/TS 3632-2 UV-Vis spectrometric method for saffron classification, making experiments on saffron samples to which were added increasing concentrations of common saffron spice adulterants (safflower, marigold and turmeric). The results showed that the ISO/TS 3632-2 method is not able to detect addition of up to 10-20%, w/w, of saffron adulterants. For additions from 20 to 50%, w/w, of the three adulterants, saffron was classified in a wrong category; addition of higher than 50%, w/w, determined variations in the investigated parameters that did not allow identification of the product as "saffron". In all cases, the method did not permit the recognition of the nature of the adulterant. On the contrary, the specificity of the HPLC/PDA/MS technique allowed the unequivocal identification of adulterant characteristic marker molecules that could be recognized by the values of absorbance and mass. The selection of characteristic ions of each marker molecule has revealed concentrations of up to 5%, w/w, for safflower and marigold and up to 2% for turmeric. In addition, the high dyeing power of turmeric allowed the determination of 2%, w/w, addition using exclusively the HPLC/PDA technique.

Keywords: spice, saffron, safflower, marigold, turmeric, fraud.

Saffron (*Crocus sativus* L., family Iridaceae) is the most valuable spice in the world. The species is a perennial meadow plant that reaches 10 to 25 cm and grows from bulbs. The cultivation of saffron requires a Mediterranean continental climate with cold winters, warm and dry summers, and a dry Mediterranean humidity regime. The plant is resistant to extreme temperatures in the summer and winter [1a]. Saffron is a spice that adds color, taste and aroma to various foods. The odor is related to its essential oil, a component of which is cineole. The bitter taste of saffron is due to picrocrocin and picrocrozioide, and its color from crocin esters that produce glucose and crocetin after hydrolysis [1a].

The world production of saffron is about 180 tons per year of which 90% is produced in Iran and the remainder in India, Greece, Morocco, Italy, Spain and other countries. The biggest worldwide exporters are Iran, followed by Spain. In Italy the annual production is around 400 kg [1a]. Saffron is cultivated mainly in Sardinia and Abruzzo, with about 35 ha and 7 ha, respectively, and to a lesser extent in Umbria, Tuscany, Liguria and Sicily. Some productions (i.e. Abruzzese di Navelli and San Gimignano) have been awarded the Protected Designation of Origin (PDO). The commercialization costs, including the different process stages, could exceed 1,000 €/kg.

Nowadays saffron production faces a crisis, but all nations involved are traditionally committed to saffron cultivation and preserve it actively. In the Mediterranean basin production of saffron has been decreased due to rising standards of living and, inevitably, due to the rise in labor costs. However, Mediterranean saffron bears the best quality features worldwide, which is attributed to deep cultivation knowledge and careful treatment by all European producers.

Due to its high value, saffron spice has been subjected to many adulterants throughout history, such as mixing of extraneous materials, immersing with vegetable oil or glycerin, and addition of various mineral substances, artificial colorants and less valuable colored spices [1a]. Saffron quality is determined after a series of characteristic parameters for the spice itself (moisture content, flower residue, foreign material, ash content, soluble condensate, coloring power, etc) combined with necessary external conditions (absence of parts from other plants, micro-biological flora and pesticide residues). Methods applied for quality assurance are widely known and enterprises are able to use the necessary technology in order to guarantee the product quality to consumers. Since 1980 a standard procedure (ISO/TS 3632) allows the quality for saffron classification. ISO/TS 3632 was updated in 2003 with the

Table 1: Specifications of ISO/TS 3632 "Saffron" [1b].

Characteristics	Specifications Categories I	II	III	Test method
Moisture and volatile matter, % (w/w), max.	12[a] 10[b]	12[a] 10[b]	12[a] 10[b]	ISO/TS 3632-2:2003, Clause 7
Total ash, % (w/w), on dry basis, max.	8	8	8	ISO 928:1997. Clause 8, and ISO/TS 3632-2:2003, Clause 12
Acid-insoluble ash, % (w/w), on dry basis, max.	1	1	1.5	ISO 930:1997. Clause 7, and ISO/TS 3632-2:2003, Clause 13
Bitterness, expressed as direct reading of the absorbance of picrocrocine at about 257 nm, on dry basis, min.	70	55	40	ISO/TS 3632-2:2003, Clause 14
Safranal, expressed as direct reading of the absorbance at about 330 nm, on dry wt basis min. max.	20 50	20 50	20 50	ISO/TS 3632-2:2003, Clause 14
Coloring strength, expressed as direct reading of the absorbance of crocine at about 440 nm, on dry wt basis, min.	190	150	100	ISO/TS 3632-2:2003, Clause 14
Artificial water soluble acid colorants	Absent	Absent	Absent	ISO/TS 3632-2:2003, Clause 16 and/or Clasue17

[a]filaments; [b] powder.

text that currently governs the product quality [1b]. This regulation is applicable to saffron strands, ground saffron and dust. The rule divides saffron into different categories based primarily on physical-chemical criteria (Table 1).

European saffron is considered the best in the world due to its chemical, physical and organoleptic features, as measured by certain parameters. New high quality verification standards and new evaluation methods should be introduced in order to determine accurately color and to prevent fraud, as reported by many scientific works [1c,2a,2b,3].

The present study evaluated the reliability of the ISO/TS 3632 UV-Vis spectrometric method for saffron classification, making analyses on samples of saffron blended with different concentrations of safflower, marigold and turmeric, widely used as saffron spice adulterants [1a,2a]. The results of the spectrometric method were compared and integrated with the HPLC/PDA/ESI-MS technique for the unequivocal identification of adulterants through the identification of specific marker compounds.

In Table 2 are reported the data related to the different parameters measured according to the ISO/TS 3632 (2003) spectrometric method for saffron category assignment performed on unadulterated and on spice-spiked San Gavino ISO Category II saffron. Five independent additions at different concentrations (10-67%) of adulterants (safflower, marigold and turmeric) were made.

Results showed that the ISO/TS 3632-2 spectrometric method is not able to detect the addition of up to 10-20%, w/w, of saffron adulterants, resulting in a correct

Table 2: Results of ISO/TS 3632 UV-vis spectrophotometry on the analyzed mixes.

Mix composition w/w	$E^{1\%}$ 257 nm	$E^{1\%}$ 330 nm	$E^{1\%}$ 440 nm	ISO Category
Saffron 100%	66	32	170	II
90% Saffron - 10% Turmeric	59	27	150	II
80% Saffron - 20% Turmeric	53	25	132	III
67% Saffron - 33% Turmeric	45	21	111	III
50% Saffron - 50% Turmeric	34	15	81	-
33% Saffron - 67% Turmeric	22	9	49	-
90% Saffron - 10% Safflower	63	31	152	II
80% Saffron - 20% Safflower	59	30	136	III
67% Saffron - 33% Safflower	53	29	113	III
50% Saffron - 50% Safflower	48	27	86	-
33% Saffron - 67% Safflower	42	26	60	-
90% Saffron - 10% Marigold	61	30	151	II
80% Saffron - 20% Marigold	55	29	135	III
67% Saffron - 33% Marigold	49	25	112	III
50% Saffron - 50% Marigold	39	22	83	-
33% Saffron - 67% Marigold	31	18	54	-

$E^{1\%}$ absorbency at λ_{max} for a 1% solution of the test sample for a 1cm cell

classification of the mixes as saffron ISO category II. For additions from 20 to 50%, w/w, of the three adulterants studied, the mixes were classified as worse than ISO category III saffron. Spikes higher than 50%, w/w, determined variations of the investigated parameters that did not allow the identification of the mixes as "saffron". In all cases the method did not permit the recognition of the kind of adulterant.

Therefore, the use of only the spectrometric technique may underestimate the saffron fraud occurrence due to the addition of less valuable spices. The hyphenated techniques like high performance liquid chromatography coupled with UV-Vis spectrophotometers and mass spectrometry may allow a better assessment of the quality of the saffron products.

Preliminarily, this study has characterized separately the fingerprint HPLC/PDA/ESI-MS of acidified water-methanol extracts of saffron, marigold, safflower and turmeric. Figure 1 showed the UV-Vis chromatograms of the studied spices. In Table 3 are reported the assignment of the characteristic molecules of each botanical species as a function of retention times, UV-Vis and mass properties.

Crocetin glycosides are responsible for the saffron yellow color; their UV-Vis spectra are characterized by an absorption maximum at about 440-460 nm depending on the molecule. *Trans*- and *cis*-crocetin glycoside showed a different spectroscopic behavior because *cis*-crocetins presented an additional absorption band around 325 nm in their UV-Vis spectrum in comparison with their *trans*-isomers. Six crocetin glycosides (**2-7**), together with colorless picrocrocin [4-(*α*-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde] (**1**), were identified in the analyzed San Gavino saffron. As confirmed by ESI-MS analysis, in agreement with literature data [2b,4], *trans*-crocetin di-(*β*-D-gentibiosyl) ester (**3**), *cis*-crocetin (*β*-D-glucosyl)-(*β*-D-gentibiosyl)

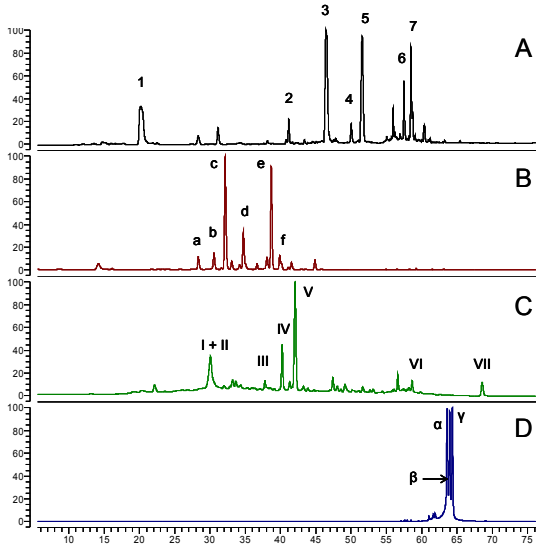

Figure 1: UV-Vis chromatograms of analyzed extracts. **A:** Saffron (λ 250 nm + λ 440 nm); **B:** Marigold (λ 350 nm); **C:** Safflower (λ 410 nm + λ 520 nm); **D:** Turmeric (λ 425 nm). For peak identification see Table 3.

Table 3: HPLC/PDA/MS chemical characterization of studied extracts.

Peak	Name	Rt (min)	UV-vis (nm)	$[M-H]^-$ (*m/z*)
Crocus sativus L. **(saffron)**				
1	4-(*α*-D-glucopyranosyl)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde (picrocrocin)	20.2	250	375
2	*trans*-Crocetin (*β*-D-neapolitanosyl)-(*β*-D-gentibiosyl) ester	41.2	260, 440	1137
3	*trans*-Crocetin di-(*β*-D-gentibiosyl) ester	46.5	260, 420, 460	975
4	*trans*-Crocetin (*β*-D-glucosyl)-(*β*-D-neapolitanosyl) ester	50.1	260, 440	975
5	*cis*-Crocetin (*β*-D-glucosyl)-(*β*-D-gentibiosyl) ester	51.6	260, 330, 435, 460	813
6	*cis*-Crocetin di-(*β*-D-gentibiosyl) ester	57.5	260, 320, 435, 460	976
7	*cis*-Crocetin di-(*β*-D-glucosyl) ester	58.8	260, 325, 440, 465	813
Calendula officinalis **(marigold)**				
a	Quercetin 3-*O*-rutinosylrhamnoside	28.4	255, 355	755
b	Quercetin 3-*O*-rutinoside	30.6	255, 355	609
c	Isorhamnetin-3-*O*-rutinosylrhamnoside	32.2	255, 350	769
d	Narcissin	34.7	255, 355	623
e	Isorhamnetin 3-*O*-neohesperidoside	38.7	255, 345	623
f	Isorhamnetin-3-*O*-glucoside	39.9	255, 355	477
Carthamus tinctorius **(safflower)**				
I	Hydroxysafflor yellow A (safflomin A)	30.1	225, 410	611
II	6-Hydroxykaempferol 3-*O*-*β*-D-glucoside	30.6	275, 340	464
III	Kaempferol 3-*O*-*β*-rutinoside	37.7	265, 350	593
IV	Safflor yellow B	40.2	225, 410	1060
V	Anydrosafflor yellow B	42.1	225, 410	1044
VI	Prechartamin	58.7	240, 405	955
VII	Chartamin	68.6	370, 520	909
Curcuma longa **(turmeric)**				
α	Demethoxycurcumin	63.6	250, 425	337
β	Bisdemethoxycurcumin	63.9	250, 420	307
γ	Curcumin	64.3	260, 430	367

ester (**5**) and *cis*-crocetin di-(*β*-D-glucosyl) ester (**7**) were the most abundant crocetin derivatives, followed by *cis*-crocetin di-(*β*-D-gentibiosyl) ester (**6**), *trans*-crocetin (*β*-D-neapolitanosyl)-(*β*-D-gentibiosyl) ester (**2**) and *trans*-crocetin (*β*-D-glucosyl)-(*β*-D-neapolitanosyl) ester (**4**).

Marigold extract was characterized by six main peaks (**a-f**), which displayed identical UV absorptions with maxima at about 255 and 350 nm, typical of flavonols. The ESI-MS $[M-H]^-$ molecular ions, together with comparison with scientific references [5], permitted their unequivocal assignation to quercetin 3-*O*-rutinosylrhamnoside (**a**), quercetin 3-*O*-rutinoside (**b**), isorhamnetin-3-*O*-rutinosylrhamnoside (**c**), narcissin (**d**), isorhamnetin 3-*O*-neohesperidoside (**e**), and isorhamnetin-3-*O*-glucoside (**f**). Among them, the isoramnetin derivatives predominated in the analyzed sample.

As reported by many scientific papers [6,7], the main component of safflower red pigments is carthamin, composed of two chalconoids with conjugated bonds; it is derived from the yellow colored precarthamin by decarboxylation. Both compounds were detected in the analyzed safflower sample as peaks **VI** and **VII**. Safflomin A (**I**), safflor yellow B (**IV**) and anhydrosafflor yellow B (**V**) were identified as the quinochalcone *C*-glycosides responsible for the yellow color of the sample. Moreover, two kaempferol derivatives were identified as 6-hydroxy-kaempferol 3-*O*-*β*-D-glucoside (**II**) and kaempferol 3-*O*-*β*-rutinoside (**III**). Identification was confirmed by spectroscopic and mass spectral data. Three molecules (**α-γ**) were identified as responsible for the yellow distinctive color of turmeric: all of them clearly possess a maximum absorption wavelength near 420 nm. According to literature data [8], these compounds were identified as the dicinnamoylmethane derivatives demethoxycurcumin (**α**), bisdemethoxycurcumin (**β**) and curcumin (**γ**) on the basis of $[M-H]^-$ molecular ions generated by the ESI-MS negative soft ionization. Subsequently, the above-mentioned analytical technique was applied to saffron samples mixed with different concentrations of turmeric, marigold and safflower in the range 2-20%, w/w. For each adulterant, marker molecules have been chosen for their unambiguous identification in the mixture: it was established that their detection was not influenced by the saffron matrix effect.

Extraction of the ion with m/z 623, corresponding to isorhamnetin 3-*O*-neohesperidoside (**e**), is able to detect the presence of marigold. In the case of safflower, the marker molecules for its identification in the mixture were anydrosafflor yellow B (**V**) and chartamin (**VII**) with ions at m/z 1044 and 909, respectively. The marker molecules could be revealed at concentrations of up to 5%, w/w, of both marigold and safflower. The characteristic turmeric curcuminoids triplet due to the presence of demethoxy-curcumin (**α**), bisdemethoxy-curcumin (**β**) and curcumin (**γ**) could easily identify its presence in the mix with saffron, and also at concentrations of 2%, even using only the UV-Vis detector. The above discussed method is currently applied by the Catania Laboratory of Central Inspectorate for Quality Control of Agricultural and Food Productions (ICQRF) on samples of saffron collected in the Italian market in the framework of the Ministry of Agriculture and Forestry institutional quality control activity.

In conclusion, the results of the present study should force the legislative authorities to release new standards for the saffron sector for the maintenance of product purity in order to avoid adulteration and fraud. The methods applied nowadays are outdated, while newer ones are not positively accepted and are rarely used, even though some of them have proven to be efficient in the field. All national and international quality control standards should be reinforced in order to limit the spread of adulterated saffron in the European market deriving from countries that affect considerably the competitiveness of European saffron.

Experimental

Plant material, reagents and standards: Strands of saffron (*Crocus sativus* L.) from San Gavino (Sardinia, Italy), turmeric (*Curcuma longa*) powder, calendula (*Calendula officinalis*) flowers, and safflower (*Carthamus tinctorius*) strands were taken in Italy by ICQRF officials. The plant material was treated in accordance with the specifications of ISO/TS 3632-2:2003 sample preparation for the official analysis [1b]. Different mixes were prepared with saffron with percentages varying from 2 to 70% by weight of each adulterant studied. HPLC-grade acetonitrile, methanol and formic acid were supplied by Romil (Milan, Italy). Distilled water was purified at 18.2 MΩ cm with a MilliQ ULTRA (Millipore, Vimodrone (MI), Italy) purification system.

UV-Vis spectrophotometry: The method used was based on the technical specification ISO/TS 3632-2:2003 [1b] and allowed determination of the main characteristics of saffron. The method is based on the spectrophotometric assessment absorption at 3 wavelengths (λmax): 257 nm (maximum absorption of picrocrocin), 330 nm (maximum absorption of safranal) and 440 nm (maximum absorption of crocin).

HPLC/PDA/ESI-MS fingerprint: 20±2 mg of sample was extracted with 2 mL of 50% aqueous methanol containing 0.1% formic acid at room temperature in an ultrasonic bath for 30 min. The mixture was centrifuged at 3000 rpm for 5 min and the supernatant decanted and used for the LC analyses, after filtration through 0.45 µm PTFE filters (LabService Analytica, Bologna, Italy). The analyses were performed with a liquid chromatograph consisting of a Finnigan Surveyor MS-pump, autosampler and photodiode-array detector (PDA), coupled with a Finnigan LCQ DECA XP MAX detector (Thermo Fisher Scientific). The analytical column was a Gemini C18 150 x 2.1 mm i.d. 3µm (Phenomenex); the flow rate was 200 µL/min, the column temperature 30°C and the injection volume 10 µL. A binary gradient of 0.3% formic acid in water (A) and 0.3% formic acid in acetonitrile (B) was employed. The mobile phase gradient was programmed as follows: 0 min, 5% B; 50 min, 28% B; 60 min, 43% B; 60-65 min, 43 % B; 70-80 min, 5% B. The range of wavelengths examined by the photodiode-array detector was 200–700 nm and the mass scan range was 100-1600 m/z. Mass spectral analyses were performed using a LCQ ion-trap mass operating in negative ion mode using an ion spray LC/MS interface. The electrospray ionization (ESI) needle voltage was 3.5 kV. The capillary voltage was 18V and the heated capillary was 250°C. A sheath gas flow rate of 36 (arbitrary units) was used and the auxiliary gas was set to 14 (arbitrary units). The main compounds of analyzed plant materials were characterized in terms of retention times, lambda max and MS data.

References

[1] (a) Kafi M, Koocheki A, Rashid MH, Nassiri N. (**2006**) *Saffron (Crocus sativus) production and processing*. Science Publishers, Enfield, NH, USA; (b) ISO/TS 3632. (**2003**) Saffron (*Crocus sativus* L.). Part 1 (specification) and Part 2 (test methods). International Organization for Standardization, Genève, Switzerland; (c) Alonzo GL, Salinas MR, Garijo J. (**1998**) Method to determine the authenticity of aroma of saffron. *Journal of Food Protection*, ***61***, 1525-1528.

[2] (a) Haghighi B, Feizy J, Kakhki AH. (**2007**) LC determination of adulterated saffron prepared by adding styles colored with some natural colorants. *Chromatographia*, ***66***, 325–332; (b) Zalacain A, Ordoudi SA, Díaz-Plaza EM, Carmona M, Blázquez I, Tsimidou MZ, Alonso GL. (**2005**) Near-infrared spectroscopy in saffron quality control: determination of chemical composition and geographical origin. *Journal of Agricultural and Food Chemistry*, ***53***, 9337-9341.

[3] Sujata V, Ravishankar GA, Venkataraman LV. (**1992**) Methods for the analysis of the saffron metabolites crocin, crocetins, picrocrocin and safranal for the determination of the quality of the spice using thin-layer chromatography, high-performance liquid chromatography and gas chromatography. *Journal of Chromatography A*, ***624***, 497-502.

[4] Carmona M, Zalacain A, Sànchez AM, Novella JL, Alonso GL. (**2006**) Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS. *Journal of Agricultural and Food Chemistry*, ***54***, 973-979.

[5] Bilia AR, Bergonzi MC, Gallori S, Mazzi G, Vincieri FF. (**2002**) Stability of the constituents of Calendula, milk-thistle and passion flower tinctures by LC-DAD and LC-MS. *Journal of Pharmaceutical and Biomedical Analysis*, ***30***, 613-624.

[6] Kazuma K, Takahashi T, Sato K, Takeuchi H, Matsumoto T, Okuno T. (**2000**) Quinochalcones and flavonoids from fresh florets in different cultivars of *Carthamus tinctorius* L. *Bioscience, Biochemistry, and Agrochemistry*, ***64***, 1588-1599.

[7] Jin Y, Zhang XL, Shi H, Xiao YS, Ke YX, Xue XY, Zhang FF, Liang XM. (**2008**) Characterization of C-glycosyl quinochalcones in *Carthamus tinctorius* L. by ultraperformance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry. *Rapid Communications in Mass Spectrometry*, ***22***, 1275-1287.

[8] He XG, Lin LZ, Lian LZ, Lindenmaier M. (**1998**) Liquid chromatography-electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in turmeric (*Curcuma longa*). *Journal of Chromatography A*, ***818***, 127-132.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1877 - 1880

Ferric Reducing, Antiradical and β-Carotene Bleaching Activities of Nicotinic Acid and Picolinic Acid Bioconjugates of Curcumin

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Received: June 4th, 2011; Accepted: October 6th, 2011

Although curcumin displays several beneficial properties, its medicinal use is limited by its low bioavailability. In the present study we report the antioxidant potentials of two bioconjugates of curcumin with nicotinic acid and picolinic acid: di-*O*-nicotinoyl curcumin [1,7-bis (4-*O*-nicotinoyl-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione] and di-*O*-picolinoyl curcumin [1,7-bis (4-*O*-picolinoyl-3-methoxyphenyl)-1,6-heptadiene-3, 5-dione], in terms of ferric reducing, radical scavenging and β-carotene bleaching abilities, and comparing the observed activity with that of curcumin. Results demonstrate that both the bioconjugates possess higher antioxidant potentials as evidenced by enhanced ferric reducing, radical scavenging and β-carotene bleaching abilities, in comparison with curcumin. On the basis of our results we conclude that these bioconjugates of curcumin may be better than curcumin for medicinal and pharmacological applications.

Keywords: Curcumin, nicotinic acid, picolinic acid, bioconjugates, antioxidant.

Curcumin, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione is a natural yellow pigment isolated from turmeric (*Curcuma longa*). Curcumin is known to exhibit anticancer, anti-inflammatory, anti-diabetic, anti-viral and neuroprotective properties [1,2]. The compound is also reported to reduce blood cholesterol, enhance wound healing, protect from liver injuries and cataract formation [3,4]. Although multiple mechanism(s) of action have been reported for curcumin, the antioxidant property associated with this molecule has been a subject of great attention and many of the associated biological and pharmacological effects attributed to it [4,5].

Pleiotropic health beneficial properties make curcumin an important pharmacologically active molecule, but low bioavailability, poor absorption and rapid metabolism in the liver and intestinal wall limits its use as a therapeutic agent [6,7]. Its highly hydrophobic nature also reduces systemic administration, and it has been shown that on intravenous administration, curcumin disappears rapidly from the blood and quickly appears as metabolites in the bile [7,8]. In order to overcome these limitations, curcumin may attach to ligands that can enhance its solubility, bioavailability, slowdown the metabolism and increase the rate of cellular uptake [9]. Keeping this rationale in mind, two bioconjugates of curcumin have been synthesized comprised of nicotinic acid (vitamin B3) and its isomer picolinic acid: di-*O*-nicotinoyl curcumin [1,7-bis (4-*O*-nicotinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (DNC) and di-*O*-picolinoyl curcumin [1,7-bis (4-*O*-picolinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (DPC, Figure 1)]. In the present study these bioconjugates have been screened for their antioxidant potential in terms of ferric reducing, radical scavenging and β-carotene bleaching activities in an effort to assess their efficacy as antioxidant moieties compared with the parent molecule i.e. curcumin.

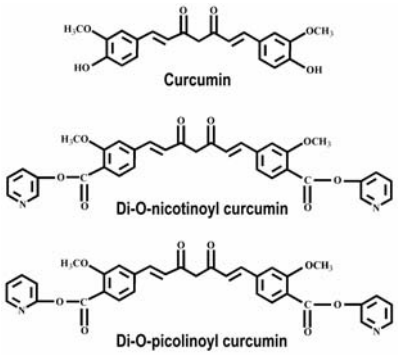

Figure 1: Chemical structures of curcumin [1, 7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], di-*O*-nicotinoyl curcumin [1,7-bis (4-*O*-nicotinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], di-*O*-picolinoyl curcumin [1,7-bis (4-*O*-picolinoyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione].

Both the synthesized bioconjugates, DNC and DPC, showed enhanced ferric reducing ability in comparison with curcumin itself ($p < 0.05$), when assayed with FRAP reagent. The ability of DNC and DPC to reduce the ferric ion was 43% and 45% higher, respectively compared with curcumin (Figure 2). Similar to the ferric reducing ability, both the bioconjugates of curcumin inhibited DPPH$^{\bullet}$ at a higher rate than curcumin ($p < 0.001$). Interestingly, DPC

showed a slightly higher radical scavenging ability/antiradical power than DNC. The ability of DNC and DPC to inhibit DPPH• was 42% and 51%, respectively higher than curcumin (Figure 3). Both the bioconjugates showed strong β-carotene bleaching capacity when tested, in comparison with curcumin. DNC elicited 20% and DPC 23% more bleaching capacity than curcumin (Figure 4).

The FRAP assay, based on ferric reducing ability of biological samples, is one of the most frequently used methods to evaluate antioxidant activity since it is independent of the enzymatic/non-enzymatic method to generate free radicals prior to evaluating the anti-radical activity of antioxidants. The ability to reduce the ferric ion at higher rates by both the bioconjugates of curcumin, confirms their higher antioxidant potentials compared with curcumin.

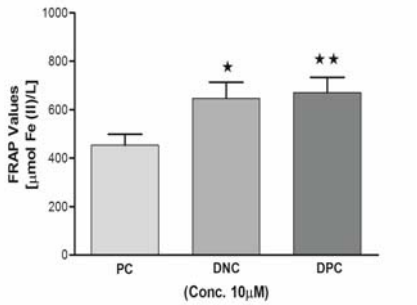

Figure 2: Comparative FRAP values of assayed bioconjugates of curcumin at a concentration of 10 µM. Values are mean ± SD of 10–12 independent experiments. PC- Curcumin; DNC-di-*O*-nicotinoyl curcumin; DPC- di-*O*-picolinoyl curcumin. *$p < 0.05$ and **$p < 0.001$, compared with curcumin.

The estimation of radical scavenging activity of curcumin along with its bioconjugates using the DPPH• method presented the advantage of using a stable and commercially available free radical. This method has been extensively applied to the study of antiradical power of biological compounds [10,11]. Easy to perform and high reproducibility adds extra advantage over other methods. Inhibition of DPPH• by DNC and DPC at higher rates than curcumin supports the results obtained from the FRAP assay.

The β-carotene bleaching assay is a reproducible and authentic method to measure the antioxidant capacity of different biological samples. The enhanced capacity of the synthesized bioconjugates to bleach β-carotene in comparison with curcumin confirms their greater antioxidant potential.

The presence of the carboxyl side chain at the *ortho* position and intramolecular coordination of the carboxylic group and nitrogen of the pyridine nuclei facilitates the proton donating ability of DPC. This may be the reason for the slightly higher DPPH• scavenging and β carotene bleaching activity of DPC in comparison with DNC.

Chemically, curcumin is a bis-α,β-unsaturated β-diketone (commonly called diferuloylmethane, Figure1), which

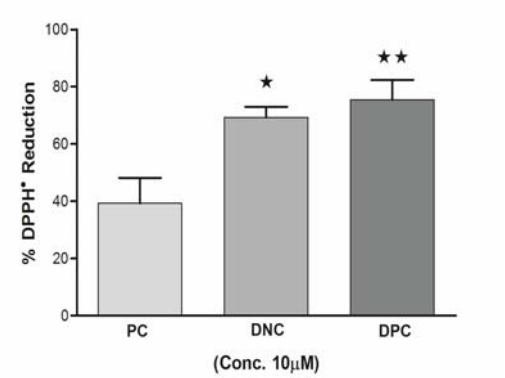

Figure 3: Comparative % DPPH• reducing activity of curcumin bioconjugates at the concentration of 10 µM. Values are mean ± SD of 10–12 independent experiments. PC- Curcumin; DNC-di-*O*-nicotinoyl curcumin; DPC- di-*O*-picolinoyl curcumin. *$p < 0.001$ and **$p < 0.0005$, compared with curcumin.

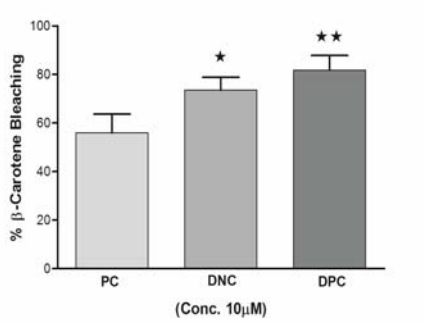

Figure 4: Comparative % β-carotene bleaching capacity of curcumin bioconjugates at the concentration of 10 µM. Values are mean ± SD of 10–12 independent experiments. PC- Pure curcumin; DNC-di-O-nicotinoyl curcumin; DPC- di-O-picolinoyl curcumin. *$p < 0.05$ and **$p < 0.001$, compared with pure curcumin.

exhibits keto–enol tautomerism, having a predominant keto form in acidic and neutral solutions and a stable enol form in alkaline media. The double bonds in curcumin provide definite conformational flexibility to the molecule, which accounts for its various properties [12,13].

The inherent disadvantage with respect to curcumin is its poor solubility in aqueous systems, which makes its use in water-based food products difficult. The alkyl and aryl portions of this molecule render it lipophilic and thus restrict its uptake into cells [14,15]. Antioxidant properties of a number of ring-substituted analogues of curcumin show that the highest antioxidant activity is obtained when the phenolic group is sterically hindered [15]. Therefore, one of the most practical approaches is to make biodegradable conjugates of curcumin with suitable ligands to enhance its cellular uptake [16]. The site of attachment of ligands to the curcumin molecule can be at phenolic hydroxyls, ketogroups (enol functions) or the active methylene group.

The masking of phenolic groups with ligands such as amino acids, nucleosides, and peptides has already been reported [7,17,18]. We are the first to select vitamin B3-complex as a ligand. The bioconjugates: DNC and DPC prepared with nicotinic acid and its isomer picolinic acid

have a biodegradable ester linkage. Since both ligands used are natural and are essential components which play a vital role in cellular physiology, the bioconjugates synthesized are predicted to enhance cellular uptake and be more efficacious than pure curcumin.

The vitamin derivatives of curcumin showed higher ferric reducing, free radical scavenging and β-carotene bleaching activities than pure curcumin, thus demonstrating that derivatization at the phenolic position of curcumin improved *in vitro* antioxidant activity. Results of our study on screening of antioxidant potential of curcumin bioconjugates may be useful in developing more efficacious conjugates of curcumin with enhanced bioavailability incorporating better pharmacological and biological effects.

Experimental

***Synthesis of di-O-nicotinoyl curcumin [1,7-bis (4-O-nicotinoyl-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione*:** Redistilled thionyl chloride (1.09 mL, 0.015 mol) was added to 1.23 g of nicotinic acid (0.01 mol) and refluxed for 30 min. The reaction mixture was evaporated to dryness on a rotary evaporator under reduced pressure. [yield = 72% (0.890 g, 6 mmol)]. One hundred mg curcumin (0.27 mmol) in 5 mL dimethyl formamide (DMF) was mixed with nicotinic chloride (90 mg ≈ 0.6 mmol). A few drops of triethylamine were added as catalyst and the reaction mixture was stirred overnight at room temperature. The completion of reaction was checked by TLC. The reaction mixture was then poured into chilled water (10 mL), shaken well, and then extracted with DCM (dichloromethane) thrice (5 mL each time). Na_2SO_4 (1g) was added to the extracted DCM, the solution kept overnight, then filtered, the filtrate evaporated to dryness, and then purified by silica gel CC using DCM/MeOH gradient.
Yield: 42%.
Rf: 0.81 (DCM/MeOH; 9.8: 0.2).
UV λmax (MeOH): 260 (s), 290,320 (s) nm.
^{1}H NMR (DMSO-d_6 and D_2O): δ 3.75 (s, 6H, two- OCH_3), 4.52 (s, 2H, C_4 of cur), 6.53 (d, 2H, C_2 and C_6 of cur), 6.80 - 6.95 (m, 6H, Ar-cur), 6.99 (m, 2H, C_5 of pyridine ring), 7.00 (d, 2H, C_4 of pyridine ring), 7.52 (d, 2H, C_1 and C_7 of cur), 8.20 (d, 2H, C_6 of pyridine ring), 8.50 (s, 2H, C_2 of pyridine ring).
Elemental analysis: observed C, 68.49; H, 4.51; N, 4.89% calculated for $C_{33}H_{26}O_8N_2$: C, 68.51; H, 4.49; N, 4.84%.

Synthesis of di-O-picolinoyl curcumin [1,7-bis (4-O-picolinoyl-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione]: Di-*O*-picolinoyl curcumin was prepared similarly to di-*O*-nicotinoyl curcumin, but by replacing nicotinic chloride by picolinic chloride.
Rf: 0.80 (DCM/MeOH; 9.8: 0.2).
UV λmax (MeOH): 260 (s), 292,326 nm.
^{1}H NMR (DMSO-d_6 and D_2O): δ 3.72 (s, 6H, two OCH_3), 4.58 (s, 2H, C_4 of cur), 6.67 (d, 2H, C_2 and C_6 of cur), 6.78 - 6.91 (m, 6H, Ar-cur), 6.80 (m, 2H, C_4 of pyridine ring), 6.99 (m, 2H, C_5 of pyridine ring), 7.00 (d, 2H, C_3 of pyridine ring), 7.54 (d, 2H, C_1 and C_7 of cur), 8.20 (d,2H, C_6 of pyridine ring).
Elemental analysis: observed C, 68.50; H, 4.48; N, 4.86% calculated for $C_{33}H_{26}O_8N_2$: C, 68.51; H, 4.49; N, 4.84%.

***Ferric reducing antioxidant potential (FRAP) assay*:** The antioxidant capacity of the bioconjugates and curcumin was determined by the ferric reducing antioxidant potential (FRAP) assay, following the method of Benzie and Strain [19]. Working FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 2, 4, 6-tri [2-pyridyl]-s-triazine (10 mM in 40 mM HCl) solution and $FeCl_3.6H_2O$ (20 mmol/L) solution in 10:1:1 ratio, respectively. Three mL of FRAP reagent was mixed with 10 µM of each sample solution and the content mixed vigorously. The absorbance was read at 593 nm at intervals of 30 sec for 4 min. An aqueous solution of Fe^{2+} in the range of 100 -1000 µmol/L was used for calibration. Using the regression equation, the FRAP values (µmol Fe (II)/L) of the compounds were calculated.

***1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH$^{\bullet}$) assay*:** Anti-radical activity of curcumin and both the bioconjugates was estimated by the procedure described by Miliauskas *et al*.[20]. Briefly, 0.1 mL of each compound was incubated in a methanolic solution of DPPH$^{\bullet}$ (0.1 mM). Absorbance at 517 nm was measured after 30 min of incubation with vigorous shaking. Methanol was used as blank reference. All the measurements were performed in triplicate. The free radical DPPH $^{\bullet}$ scavenging (i.e. reduction) activity was calculated from the equation: Activity [% of DPPH reduction] = [(A-Ax)/A] × 100%, where A – absorbance of DPPH$^{\bullet}$ solution with methanol, Ax - absorbance of a DPPH $^{\bullet}$ solution with assayed compounds.

β-Carotene bleaching assay: β-Carotene bleaching capacity of the synthesized bioconjugates was determined according to the method of Burda and Oleszek [21]. Briefly, 1 mL of β-carotene (0.2 mg/mL) was dissolved in chloroform containing 0.02 mL linoleic acid and 0.02 mL Tween 20. The mixture was then dosed with 0.2 mL of compound solution (final concentration 10 µM). Fifty mL of distilled water, saturated for 15 min with oxygen, was added to the flask. The resulting mixture was shaken and kept for 2 h at 50°C. The absorbance of the samples was measured at 470 nm, immediately after their preparation (t = 0 min) and at the end of the experiment (t = 120 min). Antioxidant activity was calculated as percent inhibition of oxidation versus control, using the equation: % β-carotene bleaching = 100 [1- (As^0-As^t)/ (Ac^0-Ac^t)] where As^0: absorbance of sample at 0 min, As^t: absorbance of the sample at 120 min, Ac^0: absorbance of control at 0 min and Ac^t: absorbance of control at 120 min.

Statistical analyses were performed using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. The results are reported as means ± SD.

Acknowledgments - The authors AP and SIR wish to acknowledge University Grants Commission, New Delhi for financial support in the form of MRP: F-38-38/2009 SR and MRP: F-37-392/2009 SR.

References

[1] Pandey A, Gupta RK, Srivastava R. (**2011**) Curcumin- The yellow magic. *Asian Journal of Applied Science*, ***4***, 343-354.
[2] Zandi K, Ramedani E, Mohammadi K, Tajbakhsh S, Deilami I, Rastian Z, Fouladvand M, Yousefi F, Farshadpour F. (**2011**) Evaluation of antiviral activities of curcumin derivatives against HSV-1 in Vero cell line. *Natural Product Communications*, ***12***, 1935-1938.
[3] Goel A, Kunnumakkara AB, Aggarwal BB. (**2008**) Curcumin as "Curecumin": from kitchen to clinic. *Biochemical Pharmacology, **75***, 787-809.
[4] Ali BH, Marrif H, Noureldayem SA, Bakheit AO, Blunden G. (**2006**) Some biological properties of curcumin: A review. *Natural Product Communications*, ***1***, 509-521.
[5] Zhou H, Beevers CS, Huang S. (**2011**) The targets of curcumin. *Current Drug Targets*, ***12***, 332-347.
[6] Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB. (**2007**) Bioavailability of curcumin: problems and promises. *Molecular Pharmacology*, ***4***, 807-818.
[7] Singh RK, Rai D, Yadav D, Bhargava A, Balzarini J, De Clercq E. (**2010**) Synthesis, antibacterial and antiviral properties of curcumin bioconjugates bearing dipeptide, fatty acids and folic acid. *European Journal of Medicinal Chemistry*, ***45***, 1078-1086.
[8] Priyadarsini KI, Maity DK, Naik GH, Kumar MS, Unnikrishnan MK, Satav JG, Mohan H. (**2003**) Role of phenolic O-H and methylene hydrogen on the free radical reactions and antioxidant activity of curcumin. *Free Radical Biology & Medicine*, ***35***, 475-484.
[9] Pandey A, Dwivedi V, Misra K. (**2010**) Drug sensitivity of curcumin analogues and bioconjugates. In *Structural modification and drug development*. Vol *7*, Gupta VK, Taneja SC, Gupta BD. (Eds). Studium Press, LLC US. 267-298.
[10] Sánchez-Moreno C, Larrauri JA, Saura-Calixto F. (**1998**) A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, ***76***, 270-276.
[11] Villano D, Fernández-Pachón MS, Moyá ML, Troncoso AM, García-Parrilla MC. (**2007**) Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta*, ***71***, 230-235.
[12] Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL. (**2005**) Anti-oxidant activities of curcumin and related enones. *Bioorganic & Medicinal Chemistry*, ***13***, 3811-3820.
[13] Barclay LR, Vinqvist MR, Mukai K, Goto H, Hashimoto Y, Tokunaga A, Uno H. (**2000**) On the antioxidant mechanism of curcumin: classical methods are needed to determine antioxidant mechanism and activity. *Organic Letters*, ***2***, 2841-2843.
[14] Kumar S, Dubey KK, Tripathi S, Fujii M, Misra K. (**2000**) Design and synthesis of curcumin-bioconjugates to improve systemic delivery. *Nucleic Acids Symposium Series*, ***44***, 75-76.
[15] Parvathy KS, Negi PS, Srinivas P. (**2010**) Curcumin–amino acid conjugates: Synthesis, antioxidant and antimutagenic attributes. *Food Chemistry*, ***120***, 523–530.
[16] Kumar S, Misra A, Tripathi S, Misra K. (**2001**) Study on curcumin-oligonucleotide conjugate as a probable anticancer agent: its hybridisation with telomere target sequence 5'-GGGATTGGGATT-3'. *Nucleic Acids Research Supplement*, ***1***, 137-138.
[17] Elias EJ, Anil S, Ahmad S, Daud A. (**2010**) Colon targeted curcumin delivery using guar gum. *Natural Product Communications*, ***6***, 915-918.
[18] Dubey SK, Sharma AK, Narain U, Misra K, Pati U. (**2008**) Design, synthesis and characterization of some bioactive conjugates of curcumin with glycine, glutamic acid, valine and demethylenated piperic acid and study of their antimicrobial and antiproliferative properties. *European Journal of Medicinal Chemistry*, ***43***, 1837-1846.
[19] Benzie IF, Strain JJ. (**1996**) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry*, ***239***, 70-76.
[20] Miliauskas G, Venskutonis PR, van Beek TA. (**2004**) Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chemistry*, ***85***, 231–237.
[21] Burda S, Oleszek W. (**2001**) Antioxidant and antiradical activities of flavonoids. *Journal of Agricultural and Food Chemistry*, ***49***, 2774-2779.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1881 - 1884

Antiviral Activities of Diarylheptanoids Isolated from *Alpinia officinarum* against Respiratory Syncytial Virus, Poliovirus, Measles Virus, and Herpes Simplex Virus Type 1 *in vitro*

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Received: August 16th, 2011; Accepted: October 15th, 2011

Alpinia officinarum has been used as a folk medicine and contains diarylheptanoids that have various biological activities. However, their antiviral activities are less elucidated. We examined the antiviral activities of nine diarylheptanoids isolated from *A. officinarum* against respiratory syncytial virus (RSV), poliovirus, measles virus, and herpes simplex virus type 1 (HSV-1) using a plaque reduction assay. The 50% inhibitory concentrations of seven of the nine diarylheptanoids for RSV were moderately but significantly lower than their 50% cytotoxic concentrations, as determined by a trypan blue exclusion assay. Four diarylheptanoids with anti-RSV activity also showed anti-poliovirus and anti-measles virus activities and three of the four exhibited anti-HSV-1 activity. Thus, seven of the nine diarylheptanoids examined exhibited potential antiviral activity against RSV, and most of the diarylheptanoids with anti-RSV activity, including two diarylheptanoids without anti-RSV activity, were effective against poliovirus, measles virus, and/or HSV-1 *in vitro*. Diarylheptanoids were suggested to have a broad spectrum of antiviral activity.

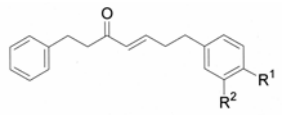
Keywords: diarylheptanoids, antiviral activity, RSV, HSV-1, measles virus, poliovirus.

Alpinia officinarum (*A. officinarum*), family Zingiberaceae, is known as lesser galangal. This rhizome has been used in various Asian cuisines and as a traditional medicine, such as an antiemetic, stomachic, and analgesic in Asia from ancient times.

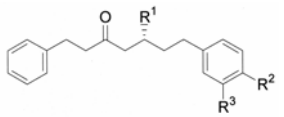
Respiratory syncytial virus (RSV) infection is very common in children less than 2 years old and sometimes causes serious bronchilitis and pneumonia [1]. In elderly and high-risk adults, RSV infection is an important illness [2]. Ribavirin, palivizumab, and motavizumab are used for the treatment and prevention of RSV infection [3–6], but there are few clinically specific and effective anti-RSV drugs.

In a series of studies on the development of bioactive components from natural sources, we found that a methanol extract from the rhizome of *A. officinarum* is effective in inhibiting tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in mouse skin [7]. An extract of *A. officinarum* was previously shown to exhibit therapeutic efficacy against herpes simplex virus type 1 (HSV-1) infection in mice [8]. Diarylheptanoids isolated from *A. officinarum* have been shown to exhibit cytotoxic activity [9], suppressive activity of inducible nitric oxide synthase expression [10], inhibitory activity of biosynthesis of prostaglandin and leukotrienes [11,12], and inhibitory activity of proinflammatory mediators [13]. Although a variety of biological activities associated with diarylheptanoids have been demonstrated, antiviral activity of diarylheptanoids has been reported only against influenza virus [14,15]. In the present study, we examined the potential anti-RSV activity of diarylheptanoids *in vitro*. Their anti-RSV activities were compared with antiviral activities against poliovirus, measles virus, and HSV-1 to characterize the anti-RSV activity.

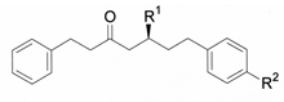
Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were examined for their anti-RSV activity and cytotoxicity *in vitro*. As shown in Table 1, the EC_{50} values of seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7**-**9**) were significantly lower than their CC_{50} values. DMSO at 1%, the maximum concentration used to dissolve diarylheptanoids in the culture medium, was not cytotoxic. The therapeutic indexes (CC_{50}/EC_{50}) of 7-(4”-hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-2**) and (5*S*)-5-methoxy-1,7-diphenyl-3-heptanone (**AO-7**) were 4.6 and more than 6.1, respectively, and RSV was more



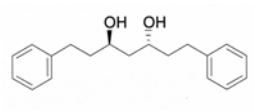
	R^1	R^2
AO-1	H	H
AO-2	OH	H
AO-3	OH	OCH_3



	R^1	R^2	R^3
AO-4	OH	H	H
AO-5	OH	OH	OCH_3
AO-6	OCH_3	OH	OCH_3



	R^1	R^2
AO-7	OCH_3	H
AO-8	OCH_3	OH



AO-9

Figure 1: Structures of diarylheptanoids (**AO-1** to **9**) from *A. officinarum*.

susceptible to **AO-2** and **AO-7** than to the other diarylheptanoids examined. In this assay, the EC_{50} value of ribavirin, used as a control, was similar to the results reported previously [16,17]. Thus, seven diarylheptanoids (**AO-1**, **2**, **4**, **5**, and **7**-**9**) were demonstrated to show moderate, but potential antiviral activity against RSV *in vitro*. This is the first evidence demonstrating the anti-RSV activity of diarylheptanoids *in vitro*.

To evaluate the antiviral spectrum of diarylheptanoids with anti-RSV activity, four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) with anti-RSV activity were examined for anti-poliovirus, -measles virus, and -HSV-1 activities. However, three diarylheptanoids (**AO-2**, **8**, and **9**) with anti-RSV activity were not used, because there were not sufficient amounts to perform a plaque reduction assay. As shown in Table 2, the EC_{50} values of four diarylheptanoids (**AO-1**, **4**, **5**, and **7**) for poliovirus and measles virus were significantly lower than their CC_{50} values. The EC_{50} values of three (**AO-4**, **5**, and **7**) of the four for HSV-1 were also significantly lower than their CC_{50} values. Therefore, among the four diarylheptanoids, three (**AO-4**, **5**, and **7**) with anti-RSV activity exhibited anti-poliovirus, -measles virus, and -HSV-1 activities. **AO-1** exhibited anti-poliovirus and -measles virus activities, but not anti-HSV-1 activity. Because the three diarylheptanoids (**AO-4**, **5**, and **7**) showed antiviral activity against all viruses used in this study, they were suggested to have broad spectrum antiviral activity.

We also examined anti-poliovirus, -measles virus, and -HSV-1 activities of **AO-3** and **6** that did not exhibit anti-RSV activity *in vitro*. As shown in Table 2, **AO-3** was significantly effective for measles virus, but not for poliovirus and HSV-1. However, all three viruses examined were significantly susceptible to **AO-6**. Only measles virus was susceptible to all of the six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) without relation to anti-RSV activity. Diarylheptanoids without anti-RSV activity were also effective against poliovirus, measles virus, and/or HSV-1 *in vitro* and the broad spectrum of antiviral activity was confirmed.

RSV has a different virus structure and replication cycle from poliovirus and HSV-1. However, it has a similar virus structure and replication cycle to measles virus as some paramyxoviruses. In Table 2, six diarylheptanoids (**AO-1**, **3**, **4**, **5**, **6**, and **7**) exhibited anti-measles virus activity. However, two (**AO-3** and **6**) of them had no anti-RSV activity (Table 1). It is possible that **AO-3** and **6** interfered with a replication step specific to measles virus but not RSV in paramyxoviruses. Although we focused on diarylheptanoids with anti-RSV activity in this study, **AO-3** and **6** were suggested to be potent candidates as anti-measles virus compounds. In our screening of anti-RSV activity *in vitro*, the CC_{50}/EC_{50} value (>6.1) of **AO-7** was highest (Table 1). **AO-7** also exhibited anti-poliovirus, -measles virus, and -HSV-1 activities (Table 2) and may be characterized as a candidate for an anti-RSV compound with a broad antiviral spectrum. Studies of the structure-antiviral activity relationships of many diarylheptanoids isolated from *A. officinarum* [18–20] against various kinds of viruses may be worthwhile to analyze the antiviral actions and to obtain more effective antiviral diarylheptanoids.

RSV was significantly susceptible to seven of the nine diarylheptanoids isolated from *A. officinarum*. Of the nine, six (**AO-1**, **3**, **4**, **5**, **6**, and **7**) with or without anti-RSV activity were effective against poliovirus, measles virus, and/or HSV-1.Thus, diarylheptanoids were suggested to possess a broad spectrum of antiviral activity.

Table 1: Anti-RSV activity and cytotoxicity of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)	CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}
1,7-Diphenyl-4*E*-hepten-3-one(**AO-1**)	36.3 ± 4.2 [c]	47.3 ± 1.3	1.3
7-(4”-Hydroxyphenyl)-1-phenyl-4*E*-hepten-3-one(**AO-2**)	5.0 ± 0.0 [c]	22.8 ± 2.5	4.6
7-(4”-Hydroxy-3”-methoxyphenyl)-1-phenyl-4*E*-hepten-3-one (**AO-3**)	42.7 ± 3.5	39.3 ± 6.4	0.9
(5*R*)-5-Hydroxy-1,7-diphenyl-3-heptanone (**AO-4**)	21.7 ± 0.6 [c]	38.3 ± 3.4	1.8
(5*R*)-5-Hydroxy-7-(4”-hydroxy-3”-methoxyphenyl)-1-phenyl-3-heptanone(**AO-5**)	37.0 ± 7.2[c]	84.8 ± 3.8	2.3
(5*R*)-5-Methoxy-7-(4”-hydroxy-3”-methoxyphenyl)-1-phenyl-3-heptanone (**AO-6**)	13.3 ± 3.8	17.0 ± 0.8	1.3
(5*S*)-5-Methoxy-1,7-diphenyl-3-heptanone (**AO-7**)	16.3 ± 3.5 [c]	>100	>6.1
(5*S*)-5-Methoxy-7-(4”-hydroxyphenyl)-1-phenyl-3-heptanone (**AO-8**)	21.7 ± 0.6 [c]	31.5 ± 6.6	1.5
(3*R*,5*R*)-1,7-Diphenylheptan-3,5-diol (**AO-9**)	22.3 ± 0.6 [c]	56.3 ± 3.1	2.5
Ribavirin	0.67 ± 0.08	NT [d]	NT [d]

The structures of these diaryheptanoids are shown in Figure 1.
[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]$P<0.05$ vs. CC_{50}; [d]Not tested.

Table 2: Anti- poliovirus, -measles virus, and -HSV-1 activities and cytotoxicities of diarylheptanoids.

Compounds	EC_{50}[a] (μg/mL)			CC_{50}[b] (μg/mL)	CC_{50} / EC_{50}		
	Poliovirus	Measles virus	HSV-1		Poliovirus	Measles virus	HSV-1
AO-1	8.3±2.3[c]	17.3±1.2[c]	53.7±4.7	45.8±1.7	5.5	2.6	0.9
AO-3	64.3±4.9	47.0±4.6[c]	59.7±0.6	63.0±10.4	1.0	1.3	1.1
AO-4	22.7±1.5[c]	17.0±2.0[c]	54.0±5.6[c]	69.5±5.2	3.1	4.1	1.3
AO-5	44.3±4.0[c]	18.3±1.2[c]	58.7±1.5[c]	>100	2.3	5.5	1.7
AO-6	3.7±0.6[c]	6.3±0.6[c]	5.7±0.6[c]	10.8±1.3	2.9	1.7	1.9
AO-7	16.7±2.1[c]	18.0±1.0[c]	18.3±0.6[c]	40.5±5.4	2.4	2.3	2.2
Acyclovir	NT[d]	NT[d]	0.23±0.04	NT[d]	NT[d]	NT[d]	NT[d]

[a]Means ± SD for four independent experiments; [b]Means ± SD for three independent experiments; [c]$P<0.05$ vs. CC_{50}; [d]Not tested.

Experimental

Chemicals: Dimethyl sulfoxide (DMSO) and ribavirin were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Acyclovir was purchased from GlaxoSmithKline, Tokyo, Japan. Diarylheptanoids (**AO-1** to **9**, Figure 1 and Table 1) were isolated from the rhizome of *A. officinarum*, as described previously [9,20].

Cells and viruses: Human epidermoid carcinoma (HEp-2) cells (American Type Culture Collection CCL-23) were purchased from Dainippon Pharmaceutical, Osaka, Japan, and grown and maintained in Eagle's minimum essential medium (EMEM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% and 2% heat-inactivated fetal calf serum, respectively. Vero E6 cells were provided by Dr K. Shiraki (Toyama University, Japan) and grown and maintained in EMEM supplemented with 8% and 2% heat-inactivated calf serum, respectively. The A2 strain of RSV was obtained from American Type Culture Collection (Rockville, MD) and grown in HEp-2 cell cultures. Poliovirus type 1 (Sabin strain), measles virus (Tanabe strain), and HSV-1 (7401H strain) were provided by Dr K. Shiraki (Toyama University, Japan) and propagated in Vero cells [8].

Antiviral and cytotoxic assays: The anti-RSV activities of 9 diarylheptanoids were examined by a plaque reduction assay using HEp-2 cells [21,22]. Briefly, HEp-2 cells grown in 24-well plates were infected with 100 plaque-forming units (PFU)/0.2 mL of RSV at 37ºC for 1 h. The cells were overlaid with 1 mL of maintenance EMEM containing 0.8% methylcellulose and various concentrations of either diarylheptanoids or ribavirin and maintained in a humidified atmosphere containing 5% CO_2 for 4–5 days.

The anti-poliovirus, -measles virus, and -HSV-1 activities were also examined by a plaque reduction assay using Vero cells [8]. Duplicate cultures of Vero cells in 60-mm plastic dishes were infected with 100 PFU/0.2 mL of poliovirus, measles virus, or HSV-1 for 1 h. Then the cells were overlaid with 5 mL of nutrient 0.8% methylcellulose medium containing various concentrations of either diarylheptanoids or acyclovir. The virus-infected cultures were incubated for 2–5 days at 37ºC. The infected cells were fixed and stained, and the plaques were counted [8]. All diarylheptanoids were dissolved in DMSO and diluted with culture medium to make the various final concentrations. The concentration of DMSO in each medium was less than 1%. Ribavirin and acyclovir were dissolved in distilled water and DMSO, respectively, and used as controls. The 50% effective antiviral concentration (EC_{50}) was the concentration that reduced virus-induced cell destruction by 50%, as described previously [8,21].

The cytotoxicity of diarylheptanoids was assessed by trypan blue exclusion assays using mock-infected HEp-2 or Vero cells. The cells were seeded at a concentration of 5 x 10^4 cells/mL in 24-well plates. After incubation at 37ºC for 24 h, the culture medium was replaced with fresh medium containing one of the diarylheptanoids at various concentrations and the cells were further incubated for 48 h. After 48 h, the cells were trypsinized and the number of viable cells was determined by a trypan blue exclusion assay. The 50% cytotoxic concentration (CC_{50}) was determined as the concentration that reduced cell destruction by 50% [21].

Statistical analysis: Statistical significances of differences between the EC_{50} and CC_{50} values were evaluated using Student's *t*-test. A *P* value of 0.05 or less was considered to be significant statistically.

Acknowledgments - We thank Ms Akane Hino for her excellent technical assistance and Ms Katherine Ono for her editorial assistance. This study was partly supported by Grant-in-Aid for Scientific Research (No. 20590131) from the Japan Society for the Promotion of Science, The Uehara Memorial Foundation, and Health and Labor Sciences Research Grants (Research on Risk of Chemical Substances) from the Ministry of Health, Labor and Welfare of Japan.

References

[1] Villarruel GR, Langley GE, Abedi GR, Anderson LJ. (**2009**) Respiratory syncytial virus activity-United States, July 2008–December 2009, *MMWR Morbidity and Mortality Weekly Report*, ***59***, 230-233.

[2] Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. (**2005**) Respiratory syncytial virus infection in elderly and high-risk adults. *The New England Journal of Medicine*, ***352***, 1749-1759.
[3] Cingoz O. (**2009**) Motavizumab. *MAbs*, ***1***, 439-442.
[4] Gill MA, Welliver RC. (**2009**) Motavizumab for the prevention of respiratory syncytial virus infection in infants. *Expert Opinion on Biological Therapy*, ***9***, 1335-1345.
[5] Pelaez A, Lyon GM, Force SD, Ramirez AM, Neujahr DC, Foster M, Naik PM, Gal AA, Mitchell PO, Lawrence EC. (**2009**) Efficacy of oral ribavirin in lung transplant patients with respiratory syncytial virus lower respiratory tract infection. *Transplantation infection*, ***28***, 67-71.
[6] Weisman LE. (**2009**) Respiratory syncytial virus (RSV) prevention and treatment: past, present, and future. *Cardiovascular & Hematological Agents in Medicinal Chemistry*, ***7***, 223-233.
[7] Yasukawa K. Sun Y, Kitanaka S, Tomizawa N, Miura M, Motohashi S. (**2008**) Inhibitory effect of the rhizomes of *Alpinia officinarum* on TPA-induced inflammation and tumor promotion in two-stage carcinogenesis in mouse skin. *Journal of Natural Medicines*, ***62***, 374-378.
[8] Kurokawa M, Ochiai H, Nagasaka K, Neki M, Xu H, Kadota S, Sutardjo S, Matsumoto T, Namba T, Shiraki K. (**1993**) Antiviral traditional medicines against herpes simplex virus (HSV-1), poliovirus, and measles virus *in vitro* and their therapeutic efficacies for HSV-1 infection in mice. *Antiviral Research*, ***22***, 175-188.
[9] Sun Y, Tabata K, Matsubara H, Kitanaka S, Suzuki T, Yasukawa K. (**2008**) New cytotoxic diarylheptanoids from the rhizomes of *Alpinia officinarum*. *Planta Medica*, ***74***, 427-431.
[10] Lee HJ, Kim JS, Ryu J-H. (**2006**) Suppression of inducible nitric oxide synthase expression by diarylheptanoids from *Alpinia officinarum*. *Planta Medica*, ***72***, 68-71.
[11] Kiuchi F, Shibuya M, Sankawa U. (**1982**) Inhibitors of prostaglandin biosynthesis from *Alpinia officinarum*. *Chemical & Pharmaceutical Bulletin*, ***30***, 2279–2282.
[12] Kiuchi F, Iwakami S, Shibuya M, Hanaoka F, Sankawa U. (**1992**) Inhibition of prostaglandin and leukotriene biosynthesis by gingerols and diarylheptanoids. *Chemical & Pharmaceutical Bulletin*, ***40***, 387-391.
[13] Yadav PN, Liu Z, Rafi MM. (**2003**) A diarylheptanoid from Lesser Galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen-activated protein kinase, p44/42, and transcription factor nuclear factor-κB. *The Journal of Pharmacology and Experimental Therapeutics*, ***305***, 925-931.
[14] Sawamura R, Sun Y, Yasukawa K, Shimizu T, Watanabe W, Kurokawa M. (**2010**) Antiviral activities of diarylheptanoids against influenza virus *in vitro*. *Journal of Natural Medicines*, ***64***, 117-120.
[15] Sawamura R, Shimizu T, Sun Y, Yasukawa K, Miura M, Toriyama M, Motohashi S, Watanabe W, Konno K, Kurokawa M. (**2010**) Anti-influenza virus activity *in vitro* and *in vivo* of diarylheptanoids isolated from *Alpinia officinarum*. *Antiviral Chemistry and Chemotherapy*, ***21***, 33-41.
[16] Kawana F, Shigeta S, Hosoya M, Suzuki H, De Clercq E. (**1978**) Inhibitory effects of antiviral compounds on respiratory syncytial virus replication *in vitro*. *Antimicrobial Agents and Chemotherapy*, ***31***, 1225-1230.
[17] Xie YY, Zhao XD, Jiang LP, Liu HL, Wang LJ, Fang P, Shen KL, Xie ZD, Wu YP, Yang XQ. (**2006**) Inhibition of respiratory syncytial virus in cultured cells by nucleocapsid gene targeted deoxyribozyme (DNAzyme). *Antiviral Research*, ***71***, 31-41.
[18] An N, Xu L-Z, Zou Z-M, Yang S-L. (**2006**) Diarylheptanoids from *Alpinia officinarum*. *Journal of Asian Natural Products Research*, ***8***, 637–641.
[19] Shin D, Kinoshita K, Koyama K, Takahashi K. (**2002**) Antiemetic principles of *Alpinia officinarum*. *Journal of Natural Products*, ***65***, 1315–1318.
[20] Sun, Y, Matsubara H, Kitanaka S, Yasukawa K. (**2008**) Diarylheptanoids from the rhizomes of *Alpinia officinarum*. *Helvetica Chimica Acta*, ***91***, 118-123.
[21] Watanabe W, Shimizu T, Hino A, Kurokawa M. (**2008**) A new assay system for evaluation of developmental immunotoxicity of chemical compounds using respiratory syncytial virus infection to offspring mice. *Environmental Toxicology and Pharmacology*, ***25***, 69-67.
[22] Watanabe W, Konno K, Ijichi K, Inoue H, Yokota T, Shigeta S. (**1994**) MTT colorimetric assay system for the screening of anti-orthomyxo- and anti-paramyxoviral agents. *Journal of Virological Methods*, ***48***, 257-265.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1885 - 1888

Isolation of *C*-glycosyl Xanthones from *Coffea pseudozanguebariae* and Their Location

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Received: October 21st, 2010; Accepted: July 17th, 2011

The biochemical composition of leaves from *Coffea pseudozanguebariae*, a wild caffeine-free coffee species, was determined. Two phenolic compounds were extracted from leaves, separated and characterized. Their structures were elucidated by mass spectrometry, and 1D and 2D NMR spectroscopy and were shown to be mangiferin (**1**) and isomangiferin (**2**), which were the main polyphenol products. Multiphoton fluorescence imaging was performed to visualize polyphenol distribution in leaf cross sections. Consistent biochemical analysis cell imaging techniques on leaves revealed yellow fluorescence in the epidermis and parenchyma cells corresponding to xanthone compounds.

Keywords: *Coffea pseudozanguebariae* Bridson, xanthones, fluorescence, mangiferin, isomangiferin, Rubiaceae.

Abbreviations: hydroxycinnamoyl quinic acid, HQA, caffeoylquinic acids (3,4,5), CQA; di-caffeoyl quinic acids (3,4; 3,5; 4,5), diCQA.

Coffea pseudozanguebariae Bridson, native to East Africa, is a wild species of coffee tree and the first caffeine-free species discovered in tropical East Africa. It exhibits different morphological and physiological characteristics from other species of coffee: one of the shortest fruiting times [1a], low hydroxycinnamoyl quinic acid (HQA) content [1.2% dmb (dry matter basis)] [1b], morphological aspects (small purple fruits on complete ripeness and small sized leaves), and a small sized genome (1.13 pg). Previous phytochemical investigations described the presence of alkaloids, with the major component trigonelline, diterpenes (cafestol, kahweol), and phenolic compounds (HQA, hydroxycinnamoyl quinic acid, or chlorogenic acids) in *C. pseudozanguebariae.* The unsaponifiable lipid fraction of green beans contains a mozambioside, a diterpene glycoside [2], high cafestol, kahweol, and four unknown diterpenes [3]. The diterpene glycoside, which is not present in the other species of commercial coffee trees, is the origin of the strong bitterness of this coffee [2]. In the soluble fraction, two classes of secondary metabolites have been particularly studied in green coffee beans: alkaloids and phenolic compounds. The content of the major alkaloid, caffeine, varies markedly between species, from 0%, dry wt, in *C. pseudozanguebariae* to 3.2%, dry wt, in *C. canephora* [4]. Trigonelline, a major coffee aroma compound, forms 1.02%, dry wt, of *C. pseudozanguebariae*, 0.67%, dry wt, of *C. canephora*, and 0.57%, dry wt, of *C. liberica* var. *dewevrei* [5]. Only two classes of phenolic compounds have been described in coffee plants: a major one, HQA and a minor one, proanthocyanins, from the flavonoid class [6]. Other phenolic compounds are polymeric, such as tannins. Lignans are also present in coffee seeds, although in minor amounts. This main family is formed from esters between hydroxycinnamate and quinic acid. No previous study on *C. pseudozanguebariae* has been reported in the literature. In this paper, we report the first chemical investigation of *C. pseudozanguebariae* leading to the isolation of compounds including the representative phenolic compounds and the visualization of their accumulation in the tissue.

A methanolic extract of *C. pseudozanguebariae* leaves was chromatographed on a RP-18 column and showed a major peak with a retention time at 10.1 min. This peak exhibited a spectrum with four maximum UV absorbances at 240 nm, 257 nm, 316 nm, and 365 nm. The minor peak at 10.9 min had the same UV characteristics. A peak at 7.3 min was identified, with the help of UV spectra, as 5-*O*-caffeoylquinic acid (or chlorogenic acid). Semi-preparative chromatography was used to isolate the unknown compounds. After evaporation, a yellow powder

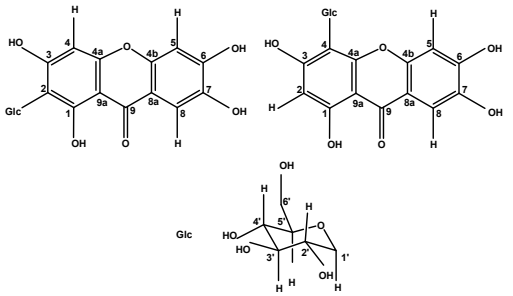

Figure 1: Structure of *C*-glucosyl xanthones. **1**: C2-β–D-glucoside 1,3,6,7 tetrahydroxyxanthen-9-one (or mangiferin), **2**: C4-β–D-glucoside 1,3,6,7 tetrahydroxyxanthen-9-one (or isomangiferin).

was obtained and used to carry out the first analysis for structure elucidation. Another separation system using a combination of cellulose column and Sephadex LH-20 gel filtration gave yellow crystalline materials (compounds **1**, **2**). From their physical, EI mass spectrometric and NMR spectroscopic features compound **1** was unambiguously established as 2-*C*-β-D-glucopyranosyl- 1,3,5,7 tetrahydroxy-xanthen-9-one (mangiferin) [7a,7b], and **2** as 4-*C*-β-D-glucopyranosyl- 1,3,5,7 tetrahydroxyxanthen-9-one (isomangiferin) [8]. Compound **2** led to a slight hypsochrome UV spectrum displacement at 318 nm. The ^{1}H spectrum of **2** is very close to that of **1** except for signals of two aromatic protons at δ_H 6.02 and 6.17.

Cross-sections of fresh *C. pseudozanguebariae* leaves at two stages of development were observed by epifluorescence microscopy. The young leaves (maroon color, and less than 4 cm long) showed a yellow and orange fluorescence in all cells (Figure 2A). The mature leaves (green color) did not show such a fluorescence, except for some cells in the mesophyll, which gave an orange fluorescence (Figure 2B). It is known that phenolic compounds naturally have the ability to emit fluorescence under ultraviolet light. The autofluorescence of polyphenols was used to localize them in tissues of the leaves. In order to visualize the accumulation of phenolic compounds, a spectral analysis was developed on the same fresh leaf sections. This technique was achieved with a special detection system, which enabled separation of the signals from different fluorophores. We used this technique with a multiphotonic microscope because the infra-red laser associated with this microscope gave the possibility of exciting molecules like a UV laser. With an excitation wavelength between 700 and 800 nm, it was possible to excite like a UV laser between 350 and 400 nm. In these optical conditions, spectral signatures were acquired for each pixel of the scanned image (from either leaf section or purified compound) and could subsequently be used for digital separation into component dyes. Then, from these spectral signatures, the Linear Unmix method was used to discriminate between various fluorescence signals, even with widely overlapping emission spectra. The emission spectral curve data obtained from the compounds were recorded in a spectral library and applied on cross-sections of leaves to localize them. The calculation was realized using a high number of iterations and by keeping a residual channel dedicated to pixels not corresponding to the selected spectral signatures. The spectral acquisition obtained on young leaf cross-sections showed a complex pattern of autofluorescence resulting from various fluorescent compounds. The fluorescence observed in young leaves, identified as the spectrum emission of mangiferin, was found after Linear Unmixing calculation in the upper epidermis and in some mesophyll cells (Figure 2C). The same technique was used with the spectrum emission of 5-*O*-caffeoylquinic acid (chlorogenic acid), the main phenolic compound in coffee, but no signal was detected on the leaves (data not shown). There were other uncharacterized fluorescent compounds in the mesophyll, which did not correspond to the known compounds and which seems to belong to other families of secondary metabolites. These compounds are not accumulated in the leaves of *C. arabica* and *C. canephora,* the commercial species of coffee. The decrease in yellow fluorescence observed between the very young and mature leaves may tally with biochemical analysis [9], in particular, during leaf development; there is a sharp decrease in the main phenolic compound, mangiferin. Consequently, tissue-specific localization could provide valuable information for understanding the actual role in the mechanisms of acclimatization to several environmental agents such as UV radiation or against pathogen and predator attacks. The fluorescent technologies now available allow researchers to study such dynamic processes in living cells.

Experimental

General experimental procedures: Chromatographic separation was performed using a HPLC series (Shimadzu, Prominence LC) equipped with software, a DGU-20A$_3$ degasser, an LC-20AD binary gradient pump, a SIL-20AC thermoautosampler, and a SPD-M20A diode array detector. The column used was a LiChrospher 5 µm RP18 (250X4 mm i.d.) from Merck (Darmstadt, Germany), and a guard column of the same material. The mobile phase consisted of 2mM phosphoric acid in water (eluent A) and MeOH (eluent B). The gradient program was as follows: a 25-80% MeOH gradient over 40 min at a flow rate of 0.8 mL.min^{-1}. Separation was at room temperature and the injection volume was 500 µL for isolation and 20 µL for identification. Phenolic compounds were identified by comparing their retention times with appropriate standards: 5-caffeoyl quinic acid, 3-caffeoyl quinic acid and 3,5-dicaffeoylquinic acid. A Q-TOF Micromass (Waters, Milford, MA, USA) mass spectrometer was used to obtain the MS data. The sample was solubilized in 50% water-acetonitrile solution, acidified using 0.1% trifluoroacetic acid solution and introduced at a flow rate of 5 µL min^{-1}. All the analyses were performed using an electrospray ionisation source (ESI) set to 100°C in positive ion mode with the following settings: capillary voltage -3000 V, cone voltage = 30 and 50 V, nebuliser gas (N_2) 400 L/h, desolvation temperature 120°C, drying gas (N_2) 20 l/h.

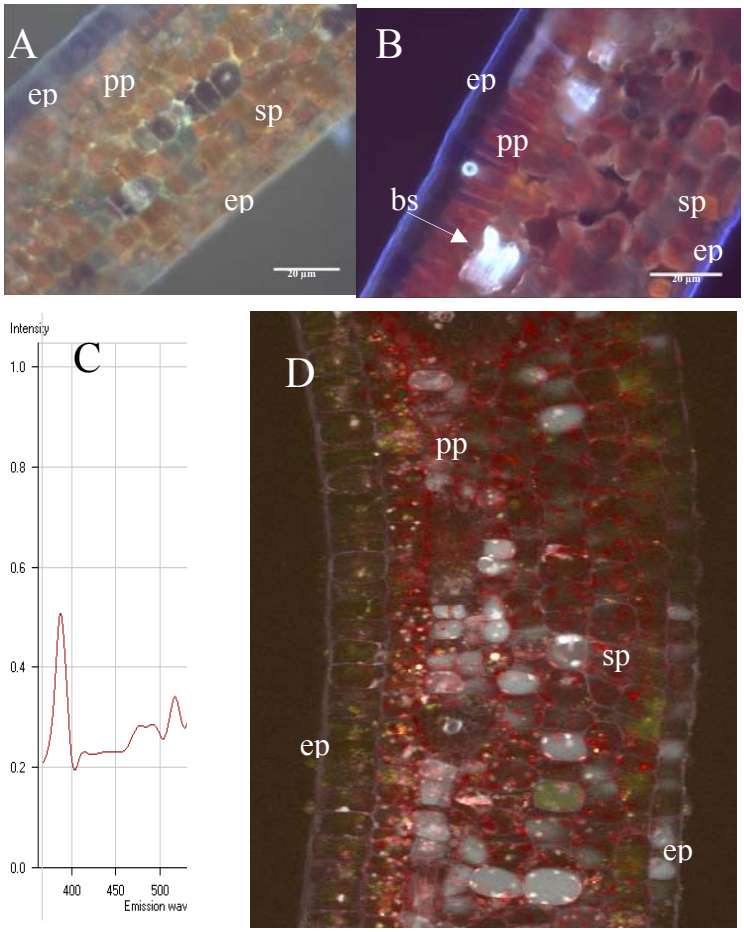

Figure 2: Histological imaging of fluorescent compound accumulation in the leaves of C. *pseudozanguebariae* . Cross-sections of young leaves (A) and mature leaves (B) by epifluorescence microscopy under UV light (long-pass filter 425 nm, real colours). Cross-sections of young leaves by spectral analysis and linear unmixing (C) on a multiphotonic microscope (false colors, yellow: mangiferin, red: chlorophyll, white: unknown). ep: epidermis, pp: palisade parenchyma, sp: spongy parenchyma, bs: bundle sheath cell, bar = 20 µm.

Mass spectra were monitored using MassLynx4.0. software, with an acquisition rate of 2 spectra s^{-1} in the range *m/z* = 70-600. NMR spectra were measured using a Bruker DRX-400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C).

Histology: Thick cross-sections (50 µm) were obtained from young leaves of *Coffea pseudozanguebariae* using a vibratome (MICROM) and then dipped in 10 mM phosphate buffer saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 120 mM NaCl, 2.7 mM KCl). Epi-fluorescence microscopy was carried out on a Leica DMRXA equipped with a Q- Imaging camera (long-pass filter 425 nm). A Zeiss 510 META NLO multiphoton microscope equipped with a Coherent Chameleon Ultra II laser was used to obtain emission fluorescence from fresh leaves. Spectral analysis was carried out using the autofluorescence properties of polyphenol compounds without any dyes. Reference spectra on purified powder of polyphenol compounds (HQA and xanthones) were obtained from spectral acquisition with excitation at 780 nm and emission between 400 and 700 nm. The Linear Unmixing Function of the microscope (method of Emission Finger printing from Zeiss) was used to visualize the fluorescence of polyphenol compounds in cells from reference spectra.

Plant material: In these experiments we used *Coffea* plants from the collection cultivated at the IRD research centre in Montpellier (France). Leaves of *C. canephora* (DB56, DB57) and *C. pseudozanguebariae* (H65, H70) of various genotypes were taken from trees maintained in a tropical greenhouse (natural daylight, 25°C, 28°C day, 78-82% humidity). The young leaves, under 4 cm long, were cut from the tips of branches. Leaves were harvested from 5 different genotypes and 500 g of the collected leaves were frozen in liquid nitrogen immediately after collection then stored at -80°C. Each batch of leaves was freeze-dried for 48 h then stored in a cold room. *C. pseudozanguebariae* fruits were harvested at the CNRA Station in Divo (Ivory Coast) from field-grown trees.

Extraction and isolation: Fine powder of leaves and beans was obtained in an analytical grinder (IKA, yellow, A10) by grinding for 1 min, repeated 3 times. The leaf powders (2 g) were extracted with 70% MeOH solution (30 mL, X3) at 4°C and bean powders (50 mg) in 5 mL of 70%

MeOH solution. The MeOH extracts were combined, filtered and evaporated under reduced pressure to give a residue. This was suspended in MeOH and filtered through a 0.2-µm disposable filter tip-syringe assembly and directly analysed by HPLC for separation and identification. A second technique was used with 80 g of freeze-dried leaves suspended in 700 mL of MeOH-H_2O (8:2) solution at room temperature for 20 min with sonication (20 min, 24 KHz, R.E.U.S-GEX 180, Contes, France), repeating 3 times. MeOH was removed in a rotavapor. After freeze-drying, the aqueous phase was subjected to a Medium Pressure (MP) column (400X47 mm) packed with cellulose (microcristallin Avicel, Darmstadt, Germany) and successively eluted with H_2O and H_2O-MeOH (1:9). Collected fractions were purified on a Sephadex LH-20 (Fluka) column (500 X 25 mm, Fluka, Basel, Switzerland) and eluted with H_2O for compound **1**. For compound **2**, fractions were purified on a MP column (210 X 30 mm, Buchi) eluted with EtOH- H_2O (8:2). After freeze-drying, both compounds (**1** and **2**) were obtained.

Acknowledgements - The authors thank Dr Maryse Bejaud for support in NMR analysis and helpful discussion. We wish to thank Elisabeth Ambert for assistance in checking bibliographic databases and for valuable help.

References

[1] (a) Akaffou DS, Ky CL, Barre P, Hamon S, Louarn J, Noirot M. (**2003**) Identification and mapping of a major gene (Ft1) involved in fructification time in the interspecific cross *Coffea pseudozanguebariae* x *C. liberica* var. 'dewevrei': impact on caffeine content and seed weight. *Theoretical Applied Genetics*, ***106***, 1486-1490; (b) Ky CL, Louarn J, Guyot B, Charrier A, Hamon S, Noirot M. (**1999**) Relations between and inheritance of chlorogenic acid contents in an interspecific cross between *Coffea pseudozanguebariae* and *C. liberica* var. 'dewevrei'. *Theoretical Applied Genetics*, ***98***, 628-637.

[2] Prewo R, Guggisberg A, Lorenzi-Riatsch A, Baumann TW, Wettstein-Bättig M. (**1990**) Crystal structure of mozambioside, a diterpene glycoside of *Coffea pseudozanguebariae. Phytochemistry*, ***29***, 990-992.

[3] de Roos B, van der Weg G, Urgert R, van de Bovenkamp P, Charrier A, Katan MB. (**1997**) Levels of cafestol, kahweol, and related diterpenoids in wild species of the coffee plant *Coffea. Journal of Agricultural and Food Chemistry*, ***45***, 3065-3069.

[4] Anthony F, Noirot M, Clifford MN. (**1993**) Biochemical diversity in the genus *Coffea* L.: Chlorogenic acids, caffeine, and mozambioside contents. *Genetic Resources and Crop Evolution*, ***40***, 61–70.

[5] Ky CL, Guyot B, Louarn J, Hamon S, Noirot M. (**2001**) Trigonelline inheritance in the interspecific cross between *Coffea pseudozanguebariae* x *C. liberica* var. dewevrei cross. *Theoretical Applied Genetics*, ***102***, 630-634.

[6] Clifford MN, Gibson CL, Rakotomalala JJ, Cros E, Charrier A. (**1991**) Caffeine from green beans of *Mascarocoffea*. *Phytochemistry*, ***30***, 4039-4040.

[7] (a) Fujita, M., Inoue, T. (**1982**) Studies on the constituents of *Iris florentina* L.II.- *C*-Glucosides of xanthones and flavones from the leaves. *Chemical & Pharmaceutical Bulletin*, ***30***, 2342-2348; (b) Catalano S, Lushi S, Flamini G, Cioni PL, Nieri EM, Morelli I. (**1996**) A xanthone from *Senecio mikanioides* leaves. *Phytochemistry*, ***42***, 1605-1607.

[8] de Beer D, Jerz G, Joubert E, Wray V, Winterhalter P. (**2009**) Isolation of isomagiferin from honeybush (*Cyclopia subternata)* using high-speed-counter-current chromatography and high-performance liquid chromatography. *Journal of Chromatography A*, ***1216***, 4282-4289.

[9] Bertrand C, Noirot M, Doulbeau S, de Kochko A, Hamon S, Campa C. (**2003**) Chlorogenic acid content swap during fruit maturation in *Coffea pseudozanguebariae.* Qualitative comparison with leaves. *Plant Science*, ***165***, 1355-1361.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1889 - 1892

Antifungal Activity and Isomerization of Octadecyl *p*-coumarates from *Ipomoea carnea* subsp. *fistulosa*

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Received: June 6th, 2011; Accepted: October 25th, 2011

Bioassay monitored HPLC assisted isolation and purification of the chief antifungal fraction of the leaves of *Ipomoea carnea* subsp. *fistulosa* (Convulvulaceae) were achieved using *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* as test organisms. The activity of the purified fraction was further confirmed by the dose dependent inhibition of the spore germination of *Alternaria alternata* and *A. porri.* The active fraction was identified as a mixture of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate. The two isomers were detected on an HPLC column with substantially different retention times, but once eluted from the column, one form was partly converted to the other in daylight. Conclusive evidence for the structures and their isomerization were obtained from the HPLC behavior, IR, UV, HRESIMS, CIMS and NMR spectral data. Important ^{1}H NMR and ^{13}C NMR signals could be separately assigned for the isomers using 2D NMR techniques.

Keywords: *Ipomoea carnea*, *Ipomoea fistulosa*, Convulvulaceae, antifungal, (*E*) octadecyl *p*-coumarate, (*Z*) octadecyl *p*-coumarate, isomerization, 2D NMR techniques.

Ipomoea carnea subsp. *fistulosa* (Mart. ex Choisy) D.F.Austin (Convulvulaceae) is a plant native to South America, but sparsely distributed in India and Bangladesh. It is used in hedgerows along cattle crossings, to fight erosion and as an ornamental. Isolation and chemical characterization of resinous glycosides [1], flavonol glycosides [2] and alkaloids [3] from the leaves, and anthocyanin from the flowers [4] of *I. carnea* have been reported. The leaves are toxic to cattle and the toxicity is attributed to polyhydroxy alkaloids such as swainsonine and calystegines [5]. Recently, a chitinase has been identified in the plant [6]. Antibacterial and antifungal activities of the extractives of the plant have been reported [7], but bioassay monitored isolation and characterizations of the antifungal compounds present in the plant have not yet been carried out. We hereby report the bioassay monitored isolation and characterization of the chief antifungal fraction. The fraction was isolated using *Colletotrichum gloeosporioides* and *Cladosporium cucumerinum* as test organisms and the activity was further confirmed against the spore germination of *Alternaria alternata* and *A. porri.* The active fraction was found to be a mixture of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate. The two isomers were detected on the HPLC column with substantially different retention times, but once eluted from the column; one form was partly converted to the other. Conclusive evidence for the structures and their isomerization were obtained from the HPLC behavior, IR, UV, HRESIMS, CIMS, ^{1}H NMR, ^{13}C NMR, DEPT and 2D NMR spectral data [8]. Survey of the literature showed several reports on the isolation of octadecyl *p*-coumarates without any mention of its antifungal activity [9]. In all these studies, characterization was achieved without resorting to detailed analysis of ^{13}C NMR, DEPT and 2D NMR spectra and, for the same reason, isomerization of the (*E*) and (*Z*) forms was not reported. The sole report on antifungal activity [10] is about a mixture of stearyl esters and not of any individual compound.

Thus, this is the first report on the antifungal activity and isomerization of octadecyl *p*-coumarates. This is also the first report in which important ^{1}H and ^{13}C NMR signals have been separately assigned for the (*E*) and (*Z*) isomers of octadecyl *p*-coumarates (Table 1). This may also be the first report in which HSQC data have been used to confirm the isomerization of alkyl *p*-coumarates.

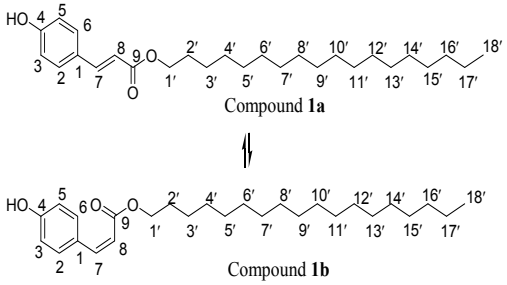

Figure 1: Isomerization of (*E*)-octadecyl-*p*-coumarate (**1a)** and (*Z*)-octadecyl-*p*-coumarate (**1b**).

Antifungal activity assay of the crude extractives showed that the ethyl acetate extractive possessed highest activity. Column chromatography of this revealed that the most active fraction was that obtained by elution with *n*-hexane-

Table 1: NMR spectroscopic data (400 MHz, $CDCl_3$) of *E*-octadecyl *p*-coumarate (**1a**) and *Z*-octadecyl *p*-coumarate (**1b**).

Sl. No.	δ_C, mult	Carbon number in the figure	δ_H, mult. (*J* in Hz)	Assigned to *E/Z*/Both
1.	167.56 C	9	-	*E*
2.	166.73 C	9	-	*Z*
3.	157.59 C	4	-	*E*
4.	156.62 C	4	-	*Z*
5.	144.22 CH	7	7.62,d, *J*=12.8 Hz	*E*
6.	143.18 CH	7	6.84, d, *J*=10.0 Hz	*Z*
7.	132.30 CH	6,2	7.63,d, *J*= 6.8 Hz	*Z*
8.	129.92 CH	6,2	7.43, d, *J*=6.8 Hz	*E*
9.	127.52 C	1	-	*Z*
10.	127.35 C	1	-	*E*
11.	117.33 CH	8	5.83,d, *J*=10.0 Hz	*Z*
12.	115.84 CH	3,5	6.84, d, *J*=6.8 Hz	*E*
13.	115.78 CH	8	6.30, d, *J*=12.8 Hz	*E*
14.	114.94 CH	3,5	6.80, d, *J*=6.8 Hz	*Z*
15.	64.67 CH_2	1’	4.19, t, *J*=5.2 Hz	*E*
16.	64.35 CH_2	1’	4.12,t, *J*=5.2 Hz	*Z*
17.	31.92 CH_2	16’	1.25-1.39,m	Both
18.	29.69* CH_2	6’,13’	1.25-1.39,m	Both
19.	29.65* CH_2	7’,12’	1.25-1.39,m	Both
20.	29.59* CH_2	8’,11’	1.25-1.39,m	Both
21.	29.54* CH_2	9’, 10’	1.25-1.39,m	Both
22.	29.36* CH_2	4’,15’	1.25-1.39,m	Both
23.	29.29* CH_2	5’,14’	1.25-1.39,m	Both
24.	28.76 CH_2	2’	1.69, quintet, *J*=5.6 Hz	Both
25.	25.98 CH_2	3’	1.25-1.39,m	*Z*
26.	25.97 CH_2	3’	1.25-1.39,m	*E*
27.	22.69 CH_2	17’	1.25-1.39,m	Both
28.	14.11 CH_3	18’	0.88, t, *J*=5.2 Hz	Both
29.	OH (phenolic)	-	5.41	*E*
30.	OH (phenolic)	-	5.34	*Z*

*Assignments interchangeable

ethyl acetate (7:3) (Tables 2 and 3). This fraction was subjected to HPLC purification using dichloromethane as eluent and two main peaks (first with t_R=34 min and second t_R=48 min) were detected. Bioassay using *Cladosporium cucumerinum* revealed that fractions corresponding to these two peaks possessed antifungal activity. If the fraction were injected immediately after elution, HPLC showed that peak alone, but after exposure to daylight for a few hours, each of the peaks showed the presence of the other one indicating isomerization of the compounds. Since it was clearly known that the two isomers could not be separated under normal conditions, the compound corresponding to the major peak (t_R=48 min) was collected for further characterization. This purified fraction gave an approximate minimum inhibitory dose of 0.3 mg against the spore germination of *Cladosporium cucumerinum* on a TLC plate (Table 3). Antifungal activity of the purified fraction was further confirmed by the spore germination inhibition of *Alternaria alternata* and *A. porri* (Table 4). Attempts to obtain good quality crystals for X-ray diffraction studies failed. Powder diffraction also did not give any useful information.

Spectral characterization of the fraction having a m.p. 79-80°C (containing **1a** as the main constituent) was achieved using UV, IR, HRESIMS, CIMS, ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC and C, H analysis. The CIMS showed a $[M+H]^+$ ion at *m/z* 417, the HRESIMS a $[M+Na]^+$ ion at

Table 2: Antifungal activity of the extractives of *Ipomoea carnea* subsp. *fistulosa* against the mycelial growth of *Colletotrichum gloeosporioides*.

Extractive/Fraction/Compound	Conc.	% mycelial growth inhibition
n-Hexane	0.5%	0.0
Ethyl acetate	0.5%	24.2 (±0.8)
Methanol	0.5%	20.4 (±1.4)
Active fraction from column*	0.5%	68.5 (±0.7)
Phenol (standard)	0.05%	78.3 (±0.4)

* Active fraction from column was obtained by elution with *n*-hexane-ethyl acetate (7:3) mixture

Table 3: Antifungal activity of the compounds of *Ipomoea carnea* subsp. *fistulosa* against *Cladosporium cucumerinum* by TLC bioautography.

Extractive/Fraction/Compound	Dose (mg)	Inhibition**	MID
n-Hexane	5.0	-	ND
Ethyl acetate	5.0	+	ND
Methanol	5.0	++	ND
Active fraction from column*	5.0	+++	ND
Octadecyl *p*-coumarate	0.3	+	0.3 mg
Phenol (standard)	0.3	+	0.3 mg

* Active fraction from column was obtained by elution with *n*-hexane-ethyl acetate (7:3).

** The observations on TLC plate for *n*-hexane, ethyl acetate and methanol extractives and active column fraction* were made after elution with ethyl acetate (R_f value of the inhibition spot ca 0.8). Observations on phenol and octadecyl *p*-coumarate were made by direct bioautography without elution after spotting the compounds quantitatively. ‘+’ indicates observable inhibition, ‘++’ indicates clear inhibition, ‘+++’ indicates very clear inhibition and ‘-’ indicates no inhibition. MID=minimum inhibitory dose; ND=Not determined.

m/z 439.3198, corresponding to a molecular weight of 416.3300, and C, H analysis gave C, 76.71%; H, 10.40%. These data gave the molecular formula as $C_{27}H_{44}O_3$ (required C, 77.8%; H, 10.45% and M^+ 416.3291). The ^{1}H NMR spectrum showed two sets of closely related signal patterns indicating the presence of two isomers in the sample. The ratio of intensity of the peaks based on their coupling constants showed that the *E* and *Z* isomers exist in the ratio 2:1. This was also consistent with the 2:1 ratio of the areas of HPLC peaks with t_R=48 min and t_R=34. ^{1}H-^{1}H COSY showed two sets of signal correlations for each of the isomers. In the first set, the signal at δ 7.62 (1H, d, *J*=12.8 Hz) correlated with that at δ 6.30, δ 7.43 (2H, d, *J*=6.8 Hz) with δ 6.84 and δ 4.19 (2H, t, *J*=5.2 Hz) with δ 1.69. In the second set, the signal at δ 7.63 (2H, d, *J*=6.8 Hz) correlated with that at δ 6.84, δ 6.83 (1H, d, *J*=10.0 Hz) with δ 5.83, and δ 4.12 (2H, t, *J*=5.2 Hz) with δ 1.63. ^{1}H-^{13}C HSQC also showed two sets of correlations. In the first set, signal at δ_C 144.22 correlated with that at δ_H 7.62, δ_C 129.92 with δ_H 7.43, δ_C 115.84 with δ_H 6.84, δ_C 115.78 with δ_H 6.30 and δ_C 64.67 with δ_H 4.19. In the second set, signal at δ_C 143.18 correlated with that at δ_H 6.83, δ_C 132.30 with δ_H 7.63, δ_C 117.33 with δ_H 5.83, δ_C 114.94 with δ_H 6.80 and δ_C 64.35 with δ_H 4.12. The ^{13}C NMR spectrum showed 28 signals, which were assigned for both *E/Z* isomers taking into consideration DEPT, COSY and HSQC data (Table 1). All these results led to the conclusion that the active fraction is a mixture of (*E*)-octadecyl *p*-coumarate (**1a**) and (*Z*)-octadecyl *p*-coumarate (**1b**).

The isomerization of (*E*)-octadecyl *p*-coumarate and (*Z*)-octadecyl *p*-coumarate is shown in Figure 1. Our finding of the isomerization of octadecyl *p*-coumarate corroborates

the reports on the isomerization of structurally related eicosanyl *p*-coumarates isolated from *Psiadia punctulata* [11] and 21'-hydroxyheneicosanyl-4-hydroxy-(*cis*- and *trans*) *p*-coumarate isolated from *Tanacetum longifolium* [12].

The fact that four earlier reports [9b-9e] on the isolation of octadecyl *p*-coumarates were from the genus *Ipomoea* may be of chemotaxonomic interest.

Experimental

General: UV spectra were obtained with a Spectronic UV-Visible spectrophotometer. IR spectra were obtained on a Perkin-Elmer spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer operating at 400 MHz and 100 MHz, respectively (Table 1). HRESIMS were obtained on a Micromass Q-TOF apparatus. Carbon-Hydrogen-Nitrogen analysis was obtained using a ThermoFinnigan Flash EA 1112 CHNS analyzer. HPLC purification was achieved using a Waters HPLC system (515 pump, 7725 Rheodyne injector, Waters 2487 Dual λ absorbance detector) under conditions as follows: Column Prep Nova Pak HR Silica 7.8 x 300 mm, flow rate 1 mL/min, UV detection at 254 nm, eluent CH_2Cl_2. TLC bioassay was achieved with silica gel plates (0.5 mm thickness). Observation of spore germination inhibition was made using a Carl Zeiss Axio Imager AI microscope.

Plant material: The leaves of *Ipomoea carnea* subsp. *fistulosa* were collected from the farm of the Indian Institute of Horticultural Research, Hessaraghatta Lake P.O., Bangalore – 560089, India and a voucher specimen is kept at the Section of Medicinal Crops of the Institute.

Extraction and isolation: The dried plant material (2 Kg) was extracted first with *n*-hexane, then with ethyl acetate and finally with methanol. Column chromatography of the ethyl acetate extractive utilizing silica gel with *n*-hexane-ethyl acetate mixtures with increasing percentages of ethyl acetate and TLC bioassay were conducted side by side. The fraction which showed maximum activity was taken for HPLC purification. Two main peaks (t_R=34 min and t_R=48 min) were detected and the eluents corresponding to these peaks were collected separately for further investigation.

Antifungal activity assays

Poisoned food technique: Pure culture of *Colletotrichum gloeosporioides* ITCC 4573 obtained from Indian Type Culture Collections, Indian Agricultural Research Institute, New Delhi, India was used for this study [13]. Percent mycelial growth inhibition values presented in Table 2 are the averages of 2 replications, standard deviation being presented in parenthesis. The purified HPLC fraction was not used in this technique because of the paucity of material and its poor solubility both in water and solvents miscible with water.

Table 4: Spore germination inhibition of *Alternaria alternata and A. porri* by octadecyl *p*-coumarates (*E* and *Z* isomers in ratio 2:1).

Treatment	*Alternaria alternata*		*Alternaria porri*	
	% germination	% inhibition w.r.t. control	% germination	% inhibition w.r.t. control
Control (3% *n*-propanol in water)	92.0 (±0.5)	-	95.0 (±0.0)	-
Octadecyl coumarates (100 mg/L)	67.0 (±1.4)	26.6 (±0.8)	71.5 (±0.7)	24.8 (±0.8)
Octadecyl coumarates (500 mg/L)	51.5 (±2.1)	44.1 (±2.3)	55.0 (±2.8)	42.1 (±3.0)
Phenol (standard) (100 mg/L)	86.0 (±0.0)	6.5 (±0.0)	91.0 (±1.4)	4.3 (±1.5)
Phenol (standard) (500 mg/L)	71.5 (±0.7)	22.3 (±0.8)	79.0 (±2.8)	16.8 (±3.0)

TLC bioautography: A pure culture of *Cladosporium cucumerinum* IMI 249540 obtained from the International Mycological Institute, U.K., maintained on a potato-dextrose-agar (PDA) medium was used for this assay [14].

Spore germination inhibition study: For this study [13], spores of *Alternaria alternata* from infected tomato fruits and *A. porri* from infected onion leaves collected from the IIHR experimental farm in Hessaraghatta, Bangalore, India were used. Spores were added to a solution of the compound in 3% *n*-propanol in water kept in cavity slides by the hanging drop method. Observation on spore germination was recorded after incubation for 3 h. Percent spore germination inhibition values presented in Table 4 are the averages of 2 replications, standard deviations being given in parenthesis.

(*E*)-Octadecyl *p*-coumarate (1a): It was collected at t_R of 48 min. as major peak during HPLC separation. The compound got partly converted to (*Z*)-octadecyl *p*-coumarate after a few hours. White solid with a faint yellowish to greenish tinge (20 mg).
MP: 79-80ºC.
IR: 3393 (OH stretching), 2921(C-H stretching), 2880 (C-H stretching), 1713 (α, β unsaturated ester), 1674 (C=C of phenol), 1604 (C=C of α, β unsaturation), 1586 (aromatic C=C), 1516 (aromatic C=C), 1468 (C-H), 1377 (CH_3), 1307 (C-O stretching) 1274 (C-O stretching), 1170 (C-O stretching), 982 (C=C conjugated to C=O), 835 (C=C-H), 722 (CH_2), 517 cm^{-1}.
UV (MeOH) λ_{max}: 225, 308 nm. Second peak showed bathochromic shift on addition of NaOH.
^{1}H NMR and ^{13}C NMR ($CDCl_3$): Table 1.
CIMS: 417 $[M+H]^+$, 164 (HO-C_6H_4 CH=COOH) 147 (HO-C_6H_4CH=CO), 129,120,107.
HRESIMS $[M+Na]^+$ 439.3198 (required for $C_{27}H_{44}O_3Na$ 439.3189).

Elemental analysis: Found C, 76.71; H, 10.40 ($C_{27}H_{44}O_3$ requires C, 77.80; H, 10.45).

Direct bioautography on TLC plate was done using *Cladosporium cucumerinum* (Table 3). The activity was further confirmed by spore germination inhibition of *Alternaria alternata* and A. *porri* (Table 4).

(*Z*)-Octadecyl *p*-coumarate (1b): It was collected at t_R of 34 min as minor peak during HPLC separation. It got partly converted to *E*-octadecyl *p*-coumarate after a few hours. White solid with a yellowish to greenish tinge (10 mg).
MP: 79-80ºC.
^{1}H NMR and ^{13}C NMR ($CDCl_3$): Table 1.

Acknowledgements - The authors thank Dr A.S. Sidhu, Director, I. I. H. R. for encouragement. They are grateful to NMR Research Centre, Indian Institute of Science, Bangalore, India for spectral data.

References

[1] Legler U. (**1965**) Die bestandtile des giftigen glykosidharzes aus *Ipomoea fistulosa* Mart. ex Choisy. *Phytochemistry*, ***4***, 29-41.
[2] (a) Lamidi M, Rondi ML, Oliver E, Faure R, Nze EL, Balansard G. (**2000**) Constituents of *Ipomoea fistulosa* leaves. *Fitoterapia*, ***71***, 203-204; (b) Dubey P, Khare N, Gupta PC. (**1982**) A new flavonoid glycoside from the leaves of *Ipomoea fistulosa*. *Current Science*, ***51***, 351-352.
[3] Umar S, Junior P, Wichtl M. (**1980**) Isolation and identification of agroclavin and α-dihydrolysergol from the leaves of *Ipomoea fistulosa*. *Planta Medica*, ***40***, 328-332.
[4] Gupta OCD, Gupta R, Gupta PC. (**1980**) Chemical examination of the flowers of *Ipomoea fistulosa*. *Planta Medica,* ***38***, 147-150.
[5] Haraguchi M, Gorniak SL, Ikeda K, Mikami Y, Kato A, Watson AA, Nash RJ, Molyneux, RJ, Asano N. (**2003**) Alkaloidal components in the poisonous plant *Ipomoea carnea* (Convulvulaceae). *Journal of Agricultural and Food Chemistry*, ***51***, 4995-5000.
[6] Patel AK, Singh VK, Yadav, RP, Moir, AJG, Jagannadham, MV. (**2009**) ICChI, a glycosylated chitinase from the latex of *Ipomoea carnea*. *Phytochemistry*, ***70***, 1210-1216.
[7] (a) Reza MS, Khan MOF, Islam MA, Chowdhury AKA. (**1994**) *In vitro* antimicrobial activity of *Ipomoea fistulosa*. *Fitoterapia*, ***65***, 465-466; (b) Chowdhury AKA, Ali MS, Khan MOF. (**1997**) Antimicrobial activity of *Ipomoea fistulosa* extractives. *Fitoterapia*, ***68***, 379-380; (c) Mahmood YAG, Ebrahim MKH, Mageda MA. (**2004**) Influence of some plant extracts and microbioagents on some physiological traits of faba bean infected with *Botrytis fabae*. *Turkish Journal of Botany*, ***28***, 519-528.
[8] Williams DH, Fleming I. (**1988**) Spectroscopic methods in organic chemistry. Tata McGraw Hill Publishing Co. Ltd., New Delhi, 1-198.
[9] (a) Gunatilake AAL, Sultanbawa M, Uvais S. (**1973**) Chemical investigation of Ceylonese plants. III. Extractives of the fruits of *Argyreia populifolia* (Convolvulaceae). *Journal of the Chemical Society*, ***11***, 1155-1157; (b) Dai H, Xiong J, Zhou J, Ding Z. (**2000**) Chemical constituents from root of *Ipomoea digitata*. *Yunnan Zhiwu Yanjiu*, ***22***, 166-168; (c) Ishiguro K, Yoshimoto M, Suzuki M, Yahara S. (**2008**) Anti-oxidative activity in the lipophilic fraction of sweet potato tubers. *Acta Horticulturae*, ***768***, 571-577; (d) Snook ME, Data ES, Kays SJ. (**1994**) Characterization and quantitation of hexadecyl, octadecyl, and eicosyl esters of *p*-coumaric acid in the vine and root latex of sweet potato [*Ipomoea batatas* (L.)]. *Journal of Agricultural and Food Chemistry*, ***42***, 2589-2595; (e) Stevenson PC, Muyinza H, Hall DR, Porter EA, Farman DI, Talwana HM, Robert OM. (**2009**) Chemical basis for resistance in sweet potato *Ipomoea batatas* to the sweet potato weevil *Cylas puncticollis*. *Pure and Applied Chemistry*, ***81***, 141-151.
[10] Norton RA, Dowd PF. (**1996**) Effect of stearyl cinnamic acid derivatives from corn bran on *Aspergilus flavus*, corn earworm larvae and dried fruit beetle larvae. *Journal of Agricultural and Food Chemistry*, ***44***, 2412-2416.
[11] Keriko JM, Nakajima S, Baba N, Iwasa J. (**1997**) Eicosanyl *p*-coumarates from Kenyan plant, *Psiadia punctualata*: plant growth inhibitors. *Bioscience, Biotechnology and Biochemistry*, ***61***, 2127-2128.
[12] Mahmood U, Kaul VK, Acharya R, Jirovetz L. (**2003**) Sesquiterpene and long chain ester from *Tanacetum longifolium*, *Phytochemistry*, ***64***, 851-853.
[13] Nene YL, Thapliyal PN. (**2002**) Fungicides in plant disease control. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, p. 531.
[14] Homans AL, Fuchs A. (**1970**) Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. *Journal of Chromatography,* ***51***, 327-329.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1893 - 1896

New Glucose Esters from the Fresh Leaves of *Jacaranda mimosaefolia*

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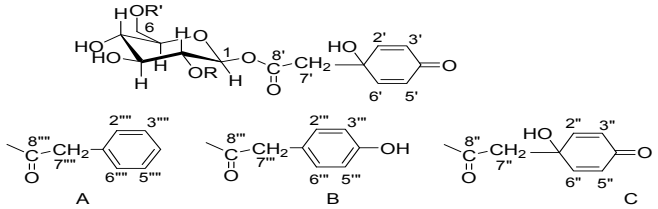
Received: November 21st, 2010; Accepted: August 16th, 2011

From the fresh leaves of *Jacaranda mimosaefolia* were isolated Phytoquinoids **1-4** established as β-D-glucopyranose 2-benzeneacetate1,6-bis(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate), for which the name Jacaranoside is proposed; β-D-glucopyranose 2-(4-hydroxybenzeneacetate) 1,6-bis(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate), for which the name Jacarandol is proposed; β-D-glucopyranose 2-benzeneacetate 1-(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate) and β-D-glucopyranose 1,6-bis (1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate) respectively.

Keywords: *Jacaranda mimosaefolia*, leaves, glucose esters, jacaranoside, jacarandol, phytoquinoids.

The genus *Jacaranda* (Bignoniaceae) is well known in traditional medicine [1]. *J. mimoseafolia* D. Don, in particular, has been classified as the World's most widely planted ornamental species [2]. A decoction of the plant is used for the treatment of parasites and for dermatological conditions [3], in the treatment of venereal diseases and for blood purification [4], and an infusion of the bark is applied as a lotion for ulcers [5]. Aqueous extracts of the twigs and seeds of *J. mimosaefolia* have been reported to show antibacterial properties [6], while the methanol extracts of the leaves showed hypothermic and cardiovascular activities [1]. Antimicrobial activity of the hexane, ethanol and aqueous extract of the leaves has also been reported [7]. Compounds previously isolated from *J. caucana* Pittier include the anticancer agents, jacaranone, its ethyl ester, scutellarein-7-glucuronide, and other triterpenoids with antitumor and cytotoxic activity [8,9]. From the leaves of *J. mimosaefolia* were isolated jacaranone, phenylacetic-β-glucoside, verbascoside, and jacaranose, with hypotensive and analgesic properties [1,6]. A phenylethanoid dimer, jacaraninoside, was more recently reported from the same species [10].

Compound **1** was isolated as a light yellowish resinous material. The positive ion ESI-MS showed a pseudo-molecular ion at *m/z* 597 $[M-H]^-$, consistent with the molecular formula $C_{30}H_{28}O_{13}$. The presence of a sugar with acylation at positions 1, 2 and 6 was apparent from the downfield shifts of the corresponding proton signals at δ 5.68, 4.93, and 4.44, 4.21, respectively. The presence of two 1-hydroxy-4-oxo-2,5-cyclohexadien-1-acetyl- (CHD) units was established through the observation of carbon NMR absorptions at δ 184.65 and 184.73 and characteristic *p*-quinoid IR absorptions at 1633.5 and 1673.1 cm^{-1} [9], with successive links to one methylene group, each observed at δ 44.3 and δ 44.9, and one ester group, each observed at δ 167.1 and δ 168.6. The third acyl group was established as a phenylacetyl unit from the ^{13}C NMR spectroscopic data with the corresponding methylene group at δ 40.6 and ester carbonyl at δ 170.1. Compound **1** bears remarkable resemblance to jacaranose [5] in all NMR spectral details except for the non-equivalence of one group of CH_2 protons in **1** ascribable to the CHD unit attached to position 1 of glucose. The AB quartet feature at this position in acetone and methanol was consistent for compounds **1** (δ 2.59, 2.69, J = 14.7 Hz); **2** (δ 2.59, 2.69, J = 14.7 Hz) and **3** (δ 2.50, 2.64, J = 14.7 Hz). Jacaranose was reported in 1988 from the leaves of *J. mimosaefolia* as a trisubtituted glucose unit with one phenylacetyl moiety and two CHD units. The report gave



	R	R'
1	A	C
2	B	C
3	A	H
4	H	C

Figure 1: Phytoquinoids **1-4** isolated from fresh leaves of *J. mimosaefolia*.

Table 1: ^{13}C NMR and DEPT spectral data, for compounds 1, 2, 3 and 4 in acetone-d_6 (75.5 MHz) with assignments supported by COSY, DEPT, HSQC and HMBC.

	δ $_C$ ppm							
Position	1		2		3		4	
Glucose								
1	92.1	CH	92.1	CH	92.3	CH	94.5	CH
2	72.7	CH	72.6	CH	72.9	CH	72.6	CH
3	74.4	CH	74.4	CH	74.6	CH	76.6	CH
4	70.3	CH	70.3	CH	70.2	CH	70.0	CH
5	74.9	CH	74.8	CH	77.7	CH	74.7	CH
6	63.4	CH_2	63.4	CH_2	61.2	CH_2	63.6	CH_2
4-oxocyclo hexadiene acetyl-								
1’	66.8	C	66.8	C	66.9	C	67.0	C
1”	67.0	C	67.0	C	-	-	67.0	C
2’	150.1	CH	150.0	CH	150.2	CH	150.3	CH
6’	150.0	CH	150.2	CH	150.4	CH	150.8	CH
2”	150.5	CH	150.6	CH	-	-	150.5	CH
6’’	150.5	CH	150.6	CH	-	-	150.8	CH
3’	127.2	CH	127.2	CH	127.4	CH	127.2	CH
5’	127.4	CH	127.2	CH	127.4	CH	127.4	CH
3”	127.2	CH	127.4	CH	-	-	127.2	CH
5”	127.4	CH	127.4	CH	-	-	127.4	CH
4’	184.7	C	184.8	C	185.0	C	185.1	C
4”	184.7	C	184.9	C	-	-	185.1	C
7’	44.3	CH_2	44.3	CH_2	44.4	CH_2	44.7	CH_2
7”	44.9	CH_2	44.9	CH_2	-	-	44.9	CH_2
8’	167.1	C	167.1	C	167.1	C	167.6	C
8”	168.6	C	168.6	C	-	-	168.7	C
Arylacetyl-								
1”’	-	-	125.0	C	-	-	-	-
1””	134.4	C	-	-	134.5	C	-	-
2”’, 6’’’	-	-	130.3x2	CHx2	-	-	-	-
2””, 6’’’’	129.3x2	CHx2	-	-	129.3x2	CHx2	-	-
3”’, 5’’’	-	-	115.2x2	CHx2	-	-	-	-
3””, 5’’’’	128.4x2	CHx2	-	-	128.4x2	CHx2	-	-
4”’	-	-	156.4	C	-	-	-	-
4””	126.9	CH	-	-	126.8	CH	-	-
7”’	-	-	39.8	CH_2	-	-	-	-
7””	40.6	CH_2	-	-	40.6	CH_2	-	-
8”’	-	-	170.6	C	-	-	-	-
8’’’’	170.1	C	-	-	170.1	C	-	-

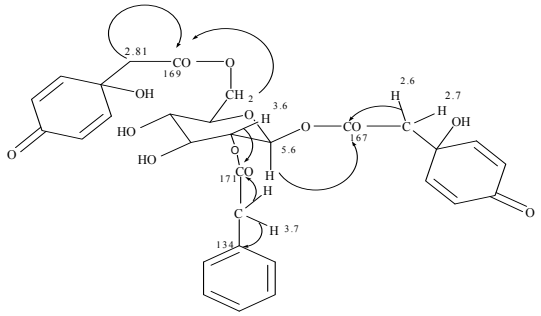


Figure 2: HMBC Correlation of compound **1**.

all singlets for the three CH_2 groups in acetone. Although the positions of esterification were reported as positions 1, 2, and 6 of β-glucose, the precise location of the different acyl groups was not established, hence the complete structure of jacaranose remained undetermined.

In the present study, the definite sites of esterification on the glucose molecule of compound **1** were, however, made possible by the combination of COSY, ^{1}H, ^{13}C, HSQC and HMBC experiments. The phenylacetyl group was located at C-2 of glucose on the basis of the downfield signal observed for H-2 (δ 4.92) and its HMBC correlation to the corresponding ester carbonyl (δ170.1). A prominent HMBC correlation of the anomeric proton to the carbonyl carbon, C-8^1 (δ 167.1), established an acylation at the anomeric C-1 position by one CHD unit. The HMBC correlation observed between position 6 protons of glucose δ_{H6a} 4.44 (dd, J = 2.1, 12.3) and δ_{H6b} 4.21 (dd, J = 5.7, 12.3) with an ester carbonyl carbon C-8^{11} (δ 168.5), indicated that the second unit of the CHD moiety was attached at C-6 of glucose. Therefore, the phytoquinoid **1** was established as β-D-glucopyranose 2-benzeneacetate1,6-bis(1-hydroxy-4-oxo-2,5 cyclohexadiene-1-acetate). Although compound **1** has a similar optical rotation {$[\alpha]^D_{25}$ +6.5 (*c* 0.05, MeOH)} as that reported for jacaranose {$[\alpha]^D_{20}$ +6.8 (*c* 0.9, MeOH)}, a repeat collection of plant material gave compound **1** with no isomer isolated showing all three singlet methylene groups as previously described for jacaranose [5]. The ABq feature at ambient temperature was consistent at 200, 300 and 500 MHz in acetone and methanol. We propose the name jacaranoside for compound **1**.

Compound **2** shows significant IR and NMR similarities to compound **1**. Thus, two CHD units at positions 1 and 6 of glucose were readily established with the major difference in the acyl group present at position 2. The presence of an extra oxygen ($[M+H]^+$ = 613), a phenolic reaction to ferric chloride, a broad down-field signal in acetone at δ 8.4, a *p*-substituted aromatic ring with an ortho- coupled proton at 6.77 (d, J = 8.4) and a quaternary carbon at δ 156 established the presence of a *p*-hydroxy phenylacetyl group instead of the phenylacetyl group found in compound **1**. HMBC correlation of H-2 of glucose with the corresponding ester carbonyl at δ 170.1 and a methylene at δ 39.8 confirmed this location. Thus, compound 2, for which the name jacarandol is proposed, was established as β-D-glucopyranose 2-(4-hydroxy benzeneacetate) 1,6-bis(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate).

Compound **3** showed the presence of only two acyl groups (ester C=O at δ 167.1 and 170.1). In compound **3**, the lack of acylation at position 6 of the sugar is apparent from the relative upfield shift of the protons (δ 3.78 and δ 3.52). An ABq (δ 2.50, 2.64, J = 14.7 Hz) was observed for the CH_2 of the CHD unit attached to position 1 of the sugar, as for compounds **1** and **2**, while evidence for the presence of phenylacetyl substitution at position 2 was apparent from the downfield shift of sugar protons at position 2 and HMBC correlations. EI-MS did not show the molecular ion, but showed ions diagnostic of a CHD residue (*m/z* 150 and 122) [9] and a phenylacetyl group (*m/z* 136, $PhCH_2COOH$).

Compound **4** showed the presence of only two acyl groups (ester C=O at δ 167.6 and 168.7). The evidence for two CHD units is borne out from two ketonic carbon resonances at δ 185.09 and 185.13. The lack of an ABq for the CHD unit on position 1 of the sugar is consistent with the lack of acylation at position 2, which showed an upfield proton shift (δ 3.40). On the other hand, the presence of the second CHD unit at position 6 of the sugar is evident from the downfield shift of the sugar protons at 6 (δ 4.03 and 4.16) and corroborated the HMBC correlations. The EI-MS showed CHD residues at *m/z* 150 and 122.

Table 2: ^{1}H NMR spectral data for compounds **1-4** (δ in ppm, acetone-d_6)

	δ_H ppm			
H	**1**	**2**	**3**	**4**
Glucose				
1	5.68,1H, d, (8.4)	5.66, 1H, d, (8.4)	5.64, 1H, d, (8.4)	5.50, 1H, d, (8.1)
2	4.93, 4.90,1H,dd,(8.4;8.4)	4.89, t, (8.4)	4.88, 1H, t, (9.0)	3.40
3	3.75, 1H, t, (9.6)	3.75 (obs)	3.74 (obs)	3.50 (obs)
4	3.52, 1H, dd, (9.3, 9.3)	3.45 (obs)	3.60 (obs)	3.40 (obs)
5	3.70 (obs)	3.70 (obs)	3.50, m	3.60 (obs)
6a	4.44, dd, (2.1, 12.3)	4.43, d, (12.0)	3.78, dd, (12.9)	4.03, dd, (1.8; 12.0)
6b	4.21, dd, (5.7, 12.3)	4.20, dd, (5.7, 12.0)	3.52 (obs)	4.16, dd, (6.0; 12.0)
4-oxocyclo hexadiene acetyl-				
2’	7.00, m	7.00, m	7.07, 7.04, dd, (10.3; 3.3)	
2’’	7.00, m	7.00, m	-	
6’	7.00, m	7.00, m	6.99, 7.03, dd, (10.2; 3.3)	
6’’	7.00, m	7.00, m	-	
3’	6.10, m	6.10, m	6.10, d, (10.2)	
3’’	6.10, m	6.10, m	-	
5’	6.10, m	6.10, m	6.10, d, (10.2)	
5”	6.10, m	6.10, m	-	
7’	ABq, 2.59, 2.69, (14.7)	ABq, 2.59,2.69, (14.7)	ABq, 2.50, 2.64, (14.7)	2.90, 2H, s
7”	2.81, 2H, s	2.81, 2H, s	-	2.80, 2H, s
Arylacetyl-				
2”’, 6’’’	-	7.14, d, (8.4)	-	-
3”’, 5’’’	-	6.77, d, (8.4)	-	-
2””, 6’’’’	7.30, m	-	7.29, m	-
3””, 5’’’’	7.30, m	-	7.29, m	-
4’’’’	7.30, m	-	7.29, m	-
7”’	-	3.56, 2H, s	-	-
7””	3.68, 2H, s	-	3.67, 2H, s	-

Experimental

General experimental procedures: The IR spectra (V_{max}) were determined on a Perkin–Elmer 2000 FT-IR Spectrophotometer. ESI-MS (negative ion mode) were recorded on an Agilent 1100 series LC/MSD Trap mass spectrometer. NMR measurements were recorded on Varian 200, 500 and Bruker 300 spectrometers. Silica gel 60 (230-400 µm, Merck) was used for vacuum liquid (VLC) and accelerated gradient (AGC) chromatographic separations. Final purifications were performed by medium pressure reverse phase liquid chromatography (MPLC) using a pre-packed Lichroprep RP-18 (40-63 µm) column. All fractions were collected in 15 mL test tubes. Pre-coated silica gel 60 F_{254} aluminum plates (0.2 mm, Merck) and pre-coated silica gel 60 RP-18 $F_{254}S$ aluminum TLC plates (Merck) were used for TLC monitoring. TLC mobile phases were 50% methanol and toluene: acetone: water (10:30:1) for reverse phase and normal phase, respectively. TLC plates were visualized by UV light, 1% ferric chloride in methanol, and 1% vanillin in H_2SO_4, followed by heating at 105°C for 1-2 min.

Plant material: The leaves of *J. mimosaefolia* D. Don, were collected in May 2007 from the Obafemi Awolowo University campus and identified at the Department of Botany. A voucher sample is lodged in the herbarium of the University as no. 16412.

Extraction and isolation: Fresh leaves (950 g) were immediately macerated in ethanol (4L) for 24 h at room temperature. After filtration, the extract was evaporated to complete dryness *in vacuo* at 40°C, yielding a crude ethanolic leaf extract (JL, 71 g). This was fractionated by VLC on silica gel using EtOAc: MeOH mixtures in gradient to give 5 fractions (Fr 1 = 35.5 g; Fr 2 = 6.14 g; Fr 3 = 2.08 g; Fr 4 = 2.10 g and Fr 5 = 1.06 g). Fraction 1 (16.0 g) was subjected to repeated AGC separation on silica using *n*-Hex: EtOAc: MeOH in gradient to yield 6 sub-fractions (1a - f). Sub-fraction 1d (1.1 g), which eluted from EtOAc: MeOH (9.5: 0.5), was subjected to final purification on reverse phase MPLC using a H_2O: MeOH gradient. Elution with 45% MeOH afforded compound **1** (232 mg). Final purification of sub-fraction 1c (2.4 g), which eluted from EtOAc: MeOH (9.0: 1.0), on reverse phase MPLC, afforded compound **2** (508 mg), eluted with 35% MeOH, compound **3** (63 mg) eluted with 30% MeOH and compound **4** (588 mg) eluted with 45% MeOH. Sub-fraction 1b (1.1 g), which eluted from EtOAc: MeOH (8.5:1.5), was similarly purified by reverse phase MPLC using MeOH: H_2O gradient. Elution with 25% and 40% MeOH afforded compound **4** (120mg) and verbascoside (141 mg), respectively.

Compound 1 [**β-D-glucopyranose-2-benzeneacetate1,6-bis(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate)**]

Light yellow resinous material.
FTIR: 3396.7, 3440.2, 1673.1, 1633.5 cm^{-1}
$[\alpha]^D_{25}$: +6.5 (*c* 0.05, MeOH).
R_f: 0.34 (MeOH: H_2O, 1:1)
^{1}H NMR and ^{13}C NMR (acetone-d_6): Tables 1 and 2
ESI-MS: *m/z* 597 [M-H]$^-$, 447
Yield: 232 mg, 1.45 %

Compound 2 [**β-D-glucopyranose2-(4-hydroxy benzene acetate) 1,6-bis (1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate)**]

Brownish resinous compound
FTIR: 3395.1, 3445.6, 1672.7, 1633.4 cm^{-1}

$[\alpha]^{D}_{25}$: +9.8 (*c* 0.06, MeOH).
R_f : 0.54 (MeOH: H_2O, 1:1)
^{1}H NMR and ^{13}C NMR (acetone-d_6): Tables 1 and 2.
ESI-MS: *m/z* 613 $[M-H]^-$, 463
Yield: 508 mg, 3.18 %

Compound 3 [β-D-glucopyranose 2-benzeneacetate 1-(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate)]

Brown gum.
R_f : 0.36 (MeOH: H_2O, 1:1)
^{1}H NMR and ^{13}C NMR (acetone-d_6): Tables 1 and 2.
EI-MS: *m/z* 150 (62), 136 (100), 122 (65)
Yield: 63 mg, 0.39%

Compound 4 [β-D-glucopyranose 1,6-bis(1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetate)]

Brown gum
R_f : 0.76 (MeOH: H_2O, 1:1)
^{1}H NMR and ^{13}C NMR (acetone-d_6): Tables 1 and 2.
EI-MS: *m/z* 150 (90), 122 (100)
Yield: 708 mg, 4.43 %

Verbascoside

R_f : 0.56 (MeOH: H_2O, 1:1)
NMR data were identical to those reported in literature [11]
Yield: 141 mg, 0.88%

Acknowledgments - The authors acknowledge the support of the Carnegie Corporation of New York for OAU Central Science Laboratory, Prof. B. Abegaz, NABSA, Gaborone, for HMBC experiments and EI MS and Rolf Blattler and Uta Wollrab of Varian Inc. for 500 NMR spectra.

References

[1] Nicasio P, Meckes M. (**2005**) Hypotensive effect of the hydroalcoholic extract from *Jacaranda mimosaefolia* leaves in rat. *Journal of Ethnopharmacology*, ***97***, 301-304.
[2] Gentry AH. (**1992**) A synopsis of Bignoniaceae ethnobotany and economic botany. *Annals of the Missouri Botanical Garden*, ***79***, 53-64.
[3] Tene V, Malagon O, Finzi PV, Vidari G, Armijos C, Zaragoza T. (**2007**) An ethnobotanical survey of medicinal plants used in Loja and Zamara-Chinchipe, Ecuador. *Journal of Ethnopharmacology*, ***111***, 63-81.
[4] Gachet MS, Schuhly W. (**2009**) Jacaranda – An ethnopharmacology and phytochemical review. *Journal of Ethnopharmacology*. ***121***, 14-27.
[5] Gambaro V, Garbarino JA, Galeffi C, Nicoletti M, Messana I, Marini-Bettolo GB. (**1988**) Constituents of *Jacaranda mimosaefolia. Revista Latinoamericana de Quimica*, ***19***, 17-19.
[6] Mahran GH, El-Fishwy AM, Hosny AMS, Hilal AM (**1991**) Phytochemical and antibacterial study of *Jacaranda mimosaefolia* D. Don grown in Egypt. *Herba Hungarica*, ***30***, 98-104.
[7] Rojas JJ, Ochoa VJ, Ocampo SA, Munoz JF. (**2006**) Screening for antimicrobial activity of ten medicinal plants used in Colombian folkoric medicine: a possible alternative in the treatment of non-nosocomial infections. *BioMed Central Complementry and Alternative Medicine*, ***6***, 1-6.
[8] Ogura M, Cordell GA, Farnsworth NR. (**1976**) Potential anticancer agents. III. Jacaranone, a novel phytoquinoid from *Jacaranda caucana*. *Lloydia*, ***39***, 255-257.
[9] Ogura M, Cordell GA, Farnsworth NR. (**1977**) Potential anticancer agents. IV. Constituents of *Jacaranda caucana* Pittier (Bignoniaceae). *Lloydia*, ***40***, 157-168.
[10] Moharram FA, Marzouk MSA. (**2007**) A novel phenylethanoid dimer and flavonoids from *Jacaranda mimosaefolia*. *Zeitschrift fur Naturforschung* B. ***62***, 1213-1220.
[11] Franzyk H, Husum TL, Jensen SR (**1998**) A caffeoyl phenyl-ethanoid glycoside from *Plantago myosuros*. *Phytochemistry*, ***47***, 1161-1162.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1897 - 1900

Shamiminol: A New Aromatic Glycoside from the Stem Bark of *Bombax ceiba*

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Dedicated to the fond memory of Professor Salimuzzaman Siddiqui *FRS*, (1897–1994), the founding director of HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan

Received: July 30th, 2011; Accepted: October 18th, 2011

A new aromatic glycoside, shamiminol was isolated from the stem bark of *Bombax ceiba* along with the known constituents stigmasta-3,5-diene, lupenone, (±)-lyoniresinol 2a-*O*-β-D-glucopyranoside and opuntiol, obtained for the first time from this plant. The structure of shamiminol was elucidated on the basis of extensive 1D- and 2D-NMR spectroscopic and mass spectrometric studies as 3,4,5-trimethoxyphenol 1-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside (**1**).

Keywords: *Bombax cieba*, Bombacaceae, aromatic glycoside, shamiminol.

Bombax cieba Linn. (Syn. *B. malabaricum* D.C. and *Salmalia malabarica* D.C.) commonly known as simbal or red silk-cotton tree, belongs to the family Bombaceaeae. It grows in the hotter forest regions of India, Sri Lanka, Malayasia, China, Java and Sumatra. In Pakistan, it is found in Hazara and other parts of the North West frontier province, and also in Sindh province [1]. Various parts of the plant are highly reputed in traditional medicine for the treatment of a variety of ailments [1]. This medicinal plant has immense therapeutic importance, and pharmacological studies showed that it possesses hypoglycemic, hypotensive, antiangiogenic, analgesic, antioxidant, vibriocidal, antimicrobial, anti-inflammatory and antidiabetic activities [2]. Phytochemical investigation of *B. ceiba* has resulted in the isolation of a number of constituents belonging to different classes of compounds [3]. In this communication, we report the isolation and structure elucidation of a new natural product, shamiminol (**1**), along with four known compounds, stigmasta-3,5-diene [4], lupenone [5], (±)-lyoniresinol 2a-*O*-β-D-glucopyranoside [6] and opuntiol [7], which were obtained for the first time from *B. ceiba*.

Shamiminol (**1**) was obtained as a colorless gum from the stem bark of *B. ceiba*. Its IR spectrum indicated the presence of a hydroxyl group (3405 cm^{-1}) and an aromatic ring (1595 and 1507 cm^{-1}), while the UV spectrum showed absorption at 215 and 265 nm, indicative of its aromatic nature. Shamiminol (**1**) has the composition $C_{20}H_{30}O_{13}$, MW = 478, as derived from positive and negative FABMS and ^1H and ^{13}C NMR spectroscopic data (Table 1). It has twenty carbons in the ^{13}C NMR spectrum (three methyls, two methylenes, nine methines, two sp^2 CH, and four sp^2 quaternary carbons).

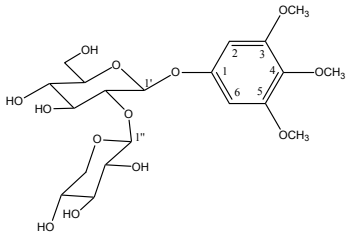

Figure 1: 3,4,5-Trimethoxyphenol 1-*O*-β-D-xylo-pyranoside-(1→2)-β-D-glucopyranoside [shamiminol (**1**)].

The ^1H NMR spectrum of **1** in CD_3OD (Table 1) showed the presence of two equivalent methoxy groups (δ 3.80, 6H, s) and two aromatic protons (δ 6.45, s), along with another methoxy group at δ 3.69, as a three protons singlet, suggesting that the molecule contained an aromatic ring with a symmetrical substitution pattern [8] (Figure 1). The presence of the 3,4,5-trimethoxyphenol system was supported by the ^{13}C NMR spectrum which showed signals for three aryl methoxy groups at δ 56.62 (3- and 5-OCH_3) and 61.23 (4-OCH_3), four quaternary carbons at δ 155.93

Table 1: NMR spectroscopic characteristics [δ (ppm) and *J* (Hz)] of shamiminol (**1**)

Assignments	δ_C	δ_H
1	155.93	
2	96.39	6.45 (s)
3	154.78	
4	134.01	
5	154.78	
6	96.39	6.45 (s)
1′	102.09	4.92 (d, 7.0)
2′	83.74	3.64 (m)
3′	77.89	3.36 (t, 8.7)
4′	71.38	3.25 (m)
5′	78.18	3.46 (ddd, 1.6, 4.0, 9.4)
6_a′	62.63	3.90 (dd, 1.6, 11.1)
6_b′		3.68 (dd, 4.0, 11.1)
1′′	106.28	4.62 (d, 7.4)
2′′	75.76	3.21 (t, 7.4)
3′′	77.39	3.62(t, 7.4)
4′′	71.12	3.49 (m)
5_a′′	67.24	3.85 (dd, 5.9, 10.9)
5_b′′		3.25 (m)
3-OCH_3	56.62	3.80 (s)
4-OCH_3	61.23	3.69 (s)
5-OCH_3	56.62	3.80 (s)
OH		

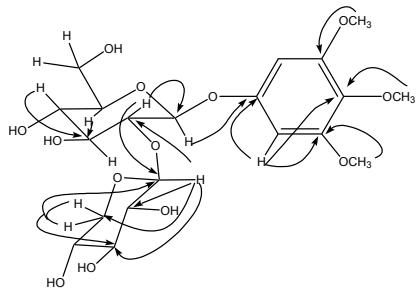

Figure 2: Important HMBC correlations of shamiminol (**1**).

(C-1), 154.78 (C-3 and 5) and 134.01 (C-4), and two methine carbons at δ 96.39 (C-2 and 6). The HSQC spectrum showed one bond correlation of H-2,6 at δ 6.45 with C-2,6, while the HMBC spectrum showed ^3J correlation of 3- and 5-OCH_3 protons (δ 3.80) with C-3 and C-5, respectively; 4-OCH_3 protons (δ 3.69) showed correlation with C-4; H-2,6 with C-4, which also showed ^2J connectivities with C-1, C-3 and C-5 (Figure 2). These spectral data confirmed that a 3,4,5-trimethoxyphenol moiety was present in the molecule [8–10], which was corroborated by the fragment ions at *m/z* 184 ($C_9H_{12}O_4$) and 167 ($C_9H_{11}O_3$) in the EIMS.

The presence of a disaccharide moiety was also revealed by the ^{1}H NMR spectrum, which exhibited two anomeric proton signals at δ 4.92 (d, *J* = 7.0, H-1′) and 4.62 (d, *J* = 7.4, H-1″) attributable to those of glucose and xylose units, respectively [11]. The spectrum analyzed with the aid of ^{1}H-^{1}H shift correlation spectroscopy (COSY) further exhibited signals due to four methines at δ 3.64 (m, H-2′), 3.36 (t, *J* = 8.7, H-3′), 3.25 (m, H-4′), and 3.46 (ddd, *J* = 1.6, 4.0, 9.4, H-5′), and one oxymethylene at δ 3.90 (dd, 1.6, 11.1, $H\text{-}6_a$′) and 3.68 (dd, *J* = 4.0, 11.1, $H\text{-}6_b$′), which were ascribed to glucose protons. The ^{13}C NMR spectrum also showed signals corresponding to the sugar unit. In the HMQC plot, chemical shift values at δ 102.09 (C-1′), 83.74 (C-2′), 77.89 (C-3′), 71.38 (C-4′), 78.18 (C-5′) and 62.63 (C-6′) showed direct one bond correlations with H-1′, H-2′, H-3′, H-4′, H-5′ and H-6′, respectively (Table 1).

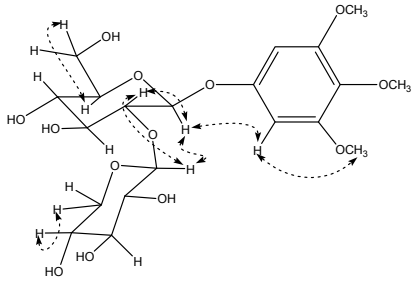

Figure 3: Important NOESY correlations for shamiminol (**1**).

Additionally, the ^{1}H NMR spectrum exhibited two multiplets at δ 3.49 and 3.25, two triplets at δ 3.21 and 3.62 with the same coupling constant of 7.4 Hz and a double doublet at δ 3.85 (*J* = 5.9, 10.9) which were assigned to H-4″, $H\text{-}5_b$″, H-2″, H-3″ and $H\text{-}5_a$″, respectively, of the xylose moiety. In the COSY-45^o plot the anomeric proton of xylose at δ 4.62 (H-1″) correlated with H-2″; H-3″ with H-4″ and H-2″; and H-4″ with $H\text{-}5_a$″, $H\text{-}5_b$″ and H-3″, while in the HMQC spectrum H-1″, H-2″, H-3″, H-4″ and H-5″ showed direct connectivity with δ 106.28 (C-1″), 75.76 (C-2″), 77.39 (C-3″), 71.12 (C-4″) and 67.24 (C-5″), respectively.

The glycosidic linkages in both glucose and xylose was determined to be β, based on the large coupling constants of the respective anomeric proton signals (7.0 and 7.4 Hz) [11]. That the glucose is attached *via* its anomeric carbon with the oxygen of the trimethoxy aromatic ring was indicated by the high frequency chemical shift of the anomeric proton (δ 4.92) and confirmed by the long-range correlation between the anomeric proton (δ 4.92) and C-1 (δ 155.93) of the benzene ring in the HMBC spectrum (Figure 2). It was reaffirmed by the cross peak observed in the NOESY-plot for the spatial connectivity of the anomeric proton with the aromatic H-2,6 (δ 6.45) (Figure 3).

The ^{13}C NMR spectral data suggested the attachment of xylose with the C-2′ of the glucose residue, as it demonstrated marked glycosidation shifts, showing an α-effect on C-2′ and β-effects on C-1′and C-3′. Thus C-2′ resonated at higher frequency (δ 83.74), and C-1′ and C-3′ appeared at δ 102.09 and 77.89, respectively. A literature search revealed that when a sugar (xylose or apiose) has a linkage with the C-6 of glucose, that carbon showed a marked α glycosidation shift, while C-5 resonated at a low frequency [9,11a]. When the same sugar was located on C-2 of glucose then this carbon resonated at high frequency, showing the α-effect [10,11b]. Consequently, the disaccharide moiety was identified as β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl, which was

supported by the mass fragments at *m/z* 185 and 183 in the positive and negative ion FABMS, respectively, which arose due to the loss of glucose and xylose moieties from the molecular ion. This was confirmed by the 2D NMR spectral data. Thus, in the HMBC spectrum, H-2′ showed crucial long-range coupling with the anomeric carbon (C-1″) of xylose and H-1″ in turn has connectivity with the C-2′ of glucose (Figure 2). The NOESY spectrum exhibited an important cross peak for the spatial proximity of H-2′ of glucose with H-1″ of xylose (Figure 3).

All the evidence discussed above led to the formulation of shamiminol as 3,4,5-trimethoxyphenol 1-*O*-β-D-xylopranosyl-(1→2)-β-D-glucopranoside (**1**), which was corroborated by the important fragment ions in the positive and negative ion FABMS at *m/z* 461 (M^++1–H_2O) and 459 (M^+-1-H_2O), and at *m/z* 364 (M^+–xylose), 432 (M^+–$3CH_3$–H), 414 and 137 in the EIMS. The complete ^1H and ^{13}C NMR spectral assignments for **1** has been made through the help of 2D NMR spectroscopy. Furthermore, the ^1H NMR spectrum of **1**, in C_5D_5N, showed no resonances for the aromatic hydroxyls. However, it exhibited a broad hump at δ 5.20 for the sugar hydroxyprotons, which disappeared on shaking with D_2O. Additionally, all the chemical shifts of the compound shifted to high frequency in C_5D_5N as compared with those in CD_3OD, except for those of 3-OCH_3 and 5-OCH_3, which resonated at low frequency, and 4-OCH_3, which appeared at high frequency (Table 1). It is important to mention that the regioisomer of **1**, 3,4,5-trimethoxyphenyl 1-*O*-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside, has been reported earlier from *Ailanthus integrifolia* [11a].

Stigmasta-3,5-diene [4], lupenone [5], (±)-lyoniresinol 2a-*O*-β-D-glucopyranoside [6] and opuntiol [7] were also isolated from the stem bark and identified by comparison with reported data. The literature search revealed that these compounds are mostly found in the stem bark of plants [4–7]. Moreover, *Cinnamomum cassia* (family Lauraceae) and *B. ceiba*, belonging to two different families, contained glucosides of lyoniresinol and 3,4,5-trimethoxyphenol, as well as catechin derivatives in their stem bark [9,2c].

Experimental

General: The IR (in KBr disc) spectrum was recorded on a JASCO A 302 spectrophotometer, and the EI and FAB mass spectra were measured on Finnigan MAT 112 and JMS HX-110 spectrometers. The ^1H NMR spectra were run in CD_3OD and C_5D_5N on Bruker Aspect AM-500 and AM-400 spectrometers operating at 500 and 400 MHz, respectively, while ^{13}C NMR spectra were recorded at 125 MHz. Purity of the compounds was checked on silica gel $60GF_{254}$ precoated cards (0.2 mm thickness), while for flash column chromatography (FCC) (Model Aldrich), silica gel 9385 (E. Merk) was used. For vacuum liquid chromatography (VLC), silica gel 60 GF_{254} was used. Recycling HPLC (LC-908w): Semipreparative ODS (C4 column) used a mixed solvent of MeOH/H_2O (1:1) at a flow rate of 4.0 mL/min.

Plant material: The stem bark of *B. ceiba* was collected in February 2000 from the Karachi University Campus. The plant was authenticated by Prof. Dr Surraya Khatoon of the Department of Botany, University of Karachi and a voucher specimen (No. 66854 KUH) was deposited in the same department.

Extraction and isolation of chemical constituents: Fresh, uncrushed and undried stem bark (2.5 kg) of *B. ceiba* was extracted twice with light petroleum at room temperature. The extracts were combined and freed of the solvent *in vacuo* to a residue (BCBP, 5 g), which on treatment with methanol, followed by filtration, gave soluble (BCBPM, 3.47 g) and insoluble (BCBPMX) fractions. The filtrate (BCBPM, 30 mg) was subjected to preparative thin layer chromatography (PTLC) over silica gel (light petroleum: EtOAc, 7.5:2.5), which afforded 10 bands (BCBPM1–10). Spectral studies (mass, peak matching and ^1H NMR) on bands 2 and 4 revealed their structures as stigmasta-3,5-diene [4] and lupenone [5].

The marc left after light petroleum extraction was extracted with methanol 3 times. The combined methanolic extracts were freed of the solvent *in vacuo* to give a thickish mass (BCBM) in which insoluble matter settled down on keeping in the cold. This was removed by filtration to give soluble (BCBMM, 36.5 g) and insoluble (BCBMI, 4.50 g) fractions. The former (BCBMM, 36.4 g) was subjected to VLC (silica gel 60 GF_{254}, light petroleum, EtOAc, MeOH and H_2O in order of increasing polarity by 10%, which gave 38 fractions). Fractions 1–13 (light petroleum 100% – EtOAc 100%), 14–17 (MeOH:EtOAc, 1:9 – MeOH:EtOAc, 4:6), 18–21 (MeOH:EtOAc, 1:1 – MeOH:EtOAc, 8:2), 22–26 (MeOH:EtOAc, 9:1 – MeOH, 100%) and 27–38 (MeOH:H_2O, 9:1 – H_2O, 100%) were combined on the basis of TLC.

Fractions 14–17 (3.0 g) were subjected to FCC (silica gel 9385, light petroleum, EtOAc, MeOH and H_2O in order of increasing polarity) affording 64 fractions. Fractions 28–32 (EtOAc:MeOH, 7.5:2.5 – EtOAc:MeOH, 5.5:4.5) were purified through FCC (silica gel 9385, lightg petroleum, $CHCl_3$ and MeOH in order of increasing polarity), affording 43 fractions. FCC fractions S-11 ($CHCl_3$: MeOH, 8:2, 252 mg) and S-12 ($CHCl_3$:MeOH, 7.75:2.25, 21.0 mg) were separately subjected to recycling HPLC semi-preparative ODS (C4 column), eluted with (MeOH:H_2O, 1:1) at a flow rate of 4.0 mL/min. S-11 yielded 2 fractions S11-HP1 (15.0 mg, R_t 24 min.) and S11-HP2 (4.0 mg, R_t 18 min.), which were collected and evaporated to dryness. Spectral studies, along with 2D NMR data, disclosed that S11-HP1 was a mixture of 2 known isomers of lyoniresinol [6]. Eluate S11-HP2 was characterized as a known compound, opuntiol [7]. S-12 also gave 3 sub-fractions in which only S-12HP2 (5.0 mg,

R_t 22 min.) was found to be pure and characterized as a new compound, shamiminol (**1**).

Shamiminol [3,4,5-trimethoxyphenol 1-*O*-β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside] (**1**)

Colorless gum.
$[\alpha]_D^{24}$: –25.20 (*c* 0.016, CH_3OH)
IR ν_{max} (KBr) cm^{-1}: 3405, 1595, 1507, 1415, 1165.
UV λ_{max} (MeOH) nm: 215, 265.
^1H and ^{13}C NMR: Table 1.
HR FAB MS (+ve) *m/z*: 479.4522 (M^++1, calcd. for $C_{20}H_{31}O_{13}$, 479.4583), 461.4370 (M^++1–H_2O, calcd. for $C_{20}H_{29}O_{12}$, 461.4431), 185.1981 (M^++1–glucose–xylose, calcd. for $C_9H_{13}O_4$, 185.1998).
HR FAB MS (–ve) *m/z*: 477.4363 (M^+–1, calcd. for $C_{20}H_{29}O_{13}$, 477.4425), 459.4259 (M^+–1–H_2O, calcd. for $C_{20}H_{27}O_{12}$, 459.4272), 183.1829 (M^+–1–glucose–xylose, calcd. for $C_9H_{11}O_4$, 183.1839).
HR EIMS *m/z* (rel. intensity, %): 432.0863 (M^+–3CH_3–H, calcd. for $C_{17}H_{20}O_{13}$, 432.0903, 57), 346.1260 (M^+–xylose, $C_{15}H_{22}O_9$, 15), 184.0703 (M^+–glucose–xylose, $C_9H_{12}O_4$, 35).
EIMS *m/z* (rel. intensity, %): 432 ($C_{17}H_{20}O_{13}$, 2), 414 ($C_{17}H_{18}O_{12}$, 5), 346 ($C_{15}H_{22}O_9$, M^+-xylose, 2), 311 ($C_{11}H_{19}O_{10}$, 10), 295 ($C_{11}H_{19}O_9$, 35), 184 ($C_9H_{12}O_4$, M^+–glucose–xylose, 51), 182 (72), 167 ($C_9H_{11}O_3$, 72), 154 (74), 137 (100), 124 ($C_7H_8O_2$, 45) 107 (C_7H_7O, 55).

Acknowledgments - One of the authors (Sadia Zikr-Ur-Rehman) is grateful to the Higher Education Commission Islamabad, Pakistan for financial support through the Merit Scholarship Scheme for Ph.D. studies in Science and Technology (200 Scholarships).

References

[1] (a) Nadkarni KM, Nadkarni AK. (**1976**) *Indian Materia Medica*, Nadkarni KM (ed.), Popular Parkashan, Bombay, 1; (b) Baquar SR. (**1989**) *Medicinal and Poisonous Plants of Pakistan*, Printas Karachi (Pakistan), 397; (c) Perry LM, Metzgar J. (**1980**) *Medicinal Plants of East and Southern Asia*, MIT Press, London, 59.

[2] (a) Saleem R, Ahmad M, Hussain SA, Qazi AM, Ahmad SI, Qazi MH, Ali M, Faizi S, Akthar S, Husnain SN. (**1999**) Hypotensive, hypoglycaemic and toxicological studies on the flavonol *C*-glycoside shamimin from *Bombax ceiba. Planta Medica*, ***65***, 331-334; (b) You YJ, Nam NH, Kim Y, Bae KH, Ahn BZ. (**2003**) Antiangiogenic activity of lupeol from *Bombax ceiba, Phytotherapy Research*, ***17***, 341-344; (c) Saleem R, Ahmed SI, Faizi Z, Zikr-Ur-Rehman S, Ali M, Faizi S. (**2003**) Hypotensive activity and toxicology of constituents from *Bombax cieba* stem bark, *Biological and Pharmaceutical Bulletin*, ***26***, 41-46; (d) Dar A, Faizi S, Naqvi S, Roome T, Zikr-Ur-Rehman S, Ali M, Firdous S, Moin ST. (**2005**) Analgesic and antioxidant activity of mangiferin and its derivatives: the structure activity relationship. *Biological and Pharmaceutical Bulletin*, ***28***, 596-600; (e) Shuping L. (**2007**) New formulation of traditional Chinese medicine for treating burn. *Faming Zhangli Shenqing Gongkai Shuomingshu* CN 101,032,577 (Cl. A61K36/82), (**2007**), Appl. 10,027,354, 30 Mar 2007: 4 pp. (Ch); (f) Bapat VR. (**2007**) A novel ayurvedic drug for treatment of different type of vaginitis. *(India) Indian Patent Application* IN 2007 CH 1,511 (Cl. A61K31/573), 17 Aug 2007, Appl. 2007/CH1,511,13 Jul 2007:23 pp. (Eng); (g) Debasis DK, Ali KM, Mandal S, Barik B, Ghosh D. (**2010**) Antidiabetic and antioxidative effects of hydro-methanolic extract of sepals of *Salmalia malabarica* in streptozotocin induced diabetic rats. *Journal of Applied Biomedicine*, ***8***, 23-33.

[3] (a) Shahat AA, Hassan RA, Nazif NM, Miert SV, Pieters L, Hammuda FM, Vlietinck AJ. (**2003**) Isolation of mangiferin from *Bombax malabaricum* and structure revision of shamimin. *Planta Medica*, ***69***, 1066-1068; (b) Faizi S, Zikr-Ur-Rehman S, Ali M, Naz A. (**2006**) Temperature and solvent dependent NMR studies on mangiferin and complete NMR spectral assignments of its acyl and methyl derivatives. *Magnetic Resonance in Chemistry*, ***44***, 838-844; (c) Puckhaber LS, Stipanovic RD. (**2001**) Revised structure for a sesquiterpene lactone from *Bombax malbaricum*. *Jounal of Natural Products*, ***64***, 260-261; (d) Zhang X, Zhu H, Zhang S, Yu Q, Xuan L. (**2007**) Sesquiterpenoids from *Bombax malabaricum. Journal of Natural Products*, ***70***, 1526-1528; (e) Singh P, Mewara DK, Sharma MC. (**2008**) A new ferulic ester and related compounds from *Bombax malabaricum* DC. *Natural Product Communications*, ***3***, 223-225; (f) Wu J, Zhang XH, Zhang SW, Xuan LJ. (**2008**) Three novel compounds from the flowers of *Bombax malabaricum*, *Helvetica Chimica Acta*, ***91***, 136-143; (g) Sampath KN, Madhurambal G.. (**2010**) Quercetagetin glucoside from the flowers of *Bombax cieba*. *Asian Journal of Research in Chemistry*, ***3***, 78-80.

[4] Iribarren AM, Pomilio AB. (**1989**) Steroidal glucosides, flavonoids and other components of *Bauhinia uruguayensis*. *Anales de la Asociacion Quimica Argentina*, ***77***, 461-466.

[5] Shang-jiang D, Gariboldi P, Jommi G. (**1986**) Constituents of Shashen (*Adenophora axilliflora*). *Planta Medica*, ***4***, 317-320.

[6] Achenbach H, Lowel M, Waibel R, Gupta M, Solis P. (**1992**) New lignan glucoside from *Stemmadenia minima*. *Planta Medica*, ***58***, 270-272.

[7] Qiu Y, Chen Y, Pei Y, Matsuda H, Yoshikawa M. (**2002**) Constituents with radical scavenging effect from *Opuntia dillenii*: structure of new α-pyrones and flavonol glycoside. *Chemical and Pharmaceutical Bulletin*, ***50***, 1507-1510.

[8] Saijo R, Nanaka GI, Nishioka I. (**1989**) Phenol glucoside gallates from *Mallotus japonicus*. *Phytochemistry*, ***28***, 2443-2446.

[9] Miyamura M, Nohara T, Tomimatso T, Nishioka I. (**1983**) Seven aromatic compounds from bark of *Cinnamomum cassia*. *Phytochemistry*, ***22***, 215-218.

[10] Higuchi H, Fukui K, Kinjo J, Nohara T. (**1992**) Four new glycosides from *Albizziae Cortex*. III. *Chemical and Pharmaceutical Bulletin*, ***40***, 534-535.

[11] (a) Kosuge K, Mitsunaga K, Koike K, Ohmoto T. (**1994**) Studies on the constituents of *Ailanthus integrifolia*, *Chemical and Pharmaceutical Bulletin*, ***42***, 1669-1671; (b) Suttisri R, Chung MS, Kinghorn AD, Sticher O, Yamshimoto Y. (**1993**) Periandrin V, a further sweet triterpene glycoside from *Periandra dulcis*. *Phytochemistry*, ***34***, 405-408.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1901 - 1904

Two New Phenolic Glycosides from *Viburnum plicatum* var. *plicatum* f. *plicatum*

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Received: August 31st, 2011; Accepted: October 17th, 2011

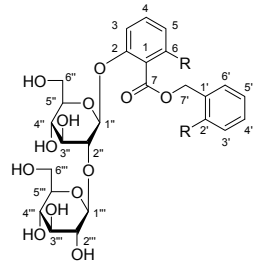
Two new glycosides, named dideoxyplicatumoside A (**1**) and *erythro*-syringylglycerol-β-*O*-4′-(+)-isoeucommin A 4‴-*O*-β-D-glucopyranoside (**2**), together with seven known compounds, were isolated from the leaves of *Viburnum plicatum* Thunb. ex Murray var. *plicatum* f. *plicatum*. Their structures were established on the basis of NMR, MS and chemical data.

Keywords: *Viburnum plicatum*, Caprifoliaceae, phenolic glycoside, sesquilignan glycoside.

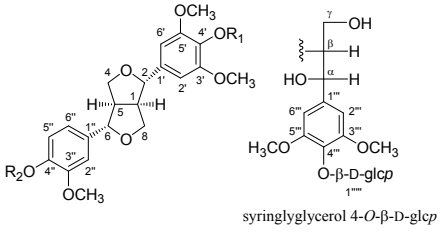
The deciduous shrub *Viburnum plicatum* Thunb. ex. Murray var. *plicatum* f. *plicatum* (Capriforiaceae) is distributed in Japan, Taiwan and China. We previously reported the isolation of two new glycosides including 13 known compounds from the leaves of *V. plicatum* var. *plicatum* f. *plicatum* [1]. In the course of further studies on the constituents of this plant, two new glycosides, named dideoxyplicatumoside A (**1**) and *erythro*-syringylglycerol-β-*O*-4′-(+)-isoeucommin A 4‴-*O*-β-D-glucopyranoside (**2**), and 7 known ones (**3**–**9**) have been isolated. This article deals with the structural elucidation and identification of these compounds.

The MeOH extract of the fresh leaves of *V. plicatum* var. *plicatum* f. *plicatum* was partitioned with $CHCl_3$, AcOEt, *n*-BuOH, and H_2O. The *n*-BuOH soluble fraction was separated by a combination of chromatographic procedures to afford two new glycosides (**1** and **2**) and seven known ones (**3**–**9**). The known compounds **3**–**9** were identified as (7*S*,8*R*)-dihydro-dehydrodiconiferyl alcohol 4-*O*-β-D-glucopyranoside (**3**) [2], (7*R*,8*S*)-dihydrodehydrodiconiferyl alcohol 4-*O*-β-D-glucopyranoside (**4**) [2], kankanoside E (**5**) [3], 7-*O*-tigloylsecologanol (**6**) [4], (+)-pinoresinol 4-*O*-β-D-glucopyranoside (**7**) [5], (7*S*,8*R*)-dihydrodehydrodiconiferyl alcohol 9′-*O*-β-D-glucopyranoside (**8**) [6] and 3′-*O*-[(2*S*)-2-methylbutanoyl] henryoside (**9**) [4], respectively, by comparison of their spectroscopic data with those previously described in the literature. This is the first record of **3**–**9** in this plant.

Compound **1** was obtained as an optically active amorphous powder. The molecular formula of **1**, $C_{26}H_{32}O_{13}$, was confirmed by HR-FAB-MS (*m/z* 575.1749 $[M+Na]^+$). The ^1H NMR spectrum of **1** showed signals due to nine aromatic protons, one CH_2 as an AB system, and



1 : R=H
Plicatumoside A : R=OH



syringlyglycerol 4-*O*-β-D-glc*p*

2 : R_1=syringlyglycerol 4-*O*-β-D-glc*p*, R_2=*O*-β-D-glc*p*
2a: R_1=syringlyglycerol 4-*O*-β-D-glc*p*, R_2=H
(+)-Isoeucommin A: R_1=H, R_2=*O*-β-D-glc*p*

Figure 1: Structures of new compounds **1** and **2**.

two anomeric protons at δ_H 4.73 (1H, d, *J*=7.6 Hz, H-1‴) and 5.34 (1H, d, *J*=7.3 Hz, H-1″). Acid hydrolysis of **1** yielded only D-glucose, which was identified by retention time and optical rotation using chiral detection by HPLC analysis. The coupling constants of the two anomeric protons indicated that the glycosyl linkages are of β-configuration. Its ^1H- and ^{13}C NMR spectra were similar to those of plicatumoside A isolated from the same plant [1]. The ^{13}C NMR spectrum of **1**, however, lacked signals from two hydroxylated aromatic quaternary carbons at C-6 and C-2′ in plicatumoside A, and instead showed signals for two aromatic CH carbons in **1** [δ_C 132.4 (s, C-6), 129.5

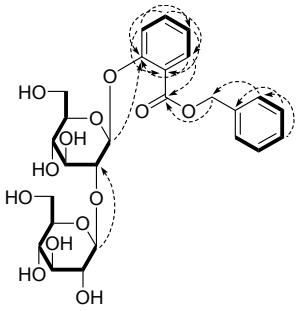


Figure 2: Main HMBCs of **1**.
Heavy lines indicate partial structures inferred from ^{1}H-^{1}H COSY.

Table 1: ^{13}C NMR spectroscopic data of **1** (100 MHz, CD_3OD).

Position	**1**	Position	**1**
1	121.6	1"	99.7
2	157.7	2"	83.2
3	116.3	3"	77.4
4	134.9	4"	70.7
5	122.6	5"	77.7
6	132.4	6"	61.5
7	167.3	1"'	104.5
1'	137.6	2"'	76.1
2', 6'	129.5	3"'	77.9
3', 5'	129.7	4"'	70.8
4'	129.4	5"'	77.6
7'	67.9	6"'	62.4

(s, C-2′, 6′)]. These spectral data, including the molecular formula, indicated that **1** is the 6, 2′-dideoxy derivative of plicatumoside A. Detailed analyses of the ^{1}H- and ^{13}C NMR spectra of **1** were undertaken with the aid of ^{1}H-^{1}H COSY and HMBC experiments (Figure 2). Consequently, the structure of **1** was elucidated as shown, and named dideoxyplicatumoside A.

Compound **2** was obtained as an optically active amorphous powder. The molecular formula of **2**, $C_{44}H_{58}O_{22}$, was confirmed by HR-FAB-MS (*m/z* 961.3316 $[M+Na]^{+}$). In the ^{1}H NMR spectrum of **2**, a signal pattern was similar to that of (+)-isoeucommin A [7], except for the presence of additional signals due to a 1,3,4,5-tetra-substituted benzene group, two methoxy groups [δ_H 3.82 (6H, s, OMe-3‴, 5‴)] and an anomeric group [δ_H 4.81 (1H, d, *J*=7.3 Hz, H-1⁗)]. Furthermore, the ^{13}C NMR spectrum exhibited the additional signals attributed to two oxygenated CH groups [δ_C 74.1 (d, C-α), 87.0 (d, C-β)] and an oxygenated CH_2 group [δ_C 61.7 (t, C-γ)]. Acid hydrolysis of **2** yielded only D-glucose, which was identified as described above. The coupling constants of the two anomeric protons indicated that the glycosyl linkages have β-configurations. Detailed analysis of the ^{1}H- and ^{13}C NMR spectra of the additional signals, with the aid of ^{1}H-^{1}H COSY, HMQC and HMBC experiments (Figure 3), suggested that the additional moiety was a syringylglycerol 4-*O*-β-D-glucopyranoside unit. From the findings presented above, compound **2** was a sesquilignan formed by (+)-isoeucommin A and syringylglycerol 4-*O*-β-D-glucopyranoside units. The additional syringylglycerol 4-*O*-β-D-glucopyranoside unit of **2** was attached to OH-4′ of (+)-isoeucommin A via an ether bridge, as shown by the HMBC cross-peak between H-β and C-4′ (Figure 3).

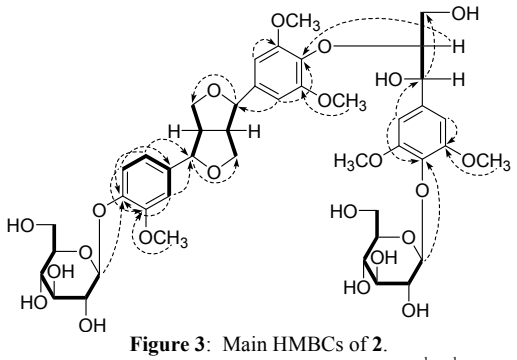


Figure 3: Main HMBCs of **2**.
Heavy lines indicate partial structures inferred from ^{1}H-^{1}H COSY.

Table 2: ^{13}C NMR spectroscopic data of (+)-isoeucommin A, **2** and **2a**.

Position	(+)-isoeucommin A*)	**2***)	**2a** #)
1	55.6	55.8	54.1
2	87.7	87.1	86.1
4	72.8	73.0	72.3
5	55.6	55.5	54.5
6	87.1	87.3	85.6
8	72.8	73.1	71.4
1'	133.2	139.0	138.0
2', 6'	104.6	104.3	102.9
3', 5'	149.4	154.6	153.4
4'	137.5	136.2	134.1
3', 5'-OCH_3	56.9	56.8	56.3
1"	137.5	137.5	132.7
2"	111.7	111.7	108.6
3"	151.0	151.0	146.7
4"	147.6	147.6	145.3
5"	118.1	118.1	114.3
6"	119.8	119.8	118.9
3"-OCH_3	56.8	57.0	56.0
α	—	74.1	72.6
β	—	87.0	86.9
γ	—	61.7	60.5
1"'	—	139.5	137.0
2"', 6"'	—	106.0	102.9
3"', 5"'	—	153.8	152.7
4"'	—	135.5	134.5
3"', 5"'-OCH_3	—	56.7	56.4
1""	102.9	102.9	—
2""	75.0	75.0	—
3""	77.9	77.9	—
4""	71.4	71.4	—
5""	78.3	78.4	—
6""	62.6	62.6	—
1""'	—	105.6	106.5
2""'	—	75.8	74.1
3""'	—	78.3	75.9
4""'	—	71.4	70.5
5""'	—	77.8	76.6
6""'	—	62.5	62.7

*) 100 Mz, CD_3OD, #) 100 MHz, $CDCl_3$.

Enzymatic hydrolysis of **2** afforded **2a**. As shown in Figure 4, the relative configuration of C-α and C-β of syringylglycerol 4-*O*-β-D-glucopyranoside unit in **2** was determined to be *erythro* by comparing the coupling constant between H-α and H-β (*J*=3.9 Hz) in the ^{1}H NMR spectrum ($CDCl_3$) of **2a** with those of the *threo* and *erythro* isomers [8–15], which was also supported by the NOESY correlations (H-α/H-β, H-α/OCH_3-3′, 5′, H-2‴, 6‴/H_A-γ, H-2‴, 6‴/H-β) of **2a**. Furthermore, NOE correlations were observed between H-1/H-2′, 6′, and H-5/H-2″, 6″. Consequently, the structure of **2** was elucidated as shown, and named *erythro*-syringylglycerol-β-*O*-4′-(+)-isoeucommin A 4‴-*O*-β-D-glucopyranoside.

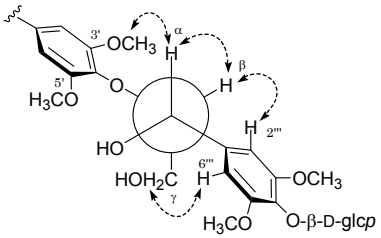


Figure 4: Main NOEs of **2**.

However, the absolute configurations of the chiral centers at C-α and C-β remain to be determined.

To the best of our knowledge, compound **1** is the first example of a naturally occurring benzyl salicylate glycoside, and there is no report of the isolation of a sesquilignan consitituent from the *Viburnum* species.

Experimental

General: Optical rotations were measured with a JASCO DIP-360 digital polarimeter. UV spectra were recorded with a Beckman DU-64 spectrometer. ^{1}H- and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 400 (400 MHz, 100 MHz, respectively) spectrometer. Chemical shifts are given on a δ (ppm) scale, with tetramethylsilane (TMS) as internal standard. FAB-MS were recorded on a JEOL JMS-DX 303 mass spectrometer. Column chromatography was carried out on Kieselgel 60 (230−400 mesh, Merck, Darmstadt, Germany) and Sephadex LH-20 (Pharmacia Fine Chemicals, Sweden). HPLC was carried out on a Tosoh HPLC system [pump, CCPS; detector, UV-8020; column, TSK gel ODS 120T (7.8 mm i.d. × 30 cm, Tosoh, Tokyo, Japan), and Cosmosil 5SL (10 mm i.d. × 25 cm, Nacalai, Tokyo, Japan).

Plant material: Leaves of *V. plicatum* Thunb. ex Murray var. *plicatum* f. *plicatum* were collected in May, 2007 in Sendai, Miyagi prefecture, Japan, and identified by one of the authors (M. K). A voucher specimen (2007-5-KM3) is held in the laboratory of M. Kikuchi.

Extraction and isolation: Fresh leaves of *V. plicatum* var. *plicatum* f. *plicatum* (860 g) were extracted with MeOH at room temp. for 3 weeks. The MeOH extract was concentrated under reduced pressure and the residue (74.5 g) was suspended in water. This suspension was successively extracted with $CHCl_3$ (28.8 g) AcOEt (14.8 g), *n*-BuOH (14.4 g) and H_2O (12.4 g). The *n*-BuOH-soluble fraction was concentrated under reduced pressure to produce a residue. This was chromatographed on a silica gel column using $CHCl_3$−MeOH−H_2O (40:10:1) and the eluate was separated into 20 fractions (frs. 1−20). Fraction 2 was chromatographed on a Sephadex LH-20 column using 50% MeOH and the eluate was separated into 18 fractions (frs. 2-1−2-18). Fraction 2-6 was subjected to prep. HPLC [column, TSK gel ODS 120T; mobile phase, MeOH−H_2O (5:6); UV detector, 205 nm; flow rate, 1.5 mL/min; column temp., 40°C: Cosmosil 5SL; mobile phase, CH_2Cl_2−MeOH− H_2O (7:10:1); UV detector, 225 nm; flow rate, 1.5 mL/min; column temp., 28°C] to give **1** (20.5 mg), **3** (2.8 mg), **4** (3.0 mg), **5** (4.5 mg) and **6** (5.0 mg), respectively. Fraction 2-7 was subjected to prep. HPLC [column, TSK gel ODS 120T; mobile phase, MeOH−H_2O (5:9); UV detector, 205 nm; flow rate, 1.5 mL/min; column temp., 40°C: TSK gel ODS 120T; mobile phase, MeOH−H_2O (2:5); UV detector, 205 nm; flow rate, 1.5 mL/min; column temp., 40°C] to give **7** (18.5 mg), **8** (2.0 mg) and **9** (5.0 mg), respectively. Fraction 4 was chromatographed on a Sephadex LH-20 column using 50% MeOH and the eluate was separated into 10 fractions (frs. 4-1−4-10). Fraction 4-1 was subjected to prep. HPLC [column, TSK gel ODS 120T; mobile phase, MeOH−H_2O (2:5); UV detector, 205 nm; flow rate, 1.5 mL/min; column temp., 40°C] to give **2** (3.2 mg).

Dideoxyplicatumoside A (**1**)
An amorphous powder.
$[\alpha]_D^{25}$: −84.3 (*c* 0.25, MeOH).
UV λ_{max} (MeOH) nm (log ε): 205 (4.5), 231 (3.9), 289 (3.4).
^{1}H NMR (400 MHz, CD_3OD) δ: 3.10–3.15 (2H, m, H-5‴, H_A-6‴), 3.19 (1H, dd, *J*=9.3, 7.6 Hz, H-2‴), 3.33–3.39 (2H, m, H-4″, 3‴), 3.43–3.49 (3H, m, H-5″, 4‴, H_B-6‴), 3.66 (1H, dd, *J*=11.7, 6.0 Hz, H_A-6 ″), 3.67 (1H, t, *J*=9.0 Hz, H-3″), 3.73 (1H, dd, *J*=9.0, 7.3 Hz, H-2″), 3.86 (1H, dd, *J*=11.7, 2.0 Hz, H_B-6″), 4.73 (1H, d, *J*=7.6 Hz, H-1‴), 5.30 (1H, d, *J*=12.5 Hz, H_A-7), 5.34 (1H, d, *J*=7.3 Hz, H-1″), 5.36 (1H, d, *J*=12.5 Hz, H_B-7), 7.06 (1H, br t, *J*=7.8 Hz, H-5), 7.25 (1H, br d, *J*=8.3 Hz, H-3), 7.36 (1H, dt, *J*=8.2, 1.4 Hz, H-4′), 7.40 (2H, t, *J*=8.2 Hz, H-3′, 5′), 7.44 (2H, dd, *J*=8.2, 1.4 Hz, H-2′, 6′), 7.51 (1H, br dt, *J*=7.8, 1.7 Hz, H-4), 7.77 (1H, dd, *J*=7.8, 1.7 Hz, H-6).
^{13}C NMR (CD_3OD): Table 1.
FAB-MS *m/z*: 575 $[M+Na]^+$. HR-FAB-MS *m/z*: 575.1749 $[M+Na]^+$ (Calcd for $C_{26}H_{32}O_{13}Na$: 575.1740).

***Erythro*-syringylglycerol-β-*O*-4′-(+)-isoeucommin A 4‴-*O*-β-D-glucopyranoside** (**2**)
An amorphous powder.
$[\alpha]_D^{25}$:−14.0 (*c* 0.20, MeOH).
UV λ_{max} (MeOH) nm (log ε): 211 (4.6), 230sh (4.3), 274 (3.5).
^{1}H NMR (400 MHz, CD_3OD) δ: 3.12 (2H, m, H-1, 5), 3.20–3.50 (8H, m, H-2⁗, 3⁗, 4⁗, 5⁗, 2⁗, 3⁗, 4⁗, 5⁗), 3.61 (1H, dd, *J*=12.0, 3.7 Hz, H_A-γ), 3.64–3.86 (4H, m, H_2-6⁗, H_2-6⁗), 3.80 (6H, s, 3′, 5′-OCH_3), 3.82 (6H, s, 3‴, 5‴-OCH_3), 3.87 (3H, s, 3″-OCH_3), 3.90 (3H, m, H_A-4, H_A-8, H_B-γ), 4.27 (3H, m, H_B-4, H_B-8, H-β), 4.74 (1H, d, *J*=4.2 Hz, H-2), 4.77 (1H, d, *J*=4.4 Hz, H-6), 4.81 (1H, d, *J*=7.3 Hz, H-1⁗), 4.87 (H-1⁗, overlapped with solvent signal), 4.91 (1H, d, *J*=5.9 Hz, H-α), 6.67 (2H, s, H-2′, 6′), 6.73 (2H, s, H-2‴, 6‴), 6.92 (1H, dd, *J*=8.3, 1.7 Hz, H-6″), 7.03 (1H, d, *J*=1.7 Hz, H-2″), 7.15 (1H, d, *J*=8.3 Hz, H-5″). ^{13}C NMR (CD_3OD): Table 2.
FAB-MS *m/z*: 961 $[M+Na]^+$. HR-FAB-MS *m/z*: 961.3316 $[M+Na]^+$ (Calcd for $C_{44}H_{58}O_{22}Na$: 961.3317).

Enzymatic hydrolysis of 2: An aqueous solution (5.0 mL) containing **2** (2.0 mg) and β-glucosidase (10 mg) was incubated at 40°C for 3 days. The reaction mixture was extracted with $CHCl_3$, and the $CHCl_3$ layer was evaporated under reduced pressure. The residue was purified by HPLC [column, Cosmosil 5SL; mobile phase, CH_2Cl_2−MeOH−H_2O (70:10:1); UV detector, 225 nm; flow rate, 1.5 mL/min; column temp., 28°C] to give **2a** as an amorphous powder (1.0 mg).

$[\alpha]_D^{25}$: +14.3 (*c* 0.15, MeOH).
UV λ_{max} (MeOH) nm (log ε): 231 (4.4), 274 (3.0).
^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.10 (1H, m, H-1), 3.16 (1H, m, H-5), 3.40–3.95 (6H, m, H-2′′′′′, 3′′′′′, 4′′′′′, 5′′′′′, H_2-6′′′′′), 3.47 (1H, br d, *J*=11.0 Hz, H_A-γ), 3.86 (6H, s, 3′′′, 5′′′-OCH_3), 3.87 (3H, m, H_A-4, H_A-8, H_B-γ), 3.90 (6H, s, 3′, 5′-OCH_3), 3.91 (3H, s, 3′′-OCH_3), 4.11 (1H, m, H-β), 4.26 (1H, dd, *J*=9.3, 6.3 Hz, H_B-8), 4.34 (1H, dd, *J*=9.0, 7.1 Hz, H_B-4), 4.53 (1H, d, *J*=7.6 Hz, H-1′′′′), 4.74 (1H, d, *J*=5.4 Hz, H-2), 4.79 (1H, d, *J*=4.6 Hz, H-6), 4.99 (1H, d, *J*=3.9 Hz, H-α), 6.62 (2H, s, H-2′′′, 6′′′), 6.64 (2H, s, H-2′, 6′), 6.82 (1H, dd, *J*=8.1, 2.0 Hz, H-6′′), 6.89 (1H, d, *J*=2.0 Hz, H-2′′), 6.90 (1H, d, *J*=8.1 Hz, H-5′′).
^{13}C NMR ($CDCl_3$): Table 2.
FAB-MS *m/z*: 799 $[M+Na]^+$. HR-FAB-MS *m/z*: 799.2787 $[M+Na]^+$ (Calcd for $C_{38}H_{48}O_{17}Na$: 799.2789).

Acid hydrolysis of 1 and 2: Each compound (*ca*. 1.0 mg) was refluxed with 1M HCl (1 mL) for 5 h. The reaction mixture was neutralized with Ag_2CO_3 and filtered. The solution was concentrated *in vacuo* and dried to give a sugar fraction. The sugar fraction was analyzed by HPLC under the following conditions: column, TSK gel Amide-80; column temperature, 45°C; mobile phase, CH_3CN−H_2O (4:1); flow rate, 1.0 mL/min; chiral detection (JASCO OR-2090). Identification of D-glucose present in the sugar fraction was carried out by comparison of the retention time and optical rotation with those of an authentic sample; t_R (min) 39.0 (D-glucose, positive optical rotation).

Acknowledgments - The authors are grateful to Mrs S. Sato and T. Matsuki of Tohoku Pharmaceutical University for conducting the NMR and MS measurements.

References

[1] Kikuchi M, Onoguchi R, Yaoita Y, Machida K. (**2011**) Two new glycosides from *Viburnum plicatum* Thunb. ex Murray var. *plicatum* f. *plicatum*. *Journal of Natural Medicines*, ***65***, 202–205.
[2] Matsuda N, Sato H, Yaoita Y, Kikuchi M. (**1996**) Isolation and absolute structures of the neolignan glycosides with the enantiometric aglycones from the leaves of *Viburnum awabuki* K. Koch. *Chemical & Pharmaceutical Bulletin*, ***44***, 1122–1123.
[3] Xie H, Morikawa T, Matsuda H, Nakamura S, Muraoka O, Yoshikawa M. (**2006**) Monoterpene constituents from *Cistanche tubulosa.* Chemical structures of kankanosides A–E and kankanol. *Chemical & Pharmaceutical Bulletin*, ***54***, 669–675.
[4] Machida K, Sagawa H, Onoguchi R, Kikuchi M. (**2010**) Three new glycosides from *Viburnum plicatum* Thunb. var. *tomentosum* MIQ. *Helvetica Chimica Acta*, ***93***, 290–297.
[5] Nishibe S, Chiba M, Hisada S. (**1977**) Studies on the Chinese crude drug "Forsythiae Fructus." I. On the constituents of Forsythiae Fructus on the market. *Yakugaku Zasshi*, ***97***, 1134–1137.
[6] Machida K, Sakamoto S, Kikuchi M. (**2009**) Two new neolignan glycosides from leaves of *Osmanthus heterophyllus*. *Journal of Natural Medicines*, ***63***, 227–231.
[7] Sugiyama M, Kikuchi M. (**1991**) Studies on the constituents of *Osmanthus* species. VII. Structures of lignan glycosides from the leaves of *Osmanthus asiaticus* Nakai. *Chemical & Pharmaceutical Bulletin*, ***39***, 483–485.
[8] Braga ACH, Zacchino S, Badano H, Sierra MG, Ruveda EA. (**1984**) ^{13}C NMR spectral and conformational analysis of 8-*O*-4′ neolignans. *Phytochemistry*, ***23***, 2025–2028.
[9] Matsuda N, Kikuchi M. (**1996**) Studies on the constituents of *Lonicera* species. X. Neolignan glycosides from the leaves of *Lonicera gracilipes* var. *glandulosa* Maxim. *Chemical & Pharmaceutical Bulletin*, ***44***, 1676–1679.
[10] Sung S-H, Huh M-S, Kim Y-C. (**2001**) New tetrahydrofuran-type sesquilignans of *Saururus chinensis* root. *Chemical & Pharmaceutical Bulletin*, ***49***, 1192–1194.
[11] Park S-Y, Hong S-S, Han X-H, Hwang J-S, Lee D, Ro J-S, Hwang B-Y. (**2007**) Lignans from *Arctium lappa* and their inhibition of LPS-induced nitric oxide production. *Chemical & Pharmaceutical Bulletin*, ***55***, 150–152.
[12] Machida K, Sakamoto S, Kikuchi M. (**2008**) Structure elucidation and NMR spectral assignments of four neolignan glycosides with enantiometric aglycones from *Osmanthus ilicifolius*. *Magnetic Resonance in Chemistry*, ***46***, 990–994.
[13] Kim K-H, Moon E, Kim S-Y, Lee K-R. (**2010**) Lignans from the tuber-barks of *Colocasis antiquorum* var. *esculenta* and their antimelanogenic activity. *Journal of Agricultural and Food Chemistry*, ***58***, 4779–4785.
[14] Wang W, Ali Z, Li X-C, Khan I-A. (**2010**) Neolignans from the leaves of *Casearia sylvestris* SWARTZ. *Helvetica Chimica Acta*, ***93***, 139–146.
[15] Li S-F, Di Y-T, Wang Y-H, Tan C-J, Fang X, Zhang Y, Zheng Y-T, Li L, He H-P, Li S-L, Hao X-J. (**2010**) Anthraquinones and lignans from *Cassia occidentalis*. *Helvetica Chimica Acta*, ***93***, 1795–1802.

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2011
Vol. 6
No. 12
1905 - 1906

Antimicrobial Chemical Constituents from the Endophytic Fungus *Phomopsis* sp. from *Notobasis syriaca*

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Received: May 14th, 2011; Accepted: October 31st, 2011

Phomosine K (**1**), a new phomosine derivative, has been isolated from *Phomopsis* sp., in addition to six known compounds: phomosine A (**2**), phenylalanine amide (**3**), 2-hydroxymethyl-4β,5α,6β-trihydroxycyclohex-2-en (**4**), (-)-phyllostine (**5**), (+)-epiepoxydon (**6**), and (+)-epoxydon monoacetate (**7**). Preliminary studies showed that compound **1** had strong antibacterial activity, while compounds **4-7** showed good antifungal, antibacterial, and algicidal properties, except compounds **4** and **6**, which lacked antifungal activity.

Key words: Fungal metabolites, *Phomopsis* sp., phomosines, epoxydones, antimicrobial activity.

Endophytic fungi are a rich source of novel organic compounds with interesting biological activities [1]. As part of a program on the isolation of biologically active compounds, we have now investigated *Phomopsis* sp. (internal strain no. 8966), isolated from the plant *Notobasis syriaca* and isolated a number of metabolites (Figure 1).

Phomosine K (**1**), a white solid, showed an $[M]^+$ at *m/z* = 332.1267 (HREIMS), corresponding to the molecular formula $C_{18}H_{20}O_6$. The IR spectrum of **1** showed the presence of an ester carbonyl group (1647 cm^{-1}) and a hydroxyl group (3410 cm^{-1}). The ^1H NMR spectrum showed signals for four methyl groups at δ 2.14 (s, 3H, CH_3-2'), 2.15 (s, 3H, CH_3-5), 2.16 (s, 3H, CH_3-5'), and 2.30 (s, 3H, CH_3-2), three phenolic OH groups at δ 2.14 (s, 3H, CH_3-2'), 2.15 (s, 3H, CH_3-5), and 2.16 (s, 3H, CH_3-5'), 2.30 (s, 3H, CH_3-2), three phenolic OH groups at δ 11.87, 7.29, and 6.54 (s, 1H, OH), one methoxy signal at δ 3.94 (s, 3H, CO_2CH_3) and two aromatic protons at δ 5.84 (s, 1H, 6'-H), and 6.46 (s, 1H, 4'-H). The ^{13}C NMR and DEPT spectrum indicated the presence of five CH_3, two CH, and eleven quaternary carbons. The structure of **1** was determined by comparison of its NMR spectroscopic data with those of phomosine A (**2**) [2]. A fourth methyl group appeared at δ 2.14 (s, 3H), instead of an aldehydic proton at δ 10.43, as found in compound **2**. The fourth methyl group at C-2′ was further confirmed from HMBC correlations of CH_3-2′ to C-1′, C-2′, and C-3′. Thus, the structure of **1**, was established as methyl 2,4-dihydroxy-5-(3-hydroxy-2,5-dimethylphenoxy)-3,6-dimethylbenzoate. The known compounds phomosine A (**2**) [2], phenylalanine amide [3], 2-hydroxymethyl-4β,5α,6β-trihydroxycyclohex-2-en (**4**) [4], (-)-phyllostine (**5**) [5a], (+)-epiepoxydon (**6**) [5], and (+)-epoxydon monoacetate (**7**) [5b] were identified by comparison with published data.

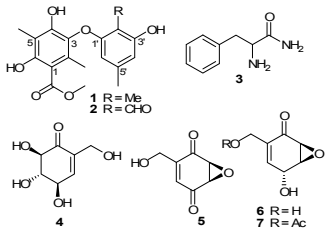

Figure 1: Chemical structure of compounds **1**-**7**.

The isolated compounds **4-7** were tested in an agar diffusion assay for their antifungal, antibacterial, and algicidal properties towards *Microbotryum violaceum, Escherichia coli, Bacillus megaterium*, and *Chlorella fusca* (Table 1). Metabolite **1** was tested for antibacterial activity in a MIC (minimum inhibitory concentration)-assay in liquid medium against *Legionella pneumophila* Corby, *Escherichia coli* K12 and *Bacillus megaterium* (Table 2).

Experimental

Plant material: College. Experimental procedures were quite similar to those reported earlier [1b]. Microbiological methods and culture conditions were as previously described [6a,b].

Culture, extraction, and isolation: The endophytic fungus *Phomopsis* sp. was isolated from *Notobasis syriaca*. It was

Table 1: Biological activities of pure metabolites **4**-**7** against microbial test organisms in agar diffusion assay.

Compound	antibacterial	antibacterial	antialgal	antifungal
	Ec[a]	Bm	Chl	Mv
4	7	PI 8	6	0
5	10	PI 7	10	7
6	7	0	5	0
7	9	PI 7	9	5
Penicillin	14	18	0	0
Tetracycline	18	18	PI 10	0
Nystatin	0	0	0	20
Actidione	0	0	35	50
Acetone	0	0	0	0

[a]*Chlorella fusca* (Chl), *Microbotryum violaceum* (Mv), *Escherichia coli* (Ec), and *Bacillus megaterium* (Bm). Application of pure substances at a concentration of 0.05 mg (50 µL of 1 mg/mL). The radius of zone of inhibition was measured in mm. PI = partial inhibition, i.e. there was some growth within the zone of inhibition.

Table 2: Antibacterial activity of **1** in a MIC assay in liquid medium against bacterial test organisms.

Compound **1**	Test organisms		
µg / mL	*Lp*[a]	*Ec*	*Bm*
100	+	+	-
50	+	-	-
25	+	-	-
12.5	-	-	-

cultivated at room temperature for 28 days [7,8] on biomalt solid agar medium. The culture medium was then extracted with ethyl acetate to afford 5.0 g of a residue after removal of the solvent under reduced pressure. The extract was separated into 2 fractions by CC on silica gel, using gradients of dichloromethane / ethyl acetate (85:15, 50:50, 0:100). The less polar fraction 1 (1.5 g) contained mainly fatty acids and lipids. The remaining fraction was further purified by silica gel CC and preparative TLC with *n*-hexane / ethyl acetate (10:1 to 5:1) to give pure compounds **1** (11 mg), **2** (40 mg), **3** (4 mg), **4** (6 mg), **5** (5 mg), **6** (5 mg), and **7** (5 mg).

Phomosine K (**1**)

MP: 199°C.

UV ($CHCl_3$): λ_{max} (log ε) = 277 (3.70), 310 (3.50) nm.

IR (KBr): ν = 3430 cm^{-1}, 2953, 1650, 1620, 1580, 1440, 1372, 1090, 1020, 795.

^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.14 (s, 3H, CH_3-2'), 2.15 (s, 3H, CH_3-5), 2.16 (s, 3H, CH_3-5'), 2.30 (s, 3H, CH_3-2), 3.94 (s, 3H, CO_2CH_3), 5.84 (s, 1H, 6'-H), 6.46 (s, 1H, 4'-H), 6.54 (s, 1H, OH), 7.29 (s, 1H, OH), 11.87 (s, 1 H, 6-OH).

^{13}C NMR (125 MHz, $CDCl_3$): δ = 8.2 (CH_3-5), 15.4 (CH_3-2), 20.9 (CH_3-2'), 21.7 (CH_3-5'), 51.9 (CO_2CH_3), 104.5 (C-1), 105.8 (C-6'), 108.4 (C-3), 110.8 (C-4'), 112.8 (C-2'), 131.1 (C-6), 133.1 (C-5), 142.2 (C-5'), 152.5 (C-4), 156.6 (C-1'), 156.6 (C-3'), 160.5 (C-6), 172.4 (C-1a).

EIMS (70 eV, 200°C): *m/z* (%) = 332.1 (55) [M^+], 298 (100), 255(25), 180 (69), 108 (96). HREIMS: *m/z* 332.1215 (calcd. 332.1260 for $C_{18}H_{20}O_6$).

Assays for biological activity: For the agar diffusion assays, the compounds were dissolved in acetone at a concentration of 1 mg/mL. Fifty µL of the solutions (50 μg) was pipetted onto a sterile filter disk (Schleicher & Schuell, 9 mm), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism [6a]. The fungal test organisms were *Escherichia coli, Bacillus megaterium,* and *Microbotryum violaceum.* Commencing at the middle of the filter disk, the radius of the zone of inhibition was measured in mm. The MIC-assays were conducted in microtiter plates in liquid media. *Legionella pneumophila* Corby was tested in YEB medium [6c]; *Escherichia coli* and *Bacillus megaterium* in NB medium. The metabolites were dissolved in methanol. In the wells, the bacterial test organisms were initially at a concentration of 2 x 10^7 cells / mL; final concentrations of the metabolites were 12.5 – 100 µg / mL: incubation of *L. pneumophila* was for 3 days with 5% CO_2 at 37°C, and that of *B. megaterium* and *E. coli* for one day at 24°C.

Acknowledgement - We thank BASF AG and the Bundesministerium für Bildung und Forschung (BMBF), project no. 03F0360A for sponsoring our research work.

References

[1] (a) Krohn K, Ullah Z, Hussain H, Floerke U, Schulz B, Draeger S, Pescitelli G, Salvodori P, Antus S, Kurtan T. (**2007**) Massarilactones E-G, new metabolites from the endophytic fungus *Coniothyrium* sp., associated with the plant *Artimisia maritima*. *Chiralty*, ***19***, 464-470; (b) Dai J, Hussain H, Dräger S, Schulz B, Kurtán T, Pescitelli G, Flörke U, Krohn K. (**2010**) Metabolites from the fungus *Phoma* sp. 7210, associated with *Aizoon canariense. Natural Product Communications*, ***5***, 1175-1180.

[2] Krohn K, Michel A, Roemer E, Flörke U, Aust HJ, Draeger S, Schulz B, Wray V. (**1995**) Biologically active metabolites from fungi; three new biaryl ethers from *Phomopsis* sp. *Natural Product Letters*, ***9***, 309-314.

[3] Ramallal RM, Liz R, Gotor V. (**2010**) Enantiopure trans-3-arylaziridine-2-carboxamides: Preparation by bacterial hydrolysis and ring-openings toward enantiopure, unnatural amino acids. *Journal of Organic Chemistry*, ***75***, 6614-6624.

[4] Smetanina OF, Kalinovskii AI, Khudyakov YV, Moiseenko OP, Pivkin MV, Menzorova NI, Sibirtsev YT, Kuznetsova TA. (**2005**) Metabolites of the marine fungus *Asperigillus varians* KMM 4630. *Chemistry of Natural Compounds*, ***41***, 243-244.

[5] (a) Mehta G, Islam K. (**2004**) Enantioselective total synthesis of epoxyquinone natural products (-)-phyllostine, (+)-epoxydon, (+)-epiepoxydon and (-)-panepophenanthrin: access to versatile chiral building blocks through enzymatic kinetic resolution. *Tetrahedron Letters*, ***45***, 7683-7687; (b) Klemke C, Kehraus S, Wright AD, König GM. (**2004**) New secondary metabolites from the marine endophytic fungus *Apiospora montagnei*. *Journal of Natural Products*, ***67***, 1058-1063.

[6] (a) Schulz B, Sucker J, Aust HJ, Krohn K, Ludewig K, Jones PG, Doering D. (**1995**) Biologically active secondary metabolites of endophytic *Pezicula* species. *Mycological Research*, ***99***, 1007-1015; (b) Höller U, Wright AD, Matthee GF, König GM, Draeger S, Aust HJ, Schulz B. (**2000**) Fungi from marine sponges: diversity, biological activity and secondary metabolites. *Mycological Research*, 104, 1354-1365; (c) Stewart AM, Meier K, Schulz B, Steinert M, Snider BB. (**2010**) Synthesis and biological evaluation of (±)-dinemasone C and analogues. *Journal of Organic Chemistry*, ***75***, 6057-6060.

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2011
Vol. 6
No. 12
1907 - 1912

Phomosines H–J, Novel Highly Substituted Biaryl Ethers, Isolated from the Endophytic Fungus *Phomopsis* sp. from *Ligustrum vulgare*‡

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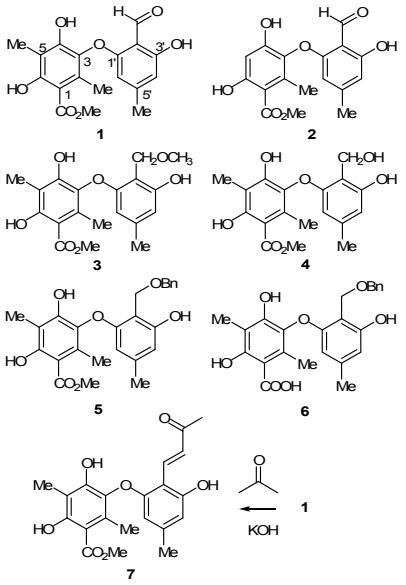
Received: May 7th, 2011; Accepted: October 29th, 2011

From the endophytic fungus *Phomosis* sp., four known phomosines A-D (**1-4**) and three new phomosines H-J (**5-7**) have been isolated. The structures of the new compounds were determined on the basis of their spectroscopic data analysis (^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HMQC, and HMBC NMR, as well as mass spectrometry). The structures of phomosine H (**5**) and J (**7**) were also confirmed by semisynthesis from phomosine A (**1**). The remaining four known compounds [phomosines A-D (**1-4**)] were identified by comparing their spectroscopic data with those reported in the literature. The four known metabolites were biologically active. Of the novel metabolites, only **6** was antifungal and antibacterial.

Key words: Endophytic fungi, fungal metabolites, phomosines, *Phomopsis* sp.

Endophytic fungi have been shown to be an excellent source of biologically active secondary metabolites [2]. We have investigated novel metabolites from an endophytic fungus, *Phomopsis* sp., which was isolated from *Ligustrum vulgare*. *Phomopsis* species have been found to synthesize a large array of structurally diverse secondary metabolites [3]. These include various classes, for example anthraquinones (e.g. altersolanol A-C [4], cytochalasins [5,6], phomopsichalasin [7], phenochalasins) [8], lactones (convolvulol, convolvulopyrone, convolvulanic acid A) [9], xanthones [10-12], nitropropionic esters [13,14], terpenoides, such as the oblongolides [13-15] and phomopsolides [16], 2,3-epoxycyclohexenes [17], isocoumarins [17], cyclic peptides, such as phomopsin A and B [18], orselliniic esters (phomozin) [19], macrolides [20,21], polyketides [22] and biaryl ethers, such as phomosines A-C [23] and D-G [24]. In this paper, we describe the isolation, physico-chemical properties, structure elucidation, semisynthesis, and biological activity of three new phomosines H-J (**5-7**), along with four known phomosines A-D (**1-4**). The fungus was cultivated in biomalt semi-solid agar culture media for 26 days at room temperature. The crude extract had good fungicidal, antibacterial, and algicidal activities. The ethyl acetate extract of the culture was separated by silica gel



Scheme 1: Chemical structure of compounds **1**-**7**.

column chromatography, resulting in the isolation of four known (**1-4**) and three new (**5-7**) biaryl ethers.

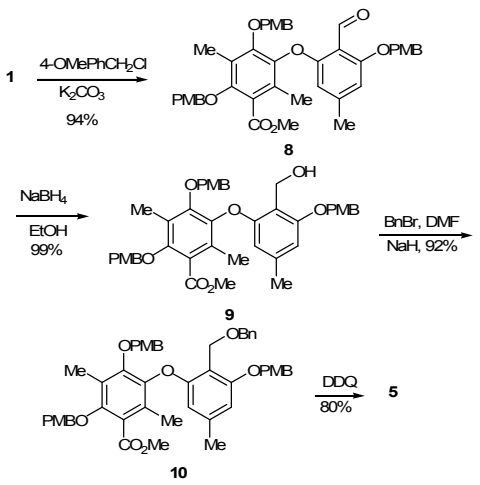
Four known metabolites, phomosines A-D (**1-4**) (Figure 1), were identified by comparison of their spectral data with those published in the literature [23,24]. The three new metabolites (**5-7**) of intermediate polarity were elucidated by direct comparison of their spectral data with those of compounds **1-4**.

Phomosine H (**5**) was isolated as a colorless solid with m.p. 163-165°C. The IR spectrum, with bands at 1648 and 3435 cm^{-1}, indicated the presence of conjugated and/or chelated ester carbonyl and hydroxyl groups. The NMR spectra with 25 signals in the ^{13}C NMR and 26 proton signals in the ^{1}H NMR spectra, together with the molecular ion at *m/z* = 438, suggested a molecular composition of $C_{25}H_{26}O_7$. Although both ^{1}H NMR and ^{13}C NMR spectra of **5** were in close correspondence to those of phomosines **1-4**, some new signals could be observed such as the resonances at δ 4.98 and 4.72 for two methylene groups and additional signals at δ 7.33-7.43 for five aromatic protons. Resonances at δ 2.33, 2.16 and 2.15 in the ^{1}H NMR spectrum were typical for three aromatic methyl groups, while two signals at δ 6.39 and 5.86 indicate aromatic protons at C-4' and C-6'. One phenolic hydroxyl was chelated with a carbonyl group and appeared at lower field (δ 11.8).

As in the phomosines A-D (**1-4**), one signal at δ 3.96 showed the presence of an ester methoxy group. The location of the benzyl ether at the benzylic methylene at C-1' was confirmed by two factors. First, the ^{13}C NMR signal for C-2'a in the free alcohol **4** is at δ 53.6, whereas in the methyl ether **3** it is at δ 63.9 and in the benzyl ether at δ 63.6. Secondly, this was unambiguously confirmed by the HMBC experiment with correlations of 2'a-H to C-3'a and 3'a-H- to C-2'a.

In order to further confirm the structure of phomosine H (**5**), chemical synthesis of **5** from **1** was performed (Scheme 2). Protection of three phenolic hydroxyl groups in **1** was achieved with *para*-methoxybenzyl chloride to form the *tris*-PMB ether **8** in 94 % yield. Treatment of **8** with $NaBH_4$ in the presence of EtOH, led to the alcohol **9** in 99 % yield. Benzylation [25] of the primary alcohol in **9** was achieved with benzyl bromide and NaH in DMS solution. Finally, deprotection of the PMB protecting groups in **10** was achieved with reaction with DDQ [26], yielding phomosine H (**5**) in 80 % yield.

The second new metabolite, phomosine I (**6**), was isolated as a colorless solid with m. p. 152-155°C. The IR spectrum showed strong bands for OH groups at 3394 cm^{-1} and for conjugated and/or chelated carbonyl groups at 1652 cm^{-1}. The structure of **6** was established mainly from its ^{1}H NMR spectrum which showed great similarities with that of methyl ester **5,** except for the missing signal for a methyl ester group. It was deduced that compound **6** was



Scheme 2: Semisynthesis of phomosine H (**5**) from phomosine A (**1**).

the corresponding acid of ester **5**. The MS and ^{13}C NMR spectra, in which 24 carbon peaks could be detected, supported this assumption.

The most polar compound, phomosine J (**7**), was isolated as a colorless solid with m.p. 225-228°C. The IR spectrum showed an absorption band for OH groups at 3510 cm^{-1} and strong carbonyl absorption at 1665 cm^{-1}. The NMR spectra with 21 signals in the ^{13}C NMR and 22 in the ^{1}H NMR spectra, together with a molecular ion at *m/z* = 386 suggested a molecular composition of $C_{21}H_{22}O_7$. Although both the ^{1}H NMR and ^{13}C NMR spectra of **7** were in close correspondence with those of **5** and **6**, some new signals could be observed, such as the two olefinic protons at δ 7.27 (d, *J* = 16.4 Hz, 2H, 2'b-H) and 8.13 (d, *J* = 16.4 Hz, 2H, 2'a-H). The *trans* relationship between the proton signals at δ 7.27 and 8.13 was evidenced from the large coupling constant (*J* = 16.4 Hz). A singlet at δ 2.33 was attributed to a conjugated methyl ketone. This connection was evident from the HMBC correlation to a carbonyl carbon appearing at δ 201.7 and long range correlations with two methylene groups at δ 8.13 and 8.13. From this evidence, structure **7** for phomosine H was proposed. It is the aldol condensation product of phomosine A (**1**) with acetone, and its structure was unambiguously proven by base-catalyzed aldol condensation of phomosine A (**1**) with acetone. However, the possibility cannot be excluded that **7** is an artifact generated during the isolation process.

Biological activity: The known phomosines A – D (**1 - 4**) and the novel phomosines H – J (**5 - 7**) were tested in an agar diffusion assay for antimicrobial activity. As displayed in Table 1, the four previously known phomosines A – D (**1 – 4**) were all active against the fungal test organism *Microbotryum violaceum*; phomosines A – C (**1 – 3)** were antibacterial against *Bacillus megaterium* and phomosines A (**1**) and D (**4**) inhibited the alga *Chlorella fusca.* Of the three new metabolites, only phomosine I (**6**) was antifungal and antibacterial.

Table 1: Biological activities of pure metabolites at a concentration of 50 µg against microbial test organisms in an agar diffusion assay [a].

Substance	*Microbotryum violaceum*	*Bacillus megaterium*	*Chlorella fusca*
1	13	10	6PI
2	6	10	0
3	6PI	7	0
4	7PI	0	7
5	0	0	0
6	9	7PI	0
7	0	0	0
Penicillin	0	18	0
Tetracycline	0	18	10PI
Nystatin	20	0	0
Actidione	50	0	35
Acetone	0	0	0

[a] Application of pure substances at a concentration of 50 µg (50 µL of 1 mg/mL). The radius of zone of inhibition was measured in mm. PI = partial inhibition, i.e. there was some growth within the zone of inhibition.

In summary, the highly antifungal and in part antibacterial group of phomosine biarylether natural products has been extensively extended by analysis of the culture extracts of Phomopsis sp., internal strain 5686. The four known metabolites were all biologically active, and the new benzyl ether **6** was antifungal as well as antibacterial.

Experimental

General experimental procedures: Column chromatography: commercial silica gel (Merck, 0.040-0.063 mm), and Sephadex LH-20 (Amersham Biosciences). Analytical and preparative thin-layer chromatography (TLC): Precoated silica gel plates (Merck, G60 F-254 or G50 UV-254), respectively. Optical rotation: Perkin–Elmer 241 MC polarimeter at the sodium D line. CD spectra were recorded on a J-810 spectropolarimeter and concentrations are given as mol/dm^3. The CD spectra were measured in millidegrees and normalized into $\Delta\varepsilon_{max}$ [l mol^{-1}cm^{-1}]/λ [nm] units. For solid-state CD protocol, see refs. [21,22]. IR spectra: Nicolet-510P spectrophotometer; ν_{max} in cm^{-1}. ^{1}H and ^{13}C NMR spectra: Bruker Avance 500 (500 MHz for ^{1}H and 125 MHz for ^{13}C) spectrometer; chemical shifts δ in ppm, coupling constant J in Hz. EI-MS and HR-EI-MS*:* MAT 8200 and Micromass LCT mass spectrometers, in *m/z*. Microbiological methods and culture conditions are as described previously [27,28].

Culture, extraction and isolation: *Phomopsis* sp., internal strain no. 5686, was isolated following surface sterilization from *Ligustrum vulgare* that had been growing in Braunschweig, Germany. The endophyte was cultivated at room temperature for 26 days on biomalt semi-solid agar media. The agar was extracted 4 times with ethyl acetate (4 x 0.6 L) to give the crude extract (19.85 g). This was subjected to CC on silica gel, using first gradients of light petroleum-dichloromethane, then dichloromethane, and then gradients of dichloromethane with up to 10% of methanol. On the basis of TLC analysis, similar fractions were combined and were re-chromatographed on silica gel to yield 9.6 g of **1** (*n*-hexane/ethyl acetate = 7:3, R_f = 0.70), 19.1 mg of **2** (*n*-hexane/ethyl acetate = 6.5:3.5, R_f = 0.65), 33.3 mg of **3** (*n*-hexane/ethyl acetate = 6.5:3.5, R_f = 0.63), 16.2 mg of **4** (*n*-hexane/ethyl acetate = 5:5, R_f = 0.68), 12.6 mg of **5** (*n*-hexane/ethyl acetate = 4:6, R_f = 0.64), 14.3 mg of **6** (*n*-hexane/ethyl acetate = 6: 4, R_f = 0.60), and 6.5 mg of **7** (*n*-hexane/ethyl acetate = 5:5, R_f = 0.68), respectively.

Phomosine H [(Methyl 3-(2-(benzyloxymethyl)-3-hydroxy-5-methylphenoxy)-4-hydroxy-2,5-dimethyl-benzoate (5)]

MP: 163-165°C.
IR (KBr): ν = 3435, 2953, 1648, 1620, 1580, 1441, 1372, 1296, 1133, 1102, 1090, 1020, 795 cm^{-1}.
UV ($CHCl_3$): λ_{max} (log ε) = 277 nm (3.70), 310 (3.50).
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.15 (s, 3 H, 5a-H), 2.16 (s, 3 H, 5'a-H), 2.33 (s, 3 H, 2a-H), 3.96 (s, 3 H, CO_2CH_3), 4.72 (s, 2 H, 2'b-H), 4.98 (s, 2 H, 2'a-H), 5.86 (s, 1 H, 6'-H), 6.39 (s, 1 H, 4'-H), 7.33-7.43 (m, 5 H, 2'd-h-H), 11.87 (s, 1 H, 6-OH).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 8.1 (C-5a), 15.4 (C-2a), 21.5 (C-5'a), 51.8 (CO_2CH_3), 63.6 (C-2'a), 72.9 (C-2'b), 104.2 (C-1), 106.2 (C-6'), 108.7 (C-2'), 110.8 (C-5), 111.5 (C-4'), 128.1-128.5 (C-2'd-h), 130.9 (C-2), 133.8 (C-3), 136.9 (C-2'c), 140.3 (C-5'), 152.8 (C-4), 156.1 (C-1'), 156.3 (C-3'), 160.3 (C-6), 172.4 (C-1a).
EIMS (70 eV, 200 °C): *m/z* (%) = 438 (71) [M$^+$], 330 (94), 298 (100), 255(25), 180 (69), 108 (96). HREIMS: *m/z* 438.1669 (calcd. 438.1679 for $C_{25}H_{26}O_7$).

Phomosine I [(3-(2-(benzyloxymethyl)-3-hydroxy-5-methylphenoxy)-4-hydroxy-2,5-dimethyl benzoic acid (6)]

MP: 152-155°C.
IR (KBr): ν = 3394, 3236, 2900, 1652, 1610, 1550, 1494, 1400, 1242, 1105, 1005, 784 cm^{-1}.
UV (MeOH): λ_{max} (log ε) = 282 nm (3.50).
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.15 (s, 3 H, 5'a-H), 2.16 (s, 3 H, 5a-H), 2.33 (s, 3 H, 2a-H), 4.73 (s, 2 H, 2'b-H), 4.98 (s, 2 H, 2'a-H), 5.86 (s, 1 H, 6'-H), 6.39 (s, 1 H, 4'-H), 7.34-7.43 (m, 5 H, 2'd-h-H), 11.87 (s, 1H, 6-OH).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 8.1 (C-5a), 15.4 (C-2a), 21.5 (C-5'a), 63.6 (C-2'a), 72.9 (C-2'b), 104.2 (C-1), 106.2 (C-6'), 108.7 (C-2'), 110.8 (C-5), 111.5 (C-4'), 128.1-128.5 (C-2'd-h), 130.9 (C-2), 133.8 (C-3), 136.9 (C-2'c), 140.3 (C-5'), 152.8 (C-4), 156.1 (C-1'), 156.3 (C-3'), 160.3 (C-6), 172.4 (C-1a).
EIMS (70 eV, 200°C): *m/z* (%) = 406 [M-H_2O]$^+$ (100), 391 (26), 378 (32), 377 (38), 360 (21), 348 (27), 327(20).
HREIMS: *m/z* 424.1509 (calcd. 424.1522 for $C_{24}H_{24}O_7$).

Phomosine I [(*E*)-methyl 4-hydroxy-3-(3-hydroxy-5-methyl-2-(3-oxobut-1-enyl)phenoxy)-2,5-dimethyl benzoate (7)]

MP: 225-228°C.
IR (KBr): ν = 3510, 2942, 1656, 1608, 1553, 1414, 1275, 1324, 1209, 1173, 1076, 822 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.08 (s, 3 H, 5'a-H), 2.12 (s, 3 H, 5a-H), 2.21 (s, 3 H, 2a-H), 2.33 (s, 3 H, 2'd-H), 3.86 (s, 3 H, CO_2CH_3), 5.73 (s, 1H, 6'-H), 6.31 (s, 1 H,

4'-H), 7.27 (d, *J* = 16.4 Hz, 2H, 2'b-H), 8.13 (d, *J* = 16.4 Hz, 2H, 2'a-H).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 8.1 (C-5a), 15.4 (C-2a), 21.7 (C-5'a), 51.8 (CO_2CH_3), 104.6 (C-1), 105.1 (C-6'), 108.3 (C-2'), 110.6 (C-5), 110.6 (C-4'), 128.6 (C-2'b), 130.9 (C-2), 133.8 (C-3), 135.6 (C-2'a), 142.9 (C-5'), 152.8 (C-4), 158.1 (C-1'), 158.5 (C-3'), 159.8 (C-6), 172.3 (C-1a), 201.7 (C-2'c).
EIMS (70 eV, 200 °C): *m/z* (%) = 386 [M^+] (57), 368 (84), 336 (100), 311 (87), 308 (37), 284 (17), 175(65).
HREIMS: *m/z* 386.1358 (calcd. 386.1366 for $C_{21}H_{22}O_7$).

Compound 8: To a stirred suspension of K_2CO_3 (1.29 g, 9.360 mmol) in dry DMF (20 mL) at room temperature was added phomosine A (**1**) (400 mg, 1.156 mmol) and a catalytic amount of TBAI (20 mg). The mixture was stirred for 30 min and then 4-methoxybenzyl chloride (1.086 g, 1.0 mL, 6.936 mmol) was added. The reaction mixture was stirred at room temperature for 8 h. The reaction was quenched with water (100 mL) and extracted 3 times with ethyl acetate (3x100 mL). The combined organic extracts were washed with water (2 ×100 mL), brine (100 mL) and dried (Na_2SO_4). After filtration, the solvent was removed to give the residue, which was purified by silica gel CC using light petroleum/EtOAc (7:1) to give **8** as a pale yellow solid in 94% yield (767 mg, 1.086 mmol).

MP: 144°C.
IR (KBr): *v* = 1648, 1620, 1580, 1295, 1100, 1090, 1027, 795 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.15 (s, 3 H, 5a-H), 2.16 (s, 3 H, 5'a-H), 2.33 (s, 3 H, 2a-H), 3.76 (s, 3 H, PMB-OCH_3), 3.82 (s, 3 H, PMB-OCH_3), 3.83 (s, 3 H, PMB-OCH_3), 3.86 (s, 3 H, CO_2CH_3), 4.84 (s, 2 H, CH_2-PMB), 5.10 (s, 4 H, CH_2-PMB), 5.90 (s, 1 H, 6'-H), 6.48 (s, 1 H, 4'-H), 6.80 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.92 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.95 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.17 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.34 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.40 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 10.2 (C-5a), 13.1 (C-2a), 22.7 (C-5'a), 52.3 (CO_2CH_3), 55.2 (PMB-OCH_3), 55.3 (PMB-OCH_3), 70.6 (CH_2-PMB), 75.2 (CH_2-PMB), 76.1 (CH_2-PMB), 107.0 (C-6'), 107.2 (C-2'), 112.2 (C-1), 113.8 (C-5), 113.9 (C-4'), 113.8 (PMB-Ar-*C*), 114.0 (PMB-Ar-*C*), 128.8 (C-2), 129.1 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 130.2 (C-3), 130.2 (PMB-Ar-*C*), 141.8 (C-5'), 151.3 (C-4), 156.1 (C-1'), 159.4 (PMB-Ar-*C*), 159.5 (C-3'), 161.8 (C-6), 168.2 (C-1a).
HREIMS: *m/z* 706.2768 (calcd. 706.2778 for $C_{42}H_{42}O_{10}$).

Compound 9: A solution of compound **8** (60 mg; 0.086 mmol) in ethanol (20 ml) was stirred at 0^o C and sodium borohydride (15 mg, 0.40 mmol) was immediately added. After the slow gas evolution, the solution was stirred overnight and then the solvent was removed. The white residue was dissolved in EtOAc and washed with water (50 mL), brine (50 mL) and dried (Na_2SO_4). After filtration, the solvent was evaporated to dryness to afford a white residue, which was purified by silica gel column chromatography using PE/EtOAc (5:1) to afford compound **9** as pale yellow solid in 99 % yield (58 mg, 0.083 mmol).

MP: 130°C.
IR (KBr): *v* = 3220, 1648, 1620, 1580, 1105, 1080, 1030, 780 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): *δ* = 2.08 (s, 3 H, 5a-H), 2.11 (s, 3 H, 5'a-H), 2.20 (s, 3 H, 2a-H), 3.77 (s, 3 H, PMB-OCH_3), 3.82 (s, 3 H, PMB-OCH_3), 3.83 (s, 3 H, PMB-OCH_3), 3.86 (s, 3 H, CO_2CH_3), 4.84 (s, 2 H, CH_2-PMB), 4.96 (s, 2 H, 2'a-H), 5.04 (s, 4 H, CH_2-PMB), 5.91 (s, 1 H, 6'-H), 6.49 (s, 1 H, 4'-H), 6.81 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.92 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.94 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.14 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.33 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.38 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*).
^{13}C NMR (125 MHz, $CDCl_3$): *δ* = 10.3 (C-5a), 13.1 (C-2a), 22.0 (C-5'a), 52.3 (CO_2CH_3), 55.2 (PMB-OCH_3), 55.3 (C-2'a), 55.4 (PMB-OCH_3), 70.0 (CH_2-PMB), 75.3 (CH_2-PMB), 76.5 (CH_2-PMB), 106.7 (C-6'), 107.0 (C-2'), 113.5 (C-1), 113.7 (C-5), 113.8 (C-4'), 113.9 (PMB-Ar-*C*), 114.2 (PMB-Ar-*C*), 125.0 (C-2), 128.8 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 130.2 (C-3), 130.2 (PMB-Ar-*C*), 139.6 (C-5'), 151.7 (C-4), 156.1 (C-1'), 159.4 (PMB-Ar-*C*), 159.5 (C-3'), 159.6 (C-6), 168.3 (C-1a).
HREIMS: *m/z* 708.2919 (calcd. 708.2934 for $C_{42}H_{44}O_{10}$).

Compound 10: To a stirred suspension of 60% NaH (20 mg, 0.833 mmol) in dry DMF (20 mL) at room temperature was added protected primary alcohol **9** (100 mg, 0.141 mmol) and a catalytic amount of TBAI (20 mg). The mixture was stirred for 15 min and then benzyl bromide (144 mg, 0.1 mL, 0.846 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. The reaction was carefully quenched with water (5 mL) and extracted 3 times with ethyl acetate (100 mL). The combined organic extracts were washed with water (2 × 100 mL), brine (100 mL) and dried (Na_2SO_4). After filtration, the solvent was removed to give a residue, which was purified by silica gel CC using light petroleum/EtOAc (7:1) to afford compound **10** as pale yellow solid in 92 % yield (103 mg, 1.29 mmol).).

MP: 118°C.
IR (KBr): *v* = 1655, 1620, 1585, 1110, 1080, 1040, 780 cm^{-1}.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.07 (s, 3 H, 5a-H), 2.10 (s, 3 H, 5'a-H), 2.20 (s, 3 H, 2a-H), 3.75 (s, 3 H, PMB-OCH_3), 3.82 (s, 3 H, PMB-OCH_3), 3.83 (s, 3 H, PMB-OCH_3), 3.85 (s, 3 H, CO_2CH_3), 4.60 (s, 2 H, CH_2-PMB), 4.83 (s, 2 H, 2'a-H), 4.84 (s, 2 H, 2'a-H), 4.85 (s, 2 H, 2'b-H), 5.04 (s, 4 H, CH_2-PMB), 5.90 (s, 1 H, 6'-H), 6.49 (s, 1 H, 4'-H), 6.81 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.74 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 6.92 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.10 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.19-

7.30 [(m, 5 H, 2'd-h-H (Bn)], 7.33 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*), 7.38 (d, *J* = 8.50 Hz, 2 H, PMB-Ar-*H*).
^{13}C NMR (125 MHz, $CDCl_3$): δ = 10.3 (C-5a), 13.1 (C-2a), 22.1 (C-5'a), 52.3 (CO_2CH_3), 55.2 (PMB-OCH_3), 55.4 (PMB-OCH_3), 60.9 (C-2'a), 70.8 (CH_2-PMB), 72.5 (C-2'b), 74.9 (CH_2-PMB), 76.2 (CH_2-PMB), 106.4 (C-6'), 107.0 (C-2'), 113.6 (C-1), 113.8 (C-5), 113.9 (C-4'), 113.9 (PMB-Ar-*C*), 114.2 (PMB-Ar-*C*), 124.8 (C-2), 128.1-128.5 (C-2'd-h), 128.8 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 129.2 (PMB-Ar-*C*), 129.6 (C-3), 130.2 (PMB-Ar-*C*), 139.0 (C-2'c), 140.1 (C-5'), 151.6 (C-4), 157.2 (C-1'), 159.3 (C-3'), 159.4 (PMB-Ar-*C*), 159.5 (C-6), 168.4 (C-1a).
HREIMS: *m/z* 708.2919 (calcd. 708.2934 for $C_{42}H_{44}O_{10}$).

Phomosine H (5): To a stirred solution of **10** (50 mg, 0.062 mmol) in CH_2Cl_2 (15 mL) containing a small amount of water (1/18-1/20 of CH_2Cl_2), DDQ (22 mg, 0.093 mmol) was added at 0°C. The reaction mixture was stirred at room temperature for 5 h. After completion of the reaction, aq. $NaHCO_3$ (10 mL) was added, and the mixture extracted with CH_2Cl_2 (100 mL). The extract was washed with aq. $NaHCO_3$ (3x100 mL) and brine (100 mL) and dried over Na_2SO_4. The solvent was evaporated and the residue was chromatographed on a silica gel column to give phomosine H (**5**) as white solid in 80% yield (21 mg, 0.050 mmol). The synthetic phomosine H (**5**) was identical in all respects (IR, UV, NMR, HREIMS) to natural phomosine H (**5**), including R_f and melting point.

Semisynthesis of phomosine H (7): A mixture of equimolar amounts of **1** (100 mg, 0.289 mmol) and acetone (170 mg, 0.2 ml, 2.890 mmol) in EtOH (10 mL) was warmed at 50°C. A 25% aqueous solution of KOH (2 mL) was added drop-wise to the reaction mixture over a period of 30 min. and further stirred for 5 h at 50°C. The stirring was terminated and the reaction mixture was kept at room temperature overnight. Ice cold water (20 mL) was added until the solid was dissolved. The reaction mixture was acidified with dilute HCl (5 mL), maintaining the temperature at 0-5°C. The precipitates formed were filtered off, dried and crystallized with aqueous ethanol to give the corresponding phomosine H (**7**) in 90 % yield (97 mg, 0.260 mmol).

Agar diffusion test for biological activity: The metabolites were dissolved in acetone at a concentration of 1 mg/mL. Fifty µL of the solution (50 µg) was pipetted onto a sterile filter disk (Schleicher & Schuell, 9 mm), which was placed onto an appropriate agar growth medium for the respective test organism and subsequently sprayed with a suspension of the test organism [19]. The test organisms were the Gram-positive bacterium *Bacillus megaterium* (grown on NB medium), the fungus *Microbotryum violaceum* and the alga *Chlorella fusca* (both grown on MPY medium). (For media recipes, see ref. [29]) Reference substances were penicillin, nystatin, actidione, and tetracycline. Commencing at the middle of the filter disk, the radius of the zone of inhibition was measured in mm. These microorgansims were chosen because (a) they are nonpathogenic and (b) they had in the past proved to be accurate initial test organisms for antibacterial, antifungal, and antialgal/herbicidal activities.

Acknowledgement - We thank the BMBF (Bundesministerium für Bildung und Forschung, Project-No.: SUA 07/003).

References

[1] Meng L, Sun P, Li L, Draeger S, Schulz B, Krohn K, Yia Y, Zhang W. (**2011**) Endophytic fungus *Penicillium chrysogenum*, a new source of hypocrellins. *Biochemical Systematics and Ecology*, ***39***, 163–165.
[2] (a) Schulz B, Boyle C, Draeger S, Römmert A, Krohn K. (**2002**) Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research*, ***106***, 996-1004; (b) Tan RX Zou WZ. (**2001**) Endophytes: a rich source of functional metabolites. *Natural Products Reports*, ***18***, 448-459.
[3] Cole RJ. (**1981**) *Handbook of Toxic Fungal Metabolites*. Cox RH. (Ed.), Academic Press, New York , London.
[4] Wheeler MM, Wheeler DMS, Peterson GW. (**1975**) Anthraquinone pigments from the phytopathogen *Phomopsis juniperovora* Hahn. *Phytochemistry*, ***14***, 288-289.
[5] Izawa Y, Shimizu T, Koyama K, Natori S. (**1989**) Six new 10-pheynl-[11]cytochalasans, cytochalasins N - S from *Phomopsis* sp. *Tetrahedron*, ***45***, 2323-2335.
[6] Tomioka T, Izawa Y, Koyama K, Natori S. (**1987**) Three 10-phenyl [11]cytochalasana, cytochalasins N, O, and P from *Phomopsis* sp. *Chemical & Pharmaceutical Bulletin*, ***35***, 902-905.
[7] Horn WS, Simmonds MSJ, Schwartz RE, Blaney WM (**1995**) Phomopsichalasin, a novel antimicrobial agent from an endophytic *Phomopsis* sp. *Tetrahedron*, ***51***, 3969-3978.
[8] Tomoda H, Namatame I, Si S, Kawaguchi K, Masuma R, Namikoshi M, Omura S. (**1999**) Phenochalasins, inhibitors of lipid droplet formation in mouse macrophages produced by *Phomopsis* sp. FT-0211. *Journal of Antibiotics*, ***52***, 851-856.
[9] Tsantrizos YS, Ogilvie KK, Watson AK. (**1992**) Phytotoxic metabolites of *Phomopsis convolvulus*, a host-specific pathogen of field bindweed. *Canadian Journal of Chemistry*, ***70***, 2276-2284.
[10] Isaka M, Jaturapat A, Rukseree K, Danwisetkanjana K, Tanticharoen M, Thebtaranonth Y. (**2001**) Phomoxanthones A and B, novel xanthone dimers from the endophytic fungus *Phomopsis* species. *Journal of Natural Products*, ***64***, 1015-1018.
[11] Elsässer B, Krohn K, Flörke U, Root N, Aust H-J, Draeger S, Schulz B, Antus S, Kurtán T. (**2005**) X-Ray structure determination, absolute configuration and biological activity of phomoxanthone A. *European Journal of Organic Chemistry*, 4563–4570.
[12] Rukachaisirikul V, Sommart U, Phongpaichit S, Sakayaroj J, Kirtikara K. (**2008**) Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. *Phytochemistry*, ***69***, 783–787.
[13] Claydon N, Grove JF, Pople M. (**1985**) Elm bark beetle boring and feeding deterrents from *Phomopsis oblonga*. *Phytochemistry*, ***24***, 937-943.

[14] Dai J, Krohn K, Gehle D, Kock I, Flörke U, Aust H-J, Draeger S, Schulz B, Rheinheimer J. (**2005**) New oblongolides isolated from the endophytic fungus *Phomopsis* sp. from *Melilotus dentata* from the shores of the Baltic Sea. *European Journal of Organic Chemistry*, 4009-4016.

[17] Hussain H, Akhtar N, Draeger S, Schulz B, Pescitelli G, Salvadori P, Antus S, Kurtán T, Krohn K. (**2009**) New bioactive 2,3-epoxycyclohexenes and isocoumarins from the endophytic fungus *Phomopsis* sp. from *Laurus azorica*. *European Journal of Organic Chemistry*, 749–756.

[18] Edgar JA, Frahn JL, Cockrum PA, Culvenor CCJ. (**1986**) Lupinosis. the chemistry and biochemistry of phompsins. *Bioactive Molecules*, 169-184.

[19] Nouguier R, Bertrand MP, Picon P, Perfetti P. (**1994**) Diastereoselective synthesis of a methylated derivative of phomozin, a phytotoxin isolated from *Phomopsis helianthi*, a phytopathogenic fungus of sunflowers. *Tetrahedron Letters*, ***35***, 8171-8172.

[20] Huang Z, Cai X, Shao C, She Z, Xia X, Chen Y, Yang J, Zhou, S Lin Y. (**2008**) Chemistry and weak antimicrobial activities of phomopsins produced by mangrove endophytic fungus *Phomopsis* sp. ZSU-H76. *Phytochemistry*, ***69***, 1604-1608.

[21] Du X, Lu C, Li Y, Zheng Z, Su W, Shen Y (**2008**) Three new antimicrobial metabolites of *Phomopsis* sp. *Journal of Antibiotics*, ***61***, 250-253.

[22] Davis RA, Andjic V, Kotiw M, Shivas RG. (**2005**) Phomoxins B and C: Polyketides from an endophytic fungus of the genus *Eupenicillium*. *Phytochemistry*, ***66***, 2771-2775.

[23] Krohn K, Michel A, Roemer E, Flörke U, Aust H-J, Draeger S, Schulz B, Wray V. (**1995**) Biologically active metabolites from fungi, 6. Phomosines A-C. Three new biaryl ethers from *Phomopsis* sp. *Natural Product Letters*, ***9***, 309-314.

[24] Dai J, Krohn K, Flörke U, Gehle D, Aust H-J, Draeger S, Schulz B, Rheinheimer K. (**2005**) Novel highly substituted biaryl ethers, phomosines D-G, isolated from the endophytic fungus *Phomopsis* sp. from *Adenocarpus foliolosus*. *European Journal of Organic Chemistry*, 5100-5105.

[25] Kanai K, Sakamoto I, Ogawa S, Suami T. (**1987**) Synthesis of 1,4-diaminocyclitol antibiotics. III. Synthesis of 4-hydroxy-purpurosamine B derivatives. *Bulletin of the Chemical Society of Japan*, ***60***, 1529-1531.

[26] Horita K, Yoshioka T, Tanaka T, Oikawa Y, Yonemitsu O. (**1986**) On the selectivity of deprotection of benzyl, mpm (4-methoxybenzyl) and dmpm (3,4-dimethoxybenzyl) protecting groups for hydroxy functions. *Tetrahedron*, ***42***, 3021-3028.

[27] Schulz B, Sucker J, Aust H-J, Krohn K, Ludewig K, Jones PG, Doering D. (**1995**) Biologically active secondary metabolites of endopyhtic *Pezicula* species. *Mycological Research*, ***99***, 1007-1015.

[28] Höller U, Wright AD, Matthée GF, König GM, Draeger S, Aust H-J, Schulz B. (**2000**) Fungi from marine sponges: Diversity, biological activity and secondary metabolites. *Mycological Research*, ***104***, 1354-1365.

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Vol. 6
No. 12
1913 - 1914

Isolation and Characterization of a new Benzofuran from the Fungus *Alternaria* sp. (HS-3) Associated with a Sea Cucumber

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Received: May 15th, 2011; Accepted: August 24th, 2011

A new compound, 4-acetyl-5-hydroxy-3, 6, 7-trimethylbenzofuran-2(3H)-one (**1**), together with two known compounds, 2-carboxy-3-(2-hydroxypropanyl) phenol (**2**) and 5-methyl- 6-hydroxy-8-methyoxy-3-methylisochroman (**3**) were isolated from the fungus *Alternaria* sp. (HS-3) associated with a sea cucumber from the Yellow Sea in China. Their structures were elucidated by spectral methods.

Keywords: sea cucumber, *Alternaria* sp, metabolism, benzofuran.

Marine-derived fungi have been the sources of novel second metabolites, a considerable number of which display promising biological and pharmacological properties [1]. Seven new indole diketopiperazines, along with twelve known ones, were isolated from the holothurian-derived fungus *Aspergillus fumigatus* [2]. Diterpene glycosides from the fungus *Acremonium striatisporum* isolated from a sea cucumber, *Eupentacta fraudatrix,* exhibited cytotoxic action against Ehrlich carcinoma cells [3-5]. In this article, we report the isolation of a new benzofuran derivative (**1**), together with two known compounds, 2-carboxy-3-(2-hydroxypropanyl) phenol (**2**) [6] and 5-methyl-6-hydroxy-8-methyoxy-3-methylisochroman (**3**) [7], from the fungus *Alternaria* sp. (HS-3) associated with a sea cucumber.

Compound **1** was assigned as $C_{13}H_{14}O_4$, as determined by HREIMS. The IR absorptions at 3385 and 1699 cm^{-1} reflected the presence of hydroxyl and carbonyl group(s). In the ^1H NMR spectrum, there was a chelating phenolic hydroxyl signal at δ 10.8, a methyl at δ 1.57 (d, 6.0 Hz), a methine at δ 4.30 (q, 7.2 Hz), and three methyl singlets at δ 2.31, 2.24 and 2.21. The ^{13}C NMR spectrum disclosed the presence of two carbonyl (δ 197.4 and 167.7), four methyl (δ 22.3, 17.3, 14.5 and 11.7), six substituted aromatic carbons (δ 159.2, 148.5, 136.6, 125.3, 124.5 and 102.7), and a methine carbon at δ 39.1. ^1H-^1H COSY revealed a contiguous sequence of coupled signals from H-9 to H-13. The HMBC data (Table 1), especially the correlations from the OH-5 (δ 10.8) to C-4, C-5 and C-6 unambiguously assigned the positions of the hydroxyl groups. Additionally, the correlations from H-9 to C-2, C-3, C-4 and C-8, indicated that the methine was attached to C-3 of the benzene ring. Thus, compound **1** was established as 4-acetyl-5-hydroxy-3, 6, 7-trimethylbenzofuran-2(3H)-one.

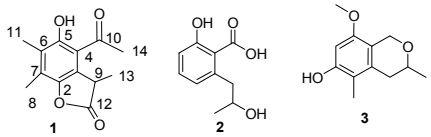

Figure 1: Structures of compounds **1**, **2** and **3**.

Table 1: ^1H and ^{13}C NMR (600 and 150 MHz, δ value, *J* in Hz) spectral data and HMBC correlations of **1** (in acetone-d_6).

Position	^{13}C NMR	^1H NMR	HMBC (C to H)
2	148.5 (C)		H-8, 9
3	136.6 (C)		H-9, 13
4	102.7 (C)		5-OH, H-9, 14
5	159.2 (C)		5-OH, H-11
6	124.5 (C)		5-OH, H-8, 11
7	125.3 (C)		H-8, 11
8	14.5 (CH_3)	2.31	
9	39.1 (CH)	4.30 (q, 7.2)	H-13
10	197.4 (C)		H-14
11	11.7 (CH_3)	2.24	
12	167.7 (C)		H-9, 13
13	17.3 (CH_3)	1.57 (d, 6.0)	H-9
14	22.3 (CH_3)	2.21 (s)	
5-OH		10.80 (s)	

Experimental

General: Melting points were obtained on a Fisher-Johns hot-stage apparatus and were uncorrected. 1D and 2D

NMR, Varian INOVA-600 MHz spectrometer; MS, VG-ZAB mass spectrometer; IR, Nicolet 5DX-FTIR spectrophotometer; CC was carried out on silica gel (200-300 mesh; Qingdao Haiyang Chemicals), octadecylsilyl silica gel (Unicorn; 45-60 μm) and Sephadex LH-20 (Amersham Biosciences). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin layer chromatography.

Fungal material: A strain (HS-3) of the fungus *Alternaria* sp. was isolated from the surface muscle of a sea cucumber from Weihai in the Yellow Sea, China. Voucher specimens are stored in the Biology Institute, Shandong Academy of Sciences, Jinan, PR China. The almost complete 18S rRNA gene sequence of strain HS-3 was deposited in GenBank under the no. JF694748.

Identification of the endophytic isolate: The endophytic fungus was grown on PDA for 5 days at 28°C. Genomic DNA was extracted and purified using the Fungal DNA Kit 50 (OMEGA, USA), according to the manufacture's instructions, suitably modified. For identification and differentiation, the Internal Transcript Spacer regions (ITS1F and ITS2) and the intervening 5.8S rRNA region was amplified and sequenced. The ITS regions of the fungus were amplified by PCR with the universal ITS primers, ITS1F (5'- CTT GGT CAT TTA GAG GAA GTAA-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). The PCR products were then purified and desalted using the EZ Spin column PCR product purification kit (BBI) and sequenced. The sequencing results were aligned with the nucleotide-nucleotide database (BLASTn) of the U.S. National Center for Biotechnology Information (NCBI) for final identification of the endophytic isolate.

Fermentation, extraction, and isolation: The fungal strain was cultivated in 50 L lipid medium at 28°C without shaking for 28 days. Two hundred and fifty 500 mL Erlenmeyer flasks each contained 2.0 g glucose, 0.4 g yeast extract and 0.4 g peptone dissolved in 200 mL of seawater. The cultures (50 L) were filtered through cheesecloth. The filtrate was concentrated to 5 L below 50°C and extracted 3 times by shaking with an equal volume of ethyl acetate. The combined organic extracts were subjected to silica gel CC, eluting with a gradient of light petroleum to ethyl acetate. Fractions of 40 mL were collected and combined by TLC examination. Fractions containing the desired compounds were further purified by Sephadex LH-20 chromatography, eluting with mixtures of light petroleum-$CHCl_3$-MeOH (2:1:1), which yielded compounds **1** (5.0 mg), **2** (31.0 mg), and **3** (6.0 mg).

4-Acetyl-5-hydroxy-3,6,7-trimethylbenzo-furan-2(3H)-one (**1**)
Colorless needles, MP: 113-115°C.
IR (KBr) ν_{max}: 3385, 3208, 3005, 2963, 2935, 1800, 1699, 1601, 1577 cm^{-1}.
^1H and ^{13}C NMR (acetone-d_6): Table 1.
EIMS: $[M]^+$ 234, $[M\text{-}CO]^+$ 206, $[M\text{-}CH_3CO]^+$ 191, HREIMS: 234.0887, relative intensity 100%, $C_{13}H_{14}O_4$.

2-Carboxy-3-(2-hydroxypropanyl) phenol (**2**)
White solid.
^1H NMR (600 MHz, acetone-d_6): δ 1.22 (3H, d, J = 6.6 Hz), 3.02 (2H, d, J = 7.4 Hz), 4.36 (1H, m), 6.78 (1H, d, J = 8.0 Hz), 7.07 (1H, dd, J = 8.0, 0.9 Hz), 7.32 (1H, dd, J = 8.0, 8.0 Hz), 11.74 (-OH, s).
^{13}C NMR (150 MHz, acetone-d_6): δ 20.9, 34.8, 76.9, 108.4, 116.4, 118.0, 136.3, 139.5, 162.4, 170.1.
ESIMS: m/z $[M\text{-}1]^-$ 195.

5-Methyl-6-hydroxy-8-methyoxy-3-methylisochroman (**3**)
White solid.
^1H NMR (600 MHz, $CDCl_3$): δ 1.38 (3H, d, J = 6.3 Hz), 2.04 (3H, s), 2.42 (1H, dd, J = 16.5, 10.6 Hz), 2.61 (1H,dd, J = 16.5, 2.3 Hz), 3.73 (1H, m), 3.70 (3H, s), 4.57 (1H, d, J = 15.0 Hz), 4.89 (1H, d, J = 15.0 Hz), 6.23 (1H, s).
^{13}C NMR(150 MHz, $CDCl_3$): δ 10.0, 21.7, 34.0, 55.0, 64.6, 70.6, 96.2, 112.9, 115.2, 134.1, 152.7, 153.9.
FABMS: m/z $[M+1]^+$ 209.

Acknowledgement - We wish to acknowledge the financial support of the National Natural Science Foundation of China (31000008), Prize Fund for Excellent Middle Aged and Young Scientists of Shandong Province (BS2009HZ004), Doctoral Prize of Shandong Academy of Sciences (2008, No. 25).

References

[1] Rateb ME, Rainer E. (**2011**) Secondary metabolites of fungi from marine habitats. *Natural Product Report*, ***28***, 290-344.
[2] Wang FZ, Fang YC, Zhu TJ, Zhang M, Lin AQ, Gu QQ, Zhu WM. (**2008**) Seven new prenylated indole diketopiperazine alkaloids from holothurian-derived fungus *Aspergillus fumigatus*. *Tetrahedron*, ***64***, 7986-7991.
[3] Afiyatullov SS, Kalinovsky AI, Pivkin MV, Dmitrenok PS, Kuznetsova TA. (**2006**) New diterpene glycosides of the fungus *Acremonium striatisporum* isolated from a sea cucumber. *Natural Product Research*, ***20***, 902-908.
[4] Afiyatullov SS, Kuznetsova TA, Isakov V.V, Pivkin MV, Prokofeva NG, Elyakov GB. (**2000**) New diterpenic altrosides of the fungus *Acremonium striatisporum* isolated from a sea cucumber. *Journal of Natural Products*, ***63***, 848-850.
[5] Afiyatullov SS, Kalinovsky AI, Kuznetsova TA, Pivkin MV, Prokofeva NG, Dmitrenok PS, Elyakov GB. (**2004**) New glycosides of the fungus *Acremonium striatisporum* isolated from a sea cucumber. *Journal of Natural Products*, ***67***, 1047-1051.
[6] Rahman MM, Sarker SD, Byres M, Gray AI. (**2004**) New salicylic acid and isoflavone derivatives from *Flemingia paniculata*. *Journal of Natural Products*, ***67***, 402-406.
[7] Masuma R, Tabata N, Tomoda H, Haneda K, Iwai Y, Omura S. (**1994**) Arohynapenes A and B, new anticoccidial agents produced by *Penicillium* sp. Taxonomy, fermentation, and structure elucidation. *Journal of Antibiotics*, ***47***, 46-53.

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Potent Toxic Macrocyclic Trichothecenes from the Marine-Derived Fungus *Myrothecium verrucaria* Hmp-F73

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Received: July 12th, 2011; Accepted: October 24th, 2011

Activity-guided fractionation of a methanol extract from the culture broth of *Myrothecium verrucaria* Hmp-F73, a fungus associated with the sponge *Hymeniacidon perleve*, afforded six macrocyclic trichothecenes, verrucarin J (**1**), 8-hydroxyverrucarin J (**2**), verrucarin A (**3**), 8-acetoxyroridin H (**4**), isororidin E (**5**), and roridin E (**6**), along with trichoverrin B (**7**). All seven metabolites displayed potent toxicity to the brine shrimp (*Artemia salina*). In addition, compounds **2**, **3**, and **6** showed weak phytotoxic activities against lettuce seeds. A preliminary structure-activity relationship of the metabolites is also discussed.

Keywords: *Myrothecium verrucaria* Hmp-F73, *Hymeniacidon perleve*, trichothecenes, mycotoxins, brine shrimp lethality.

In connection with our ongoing screening for bioactive secondary metabolites from fungi [1], we screened in a brine shrimp lethality assay the MeOH extracts of 98 fungal strains associated with the marine sponge *Hymeniacidon perleve* collected in Dalian. The extract of a strain of *Myrothecium verrucaria* Hmp-F73 exhibited potent toxic activity to the brine shrimp. Through activity-guided fractionation, we isolated seven trichothecene derivatives (**1-7**; Figure 1), namely verrucarin J (**1**), 8-hydroxyverrucarin J (**2**), verrucarin A (**3**), 8-acetoxyroridin H (**4**), isororidin E (**5**), roridin E (**6**) and trichoverrin B (**7**) [2]. Their structures were identified by comparison of their spectroscopic data with those published [2].

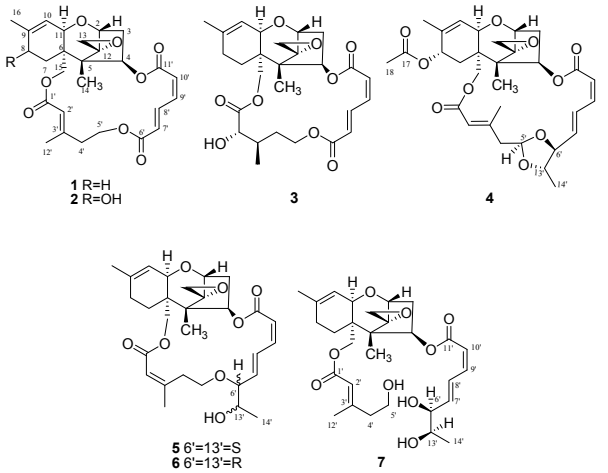

Figure1: Structures of compounds **1-7**.

Compounds **1-7** were evaluated for their toxicity toward brine shrimp larvae and phytotoxic activity against lettuce seed germination. As shown in Table 1, they exhibited relatively strong lethality activities to the brine shrimp after exposure for 24 h, with LC_{50} values ranging between 1 ng/mL and 1.1 μg/mL. Notably, verrucarins (**1**, in particular **2** and **3**) were much more toxic than roridinds (**4** and **6**) and trichoverrin B (**7**), suggesting that the presence of an ester carbonyl group at position C-6′ is essential for activity. Besides, it seems that mono-hydroxylation of a double bond at C-2′ of the macrocyclic ring of a verrucaroid (I) series is able to highly improve the toxicity (**1** and **2** vs **3**), indicating that the activity is associated with the presence of the 2′-OH substituent. For example, verrucarin A (**3**) showed 6- and 7-fold more activity than verrucarin J (**1**) and 8-hydroxyverrucarin J (**2**), respectively. Among the compounds tested, verrucarin A (**3**) exerted the best activity (LC_{50} = 0.001 μg/mL), while trichoverrin B (**7**) produced the least (LC_{50} = 1.138 μg/mL) since it was a nonmacrocyclic trichothecene, thereby indicating that an intact macrolide ring is also an important requirement for a higher toxic effect. Interestingly, verrucarin A (**3**) was reported to display no significant difference in the inhibition of protein synthesis in Vero cells and rat spleen lymphocytes. Thus, the *in vitro* cell response of a given trichothecene is not always an accurate predictor of toxicity in whole animals [3]. Our findings demonstrated for the first time that some modification of the macrolide ring moiety could highly affect the toxicity of trichothecene derivatives.

Table 1: Brine shrimp lethality and phytotoxicity of compounds **1-7**[a].

compound	brine shrimp assay LC_{50} (μg/mL)	phytotoxicity assay 100 μg/mL
verrucarin J (**1**)	0.006	inactive
isororidin E (**5**)		
8-hydroxyverrucarin J (**2**)	0.007	20%
verrucarin A (**3**)	0.001	30%
8-acetoxyroridin H (**4**)	0.15	inactive
roridin E (**6**)	0.221	50%
trichoverrin B (**7**)	1.138	inactive

[a]Compounds **1** and **5** were tested as a mixture (3:1).

Moreover, the phytotoxic activity of the compounds **1-7** toward the lettuce seed germination in a Petri dish bioassay was not observed at a concentration of 1 μg/mL. Only when the concentration was up to 100 μg/mL did the compounds **2**, **3** and **6** exerted a weak inhibiting activity.

From the present study, it can be concluded that the toxicity of the trichothecenes to brine shrimp is not related to the phytotoxicity potency. The trichothecenes **1-7**, identified from *M. verrucaria* Hmp-F73, were found to be most toxic against the brine shrimp larvae. This may constitute chemical defense of the sponge itself to protect it against predators like fishes and shrimps.

Experimental

General: ^{1}H and ^{13}C NMR spectra were acquired using a Bruker ARX 300 MHz spectrometer. ESI MS data were recorded using a Waters Micromass® ZQ mass spectrophotometer in positive ion mode.

Microorganism and culture conditions: Fungal strain Hmp-F73 was isolated from the marine sponge *Hymeniacidon perleve* collected from the Bohai Sea at the shore off Lingshui Qiao in Dalian, China, and identified as *Myrothecium verrucaria* (Genbank accession number: HQ625520). Seed culture was prepared with a 500 mL Erlenmeyer flask containing 100 mL of culture medium containing (g/L): potato 200 g, glucose 20 g and cultivated at 28°C for 48 h with shaking at 150 rpm. The seed culture was inoculated to 3 L Erlenmeyer flasks (×16) containing 1000 mL culture medium, the same as with the seed culture, and incubated for 4 days, at 28°C and 150 rpm on rotary shakers.

Extraction and isolation: To the cell-free broth (filtered, 16 L) of Hmp-F73, 60 mL of HP20 resin per L was added. After shaking at 100 rpm for 1 h, the resin was recovered and successively washed with deionized H_2O and MeOH. After evaporation of MeOH, the resulting crude extract (9.66 g) was subjected to vacuum flash chromatography over silica gel with ethyl acetate-*n*-hexane (0:10→10:0) and pure MeOH to provide Fr.1-Fr.6. Compounds **1** and **5** (26.7 mg, inseparable 3:1), **4** (7.7 mg) and **6** (15.4 mg) were purified from Fr. 3 (86.6 mg) by RP-HPLC (Dionex U3000, USA) using a YMC-pack ODS-A column (5 μm, 250 × 10 mm) eluting with 70% MeOH at a flow rate of 2.5 mL/min with UV detection at 220 nm. Similarly, semi-preparative HPLC of Fr. 4 (90.0 mg) eluting with 77% MeOH afforded **3** (6.4 mg), and purification of Fr. 5 (389.9 mg) with 55% MeOH yielded compounds **2** (6.5 mg) and **7** (13.5 mg).

Brine shrimp lethality assay: This assay was performed according to the reported method [1]. LC_{50} values for the test compounds were analyzed and calculated with SPSS 13.0.

Phytotoxicity assay: The Petri dish bioassay was carried out by the reported method [4].

Acknowledgments - This work was supported by a grant from the National High Technology Research and Development Program of China (863 Program 2007AA09Z417).

References

[1] Qin JC, Zhang YM, Hu L, Ma YT, Gao JM (**2009**) Cytotoxic metabolites produced by *Alternaria* No.28, an endophytic fungus isolated from *Ginkgo biloba*. *Natural Product Communications*, ***4***, 1473–1476.

[2] (a) Breitenstein W, Tamm C. (**1978**) Verrucarine and voridine.35. Partial synthesis of tetrahydroverrucarin-J. *Helvetica Chimica Acta*, ***61***, 1975-1983; (b) Jarvis BB, Midiwo JO, Mazzola EP. (**1984**) Antileukemic compounds derived by chemical modification of macrocyclic trichothecenes.2. Derivatives of roridin-A and roridin-H and verrucarin-A and verrucarin-J. *Journal of Medicinal Chemistry*, ***27***, 239-244; (c) Wagenaar MM, Clardy J. (**2001**) Two new roridins isolated from *Myrothecium* sp. *Journal of Antibiotics*, ***54***, 517-520; (d) Jarvis, BB, Desilva T, McAlphine JB, Swanson SJ, Whittern DN. (**1992**) New trichoverroids from *Myrothecium verrucaria* isolated by high-speed countercurrent chromatography. *Journal of Natural Products*, ***55***, 1441-1446.

[3] Thompson WL, Wannemacher RW. (**1986**) Structure-function-relationships of 12,13-epoxytrichothecene mycotoxins in cell-culture - comparison to whole animal lethality. *Toxicon*, ***24***, 985-994.

[4] (a) Abbas HK, Johnson BB, Shier WT, Tak H, Jarvis BB, Boyette CD. (**2002**) Phytotoxicity and mammalian cytotoxicity of macrocyclic trichothecene mycotoxins from *Myrothecium verrucaria*. *Phytochemistry*, ***59***, 309-313; (b) Andolfi A, Boari A, Evidente A, Vurro M. (**2005**) Metabolites inhibiting germination of *Orobanche ramosa* seeds produced by *Myrothecium verrucaria* and *Fusarium compactum*. *Journal of Agricultural and Food Chemistry*, ***53***, 1598-1603.

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2011
Vol. 6
No. 12
1917 - 1920

Synthesis and Bioactivity of Novel Coumarin Derivatives

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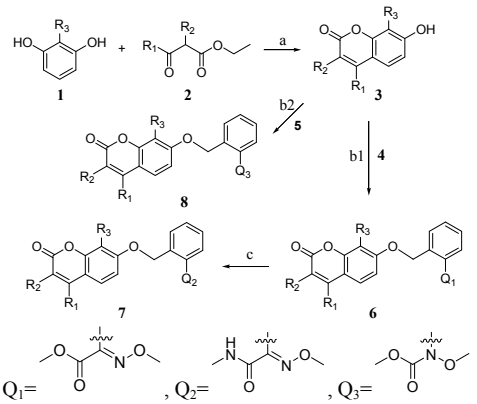
Received: July 12th, 2011; Accepted: October 26th, 2011

A series of coumarin derivatives (**6-8**) containing (*E*)-methyl 2-(methoxyimino)-2-phenylacetate, (*E*)-2-(methoxyimino)-*N*-methyl-2-phenylacetamide and methyl methoxy(phenyl)carbamate were synthesized from substituted resorcinols (**1**) and substituted *β*-keto esters (**2**) as starting material via cyclization and condensation reactions. The test results indicated that (*E*)-methyl 2-{2-[(3-hexyl-4-methyl-coumarin-7-yloxy)methyl]phenyl}-2-(methoxyimino)acetate (**6f**) was the optimal structure with good fungicidal activity against cucumber grey mold (CGM) giving 100% control at 100 mg L^{-1} concentration, much higher than that of coumoxystrobin. Methyl 2-[(3,4-dimethyl-coumarin -7-yloxy)methyl]phenyl(methoxy)carbamate (**8a**) was another optimal structure with good fungicidal activity against wheat powdery mildew (WPM) showing 100% control at 50 mg L^{-1} concentration, at the same level as that of the commercial kresoxim-methyl, and very significantly higher than that of coumoxystrobin (no control against WPM at 400 mg L^{-1}).

Keywords: Coumarins, strobilurins, intermediate derivatization method, fungicidal activity, insecticidal activity.

Substituted coumarins and methoxyacrylates derived from strobilurin A exhibit a broad spectrum of bioactivities [1-4]. A series of coumarin derivatives containing (*E*)-methyl 3-methoxy-2-phenylacrylate (MA) were synthesized and bioassayed in our previous study [5a-5c], which resulted in the discovery of coumoxystrobin (SYP-3375), (*E*)-methyl 2-{2-[(3-*n*-butyl-4-methylcoumarin-7-yloxy) methyl] phenyl}-3-methoxyacrylate, having a broad spectrum fungicidal activity against cucumber downy mildew (CDM), cucumber grey mold (CGM), wheat powdery mildew (WPM), rice sheath blight (RSB) and apple valsa canker (AVC). Other non-acrylate compounds, such as Kresoxim-methyl [5d], Dimoxystrobin [6], Orysastrobin [7], Pyraclostrobin [8] and Pyrametostrobin (SYP-4155) [9] containing related pharmacophores such as (*E*)-methyl 2-(methoxyimino)-2-phenylacetate (OE), *(E)*-2-(methoxyimino)-*N*-methyl-2-phenylacetamide (OA) and methyl methoxy(phenyl) carbamate (MC), were shown to have excellent fungicidal activity. A number of coumarin derivatives containing OE, OA and MC were synthesized using the intermediate derivatization method based on either bioisosteric replacement [5b,5c,10-12] or active substructure combination [13]. The fungicidal activity and the structure–activity relationship are also discussed in this paper.

Synthesis: As shown in Scheme 1, according to known methods [14,15,16a], substituted coumarins (**3**) were prepared from substituted resorcinols (**1**) and substituted *β*-keto esters (**2**). Then, intermediates (**3**) were first treated with 1 equiv. of (*E*)-methyl 2-(2-(bromomethyl) phenyl) -2-(methoxyimino)acetate (**4**) in butanone at refluxing



Scheme 1: Reagents and conditions: (a) c.H_2SO_4/5°C-room temperature; (b1) K_2CO_3 in butanone, reflux; (b2) NaH in DMF, room temperature; (c) CH_3NH_2 in CH_3OH, room temperature.

temperature for 5 h to afford compounds (**6**) in 80–90% yields, followed by reacting with 2.1 equiv. of methylamine in CH_3OH at room temperature for 3 h to obtain target compounds (**7**) in 85-90% yields; The desired compounds (**8**) were synthesized via the reaction of intermediates (**3**) with 1 equiv. of methyl 2-(bromomethyl)phenyl(methoxy)carbamate (**5**) in DMF at room temperature for 3 h in 65–90% yields.

Biological activities: Evaluations of biological activities of the title compounds were performed as previously reported [16b-16d]. Biological data were reported in the range 0 (indicates no control) to 100% (complete control). The test results of fungicidal activities of the title compounds are listed in Tables 1 and 2.

Table 1: Chemical structures, physical properties and fungicidal activity against CDM of synthesized coumarin analogues.

Compd	Substituents				mp (°C)	Yield (%)	Fungicidal activity (% control at given concentration mg L^{-1})					
	R_1	R_2	R_3	Q			CDM					
							400	200	100	50	25	6.25
6a	H	H	H	Q_1	106-110	80	70	60	/	/	/	/
6b	CH_3	H	H	Q_1	150-152	85	70	70	/	/	/	/
6c	CH_3	CH_3	H	Q_1	116-117	88	95	95	90	80	55	30
6d	CH_3	H	CH_3	Q_1	178-180	81	0	0	/	/	/	/
6e	CH_3	Cl	CH_3	Q_1	198-200	83	0	0	/	/	/	/
6f	CH_3	n-C_3H_7	H	Q_1	121-122	80	60	/	/	/	/	/
6g	CH_3	n-C_4H_9	H	Q_1	100-102	86	100	98	90	95	80	45
6h	CH_3	n-C_6H_{13}	H	Q_1	75-78	87	100	100	100	100	100	85
6i	n-C_3H_7	Cl	H	Q_1	136-138	83	0	/	/	/	/	/
6j	Ph	Cl	H	Q_1	166-168	81	98	80	60	50	/	/
7a	H	H	H	Q_2	129-130	88	80	50	/	/	/	/
7b	CH_3	H	H	Q_2	213-214	90	80	80	/	/	/	/
8a	CH_3	CH_3	H	Q_3	131-133	68	100	95	90	100	90	70
8b	CH_3	Cl	H	Q_3	139-141	79	100	85	70	85	60	20
8c	CH_3	F	H	Q_3	114-116	75	100	75	55	/	/	/
8d	CH_3	n-C_4H_9	H	Q_3	65-67	70	0	/	/	/	/	/
8e	$CH_2CH_2CH_2$		H	Q_3	177-179	85	0	/	/	/	/	/
8f	$CH_2CH_2CH_2$		CH_3	Q_3	150-151	88	80	/	/	/	/	/
8g	$CH_2CH_2CH_2CH_2$		H	Q_3	160-161	92	0	/	/	/	/	/
8h	$CH_2CH_2CH_2CH_2$		CH_3	Q_3	167-169	90	80	/	/	/	/	/
coumoxystrobin							100	100	100	100	100	95
kresoxim-methyl							/	/	/	/	30	0

note: "/" stands for no data

Table 2: Fungicidal activity against RSB, CGM and WPM of synthesized coumarin analogues.

Compd	Fungicidal activity (% control at given concentration mg L^{-1})								
	RSB	CGM				WPM			
	400	400	200	100	50	400	200	100	50
6a	0	0	/	/	/	50	/	/	/
6b	90	70	/	/	/	50	/	/	/
6c	80	0	/	/	/	30	/	/	/
6d	90	0	/	/	/	0	/	/	/
6e	40	0	/	/	/	0	/	/	/
6f	0	100	100	100	60	100	100	95	85
6g	90	20	/	/	/	100	50	/	/
6h	70	50	/	/	/	20	/	/	/
6i	0	30	/	/	/	95	95	85	70
6j	95	70	/	/	/	0	/	/	/
7a	0	50	/	/	/	40	/	/	/
7b	60	40	/	/	/	0	/	/	/
8a	50	0	/	/	/	100	100	100	100
8b	40	85	10	/	/	95	95	85	/
8c	0	100	55	/	/	100	30	/	/
8d	80	90	/	/	/	0	/	/	/
8e	0	80	/	/	/	0	/	/	/
8f	0	50	/	/	/	50	/	/	/
8g	50	98	/	/	/	0	/	/	/
8h	30	0	/	/	/	70	/	/	/
coumoxystrobin	70	80	80	65	/	0	/	/	/
kresoxim-methyl	/	/	/	/	/	100	100	100	100

note: "/" stands for no data

Many compounds showed 100% control against CDM at 400 mg L^{-1}, and these compounds were further tested at a lower dose range (200, 100, 50, 25, and 6.25 mg L^{-1}). The fungicidal activities of all synthesized compounds were lower than that of coumoxystrobin, except for 6h, which was comparable in potency (Table 1).

Some synthesized compounds also have fungicidal activity against RSB, CGM and WPM, besides CDM. Especially, compound **6f** showed 100% control against CGM at 100 mg L^{-1}, much higher than that of coumoxystrobin (65%). Furthermore, compounds **6f**, **6i** and **8a** all exhibited excellent activity against WPM, even at a lower dose range (200, 100, and 50 mg L^{-1}). Particularly, **8a** displayed equal fungicidal activity (100%) against WPM at 50 mg L^{-1} to kresoxim-methyl. However, coumoxystrobin had no activity against WPM at 400 mg L^{-1}, which indicated that **8a** is comparable with the commercial kresoxim-methyl (Table 2).

Based on data presented in Table 1, the title compounds (R_1 is methyl and R_2 is halogen or C_1-C_6 alkyl) seem to be more active against CDM at lower concentrations, particularly compound **6h** (R_1 is methyl and R_2 is n-C_6H_{13}), which was 85% active at 6.25 mg L^{-1}, slightly lower than that of coumoxystrobin, and significantly higher than that of kresoxim-methyl. Replacement of the methyl group at R_1 with either n-C_3H_7 or Ph resulted in loss of activity, respectively (**6i** and **6j**). There are, however, a few exceptions, such as **6e**, **6f** and **8d**. When R_1 and R_2 are combined into a five- or six-membered ring, the fungicidal activity decreases considerably. In addition, introduction of methyl to R_3 of the coumarin ring has little effect on the overall fungicidal activity. Furthermore, related pharmacophores, such as OE ($Q=Q_1$), OA ($Q=Q_2$) and MC ($Q=Q_3$) were studied. As shown in the comparison of these three groups of compounds (**6g** versus **8d; 6a** versus **7a; 6b** versus **7b**), it seemed that the fungicidal activity of OE ($Q=Q_1$) was at the same level as that of MC ($Q=Q_2$), significantly greater than that of OA ($Q=Q_3$), but an exception was noted, the pairs **6c** and **8a** showed the opposite trend.

Based on the results against WPM presented in Table 2, firstly, of the title compounds (R_1 is methyl or n-C_3H_7), all showed good fungicidal activity against WPM, but replacement of n-C_3H_7 with Ph led to loss of activity; while replacement of methyl with H had little effect on the

activity of the whole molecule. Secondly, introducing H, halogen, methyl, *n*-C_4H_9 or *n*-C_6H_{13} to R_2 to replace *n*-C_3H_7 resulted in either reduction or loss of fungicidal activity. The cyclization of R_1 and R_2 made no contribution to the activity of the target compounds. Finally, as for Q, seemingly OE (Q=Q_1) has greater fungicidal activity than OA and MC (Q=Q_2, Q_3), but there is still an exception, the pairs **6a** and **7a** showed nearly equal efficacy.

Insecticidal activity: All synthesized compounds were tested against armyworm, diamondback moth, peach aphid, carmine spider mite and common mosquito. The bioassay results showed that the tested compounds lacked activity against diamondback moth and peach aphid, while three compounds exhibited some insecticidal or acaricidal activities, specifically **6h**, with 100% control against carmine spider mite; **7b** with 50% control against common mosquito, and **8h** with 40% control against armyworm. Overall, the insecticidal activity of this series of compounds was considered modest.

Conclusion: Two compounds with improved fungicidal activity compared with coumoxystrobin, **6f** and **8a**, were discovered as the optimal structure against CGM and WPM respectively, through introducing OE (Q=Q_1), OA (Q=Q_2) and MC (Q=Q_3) to replace MA (*β*-methoxyacrylate pharmacophore) in coumoxystrobin. Compound **6f** (R_1 methyl, R_2 methyl, R_3 H, and Q Q_3) showed good control against CGM and WPM, especially against CGM (100%) at 100 mg L^{-1}, much higher than that of coumoxystrobin (65%); Compound **8a** (R_1 methyl, R_2 *n*-C_3H_7, R_3 H, and Q Q_1) exhibited excellent activity against WPM at 50 mg L^{-1} (100%), at the same level as that of the commercial kresoxim-methyl, and very significantly higher than that of coumoxystrobin (no control against WPM at 400 mg L^{-1}). Another compound, **6h** (R_1 methyl and R_2 *n*-C_6H_{13}), was also discovered, but although this compound exhibited good activity against CDM (85%) at 6.25 mg L^{-1}, it appeared to be slightly less potent than coumoxystrobin, but superior to kresoxim-methyl. Further syntheses and structure optimization studies are in progress.

Experimental

General: All starting materials and reagents were commercially available. Experimental conditions were quite similar to those reported earlier [9].

Synthesis of substituted coumarins (3): To a solution of substituted resorcinol (**1**) (1 mmol) in 10 mL of conc. H_2SO_4, ethyl 3-oxopropanoate (**2**) (1 mmol) was added drop wise at 5°C. After all the solution has been added (about 30 min), the reaction mixture was stirred at room temperature for 4–5 h and then was poured with vigorous stirring into a mixture of ice (50 g) and water (50 mL), and kept in a refrigerator overnight. The solid was filtered and thoroughly washed with cold water till the pH of the filtrate become neutral. The residue was dried over a sintered glass funnel by vacuum to afford substituted coumarins **3**.

Synthesis of compound 6b: 7-Hydroxy-4-methylcoumarin (0.43 g, 2.45 mmol) was dissolved in 5 mL of butanone, and anhydrous potassium carbonate (0.68 g, 4.90 mmol) was added to the solution. The solution was stirred for 30 mins, and methyl (*E*)-methyl 2-[2-(bromomethyl)phenyl]-2-(methoxyimino)acetate (0.70 g, 2.45 mmol) was then added. The reaction mixture was stirred at refluxing temperature for 5 h and then was cooled, diluted with 50 mL water and extracted with ethyl acetate (3 × 100 mL). The combined extracts were washed with brine, dried (anhydrous magnesium sulfate), and filtered. The filtrate was evaporated and the crude product was purified via silica gel CC, using a 1:2 (v/v) mixture of ethyl acetate and light petroleum (boiling point range: 60-90°C) as the eluting solution to obtain compound **6b**: 0.83 g.

Synthesis of compound 7b: Compound **6b** (0.43 g, 2.45 mmol) was dissolved in 10 mL of methanol, and methylamine (0.16 g, 5.14 mmol) was added to the solution. After 3 h, the mixture was concentrated, diluted with 50 mL water and extracted with ethyl acetate (3 × 100 mL). The combined extracts were washed with brine, dried (anhydrous magnesium sulfate), and filtered. The filtrate was evaporated and the crude product was purified via silica gel CC, using a 1:1 (v/v) mixture of ethyl acetate and light petroleum to obtain compound **7b**: 0.90 g.

Synthesis of compound 8a: 7-Hydroxy-3,4-dimethyllcoumarin (0.52 g, 2.73 mmol) was dissolved in 5 mL of DMF, and 60% sodium hydride (0.22 g, 5.46 mmol, washed with light petroleum) was added to the solution. The solution was stirred for 30 mins, and methyl 2-(bromomethyl)phenyl(methoxy)carbamate (0.75 g, 2.74 mmol) was then added and stirred for 3 h. The reaction mixture was added to 50 mL water and extracted with ethyl acetate (3 × 100 mL). The combined extracts were washed with brine, dried (anhydrous magnesium sulfate), and filtered. The filtrate was evaporated and the crude product was purified via silica gel CC, using a 1:2 (v/v) mixture of ethyl acetate and light petroleum to obtain compound **8a**: 0.82 g.

(*E*)-Methyl 2-(methoxyimino)-2-{2-[(4-methyl-coumarin-7-yloxy)methyl]phenyl}acetate (6b)

Yield 85% of a white solid.

MP: 150-152°C.

IR (KBr) ν: 2980, 2960 (s, C-H), 1735, 1715 (s, C=O), 1610 (m, C=C), 1390 (m, C-H), 1290 (s, C-O), 1060, 1010 (s, =C-H), 870, 830, 750 (s, Ph-H) cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.39-7.54 (4H, m, *H*-3', *H*-5', *H*-6' and *H*-5), 7.21-7.24 (1H, m, *H*-4'), 6.85 (1H, dd, *J*=2.7, 9.0 H_Z, *H*-6), 6.80 (1H, d, *J*=2.7 H_Z, *H*-8), 6.13 (1H, s, *H*-3), 5.02 (2H, s, OCH_2), 4.05 (3H, s, NOCH_3), 3.87 (3H, s, COOCH_3), 2.39 (3H, s, CH_3-4).

Anal. Calcd. (%) for $C_{21}H_{19}NO_6$: C, 66.13; H, 5.02; N, 3.67. Found: C, 66.22; H, 4.98; N, 3.64.

(*E*)-2-(Methoxyimino)-*N*-methyl-2-{2-[(4-methyl-coumarin-7-yloxy)methyl]phenyl}acetamide (**7b**)

Yield 90% of a white solid.

MP: 213-214°C.

IR (KBr) ν: 3200 (s, N-H), 2980, 2960 (s, C-H), 1720 (s, C=O), 1660 (s, C=C), 1620 (s, N=C), 1500 (s, Ph), 1420 (s, C-H), 1260, 1140 (s, C-O), 1000 (s, =C-H), 930, 750 (s, Ph-H) cm^{-1};

^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.41-7.50 (4H, m, *H*-5, *H*-3', *H*-5' and *H*-6'), 7.20-7.23 (1H, m, *H*-4'), 6.82-6.84 (2H, m, *H*-6 and *H*-8), 6.79 (1H, bs, N*H*), 6.13 (1H, s, *H*-3), 5.02 (2H, s, OCH_2), 3.97 (3H, s, NOCH_3), 2.92 (3H, d, *J*=5.1 H_Z, NHCH_3), 2.38 (1H, s, CH_3-4);

Anal. Calcd: C, 66.31; H, 5.30; N, 7.36. Found: C, 66.25; H, 5.36; N, 7.39.

Methyl 2-[(3,4-dimethyl-coumarin-7-yloxy)methyl] phenyl(methoxy)carbamate (**8a**)

Yield 76% of a white solid.

MP: 121-122°C.

IR (KBr) ν: 2940 (s, C-H), 1730, 1700 (s, C=O), 1600 (m, C=C), 1490 (w, Ph), 1430 (m, C-H), 1375, 1310, 1250, 1190 (s, C-O), 1170, 1080 (s, =C-H), 980, 805, 760 (s, Ph-H) cm^{-1};

^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.39-7.43 (1H, m, *H*-6'), 7.50 (1H, d, *J*=8.7 H_Z, *H*-5), 7.38-7.40 (3H, m, *H*-3', *H*-4' and *H*-5'), 6.91 (1H, dd, *J*=2.4, 8.7 H_Z, *H*-6), 6.85 (1H, d, *J*=2.4 H_Z, *H*-8), 5.17 (2H, s, OCH_2), 3.83 (3H, s, COOCH_3), 3.77 (3H, s, NOCH_3), 2.36 (3H, s, CH_3-4), 2.18 (3H, s, CH_3-3);

Anal. Calcd: C, 65.03; H, 5.18; N, 3.79. Found: C, 65.23; H, 5.11; N, 3.74.

Supplementary data: ^{1}H NMR, IR, MS and elemental analyses for compounds **6a**, **6c-6j**, **7a** and **8b-8h**.

Acknowledgments - The project was supported by the National Key Technology Support Program during the 12th Five-Year Plan Period (Grant No. 2011BAE06B00 and 2011BAE06B05) and the National Key Basic Research Program (973 Program) (Grant No. 2010CB735601 and 2010CB126105).

References

[1] (a) Singh R, Gupta BB, Malik OP. (**1987**) Studies on pesticides based on coumarin. I. Antifungal activity of 6-alkyl-3-*n*-butyl-7-hydroxy-4-methylcoumarins. *Pesticide Science*, ***20***, 125-130; (b) Daoubi M, Duran-Patron R, Hmamouchi M, 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*. *Pest Management Science*, ***60***, 927-932.

[2] Beriger E. (**1978**) Insecticidally active 3-*N*-(4-trifluoromethylphenyl)-carbamoyl-4-hydroxycoumarin. US4078075.

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

[4] Liu CL, Li ZM. (**2003**) Discovery of strobin fungicides. *Chinese Journal of Pesticides (Nong Yao)*, ***3***, 43-48.

[5] (a) Liu CL, Li M, Guan AY, Zhang H, Li ZM. (**2007**) Design, synthesis and fungicidal activity of novel (*E*)-methyl 2-{2-[(coumarin-7-yloxy)methyl]phenyl}-3-methoxyacrylates. *Natural Product Communications*, ***2***, 845-848; (b) Guan AY, Liu CL, Li M, Zhang H, Li ZN, Li ZM. (**2011**) Design, synthesis and structure-activity relationship of novel coumarin derivatives. *Pest Management Science*, ***67***, 647-655; (c) Guan AY, Liu CL, Li ZN, Zhang MX, Si NG. (**2011**) The discovery of fungicide coumoxystrobin. *Chinese Journal of Pesticides (Nong Yao)*, ***2***, 90-92; (d) Tomlin CDS. (**2009**) Kresoxim-methyl, In *The Pesticide Manual*. ***15***th Ed.,. British Crop Protection Council, Alton, UK. 688-690.

[6] Tomlin CDS. (**2009**) Dimoxystrobin, In *The Pesticide Manual*. ***15***th Ed., British Crop Protection Council, Alton, UK. 383-384.

[7] Tomlin CDS. (**2009**) Orysastrobin, In *The Pesticide Manual*. ***15***th Ed., British Crop Protection Council, Alton, UK. 840-841.

[8] Tomlin CDS, (**2009**) Pyraclostrobin, In *The Pesticide Manual*. ***15***th Ed.,. British Crop Protection Council, Alton, UK. 971-972.

[9] Li M, Liu CL, Yang JC, Li L, Li ZN, Zhang H, Li ZM. (**2009**) Design, synthesis and biological activity of new strobilurin derivatives with a 1*H*-Pyrazol-5-oxy side chain. *Natural Product Communications*, ***4***, 1215-1220.

[10] Liu CL. (**2011**) New approach for agrochemical discovery and application (1)—Intermediate derivatization method. *Chinese Journal of Pesticides (Nong Yao)*, ***1***, 20-22.

[11] Liu CL. (**2009**) New agrochemical candidates from intermediate derivatization method based on bioisosteric replacement. In *Frontiers of Modern Chemical Engineering, Metallurgy, and Material Technologies-7th Academic Conference of Chemical, Metallurgy and Material Engineering Department, CAE*, Wang JK. (Ed). Chemical Industry Press Publishers, Beijing. 86-94.

[12] (a) Liu CL. (**2008**) Innovation of pesticide discovery methods and candidate compounds. *High-technology & Industrialization (Chinese)*, ***9***, 79-81; (b) Chai BS, Liu CL, Li HC, He XM, Luo YM, Huang G, Zhang H, Chang JB. (**2010**) Design, synthesis and acaricidal activity of novel strobilurin derivatives containing pyrimidine moieties. *Pest Management Science*, ***66***, 1028-1214.

[13] Menn JJ. (**1980**) Contemporary frontiers in chemical pesticide research. *Journal of Agricultural and Food Chemistry*, ***28***, 2-8.

[14] (a) Iqbal PF, Bhat AR, Azam A. (**2001**) Antiamoebic coumarins from the root bark of *Adina cordifolia* and their new thiosemicarbazone derivatives. *Journal of Medicinal Chemistry*, ***44***, 664-671; (b) *Khan KM, Saify ZS*, *Begum S, Noor F, Khan MZ*, *Hayat S, Choudhary M*I, *Perveen* S, *Rahman AU, Ullah Z*. (**2003**) Synthesis and biological screening of 7-hydroxy-4-methyl-2*H*-chromen-2-one, 7-hydroxy-4,5-dimethyl-2*H*-chromen-2-one and some derivatives. *Natural Product Research*, ***17***, 115-125.

[15] Garazd YL, Panteleimonova TN, Garazd MM, Khilya VP. (**2002**) Modified coumarins. 7. Synthesis and biological activity of mannich bases of substituted 7,8,9,10-tetrahydrobenzo[*c*]chromen-6-ones. *Chemistry of Natural Compounds*, ***38***, 532-538.

[16] (a) Bender DR, Kanne D, Frazier JD, Rapoport H. (**1983**) Synthesis and derivatization of 8-acetylpsoralens. Acetyl migrations during Claisen rearrangement. *The Journal of Organic Chemistry*, ***48***, 2709-2719; (b) Ross R, Fujimoto TT, Shaber SH. (**1997**) Benzyloxy substituted aromatics and their use as fungicides and insecticides. EP0811614; (c) Jacobson RM, Nguyen LT. (**2000**) Phosphoryl hydrazine insecticides. US6147062; (d) Li M, Liu CL, Li L, Yang H, Li ZN, Zhang H, Li ZM. (**2010**) Design, synthesis and biological activities of new strobilurin derivatives containing substituted pyrazoles. *Pest Management Science*, ***66***, 107-112.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1921 - 1924

Kinase Inhibitory, Haemolytic and Cytotoxic Activity of Three Deep-water Sponges from North Western Australia and their Fatty Acid Composition

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Received: August 10th , 2011; Accepted: October 10th, 2011

The c-AMP dependent protein kinase (PKA) inhibition, haemolytic activity, and cytoxicity of 21 extracts obtained from North Western Australian sponges collected from depths of 84-135 m were investigated. Hexane extracts from *Ircinia*/*Sarcotragus* sp. and *Geodia* sp. displayed PKA inhibitory activities of 100 and 97% respectively (at 100 μg/mL), while aq. methanol extracts from *Haliclona* sp. exhibited potent haemolytic activity (75%) and hexane extracts from *Geodia* sp. were highly toxic (88%) to the brine shrimp *Artemia franciscana*. As the non-polar extracts gave the greatest PKA inhibition, these were further analysed by GC-MS and 29 fatty acids were identified in the highest proportions in *Ircinia/Sarcotragus* sp. > *Haliclona* sp. > *Geodia* sp. In contrast to shallow-water sponges that are dominated by polyunsaturated fatty acids with a high percentage of long chain fatty acids, LCFAs (C_{24}-C_{30}), the deep-sea sponges investigated herein were all found to be rich in saturated fatty acids, in particular C_{14}-C_{20} fatty acids, including odd and branched chain fatty acids, with only low levels (0-10%) of LCFAs. Screening of the PKA inhibitory activity of a series of commercially available fatty acids identified C_{14}-C_{18} fatty acids as possessing significant PKA inhibitory activity that may contribute to the activity observed in the sponges studied.

Keywords: deep-water sponges, fatty acids, enzyme inhibitors, protein kinase A, haemolysis, brine shrimp toxicity, cytotoxicity.

The deep sea is one of the most biodiverse and species-rich habitats on the planet, but also one of the least explored. In recent years, deep-sea organisms have emerged as an important new source of unexplored chemical, genetic and biological diversity [1a]. Yet of the 30,000 marine natural products reported to-date, less than 2% derive from deep-sea organisms [1b]. Moreover, metabolites from deep-sea organisms have exhibited a significantly higher 'hit rate' than their shallow-water counterparts in a wide number of bioassays [1c], including cytotoxicity towards both murine and human cancer cell lines [1b,2]. Of particular interest, are the metabolites from deep-sea sponges, which account for over 60% of the deep-sea natural products reported [1b].

c-AMP Dependent protein kinase (protein kinase A, PKA) is an important enzyme involved in the regulation of an increasing number of physiological processes including immune, cardiovascular and reproductive functions; steroid biosynthesis; adipocyte metabolism; and exocytotic processes [3a]. As such, the inhibition of PKA has become an attractive drug target in a number of areas, in particular in immune function [3b] and for memory disorders such as Alzheimer's and Parkinson's disease and schizophrenia [3c]. Interestingly, despite PKA being one of the most well characterized of all protein kinases [3d], there are as yet no PKA inhibitors reported from any marine sources [4]. In addition to screening for specific enzyme inhibitors, the brine shrimp lethality assay is a simple and inexpensive way to screen natural product extracts for pharmacological activity [5a]. A strong correlation has been found between brine shrimp toxicity and cytotoxicity in human cancer cell lines [5b], and cytotoxicity is the most commonly reported biological activity in marine fauna, in particular marine sponges [5c]. Haemolytic activity is also prevalent in Porifera, and is another general indicator of bioactivity, in particular membrane-directed cytotoxicity, which is desirable in the development of bactericidal molecules and for targeted intracellular drug delivery.

Herein, we describe the biological screening of 21 extracts obtained from three NW Australian deep-sea sponge samples. The sponge extracts described herein were tested for their ability to inhibit PKA; to lyse equine erythrocytes; and for their toxicity towards the brine shrimp *Artemia francescana*. Furthermore, the fatty acid composition of the bioactive, non-polar extracts of each sample was identified by GC-MS. Using remotely operated vehicles, a range of deep-sea organisms including sponges, cnidarians, echinoderms, crustaceans and fish were

collected in the North West Shelf of Australia from depths of 84-135 m, as part of wider study on deep-sea ecosystems [6a,6b]. From this diverse collection, the three largest sponge samples were chosen for investigation and identified as *Haliclona* sp. (Order Haplosclerida, Family Chalinidae), *Geodia* sp. (Order Astrophorida, Family Geodiidae) and an intertwined sample of *Sarcotragus* sp. and *Ircinia* sp. (both belonging to the Order Dictyoceratida, Family Irciniidae). Crude extracts were prepared from each sample with dichloromethane and methanol and further partitioned using a modified Kupchan method to give seven partially purified extracts in water, butanol, 50% aq. methanol, 10% aq. methanol, dichloromethane and two hexane extracts, one from the original crude methanol extract (referred to as hexane 1) and the other from the original dichloromethane extract (referred to as hexane 2). This gave a total of 21 extracts that were tested at a final concentration of 100 μg/mL.

PKA inhibitory activity of 97-100% was observed for the hexane 1 extracts of all sponges investigated except *Haliclona*. Other non-polar extracts, including the hexane 2 from *Geodia* sp. and the dichloromethane extract from *Iricinia/Sarcotragus* sp. displayed significant activity (41-84%, Fig. 1 A-C). This is consistent with the large number of non-polar kinase inhibitors described from marine sponges, which include sesterterpenoid mitogen activated protein kinase MSK1 and MAPKAPK-2 inhibitors from *Ircinia* sp. [7a] and a series of prenylhydroquinones isolated from *Sarcotragus muscarum* and *Ircinia fasciculata* that inhibited multiple protein kinases [7b]. Although marine-derived PKA inhibitors are yet to be reported, PKA and related enzymes have been identified from marine sources (mollusks, microalgae), where they play similar roles to their mammalian counterparts [7c].

Haemolytic activity was found in over half of the extracts, with the greatest activity (75%) observed for the 50% aq. methanol extract of the *Haliclona* sp. Extracts showing the highest PKA inhibitory activity (Figure 1, hexane 1 panels B-C) displayed low haemolysis, while almost all *Haliclona* extracts exhibited significant haemolytic activity (Figure 1, panel D), consistent with reports of such activity for this genus [8]. Of the sponges under investigation, haemolytic activity has previously been reported for both *Haliclona* sp. and *Geodia corticostylifera* [9a]. The hexane 1 extract from *Geodia* sp. exhibited 88% toxicity towards the brine shrimp *A. franciscana*; the first report of such activity for this genus (see Supplementary data). Although not observed here, brine shrimp toxicity has been reported previously for the other two sponge genera studied here, including a family of cerebrosides from *Haliclona* sp. [9b] and three sulphated polyprenylhydroquinones from *Ircinia spinosula* [9c].

A diverse range of bioactive metabolites have been reported from shallow-water species from the four sponge genera under investigation. There is, however, a paucity of reported metabolites from related deep-water species, with only a small handful to-date including: triterpenoids [10a]

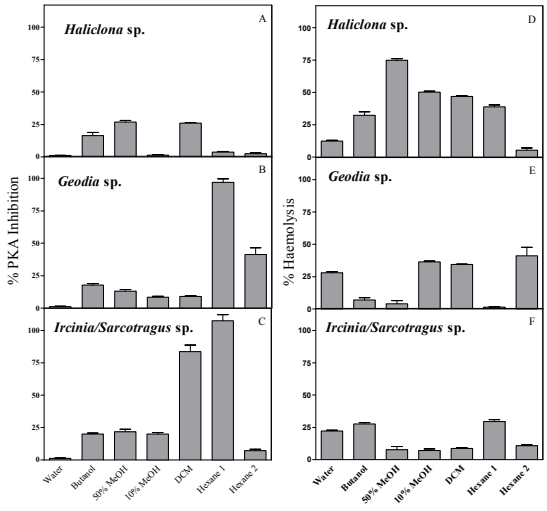

Figure 1: % PKA inhibition and haemolysis activity of deep-sea sponge extracts. Measurements were performed in triplicate at 100 μg/mL and data are presented as means ± S.E.M. DCM, dichloromethane.

and sesterterpene sulfates from the deep-water sponges *Sarcotragus spinulosus* and *Ircinia* sp. [10b]; and the nematocidal polyketide geodin [10c] and antibacterial alkaloid barettin [10d] from two deep-water *Geodia* species. The results described herein are the first report on biological activity in deep-water variants of *Haliclona*.

GC-MS analysis on the lipid component of each sponge was performed (see Table 1), and a total of 23 fatty acids identified for the *Haliclona* sp. as 23-67% of each extract. The major fatty acids were the PUFA C20:4 *n*-5 (21%) and 9-octadecenoic acid (7%) in the hexane 1 extract; hexadecanoic acid (8%) and 12-methyltetradecanoic acid (6%) in the hexane 2 extract; and 3-methylbutanoic acid (14%) and eicosanoic acid (7%) in the dichloromethane extract.

A total of 17 fatty acids were identified for the *Geodia* sp. as 4-24% of each extract: significantly lower than in the other sponges. The C18 fatty acids, 9-octadecenoic acid (5%) and octadecanoic acid (5%) in the dichloromethane extract were the most abundant fatty acids in this sponge, with all other fatty acids present as <5% of each extract. In contrast, the *Ircinia/Sarcotragus* sp. were particularly rich in fatty acids, with a total of 22 identified as 42-83% of each extract. The major fatty acids, accounting for 10% of each extract, were 7-methylhexadecanoic acid/PUFA C20:4 *n*-5, 11-octadecenoic acid, and octadecanoic acid in the hexane 1, hexane 2 and dichloromethane extracts.

A diverse range of fatty acids were identified for the deep-sea sponges including long, branched and odd chain fatty acids common to the phylum Porifera [11a]. The fatty acid profile was dominated by saturated C_{14}-C_{20} fatty acids, but also varied across species either in type or in relative percentages, including extracts exhibiting similar activities. There were no apparent correlations amongst the bioassay results and/or their lipid profiles. For example, although the bioactivity profiles of the *Geodia* and *Ircinia/*

Table 1: Fatty acid composition of the non-polar extracts from the deep water sponges *Haliclona* sp, *Geodia* sp. and *Ircinia/Sarcotragus* sp.

			***Haliclona* sp.**			***Geodia* sp.**			***Ircinia/Sarcotragus* sp.**		
	Fatty acids	**Type**	***A***	***B***	***C***	***A***	***B***	***C***	***A***	***B***	***C***
1	2-Methylbutanoic acid	5:0	-	-	2.38	-	-	-	-	-	-
2	3-Methylbutanoic acid	5:0	-	-	13.52	-	-	-	-	-	-
3	Pentanoic acid	5:0	-	-	5.29	-	-	-	-	-	-
4	Hexanoic acid	6:0	-	-	0.32	1.54	-	1.24	-	-	-
5	Decanoic acid	10:0	2.18	4.75	-	-	0.65	-	2.46	3.66	-
6	10-Methylundecanoic acid	12:0	-	-	-	-	-	-	-	-	0.21
7	Dodecanoic acid	12:0	-	-	-	3.65	-	-	2.58	5.85	-
8	Tridecanoic acid	13:0	-	-	-	-	0.39	-	-	-	-
9	Tetradecanoic acid	14:0	-	1.58	5.32	2.03	0.49	2.03	5.72	6.39	-
10	12-Methyltetradecanoic acid	15:0	6.42	3.77	5.72	2.51	0.63	1.40	5.78	8.27	6.23
11	5,9,13-Trimethyltetradecanoic acid	17:0	-	-	-	-	-	-	-	4.37	-
12	Pentadecanoic acid	15:0	0.5	1.53	1.30	1.57	0.60	-	-	-	6.36
13	Hexadecanoic acid	16:0	7.74	2.21	3.39	-	0.25	-	0.32	3.66	4.39
14	7-Methylhexadecanoic acid	17:0	-	1.31	-	-	-	2.71	-	9.65	-
15	9-Hexadecenoic acid	16:1 *n*-9	0.84	5.89	2.63	-	-	1.77	0.44	-	-
16	Heptadecanoic acid	17:0	-	-	3.33	-	0.35	-	-	7.31	-
17	10-Methylheptadecanoic acid	17:0	-	-	-	-	-	-	-	-	8.51
18	Octadecanoic acid	18:0	-	3.85	6.60	-	-	4.96	-	-	9.75
19	17-Methyloctadecanoic acid	19:0		-	0.24	-	-	-	-	-	3.41
20	9-Octadecenoic acid	18:1 *n*-9	0.41	6.47	2.26	-	0.41	5.21	-	-	7.84
21	11-Octadecenoic acid	18:1 *n*-7	-	-	-	1.90	-	-	9.53	-	7.98
22	9,12,15-Octadecatrienoic acid	18:3 *n*-9	-	-	1.24	-	-	-	-	-	8.16
23	Nonadecanoic acid	19:0	2.98	1.83	-	-	-	-	-	-	-
24	Eicosanoic acid	20:0	1.98	2.18	7.14	-	-	0.52	3.69	6.75	8.22
25	11,14-Eicosadienoic acid	20:2 *n*-6	-	3.47	-	-	-	3.16	-	-	-
26	5,8,11,14-Eicosatetranoic acid	20:4 *n*-6	-	21.15	-	-	-	-	3.23	9.62	-
27	Docosanoic acid	22:0	-	-	1.67	-	-	0.78	2.55	-	3.16
28	Tetracosanoic acid	24:0	0.15	-	3.69	-	-	-	2.54	-	4.65
29	Hexacosanoic acid	26:0	-	1.75	1.19	-	-	-	2.87	-	3.96
		% of total extract	**23.20**	**61.74**	**67.23**	**13.20**	**3.77**	**23.78**	**41.71**	**65.53**	**82.83**

A: dichloromethane-derived hexane extract (hexane 2); **B**: methanol-derived hexane extract (hexane 1); **C**: dichloromethane extract.

Sarcotragus species were similar, their fatty acid profiles were entirely different. For the latter, even extracts displaying similar PKA inhibition activities comprised entirely different fatty acids (*cf.* the *Ircinia* hexane 1 and dichloromethane extracts in Figure 1, panel C and Table 1, columns B and C). Likewise, two of the samples obtained from the identical location and depth (*Geodia* and *Haliclona)* displayed both different bioactivities and fatty acid profiles, indicating that these attributes are largely species dependent. Of the sponge genera investigated, only the fatty acid composition for *Ircinia* sp. has been reported in detail previously, which showed a similar predominance of straight and branched C_{14}-C_{18} fatty acids to that obtained. Our *Ircinia/Sarcotragus* sp. from 84 m depth contained a greater proportion of saturated fatty acids than reported *Ircinia* specimens from ~10 m, and also lacked the C_{24}-$C_{25}\Delta^{5,9}$ demospongic acids characteristic of shallow-water *Ircinia sp.* [11b]. However, whether these differences are depth related will require further study.

To test which of the fatty acids may contribute to the observed bioactivity, a series of commercially available fatty acids (C14:0, C16:0, C18:0, C18:1, C14-22, C18-22) were examined for their ability to inhibit PKA. Several of these exhibited high levels of PKA inhibition at 100 µg/mL in the order C14:0 (64%), C16:0 (34%), C18:1 (33%) and C18:0 (26%) (see Supplementary data). As these are the dominant fatty acids in the sponges investigated, they may contribute to the observed PKA inhibitory activities, along with other non-polar metabolites, the identification of which is currently underway. In conclusion, these results indicate that deep-sea sponges of NW Australia are a rich source of PKA inhibitory, haemolytic and cytotoxic substances.

Experimental

Collection: The deep-sea sponges, were collected in the North West Shelf of Australia in June-August of 2008 and identified as: *Haliclona* sp., *Geodia* sp., and a mixed sample of intertwined *Sarcotragus* sp. and *Ircinia* sp., the former two from the same location (depth 135 m), and the latter from a location 80 km away (depth 84 m). Samples were collected under permit AU-COM2008032 issued by the Australian Government and vouchers (WAMZ45792-WAMZ45795) are at the Western Australian Museum.

Extracts: Sponge extracts were prepared from methanol and dichloromethane, followed by fractionation using a modified Kupchan partitioning method [12] to give seven extracts: water, butanol, 50% aq. methanol, 10% aq. methanol, dichloromethane, hexane (1) and hexane (2), where hexane (1) was derived from the crude methanol extract and hexane (2) from the crude dichloromethane extract (see Supplementary data). All assays were conducted at 100 µg/mL (in 2% DMSO or 2% ethanol).

Protein kinase A inhibition: This was determined using the Kinase-Glo® Luminescent Kinase Assay (Promega Corporation, Madison, USA). Each well of an opaque white 96 well plate (Corning) contained 5 µL of sample, 25 µL of ATP (20 µM), and 20 µL of a mixture of kinase (0.1unit/µL) and Kemptide substrate (140 µM) in reaction buffer (40 mM Tris, 20mM $MgCl_2$, BSA 0.1mg/mL, pH 7.4). After incubation (1 h, rt), 50 µL of the Kinase-Glo® Reagent was added, the mixture incubated for 15 min and luminescence measured using a BMG Labtech FLUOstar Optima® luminometer. Data were determined relative to positive controls containing only solvent, while negative controls contained no substrate and staurosporine (IC_{50} = 7nM against PKA) was used as an internal standard.

Haemolytic activity: Reaction mixtures containing 100 µL of sample and 900 µl of a 0.5% haemocyte suspension (from defibrinated horse blood, Oxoid, Australia) in PBS were incubated for 30 min at 37°C, centrifuged (5 min, 3000 rpm) and 100 µL of supernatant from each sample transferred to a 96 well plate and absorbance measured at λ 550 nm on a Spectramax PLUS. Data were determined relative to positive controls containing distilled water, and negative controls contained PBS in place of the sample.

Cytotoxicity: Brine shrimp eggs (*Artemia franciscana*) were hatched at 26-28°C for 48 h in brine using a light source and constant aeration. Ten animals were transferred to individual wells of a 96 well plate, along with 20 µL of sample and 180 µL of salt solution. Plates were incubated for 48 h under a light source, then surviving animals counted and percent mortality calculated. Control tests accounted for natural death [5a].

GC/MS Analysis: Performed on a Shimadzu QP-5050A GC-MS system equipped with a BP-5 fused silica Rxi-5ms capillary column (5% phenyl/95% dimethyl polysiloxane, 30 m x 0.25 mm, 0.25 µm film thickness, Resrek), using a helium carrier gas (1.0 mL/mn) and samples of 1 µL over the temperature range 80-300°C. Fatty acids were detected as their methyl esters after treatment with methanolic BF_3 and their structural identification was based on comparisons to the NIST 08 mass spectral database.

Supplementary data: Further experimental details are available on-line.

Acknowledgments – We thank D Cummings and A Fowler for sample collections; J Korth and V Valenzuela for technical assistance; and the Australian Research Council (Linkage grant, LP0775183) and Woodside Energy Ltd for financial and logistical support.

References

[1] (a) Gage JD. (**1996**) Why are there so many species in deep-sea sediment? *Journal of Experimental Marine Biology and Ecology* ***200***, 257-286; (b) Skropeta D. (**2008**) Deep-sea natural products. *Natural Product Reports*, ***25***, 1131-1166; (c) Schupp PJ, Kohlert-Schupp C, Whitefield S, Engemann A, Rohde S, Hemscheidt T, Pezzuto JM, Kondratyuk TP, Park EJ, Marler L, Rostama B, Wright AD. (**2009**) Cancer chemopreventive and anticancer evaluation of extracts and fractions from marine macro- and micro-organisms collected from twilight zone waters around Guam. *Natural Product Communications*, ***4***, 1717-1728.

[2] Dumdei EJ, Blunt JW, Munro MHG, Pannell LK. (**1997**) Isolation of calyculins, calyculinamides, and swinholide H from the New Zealand deep-water marine sponge *Lamellomorpha strongylata*. *Journal of Organic Chemistry*, ***62***, 2636-2639.

[3] (a) Tasken K, Aandahl EM. (**2004**) Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiological Reviews*, ***84***, 137-167; (b) Torgersen KM, Vang T, Abrahamsen H, Yaqub S, Tasken K. (**2002**) Molecular mechanisms for protein kinase A-mediated modulation of immune function. *Cellular Signalling*, ***14***, 1-9; (c) Arnsten AFT, Ramos BP, Birnbaum SG, Taylor JR. (**2005**) Protein kinase A as a therapeutic target for memory disorders: rationale and challenges. *Trends in Molecular Medicine*, ***11***, 121-128; (d) Taylor SS, Yang J, Wu J, Haste NM, Radzio-Andzelm E, Anand G. (**2004**) PKA: a portrait of protein kinase dynamics. *Biochimica Et Biophysica Acta-Proteins and Proteomics*, ***1697***, 259-269.

[4] Skropeta D, Pastro NJ, Zivanovic A. (**2011**) Kinase inhibitors from marine sponges. *Marine Drugs*, ***9***, 2131-2154.

[5] (a) Solis PN, Wright CW, Anderson MM, Gupta MP, Phillipson JD. (**1993**) A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). *Planta Medica*, ***59***, 250-252; (b) Carballo J, Hernández-Inda Z, Pérez P, García-Grávalos M. (**2002**) A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotechnology*, ***2***, 17-21; (c) Blunt JW, Copp BR, Munro MHG, Northcote PT, Prinsep MR. (**2006**) Marine natural products. *Natural Product Reports*, ***23***, 26-78.

[6] (a) Cummings DO, Booth DJ, Lee RW, Simpson SJ, Pile AJ. (**2010**) Ontogenetic diet shifts in the reef fish *Pseudanthias rubrizonatus* from isolated deep water populations. *Marine Ecology Progress Series*, *submitted*; (b) Cummings DO, Lee RW, Simpson SJ, Booth DJ, Pile AJ, Holmes SP. Resource partitioning amongst co-occurring decapods on wellheads from Australia's North West Shelf. An analysis of carbon and nitrogen stable isotopes. *Journal of Experimental Marine Biology and Ecology*. *submitted*

[7] (a) Buchanan MS, Edser A, King G, Whitmore J, Quinn RJ. (**2001**) Cheilanthane sesterterpenes, protein kinase inhibitors, from a marine sponge of the genus *Ircinia*. *Journal of Natural Products*, ***64***, 300-303; (b) Watjen W, Putz A, Chovolou Y, et al. (**2009**) Hexa-, hepta- and nonaprenylhydroquinones isolated from marine sponges *Sarcotragus muscarum* and *Ircinia fasciculata* inhibit NF-kappa B signalling in H4IIE cells. *Journal of Pharmacy and Pharmacology*, ***61***, 919-924; (c) Dawson JF, Wang KH, Holmes CFB. (**1996**) Identification and characterization of cAMP-dependent protein kinase and its possible direct interactions with protein phosphatase-1 in marine dinoflagellates. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire*, ***74***, 559-567.

[8] Mebs D, Weiler I, Heinke HF. (**1985**) Bioactive proteins from marine sponges - screening of sponge extracts for hemagglutinating, hemolytic, ichthyotoxic and lethal properties and isolation and characterization of hemaglutinins. *Toxicon*, ***23***, 955-962.

[9] (a) Rangel M, Konno K, Brunaldi K, Procopio J, De Freitas JC. (**2005**) Neurotoxic activity induced by a haemolytic substance in the extract of the marine sponge *Geodia corticostylifera*. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology*, ***141***, 207-215; (b) Mansoor TA, Shinde PB, Luo X, Hong J, Lee CO, Sim CJ, Son BW, Jung JH. (**2007**) Renierosides, Cerebrosides from a marine sponge *Haliclona* (*Reniera*) sp. *Journal of Natural Products*, ***70***, 1481-1486; (c) DeRosa S, Crispino A, Degiulio A, Iodice C, Milone A. (**1995**) Sulfated polyprenylhydroquinones from the sponge *Ircinia spinosula* *Journal of Natural Products-Lloydia*, ***58***, 1450-1454.

[10] (a) Ponomarenko LP, Makar'eva TN, Stonik VA. (**1998**) A new nortriterpenoid from the deep-sea sponge *Sarcotragus spinulosus*. *Russian Chemical Bulletin*, ***47***, 2017-2019; (b) Wright AE, McCarthy PJ, Schulte GK. (**1989**) Sulfircin: a new sesterterpene sulfate from a deep-water sponge of the genus Ircinia. *Journal of Organic Chemistry*, ***54***, 3472-3474; (c) Capon RJ, Skene C, Lacey E, Gill JH, Wadsworth D, Friedel T. (**1999**) Geodin A magnesium salt: A novel nematocide from a southern Australian marine sponge *Geodia*. *Journal of Natural Products*, ***62***, 1256-1259; (d) Lidgren G, Bohlin L. (**1986**) Studies of swedish marine organisms VII. A novel biologically active indole alkaloid from the sponge *Geodia baretti Tetrahedron Letters*, ***27***, 3283-3284.

[11] (a) Bergquist PR, Lawson MP, Lavis A, Cambie RC. (**1984**) Fatty-acid composition and the classification of the Porifera. *Biochemical Systematics and Ecology*, ***12***, 63-84; (b) Carballeira NM, Shalabi F, Cruz C, Rodriguez J, Rodriguez E. (**1991**) Comparative study of the fatty acid composition of sponges of the genus *Ircinia* - identification of the new 23-methyl-5,9-tetracosadienoic acid. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, ***100***, 489-492.

[12] Thale Z, Johnson T, Tenney K, Wenzel PJ, Lobkovsky E, Clardy J, Media J, Pietraszkiewicz H, Valeriote FA, Crews P. (**2002**) Structures and cytotoxic properties of sponge-derived bisannulated acridines. *Journal of Organic Chemistry*, ***67***, 9384-9391.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1925 - 1928

Antimicrobial and Cytotoxic Effects of Mexican Medicinal Plants

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Received: May 28th, 2011; Accepted: October 3rd, 2011

The antimicrobial effects of the Mexican medicinal plants *Guazuma ulmifolia*, *Justicia spicigera*, *Opuntia joconostle*, *O. leucotricha*, *Parkinsonia aculeata*, *Phoradendron longifolium*, *P. serotinum*, *Psittacanthus calyculatus*, *Tecoma stans* and *Teucrium cubense* were tested against several human multi-drug resistant pathogens, including three Gram (+) and five Gram (-) bacterial species and three fungal species using the disk-diffusion assay. The cytotoxicity of plant extracts on human cancer cell lines and human normal non-cancerous cells was also evaluated using the MTT assay. *Phoradendron longifolium*, *Teucrium cubense*, *Opuntia joconostle*, *Tecoma stans* and *Guazuma ulmifolia* showed potent antimicrobial effects against at least one multidrug-resistant microorganism (inhibition zone > 15 mm). Only *Justicia spicigera* and *Phoradendron serotinum* extracts exerted active cytotoxic effects on human breast cancer cells ($IC_{50} \leq 30$ µg/mL). The results showed that *Guazuma ulmifolia* produced potent antimicrobial effects against *Candida albicans* and *Acinetobacter lwoffii*, whereas *Justicia spicigera* and *Phoradendron serotinum* exerted the highest toxic effects on MCF-7 and HeLa, respectively, which are human cancer cell lines. These three plant species may be important sources of antimicrobial and cytotoxic agents.

Keywords: Mexican medicinal plants, antimicrobial, multidrug-resistance, cancer, cytotoxic, peripheral blood mononuclear cells.

Plant extracts have been an important source of anticancer [1] and antimicrobial [2] agents currently used in clinics, and Mexican medicinal plants play an important role in the search for new treatments. *Guazuma ulmifolia* (Sterculiaceae), *Justicia spicigera* (Acanthaceae), *Opuntia joconostle* (Cactaceae), *O. leucotricha* (Cactaceae), *Parkinsonia aculeata* L. (Fabaceae), *Phoradendron longifolium*, *P. serotinum* (Viscaceae), *Psittacanthus calyculatus* (Loranthaceae), *Tecoma stans* (Bignoniaceae) and *Teucrium cubense* (Lamiaceae) are plants traditionally used to treat infections (Table 1). The antimicrobial effects of *Guazuma ulmifolia*, *Justicia spicigera*, *Tecoma stans*

Table 1: Collecting data and ethnomedical information on Mexican plants for antimicrobial and cytotoxic studies.

Plant name (Family)	Native	Collection information	Location collection	Part Used	Ethnomedical uses	References
Guazuma ulmifolia Lam. (Sterculiaceae)	From Mexico to South America	M. Gómez 705, QMEX	Corregidora, Qro., México	Bark	Diarrhea, stomachache, fever, urinary infections,	[4]
Justicia spicigera Schltdl. (Acanthaceae)	From Mexico to South America	Garcia-Perez 46450, SLPM	Ciudad Valles, Slp, México	Leaves	Fever, diarrhea, dysentery,	[7]
Opuntia joconostle F.A.C Weber (Cactaceae)	Mexico	M. Gómez 705, QMEX	San Miguel de Allende, Gto., México	Fruit	Anti-inflammatory, wounds, fever	[8]
Opuntia leucotricha DC. (Cactaceae)	Mexico	M. Gómez 706, QMEX	Cerritos, Slp., México	Fruit	Anti-inflammatory, wounds, fever	[8]
Parkinsonia aculeata L. (Fabaceae)	From south United States to South America and Caribbean	M. Gómez 705, QMEX	Querétaro, Qro., México	Flowers	Urinary tract infections, fever, stomachache	[9]
Phoradendron longifolium Eichler ex. Trel. (Viscaceae)	Mexico	M. Gómez 705, QMEX	Cerro de San Pedro, SLP., México	Leaves	Anti-inflammatory, urinary tract infections	[8]
Phoradendron serotinum (Raf.) M. C. Johnst. (Viscaceae)	From eastern United States to Mexico	E Estrada 20768, CFNL	Santa Catarina, NL, Mexico	Leaves	Body pain, urinary tract infections, dysmenorrheal	[10]
Psittacanthus calyculatus (DC.) G. Don (Loranthaceae)	Mexico	M. Gómez 754, QMEX	Querétaro, Qro., México	Leaves	Wounds, fever	[9]
Tecoma stans (L.) Juss. ex Kunth (Bignoniaceae)	From southeastern United States to South America and Caribbean	M. Gómez 700, QMEX	Querétaro, Qro., México	Leaves	Gastritis, diarrhea, stomachache	[5]
Teucrium cubense Jacq. (Lamiaceae)	From south United Sates to South America	M. Gómez 701, QMEX	Querétaro, Qro., México	Leaves and branches	Skin infections, anti-inflammatory,	[9]

QMEX Herbarium of Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro
CFNL: Herbarium of Facultad de Ciencias Forestales, Universidad Autónoma de Nuevo León
SLPM: Herbarium Isidro Palacios of Instituto de Investigación de Zonas Desérticas, Universidad Autónoma de San Luis Potosí

Table 2: Antimicrobial activites of Mexican plant extracts against human pathogens.

	Microorganism/ Concentration (µg/disk)	***Enterococcus faecalis*** 7140203#	***Staphylococcus aureus*** 3090014	***Staphylococcus haemolyticus*** 309220	***Acinetobacter baumannii*** 130412	***Acinetobacter lwoffii*** 130415	***Burkholderia cepacia*** 160255	***Escherichia coli*** 412352	***Pseudomonas aeruginosa*** 160239	***Candida albicans*** 3100268	***Candida tropicalis*** 120120	***Trichosporon belgeii*** 80145
GU	75	NA	NA	NA	NA	NA	NA	NA	NA	26 ± 2	NA	NA
	375	NA	NA	NA	NA	19 ± 1	NA	NA	NA	26 ± 3	NA	NA
	750	16 ± 2 *	NA	NA	NA	NA	NA	NA	10 ± 3	NA	NA	NA
JS	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	10 ± 1	NA
	375	NA	NA	NA	NA	11 ± 2	NA	NA	12 ± 3	NA	NA	11 ± 2
	750	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
OJ	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	12 ± 3	NA	NA	NA	18 ± 3	NA	NA	NA	NA	NA	NA
	750	11 ± 2	NA	NA	NA	NA	NA	13 ± 2	NA	NA	NA	NA
OL	75	12 ± 2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	12 ± 3	NA	NA	NA	NA	NA	NA	10 ± 2	NA	NA	NA
	750	NA	NA	NA	NA	16 ±1	NA	NA	10 ± 1	NA	NA	NA
PA	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	750	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PL	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	22 ± 2	NA	NA	NA	NA	NA	NA
	750	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PS	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	750	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
PC	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	NA	NA	NA	8 ±2	NA	NA	NA
	750	NA	NA	NA	NA	16 ± 3	16 ± 1	NA	8 ± 2	NA	NA	NA
TS	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	750	NA	NA	NA	NA	NA	14 ± 2	NA	NA	NA	10 ± 2	NA
TC	75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	375	NA	NA	NA	NA	22 ± 3	NA	NA	NA	NA	NA	NA
	750	NA	NA	NA	NA	NA	NA	NA	10 ± 2	12 ± 3	NA	NA
NEO	25	20 ± 3	19 ± 2	20 ± 2	15 ± 2	21± 2	17 ± 3	20 ± 3	15 ± 2	23 ± 3	19 ± 2	20 ± 2

*Diameter of clear zone includes the diameter of the 6 mm disk. # Reference number. Values are shown as the mean ± standard deviations of at least three experiments in triplicate. NA=No inhibitory activity. GU, *Guazuma ulmifolia*; JS, *Justicia spicigera*; OJ, *Opuntia joconostle*; OL, *Opuntia leucotricha*; PA, *Parkinsonia aculeata*, PL, *Phoradendron longifolium*, PS, *Phoradendron serotinum*; PC, *Psittacanthus calyculatus;* TS, *Tecoma stans;* TC, *Teucrium cubense*

and *Parkinsonia aculeata* have been recorded against drug-sensitive bacteria and fungi [3-5].

In traditional medicine the term "cancer" is an ill-defined concept, and plant extracts with active cytotoxic effects are employed for the empirical treatment of dermatological diseases and as anti-inflammatory agents [6]. On this basis, and since the cytotoxicity of *Guazuma ulmifolia*, *Justicia spicigera*, *Opuntia joconostle*, *O. leucotricha*, *Parkinsonia aculeata*, *Phoradendron longifolium*, *P. serotinum*, *Psittacanthus calyculatus*, *Tecoma stans* and *Teucrium cubense* for human cancer cell lines remain unexplored, we also tested the cytotoxic effects of these plant extracts on human cancer cells used empirically for the treatment of dermatological and inflammatory illnesses (Table 1).

This work demonstrates that *Guazuma ulmifolia*, *Justicia spicigera*, *Opuntia joconostle*, *O. leucotricha*, *Parkinsonia aculeata*, *Phoradendron longifolium*, *P. serotinum*, *Psittacanthus calyculatus*, *Tecoma stans* and *Teucrium cubense* exert antimicrobial and cytotoxic effects and may be important sources of cytotoxic and antimicrobial agents.

Table 3: Cytotoxic activity of Mexican medicinal plants on human cancer cell lines with IC_{50} < 200 µg/mL.

	Cancer cell lines_(IC_{50} µg/mL)				
	MCF-7	HeLa	SW-480	DU-145	SKOV-3
Justicia spicigera	48 ± 5.1	17 ± 2.6	49 ± 3.4	> 200	43 ± 4.5
Parkinsonia aculeata	189 ± 25	>200	>200	>200	137 ± 29
Phoradendron serotinum	28 ± 4.1	46 ± 3.2	79 ± 8.7	87 ± 7.7	37 ± 5.2
Cisplatin	7.1 ±1.1	3.21 ±0.9	13.68 ± 1.3	2.56 ± 0.7	10.7±1.3

We examined aqueous extracts of several Mexican native plants normally used to treat infections or illnesses with cancer symptoms to explore their antimicrobial and cytotoxic effects. In the case of *Justicia spicigera*, we used ethanolic extracts because this is the traditional manner of extraction for this plant [7].

Phoradendron longifolium, *Teucrium cubense*, *Opuntia joconostle* (against *A. lwoffii*), *Tecoma stans* (against *A. lwoffii* and *B. cepacia*), and *Guazuma ulmifolia* (against *A. lwoffii*, *E. faecalis* and *C. albicans*) exhibited high antimicrobial potentials, with IZ ≥ 15 mm [11]. *Guazuma ulmifolia* showed the highest antimicrobial effects, and tested at 75 µg/ disk (IZ=26 mm) displayed a higher (P≤0.05) antimicrobial potency than the positive control neomycin at 25 µg/ disk (IZ=23 mm) against *C. albicans* (Table 2).

The most susceptible microorganism was *A. lwoffii*; eight out of ten plant extracts showed antimicrobial effects against this bacterium. In contrast, the most resistant microorganisms were *S. aureus*, *S. haemolyticus*, *A. baumannii* and *T. belgeii,* which were resistant to all plant extracts (Table 2). The results showed that there was a positive correlation between their traditional use against infections and the antimicrobial experimental effects observed. Experiments are currently being carried out in our laboratory to test fractions of *Phoradendron longifolium, Teucrium cubense, Opuntia joconostle, Tecoma stans* and *Guazuma ulmifolia* on multidrug-resistant microorganisms using the broth dilution method.

The National Cancer Institute (NCI) establishes that plant extracts with cytotoxic ED_{50} (Effective Dose 50) values ≤ 30 µg/mL are considered to be active [12]. According to these criteria, only *Phoradendron serotinum* and *Justicia spicigera* exerted active toxic effects against MCF-7 (IC_{50}=28 µg/mL) and HeLa (IC_{50}=17 µg/mL) cancer cells, respectively (Table 3). Table 3 only shows plant extracts that exerted toxic effects (IC_{50}<200 µg/mL); the other extracts lacked cytotoxicity on the tested human cancer cells. Although the IC_{50} values exhibited by *Phoradendron serotinum* and *Justicia spicigera* were higher when compared with CDDP, these plants did not display toxic effects on PBMCs, and in concentrations of 200 µg/mL also increased the proliferation of these cells in a similar manner to PHA 1 µg/mL (Fig. 1). The results are of great interest because it is very desirable to find new cytotoxic agents with similar or higher potency and less toxic effects than currently used agents.

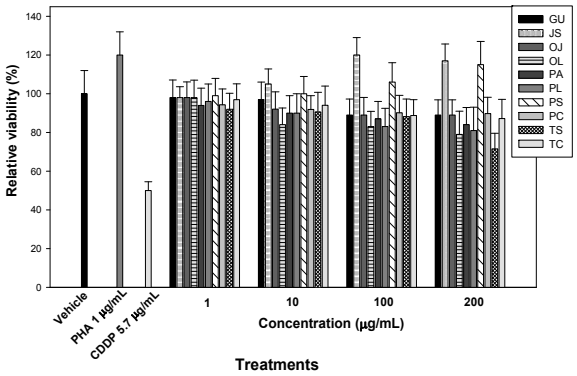

Figure 1: Effect of Mexican medicinal plant extracts on the viability of human peripheral blood mononuclear cells. PBMCs were seeded and after 1 day, cultures were fed with different concentrations of plant extracts, cisplatin (CDDP) (5.7 µg/mL) or phytohemagglutinin (PHA) (1 µg/mL) as control. After 2 days, cell viability was determined by MTT assay. GU, *Guazuma ulmifolia*; JS, *Justicia spicigera*; OJ, *Opuntia joconostle*; OL, *Opuntia leucotricha*; PA, *Parkinsonia aculeata*, PL, *Phoradendron longifolium*, PS, *Phoradendron serotinum*; PC, *Psittacanthus calyculatus;* TS, *Tecoma stans;* TC, *Teucrium cubense*

In summary, these results provide a scientific validation of the ethnomedicinal uses of the plant extracts tested. Thus, it is necessary to perform further phytochemical studies on the isolation and identification of active compounds, mainly *Guazuma ulmifolia*, *Justicia spicigera* and *Phoradendron serotinum*. Also, the mechanisms of action by which these plant extracts exert their antimicrobial and cytotoxic effects should be studied.

Experimental

Microorganisms: Eleven human pathogenic microorganism isolates, obtained and identified as previously described [13], were used for the antimicrobial tests (Table 2). The human pathogens were resistant (diameter of inhibition zones of ≤ 7 mm) to chloramphenicol (30 µg/disk), tetracycline (30 µg/disk), penicillin (10 µg/disk), and erythromycin (15 µg/disk), but sensitive to neomycin (25 µg/disk), as evaluated by the disk diffusion assay (see below).

Plant material and extraction: Plants were collected, taxonomically validated and preserved at the herbaria indicated in Table 3. The aqueous extracts were obtained as described previously [14]. *Justicia spicigera* extracts were obtained as follows: powdered dry leaves (10 g) were subjected to exhaustive Soxhlet extraction with ethanol for 2 h at 180°C. The extract was filtered and concentrated under reduced pressure until dry. All the supernatants were lyophilized (Freezone 4.5, Labconco, Kansas City, MO).

Cell lines and culture conditions: Human cervical carcinoma (HeLa), colorectal adenocarcinoma (SW-480) and breast carcinoma (MCF-7) cells were maintained in DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 7% fetal bovine serum (GIBCO) and antibiotics (100 units/mL penicillin and 100 pg/mL streptomycin), whereas ovarian carcinoma (SKOV-3) and prostate carcinoma (DU-145) cells were maintained in

RPMI with 7% FBS. All human cancer cell lines were obtained from ATCC (Manassas, VA. USA) and grown at 37°C in 5% CO_2.

Antimicrobial assay: Antimicrobial tests were carried out with the disk diffusion method. Filter-paper discs (6 mm diameter) were impregnated with the plant extracts in concentrations ranging from 75 to 750 µg/disk. Negative controls were prepared using impregnated discs with vehicle and virgin discs (blank control). Neomycin (25 µg/disk) was employed as a positive control. Antibacterial activity was defined by measuring the diameter of the growth inhibition zone (IZ) around a disk after 24 h of incubation.

Cytotoxicity assay: Human cancer cells (5 x 10^3 cells/well) and PBMCs (2.5 x 10^4 cells/well), obtained as previously described [13], were seeded in 96 well plates. After 24 h of incubation, plant extracts at concentrations ranging from 1 to 200 µg/mL were added to the cells. Phytohemagglutinin (Sigma) was used as a positive control. The assay was performed as described previously [13] and the optical density (O.D.) was measured at 590 nm in an ELISA reader (Biorad Laboratories, Hercules, CA, USA). The viability of the treated cells was estimated from the relative growth as follows:

$$\text{relative viability} = \frac{\text{control O.D.} - \text{sample O.D.}}{\text{control O.D.}} \times 100$$

The concentration leading to 50% inhibition of viability (IC_{50}) was also calculated by regression analysis (% survival versus log concentration).

Statistical analysis: Experimental values are expressed as the mean ± the standard deviation of at least 3 experiments, in triplicate. Data were analyzed using the one-way ANOVA and Student´s-*t* test when necessary. The level of $p \leq 0.05$ was used to determine statistical significance. All calculations were performed using the JMP 5.1 program (SAS Institute Inc).

Acknowledgments - AJAC (174493) and RZB (233280) were endowed with graduate fellowships from CONACYT. We thank Elizabeth Ortiz-Sanchez, Miriam Guido and Rocio Méndez for their technical assistance.

References

[1] Saklani A, Kutty SK. (**2008**) Plant-derived compounds in clinical trials. *Drug Discovery Today*, ***13***, 161–171.
[2] Cowan MM. (**1999**) Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, ***12***, 564-582.
[3] Heinrich M, Kuhnt M, Wright CW, Rimpler H, Phillipson JD, Schandelmaier A, Warhurst DC. (**1992**) Parasitological and microbiological evaluation of Mixe Indian medicinal plants (Mexico). *Journal of Ethnopharmacology*, ***36***, 81-85.
[4] Navarro V, Villarreal ML, Rojas G, Lozoya X. (**1996**) Antimicrobial evaluation of some plants used in Mexican traditional medicine for the treatment of infectious diseases. *Journal of Ethnopharmacology*, ***53***, 143-147.
[5] Kumar VP, Chauhan NS, Padh H, Rajani M. (**2006**) Search for antibacterial and antifungal agents from selected Indian medicinal plants. *Journal of Ethnopharmacology*, ***107***, 182-188.
[6] Alonso-Castro AJ, Villarreal ML, Salazar-Olivo LA, Gómez-Sánchez M, Domínguez F, García-Carranca A. (**2011**) Mexican medicinal plants used for cancer treatment: pharmacological, phytochemical and ethnobotanical studies. *Journal of Ethnopharmacology*, ***133***, 945-972.
[7] Domínguez XA. Alcorn JB. (**1985**) Screening of medicinal plants used by Huastec Mayans of northeastern Mexico. *Journal of Ethnopharmacology*, ***13***, 139-156.
[8] González-Elizondo M, López-Enriquez IL, González-Elizondo MS, Tena-Flores JA. (**2004**) *Plantas Medicinales del Estado de Durango y Zonas Aledañas*. Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional Unidad Durango. Instituto Politécnico Nacional, México, pp. 210.
[9] López R, Hinojosa A. (**1988**) *Catalogo de plantas medicinales sonorenses*. Universidad de Sonora, Hermosillo Sonora, México, pp. 143.
[10] Adame J, Adame H. (**2000**) *Plantas curativas del Noreste Mexicano: Nuevo León, Coahuila, Tamaulipas y altiplano potosino*. Monterrey, Nuevo León, México. Ediciones Castillo, pp. 386.
[11] Mothana RA, Lindequist U, Lindequist U, Gruenert R, Bednarski PJ. (**2009**) Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *Complementary and Alternative Medicine*, ***9***, 7-17.
[12] Suffness M, Pezzuto JM. (**1990**) Assays related to cancer drug discovery. In *Methods in Plant Biochemistry: Assays for Bioactivity*, Hostettmann, K. (Ed.), ***6***, 71-133.
[13] Jacobo-Salcedo MdR, Gonzalez-Espindola LA, Alonso-Castro AJ, Gonzalez-Martinez MdR, Dominguez F, Garcia-Carranca A. Antimicrobial and cytotoxic effects of *Magnolia dealbata* Zucc and its active compounds. *Natural Product Communications*, ***6***, 1121-1124.
[14] Alonso-Castro AJ, Salazar-Olivo LA. (**2008**) The anti-diabetic properties of *Guazuma ulmifolia* Lam. are mediated by the stimulation of glucose uptake in normal and diabetic adipocytes without inducing adipogenesis. *Journal of Ethnopharmacology*, ***118***, 252-256.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1929 - 1932

Chemometrics Evaluation of the Herbal Drug *Andrographis paniculata*

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Received: January 14th, 2011; Accepted: July 22nd, 2011

A fingerprint was developed to evaluate ten genotypes of *Andrographis paniculata* (a herbal drug) collected from different geographical locations. The genotypes were assessed by HPLC and the chromatogram areas were used to construct fingerprints. Between the two fingerprints, vector of differences was defined. The scalar mean of the deviated vector was used for statistical analysis. By incorporating appropriate chemometric methods better differentiation of samples was achieved. This provides tools for extracting relevant chemical information from the obtained data. The developed method can be used as a quality control tool for rapid authentication, identity, assessment and selection of good quality natural drug from a wide variety of herbal samples.

Keywords: *Andrographis paniculata*, quality control, andrographolide, HPLC, fingerprinting, chemometrics.

In recent years, there has been a great demand for plant derived products. These are increasingly being sought as medicinal products, nutraceuticals and cosmetics [1]. *Andrographis paniculata* Nees (family Acanthaceae), one of the most promising herbal medicines in South East Asia, is claimed to have anti-inflammatory [2a], anti-platelet aggregation [2b], antihyperglycemic [2c], hepatoprotective [2d], antitumor [2e], and anti-human immunodeficiency virus activities [2f,2g]. Andrographolide is a principal medicinal component of *A. paniculata* [2h]. It is well known that the therapeutic effects of this herbal drug are based on the synergistic effect of its constituents and, therefore, it has become essential to develop a reliable, specific and sensitive detection and quantification technique for obtaining good quality raw and processed materials.

Conventional chromatographic study focuses mainly on the determination of the active components, while fingerprinting can offer integral characterization of a complex system with a quantitative degree of reliability. In this respect, fingerprinting has gained more attention than any of the other quality control systems [3a-3d]. Fingerprinting is a method that provides chemical information about medicines from spectrograms, chromatograms and other information obtained by analytical techniques [4a,4b]. There are always chances of wide variations with respect to the chemical contents of crude drugs/raw materials of plant origin due to various reasons such as climatic conditions, geographical distribution, source and season. For this reason, it is necessary to establish a fingerprint for a medicinal herb for its quality control. The advancement of chemometric methods has become a leading tool towards faster analytical results and shorter product development times [5a]. Hence chromatographic fingerprinting with chemometric analysis for controlling the quality of herbal medicines and their products has been accepted by many countries and organizations [5b,5c]. The objective of this work was to establish a simple and reliable technique for quantification of andrographolide and develop fingerprinting for ten genotypes of *A. paniculata* collected from different geographical locations of India.

The quantitative estimation of andrographolide was performed with regard to specificity, precision, accuracy and linearity. This was applicable in fingerprinting and variation analysis within genotype [6a,6b]. The composition of the HPLC mobile phase was optimized to achieve good resolution. The best resolution and peak shape was obtained using acetonitrile, methanol and water (65:20:15 v/v). The HPLC profile of the standard compounds was prepared. Andrographolide had a stable and high content and was completely separated from the other peaks. The presence of andrographolide in *A. paniculata* samples was verified (peak 'd') by comparison of its retention time with that of the standard chromatogram (Figure 1). Specificity was ascertained by comparing the area of the standard peak with that of the sample peak. The precision was evaluated by analysis of the sample in three replications to maintain reproducibility and repeatability. There was no significant difference in the quantification of each sample. The correlation coefficient between the standard and sample graphs was 0.9963 and the R.S.D. value of the peak areas was 1.8%. Also, the sample recoveries were determined by adding reference solutions of variable concentrations. The average value obtained was 99.4% (n = 3), with an R.S.D. less than

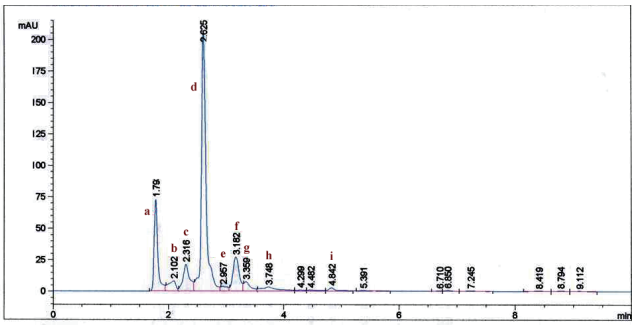

Figure 1: HPLC profile of crude extract of *A. paniculata* and selected peaks for fingerprinting (a-i).

2%. The results indicate that the method developed has acceptable precision, reproducibility and stability.

The fingerprints were constructed by the peak areas of the HPLC chromatograms of ten *A. paniculata* genotypes presented in Table 4. The chromatogram was represented as vector a = [a_1, a_2, . . . , a_n], where a_i (i = 1, 2, . . . , n) is the area of the i^{th} chromatographic peak and n is the number of chosen peaks. The nine selected common peaks are shown in Fig 1 (a – i). The differences between the two fingerprints can be defined as $r = a_1 - a_2$, where a_1 and a_2 represent two fingerprints of the same sample. If measurement errors are only from random noise, each peak will have a distribution of N (μ_i, σ_i^2), where μ_i and σ_i are the original value and the standard variance of the i^{th} peak area, respectively. The values for μ_i and σ_i were estimated by repeated measurements on the same sample. With the peak area distribution transformation into $\mu`_i = \mu_i/\sigma_i$, each peak area is divided by its standard variance. If $a_1 = a_2$, then r = 0, and the two fingerprints are clearly identical. If r is sufficiently small to be statistically compared with a zero vector, then it can be concluded that there is no significant difference between the two fingerprints and they are considered to be the same. On the basis of r, the similarity matrix was constructed. When $\bar{r} = 0$, it means that two fingerprints have no significant difference, if $\bar{r} \neq 0$, it means that there is a difference between them, where $\bar{r} = \frac{1}{n}\sum_{i=1}^{n} r_i$ and r^i is the i^{th} element of r. The relative standard deviation (RSD) values of the nine common peaks of the HPLC chromatograms among five batches of samples are shown in Table 1.

RSD values were found to be less than 0.98% for retention time and less than 4.28% for the peak area, which meant the common peaks were in good correspondence in five batches of every sample. This indicates that the pick of same batch was similar. Both the correlation coefficient and the vector cosine were calculated between five batches of fingerprints from same producing area and the results were about 0.9637 (table-2).

The fingerprints were assessed by analysis of PCA. The two principal components represent almost 92% of the total variance in the data set. The first principal components (PCs) consisted of 71% of the total variability

Table 1: RSD values of nine common peaks in HPLC chromatograms among five batches of samples.

Genotypes	No. of Samples		Peak a	Peak b	Peak c	Peak d	Peak e	Peak f	Peak g	Peak h	Peak i
AP1	5	RT	0.69	2.21	1.84	0.48	4.78	2.06	3.16	3.86	2.88
		Area	31.93	44.92	35.63	35.83	57.57	33.75	34.40	51.71	36.78
AP2	5	RT	0.76	2.20	1.82	0.55	5.89	2.00	3.02	3.61	5.05
		Area	83.15	79.44	90.46	122.35	51.05	134.38	71.87	26.39	53.60
AP3	5	RT	0.93	1.55	2.06	0.59	4.07	1.98	3.06	2.64	6.58
		Area	43.16	36.57	41.01	51.51	63.22	39.98	49.11	32.78	37.34
AP4	5	RT	0.86	1.44	0.49	0.59	4.74	2.09	3.07	2.51	7.27
		Area	36.13	22.67	29.45	47.61	46.82	31.39	36.52	25.13	27.85
AP5	5	RT	1.05	1.60	0.47	0.62	4.32	2.19	3.97	1.50	7.75
		Area	135.60	21.12	213.34	152.06	123.29	35.98	82.19	28.26	54.62
AP6	5	RT	1.02	1.64	0.41	0.43	3.61	1.56	3.85	1.49	7.44
		Area	58.10	17.11	23.09	72.93	56.91	32.88	21.62	27.05	24.71
AP7	5	RT	1.05	1.73	0.40	0.42	1.88	1.54	3.83	1.80	7.87
		Area	88.97	66.07	69.58	92.37	49.66	123.68	77.14	66.99	73.62
AP8	5	RT	0.83	1.64	0.31	0.40	3.12	1.13	3.23	1.72	8.07
		Area	55.84	103.13	103.74	48.13	168.45	75.65	83.35	156.40	108.37
AP9	5	RT	0.83	2.49	1.78	0.43	2.29	1.12	1.73	1.68	7.98
		Area	78.03	89.51	70.94	81.64	102.24	120.56	69.42	104.19	88.10
AP10	5	RT	0.66	2.46	2.26	0.41	3.15	1.33	2.23	3.12	5.34
		Area	25.69	87.97	45.06	27.52	87.33	66.13	70.15	98.36	65.20

Table 2: Result of similarity matrix of same batch of collected genotypes.

Genotypes	No of samples	Correlation coefficient	Cosine value
AP1	5	0.973 ± 0.011	0.932 ± 0.017
AP2	5	0.961 ± 0.021	0.952 ± 0.019
AP3	5	0.977 ± 0.027	0.971 ± 0.018
AP4	5	0.965 ± 0.021	0.937 ± 0.011
AP5	5	0.973 ± 0.023	0.961 ± 0.026
AP6	5	0.971 ± 0.023	0.986 ± 0.029
AP7	5	0.941 ± 0.02	0.976 ± 0.022
AP8	5	0.959 ± 0.021	0.978 ± 0.029
AP9	5	0.980 ± 0.024	0.923 ± 0.027
AP10	5	0.937 ± 0.016	0.969 ± 0.016

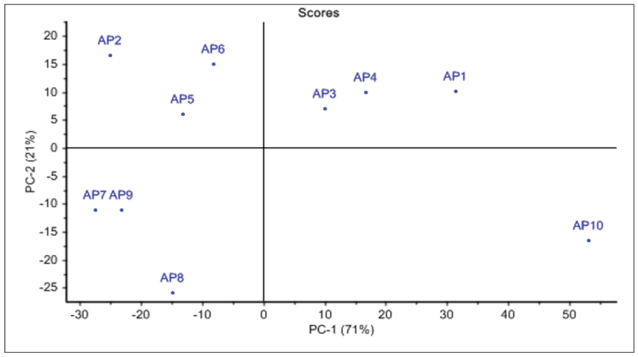

Figure 2: Principal component analysis of HPLC data.

followed by the second PCs with 21% variance. Overall, each sample was able to form a distinct cluster in the two-dimensional plot (Fig. 2). Examining the space defined by the first and second PCA of the HPLC data proves the variability found among the collected genotypes. The *t*-test statistics displayed variation which comes from the differences of $\mu`_i$ of the two corresponding peaks (Table 3). The results indicated that the region of origin will cause differences between the fingerprints of *A. paniculata*, and this can be used to formulate standard criteria for selecting the crude drug, and be a quality control standard for the planting of *A. paniculata*, as well as to provide the link for the botanical identity and quality from the number of chemical constituents present in plant [7].

SIMCA classification was employed for performing a real situation task such as assigning upcoming unknown samples [8]. SIMCA classification based on the predefined PCA model yielded 100% correct classification for all classes at 5% significance level. The SIMCA showed that the samples AP3, AP9 and AP7 maintained a higher

Table 3: Result of similarity analysis and compression of mean fingerprinting.

Fingerprint pairs	Correlation coefficient	Vector cosine			ρ
AP1,AP2	-0.04247	0.0431	1.01	3.039282771	0.00
AP1,AP3	0.983874	0.9874	1.68	5.046522	0.00
AP1,AP4	0.902356	0.9023	0.64	1.913784326	0.00
AP1,AP5	0.805445	0.8624	1.53	4.615757373	0.00
AP1,AP6	0.614506	0.6256	0.93	2.790479535	0.00
AP1,AP7	0.727364	0.7364	2.20	6.603093	0.00
AP1,AP8	0.959682	0.9682	4.78	14.34605	0.00
AP1,AP9	0.825119	0.8272	2.48	7.452129	0.00
AP1,AP10	0.939898	0.9898	0.03	0.098381*	0.00
AP2,AP3	-0.01141	0.1143	0.70	2.1134	0.00
AP2,AP4	0.122771	0.1229	0.90	2.7056	0.00
AP2,AP5	0.192286	0.1953	0.149	0.44731*	0.00
AP2,AP6	-0.2859	0.2910	0.55	1.66755*	0.00
AP2,AP7	0.181364	0.1863	0.51	1.539489*	0.00
AP2,AP8	-0.03151	0.0377	0.317	0.951809829*	0.00
AP2,AP9	0.134425	0.1373	0.395	1.187798096*	0.00
AP2,AP10	-0.19422	0.1952	0.53	1.59152*	0.00
AP3,AP4	0.916221	0.9192	0.66	2.0005	0.00
AP3,AP5	0.822485	0.8434	1.19	3.570007	0.00
AP3,AP6	0.663067	0.6630	0.46	1.38715*	0.00
AP3,AP7	0.724001	0.7286	1.85	5.535267	0.00
AP3,AP8	0.944385	0.9740	2.07	6.235882	0.00
AP3,AP9	0.842407	0.8442	2.24	6.713606	0.00
AP3,AP10	0.912364	0.9233	0.43	1.12933*	0.00
AP4,AP5	0.877401	0.8812	1.47	4.432317	0.00
AP4,AP6	0.622719	0.6257	0.69	2.084494	0.00
AP4,AP7	0.908865	0.9101	3.24	9.741992	0.00
AP4,AP8	0.934218	0.9352	2.94	8.821639	0.00
AP4,AP9	0.874958	0.8749	2.66	7.994877	0.00
AP4,AP10	0.785968	0.7879	0.18	0.5403*	0.00
AP5,AP6	0.747936	0.7499	0.75	2.26173	0.00
AP5,AP7	0.740466	0.7404	1.49	2.985292	0.00
AP5,AP8	0.807512	0.8175	0.61	1.842163	0.00
AP5,AP9	0.690674	0.7907	0.76	2.303481	0.00
AP5,AP10	0.670422	0.6724	0.61	1.83601	0.00
AP6,AP7	0.540904	0.5409	1.20	3.613517	0.00
AP6,AP8	0.65615	0.6581	0.77	2.33181	0.00
AP6,AP9	0.581609	0.5886	1.09	3.285674	0.00
AP6,AP10	0.580291	0.5892	0.42	1.28251	0.00
AP7,AP8	0.865307	0.8699	0.02	0.07126*	0.00
AP7,AP9	0.902316	0.9203	0.26	0.7739*	0.00
AP7,AP10	0.584318	0.6143	0.91	2.75372	0.00
AP8,AP9	0.925151	0.9251	0.13	0.39441*	0.00
AP8,AP10	0.861045	0.8810	1.30	3.9104	0.00
AP9,AP10	0.666304	0.6734	0.90	2.7236	0.00

*Shows non-significant differences

distance than the rest of the samples. The training set ranging from 0.1305 (AP10) to 0.7784 (AP3) represents the sample to model distance. The sample distance ranges from 0.56495 (AP3) to 0.98776 (AP10). Principal component mapping by SIMCA described samples into a much lower dimensional subspace and reasonably defined the class of this herbal medicine.

Hierarchical cluster analysis was performed to generate clusters of *A. paniculata* genotypes. The data for the chromatographic peak areas of the samples were normalized to obtain an $m \times k$ matrix (where m is the number of samples and k is the number of chromatographic peaks). All the samples formed two broad categories (Fig. 3). The genotype AP10 was more diverse than the other genotypes. The established relationship between ten genotypes of *A. paniculata* by chromatographic fingerprinting and the cluster analysis

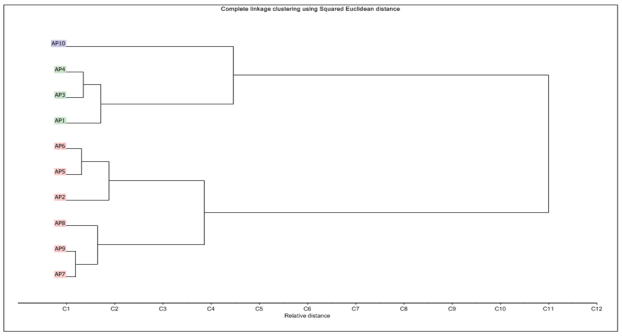

Figure 3: The hierarchical clustering of collected genotypes from different geographic locations.

Table 4: Collections of *A. paniculata* and their geographical status.

Genotype	Region	Latitude	Longitude	Mean Sea Level
AP1	Kondagaon (Chhattisgarh)	19°36` 00.00``N	81°40` 12.00``E	1934 ft.
AP2	Kanker (Chhattisgarh)	20°16` 05.78``N	81°29` 52.31``E	1597 ft.
AP3	Narayanpur (Chhattisgarh)	19°42` 53.81``N	81°14` 44.11``E	1806 ft.
AP4	Bastar (Chhattisgarh)	19°35`03.34``N	81.41` 28.16``E	1948 ft.
AP5	Jagdalpur (Chhattisgarh.)	19°05`00.73`` N	82°01` 39.45``E	1827 ft.
AP6	Raipur (Chhattisgarh)	21°14` 34.09``N	81°38` 19.35``E	981ft.
AP7	Nagpur (Maharashtra)	21°09` 14.00``N	79°04` 59.00``E	1034 ft.
AP8	Neemach (Madhya Pradesh)	24°27` 45.03``N	74°50` 37.17``E	1619 ft.
AP9	Chitrakoot (Madhya Pradesh)	24°34` 47.09``N	80°49` 49.95``E	1061 ft.
AP10	Indore (Madhya Pradesh)	22°43` 31.13``N	75`51` 56.00``E	1810 ft.

gives the preference of selection of good quality material and is applicable for adoption for a breeding programme for crop improvement [9a,9b].

In summary, the enhanced HPLC fingerprinting method can comprehensively and properly reveal the quality characteristics of medicinal herbs, and will become a very useful complementary technique for quality determination. The results give evidence that *A. paniculata* samples from different geographical origin have a varied and complex chemical mixture.

Experimental

Plant materials and crude extraction: The present study was confined to ten genotypes of *A. paniculata*. Dried leaves of samples from 10 different geographical origins were selected based on their performance and evaluated through field experimentation at the Department of Plant Molecular Biology & Biotechnology, Indira Gandhi Agricultural University, Raipur, India [10]. The collection sites and their geographical status are shown in Table 4. Young leaves were collected from plants at flowering time, but avoiding material containing flowers or damaged by insects or fungi. Leaves were then cut into small pieces to permit homogeneous sampling. Drying was carried out at an ambient temperature of 45°C, followed by grinding. Two g of fine power was extracted by reflux with 100 mL of 70% (v/v) ethanol, with 3 cycles. The ethanolic extracts were filtered and concentrated in a rotary evaporator at 60°C. The extracts were stored at 4°C until used.

HPLC condition and standard preparation: HPLC analysis was carried out on an Agilent Series 1100 chromatograph, equipped with a vacuum degasser, a quaternary pump and a diode array detection system, connected to a reversed-phase column (Diamonsil C18, particle size 5 µm, metachem (150 mm X 4.6 mm column, Agilent, USA). Data collection was performed using Chemstation software (Agilent). The temperature was fixed at 25°C. The injection volume was 20 µL and elution was monitored at 223 nm. Andrographolide (Sigma, USA) at different concentrations (22.0 mg, 11.0 mg, and 5.5 mg) was used for method validation and preparation of calibration curves. Crude extract was evaporated under reduced pressure until dryness and used for HPLC quantification.

Fingerprinting of *A. paniculata*: The 5 samples of each *A. paniculata* genotype were collected for HPLC quantification. The areas of 9 common peaks in the chromatograms were used to construct the fingerprints. The standard variance of each peak area was calculated

from the data array and then used to normalize it. The PCA calculation was based on a singular value decomposition of the data array of the fingerprints. The first 2 scores of PCA results were used to make a projection plot that provided a visual determination of the similarity among the fingerprints. Soft Independent Model Class Analogy (SIMCA) was used to classify samples based on their similarities to principal component models. The data of the training set were used to determine principal component models for each class. One of the ways of determining the appropriate number of principal components is to use the cross-validation technique. The classification of test set samples was obtained by adjusting each of these to each model and deciding at the 95% confidence level. Cluster analysis classification was used as a decision criterion for the distance between the samples. The average fingerprinting profile of 5 batches of every sample was applied to construct a cluster by measuring Euclidean distance. The cluster analysis was operated with the assistance of Unscrambler® 8.0 [11]. Samples were classified into classes that correspond to the classes of their nearest neighbours.

References

[1] Bhanu PSS, Zafar R, Panwar R. (**2005**) Herbal drug standardization. *The Indian Pharmacist*, ***4***, 19-22.

[2] (a) Sheeja K, Shihab PK, Kuttan G. (**2006**) Antioxidant and anti-inflammatory activities of the plant *Andrographis paniculata* Nees. *Immunopharmacology and Immunotoxicology*, ***28***, 129-140; (b) Amroyan E, Gabrielian E, Panossian A. (**1996**) Inhibitory effect of andrographolide from *Andrographis paniculata* on PAF-induced platelet aggregation. *Phytomedicine*, ***1***, 27-31; (c) Yu BC, Hung CR, Chen WC. (**2003**) Antihyperglycemic effect of andrographolide in streptozotocin-induced diabetic rats, *Planta Medica*, ***69***, 1075-1079; (d) Shukla B, Visen PK, Patnaik GK, Dhawan BN. (**1992**) Choleretic effect of andrographolide in rats and guinea pigs. *Planta Medica*, ***58***, 146-149; (e) Menon V, Bhat S. (**2010**) Anticancer activity of andrographolide semisynthetic derivatives. *Natural Product Communications*, ***5***, 717-720; (f) Kumar RA, Sridevi K, Kumar NV, Nanduri S, Rajagopal S. (**2004**) Anticancer and immunostimulatory compounds from *Andrographis paniculata*. *Journal of Ethnopharmacology*, ***92***, 291-295; (g) Handa SS, Sharma A. (**1990**) Hepatoprotective activity of andrographolide against galactosamine and paracetamol intoxication in rats. *Indian Journal of Medical Research*, ***98***, 284-292; (h) Samy RP, Thwin MM, Gopalakrishnakone P. (**2007**) Phytochemistry, pharmacology and clinical use of *Andrographis paniculata*. *Natural Product Communications*, ***2***, 607-618.

[3] (a) Nederkassel AM, Vijverman V, Massart DL, Heyden YV. (**2005**) Development of a *Ginkgo biloba* fingerprint chromatogram with UV and evaporative light scattering detection and optimization of the evaporative light scattering detector operating conditions. *Journal of Chromatography A*, ***1085***, 230-239; (b) Srivastava A, Misra H, Verma RK, Gupta MM. (**2004**) Chemical fingerprinting of *Andrographis paniculata* using HPLC, HPTLC and densitometry. *Phytochemical Analysis*, ***15***, 280-285; (c) Koll K, Reich E, Blatter A, Veit M. (**2003**) Validation of standardized high-performance thin-layer chromatographic methods for quality control and stability testing of herbals. *Journal of AOAC International*, ***89***, 909-915; (d) Arpini S, Fuzzati N, Giori A, Martino E, Mombelli G, Pagni L, Ramaschi G. (**2008**) HPLC-DAD-MS fingerprint of *Andrographis paniculata* (Burn. f.) Nees (Acanthaceae). *Natural Product Communications*, ***3***, 1981-1984.

[4] (a) Xu YQ, Sun SQ, Feng XF, Hu SL. (**2003**) Quick identification of scullcaps in different geographical origins using clustering analysis method and infrared fingerprint spectra. *Spectroscopic Analysis*, ***23***, 502-505; (b) Xiao LH, Wang HY, Li FM. (**2004**) Comparative study on HPLC-FPS of *Radix scutellariae* of various sources. *Journal of Shenyang Pharmaceutical University*, ***21***, 21-31.

[5] (a) Seasholtz MB. (**1999**) Making money with chemometrics. *Chemometrics and Intelligent Laboratory Systems,* ***45***, 55-64; (b) Wang S, Ma HQ, Sun YJ, Qiao CD, Shao SJ, Jiang SX. (**2007**) Fingerprint quality control of *Angelica sinensis* (Oliv.) Diels by high-performance liquid chromatography coupled with discriminant analysis. *Talanta*, ***72***, 434–436; (c) Li W, Deng Y, Dai R, Yu Y, Saeed MK, Li L, Meng W, Zhang X. (**2007**) Chromatographic fingerprint analysis of *Cephalotaxus sinensis* from various sources by high-performance liquid chromatography-diodearray detection-electrospray ionization-tandem mass spectrometry, *Journal of Pharmaceutical and Biomedical Analysis*, ***45***, 38-46.

[6] (a) Semmar N, Jay M, Farman M, Roux MA. (**2008**) New approach to plant diversity assessment combining HPLC data, simplex mixture design and discriminate analysis. *Environmental Modeling Assessment*, ***13***, 17-33; (b) Giri L, Andola CH, Purohit VK, Rawat MSM, Rawal RS, Bhatt ID. (**2010**) Chromatographic and spectral fingerprinting standardization of traditional medicines: an overview as modern tools. *Research Journal of Phytochemistry,* ***4***, 234-241.

[7] Gan F, Ye R. (**2006**) New approach on similarity analysis of chromatographic fingerprint of herbal medicine. *Journal of Chromatography A*, ***1104***, 100-105.

[8] Hatanaka RR, Flumignan DL, de Oliveira JE. (**2009**) GC fingerprints coupled to pattern-recognition multivariate SIMCA chemometric analysis for Brazilian gasoline quality studies. *Chromatographia*, ***70***, 1135-1142.

[9] (a) Qiang W, Xiao R, Jun-hua H, Ning-yi X, Qi-chuan Y. (**2006**) Intra-specific genetic relationship analyses of *Elaeagnus angustifolia* based on RP-HPLC biochemical markers. *Journal of Zhejiang University Science B*, **7**, 272-278; (b) Lonni AASG, Scarimino IS, Silva LMC, Ferreria DT. (**2003**) Differentiation of species of the *Baccharis* genus by HPLC and chemometric methods. *Analytical Science,* ***19***, 1013-1018.

[10] Sharma SN, Sinha RK, Sharma DK, Jha Z. (**2009**) Assessment of intra-specific variability at morphological, molecular and biochemical level of *Andrographis paniculata* (Kalmegh). *Current Science*, ***96***, 402-407.

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NPC Natural Product Communications

2011
Vol. 6
No. 12
1933 - 1938

Garcina cambogia Leaf and Seawater for Tannase Production by Marine *Aspergillus awamori* BTMFW032 under Slurry State Fermentation

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Received: September 15th, 2010; Accepted: October 27th, 2011

Garcinia gummi-gutta (syn. *G. cambogia, G. quaesita*), known to have medicinal properties, was evaluated as a substrate and inducer for tannase production by a marine *Aspergillus awamori* BTMFW032, under slurry state fermentation using Czapekdox-minimal medium and sea water as the cultivation medium. Among the various natural tannin substrates evaluated, *Garcinia* leaf supported maximal tannase production. The cultivation conditions and components of the cultivation medium were optimized employing response surface methodology. The experimental results were fitted to a second-order polynomial model at a 92.2% level of significance ($p<0.0001$). The maximal tannase activity was obtained in a slurry state medium containing 26.6%, w/v, *Garcinia* leaf, supplemented with 0.1% tannic acid as inducer. The optimum values of pH, temperature and inoculum concentration obtained were 5.0, 40ºC and 3%, respectively. A Box-Behnken model study of the fermentation conditions was carried out, and the best production of tannase was registered at 40ºC without agitation. Optimization strategy employing response surface methodology led to nearly 3-fold increase in the enzyme production from 26.2 U/mL obtained in unoptimized medium to 75.2 Units/mL in Box Behnken design, within 18 h of fermentation. It was observed that sea water could support maximal tannase production by *A. awamori* compared with other media suggesting that the sea water salts could have played an inducer role in expression of tannase encoding genes. To the best of our knowledge, this is the first report on production of tannase, an industrially important enzyme, utilizing *Garcinia* leaf as substrate under slurry state fermentation by marine *A. awamori* and sea water as the cultivation medium.

Keywords: Tannase, *Aspergillus awamori*, *Garcinia cambogia*, slurry state, response surface methodology, Box Behnken model.

Tannin Acyl Hydrolase (E.C.3.1.1.20), commonly referred to as tannase, catalyzes the hydrolysis of ester and depside bonds present in hydrolysable tannins to form glucose and gallic acid. Tannases are enzymes produced by microbes that are responsible for the *in vivo* hydrolysis of tannins, polyphenols that are widely present in plants. Tannase is known as a versatile enzyme with several interesting properties suitable for industrial applications. It is used for the production of gallic acid, which is used in the pharmaceutical industry for production of the anti-bacterial drug trimethoprim [1], as a substrate for the synthesis of propyl gallate, an anti-oxidant, and in the food industry [2]. Tannase is also used in the manufacture of instant tea and acorn wine, in the clarification of beer and fruit juices; the manufacture of coffee flavored soft drinks; improvement in the flavor of grape wine; as an analytical probe for determining the structure of naturally occurring gallic acid esters [3], and for cleavage of polyphenolics, such as dehydrodimer crosslinks present in the cell wall of plants, which is necessary for plant cell wall digestibility [4]. Tannase is useful for hydrolysis of tannins known to discolor the tooth surface. In animal feed, reduction of the anti-nutritional effects of tannins is minimized by tannase. Tannase can be potentially used for the degradation of tannins present in the effluents of tanneries, which represent serious environmental problems [5].

Tannases are enzymes produced by microbes and are responsible for the *in vivo* hydrolysis of tannins, polyphenols that are widely present in plants. Tannase is produced by a large number of fungi, of which terrestrial *Aspergillus awamori* Nakazawa is one [3,6]. Tannase produced by *A. awamori* was purified and characterized [6]. The fungal strain used in the present study was previously identified as *A. awamori* and as a potential lipase producer [7].

Solid-state fermentation (SSF) is generally preferred for enzyme production owing to the fact that it allows economic production of highly concentrated crude enzymes at low cost of extraction of the pure enzymes [8]. However, it was noted that during SSF production of tannase by *A. awamori* BTMFW032, there was a very high

level of spore formation and consequent difficulty in handling of the fermented moldy bran. So, slurry state fermentation (SLSF) was evaluated as an alternative to submerged fermentation (SmF) and SSF. In fact, enzyme production in SLSF has been employed in limited cases such as pectinase and single cell protein production by *A. niger* and *Trichoderma viride* on pulps from lemon juice clarification [9].

Statistical experimental designs provide an efficient approach to optimization. Response surface methodology (RSM) is a powerful technique for testing multiple process variables, because fewer experimental trials are needed as compared with studying one variable at a time. Also, significant interactions between the variables can be identified and quantified by this technique. The Box Behnken design is especially suitable for accounting for the interactions and identifying the more significant components in a medium. A combination of factors generating a certain optimal response can be identified from the use of a factorial design and response surface methodology. Tannic acid concentration, agitation speed, and pH during the fermentation were identified as important process parameters effecting cell growth and enzyme synthesis by *A.awamori*. These parameters were optimized in a laboratory bioreactor by response surface methodology using Box and Behnken factorial design to determine the optimum conditions for enzyme production and gallic acid accumulation [3].

Commercial success of any fermentation process for industrial enzyme production very much relies on a cheap substrate that is easily obtainable, and which results in a high process yield. Although several natural substrates were tried for different enzyme production, to the best of our knowledge, *Garcinia* sp.(family Clusiaceae) was never tried for any fermentation. *G. cambogia* is a moderate-sized, evergreen tree native to South India and Southeast Asia. The fruit of this species has been traditionally used in food preparation and cooking, having a distinctive taste, but the leaves were not recognized for any utility. *Garcinia* is a source of a revolutionary natural diet ingredient that is currently popular in the US, Japan, Europe, and other western countries. In this study, the prospects of utilizing naturally available *Garcinia* leaves were tried as a source of carbon and inducer of tannase production for SLSF. Previous studies of tannase production by *A. awamori* [3,6], did not explore SLSF, utilization of natural substrate, and sea water as an enzyme production medium. Here we report the role of sea water and natural substrate resources, and the prospects of SLSF in tannase production by the marine fungus *A. awamori* BTMFW032, which holds promise as a tannase producer for further industrial application.

Selection of natural substrates as carbon source: Various natural substrates were tried as a carbon source, both independently and in combination with tannic acid, for tannase production by *A. awamori* BTMFW032. It is evident from the data presented in Table 1 that, irrespective of the substrate used and whether the medium is prepared with sea water or not, tannic acid influences tannase synthesis by the fungus, whereas in the case of a few substrates, tannase production by the fungus was observed even in the absence of tannic acid. In fact, tamarind fruit in combination with tannic acid in sea water medium recorded maximum enzyme activity (374.67 U/mL), followed by grape wastes and tannic acid in Czapekdox medium (369.2 U/mL), and *Garcinia* leaves and tannic acid in sea water medium (256.0 U/mL). Moreover, it was observed that *Garcinia* leaves alone could induce tannase production by the fungus (22.1 U/mL in Czapekdox medium and 26.2 U/mL in sea water) suggesting their potential for use as a substrate for tannase production. All other substrates were not effective and recorded tannase at very low level compared with the control. Furthermore, tamarind fruit and grape wastes are not economical and not easily available, even though they showed enhanced production when used as a carbon source and inducer. Hence, *Garcinia* leaf was selected as a potential substrate for tannase production. It was also noted that *Garcinia* contains 5 mg tannic acid equivalents in 500 mg of leaf (i.e, 1% tannic acid equivalent was present in *Garcinia* leaves, by the Folin-denins method). Probably the presence of tannic acid in *Garcinia* might be the causative factor for the production of tannase enzyme by *A. awamori*. The data also provide evidence for the positive role played by sea water as a medium for enhanced production of tannase by *A. awamori* BTMFW032. Maybe the various ions and inorganic salts present in the sea water could influence the fungus by satisfying its requirements for enhanced synthesis of tannase. Of course, a detailed study is warranted to establish this fact. The data further indicate that, in spite of the fact that natural substrates could induce tannase production by the fungus, the organism requires tannic acid in the medium for enhanced synthesis, confirming the inducer role of tannic acid for tannase production by *A. awamori*.

Selection of the variables that significantly affect tannase production: Plackett-Burman (PB) design offers an effective screening procedure and computes the significance of a large number of factors in one experiment, which is time saving and maintains convincing information on each component [15]. The tannase production with PB design experiments showed a wide variation of enzyme concentrations from 26.2 to 57.4 U/mL, which indicated the importance of medium optimization to attain higher yields.

The statistical significance of the model equation, evaluated by the F-test analysis of variance (ANOVA), revealed that the obtained regression is statistically significant. The model F value of 8.36 and values of Prob>F less than 0.05 provide evidence that the model terms are significant. Furthermore, the results presented as a pareto chart (Figure 1) for the effect of individual

Table 1: Effect of various natural substrates as carbon source on tannase production by *Aspergillus awamori* BTMFW032 (enzyme activity assayed after 48 h of fermentation).

No	Natural substrate	Media	Inducer	Enzyme activity U/mL
1	Control	Seawater 50%,v/v, + Czapekdox 50%,v/v	Tannic acid (1%,w/v)	240.5
2	Mango leaves	Czapekdox Seawater	Tannic acid	13.4
3	Mango leaves	Seawater	Tannic acid	172.6
4	Grape wastes	Seawater	Tannic acid	369.2
5	*Garcinia* leaves	Seawater	Tannic acid	256.0
6	*Garcinia* leaves	Czapekdox	Tannic acid	188.7
7	*Garcinia* leaves	Czapekdox	-	22.1
8	*Garcinia* leaves	Seawater	-	26.2
9	Cashew apple	Seawater	Tannic acid	154.8
10	Tea powder	Seawater	Tannic acid	98.7
11	Tamarind fruit	Czapekdox	Tannic acid	374.7
12	Tamarind pods,	Czapekdox	Tannic acid	0.0
13	*Psdium* leaves,	Czapekdox	Tannic acid	0.0
14	Coconut fiber	Czapekdox	Tannic acid	0.0
15	Coconut pith	Czapekdox	Tannic acid	0.0
16	*Averrhoa bilimbi* fruits	Czapekdox	Tannic acid	0.0
17	*A. bilimbi* leaves	Seawater	Tannic acid	0.0

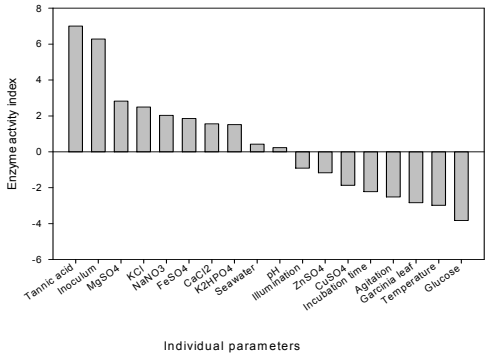

Figure 1: Pareto chart showing the positive and negative effects of individual factors.

parameters studied in PB design testify that tannic acid and inoculum concentration had a positive effect in enhancing enzyme production, along with the increase in their concentrations, whereas glucose, temperature and *Garcinia* leaves had a negative effect, along with an increase in variable. Validation of the PB design was carried out in shake flasks under conditions predicted by the model and it was noted that the experimental values were very close those predicted and hence the model was successfully validated. From the results obtained with PB design it is inferred that among the eighteen variables evaluated, five, namely *Garcinia* leaf, tannic acid, glucose, temperature and inoculum were found to be the most significant.

First order model equation: Tannase activity Y (U/mL) = +44.14250 –0.28315**Garcinia* –76550*glucose +140.0100*tannicacid. 39740*temperature +5.02600*inoculum.

RSM using Box-Behnken design was adopted towards selection of the optimal level of significant variables (*Garcinia* leaf, tannic acid, glucose, inoculum concentration and temperature), based on the PB design experiment. The results obtained were analyzed by ANOVA, with the following regression equation for the level of tannase production.

Tannase activity Y(U/mL) = +1.29328 –0.18255**Garcinia* +1.81366*glucose +541.516*tannic acid +1.97696*temperature +1.24176*inoculum –0.047544**Garcinia*^{2} –0.16906*glucose^2 –3269.474*tannicacid^2 –0.046906*temperature^2 –1.98237*inoculum^2 –0.037727**Garcinia**Glucose –0.88075**Garcinia**tannic acid +0.056280**Garcinia**temperature +0.21372**Garcinia**inoculum –14.45289*glucose*tannicacid +0.022286*glucose*inoculum+1.18215* tannic acid*temperature +51.60360*tannic acid*inoculum.

The ANOVA analysis of tannase production showed that the model F value of 14.84 and values of Prob>F less than 0.05 were significant. Three linear and six quadratic terms were significant model terms for the response.

The model coefficients estimated by multiple linear regressions and ANOVA showed that the model was significant with coefficient of determination R^2 of 0.9223. This ensured a satisfactory adjustment of the quadratic model to the experimental data and indicated that approximately 92.2% of the variability in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for comparing models with different number of variables, was 0.8601. All selected parameters were significant and varied levels of interactions were recorded for the variables in their cumulative effect on tannase production. The coefficient of variance was 6.53, and the adequate precision that measures the signal to noise ratio was 15.203. A ratio greater than 4 is desirable as it indicates an adequate signal. Thus this model could be used to navigate the design space.

Experimental data on the effect of five selected physicochemical factors on production of tannase by *A. awamori* BTMFW032 in a total of 46 experiments showed strong dependence on the presence and levels of the selected factors as the enzyme production varied between 37.4U/mL and 76.8 U/mL under the experimental conditions studied.

Analysis of factors influencing tannase production: Three dimensional response surface curves were plotted to study interaction among various physicochemical factors and to determine the optimum concentration of each individual factor for maximum tannase production. The model predicted 75.8 U/mL of tannase enzyme activity and obtained a maximum of 75.2 U/mL. It was noted that tannic acid, which is used as an inducer, has a very high influence on the enzyme production and its higher concentration supported maximum enzyme production.

Interaction between factors: The pair wise interactions among the factors in terms of tannase production under the optimized condition were assessed by examining the response surfaces. Three dimensional response surfaces were generated holding three factors constant at a time and plotting the response obtained for varying levels of the other two. With increase in tannic acid, enzyme activity increased, but at higher and lower concentrations of *Garcinia*, there was no interactive effect on the production of enzyme (Figure 2).

At higher concentrations of tannic acid, the medium level of inoculum concentration showed more enzyme activity compared with the higher and lower levels, whereas *Garcinia* showed less interaction with inoculum concentration at its higher, middle and lower levels.

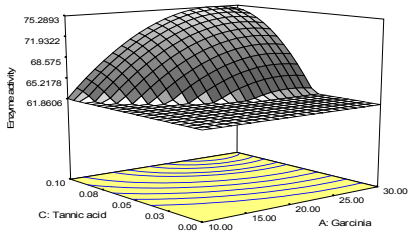

Figure 2: Interaction between tannic acid and *Garcinia* concentrations.

The parabolic nature of the graph indicates that the interactive effect of *Garcinia* and glucose was very high at higher concentrations of tannic acid; the higher and lower levels of *Garcinia* and glucose showed lesser enzyme activity compared with the medium level (Figure 3).

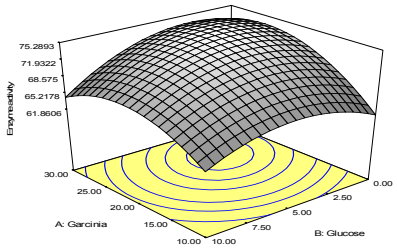

Figure 3: Interaction between glucose and *Garcinia* concentration.

At higher and lower concentrations of glucose and temperature enzyme productivity was less compared with the medium level. A total of 18 variables were checked and, out of these, five parameters were optimized by RSM. These were *Garcinia* leaf (26%), glucose (3.2 mM), tannic acid (0.1%), temperature (40ºC) and inoculum concentration (3%). Time course studies using *A. awamori* under final optimized conditions revealed that tannase production increased rapidly during the initial stages of fermentation and maximum enzyme activity was recorded at 24 h (Figure 4). However, with further progress of fermentation, the enzyme activity declined.

In an earlier study [3], tannic acid concentration, agitation speed and pH during the fermentation were identified as important process parameters effecting cell growth and enzyme synthesis by *A. awamori*, and these parameters were optimized in a laboratory bioreactor by RSM using

Box-Behnken factorial design to determine the optimum conditions for enzyme production and gallic acid accumulation. In that report, under optimum process conditions for enzyme synthesis, the fermentation run lasted 60 h with an initial tannic acid concentration of 35.0 g/L,

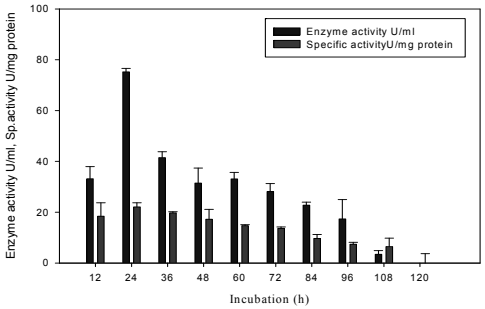

Figure 4: Time course study under optimized conditions for tannase production by *A. awamori* BTMFW 032.

yielding a biomass concentration of 7.13 g/L containing 771 IU of intracellular tannase per g dry cell weight and 19 g/L of gallic acid. However, maximum gallic acid accumulation (40.3 g/L) was obtained in 24 h with an initial substrate concentration of 45 g/L [3]. Jamun leaves and amla leaves were used for tannase production [16] where a maximum enzyme yield of 69 U/g dry substrate was obtained after 96 h of incubation. At the same time, palm kernel cake and tamarind seed powder as substrate, respectively, supported maximal enzyme activity of 13.0 U/g and 6.4 U/g dry substrate [17], whereas wheat bran enriched with 0.8% tannic acid supported higher enzyme activity of 67.5 U/g dry substrate [18].

Conclusion: Synthetic substrate tannic acid, which is expensive and difficult to obtain on a large scale, is an important issue affecting the viability of production of fungal tannases by industrial fermentation. Hence any natural substrate that can support enhanced production of tannase either alone or in combination with tannic acid would have great impact on enzyme production in the industry. In this context the results obtained in the present study with different natural substrates have strongly indicated the prospects of *Garcinia* leaves, which are normally discarded, as a substrate and inducer for tannase production by *A. awamori* BTMFW032. There was clear indication that when *Garcinia* was used in combination with tannic acid, tannase levels were at a higher level than when tannic acid alone was used as substrate. It is speculated that *Garcinia* is a potential material that can be used as a substrate for tannase production owing to its utilizable tannin content. Further, in the present study, it was observed that sea water could support maximal tannase production by *A. awamori* BTMFW032 compared with other media suggesting that the sea water salts could have played an inducer role in expression of tannase encoding genes, which of course needs further investigation. Optimization strategy employing RSM led to a nearly 3-fold increase in the enzyme production from 26.2 U/mL obtained in unoptimized media to 75.2 U/mL in Box Behnken design, within a short period of incubation. To the best of our knowledge, this is the first report on production of tannase, an industrially important enzyme, utilizing *Garcinia* leaf as substrate under slurry state fermentation by marine *A. awamori* BTMFW032 and sea water as cultivation medium.

Experimental

Strain and cultivation conditions: *A. awamori* BTMFW032, isolated from seawater from the Arabian Sea as part of an earlier investigation and available as stock culture at the Department of Biotechnology, Cochin University of Science and Technology, was used in the present study for the production of tannase. The strain was maintained on Czapekdox -minimal agar media with 1% tannic acid (w/v) as the sole carbon source. Since tannase is an inducible enzyme, pre-induced inoculum was used throughout the study. Inoculum was prepared as a spore suspension by the addition of 5 mL sterile physiological saline to a 10 day old agar slope culture, and mixed well for 30 min on a shaker, at room temperature (RT, 28±2°C). The suspension, with spores adjusted to a spore concentration of 10^8 spores per mL using sterile physiological saline, was used as inoculum.

Slurry state fermentation (SLSF)

Substrate preparation: The fresh leaves were dried at 60°C for 24 h, coarsely ground, and stored at 4°C until further use [10]. In general, substrates for SLSF were prepared with a coarsely ground matrix of the selected natural substrate. Cultivation of the fungus for tannase production was carried out in 250 mL Erlenmeyer flasks. The coarsely ground matrixes of the substrates and commercially available tea powder were moistened with Czapekdox minimal medium and seawater (10% matrix (w/v) in minimal medium) and inoculated with the prepared spore inoculum at a concentration of 1%, v/v, and incubated for 2 days at RT (28±2ºC) and 100 rpm in a rotary shaker. After incubation, the fermented slurry was centrifuged at 4ºC and 10,000 rpm for 15 min, and the supernatant was collected and assayed for tannase activity.

Selection of natural substrates as carbon source: A univariable strategy of natural carbon source was employed to screen and select the best natural substrate for induction of tannase production by the fungus as a substitute/supplement to synthetic tannic acid which is currently used for the production of the enzyme. The natural substrates tried were basically tannin containing agro residues, which included fruits, different leaves, and those in combination with tannic acid, like commercially available tea powder, freshly dried and ground mango leaves, tamarind fruit, dry tamarind pods, *Garcinia cambogia* leaves, *Psidium* leaves, coconut fiber and pith, grape waste, cashew apple, and *Averrhoa bilimbi* fruits and leaves. Their concentrations were variably fixed and induced at 10% concentration (w/v). Minimal medium and seawater with tannic acid as sole carbon source were maintained as controls.

Tannin estimation: Tannin content in the natural substrates was estimated following the Folin-Denis method [11]. Tannin-like compounds reduce phosphotungsto-molybdic acid in alkaline solution to produce a highly colored blue solution, the intensity of which is proportional to the amount of tannins. An aliquot of 0.5 g of powdered material was diluted with 75 mL distilled water, boiled for 30 min and centrifuged at 2000 rpm for 20 min. The supernatant was collected and made up to 100 mL. From this solution, 1 mL was diluted with 75 mL distilled water and to it was added 5 mL of Folin-Denis reagent, followed by 10 mL of sodium carbonate and made up to 100 mL. The intensity was measured in a spectrophotometer at 700 nm after 30 min. The tannin content of the sample was calculated as tannic acid equivalents from a tannic acid standard graph.

Tannase assay: Tannase activity was estimated based on the formation of the chromogen between gallic acid (released by the action of tannase on methyl gallate) and Rhodanine (2-thio-4–ketothiazolidine) [12].The enzyme reaction mixture was prepared by the addition of 0.05M citrate buffer, 0.01M methyl gallate and crude enzyme, and incubated at 30ºC for 5 min. Later, the reaction mixture was mixed with 0.667% methanolic rhodanine and incubated further under the same conditions. Then, 0.5M potassium hydroxide was added and incubated further. Finally the enzyme reaction mixture was diluted 5 times with distilled water and the pink color that developed was read at 520 nm using a spectrophotometer (UVShimadzu-160A). A set of blanks and controls were also maintained. One unit of tannase activity (U) is defined as the amount of enzyme required to liberate 1 μM of gallic acid per minute under defined conditions.

Selection of factors that significantly affect tannase production: Factors that significantly influence tannase production were selected statistically employing the PB method [13] in the first phase followed by RSM using Box-Behnken design. The variables and their experimental levels studied using the PB method for selection of the variables that had the largest influence on the experimental response is presented in Table 2. The variables selected by the PB method were later applied to Box-Behnken design in order to evaluate the individualized influence of each variable, as well as the effect of their interactions on enzyme production [14].

Table 2: Variables and their experimental levels adopted in Plackett –Burman method for selection of variables that influence tannase production by *Aspergillus awamori* BTMFW032.

S.No	Variable	Upper level (+)	Lower level (-)
1	*Garcinia* leaves	30%, w/v	10%
2	Seawater	75%	25%
3	Sodium nitrate	50 mM	10 mM
4	Potassium chloride	10 mM	5 mM
5	Magnesium sulfate	10 mM	5 mM
6	Zinc sulfate	0.05 mM	0
7	Copper sulfate	0.025 mM	0
8	Ferrous sulfate	0.5 mM	0.1 mM
9	Dipotassiumhydrogen phosphate	15 mM	5 mM
10	Glucose	10 mM	0
11	Tannic acid	0.1%, w/v	0
12	pH	5	2
13	Temperature	40ºC	25ºC
14	Inoculum	3%, v/v	0.5%, v/v
15	Agitation	150 rpm	0
16	Illumination	Dark	Light
17	Incubation hours	48 h	12 h
18	Calcium chloride	5 mM	0

Optimization of production by RSM: Five independent variables were used to obtain the combination of values that optimizes the response within the region of 3-dimensional (3D) observation spaces, which allows one to design a minimal number of experiments. The experiments were designed using the software, Design Expert Version 6.0 (State Ease, Minneapolis, MN, USA). The components (independent variables) selected for the optimization were: concentration of the substrate (*Garcinia* leaves), tannic acid, glucose, inoculum concentration and temperature. Box-Behnken design is a second order design for the estimation of quadratic effect and a 2 level factorial design. Each factor was studied at 3 different levels, the levels coded in units with values -1, 0, and 1 representing lower, middle, and higher values, respectively. The quadratic model chosen to represent the relationship fitted for the 5 variables was:–

$$Y=\beta o+\Sigma\,\beta iXi+\Sigma\,\beta iiXi^2+\Sigma\,\Sigma\,\beta ijXiXj$$

Y-variable enzyme yield, X1, X2, X3, X4, X5 are the independent variables selected. Analysis of variance (ANOVA) was performed and 3 dimensional response surface curves were plotted by Design Expert software to study the interaction among the various physico-chemical factors. The coded and actual values of independent variables are given in Table 3.

Table 3: Variables and their experimental levels adopted in Box-Behnken method for tannase production by *Aspergillus awamori* BTMFW032.

S.No	Variables	Low level (-1)	Medium level (0)	High level (+1)
1	*Garcinia* leaves	10%, w/v	20%, w/v	30%, w/v
2	Tannic acid	0	0.05%, w/v	0.1%, w/v
3	Glucose	0	5 mM	10 mM
4	Temperature	25 ºC	32.5ºC	40ºC
5	Inoculum	0.5%, v/v	1.75%, v/v	3%,v/v

All experiments were carried out in triplicate. Replicates at the centre of the domain in 3 blocks permit the checking of the absence of bias between several sets of experiments. The effect of variables and their interactions and all the coefficients were calculated by the software package Expert Version 6.0.

Experimental validation of the optimized conditions: The validation of the response surface was carried out through solutions obtained and a time course study conducted with the optimized parameters in shake flasks in triplicate.

Acknowledgment - The authors wish to thank Department of Science and Technology, India for their financial and technical support under SERC-DST-WOS-A Project given to Beena P.S.

References

[1] Bajpai B, Patil S. (**1996**) Tannin acyl hydrolase activity of *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma*. *World Journal of Microbiology and Biotechnology*, ***12***, 217-220.
[2] Lekha PK, Lonsane BK. (**1997**) Production and application of tannin acyl hydrolase: state of the art. *Advances in Applied Microbiology*, ***44***, 215-260.
[3] Seth M, Chand S. (**2000**) Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*, optimization of process parameters. *Process Biochemistry*, ***36***, 39–44.
[4] Garcia-Conesa MT, Kauppinen S, Williamson G. (**2001**) Hydrolysis of diethyl diferulates by a tannase from *Aspergillus oryzae*: Breaking cross-links between plant cell wall polymers, *Carbohydrate Polymer*, ***44***, 19-24.
[5] Van de Lagemaat J, Pyle DL. (**2001**) Solid-state fermentation and bioremediation: development of a continuous process for the production of fungal tannase. *Chemical Engineering Journal*, ***84***, 115-123.
[6] Kalyani M, Ranjan KN, Subhendu SB, Rintu B, Ashok P, George S. (**2005**) Purification, characterization and some studies on secondary structure of tannase from *Aspergillus awamori* Nakazawa. *Process Biochemistry*, ***40***, 3251-3254.
[7] Basheer SM. (**2008**) Lipase production by marine fungus *Aspergillus awamori* Nagazawa Ph.D. thesis, Cochin University of Science and Technology, India.236.
[8] Tao S, Peng L, Beihui L, Deming L, Zuohu L. (**1997**) Solid state fermentation of rice chaff for fibrinolytic enzyme production by *Fusarium oxysporum*. *Biotechnology Letters*, ***19***, 465-467.
[9] De Gregorio A, Mandalari G, Arena N, Nucita F, Tripodo MM, Lo Curto RB. (**2002**) SCP and crude pectinase production by slurry-state fermentation of lemon pulps. *Bioresource Technology*, ***83,*** 89-94.
[10] Zhong W, Cen P. (**2005**) Pectinase production by *Aspergillus niger* P-6021 on citrus Changshan-huyou peel in slurry-state fermentation. *Chinese Journal of Chemical Engineering*, ***13***, 510-515 .
[11] Schanderl SH. (**1970**) In *Methods in food analysis*, Academic Press, NewYork,709.
[12] Sharma S, Bhat TK, Dawra RK **(2000)** A spectrophotometric method for assay of tannase using rhodanine. *Analytical Biochemistry*, ***279***, 85-89.
[13] Plackett RL, Burman, JP. (**1946**) The design of optimum multifactorial experiments. *Biometrika*, ***33***, 305-325.
[14] Box GEP, Hunter WG, Hunter JS. (**1978**) *Statistics for Experimenters*. John Wiley & Sons, New York, USA, 653.
[15] Sharma DC, Satyanarayana T. (**2006**) A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus* Dcsr1 in submerged fermentation by using statistical methods, *Bioresource Technology*, ***97***, 727–733.
[16] Rakesh K, Jitender S, Randhir S. (**2007**) Production of tannase from *Aspergillus ruber* under solid-state fermentation using jamun (*Syzygium cumini*) leaves. *Microbiological Research*, ***162***, 384-390.
[17] Sabu A, Pandey A, Daud MJ, Szakacs G. (**2005**) Tamarind seed powder and palm kernel cake: two novel agro residues for production of tannase under solid state fermentation by *Aspergillus niger* ATCC16620. *Bioresource Technology*, ***96***, 1223-1228.
[18] Gustavo ASP, Selma GFL, Selma CT, Sonia C (**2001**) Selection of tannase-producing *Aspergillus niger* strains. *Brazilian Journal of Microbiology*, ***32***, 24-26.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1939 - 1943

Gas Chromatographic Quantitative Analysis of Methanol in Wine: Operative Conditions, Optimization and Calibration Model Choice

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Received: July 26th, 2011; Accepted: September 28th, 2011

The influence of the wine distillation process on methanol content has been determined by quantitative analysis using gas chromatographic flame ionization (GC-FID) detection. A comparative study between direct injection of diluted wine and injection of distilled wine was performed. The distillation process does not affect methanol quantification in wines in proportions higher than 10%. While quantification performed on distilled samples gives more reliable results, a screening method for wine injection after a 1:5 water dilution could be employed. The proposed technique was found to be a compromise between the time consuming distillation process and direct wine injection. In the studied calibration range, the stability of the volatile compounds in the reference solution is concentration-dependent. The stability is higher in the less concentrated reference solution. To shorten the operation time, a stronger temperature ramp and carrier flow rate was employed. With these conditions, helium consumption and column thermal stress were increased. However, detection limits, calibration limits, and analytical method performances are not affected substantially by changing from normal to forced GC conditions.

Statistical data evaluation were made using both ordinary (OLS) and bivariate least squares (BLS) calibration models. Further confirmation was obtained that limit of detection (LOD) values, calculated according to the 3σ approach, are lower than the respective Hubaux-Vos (H-V) calculation method. H-V LOD depends upon background noise, calibration parameters and the number of reference standard solutions employed in producing the calibration curve. These remarks are confirmed by both calibration models used.

Keywords: wine, methanol, distillation, gas chromatography, ordinary least squares, bivariate least squares detection limits.

Due to its cheapness and easy accessibility, methanol has been used for adulteration of alcoholic beverages and wines. In recent times, this fraudulent behavior has lead to either the death or blindness of many people in some developing countries almost every year. In 1986, Italy numbered tens of victims caused by accidental methanol intake. Methanol intake causes headache, vertigo, fatigue, nausea, vomiting, partial or irreversible blindness, and even death. The human minimum lethal dose of methanol ranges from 300 to 1000 mg/kg body weight [1]. A number of methods for the determination of methanol have been proposed. Upadhyay and Gupta developed a spectrophotometric determination of methanol [2]. The AOAC Official Methods of Analysis include a chromotropic colorimetric method [3], a titrimetric method [4], and a gas chromatographic method [5]. Enzymatic methods with spectrophotometric and chemical sensor detection were proposed by Mizgunova *et al.* [6], and Sun *et al*. [7], respectively. A method using a biosensor with chemiluminescence detection was reported by Sekine *et al*. [8]. Van der Berg *et al.* have employed near-infrared spectroscopy in the control of alcohol process quality assurance [9]. Determination of methanol in biological samples with electrochemical detection in high performance liquid chromatography was performed by Tagliaro *et al.* [10]. Mei-Ling *et al.* proposed several gas chromatographic methods for methanol [11], and simultaneous determination of ethanol and methanol [12]. Although, for methanol analysis, several methods based on different principles have been proposed in the past, a study of the effects of chromatographic conditions and sample pretreatments on its quantification has not been carried out. Therefore, a comprehensive statistical study of detection limits, quantification limits, and calibration curve model has been performed.

In analytical chemistry the detection limit is defined as the lowest concentration that provides an instrumental signal distinguishable from background noise. The Environmental Protection Agency (EPA) procedures identify this limit as three times the standard deviation of the blank. The overlap regions between the signals of the blank and the analyte are distance from zero dependent (Figure 1). The standard deviations (σ) are statistically

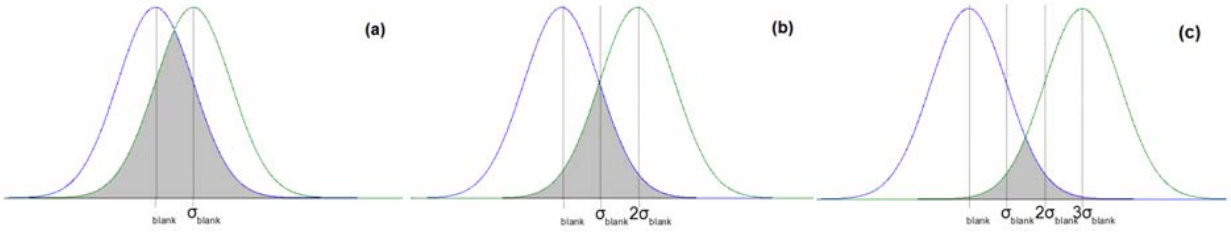

Figure 1: Overlap regions between blank signal and a response at 1σ, z = 0.3085 (**a**); 2σ z = 0.1587 (**b**); and 3σ z = 0.0688 (**c**).

compatible with concentration levels. When the instrumental response is σ times higher than the blank, the overlap between blank and analyte distributions is 30.9% (Figure 1 a). The overlap decreases to 15.9% if the blank has a 2σ distance from a blank (Figure 1 b). The percentages of overlap reduce to 6.9 and 2.3 for 3σ (Figure 1 c) and 4σ (data not shown) blank-analyte distances, respectively. The overlap region decreases in an asymptotic way with the distances from zero (Figure 2). These considerations are made under Gaussian distribution conditions.

With the limit of detection (LOD), while the 3σ approach takes into account only false positives α, Hubaux-Vos (H-V) also considers false negatives β. H-V LOD can be carried out by either graphical methods or a numerical iterative process [13]. The choice of a linear calibration model, when experimental data have to be fitted, depends on the uncertainties associated with the axes. The ordinary least squares (OLS) method is probably the most widely used regression technique. Often OLS has a limited scope, since it considers the x-axis to be free of error. An alternative to this model is the bivariate least squares (BLS) linear calibration, which takes into consideration errors in both axes [14]. Taking into consideration x-errors and y-errors, the need of a suitable linear calibration model, like BLS, becomes relevant.

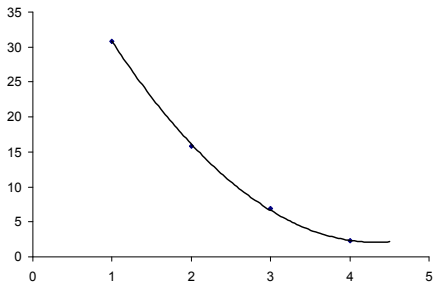

Figure 2: Asymptotic trend of % overlap (ordinate) vs distance from blank signal in σ units (abscissa).

The present work focuses on the OLS and BLS models to GC-FID determination of methanol in wine. In order to ensure the LOD estimation, both OLS and BLS models were used. By application of the two calibration models, the detection limits were calculated according to the EPA 3σ and H-V method. The aim of this work was to test the influence of sample pretreatment and chromatographic conditions on quantification of methanol in wine, referring to OLS and BLS calibration models.

An appropriate calibration curve for methanol determination in wine was set up in the range 5 – 100 mg/L, by the use of six standard reference levels. The ordinary least squares calibration equation y_{OLS} = 0.7768·x+0.0099 has a good regression coefficient R^2 (0.9994), whereas bivariate least squares provides an equation y_{BLS} = 0.7854·x+0.0063. Both equations provide limits of detection, with the 3σ approach, of 1.18 mg/L and 1.17 mg/L for OLS and BLS calibration models, respectively. On the other hand, 10.75 mg/L for OLS and 10.87 mg/L for BLS are the detection limits obtained through the H-V iterative calculation, with α = β = 0.025, n=6. Table 1 shows that methanol content of Feb 2011 (33.3 mg/L) and May 2011 (33.7 mg/L) is very close to quantification limits calculated via H-V (SEE Table 2).

The investigated wine samples were injected into the GC-FID system after simple dilution with water. With the direct injection of wine samples, the dilution operation could appear to be time consuming, but it allows improvement of column stability and shelf life enhancement of the standards. With reference to OENO 19/2004 OIV methanol content limits [15], if the wine is injected directly, the internal standard concentration has to be five times higher than the 1:5 diluted sample. Moreover, the calibration range has to be five times wider as well. Standard aqueous solutions containing methanol and acetonitrile (I.S.) in the 0 – 500 mg/L range are rather stable at room temperature. If the concentration exceeds 1000 mg/L the solution stability with time is not ensured. Results from a study performed on two different concentration ranges is shown in Figure 3, where the relative response factor (RRF) is plotted against the methanol concentration. RRF is calculated by the following formula:

$$RRF = \frac{A_{MeOH}}{A_{ACN}} / \frac{C_{MeOH}}{C_{ACN}}$$

where A_{MeOH} is the peak area of methanol, A_{ACN} is the peak area of the acetonitrile internal standard, and C_{MeOH} and C_{ACN} are the concentrations, in mg/L, of methanol and acetonitrile respectively.

The first evidence was that the change in the RRF was much higher in concentrated than dilute solutions. In the 5 mg/L reference solution the RRF after 5 months was 14% higher than the initial value. When a higher concentration is considered, i.e. 100 mg/L standard solution, after 5 months there is an increasing on RRF value from 0.75 to 1.01, this meaning an enhancement of about 35%. A variation in RRF values between 0.66 and 0.81 was shown by the 10000 mg/L reference solution after 5 days; increasing to 0.99 after 5 months. A more pronounced difference was encountered from 0.66 to 1 (up to 52%), matching 5 months time range, when a 15000 mg/L reference solution was taken into account. Probably, these variations were caused by evaporation of volatile compounds present in the reference standard solution and are, therefore, temperature dependent.

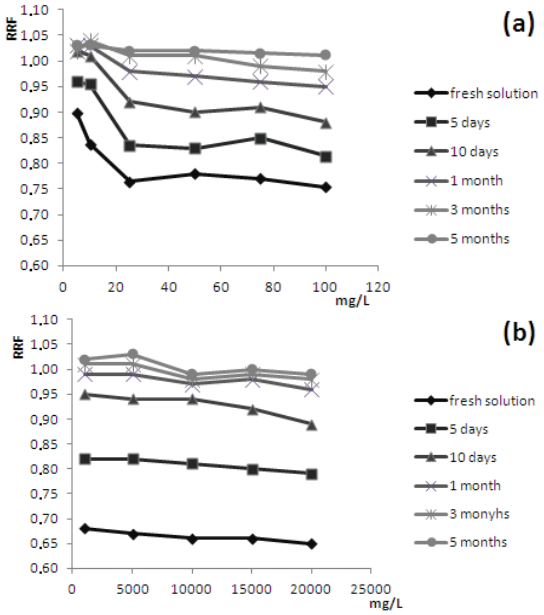

Figure 3: Relative response factors versus methanol concentration level. methanol concentration ranges (**a**) 0 – 100 mg/L; (**b**) 0 – 20000 mg/L.

In order to evaluate the influence of non volatile compounds in GC quantification of methanol, a comparative study between distilled wine and diluted wine by direct injection (DWDI) was carried out. Analyses were performed on samples provided from UIV (Unione Italiana Vini). Preliminary tests suggested slightly higher values for methanol in distilled samples. Determined methanol contents and UIV proficiency test data are reported in Figure 4. Except for the March 2011 test, the methanol content, after distillation, seemed to be slightly higher than that of the directly injected diluted samples. From a practical point of view, an exhaustive focus on these differences is worthy of investigation.

The influence of the distillation process sets the methanol recovery data slightly higher than those determined through DWDI. This apparent surplus could not be explained on the basis of background noise as the baselines

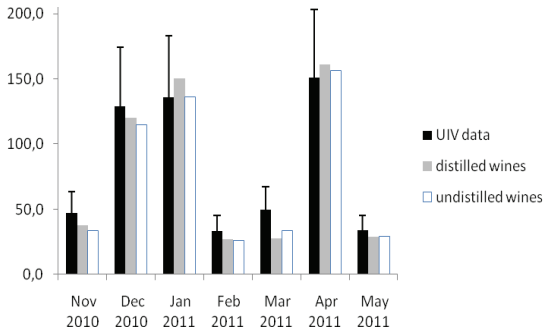

Figure 4: Methanol levels in mg/L of seven different UIV proficiency tests.

for both the distilled and undistilled wine were virtually the same. In addition, reproducibility calculated according to ISO 5725:5, provided by the UIV interlaboratory proficiency test, represents a 35-50% average range of the mean data (Table 1). Compared with DWDI, data results of methanol determination through distillation do not affect detected concentrations beyond 10%. This difference is compatible with the UIV reproducibility values.

Table 1: Methanol content and reproducibility provided by UIV interlaboratory proficiency test.

UIV proficiency test month	UIV proficiency test data[a]	Reproducibility[b]
Nov 2010	47.2	21.7
Dec 2010	129.1	49.8
Jan 2011	135.8	46.8
Feb 2011	33.3	15.3
Mar 2011	49.8	25.8
Apr 2011	150.7	52.8
May 2011	33.7	13.6

[a] methanol content in mg/L coming from UIV interlaboratory proficiency test.
[b] calculated according to ISO 5725:5, $R = 2.8 \cdot S_R$ (mg/L).

According the OENO 19/2004 OIV resolution, the maximum methanol contents in wine are 400 and 250 mg/L for red and white/rosè wines, respectively. The DWDI method provides a methanol concentration on the average of 3% less than that determined for distilled wine samples, but not more than 10%. The DWDI method is faster, and less reagents and energy consuming. So it can be used as a screening check method. When doubtful cases result and quality data assurance has to made, sample distillation can be a valid option to confirm the analytical response.

In the acetal-acetonitrile elution region, chromatograms appear very similar to each other. In the first 10 minutes, there are no differences between distilled and undistilled wine sample for normal conditions (Figure 5 a and b). However, when the temperature program and carrier flow rate are increased, the undistilled samples (Figure 5 c) show many peaks. After 6 minutes there are a number of peaks belonging to the glycol fraction of the wine. The glycol-like compounds are characterized by a high boiling point. Therefore, following a distillation process, they are removed from the samples, as shown in Figure 5 d.

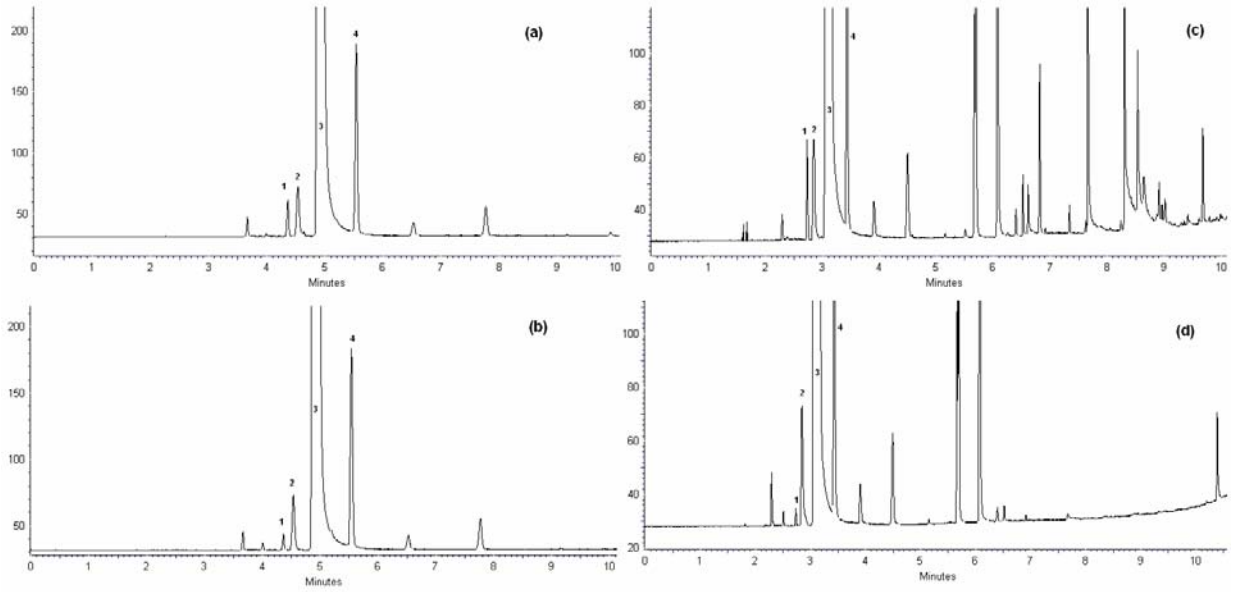

Figure 5: UIV Proficiency Test of April 2011, red dry wine gas chromatograms; sample dilution 1:5. 1) 1,1-diethoxyethane, 2) methanol, 3) ethanol, 4) acetonitrile. (**a**) normal conditions; (**b**) normal conditions, distilled sample; (**c**) forced conditions; (**d**) forced conditions, distilled sample.

Resolution between methanol and the identified acetal (1,1-diethoxyethane) peak was not less than 0.9. The asymmetry factor, calculated at 10% of the maximum height for methanol, was 1.76. The calibration curve parameters by keeping six concentration levels with normal and forced temperature/flow programs are in agreement. These latter show equations $y_{OLS} = 0.7664 \cdot x + 0.0102$ (R^2 0.9999) and $y_{BLS} = 0.7628 \cdot x + 0.0124$. Method suitability quantification is supported by peaks resolution, band width and peak shape. Recovery performances were assessed by the interlaboratory proficiency test. The method was tested with two spiked commercial red wines and with the UIV proficiency test of the April 2011 sample. In the spiked samples, the mean recoveries (n=3) were 101.3% and 103.2%. For the UIV proficiency test of April 2011, the mean recovery was 102.9%.

Table 2: Detection and quantification limits expressed in mg/L.

Detection limits calculation approach	calibration model	Normal chromatographic condition[a]		Forced chromatographic condition[b]	
		LOD	LOQ[c]	LOD	LOQ[c]
H-V	OLS	10.8 (4.5)[d]	35.8	9.1	30.2
	BLS	10.9 (4.5)[d]	36.2	9.0	30.1
3σ	OLS	1.2 (1.1)[d]	3.9	1.2	4.0
	BLS	1.2 (1.1)[d]	3.9	1.2	4.0

[a] He 2 mL/min; oven 50°C × 6 min; ramp 10°C/min, isotherm 210°C × 15 min.
[b] He 4 mL/min; oven 50°C × 3 min; ramp 20°C/min, isotherm 210°C × 7.5 min.
[c] Limit of quantification is expressed as (10/3)×LOD.
[d] In parenthesis, limits of detection obtained with fifteen data points, reported in [16].

In Table 2 quantification and detection limits for both normal and forced chromatogram condition are reported. When forced GC-FID conditions were employed, the analytical method performance remained almost identical to the normal GC-FID conditions. In both cases, temperature/flow and total running time did not affect the determination of calibration parameters, LOD and LOQ.

Limits of detection calculated according to the Hubaux-Vos recursive formula are higher than those using the 3σ approach, in both calibration models BLS and OLS. This is more pronounced when limits of quantification are considered. Comparing the calibration curve made up of fifteen data points [16], while the 3σ LOD is substantially unvaried, if six levels are employed, the H-V detection limits are rather different. The calibration curve built up on the 5 – 100 mg/L concentration range shows a LOD approximately 2.5 times higher than that of the 0.3 – 510.6 mg/L range [16].

Experimental

Compounds and analysis: Acetonitrile (99.8%) (Sigma-Aldrich) was employed as internal standard (IS). Methanol (99%) (Sigma-Aldrich) was used for standard solution preparation. Ethanol (96% v/v) (VWR™) was added to standard solution at 3% vol. Stock standard solution was stored at -4°C. Methanol standard solution concentration range 0 – 500 mg/L. IS concentration 76 mg/L, in both standard solution and samples. Wine samples were supplied from UIV (Unione Italiana Vini, Verona, Italy). Analysis was performed in a Trace GC Ultra instrument, equipped with a flame ionization detector (ThermoFisher Scientific, Milan, Italy). Column: retention gap 1 m × 0.32 mm I.D. (deactivated polyethylene glycol, Varian BV, PL Middelburg, Netherlands) connected to 50 m WCOT fused silica CP-WAX 57 CB capillary column (0.32 mm I.D., film thickness 0.2 m, stabilized polyethylene glycol, Varian BV, PL Middelburg, Netherlands). GC conditions: carrier He 2 mL/min, FID temperature 250°C (H_2 35 mL/min, air 350 mL/min, makeup gas N_2 30 mL/min), injector temperature 240°C. Oven temperature 50°C (6 min isotherm), ramp program 10°C/min rate to 210°C (15 min isotherm). Injection volume 1 μL. Split ratio 1:20. Column contamination by non volatile compounds was

avoided by using a Split Precision® Liner 5 mm × 8.0 × 105 filled with deactivate wool (Restek). H-V detection limit calculation was carried out as described by Hubaux and Vos [13]. BLS calibration was accomplished through calculation proposed by Lisý *et al.* [17] and Riu *et al*. [14].

References

[1] International Programme on Chemical Safety. (**1997**) Methanol. *Enviromental Health Criteria, World Health Organization*, Geneva ***196***.

[2] Upadhyay MS, Gupta VK. (**1984**) Reagent system for the spectrophotometric determination of methanol in environmental and biological samples. *Analyst*, ***109***, 1427–1429.

[3] AOAC O□cial Method 958.04 (**1998**) Methanol in distilled liquors – chromotropic acid colorimetric method. *AOAC Official Method of Analysis*, Code No. 26.1.34.

[4] AOAC Official Method 920.132 (**1998**) Methanol in vanilla extract – titrimetric method. *AOAC Official Method of Analysis*, Code No. 36.2.17.

[5] AOAC Official Method 972.11 (**1998**) Methanol in distilled liquors – gas chromatographic method. *AOAC Official Method of Analysis*, Code No. 26.1.36.

[6] Mizgunova UM, Zolotova GA, Dolmanova IF. (**1996**) Enzymatic method for the determination of ethanol and methanol with spectrophotometric detection of the rate of the process. *Analyst*, ***121***, 431–433.

[7] Sun LX, Okada T. (**2000**) Simultaneous determination of the concentration of methanol and relative humidity based on a single Nafion(Ag)-coated quartz crystal microbalance. *Analytica Chimica Acta*, ***421***, 83–92.

[8] Sekine Y, Suzuki M, Takeuchi T, Tamiya E, Karube I. (**1993**) Selective flow-injection determination of methanol in the presence of ethanol based on a multi-enzyme system with chemiluminescence detection. *Analytica Chimica Acta*, ***280***, 179–184.

[9] Van den Berg FWJ, van Osenbruggen WA, Smilde AK. (**1997**) Process analytical chemistry in the distillation industry using near-infrared spectroscopy. *Process Control Quality*, ***9***, 51–57.

[10] Tagliaro F, Dorizzi R, Ghielmi S, Marigo M. (**1991**) Direct injection high performance liquid chromatographic method with electrochemical detection for the determination of ethanol and methanol in plasma using an alcohol oxidase reactor by gas chromatography with direct on-column injection. *Journal of Chromatography*, ***566***, 333–339.

[11] Mei-Ling W, Jih-Terng W, Youk-Meng C. (**2004**) A rapid and accurate method for determination of methanol in alcoholic beverage by direct injection capillary gas chromatography. *Journal of Food Composition Analysis*, ***17***, 187-196.

[12] Mei-Ling W, Jih-Terng W, Youk-Meng C. (**2004**) Simultaneous quantification of methanol and ethanol in alcoholic beverage using a rapid gas chromatographic method coupling with dual internal standards. *Food Chemistry*, ***86***, 609-615.

[13] Hubaux A, Vos G. (**1970**) Decision and detection limits for linear calibration curves. *Analytical Chemistry*, ***42***, 849-855.

[14] Riu J, Rius FX. (**1996**) Assessing the accuracy of analytical methods using linear regression with errors in both axes. *Analytical Chemistry*, ***68***, 1851-1857.

[15] OIV Resolution OENO 19/2004. (**2004**) Maximum content limits of methanol in wines, http://news.reseau-concept.net/images/oiv_uk/Client/Resolution_OENO_EN_2004_19.pdf

[16] Caruso R, Scordino M, Traulo P, Gagliano G. (**2011**) GC-FID determination of volatile compounds in wine: comparison between EPA 3σ approach and Hubaux-Vos calculation of detection limits, using ordinary and bivariate least-squares. *Journal of AOAC International*, *in press.*

[17] Lisý JM, Cholvadová A, Kutej J. (**1990**) Multiple straight-line least-squares analysis with uncertainties in all variables. *Computers & Chemistry*, ***14***, 189-192.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1945 - 1948

Composition and Biological Potential of Essential Oil from *Thelechitonia trilobata* Growing in South Africa

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Received: May 20th, 2011; Accepted: October 4th, 2011

Thelechitonia trilobata is regarded as a troublesome weed that grows to form a dense blanket over the soil preventing the growth of other crops in farmland. Although the plant is regarded as a notorious, invasive plant, its chemical composition and biological potential have not been reported. The essential oil was isolated from the fresh leaves of *T. trilobata* using hydrodistillation. α-Pinene (21.6%), α-phellendrene (21.0%), limonene (12.8%) and germacrene D (7.5%) were the major constituents of the oil. The essential oil was screened against agricultural pests. The anti-tick properties were tested on *Ripicephalus e. ervertsi* found on sheep, while repellency, fumigation, and contact toxicity tests were carried out with maize weevils. Except for the contact toxicity test, all other bioassays gave positive results.

Keywords: *Thelechitonia trilobata*, Asteraceae, essential oil, α-pinene, α-phellendrene, limonene, germacrene D, fumigant, contact toxicity, repellency.

Weeds are classified as plants that are considered by the user to be a nuisance, which interfere with the management objectives for a given area of land at a given point in time. The word weed can therefore be said to be unwanted plants in human-made settings such as garden, farmland or uncultivated land space [1,2].

Thelechitonia trilobata (L.) H. Rob. & Cuatrec. {synonyms *Complaya trilobata* (L.) Strother; *Silphium trilobatum* L.; *Wedelia trilobata* (L.) Hitchc} is a member of the Asteraceae family and considered to be a very noxious weed. It is native not only to Southern Africa but Central America as well. This plant resembles a daisy, which is why its common name is the Singapore Daisy [3,4]. *T. trilobata* is a creeping, mat-forming, perennial plant. The leaves are about 4-9 cm long and 2-5 cm wide. Flowers are yellow in color with 8-13 rays per head [3-10]. It is considered one of the World's one-hundred most alien invasive species, and causes huge problems to the surrounding vegetative life, as well as to farmers. It can, therefore, be said to be a threat to the South African economy and environment. However, this plant is being overlooked as just a noxious weed without investigating its biological potential. Scientific research was therefore imperative to find end usage for this invading weedy plant, and this stimulated our interest in investigating its essential oil composition and biological potential. To the best of our knowledge, there is no previous report on the chemical composition and biological activities of the essential oils of *T. trilobata*. This paper is, therefore, reporting for the first time the fresh leaf volatile oil composition, its anti-tick property on sheep, and fumigative toxicity, contact toxicity and repellency properties against maize weevils.

Hydrodistillation of the leaves of *T. trilobata* gave a colorless oil with an aromatic pungent smell (yield 0.12%, w/w). Thirty-seven compounds were identified in the oil mixture accounting for 91.9% of the total oil composition. Analysis of the oil revealed a dominance of monoterpenes (65.8%), of which α-pinene, α-phellandrene, *p*-cymene, and limonene were the major components (Table 1). No oxygenated monoterpene was detected in the oil mixture. The percentage composition of sesquitepenoids in the oil

Table 1: Chemical composition of *T. trilobata* essential oil.

Serial No	RI	Compound	Percent composition
1	941	α-Pinene	21.6
2	953	Camphene	0.5
3	975	Sabinene	0.5
4	977	β-Pinene	2.3
5	991	Myrcene	1.0
6	1005	α-Phellandrene	21.0
7	1025	*p*-Cymene	4.5
8	1029	Limonene	12.8
9	1039	(*Z*)-β-Ocimene	0.9
10	1049	(*E*)-β-Ocimene	0.7
11	1392	β-Elemene	0.3
12	1419	(*E*)-Caryophyllene	3.5
13	1453	α-Humulene	2.8
14	1481	Germacrene D	7.5
15	1497	Bicyclogermacrene	3.4
16	1511	(*E*,*E*)-α-Farnesene	0.2
17	1516	γ-Cadinene	0.1
18	1525	δ-Cadinene	0.2
19	1566	(*E*)-Nerolidol	0.2
20	1576	Germacrene D-4-ol	0.1
21	1578	Spathulenol	1.4
22	1581	Caryophyllene oxide	0.3
23	1609	Humulene epoxide II	0.1
24	1641	τ-Muurolol	0.1
25	1689	Shyobunol	0.2
26	2082	*n*-Octadecanol	0.1
27	2100	Heneicosane	0.1
28	2200	Docosane	0.1
29	2300	Tricosane	0.2
30	2400	Tetracosane	0.4
31	2500	Pentacosane	0.6
32	2600	Hexacosane	0.8
33	2700	Heptacosane	0.8
34	2800	Octacosane	0.9
35	2900	Nonacosane	0.8
36	3000	Triacontane	0.5
37	3100	Untriacontane	0.5
		Total Identified	91.9

was 21.1% of which ß-caryophyllene, α-humulene, germacrene D and bicyclogermacrene were the dominant ones. Fatty acids formed 5.7% of the oil.

The essential oil of *T. trilobata* was effective in killing *Ripicephalus e. ervertsi* (ticks) found at the anus, udder and tail parts of sheep within one hour at concentrations of both 5 mg/mL and 10 mg/mL (Table 2). The ticks at these parts were nymphs, while those found at the lower part of the stomach toward the rear leg were adults. This means that the oil extract is more effective on the nymphs than the grown adult tick. This is noteworthy as Triatix 125, a synthetic insecticide (also used as control), was only active after 48 hours.

Table 2: Mortality of ticks (number) vs. time of exposure at different concentrations of essential oils.

Time (mins)	Concentrations and body parts where ticks found				
	5 mg/mL		10 mg/mL		
30	Stomach	Anus	Udder	Anus	Tail
30	1	7	1	1	5
45	1	7	0	1	5
60	1	0	0	1	0
75	1	0	0	0	0
90	1	0	0	0	0
120	1	0	0	0	0
720	1	0	0	0	0

Table 3: Percentage repellency of maize weevils to the fresh leaf oil.

Exposure time (h)	(0.47 µL/cm^2 =15 µL of the oil)	
	% repellence	No. repelled
1	14.3	12
2	61.8	15
4	62.9	16
20	80.9	19
24	66.6	17
48	42.9	15

The repellency was best at 0.47 µL/cm^2 (15 µL) of the oil (Table 3). Neither lower nor higher concentrations were effective. Furthermore, it was observed that after 20 hours the number of insects in the treated zone increased, thereby reducing the percentage repellency. It can, therefore, be concluded that the oil is most effective at 15 µL/mL within the first 20 hours as a weevil repellant.

The result of the fumigative toxicity test is presented in Table 4. The oil extract was most effective after 4 days at low concentrations and effective on the first day at higher concentrations. This result is very promising as the oil can be used as a fumigant against maize weevils during storage. The contact toxicity was very poor and hence not reported here.

Experimental

Plant material and isolation of essential oil: Fresh leaves of *Thelechitonia trilobata* (L.) H. Rob. & Cuatrec. were collected from around the Agricultural farm of the University of Zululand in the northern part of Kwa-Zulu Natal Province, South Africa and a voucher specimen (JPKN 1) was deposited in the herbarium at the University of Zululand. The leaves (500 g) were subjected to hydrodistillation using a Clevenger-type apparatus [11,12]. The essential oil was collected 4 h after boiling, weighed and kept at 4^{o}C till used for bioassays.

Table 4: Percentage mortality using fumigation vs. exposure time for maize weevils (*Sitophilus zeamais*) at different concentrations of leaf oil.

Conc.	Day 0		Day 2		Day 4		Day 6		Day 8		Day 12	
	%	No of dead insects	%	No of dead insects	%	No of dead insects	%	No of dead insects	%	No of dead insects	%	No of dead insects
4µL/L	0	0	0	0	0	0	8.3	2	41.6	10	79.2	19
8µL/L	0	0	0	0	0	0	41.6	10	50	12	79.2	19
16µL/L	0	0	20.8	5	41.6	10	79.2	19	83.3	20	87.5	21
32µL/L	20.8	5	20.8	5	58.3	14	79.2	19	95.8	23	100	24
40µL/L	20.8	5	79.2	19	100	24	100	24	100	24	100	24

% = Percentage mortality

GCMS analysis of the essential oil: The essential oil of *T. trilobata* was analyzed by GC-MS using an Agilent 6890 GC with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and an Agilent ChemStation data system. The GC column was a HP-5ms fused silica capillary with a (5% phenyl)-polymethylsiloxane stationary phase, film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. Injector temperature was 200°C and detector temperature 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, held for 10 min; increased at 3°C/min to 200°C; and increased at 2°/min to 220°C. A 1%, w/v, solution of the sample in CH_2Cl_2 was prepared and 1 μL was injected using a splitless injection technique.

Identification of the oil components was based on their retention indices determined by reference to a homologous series of *n*-alkanes (C_8-C_{30}), and by comparison of their mass spectral fragmentation patterns with those reported in the literature [13-15] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Parasite identification and bioassay: The anti-tick bioassay was carried out on a pool of sheep at the University of Zululand's Dairy Unit. The ticks were identified as adult and nymph types of *Ripicephalus e. ervertsi* by Holeise Heyne of the Parasitology Department, Veterinary Institute, Agricultural Research Council, South Africa. Sheep were caught, identified, and searched for ticks, ears and genital/anal areas being the most popular areas of attachment. Attached ticks in these particular areas were counted and recorded. Essential oil (5 mg/mL and 10 mg/mL) in a 1 mL mixture of water, Tween-20 (Polysorbate 20) and *n*-hexane for an even consistency of oil were prepared. Two drops of each concentration were applied to the area of tick attachment. Triatix 125, a synthetic insecticide, was used as control. The ticks were observed each day to note if any fell off the sheep. This experiment was conducted over a period of 3 days using 3 sheep with similar *Ripicephalus e. ervertsi* (ticks) attachment. Average results were recorded.

Rearing of test insects: Adults of *Sitophilus zeamais* were obtained from a colony maintained by the Plant Protection Research Institute, Pretoria, South Africa. These were mass reared on whole maize grains in 5 L glass jars in a controlled chamber, at 28 ± 2°C and 56-65% RH in the Department of Chemistry, University of Zululand. Newly emerged, one-week-old insects were used in the bioassay [16]. 21-24 maize weevils were used for the study.

Repellency assay of the essential oil: The repellent effect of *T. trilobata* essential oil against *S. zeamais* was studied using a modified area preference method [17]. The test area consisted of a 9 cm Whatman No.1 filter paper cut into 2 halves. Different oil concentrations were prepared by diluting 2, 4, 8, 10 and 40 μL of the oil in 1 mL *n*-hexane and these corresponded to concentrations of 0.314, 0.472, 0.94 and 1.26 μL of oil/cm^2 of the filter paper, respectively. The other half was treated with 0.5 mL *n*-hexane alone and this served as a control. Both essential oil treated and *n*-hexane treated filter paper halves were air dried for 10 min to evaporate the solvent. With the aid of a clear adhesive tape, both halves were later joined together into full discs and placed in 9 cm glass Petri dishes. Twenty one-week old, unsexed adult insects were released at the centre of the rejoined filter paper disc and the Petri dish was covered. Each treatment was replicated 4 times for both *S. zeamais*. The number of insects present on the control (Nc) and treated (Nt) areas of the filter paper were recorded for 1, 2, 3, 4, 20, 24 and 48 h. 24 insects were used. Percentage repellency (PR) was calculated as follows:

$$PR = ((Nc - Nt)/(Nc + Nt)) \times 100$$

The weevils were exposed to a filter paper with half the filter paper being oil and the other half being acetone (control) and they were observed to see whether the concentrated oil repelled them. Concentration was calculated by dividing the oil quantity (μL) by the surface area of half the filter paper (31.81 cm²) [17].

Fumigant assay of the essential oil: The fumigation chambers consisted of 500 mL glass jars with screw-on lids. For the bioassay, solutions of 0, 4, 8, 16, 32 and 40 μL of the oil were each diluted with 1 mL *n*-hexane to correspond to concentrations of 0 (control), 4, 8, 16, 32 and 40 μL/mL One mL of each concentration was then separately applied to 7 mm discs of Whatman No.1 filter paper, air-dried for 10 min and placed at the bottom of the jars. Twenty one-week old adult insects were placed on muslin cloths 21 × 29 mm each with 40 g whole maize grains. The cloths were tied closed with rubber bands and hung at the centre of the jars, which were then sealed with air-tight lids. There were 4 replicates for each concentration. Fumigation was carried out for 24 h, after which the insects were transferred from the fumigation chambers onto clean maize, and mortality was checked for a further 12 days [17].

Contact toxicity of the essential oil: The contact effect of the essential oil of *T. trilobata* on the adults of *S. zeamais* was investigated [16, 17]. Maize grains were treated with concentrations of 0, 25, 50, 100, 200 and 300 μL of essential oil in 1 mL *n*-hexane. The different concentrations of the oil were mixed with 40 g of maize grain respectively. These were thoroughly stirred to allow for homogeneity of the oil on the treated grains. Treated

samples were air dried for 1 h in order to remove the solvent. The grains were thereafter infested with 21-week old *S. zeamais* adults per jar and each jar was covered with a cotton mesh held in place by cover rims. There were 4 replicates per treatment. Dead insects in the jar were counted daily for 6 days.

Acknowledgments – The project described was supported in part by Award Number T37MD001810 from the National Institute on Minority Health and Health Disparities. The authors are also grateful to NRF South Africa, the Directorate of Research, Walter Sisulu University, Govan Mbeki Research and Development Centre, University of Fort Hare and Research Committee of the University of Zululand for research funding.

References

[1] Janick J. (**1979**) *Horticultural Science* (3rd ed.). San Francisco, W.H. Freeman. p. 308.
[2] Whitson T. (Ed.) (**1992**) *Weeds of the West*. Western Society of Weed Science. p. ix.
[3] Cuatrecasas J, Robinson H. (**1992**) *Thelechitonia cuatrecasas*, an older name for *Complaya strother* (Ecliptinae-Heliantheae-Asteraceae). *Phytologia*, ***72***, 141-143
[4] PIER (Pacific Island Ecosystem at Risk) (**2003**) *Wedelia trilobata*, http://www.hear.org/pier/species/wedelia_tribota.htm [Accessed 13 May 2011]
[5] Santos I. (**1998**) *Wedelia trilobata* on Pohnpei. *Aliens*, *7*, 3.
[6] Thaman RR. (**1999**) *Wedelia trilobata: Daisy invader of the Pacific Island*. IAS Technical Report, 99/2. Institute of Applied Science, University of the South Pacific, Suva, Fiji Island.
[7] Henderson L. (**2001**) *Alien Weeds and Invasive Plants.* Plant Protection Institute Handbook No. 12. Plant Protection Research Institute, Agriculture Research Council, South Africa, p 58.
[8] Pienaar K. (**1984**) *The South African what flower is that? (A-Z of Garden flowers in South Africa)*. 2nd Impression, Struik, Cape Town, p 347.
[9] Sheat WG, Schofield G. (**1995**) *Complete Gardening in South Africa*. Struik, South Africa, p 285.
[10] www.sanparks.org/parks/Kruger/conservation [Accessed 16 May 2011]
[11] Oyedeji AO, Afolayan AJ, Hutching A. (**2009**) Compositional variation of the essential oils of *Artemisia afra* Jacq. from three provinces in South Africa – A case study of its safet y. *Natural Product Communications*, ***4***, 849-852.
[12] *British Pharmacopoeia* (**1980**) ***11***, P. A. 109, HMSO.
[13] Adams RP. (**2007**) *Identification of Essential Oil Components by Gas Chromatography / Mass Spectrometry*, 4th Ed. Allured Publishing, Carol Stream, Illinois.
[14] Joulain D, Koenig WA. (**1998**) *The Atlas of Spectra Data of Sesquiterpene Hydrocarbons.* E.B.-Verlag, Hamburg.
[15] ESO 2000. (**1999**) *The Complete Database of Essential Oils.* B.A.C.I.S., The Netherlands.
[16] Odeyemi OO, Masika P, Afolayan AJ. (**2008**) Insecticidal activities of essential oil from the leaves of *Mentha longifolia* L. subsp. *capensis* against *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae). *African Entomology*, ***16***, 220-225.
[17] Tapondjou AL., Adle C, Bouda H, Fontem DA. (**2002**) Efficacy of powder and essential oil from *Chenopodium ambrosioides* leaves as post-harvest grain protectants against six-stored beetles. *Journal of Stored Products Research*, ***38***, 395-402.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1949 - 1952

Chemical Composition and Antibacterial Activity of Essential oil from *Salvia mukerjeei*

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Received: August 22nd, 2011; Accepted: October 4th, 2011

The composition of steam volatile oil from aerial parts of *Salvia mukerjeei* Bennet & Raizada (Lamiaceae) was analyzed by capillary GC and GC-MS. The oil was rich in sesquiterpene hydrocarbons (67.3%). Among 71 identified constituents representing 91.7% of the oil, β-caryophyllene (28.7%), γ-muurolene (15.5%) and dehydro-aromadendrane (9.5%), were the principal constituents. The oil was tested against ten bacterial strains and was active against *Enterococcus faecalis*, *Erwinia chrysanthemi* and *Agrobacterium tumefaciens*.

Keywords: *Salvia mukerjeei*, Lamiaceae, essential oil composition, β-caryophyllene , γ-muurolene, antibacterial activity.

The genus *Salvia* Linn. contains about 700 species widely distributed in the temperate and warm regions of both the hemispheres. Seven species of *Salvia* have been reported to grow in the central Himalayan region of India [1,2]. Two species have been investigated thoroughly for their steam volatile components [3,4]. The roots of *Salvia mukerjeei* are reported to cure cold and cough, the leaves are applied to wounds and the seeds are used for dysentery and colic [5].

The literature contains several reports on the essential oil composition of *Salvia* species growing worldwide [6] but none for *S. mukerjeei.* The present communication describes a detailed analysis of the oil and its antibacterial activity.

The percentage oil yield from the plant was 0.34% by weight. The GC analysis revealed more than eighty components. Seventy one identified constituents represented 91.7% of the oil, several in trace amounts. The compounds were identified by comparison of their Retention Indices and mass spectra with the literature [7]. The oil was rich in sesquiterpene hydrocarbons (67.3%) including β-caryophyllene (28.7%), γ-muurolene (15.5%), dehydro-aromadendrane (9.5%) and α-guainene (5.4%) in addition to monoterpene hydrocarbons (4.5%) and oxygenated sesquiterpenoids (7.8%) [Table 1].

Since *S. mukerjeei* produced a relatively satisfactory yield of sesquiterpene rich oil, in contrast to the generalization [8] that only the oil poor (<0.2%) Lamiaceae taxa are rich in sesquiterpene hydrocarbons. A comparison between the essential oil composition of *S. mukerjeei* with those of other *Salvia* species indicates it is closely related to those from *S. aethiopis* (β-caryophyllene 23.3%, α-copaene 23.3%) [9], *S palanestina* (β-caryophyllene 36.4%) [10], *S. canariensis* (β-caryophyllene 30.2%, α-pinene 9.5%) [11], *S. hydrangea* (β-caryophyllene 33.4%, caryophyllene oxide 25.4%) [12] and *S. bracteata* β-caryophyllene 10.7-41.6%, γ-muurolene 1.8 - 9.9%) [13]. In terms of the essential oil composition, *S. mukerjeei* can also be compared with *S. angulata* from Venezuala which is also rich in sesquiterpene hydrocarbons: β-caryophyllene (27.7%), germacrene D (19.5%), bicyclogermacrene D (18.3%) [14]. However, germacrene D is completely absent in the essential oil from *S. mukerjeei.*

Antibacterial testing of the essential oil showed a maximum zone of inhibition (20mm) against the Gram-positive bacteria, *Enterococcus faecalis* followed by the Gram-negative, *E. chrysanthemi* (18 mm), *A. tumefaciens* (17 mm), *E. coli* (15 mm) and the lowest (10 mm) against *X. phaseoli*. The zone of inhibition values are summarized in Table 2. All bacterial strains were further tested at different concentrations to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values.

Erwinia chrysanthemi showed the lowest MIC at a concentration of 31.25 μL/mL, while the MIC values against *E. coli, A. tumefaciens* and *B. subtilis* were at 62.5 μL/mL and the minimum bactericidal concentration of 125.0 μL/mL was against *A. tumefaciens*. The results are summarized in Table 3. The *S. palaestina* Oil, with β-caryophyllene as a principal constituent, also showed better activity against the Gram-positive bacteria *S. aureus*, than Gram-negative bacteria (*E. coli, P. aeruginosa*.) [10]. The oil from *S. canariensis,* with β-caryophyllene

Table 1: Chemical composition of the essential oil from aerial parts of *S. mukerjeei*.

Compound	Percentage in the oil	RI observed
α-Pinene	0.4	933
Camphene	0.4	953
Benzaldehyde	0.1	967
Sabinene	1.0	978
β-Pinene	1.7	979
δ-2-Carene	0.1	1000
Isosylvestrene	0.1	1005
α-Terpinene	0.1	1017
o-Cymene	0.2	1026
Limonene	0.1	1029
β-Phellandrene	5.0	1031
1,8-Cineole	0.1	1034
(*Z*)-β-Ocimene	0.3	1036
(*E*)-β-Ocimeme	1.0	1054
γ-Terpinene	0.3	1062
p-Mentha-3,8-diene	0.1	1071
p-Mentha-2,4(8)-diene	0.1	1089
Terpinolene	0.5	1100
n-Nonanal	0.5	1105
Dehydrosabinaketone	0.2	1128
Camphor	0.5	1147
Pinocarvone	0.1	1165
Karahanaenone	0.9	1168
Terpinen-4-ol	0.3	1179
α-Terpineol	0.2	1192
γ-Terpineol	0.1	1197
p-Cymen-9-ol	0.3	1203
Linalyl formate	0.1	1207
Carvone	0.1	1243
Carvacrol methyl ether	0.1	1245
Thymol	1.2	1293
δ-Elemene	0.2	1340
Thymol acetate	trace	1358
α-Copaene	0.7	1378
β-Bourbonene	0.5	1380
(*E*)-β-Damascenone	0.2	1392
β-Elemene	0.3	1394
Longifolene	trace	1408
β-Doprezianene	0.1	1418
β-Caryophyllene	28.7	1422
γ-elemene	0.1	1432
α-Guaiene	5.4	1447
allo-Aromadendrene	0.2	1452
Dehydro-aromadendrane	9.5	1457
γ-Gurjunene	0.1	1480
γ-Muurolene	15.5	1484
γ-Himachalene	0.2	1489
α-Amorphene	0.2	1491
δ-Selinene	0.1	1494
cis-β-Guaiene	0.3	1497
trans-Muurola-4-(14),5-diene	0.2	1498
α-Muurolene	0.2	1500
(*Z*)-α-Bisabolene	0.2	1507
δ-Amorphene	0.7	1510
(*Z*)-γ-Bisabolene	0.1	1514
γ-Cadinene	1.1	1517
δ-Cadinene	1.5	1527
Zonarene	0.1	1529
trans-Cadina-1(2),4-diene	0.1	1530
cis-Calamenene	1.1	1539
α-Cadinene	0.3	1542
Furopelargone A	0.2	1547
Germacrene B	0.3	1557
Globulol	0.2	1561
Spathulenol	3.9	1587
Guaiol	0.1	1596
trans-Isolongifolone	0.9	1613
Eremoligenol	1.5	1625
Hinesol	0.2	1634
β-Atlantone	0.1	1636
Guaia-3,10(14)-die-11-ol	0.2	1687
Total identified	91.7 %	

trace= less than 0.1%

Table 2: Antibacterial screening of *S. mukerjeei* (1000 µL/mL).

Microorganism (Bacteria)	Zone of Inhibition (mm)		
	Oil	Streptomycin	Erythromycin
Erwinia chrysanthemi	18	12	12
Escherichia coli	15	18	18
Agrobacterium tumefaciens	17	30	28
Xanthomonas phaseoli	10	25	12
Bacillus subtilis	15	22	13
Staphylococcus aureus	15	18	10
Klebsiella pneumoniae	14	22	25
Enterococcus faecalis	20	25	12
Salmonella enterica enterica	14	16	12
Pasteurella multocida	13	23	18

Table 3: MIC/MBC values of *S. mukerjeei* oil.

Microorganism (Bacteria)	Concentration (µL/mL)				
	500.0	250.0	125.0	62.5	31.25
E. chrysanthemi	15*	11	10	8	6▲
E. coli	13	9*	7	7▲	-
A. tumefaciens	14	11	9*	7▲	-
X. phaseoli	7	7	-	-	-
B. subtilis	9	8	7	7▲	-
S. aureus	12	10	7	-	-
K. pneumoniae	11	7▲	-	-	-
E. faecalis	17	12*	9	9▲	-
S. enterica enterica	12	9	7	-	-
P. multocida	10	7▲	-	-	-

*MBC, ▲ MIC

and viridiflorol as the major components, had limited activity against the Gram-positive bacteria *S. aureus* and *S. epidermis*, and were inactive against the Gram-negative bacteria (*E. coli, P. aeruginosa, Salmonella spp*. and *Protes mirabilis*) [11]. The essential oil of *S. mukerjeei* showed significant activity against all Gram-positive as well as Gram-negative bacteria, thus indicating the importance of this plant for the treatment of infectious diseases.

Experimental

Plant material: The plant material was collected from Lamgara, district Almora, India at an elevation of 1,900 m in March, when the plant was flowering. The aerial part of the plant was used for the extraction of oil. The plant was identified by the Botany Department, Kumaun University, Nainital and its identity was further confirmed by the Botanical Survey of India, Dehradun, where a herbarium specimen was deposited (Herbarium Voucher No. [BSI (NC) Acc No.112203 BSD].

Extraction and isolation of oil: The oil was obtained by the steam distillation of fresh plant material (2.0 kg) using a copper still fitted with spiral glass condensers. The aqueous distillate was saturated with NaCl and the oil was extracted with hexane. The organic layer was separated and dried over anhydrous sodium sulphate. The solvent was removed using rotovap at reduced pressure and at 30°C.

Gas Chromatography: The GC analysis was performed using a Hewlett Packard 6980 gas chromatograph fitted with an HP-5MS capillary column (30m x 0.25mm, film thickness 0.25µm), and a flame ionization detector. The oven was programmed at 50^0C for 10 min and then raised

to 240°C (3°C/min) where it was maintained for 5 min. The composition data were taken from area percent data without the use of correction factors and rounded off to the first decimal place. Co-injection of the oil and n-alkanes (C_8 - C_{23}) allowed calculation of the Retention Indices [7].

Gas Chromatography-Mass Spectrometry: GC-MS analysis was carried out using an AGILENT 5973 Network Mass Selective Detector interfaced with an AGILENT 6856 GC system fitted with an HP-5MS capillary column (30m X 0.25mm, film thickness 0.25 µm) at 50°C for 10 min then raised by 3°C/ min to 230°C where it was maintained for final 10 min. The mass spectra were acquired at 70 eV, at 2.41 scan/second and a mass range of 41-350. Helium gas was used as a carrier gas in both the analyses.

Antibacterial assay

Microbial culture: The test organisms were obtained from the Institute of Microbial Technology, Chandigarh and the Botany Department, Kumaun University, Nainital, India as laboratory isolates of the pure culture of Gram positive *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 737), *Enterococcus faecalis* (MTCC 439), and Gram negative *Agrobacterium tumefaciens* (MTCC 609), *Erwinia chrysanthemi* (KUMSCC 328), *Xanthomonas phaseoli* (KUMSCC 327) *Escherichia coli* (MTCC 443), *Salmonella enterica enterica* (MTCC 3223), *Klebsiella pneumoniae* (MTCC 109) and *Pasteurella multocida* (MTCC1148). The isolates were separately cultured on nutrient agar plates for 24 hours. Twenty mL of the medium was poured into a sterile Petri plate (80 mm diameter) and allowed to solidify. A colony of each test organism was sub-cultured on ten mL nutrient broth and incubated at 37° ±1°C for 8 hours. One mL of the broth culture was then used to flood the agar plates.

Antibacterial testing: The preliminary screening of antibacterial activity was conducted at 1000 µL/mL concentration by using disc-diffusion method. [15] Streptomycin 30 µg/disc (Himedia) and Erythromycin 15 µg/disc (Himedia) were used as positive control while hexane was used as a negative control on all the bacteria to compare the zones of inhibition with that of the oil sample. The MIC values were determined at five different concentration of the oil (500, 250, 125, 62.5, and 31.25 µL/mL) and each set of experiment was done in triplicate.

Nutrient agar with a pH value of 7.3±0.2 was autoclaved at 121°C for 30 min at 15 psi. Whatman filter paper no. 1 was used to prepare discs of 5 mm diameter. Micro-organism were streaked in a radial pattern on the agar plate [16], rotating the plate approximately 60°C each time to ensure an even distribution of inoculum. Once the plates were inoculated with the micro-organism, four filter discs of 5 mm diameter were applied in a clockwise pattern, one containing plant oil (15 µL), another one of solvent (negative control, *n*-hexane) and two discs of standard antibiotics (positive controls). The plates were inverted and placed in an incubator (37°± 1°C). After 24 hours of incubation, each plate was examined for growth and inhibition. The diameter of the complete inhibition zone was measured to the nearest whole millimeter.

Acknowledgements - We are thankful to Dr. Y.P.S. Pangtey, Botany Department, Kumaun University, Nainital and Botanical Survey of India, Dehradun, India for the identification of plant herbaria specimen. Lalit Mohan is thankful to Uttarakhand Council for Science & Technology, Dehradun, India for financial assistance through a project grant(No.UCS&T/ R&D/CHEM-10/07-08/1835/1).

References

[1] Stracheyi R. (**1974**) *Catalogue of the plant of Kumaon and the portions of Garhwal and Tibet,* reprint ed., Periodical Experts Book Agency, New Delhi, India, 140.

[2] Gupta RK. (**1968**) Flora of Nainitalensis, Navyug Traders. New Delhi, India.

[3] Negi A, Javed MS, Melkani AB, Dev V, Beauchamp PS. (**2007**) Steam volatile terpenoids from *Salvia leucantha. Journal of Essential Oil Research*, ***19***, 401-403.

[4] Melkani AB, Negi A, Sati SC, Khulbe K, Dev V. (**2010**) Terpenoid composition and antimicrobial activity of the essential oil from *Salvia nubicola* Wall ex Sweet. *Journal of Essential Oil Research*, ***22***, 575-577.

[5] Raizada MB, Saxena HO. (**1978**) Flora of Mussoorie ***V-I***, p. 593-595.

[6] For e.g., (a) Formisano C, Senatore F, Arnold NA, Piozzi F, Rosselli S. (**2007**) GC and GC/MS analysis of the essential oil of *Salvia hierosolymitana* Boiss. growing wild in Lebanon, *Natural Product Communications*, ***2***, 181-184; (b) Rustaiyan A, Masoudi S, Tabatabaei-Anaraki M. (**2007**) Terpenoids from Iranian *Salvia* species, *Natural Product Communications*, ***2***, 1031-1042; (c) Guy P, Kamatou GPP, van Zyl RL, Davids H, van Vuuren SF, Viljoen AM (**2008**) Synergistic and antagonistic interactions of essential oils on the biological activities of the solvent extracts from three *Salvia* species, *Natural Product Communications*, ***3***, 1111-1115; (d) Schmiderer C, Grassi P, Novak J, Franz C. (**2008**) Diversity of essential oil glands of Spanish Sage (*Salvia lavandulifolia* Vahl, Lamiaceae), *Natural Product Communications*, ***3***, 1155-1160; (e) Öztürk M, Tel G, Duru ME, Harmandar M, Gülaçtı Topçu G. (**2009**)The effect of temperature on the essential oil components of *Salvia potentillifolia* obtained by various methods, *Natural Product Communications*, ***4***, 1017-1020; (f) Rojas LB, Visual T, Morillo M, de Rojas YC, Arbola JC, Usubillaga A. (**2010**) The volatile constituents of *Salvia leucantha*. *Natural Product Communications*, ***5***, 937-938; (g) Tabanca N, Demirci B, Turner JL, Pounders C, Demirci F, Başer KHC,Wedge DE. (**2010**) Microdistillation and analysis of volatiles from eight ornamental *Salvia* Taxa. *Natural Product Communications*, ***5***, 1421-1426; (h) Canzoneri M, Bruno M, Rosselli S, Russo A, Cardile V, Formisano C. (**2011**) Chemical composition and biological activity of *Salvia verbenaca* essential oil. *Natural Product Communications*, ***6***, 1023-1026.

[6] Adams RP. (**2007**) *Identification of essential oil component by Gas Chromatography /Quadrupole Mass Spectrometry,* 4th Ed., Allured Publishing Co., Carol stream, Illinois, USA.
[7] Lawrence BM. (**1992**) Chemical composition of Labiatae oils and their exploitation. In: *Advances in Labiatae Science*. Edts. R.M Harley and T. Reynolds, Royal Botanical Gardens, Kew. UK.
[8] Chalchat JC, Gorunovic MS, Petrovic SD, Maksimovic ZA. (**2001**) Chemical composition of two wild Species of the genus *Salvia* L. from Yugoslavia: *Salvia aethiopis* and *Salvia Verticillata*. *Journal of Essential Oil Research*, ***13***, 416-418.
[9] Sennatore F. Formisanno C, Arnold NA , Piozzi F. (**2005**) Essential Oil from *Salvia* sp. (Lamiaceae). III. Composition and antimicrobial activity of the essential oil of *Salvia palanestina* Benth. growing in Lebanon. *Journal of Essential Oil Research*, ***17***, 419-421.
[10] Garcia Vallejo MC, Moujir L, Burillo J, Guerra LL, Gonzalez M, Penate RD, Andres LS, Luis JG, Blanco FL, de Galarreta CMR. (**2006**) Chemical composition and biological activities of the essential oil of *Salvia canariensis*. *Flavour and Fragrance Journal*, ***21***, 72-76.
[11] Barazandeh MM. (**2004**) Volatile constituents of the oil of *Salvia hydrangea* DC ex Benth. from Iran. *Journal of Essential Oil Research*, ***16***, 20-21.
[12] Sefidkon F, Hooshidary F, Jamzad Z. (**2007**) Chemical variation in the essential oil of *Salvia bracteata* Banks & Soland from Iran. *Journal Essential Oil Bearing Plants*, ***10***, 265-272.
[13] Colmenares NG, Eymargarcia M, Alfredo U. (**2006**) Volatile constituents from the aerial parts of *Salvia angulata* H.B.K of Venezuela. *Journal of Essential Oil Research*, ***18***, 486-488.
[14] Bauer AW, Kirby WMM, Sherris JC, Turek M. (**1966**) Antibiotic susceptibility testing by standardized single disc method. *American Journal of Clinical Pathology*, ***45***, 493-496.
[15] Afolayan AJ. (**2003**) Extracts from the shoots of *Aretotis artotoides* inhibit the growth of bacteria and fungi. *Pharmaceutical Biology*, ***41***, 22-25.

NPC Natural Product Communications

2011
Vol. 6
No. 12
1953 - 1966

Revealing Indigenous Indonesian Traditional Medicine: Anti-infective Agents

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Received: May 29th, 2011; Accepted: August 25th, 2011

Indonesia is rich in medicinal plants which the population has used traditionally from generation to generation for curing diseases. Our interest in the treatment of infectious diseases has lead to the investigation of traditional Indonesian treatments. In this review, we present a comprehensive review of ethnopharmacologically directed screening in Indonesian medicinal plants to search for new anti-viral, anti-malarial, anti-bacterial and anti-fungal agents. Some potent drug leads have been isolated from Indonesian medicinal plants. Further research is still required for the lead development as well as the search for new bioactive compounds from the enormous medicinal plant resources.

Keywords: Indonesian medicinal plants, anti-viral, anti-malaria, anti-bacteria, anti-fungi.

Indonesia is an archipelago consisting of approximately 17,508 islands and is covered by tropical rain forest, seasonal forest, mountain vegetation, subalpine shrub vegetation, swamp and coastal vegetation. With its reflective mixture of Asian and Australian native species, Indonesia is said to possess the second largest biodiversity in the world, with around 40,000 endemic plant species including 6,000 medicinal plants [1-5].

Medicinal plants have accompanied the development of indigenous Indonesian traditional treatments which is a combination of physical and spiritual aspects to form a holistic medication. This is heavily influenced by the Indian Ayurveda, ever since the early civilization in Indonesia when Hinduism spread from India to Asia [6,7]. Conversely, the incorporation of indigenous medicinal plants not found in India enhanced the development of local Indonesian traditional medication [6] which has been further enriched by influences from Chinese and Arabian traders [8]. The long indigenous history and the variety of geographical conditions have created a variety of unique Indonesian cultures, of which only a few have recorded their traditional medication; these include the indigenous people of Sumatra, Java and Bali [9-13]. Much of this knowledge was not recorded and was verbally passed from generation to generation, which is common in tribes living in remote areas [14]. The knowledge is commonly practiced which leads to most of the population still relying upon medicinal plants [15,16].

The settlement of Europeans in Indonesia in the early 17th century intervened with the local medication [17]. The lack of knowledge of European physicians of the unique tropical diseases and the limited western medication forced the Western scientists to explore the Indonesian medicinal plants [17]. They subsequently used and published indigenous Indonesian traditional herbal medicine treatments showing them to be understandable and legitimate [8,17]. Findings were published in notable books, including "De medicina Indorum" by Bontius in 1642 [18], "the Ambonese herbal" by Rumphius in 1741 [19], "Materia Indica" by van der Burg in 1885 [17], "De nuttige planten van Nederlansch Indie" by Heyne in 1927 [20] and "select Indonesian medicinal plants" by Steenis-Kruseman in 1953 [21]. The outcomes were also reported in the Medical Journal of the Dutch East-Indies (1894-1925) [17].

Common infectious diseases occuring in traditional circumstances were able to be correlated with the then modern clinical pathology [19]. This made it easier for investigators, when reading ancient texts and listening to local informers (healers), to re-collect the knowledge through expedition which now helps scientists to perform narrowed pharmacochemical screening in finding new anti-infectious drug leads [22-26]. Since the 1970's, with the use of laboratory based experiments and the development of analytical technology, many bioactive compounds from Indonesian medicinal plants have been revealed. These include anti-viral, anti-malarial, anti-bacterial and anti-fungal agents. This review examines

these anti-infective natural products and lists plant extracts that require further analysis.

Anti-Viral Agents: Traditional knowledge was clearly unable to describe a viral pathology, however ethnopharmacological studies show the Indigenous people could diagnose viral infections and could cure them by using specific plant preparations [27]. Common viral infections known in Indonesian traditional circumstances include human herpes and rhino virus [12,28]. Later diseases such as poliovirus, human immunodeficiency virus, and avian myeoblastosis virus have resulted in extensive screening of Indonesian medicinal plants for activities against these viruses. This section will consider developments in targeting herpes simplex virus (HSV), poliovirus, rhinovirus, human immunodeficiency virus (HIV) and avian myeoblastosis virus-reverse transcriptase (AMV-RT).

Table 1: Extracts of selected Indonesian medicinal plants which have potential anti-HSV-1 and poliovirus activity [33, 34].

Plant	Part of plant	Virus type	
		HSV-1 EC_{50}, µg/mL	Poliovirus IC_{50}, µg/mL
Guttiferae			
Garcinia griffithii T. A.	-	781	600
Garcinia mangostana L.	LF	40*	
Melastomataceae			
Melastoma malabathricum L.	LF	192	111
Loranthaceae			
Elytranthe globosa B.	LF	336	217
Elytranthe maingayi V.T.	LF	233	41
Elytranthe tubaeflora R.	LF	176	56
Scurrula ferruginea D.	LF	i	62
Meliaceae			
Toona sureni (Bl.) Merr	LF	37*	
Piperaceae			
Piper aduncum L.	FL	344	105
Piper aduncum L.	LF	720	105
Punicaceae			
Punica granatum L.	PR	64*	
Sapindanaceae			
Filicium decipiens T.	SB	68*	
Nephelium lappaceum L.	PR	70*	
Simaroubaceae			
Eurycoma longifolia Jack.	ST	62*	
Verbaneceae			
Vitex pubescens V.	BK	i	i

LF: Leaf; SB: Stem bark; FL: Flower; PR: Pericarp; ST: Stem; HSV-1: Herpes simplex virus type-1; i: inactive. All the samples are methanol extracts. EC_{50}: 50% effective concentration *Expressed as IC_{50}: concentration that inhibits 50%.

Anti-HSV and anti-poliovirus: Selected Indonesian medicinal plant extracts have been tested against HSV-1 and poliovirus (Table 1) [29]. Quassionoid compounds might be responsible for the anti-HSV-1 activity although a drawback to further development is their cytotoxicity [30]. Another study proposed the essential oil and tannin constituents were responsible for the anti-HSV-1 activity [31, 32]. This was based on data suggesting plants that do not contain essential oils or tannins, e.g. *Garcinia* sp, have lower activity against HSV-1 than high essential oil containing plants, e.g. Sapindanaceae plants.

Anti-Rhinovirus: Ginger rhizome is commonly prepared into a traditional hot drink for common cold relief which can be correlated with rhinovirus infection. Many rhino anti-virals from natural products have been found from higher plants including lipophilic flavonoid type molecules (i.e flavan **1** and 4',6-dichloroflavan **2**) [35]. An investigation into the Indonesian ginger rhizome resulted in the isolation of four lipophilic sesquiterpenes, ar-curcumene **3**, β-sesquiphelandrene **4**, α-zingiberene **5**, β-bisabolene **6** (Figure 1). These compounds possessed rhinovirus IB inhibitor activity at an ED_{50} of 20.4, 0.9, 1.90, 14.3 µg/10 mL, respectively. The most active is the β-sesquiphellandrene **4** with IC_{50} of 0.44 µM. A structure analysis would suggest that the sesquiterpene and flavan compounds are equal in size and polarity [36]. However, a minor change in structure results in a significant difference in bioactivity and further studies are therefore required to determine the target of the flavan and the sesquiterpenes molecules.

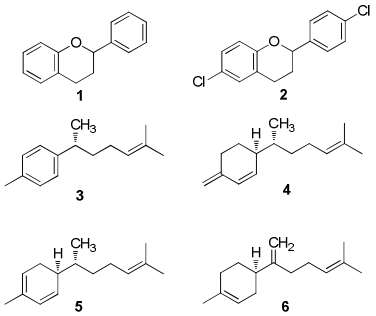

Figure 1: Rhino anti-viral sesquiterpenes (**3**-**6**) isolated from *Zingiber officinale* [36].

Anti-HIV agents: Although HIV is an imported disease to Indonesia, it is highly prevalent. While there are no specific traditional medicines to treat HIV, traditional anti-viral concoctions may lead to new potential anti-HIV leads. Therefore at least twenty one Indonesian medicinal plants were screened against HIV-1 protease and HIV-1 replication (Table 2) [37-39].

The methanol extracts of some medicinal plants are more active against HIV-1 protease while the more polar water extracts are more active against HIV-1 replication. There are four methanol extracts of *Terminalia belerica* Roxb, *Swiettenia mahagoni L.*, *Woodfordia floribunda* Salisb. and *Garcinia mangostana* L. which may contain potential anti-HIV-1 protease inhibitors with IC_{50} values of 50, 40, 40, 50 µg/mL, respectively [37]. However, there was no further exploration of these plants except *Garcinia mangostana* L. This study isolated mangostin **7** and γ-mangostin **8** (Figure 2) which showed non-competitive inhibition against HIV-1 protease with IC_{50} values of 5.12 and 4.81 mM, respectively [40].

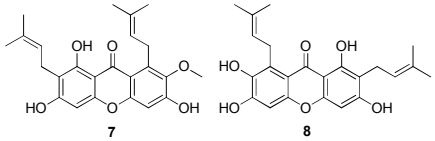

Figure 2: Mangostins isolated from Garcinia mangostana [40].

Table 2: Extracts of selected Indonesian medicinal plants which show anti-HIV-1 protease and replicase activity [37,38].

Plant	Part of plant	HIV-1 protease IC_{50}, µg/mL		HIV-1 rep. IC_{50}, µg/mL
		MeOH ext.	H_2O ext.	
Acanthaceae				
Andrographis paniculata Nees.	LF	500	500	≥170*
Apiaceae				
Foeniculum vulgare Mill	SD	100	>500	
Apocynaceae				
Parameria laevigata Moldenke.	BK	100	>500	
Clusiaceae				
Garcinia mangostana L	PL	50	100	
Combretaceae				
Terminalia belerica Roxb.	FR	50	220	
Compositae				
Elephantopus scaber L.	WP	500	>500	
Elaeocarpaceae				
Elaeocarpus grandiflorus Smith	FL	100	100	
Fabaceae				
Caesalpinia sappan L.	BK	280	320	
Sindora sumatrana Miq.	FR	260	360	≥134*
Hypoxidaceae				
Curculigo orchioides Gaertn.	FR	400	400	
Lauraceae				
Cinnamomum sintok Bl.	BK	220	320	
Loganiaceae				
Strychnos nux-vomica L.	BK	500	>500	
Loranthaceae				
Loranthus parasiticus (L.) Merr	ST	100	260	≥79.4*
Lythraceae				
Woodfordia floribunda Salisb.	FL	50	50	
Malvaceae				
Helicteres isora L.	FL	380	>500	≥65*
Meliaceae				
Swietenia mahagoni L.	BK	40	100	≥28.4**
Parmeliaceae				
Usnea misaminensis Vain.	WP	220	>500	
Poaceae				
Andropogon zizanioides (L.) Urban	RT	500	>500	
Solanaceae				
Physalis angulata L.	AP	340	>500	
Zingiberaceae				
Curcuma aeruginosa Roxb.	RZ	500	>500	≥323*
Curcuma xanthorriza Roxb.	RZ	300	>500	

RT:Root; SD:Seed; FL:Flower; LF: Leaf; AP:Aerial part; BK: Bark; WP:Whole plant; ST: Stem; HSV-1: Herpes simplex virus type 1. * H_2O extract. ** MeOH extract. IC_{50}: concentration that inhibits 50%

Anti-AMV-RT: The fruit of the Indonesian medicinal plant *Helicteres isora* L, was screened for potential anti-AMV-RT activity. From these four secondary metabolites were reported: rosmarinic acid **9**, 4'-*O*-D-glucopyranosyl rosmarinic **10**, 4,4'-*O*-di-*β*-D-glucopyranosyl rosmarinic acid **11** and 4'-*O*-D-glucopyranosil isorinic acid **12**, which exhibited xanthine oxidase inhibition [41]. Additional work revealed the flavonoid glucoronides, 3',5,7,8-tetrahydroxy-4'-methoxyflavone 8-*O*-*β*-D-glucopyranosiduronic acid methyl ester **13**, 4',5,7,8-tetrahydroxyflavone 8-*O*-*β*-D-glucopyranosiduronic acid methyl ester **14** and the first neolignans, helicterins A-F (**15-20**), helisterculin A **21**, helisterculin B **22** and helisorin **23**, isolated from *Helicteres isora* L obtained from Indonesia (Figure 3) [42-43].

The helicterins were tested against avian myeoblastosis virus-reverse transcriptase (AMV-RT) and presented weak

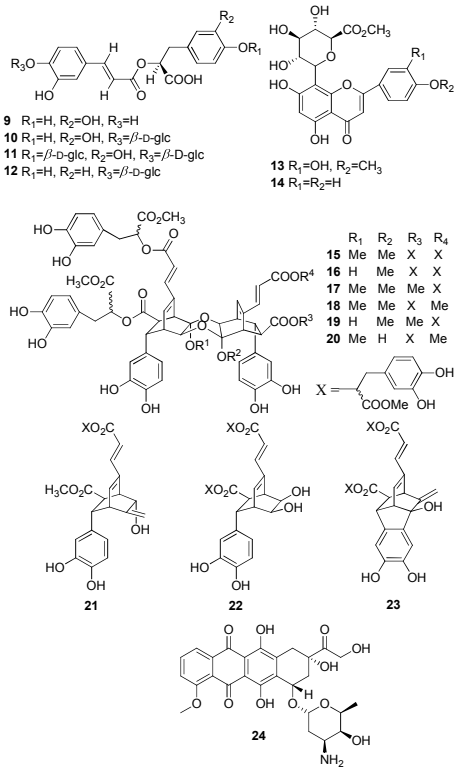

Figure 3: Glycosidic and non glycosidic metabolites isolated from *Helicteres isora* [41-43].

Table 3: Bioactivity of helicterins A-F **15**-**20**, helisterculin A **21**, helisterculin B **22**, helisorin **23** and adriamicyn **24** against AMV-RT [42,43].

Compounds	AMV-RT, IC_{50} µM
15	66
16	172
17	417
18	372
19	120
20	226
21	1600
22	1000
23	460
24	66

activity (Table 3). However, compared to the standard drug for AMV infection, adriamycin **24**, and helicterins A **15** possessed equal activity [43]. There is no specific information regarding the mechanism of inhibition of this compound.

Figure 4: Map of Indonesia [46]. * The sites of ethnophamarcological field trips in endemic malaria regions in some islands. Each region is discussed separately in the following sections.

Anti-malarial agents: Ethnopharmacological studies revealed particular medicinal plants were used for malarial

fever therapy in some regions (Figure 4). Further experiments produced the extracts responsible for the claimed activity (Table 4) with only a small number investigated further to reveal individual compounds [44,45].

Table 4: Anti-malarial activity of extracts of some Indonesian medicinal plants [44-45].

Plants	Part of plant	Inhibition rates against *Plasmodium falciparum*, %
Apocynaceae		
Catharanthus roseus L.	AP	52
Rauvolfia serpentine (L.) Benth.	BK	66
Asteraceae		
Achillea millefolium L.	WP	98
Ageratum conyzoides L	WP	62
Euphorbiaceae		
Phyllanthus niruri L.	WP	50*
Loganiaceae		
Strycthnos lucida R.Br.	WD	100
Meliaceae		
Azadirachta indica Juss	LF	60
Lansium domesticum Corr.	BK	66
Swietenia macrophylla King.	SD	98
Myrtaceae		
Baeckea frutenscen L.	LF	90
Rubiaceae		
Morinda citrifolia L.	FR	56
Zingiberaceae		
Curcuma xanthorrhiza Roxb.	RZ	100

AP: Aerial part; BK: Bark; WP: Whole plant; WD: Wood; LF: leaf; SD: Seed; FR: Fruit; RZ: Rhizome. All materials were prepared as a water extract. Concentration for assay, 1 mg/mL. *50% of inhibition at 3.5 µg/mL.

Sumatra Island: An expedition in Sumatra Island revealed *Anthocephalus chinensis*, *Beilschmiedia madang* BL. and *Brucea javanica* L. Merr to be used in malarial therapy [3].

Anthocephalus chinensis (Rubiaceae). The people in Indragiri Hulu area, Riau Province have used the bark, root and leaf of *Anthocephalus chinensis* in malaria therapy. Fourteen compounds were successfully isolated including glycosides, 3'-*O*-caffeoylsweroside **25**, sweroside **26**, loganic acid **27**, loganin **28**, loganol **29**, kelampayoside A (derived from "kelampayan", the local species' name) **30**, 8-epikingiside **31**, kelampayoside B **32** (Figure 5), and alkaloids vallesiachotamine **33**, isovallesiachotamine **34**, cadambine **35**, strictosidine lactam **36**, desoxycordifoline **37**, 5α-carboxystrictosidine **38** (Figure 6) [47]. Compounds **26**-**29**, **31**, **33**-**37** were previously identified from various Rubiaceous species of different origins [48-52].

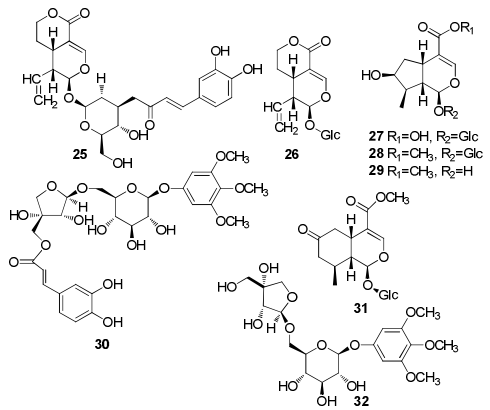

Figure 5: Glycosides isolated from the bark of *Anthocephalus chinensis* [47].

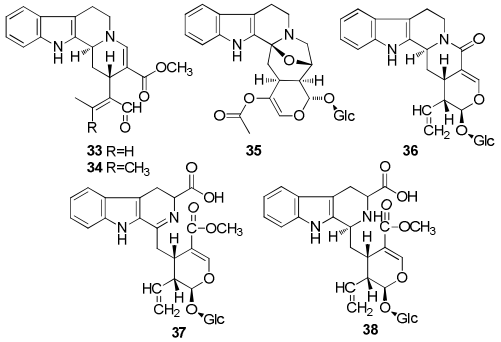

Figure 6: Alkaloids isolated from the bark of *Anthocephalus chinensis* [47].

Among the fourteen compounds (Figures 5 and 6), only eight were subjected to *in vitro* testing against the *Plasmodium falciparum* K1 strain. The results indicated no anti-malarial activity except the indole type compound cadambine **35**, which showed a moderate inhibitory activity at IC_{50} of 6.77 µM and IC_{90} 9.85 µM [47].

Beilschmiedia madang BL. (Lauraceae). The decocted wood of *B. madang* (locally called as 'medang kohat') has been used by people in Kepahiang to treat malaria fever [53]. From this was isolated dehatrine **39** (Figure 7) which was found to have anti-malarial activity with an IC_{50} of 0.17 µM against the *Plasmodium falciparum* K1 strain, a chloroquine resistant strain [53].

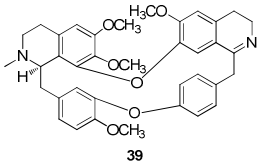

Figure 7: Molecular structure of dehatrine [53].

Brucea javanica L. Merr. (Simaroubaceae). This species was found through an expedition to Rejang Lebong where the plant has been traditionally used to treat malaria. The same species is used by the Bugisse, 'people of Makasasar' for the same pupose. Successfully identified were eleven triterpenoids including, bruceajavanin A **40**, dihydrobrucejavanin A **41**, bruceajavanin B **42**, bruceantinol **43**, bruceantinol B **44**, bruceine A **45**, bruceine B **46**, bruceine C **47**, bruceine D **48**, bruceine J **49**, yandaziolide A **50** [54] and five alkaloids including 11-hydroxy-canthine-6-one **51**, 11-hydroxy-1-methoxy-canthin-6-one **52**, canthine-6-one **53**, canthine-6-one-3-*N*-oxide **54** and bruceacanthinoside **55** (Figure 8) [55], of which the last four compounds **51**-**54** were previously reported from non-Indonesian Simaroubaceous medicinal plants [56-58].

Compounds **40** and **42** possessed moderate anti-malarial activity, e.g. IC_{50} 1.1 µM and 4.4 µM against the *Plasmodium falciparum* K1 strain, respectively [55]. On the other hand, compound **43** presented only modest anti-malarial activity (IC_{50}, 25 µM) [55]. Some of the

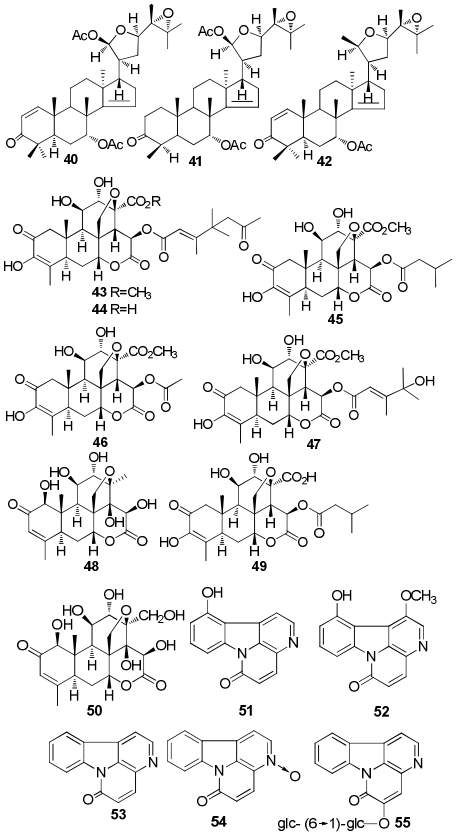

Figure 8: Metabolites isolated from *Brucea javanica* [55].

compounds were also tested against *Babesia gibsoni* parasite of dogs which brucein A **45**, bruceinthinol **43** and B **44** possessed potent activities with IC_{50} values of 4, 12 and 12 ng/mL, respectively [54].

Java and Lombok Islands: The Javanese and Lomboknesse native people have been infected with malarial fever for centuries. However, they survive by consuming young leaves of *Alstonia scholaris* [59]. Investigation on samples taken from Java and Lombok Island isolated 21 alkaloids, including, picraline **56**, N_1-methylburnamine **57**, akuammidina **58**, *φ*-akuammigine **59**, akuammiline **60**, akuammiline N_4-oxide **61**, deacetylakuammiline **62**, 5α-methoxy-akuammiline **63**, tubotaiwinine **64**, tubotaiwinine N_4-oxide **65**, scholaricine **66**, N_4-methylscholaricine **67**, vallesamine **68**, alstonamine **69**, 6,7-*seco*-alstonamine **70**, 6,7-*seco*-19,20*α*-epoxy-alstonamine **71**, leuconolam **72**, mataranine A **73**, mataranine B **74**, kotarajine **75** and (15*S*,16*S*)-losbamine **76** (Figure 9) [59, 60]. Tubotaiwine **64** and mataranine A **73** and B were claimed to be responsible for the anti-malarial activity (Table 5) [60,61].

Flores Island: An ethnopharmacological expedition reported that the people of Flores Island have used *Fagara rhetza* (Roxb.) DC, locally named as Haleza, to treat malarial fever. Eleven compounds were isolated in this study. The bark contains rutaecarpine **77**, evodiamine **78**, skimmianine **79**, zanthobungeanine **80**, *O*-geranylsinapyl

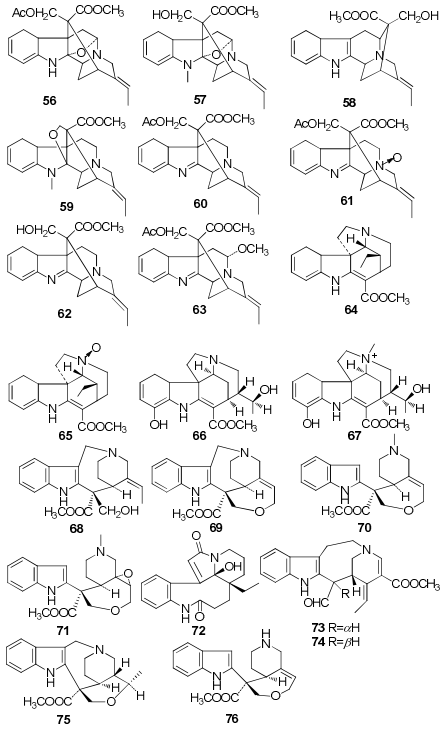

Figure 9: Alkaloids isolated from *Alstonia scholaris* [59, 60].

Table 5: Anti-malarial activity of several alkaloids isolated from *Alstonia scholars* [60,61].

Alkaloids	EC_{50}, μM
60	18
62	36
63	7
64	[a]
69	24
70	17
73	7.4[b]
74	9.7[c]

[a]No potency. [b]Bioactivity against *Plasmodium falciparum* K1 (an antifolate resistant parasite strain). [c] Against *Plasmodium falciparum* TM4 (an anti-folate sensitive parasite strain)

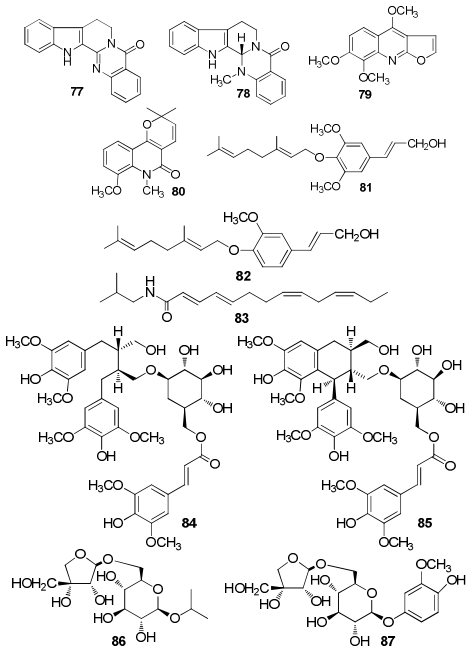

Figure 10: Metabolites isolated from *Fagara rhetza* [62-67].

alcohol **81**, *O*-geranylconiferyl alcohol **82**, hazaleamide **83**, hazaleanin A **84**, hazaleanin B **85**, isopropyl apioglucoside **86** and 4-hydroxyguaiacol apioglucoside **87**, of which compounds **77**-**80** were previously identified from other plants however no bioactivity data was reported (Figure 10) [62-67]. In this study, only hazaleamide **83** showed a moderate activity against *Plasmodium falciparum* with an IC_{50} value of 43 μM [62-67].

Kalimantan (Borneo) Island: Malarial fever is a long lasting health problem of Dayak tribes that inhabit the isolated rainforests of Kalimantan Island. An expedition found two plants species, *Eurycoma longifolia* Jack. and *Lansium domesticum* Corr. Ser. have been intensively used for malarial fever treatment [68-69].

Eurycoma longifolia Jack. (Simaroubaceae). This plant is locally called "pasak bumi" by people of Borneo Island where it has been used to treat malaria, dysentery, glandular swelling and persisten fever [68]. Eleven molecules, 9-methoxycanthin-6-one **88**, 9-hydroxycanthin-6-one **89**, 9-methoxycanthin-6-one-*N*-oxide **90**, 9-hydroxycanthin-6-one-*N*-oxide **91**, *β*-carboline-1-propionic acid **92**, 7-methoxy-*β*-carboline-1-propionic acid **93**, eurycomanone **94**, 13,21-dihydroeurycomanone **95**, 13*β*,21-dihydroeurycomanone **96**, eurycomanol **97** and longilactone **98**, were isolated of which only the compounds **93** and **94** were shown to have weak anti plasmodium activity [68].

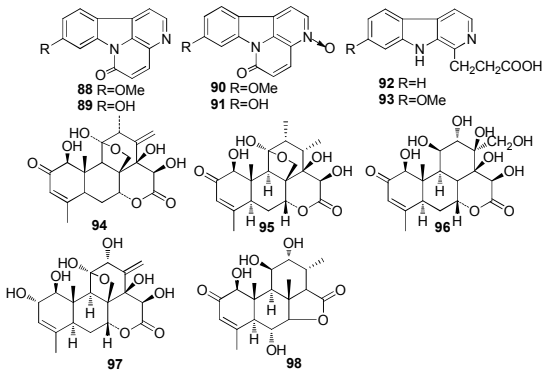

Figure 11: Alakaloids and terpenes isolated from *Eurycoma longifolia* [68].

Lansium domesticum Corr. Ser. (Meliaceae). Isolation revealed five compounds, 3-keto-22-hydroxyonoceradiene **99**, onoceradienedione **100**, methyl lansiolate **101**, methyl lansiolate A **102** and methyl 15-acetoxylansiolate **103** (Figure 12) [69]. The anti-malarial activity of the isolates were tested against *Plasmodium berghei* (Table 6).

Table 6: Anti malarial activity of isolates from *Lansium domesticum* Corr. Ser [69].

Quassinoids	IC_{50}, μM
99	2.41
100	1.66
101	0.65
102	0.69
103	0.17

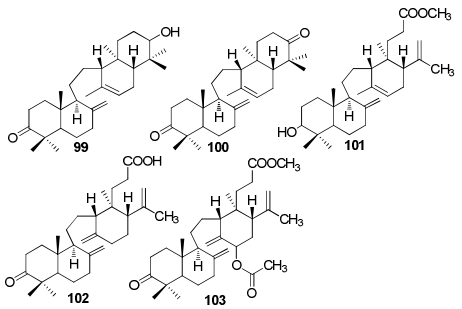

Figure 12: Anti-malarial agents isolated from *Lansium domesticum* [69].

The most active compound isolated was the methyl ester 15-acetoxylansiolate **103**. This *in vitro* test result agreed with the *in vivo* test by using infected *Plasmodium berghei* mice where **103** possessed 44% parasetimia inhibition at 50 mg/kg daily doses [69].

Sulawesi Island: There are two traditionally prepared medicines for malarial therapy; the seed kernel of *Caesalpinia crista* species (called 'Bagor') and decocted root bark and stem of *Quassia indica* (Gaertn.) Nooteboom, named 'Tobello' by central Sulawesi people.

Caesalpinia crista (Fabaceae). From this species were isolated at least 24 cassane and norcasssane type compounds. The quassionoids are caesalpinins C-P **104**-**117**, norcaesalpinins A-F **118**-**123**, caesalmin B **124**, E **125** and G **126**, caesalpin F **127**, 14(17)-dehydrocaesalpin F **128**, caesaldekarin E **129**, 2-acetoxy-3-deacetoxycaesaldekarin E **130**, 1-deacetoxy-1-oxocaesalminin C **131**, 3-deacetoxy-6-acetoxycaesaldekarin E**132**, 2-acetoxycaesaldekarin E **133**, bonducellpins A-C **134**-**136** and 7-acetoxybonducellpin C **137** (Figure 13) [70-73].

The preliminary anti-malarial testing in mice infected with chloroquinene-resistant *Plasmodium berghei*, showed that norcaesalpinin A **118** suppressed the parasitemia by 48.0, 40.9 and 33.0% at doses of 10, 1 and 0.1 mg/kg, respectively [70].

Most of the isolates were also tested against *Plasmodium falciparum* culture (see Table 7) with **122** as the most active quassinoid with an IC_{50} value of 0.09 μM [74].

A QSAR study on the cassane and norcassane-type diterpenes observed that the presence of an acetoxy group resulted in a higher anti-malarial activity than when a hydroxyl substituent was present. On the other hand, any additional functional group on the C-ring in 17-norcassane-type diterpenes reduced the activity [74].

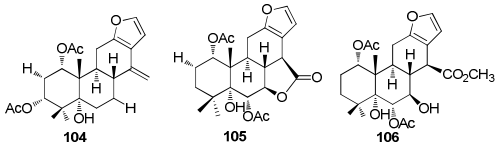

Figure 13: Quassionoids isolated from *Caesalpinia crista* [70-73].

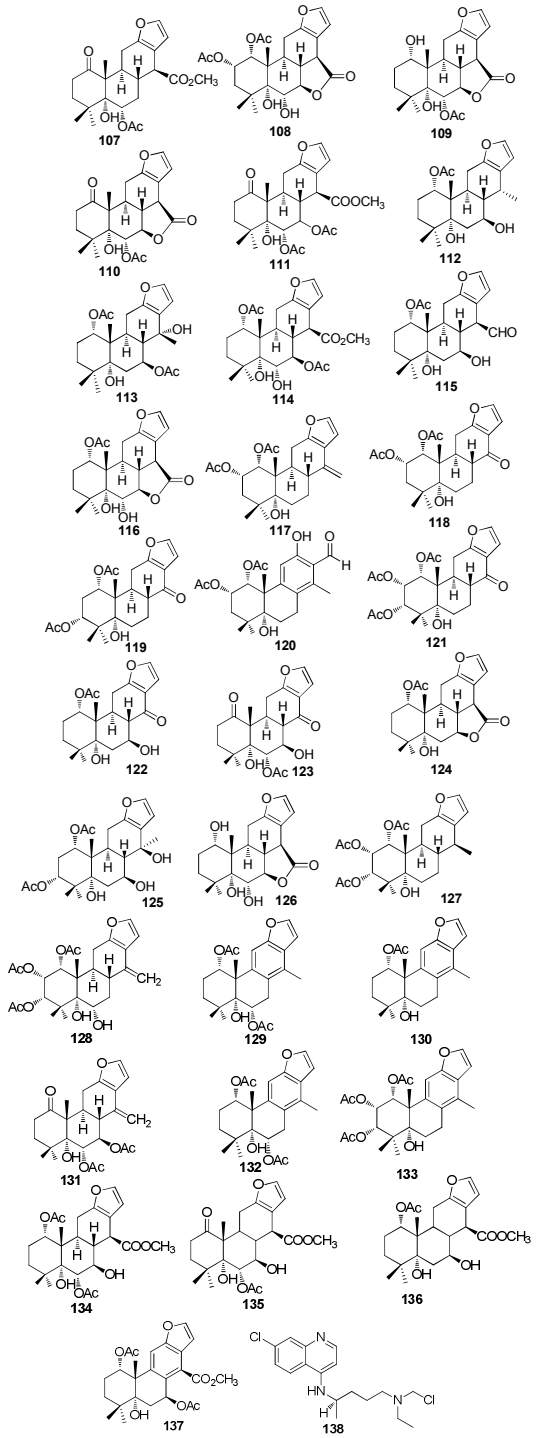

Figure 13 (*continued*): Quassionoids isolated from *Caesalpinia crista* [70-73].

Quassia indica (Gaertn.) Nooteboom. (Simaroubaceae). The chemical screening of the species isolated eight quassinoids, samaderine B-C, E, X-Z, **139**-**144**, indaquassin C, X **145**-**146**, simarinolide **147** and 2-*O*-glucosylsamaderine C **148** (Figure 14). Compounds **139**-**141**, **145**, **147** were previously isolated from other simaroubaceous [76]. Four of the isolates were tested for anti-malarial activity against *Plasmodium falciparum* K1 (Table 8) which showed that samaderine **142** was the most active compound [76].

Table 7: Isolated quassinoids activity against *Plasmodium falciparum* [73,74].

Quassinoids	IC_{50} µM	Quassinoids	IC_{50} µM
104	0.76	**121**	2.0
105	0.80	**122**	0.09
106	6.50	**123**	0.14
107	0.65	**124**	0.80
109	>10	**125**	>10
110	>10	**126**	>10
111	1.00	**128**	0.20
112	0.4	**129**	4.0
113	0.65	**130**	0.098
114	>10	**131**	2.9
115	0.12	**133**	6.5
116	>10	**135**	0.24
117	1.7	**136**	0.12
118	0.80	**137**	0.60
119	0.26	**138***	0.29
120	5.0		

* Chloroquine **138**, positive control, was discovered in 1934 and it has been used as anti-malarial agent which also shows anti-viral effects [75].

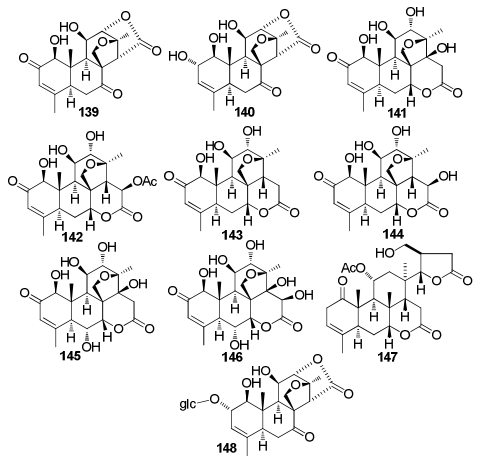

Figure 14 : Quassionoids isolated from *Quassia indica* [76].

Table 8: Anti-malarial activity of isolates from *Quassia indica* (Gaertn.) Nooteboom [76].

Quassinoids	IC_{50}, nM
139	210
141	56
142	14
144	71

Anti-bacterial agents: Indigenous Indonesian people has treated diarrhea, swelling, redness and fever with medicinal plant preparations [11,77] in which these symptoms have modern clinical correlations with bacteria causing diseases. Most of the anti-bacterial outcomes were preliminary studies of extracts (see Table 9), which only gave indications of activity. Several extracts such as those from *Terminalia catappa, Swietenia mahagoni* Jacq., *Phyllanthus acuminatus, Ipomoea spp., Tylopora asmathica* and *Hyptis brevipes* possessed high activity which might provide a stimulus for further research [78]. Additional work was performed on a few species resulting in the following:

Clerodendron calamisotum L. and *Clerodendron paniculatum* L. (Verbenaceae): At least five alkaloids were present in *Clerodendron calamisotum* L. and *Clerodendron paniculatum* L. however the alkaloids were not responsible for the anti-bacterial activity [61].

Table 9: Anti-bacterial activities of extracts of Indonesian medicinal plants [24,78].

Plant	Part of plant	Anti-microbial activities** BS	SA	EC	SC	FO	XC
Acanthaceae							
Gendarusa vulgaris Nees	LF, RT, ST		+				
Graptophyllum pictum (L.) Griff.*							DG
Staurogyne sp	LF, RT, ST		+		+	+	
Amaranthecеae							
Celosia argentea L. fma	FR, LF, ST		++	+		+	
Celosia plumose (Voss) Back	FR, LF, ST		++	+		+	
Amaryllidaceae							
Crinium asiaticum	LF		++	+	+		
Crinium lineare L.	LF				++		
Curculigo sp.	LF, RT, ST		++	+			
Anarcadiaceae							
Mangifera foetida Lour.	LF		+				
Mangifera indica L.	BK		++				
Apocynaceae							
Alstonia scholaris (L) R. Br.	LF, ST		++				
Cantharanthus roseus (L.) G. Don*			+				
Araceae							
Acorus calamis L.	LF, RT,RZ		+	+			
Araliaceae							
Aralidium pinnatifidum Miq.	LF		++	+			
Balsaminaceae							
Impatiens balsamina L.	LF, ST		+				
Begoniaceae							
Begonia isopera Bl.	LF, ST		+		+	+	
Bombaceae							
Ceiba petandra (L.) Gaertn.	LF, ST		++				
Bromeliaceae							
Ananas comosus (L.) Merr.	LF		+	+		+	
Capparaceae							
Crateva religiosa Forst.	LF, ST		+	+			
Clusiceae							
Cratoxylon formosum (Jack)	BK		+				
Garcinia atroviridis Griff.	LF, ST		++	+			
				+			
Garcinia mangostana L.	BK		+				
Garcinia parvifolia Miq.	LF, ST		++	+			
				+			
Compositae							
Blumea balsamifera DC.	LF, RT, ST		+	+		+	
Plucea indica (L.) Less.	LF, ST		++	+		+	
Connaraceae							
Rourea mimosoides (Vahl)	LF, ST		+	+			
Convulaceae							
Ipomoea batatas (L.) Lamk.	LF, ST		+				
Merremia umbellata (L.) Hall.	LF, ST		+				
Cyperaceae							
Scleria purpurascens Steud.	LF, ST		+				
Dillenaceae							
Dillenia meliosmifolia Hook.	LF		+				
Tetracia asiatica (Lour.)	LF		+		+		
Tetracera sp.	LF		+				
Ebenaceae							
Diospyros sp.	LF, ST		++	+			
Euphorbiaceae							
Breynia racemosa M.	LF, ST		+				
Croton cf. caudatus Geisel.	LF, ST, BK		++				
Galearia filiformis Boerl.	LF				+		
	BK		++	+	+		
Galearia sp.	LF, ST		++		+	+	
Jatropha curcas L.	LF				+		
Euphorbiaceae							
Macaranga gigantea M.	LF		++	+			
Macaranga gigantea M.	BK		+	+	+		
Macaranga triloba M.	LF		+	+		+	
Mallotus ricinoides M.	LF		+				
Euphorbiaceae							
Phyllanthus emblica L.	LF, ST		+	+			
Pimeliodendron papaveriodes	LF, ST		+	+			

Table 9 (*continued*): Anti-bacterial activities of extracts of Indonesian medicinal plants [24,78].

Plant	Part of plant	Anti-microbial activities** BS	SA	EC	SC	FO	XC
Gnetaceae							
Gnetum latifolium Bl.	LF, ST		+			+	
Hanguanaceae							
Hanguana malayana (Jack) Merr.	LF, ST		++				
Labiatae							
Coleus scutellarioides (L.) Bth.	LF, ST		++			+	
*Hyptis brevipes**			+++	+			+
Ocimum basilicium L.	FL, LF, ST		+	+			
Ocimum tenuiflorum L.	FL, LF, ST		+	+			
Orthosiphon aristatus (Bl.) Miq.	LF, ST		+				
Lauraceae							
Litsea elliptica Bl.	LF		+				
Litsea robusta Bl.	LF		++				
Lecythidaceae							
Barringtonia lanceolata (Bl.)	LF		++				
Barringtonia sp.	BK		++				
Leguminosae							
Bauhinia sp	LF, ST				+		
Crotalaria juncea L.*		DG					
Cassia cf. nodosa Buch. Ham.	BK		++	++		+	
Flemingia strobilifera (R.Br.)	FR, LF, ST		+				
Mimosa pigra L.	LF, ST		+				
Sesbania aculeate Poir.	LF, RT, ST		++				
Tamarindus indica L.	LF, ST		++	++			
Longaniaceae							
Fagraea cfrf. auriculata Jack	LF		+	+	+		
Lythraceae							
Lagerstromia speciosa (L.)	LF, ST		++	++			
Malvaceae							
Abelmoschus ficulneus W. et H.	LF, ST		++		++		
Abelmoschus moschatus Medik.	LF, ST		+				
Gossypium barbadense L. var.	LF, ST		++				
Hibiscus x archeri Wats.	LF, ST		++				
Hibiscus sabdariffa L.	LF, ST		++				
Hibiscus tiliaceus L.	BK		++				
	LF, ST		+				
Melastomataceae							
Dissochaeta gracilis (jack) Bl.	LF, ST		+			+	
Melastoma affine D.Don	LF, ST		++	+	+		
Melastoma malabathricum L.	FL,ST,LF		++		+	+	
Phyllagathis rotundifolia (Jack) Bl.	LF, ST		+	+	+		
Meliaceae							
Aglaia eximia Miq.	BK		+	+		+	
Swietenia mahagoni Jcq.*		+	++	DG			+
		+					+
*Swietenia mahagoni*L. *	PC	+	+				
Menispermaceae							
Arcangelisia flava (L.) Merr.	LF, RT, ST		++		++	+	
Tinomiscium petiolare Hook. F. et Thoms.	BK		+				
Moraceae							
Artocarpuis elasticus Reinw. Ex Bel.	BK		++			+	
						+	
Artocarpus nitidus Trec.	BK			+			
Ficus grossularioides Burm. f.	LF, ST		+	+			
Ficus macrocarpa Kurz	LF, ST		++	+			
Ficus pandana Burm. f.	LF, ST		++	+			
Myristicaceae							
Horsfieldia glabra (Bl.) Warb.	BK		+				
Horsfieldia wallichii (Hook. F. et Thunb.) Warb.	BK		++				
Myrtaceae							
Eugenia polyantha Wight	LF, ST		+			+	

Table 9 (*continued*)**:** Anti-bacterial activities of extracts of Indonesian medicinal plants [24,78].

Plant	Part of plant	Anti-microbial activities**					
		BS	SA	EC	SC	FO	XC
Myrtaceae							
Psidium guajava L.	LF, ST		++	+			
Rhodmania cinerea Jack	LF, ST		++	+			
Pandanaceae							
Pandanus sp.	LF		+				
Passifloraceae							
Adenia cordifolia ENgl.	LF, ST		++	+			
Rhamnaceae							
Gouania lepostachya DC.	LF, ST		++		+	+	
Rhizophoraceae							
Anisophyllea disticha (Jack) Baill.	LF, ST		+		+		
Rubiaceae							
Hedyotis capitellata Wall.	LF, ST		+				
Hedyotis leucocarpa Elm.	LF, ST		+				
Mussaenda frondosa L.	LF, ST		+				
Uncaria gambir (Hunt.) Roxb.	LF, ST		++			+	
Rutaceae							
Citrus aurantifolia Swingle	FR		++	++			
Simaroubaceae							
Brucea javanica (L.) Merr.	LF, ST		++		+		
Staphylaceae							
Turpinia sphaerocarpa Hassk.	LF, ST		++	++			
Symplocaceae							
Symplocos cochinchinensis (Lour.) Moore	LF, ST		+	+	++	+	
Theaceae							
Eurya acuminata DC.	LF, ST		+				
Thymelaeceae							
Aqularia malaccensis Lamk.	LF, ST		+				
Tiliaceae							
Elaeocarpus cf. mastersii King	LF, ST, BK		++	++			
Grewia acuminata Juss.	BK		++			+	
Ulmaceae							
Trema tomentose (Roxb.) Hara	LF, ST		+				
Urticaceae							
Dendrocnide stimulans (L.f.) Chew	RT		++				
Violaceae							
Rinorea anguifera (lour.) O.K.	BK		+	+			
Zingiberaceae							
Boesenbergia rotunda (L.) Mansf.	LF, RZ, ST		+				
Costus sp.	LF, ST		+				
Curcuma cf. heyneana Val. Et v. Zijp	RZ					+	
Curcuma sp.	RZ		+		+		
Zingiber purpureum Roxb.	RZ		+		+		

LF: Leaf, ST: Stem, BK: Bark, PC: Pericarp, RZ: Rhizome, BS: *Bacillus subtilis*, SA: *Staphylococcus aureus*, EC: *Escherichia coli*, SC: *Saccharomyces cerevisiae*, FO: *Fusarium oxysporum*, XC: *Xanthomonas campestris*. All samples are methanol extracts except indicated by * extract of DCM. ** + is 25% or less than control; ++, equal to the control; +++, 25-50% more than the control; ++++, 50% and more than the control.

Curcuma xanthorrhiza Roxb.: The species sample collected from Yogyakarta contains sesquiterpenes, α-curcumene **149**, germacrone **150**, zederone **151**, xanthorrizol **152** and curcumin **153** (Figure 15). The crude extracts possessed moderate antibacterial activity (MRSA) while the isolated curcumene showed less activity [79]. The extract has potential against *Staphylococcus* mutans for dental caries prevention [80].

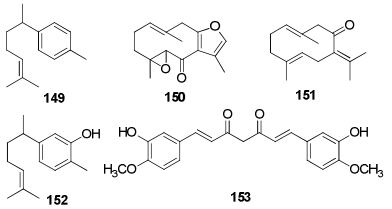

Figure 15: Sesquiterpenes isolated from Curcuma xanthorrhiza [79].

Mitrephora celebica (Annonaceae): Two oropheic acid and four diterpenes were isolated from the bark of the species, oropheic acid **154**, 13,14-dihydrooropheic acid **155**, *ent*-trachyloban-19-oic acid **156**, *ent*-kaur-16-en-19-oic acid **157**, 8(14),15-pimaradien-18-oic acid **158** and 7,15-pimaradien-18-oic acid **159** (Figure 16) [81, 82]. These compounds are proposed to be responsible for the antibacterial activities of the stem bark extracts against methicillin-resistant *Staphylococcus aureus* and *Mycobacterium smegmatis*.

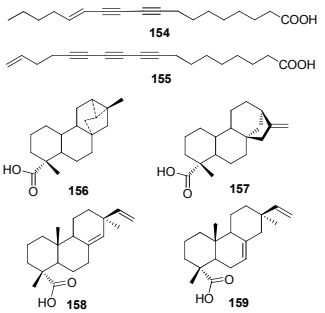

Figure 16: Oropheic acids and diterpenes isolated from *Mitrephora celebica* [81,82].

Among the compounds, **154**, **155**, **156** and **157** exhibited significant inhibitory activity with an MIC of 25, 12.5, 6.25, 6.25 µg/mL, respectively while the others showed an MIC of more than 100 µg/mL [81,82]. This difference might be correlated with the existence of an extra bridged ring in ring C of the structure.

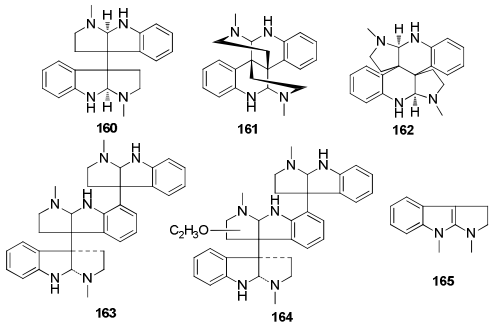

Figure 17: Alakloids isolated from *Psychotria malayana* [61].

Psychotria malayana Jack (Rubiaceae): The aqueous extracts of the leaves or bark of the species has been used traditionally to treat infections on open wounds by the Lomboknesse. Chemical investigation on the locally named as "lolon jalun" plant successfully identified six alkaloids, *meso*-chimonanthine **160**, calcycanthine **161**, *iso*-calcycanthine **162** and hodgkinsine **163** and two

probable new alkaloids named LPM-574 **164** and LMP-186 **165** (Figure 17) [61]. Hodgkinsine **163** presents the major alkaloid while *meso*-chimonanthine **160** exists as a minor alkaloid constituent along with two others which were still under investigation [59]. Further chemical investigation on their activities are discontinued [59]. However, initial bacterial testing (Table 10) revealed that isolate **164** can caused bacteriolysis at 1.0 mg/mL but suppressed the growth at 0.5 mg/mL [61].

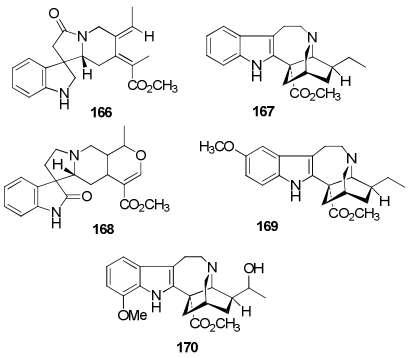

Figure 18: Alkaloids isolated from *Voacanga foetida* [84].

Voacanga foetida (Bl.) Rolfe (Apocynaceae): A phytochemical study on a Lombok Island sample isolated seven alkaloids, lombine **166** and coronaridine **167** [84], uncarine **168** voacangine **169**, voacristine **170**, mataranine A **73** and B **74** (Figure 18). The latter two compounds also exist in *Alstonia scholaris* collected from the same region.

Table 10: Bacteriostatic activity of alkaloids isolated from *Psychotria malayana* Jack [61].

Sample	C, mg/mL	Bacteriostatic activity	
		SA	EC
163	1.0	-	-
	0.5	-	-
164	1.0	++	++
	0.5	+	+

Note: C : Concentration ; SA: *Staphylococcus aureus*; EC: *Eschiricia coli*.

Lombine **166** is responsible for the antibacterial activity of the original extract as this alkaloid exhibited bactericidal activity against *Staphylococcus aureus* and *Escherichia coli* resulting in 94% and 95% cell death, respectively at a concentration of 0.5 mg/mL compared to crude extracts which only partially inhibited at the same concentration [83]. Voacristine **170** showed lower activity with 87% of bacterial cell death at 1.0 mg/mL [61].

Antifungal agents: Indonesian medicinal plants have also been prepared traditionally for fungi caused diseases [84, 85]. Investigations resulted in several species extracts showing significant activities (see Tables 11 and 12) [86, 87]. For example, the extracts of *Tylophora asthmatica*, *Phyllanthus acuminatus* Vahl. and *Swietenia mahogany* Jacq. contain potent compounds against *Phytium ultimum*. On the other hand, extracts of *Ipomoea spp* and *Swietenia mahogany* Jacq. gave significant inhibition against *Scleretonium rolfsii*. Other anti-fungal studies showed *Wangiellia dermatitidis* and *Microsporum gypseum* are more sensitive against Zingiberacea extracts.

In summary, this review has highlighted the investigations which studied 181 of around six thousand Indonesian medicinal plants which revealed 165 isolates. Some potent drug leads were isolated, eg β-sesquiphellandrene as an anti-rhinoviral agent, dehatrine, mataranine A and B as anti-malarial agents and lombine and LPM-574 as anti-bacterial agents. The remaining preliminary studies on these Indonesian medicinal plants indicate that they are prospective sources of potent anti-microbial constituents and require further studies to establish these outcomes.

Acknowledgments: ASN would like to thank the AusAID program and the University of Wollongong, Australia for ADS and HDR scholarships.

Table 11: Anti-yeast like fungal activity of extracts of medicinal plants [87].

Plants	Part of plant	Yeast like fungal species				
		CN	SC	CA	WD	AA
Zingiberaceae						
Alpinia galanga	ST				11.9± 0.8	
Alpinia galanga	RZ	10.2±0.7			27.3 ±7.5	9.5 ±0.0
Alpinia mutica	ST				8.2 ±0.7	
Curcuma zedoaria	RZ	14.8±2.1	13.3±0.2	13.5± 0.0	11.5 ±1.0	
Zingiber purpureum	RZ	11.4±0.5		9.3± 0.9	11.3± 1.2	11.1± 0.4
Zingiber officinale	RZ			11.5± 0.0		

CN: *Cryptococcus neoformans*; SC: *Sacharomyces cerevisiae*; CA: *Candida albicans*; WD: *Wangiellia dermatitidis*; AA: *Alternaria alternate*; ST: Stalk; RZ: Rhizome. All sample are ethanolic extracts; Antifungal activity showed by mean diameter (mean ± SE, mm) of fungal growth inhibition zones. Sterile paper disc (7.5 mm diameter) was impregnated into 0.2 mg/µL ethanolic extract for disk diffusion assays.

Table 12: Anti-filamentous fungal activity of extracts of medicinal plants [78,87].

Plants	Filamentous like fungal species									
	PU	RS	SR	AF	PP	FO	MG	PB	Rh	TM
Acanthaceae										
Graptophyllum pictum (L.) Griff.*	+	++	+		+					
Graptophyllum pictum (L.) Griff.**			+							
Apoctnaceae										
Alstonia scholaris (L.) R.Br.**	+		+							
Cantharanthus roseus (L.) G. Don**	+		+							
Asclepiadaceae										
*Tylophora asthmatica**					DG					

Table 12 (*Continued*): Anti-filamentous fungal activity of extracts of medicinal plants [78,87].

Plants	Filamentous like fungal species									
	PU	**RS**	**SR**	**AF**	**PP**	**FO**	**MG**	**PB**	**Rh**	**TM**
*Tylophora asthmatica***	+++									
Convolvulaceae										
*Ipomoea spp.**	++	+	+							
*Ipomoea spp.***	++	++	++++							
Combreatceae										
Terminalia catappa L.*	+	+	+	+						
Terminalia catappa L.**	++++				++					
Elaeocarpaceae										
Elaeocarpus grandiflorus S.*			+	+						
Elaeocarpus grandiflorus S.**	++									
Euphrobiaceae										
Phyllanthus acuminatus Vahl.*	++		+	++						
Phyllanthus acuminatus Vahl.**	+++	+								
Fabaceae										
Erythrina variegata L.*	+	+		+						
Antidesma bunius (L.) Spreng*		+	+	+						
Fibaceae										
Sesbania grandiflora Pers*	++			+						
Labiatae										
*Hyptis brevipes**	+	+	+	+	++					
Leguminosae										
Crotalaria juncea L.*		+			+					
Crotalaria juncea L.**	+									
Meliaceae										
Swietenia mahagoni Jacq.*	+++	++	+++							
Swietenia mahagoni L. (leaf)*				++	+					
Swietenia mahagoni L. (Pericarp)*	++	+	+	+						
Swietenia mahagoni L. (Pericarp)**		+		+						
Moringaceae										
Moringa oleifera Lam.**	+									
Rubiaceae										
Mussaenda pubescens Ait. f.**	+	++		+						
Morinda citrifolia L.*		+								
Morinda citrifolia L.**	++	+	+	+						
Zingiberaceae										
Alpinia galanga (ST)***								8.2		
*Alpinia galanga****				14.4		11.9	20.6	31.1	12.2	16.6
*Curcuma globosus****				9.5			25.3	9.5	14.2	9.5
*Curcuma zedoaria****							27.9	8.2		26.4
*Etlingera elatior ****							9.5			
*Etlingera littoralis****				8.8						
*Zingiber purpureum****				14.2		9.5	27.3	17.1	11.5	20.6
*Zingiber officinale****							20.8	12.2	8.8	18.2

PU: *Phytium ultimum*; RS: *Rhizoctonia solani*; SR: *Scleretonium rolfsii*; AF: *Aspergillus fumigatus*; PP: *Phytophthora parasitica*; FO: *Fusarium oxyporum*; MG: *Microsporum gypseum*; PB: *Pseudallescheria*; RH: *Rhizopus s*p; TM: *Trichophyton mentagrophytes*; DG: Decrease growth. All extracts were obtained from the aerial part of plants except, *S. Mahogany*, whose seed was used and the Zingiberaceae, whose rhizome was used unless stated as ST (stalk). * DCM extract; **MeOH extract; ***EtOH extract. Activity shown as symbol + 25% less than control, ++ equal to the control, +++, 25-50% more than control, ++++ 50% than control. In case of Zingiberaceae extracts, result was showed as mean diameter (in mm) of fungal growth inhibition zones. Sterile paper disc (7.5 mm diameter) was impregnated into 0.2 mg/µL ethanolic extract for disk diffusion assays.

References

[1] Younce WC. (**2001**) *Indonesia: issues, historical background and bibliography*. Nova Science Publishers, New York.
[2] Witton P, Elliott M, Greenway P, Jealous, V. (**2003**) *Indonesia*. Lonely Planet Publication, Singapore.
[3] Kitagawa I, Shibuya H. (**1995**) Pharmacochemical investigation of Indonesian medicinal plants. *Proceedings of the Phytochemical Society of Europe*, ***37***, 335-358.
[4] Government of the Republic of Indonesia - State Ministry for Environment. (**1997**) *Agenda 21-Indonesia. A national strategy for sustainable development*. Government of the Republic of Indonesia - State Ministry for Environment, Jakarta.
[5] Government of the Republic of Indonesia. (**1997**) *Country paper on the implementation of biodiversity management in Indonesia*. Government of the Republic of Indonesia, Jakarta.
[6] Beers S. (**2001**) *Jamu: The ancient art of herbal healing.* Periplus Editions, Singapore.
[7] Williamson E. (**2006**) Systems of traditional medicine from South and Sout East Asia. *The Pharmaceutical Journal*, ***276***, 539-540.
[8] Pols H. (**2010**) The triumph of jamu. *Inside Indonesia*, ***100***, 1-6.
[9] Susanto YW. (**1996**) *Sejarah jamu gendong*. Departemen Kesehatan Republik Indonesia, Jakarta.
[10] Kozok U. (**1999**) *Warisan leluhur: sastra lama dan aksara Batak*. Kepustakaan Populer Gramedia, Jakarta.
[11] Masyarakat Pernaskahan Nusantara. (**2000**) *Naskah sebagai sumber pengetahuan budaya : kumpulan makalah simposium internasional pernaskahan nusantara II, 26 November 1998*. Masyarakat Pernaskahan Nusantara, Jakarta.
[12] Ranggasutrasna KN, Pakubuwana V, Marsono D. (**1991**) *Centhini: Tambangraras-among raga*. Balai Pustaka, Jakarta.
[13] Tinggen IN. (**1996**) *Sarining usada bali pusaka leluhur*. Indra Jaya, Singaraja.
[14] Leaman DJ. (**1996**) *The medicinal ethnobotany of the Kenyah of East Kalimantan (Indonesian Borneo)*. Ph.D thesis, University of Ottawa, Canada, 1-385.

[15] Riswan S, Sangat-Rumantyo H. (**1991**) The opportunity, technical and practical constraints of the plant derived drug development in Indonesia, in *Workshop on drug development, biological diversity and economic growth*. Bethesda, MD, USA, 2-8.
[16] Stevensen C. (**1999**) Jamu: an Indonesian herbal tradition with a long past, a little known present and an uncertain future. *Complement Therapy Nursing and Midwifery*, ***5***, 1-3.
[17] Pols H. (**2009**) European physician and botanists, indigenous medicine in the Dutch East Indies, and colonial network of mediation. *East asia sciences, technology and society: an international journal*, ***3***, 173-208.
[18] Bontius J. (**1642**) *De medicina Indorum*. Francisus Hackius, Lugduni Batavorum, Leiden.
[19] Buenz EJ, Johnson HE, Beekman EM, Motley TJ, Bauer BA. (**2005**) Bioprospecting Rumphius's Ambonese Herbal: Volume I. *Journal of Ethnopharmacology*, ***96***, 57-70.
[20] Heyne K. (**1927**) *De nuttige planten van der Nederlansch Indie (revised and enlarged edition)*. Buitenzorg, Batavia.
[21] van Steenis-Kruseman MJ. (**1953**) Select Indonesian medicinal plants. *Organisation for Scientific Research in Indonesia Bulletin*, ***18***, 1-90.
[22] WHO. (**2000**) *General guidelines for methodologies on research and evaluation of traditional medicine*. World Health Organisation.
[23] Elliott S, Brimacombe J. (**1987**) The medicinal plants of Gunung Leuser National Park, Indonesia. *Journal of Ethnopharmacolgy*, ***19***, 285-317.
[24] Grosvenor PW, Supriono A, Gray DO. (**1995**) Medicinal plants from Riau province, Sumatra, Indonesia. Part 1: Uses. *Journal of Ethnopharmacology*, ***45***, 75-95.
[25] Grosvenor PW, Supriono A, Gray DO. (**1995**) Medicinal plants from Riau province, Sumatra, Indonesia. Part 2: Antibacterial and antifungal activity. *Journal of Ethnopharmacolgy*, ***45***, 97-111.
[26] Roosita K, Kusharto CM, Sekiyama M, Fachrurozi Y, Ohtsuka R. (**2008**) Medicinal plants used by the villagers of a Sundanese community in West Java, Indonesia. *Journal of Ethnopharmacolgy*, ***115***, 72-81.
[27] Balick MJ. (**1994**) Ethnobotany, drug developement and biodiversity conservation-exploring the linkages. In *Ethnobotany and the search for new drugs*. Chadwick, DJ, Marsh, J (Eds). John Wiley & Sons, England.
[28] Limananti AI, Triratnawati A. (**2003**) Jamu Cekok Components for Treating Children Have No Appetite: An Ethnomedicine Approach. *MAKARA of Health Series*, *7*, 11-20.
[29] Kurokawa M, Ochiai H, Nagasaka K, Neki M, Xu H, Kadota S, Sutardjo S, Matsumoto T, Namba T, Shiraki K. (**1993**) Antiviral traditional medicines against herpes simplex virus (HSV-1), poliovirus, and measles virus *in vitro* and their therapeutic efficacies for HSV-1 infection in mice. *Antiviral Research*, ***22***, 175-88.
[30] Morita H, Kishi E, Takeya K, Itokawa H, Tanaka O. (**1990**) New quassinoids from the roots of Eurycoma longifolia. *Chemical letter*, ***59***, 749-752.
[31] Hattori M, Nakabayashi T, Lim Y. (**1995**) Inhibitory effects of various Ayurvedic and Pnamanian medicinal plants on the infection of herpes simplex virus-1 *in vitro* and *in vivo. Phytoteraphy Research*, ***9***, 270-276.
[32] Fukuchi K, Sakagami H, Okuda T. (**1989**) Inhibition of herpes simplex virus infection by tannins and related compounds. *Antiviral research*, ***11***, 285-298.
[33] Lohezic-Le Devehat F, Bakhtiar A, Bezivin C, Amoros M, Shiraki K. (**1999**) Antiviral and cytotoxic activities of some Indonesian plants. *Fitoterapia*, ***73***, 400-405.
[34] Nawawi A, Nakamura N, Hattori M, Kurokawa M, Shiraki K. (**1999**) Inhibitory effects of Indonesian medicinal plants on the infection of herpes simplex virus type 1. *Phytotherapy Research*, ***13***, 37-41.
[35] Bauer DJ, Selway JWT, Batchelor JF, Tisdale M, Caldwell IC, Young DAB. (**1981**) 4',6-Dichloroflavan, a new antirhinovirus compound. *Nature*, ***292***, 369-370.
[36] Denyer CV, Jackson P, Loakes DM. (**1994**) Isolation of antirhinoviral sesquiterpenes from ginger *(Zingiber officinale). Journal of Natural Products*, ***57***, 658-662.
[37] Kusumoto IT, Kakiuchi N, Hattori M, Namba T, Sutardjo S, Shimotohno K. (**1992**) Screening of some Indonesian medicinal plants for inhibitory effects on HIV-1 protease. *Shoyakugaku Zasshi*, ***46***, 190-193.
[38] Otake T, Mori H, Morimoto M, Ueba N, Sutradjo S, Kusumoto IT, Hattori M, Namba T. (**1995**) Screening of Indonesian plant extracts for anti-human immunodeficiency virus type 1 (HIV-1) activity. *Phytotherapy Research*, ***9***, 6-10.
[39] Ma C, Nakabayashi T, Miyashiro H, Hattori M, El-Meckkawy S, Namba T, Shimohtohno K. (**1994**) Screening of traditional medicines for their inhibitory effects on human immunodeficiency virus protease. *Wakan Iyakugaku Zasshi*, ***11***, 416-417.
[40] Chen SX, Wan M, Loh BN. (**1996**) Active constituents against HIV-1 protease from *Garcinia mangostana. Planta Medica*, ***62***, 381-382.
[41] Satake T, Kamiya K, Saiki Y, Hama T, Fujimoto Y, Kitanaka S, Kimura Y, Uzawa J, Endang H, Umar M. (**1999**) Studies on the constituents of fruits of *Helicteres isora L. Chemical & Pharmaceutical Bulletin*, ***47***, 1444-1447.
[42] Tezuka Y, Terazono M, Kusumoto TI, Kawashima W, Hatanaka Y, Kadota S, Hattori M, Namba T, Kikuchi T, Tanaka K, Supriyatna S. (**1999**) Helisterculins A and B, 2 new (7.5',8.2')-neolignans, and helisorin, the 1st (6.4',7.5',8.2')-neolignan, from the Indonesian medicinal plant *Helicteres isora. Helvetica Chimica Acta*, ***82***, 408-417.
[43] Tezuka Y, Terazono M, Kusumoto TI, Hatanaka Y, Kadota S, Hattori M, Namba T, Kikuchi T, Tanaka K, Supriyatna S. (**2000**) Helicterins A - F, six new dimeric (7.5',8.2')-neolignans from the Indonesian medicinal plant *Helicteres isora. Helvetica Chimica Acta*, ***83***, 2908-2919.
[44] Murnigsih T, Subeki Matsuura H, Takahashi K, Yamasaki M, Yamato O, Maede Y, Katakura K, Suzuki M, Kobayashi S, Chairul, Yoshihara T. (**2005**) Evaluation of the inhibitory activities of the extracts of Indonesian traditional medicinal plants against *Plasmodium falciparum and Babesia gibsoni. The Journal of Veterinary Medical Science*, ***67***, 829-831.
[45] Mustofa ET, Sholikhah Wahyuono S. (**2007**) In vitro and invivo antiplasmodial activity and cytotoxicity of extracts of *Phyllanthus niruri L.* herbs traditionally used to treat malaria in Indonesia. *Southeast Asian Journal of Tropical Medicine and Public Health*, ***38***, 609-615.
[46] Fédération Internationale de Géomètres. (**2011**) *Facts about Indonesia*.

[47] Kitagawa I, Wei H, Nagao S, Mahmud T, Hori K, Kobayashi M, Uji T, Shibuya H. (**1996**) Indonesian medicinal plants. XIV. Characterization of 3'-*O*-caffeoylsweroside, a new secoiridoid glucoside, and kelampayosides A and B, two new phenolic apioglucosides, from the bark of *Anthocephalus chinensis* (Rubiaceae). *Chemical & Pharmaceutical Bulletin*, ***44***, 1162-1167.

[48] Garcia J, Lavaitte S, Gey C. (**1989**) 8-Epikingiside and its vanillate ester, isolated from *Gentiana pyrenaica. Phytochemistry*, ***28***, 2199-2201.

[49] El-Naggar LJ, Beal JL. (**1980**) Iridoids. A Review. *Journal of Natural Products*, ***43***, 649-707.

[50] Brown RT, Chapple CL, Lashford AG. (**1977**) Isolation of strictosidine (isovincoside) lactam from *Nauclea latifolia. Phytochemistry*, ***16***, 1619-1620.

[51] Brown RT, Warambwa BFM. (**1978**) Adina alkaloids: Isolation of desoxycordifoline. *Phytochemistry*, ***17***, 1686-1687.

[52] Unknown. (**1989**) Short Communication. *Phytochemistry*, ***28***, 2503-2504.

[53] Kitagawa I, Minagawa, K Zhang RS, Hori K, Doi M, Inoue M, Ishida T, Kimura M, Uji T, Shibuya H. (**1993**) Dehatrine, an antimalarial bisbenzylisoquinoline alkaloid from the Indonesian medicinal plant *Beilschmiedia madang,* isolated as a mixture of two rotational isomers. *Chemical & Pharmaceutical Bulletin*, ***41***, 997-999.

[54] Matsuura H, Takahashi K, Nabeta K, Yamasaki M, Maede Y, Katakura K. (**2007**) Screening of Indonesian Medicinal Plant Extracts for Antibabesial Activity and Isolation of New Quassinoids from *Brucea javanica. Journal of Natural Products*, ***70***, 1654-1657.

[55] Kitagawa I, Mahmud T, Simanjuntak P, Hori K, Uji T, Shibuya H. (**1994**) Indonesian medicinal plants. VIII. Chemical structures of three new triterpenoids, bruceajavanin A, dihydrobruceajavanin A, and bruceajavanin B, and a new alkaloidal glycoside, bruceacanthinoside, from the stems of *Brucea javanica* (Simaroubaceae). *Chemical & Pharmaceutical Bulletin*, ***42***, 1416-1421.

[56] Anderson LA, Harris A, Phillipson JD. (**1983**) Production of cytotoxic canthin-6-one alkaloids by *Ailanthus altissima* plant cell cultures. *Journal of Natural Products*, ***46***, 374-378.

[57] Lassak EV, Polonsky J, Jacquemin H. (**1977**) 5-Hydroxycanthin-6-one from *Simarouba amara. Phytochemistry*, ***16***, 1126-1127.

[58] Fukamiya N, Okano M, Aratani T, Negoro K, McPhail AT, Ju-Ichi M, Lee KH. (**1986**) Antitumor Agents, 79. Cytotoxic Antileukemic Alkaloids from *Brucea antidysenterica. Journal of Natural Products*, ***49***, 428-434.

[59] Hadi S, Bremner JB. (**2001**) Initial studies on alkaloids from Lombok medicinal plants. *Molecules*, ***6***, 117-129.

[60] Yamauchi T, Abe F. (**1998**) Regional differences of indole alkaloids in *Alstonia scholaris* and *Alstonia macrophylla*. In *Towards natural medicine research in the 21st century*. Ageta, H, Aimi, N, Ebizuka, Y, Fujita, T, Honda, G (Eds). Elsevier Science & Technology Books, 51-58

[61] Hadi S. (**2002**) *Bioactive Alkaloids from Medicinal Plants of Lombok*. PhD thesis, School of Chemistry, University of Wollongong, Wollongong.

[62] Abe F, Furukawa M, Nonaka G, Okabe H, Nishioka I. (**1973**) Studies on *Xanthoxylum spp.* I. Constituents of the root of *Xanthoxylum piperitum* DC. *Yakugaku Zasshi*, ***93***, 624-628.

[63] Ishii H, Chen IS, Akaike M, Ishikawa T, Lu ST. (**1982**) Studies on the chemical constituents of rutaceous plants. XLIV. the chemical constituents of *Xanthoxylum integrifolium* (Merr.) Merr. (*Fagara integrifoliola* Merr.). The constituents of the root wood. *Yakugaku Zasshi*, ***102***, 182-188.

[64] Shoji N, Umeyama A, Takemoto T, Kajiwara A, Ohizumi Y. (**1986**) Isolation of evodiamine, apowerful cardiotonic principle, from *Evodia rutaecarpa* benthan (Rutaceae). *Journal of Pharmaceutical Sciences*, ***75***, 612-613.

[65] Ren LJ, Xie FZ. (**1981**) Alkaloids from the root of *Zanthoxylum bangeaum* Maxim. *Yaoxue Xuebao*, ***16***, 672-677.

[66] Shibuya H, Takeda Y, Zhang RS, Tanitame A, Tsai YL, Kitagawa I. (**1992**) Indonesian medicinal plants. IV. On the constituents of the bark of *Fagara rhetza* (Rutaceae). (2). Lignan glycosides and two apioglucosides. *Chemical & Pharmaceutical Bulletin*, ***40***, 2639-2646.

[67] Shibuya H, Takeda Y, Zhang RS, Tong RX, Kitagawa I. (**1992**) Indonesian medicinal plants. III. On the constituents of the bark of *Fagara rhetza* (Rutaceae). (1): Alkaloids, phenylpropanoids, and acid amide. *Chemical & Pharmaceutical Bulletin*, ***40***, 2325-2330.

[68] Kardono LBS, Angerhofer CK, Tsauri S, Padmawinata K, Pezzuto JM, Kinghorn AD. (**1991**) Studies on Indonesian medicinal plants. IV. Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia. Journal of Natural Products*, ***54***, 1360-1367.

[69] Omar S, Zhang J, MacKinnon S, Leaman DJ, Durst T, Philogene BJR, Arnason JT, Sanches-Vindas PE, Poveda L, Tamez PA, Pezzuto JM. (**2003**) Traditionally-used antimalarials from Meliaceae. *Current Topics in Medicinal Chemistry*, ***3***, 137-139.

[70] Banskota AH, Attamimi F, Usia T, Linn TZ, Tezuka Y, Kalauni SK, Kadota S. (**2003**) Novel norcassane-type diterpene from the seed kernels of *Caesalpinia crista. Tetrahedron Letters*, ***44***, 6879-6882.

[71] Awale S, Linn TZ, Tezuka Y, Kalauni SK, Banskota AH, Attamimi F, Ueda J, Kadota S. (**2006**) Constituents of *Caesalpinia crista* from Indonesia. *Chemical & Pharmaceutical Bulletin*, ***54***, 213-218.

[72] Linn TZ, Awale S, Tezuka Y, Banskota AH, Kalauni SK, Attamimi F, Ueda J, Asih PBS, Syafruddin D, Tanaka K, Kadota S. (**2005**) Cassane- and Norcassane-type diterpenes from *Caesalpinia crista* of Indonesia and their antimalarial activity against the growth of *Plasmodium falciparum. Journal of Natural Products*, ***68***, 706-710.

[73] Kitagawa I, Simanjuntak P, Mahmud T, Kobayashi M, Fujii S, Uji T, Shibuya H. (**1996**) Indonesian medicinal plants. XIII. Chemical structures of caesaldekarins c, d, and e, three additional cassane-type furanoditerpenes from the roots of *Caesalpinia major* (Fabaceae). Several interesting reaction products of caesaldekarin a provided by N-bromosuccinimide treatment. *Chemical & Pharmaceutical Bulletin*, ***44***, 1157-1161.

[74] Kalauni SK, Awale S, Tezuka Y, Banskota AH, Linn TZ, Asih PBS, Syafruddin D, Kadota S. (**2006**) Antimalarial activity of cassane- and norcassane type diterpenes from *Caesalpinia crista* and their structure-activity relationships. *Biological & Pharmaceutical Bulletin*, ***29***, 1050-1052.

[75] Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. (**2003**) Effects of chloroquine on viral infections: an old drug against today's diseases. *The Lancet Infectious Diseases*, ***3***, 722-727.

[76] Kitagawa I, Mahmud T, Yokota K, Nakagawa S, Mayumi T, Kobayashi M, Shibuya H. (**1996**) Indonesian medicinal plants. XVII. Characterization of quassinoids from the stems of *Quassia indica. Chemical & Pharmaceutical Bulletin*, ***44***, 2009-2014.

[77] Sudirga SK. (**2004**) Pemanfaatan tumbuhan sebagi obat tradisional di Desa Trunyan Kecamatan Kintamani Kabupaten Bangli. *Journal of Enviroment*, ***4***, 1-12.
[78] Goun E, Cunningham G, Chu D, Nguyen C, Miles D. (**2003**) Antibacterial and antifungal activity of Indonesian ethnomedical plants. *Fitoterapia*, ***74***, 592-596.
[79] Sukari MA, Rashid MA, Tang NY, Rahmani M, Lajis NH, Kalid K, Yusuf UK. (**2008**) Chemical constituents and bioactivity of *Curcuma xanthorrhiza* roxb. *Journal of Ultra Scientist of Physical Sciences*, ***20***, 605-610.
[80] Kim JE, Kim HE, Hwang JK, Lee HJ, Kwon HK, Kim BI. (**2008**) Antibacterial characteristics of *Curcuma xanthorrhiza* extract on *Streptococcus* mutans biofilm. *Journal of Microbiology*, ***46***, 228-232.
[81] Zgoda JR, Freyer AJ, Killmer LB, Porter JR. (**2001**) Polyacetylene Carboxylic Acids from *Mitrephora celebica. Journal of Natural Products*, ***64***, 1348-1349.
[82] Zgoda-Pols JR, Freyer AJ, Killmer LB, Porter JR. (**2002**) Antimicrobial diterpenes from the stem bark of *Mitrephora celebica. Fitoterapia*, ***73***, 434-438.
[83] Hadi S, Bremner JB. (**2006**) Constituents of medicinal plants of lombok: a new antibacterial alkaloid from *Voacanga foetida* (Bl.) Rolfe. *Natural Product Communications*, ***1***, 825-829.
[84] Widyawati Subakir. (**2006**) *Comparison the effectiveness of 2% Alpinia galanga with 2% ketokonazole in vitro to the growth of Malassezia furfur in Pitriasis versicolor*. Hns thesis, Department of Microbiology, Diponegoro University, Semarang.
[85] Filet GJ. (**1855**) *De planten in den botanischen tuin bij het Groot militair hospitaal te Weltevreden: hunne inl. benamingen, groeiplaats en gebruik.* Naar de beste schrijvers en inlandsche opgaven zamengesteld.
[86] Omar S, Lemonnier B, Jones N, Ficker C, Smith ML, Neema C, Towers GH, Goel K, Arnason, JT. (**2000**) Antimicrobial activity of extracts of eastern North American hardwood trees and relation to traditional medicine. *Journal of Ethnopharmacology*, ***73***, 161-170.
[87] Ficker CE, Smith ML, Susiarti S, Leaman DJ, Irawati C, Arnason, JT. (**2003**) Inhibition of human pathogenic fungi by members of Zingiberaceae used by the Kenyah (Indonesian Borneo). *Journal of Ethnopharmacology*, ***85***, 289-293.

Natural Product Communications

2011 Volume 6

Natural Product Communications 6 (1-12) 1-1968 (2011)

ISSN 1934-578X (print)
ISSN 1555-9475 (online)

NPC Natural Product Communications

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