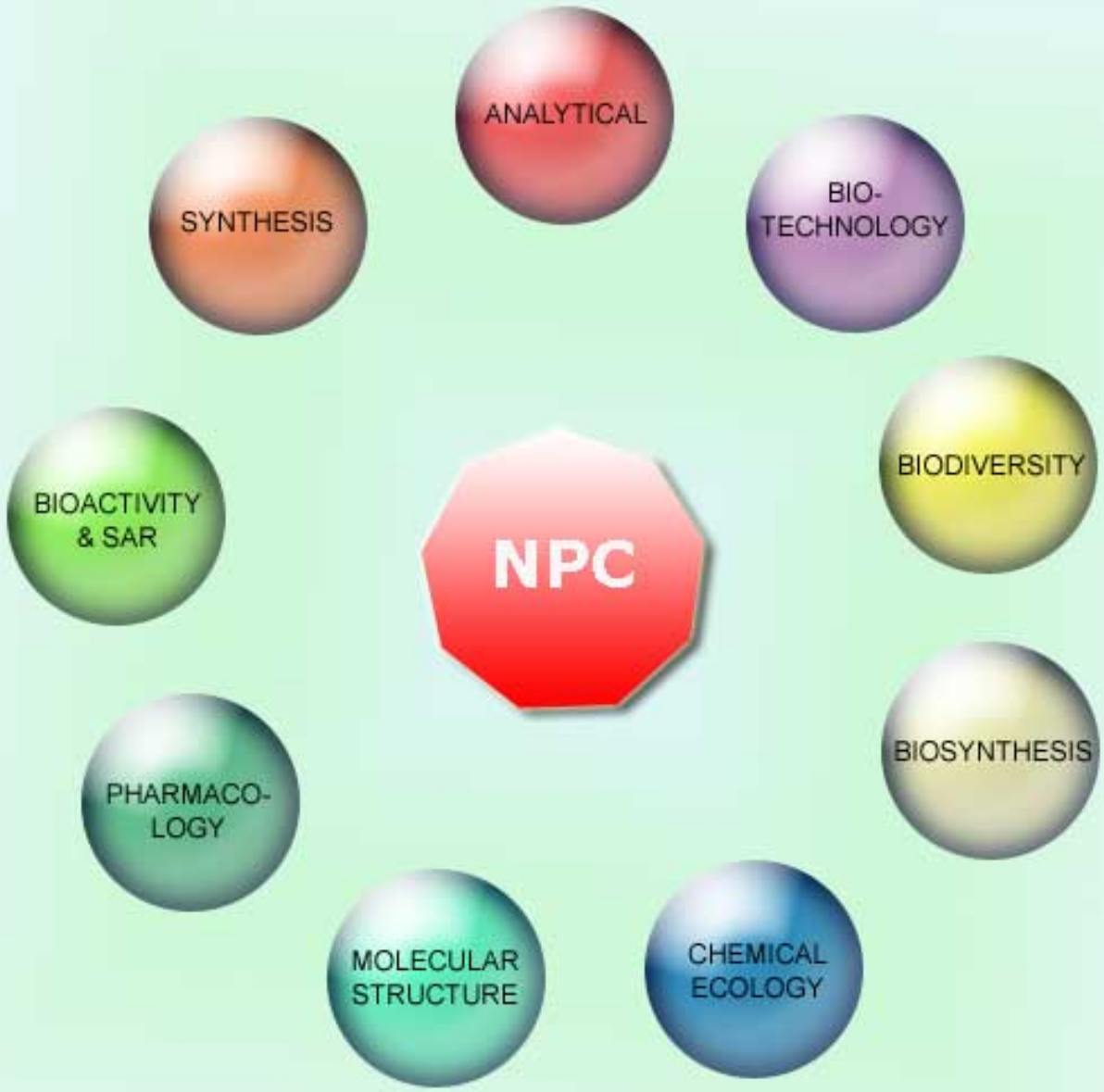
NATURAL PRODUCT COMMUNICATIONS

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



This Issue is Dedicated to the Memory of Professor Ivano Morelli

Volume 1. Issue 12. 2006
ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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Natural Product Communications Vol. 1 (12) 2006
Published online (www.naturalproduct.us)

Editorial

Special Issue in Memory of Professor Ivano Morelli

The December issue of *Natural Product Communications* is dedicated to Professor Ivano Morelli, an inspiration to scientists, young and old. The more enjoyable element of publishing this memorial issue comes from the recognition and tribute made to an exceptional individual by the many former students and colleagues who are authors of the papers in this issue. Indeed, Professor Morelli has played a key role in the development of phytochemical research, not only within the Italy, but also in the international arena.

Many thanks go to the authors, who have contributed some of their finest work to a timetable that has been quite challenging, and to the manuscript reviewers for their help in making this issue a success.

Finally, the editors would like to pay their tribute to the scholarly, scientific, and mentorship roles of Professor Ivano Morelli. Truly, his pursuit of scientific knowledge concerning organic chemistry and his dedication to his students and colleagues make him an exceptional individual worthy of our admiration, emulation, and the homage being paid to him in these pages of *Natural Product Communications.*

Pawan K. Agrawal
Editor-in-Chief

Natural Product Communications Vol. 1 (12) 2006
Published online (www.naturalproduct.us)

Obituary

Ivano Morelli
(1940-2005)

Prof. Ivano Morelli was born March 11, 1940, in Pisa, Italy. He graduated from Pisa University in 1965 and received an Assistant Professor position in Organic Chemistry in 1966. From 1980 until his death he was Full Professor of Phytochemistry at the Faculty of Pharmacy, Pisa University.

During his career he was appointed as Director of Bioorganic and Biopharmacy Department since 1986 when it was called Institute of Organic Chemistry; in the last years he was also member of the Academic Senate of Pisa University. He served the Italian Society of Phytochemistry as President in the years 1987-1989 and 2004-2005. He was also the Director of the School of Science and Technology of Medicinal Plants of Pisa University from 1985 until 2001 (the year of its disactivation) and Director of several PhD courses during the last 10 years.

His chemical work focused on the study of mosses and reaction mechanisms of epoxides and dibromocyclohexanes during the years 1966-1978; chemical studies of Mediterranean plants belonging to Rosaceae, Apiaceae, and Asteraceae families (1978-1990); studies of plants belonging to developing countries of Latin America, Africa, and Asia (1990-2005); analysis of essential oils of Apiaceae, Asteraceae, and Lamiaceae from 1990 until 2005.

We remember him as a very active scientist and an eclectic man: a man of few words, but open-hearted, honest, concrete, and fair. The wellbeing of his collaborators, students, and friends was for him one of the most important thoughts. The door of his office was never closed and he knew how to listen the requests of everyone. He liked to give suggestions, but at the same time, he knew how to ask suggestions, in a mutual opinions exchange. His open-mindedness, curiosity, diplomacy, and passion brought him far, to Asia, Europe, and America, taking his scientific cooperation and opening his laboratory to many young researchers from different countries. He gives the opportunity to the young scientists to work without any restriction, giving hospitality to any people and to their opinions, since he was researcher of all the "human activities". His contribution to the knowledge of the plants belonging to Italian and international flora is great but his love for the teaching, the research, the University, and the co-workers was great.

An inexorable leukemia took him far from his dear the 18 April 2005. He is survived by his wife, Iolena, and two sons, Marco and Ilaria. His love for the phytochemistry was stronger than the fear of his disease; he continued to be present among us even if he was far from the Department, during his hard time in the hospital. He had never stopped to fight: his brave heart was a daily teaching. The death obliged him to rest but never the death prevented him from teaching us his last lesson of life.

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Volume 1, NUMBER 12

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Triterpenes from *Maytenus macrocarpa* and Evaluation of Their Anti-HIV Activity

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Received: September 8th, 2006; Accepted: November 2nd, 2006

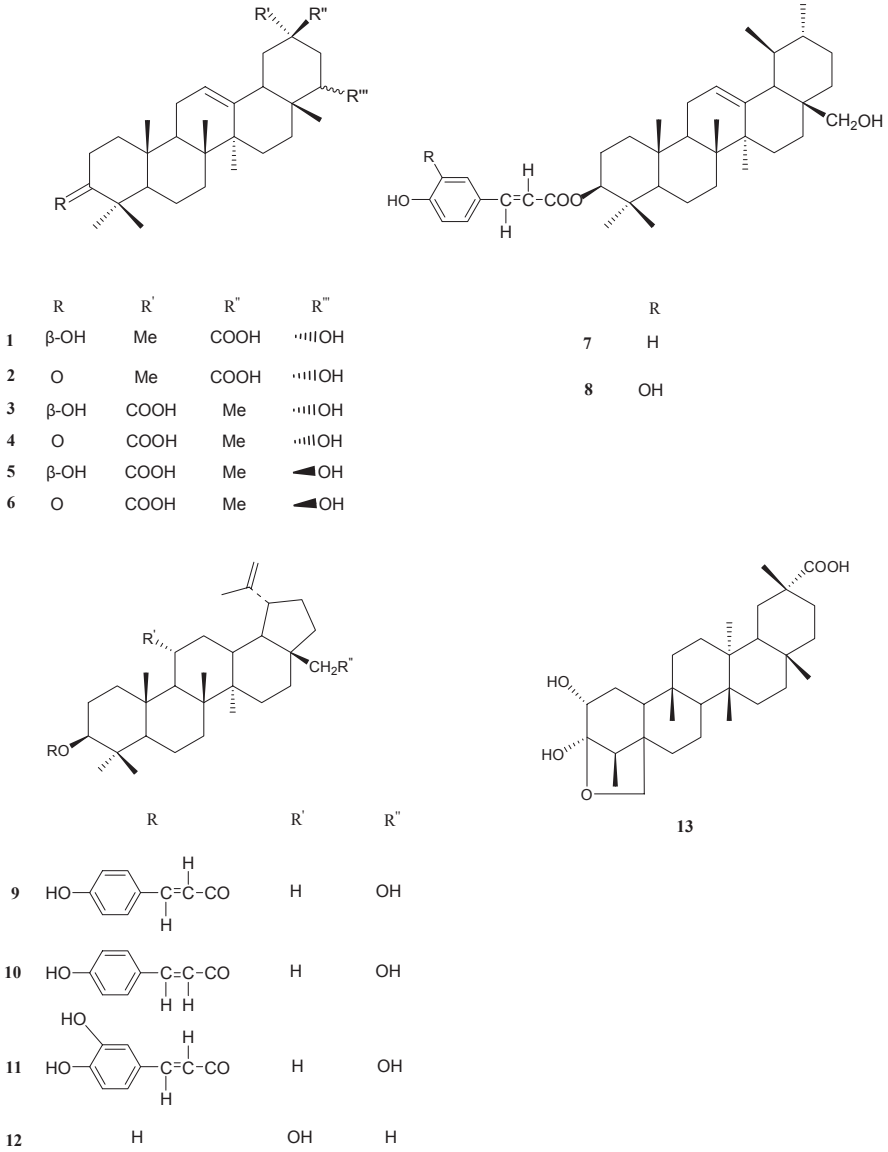
Dedicated to the memory of Professor Ivano Morelli.

A set of pentacyclic triterpenes has been isolated from the bark of *Maytenus macrocarpa* (Celastraceae). It includes two new olean-12-ene derivatives, namely 3β, 22α-dihydroxy-olean-12-en-30-oic acid (**1**) and 22α-hydroxy-olean-12-en-3-oxo-30-oic acid (**2**), and the new urs-12-ene derivative 3-(*E*)-coumaroyluvaol (**7**), along with 10 known compounds possessing olean-12-ene, urs-12-ene, lupane and friedelane skeletons. The structures have been elucidated by extensive spectroscopic methods including 1D- and 2D-NMR experiments, as well as ESI-MS analysis. All isolated compounds were tested for anti-HIV activity in C8166 cells infected with HIV-1_{MN}. The most active compound was 22α-hydroxy-12-en-3-oxo-29-oic acid (triterpenonic acid A, **4**), with an EC_{50} value of 1 μg/mL and a selectivity index of 35.

Keywords: *Maytenus macrocarpa*, Celastraceae, pentacyclic triterpenes, anti-HIV activity.

Species belonging to the genus *Maytenus* (Celastraceae) have been used as a traditional medicine in the Amazonian region against cancer, rheumatism, and inflammation [1-2]. *M. macrocarpa* (R & P) Briquet is endemic to the Amazonian region of Peru and an alcoholic infusion of its bark is used, generally in "aguardiente", for the treatment of rheumatism, influenza, gastrointestinal diseases, and as an antitumor agent for skin cancer [3]. In previous papers, dammarane [3] and friedelane triterpenes [4] from the stem bark exudates, sesquiterpene polyol esters from the leaves [5], and the nortriterpenes macrocarpins A-D from the roots [6] of *M. macrocarpa* have been reported. Furthermore a dihydro-β-agarofuran sesquiterpene isolated from the roots of *M. macrocarpa* has been reported to act as a modulator of daunomycin resistance in a multidrug-resistant *Leishmania tropica* line. [7].

Here we report the isolation and characterization of three new triterpenes from the bark of *M. macrocarpa*, namely 3β, 22α-dihydroxy-12-en-30-oic acid (**1**), 22α-hydroxy-12-en-3-oxo-30-oic acid (**2**) and 3-(*E*)-*p*-coumaroyluvaol (**7**), along with the known olean-12-ene derivatives maytenfolic acid (**3**), triptotriterpenonic acid A (**4**), 22-*epi*-maytenfolic acid (**5**), 22-*epi*-triptotriterpenonic acid A (**6**), the urs-12-ene derivative 3-(*E*)-caffeoyluvaol (**8**), the lupane-type triterpenes 3-(*E*)-*p*-coumaroylbetulin (**9**), 3-(*Z*)-*p*-coumaroylbetulin (**10**), 3-(*E*)-caffeoylbetulin (**11**), nepeticin (**12**), and friedelane orthosphenic acid (**13**). On the basis of the anti-HIV activity reported for triterpenes closely related to compounds **1-13** isolated from *M. macrocarpa* [8-10], the inhibitory activity of these compounds against HIV-1 replication in acutely infected C8166 cells has been evaluated.



	R	R'	R"	R'''
1	β-OH	Me	COOH	OH
2	O	Me	COOH	OH
3	β-OH	COOH	Me	OH
4	O	COOH	Me	OH
5	β-OH	COOH	Me	OH
6	O	COOH	Me	OH

	R
7	H
8	OH

13

	R	R'	R"
9	HO-C=C-CO	H	OH
10	HO-C=C-CO	H	OH
11	HO, HO-C=C-CO	H	OH
12	H	OH	H

Figure 1: Compounds **1-13** isolated from the bark of *Maytenus macrocarpa*.

Column chromatography of a $CHCl_3$ extract of the bark of *M. macrocarpa* on silica gel, yielded compounds **1**-**13**.

Compound **1** was obtained as an amorphous white solid, which showed in the ESI-MS in positive ion mode the quasi-molecular ion $[M+H]^+$ at *m/z* 473, corresponding to the molecular formula $C_{30}H_{48}O_4$. The ^{13}C NMR spectrum showed signals for 30 carbons. The ^1H NMR spectrum showed seven tertiary methyl singlets at δ 0.82, 0.94, 1.00, 1.03, 1.05, 1.26, and 1.33. The presence of two secondary alcoholic functions was evident from the signals at δ 3.19 (1H, dd, *J* = 3.5 and 11.5 Hz) and δ 3.62 (1H, dd, *J* = 3.0 and 11.0 Hz), which correlated in the HSQC spectrum with the carbon resonances at δ 79.7 and 78.8, respectively. An additional feature of the ^1H NMR spectrum was a signal at δ 5.24 (1H, t, *J* = 3.5 Hz) typical of H-12 of a Δ-12 oleanene. Analysis of ^1H and ^{13}C NMR spectroscopic data and comparison with those of 3β,22α-dihydroxy-12-en-29-oic acid (**3**), known as maytenfolic acid [11], suggested that the difference between the two compounds should be confined to ring E [12] [δ 39.5 (C-17), 49.9 (C-18), 44.3 (C-19), 46.2 (C-20), 40.7 (C-21), 78.8 (C-22) 34.1 (C-29), 181.1 (C-30) in **1**; δ 39.9 (C-17), 48.3 (C-18), 42.3 (C-19), 45.2 (C-20), 38.9 (C-21), 76.9 (C-22), 180.6 (C-29), 21.6 (C-30) in **3**]. Comparison of the ROESY spectra of **1** and **3**

allowed us to clarify the difference between the two compounds. While, in the case of **3**, the ROESY spectrum showed correlations between the signal at δ 1.22 (Me-30) and the signals at δ 2.36 (H-18), 3.52 (H-22), and 1.01 (Me-28), these effects were absent in the ROESY spectrum of **1**, where the signal at δ 1.26 (Me-29) showed a correlation with that at δ 1.33 (Me-27). This evidence led to the conclusion that **1** was 3β,22α-dihydroxy-olean-12-en-30-oic acid, which was named macrocarpoic acid A.

Compound **2** showed, in the ESI-MS in positive ion mode, the quasi-molecular ion $[M+H]^+$ at *m/z* 471, corresponding to the molecular formula $C_{30}H_{46}O_4$. The compound exhibited, in its ^1H NMR spectrum, seven singlet methyl groups (δ 0.95, 1.09, 1.11 x 2, 1.13, 1.26, 1.33), one olefinic proton (δ 5.25), and one methine proton (δ 3.63) linked to an oxygen bearing carbon. In the ^{13}C NMR spectrum the occurrence of signals for 30 carbons, including peaks at δ 123.4, 143.5, 181.0 and 219.0, suggested that **2** was an oxoolean-12-enoic acid [13]. By comparison of NMR data of **2** with those of **1** it was speculated that the position of the carbonyl group (δ 219.0) was at C-3 on the basis of the absence in **2** of the signals at δ 3.19 in the ^1H NMR spectrum and 79.7 in the ^{13}C NMR spectrum, observed for **1**. It was confirmed by the long range correlations observed in the HMBC spectrum between the proton signals at δ 1.09 (Me-23) and 1.11 (Me-24) and the carbon resonance at δ 219.0 (C-3). Thus **2** was assigned as 22α-hydroxy-olean-12-en-3-oxo-30-oic acid, and named macrocarpoic acid B.

The molecular formula $C_{39}H_{56}O_4$ was assigned to compound **7** on the basis of the ESI-MS spectrum, which showed the quasi-molecular ion peak $[M+H]^+$ at *m/z* 589. The ^1H NMR spectrum showed seven methyl groups at δ 0.88 (d, *J* = 6.5 Hz), 0.96 (s), 0.97 (d, *J* = 6.5 Hz), 1.01 (s), 1.08 (s), 1.09 (s), and 1.17 (s), two signals ascribable to a primary alcoholic function at δ 3.08 and 3.59 (each 1H, d, *J* = 11.0 Hz), and a signal for a methine proton linked to an oxygen-bearing carbon at δ 4.58 (dd, *J* = 3.5 and 11.0 Hz). Further features were signals due to three olefinic protons at δ 5.21 (t, *J* = 3.5), 6.32 (d, *J* = 15.9 Hz), and 7.64 (d, *J* = 15.9 Hz), and signals at δ 6.83 (2H, d, *J* = 8.0 Hz) and 7.50 (2H, d, *J* = 8.0 Hz), typical of a 1,4-disubstituted aromatic ring. The ^{13}C NMR spectroscopic data of **7** were similar to those of urs-12-en-3β,28-diol, known as uvaol [14], except for the chemical shift of C-3 and the occurrence of an acyl moiety identified as (*E*)-*p*-coumaroyl. In the HMBC spectrum, the proton at δ 4.58 (H-3) correlated with the carbon resonance at δ 167.2 (C=O), indicating that the (*E*)-coumaroyl group was located at C-3. Thus compound **7** was identified as 3-(*E*)-coumaroyluvaol, and named macrocarpol A.

The known compounds maytenfolic acid (**3**) [11], triptotriterpenonic acid A (**4**) [15], 22-*epi*-maytenfolic acid (**5**) [16], 22-*epi*-triptotriterpenonic acid A (**6**) [16], 3-(*E*)-caffeoyluvaol (**8**) [14], 3-(*E*)-*p*-coumaroylbetulin (**9**) [17], 3-(*Z*)-*p*-coumaroylbetulin (**10**) [18], 3-(*E*)-caffeoylbetulin (**11**) [18], nepeticin (**12**) [12], and orthosphenic acid (**13**) [12] were identified by comparison of their spectroscopic data with those reported in the literature.

The co-occurrence in *M. macrocarpa* of triterpenes belonging to different classes is an unusual finding. For maytenfolic acid (**3**), previously isolated from *M. diversifolia* [11], antileukemic activity has been reported [11], while compounds **9**-**11**, betulin esters, have been reported to exert antitumor promoting activity [19]. Compounds **7**-**8** are esters of uvaol, for which antiproliferative and antileukemic activity have been reported [20].

Table 1: Anti-HIV activity of compounds **1**-**13**.

Compounds	EC_{50}[a]	TC_{50}[b]
1	10	50
2	10	50
3	10	80
4	1	35
5	100	200
6	50	100
7	10	50
8	10	100
9	12	62
10	10	25
11	5	50
12	10	50
13	10	80

EC_{50}[a] = concentration (μg/mL) that reduced by 50% the production of gp120 in infected C8166 cells. TC_{50}[b] = concentration (μg/mL) that caused 50% cytotoxicity to uninfected C8166 cells.

On the basis of the anti-HIV activity reported for the lupane derivative betulinic acid [8-9], and, to a minor extent, for salaspermic acid, a friedelane derivative closely related to orthosphenic acid [10], the anti-HIV activity of compounds **1**-**13** in C8166 cells infected with HIV-1_{MN} was tested. The most active compound was **4**, with an EC_{50} value of 1μg/mL and a selectivity index above 30 (Table 1). The investigation of the mechanism of action of **4** revealed that it was more effective when added either

prior to or at the time of virus infection. Further experiments confirmed that it inhibited the binding of gp120 to sCD4 in a dose dependent manner. At a concentration corresponding to its EC_{50}, compound **4** inhibited the interaction between gp120 and CD4 by 55%.

The activity exerted by betulin esters (**9**-**11**) is very much lower than that reported for betulinic acid and its derivatives by Hashimoto *et al*. [8]. Also orthosphenic acid (**13**) showed an anti-HIV activity lower than that reported for salaspermic acid [10], from which it differs only by the occurrence of a further α-OH group at C-2.

Experimental

General procedures: Optical rotations were measured on a Jasco DIP 1000 polarimeter. UV spectra were obtained on a Beckman DU 670 spectrometer. IR measurements were obtained on a Bruker IFS-48 spectrometer. Melting points were determined using a Bausch & Lomb apparatus. Accurate molecular weights were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18-39) at 2465.1989 Da and Angiotensin III at 931.5154 Da as internal standards. ESI-MS analyses were performed using a ThermoFinnigan LCQ Deca XP Max ion trap mass spectrometer equipped with Xcalibur software. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D-NMR spectra were acquired in CD_3OD. Standard pulse sequence and phase cycling were used for DQF-COSY, HSQC, HMBC and ROESY spectra. TLC was performed on silica gel F254 (Merck) plates, and reagent grade chemicals (Carlo Erba) were used throughout.

Plant material: *Maytenus macrocarpa* was collected in the Ucayali Region (Pucallpa), Peru. A voucher specimen is deposited at the Department of Pharmaceutical Sciences, Salerno, Italy.

Extraction and isolation: The dried and powdered bark of *M. macrocarpa* (310 g) was defatted with light petroleum and then extracted by maceration at room temperature with $CHCl_3$ until exhaustion. The $CHCl_3$ extract was concentrated under reduced pressure to a syrupy consistency (2.5 g). The crude extract was chromatographed on a silica gel column using $CHCl_3$ and increasing amounts of MeOH (up to 20%). After monitoring by TLC [Si gel plates, $CHCl_3$-MeOH (9:1)], the fractions were combined to give A (350 mg), B (150 mg), C (200 mg) and D (280 mg). Fractions A-D were submitted to HPLC on a μ-Bondapack C-18 column (30 cm x 7.8 mm i.d., flow rate 2.0 mL/min) using MeOH: H_2O in the ratio 85:15 for A-C and 3:1 for D (isocratic conditions). Pure **11** (12 mg, Rt = 12 min), **9** (8 mg, Rt = 15 min) and **10** (6 mg, Rt = 19 min) were obtained from A; **12** (9 mg, Rt = 24 min) from B; **7** (5 mg, Rt = 20 min), **8** (8 mg, Rt = 19 min) from C; **1** (7 mg, Rt = 8 min), **2** (5 mg, Rt = 12 min), **3** (12 mg, Rt = 10 min), **4** (10 mg, Rt = 15 min), **5** (7 mg, Rt = 11 min), **6** (9 mg, Rt = 16.5 min), and **13** (18 mg, Rt = 7 min) from D.

Antiviral assays: The anti-HIV activity and toxicity of compounds **1**-**13** were assessed in C8166 cells infected with HIV-1_{MN}. Cells were cultured in RPMI 1640 with 10% fetal calf serum. Forty-thousand cells per microtiter plate well were mixed with 5-fold dilutions of compounds prior to addition of 10 $CCID_{50}$ units of virus and incubated for 5-6 days. Formation of syncytia was examined from 2 days post-infection. The inhibition of HIV-infection was determined by examining syncytia, by estimating antigen gp120 by ELISA, and by measuring cell viability for virus-infected cells and uninfected cell controls using the XTT-formazan method.

Virus infectivity assay: The total progeny virus was titrated in microtiter plates using double dilutions of freshly collected supernatants and C8166 cells. The end point was determined by examining syncytia formation and by the XTT-formazan method. Virus titer ($CCID_{50}$) is expressed as the reciprocal of the dilution that gave a 50% end point. To measure the effects of compounds on virus infectivity, HIV-1_{MN} (10^4-10^5 $CCID_{50}$) was incubated with test compound at 37°C for 1h, the virus was serially diluted, and the infectivity end-point determined.

Gp120-sCD4 interaction assay: Gp120-sCD4 interaction was measured by ELISA; sCD4 was bound to microtiter plate wells at a concentration of 0.05 μg/well. Various dilutions of compounds were mixed with equal volumes of recombinant gp120 (0.04 μg/mL) and added to CD4 coated wells. After incubation at 37°C for 3-5 h, the binding of gp120 was detected using human anti-HIV serum and anti-human Ig conjugated to horseradish peroxidase.

Using WIACALC (Pharmacia LKB), the percent inhibition was calculated from linear logarithmic plots using three concentrations of gp120 alone as standard.

Macrocarpoic acid A (**1**)

MP: 272-274ºC.
$[\alpha]_D$: +48.2º (*c* 0.1, $CHCl_3$).
Rf : 0.6 ($CHCl_3$-MeOH, 9:1).
IR (KBr): 3480, 2970-2880, 1690, 1450, 1360, 1230, 1070 cm^{-1}.
^1H NMR (600 MHz, MeOH): 0.82 (3H, s, Me-23), 0.94 (3H, s, Me-28), 1.00 (3H, s, Me-25), 1.03 (3H, s, Me-24), 1.05 (3H, s, Me-26), 1.26 (3H, s, Me-29), 1.33 (3H, s, Me-27), 3.19 (1H, dd, *J* = 3.5 and 11.5 Hz), 3.62 (1H, dd, *J* = 3.0 and 11.0 Hz), 5.24 (1H, t, *J* = 3.5).
^{13}C NMR (150 MHz MeOH): 15.7 (CH_3, C-25), 16.1 (CH_3, C-24), 17.3 (CH_3, C-26), 19.0 (CH_2, C-6), 24.4 (CH_2, C-11), 25.8 (CH_3, C-28), 26.3 (CH_3, C-27), 26.5 (CH_2, C-15), 28.2 (CH_2, C-2), 28.3 (CH_3, C-23), 29.0 (CH_2, C-16), 33.4 (CH_2, C-7), 34.1 (CH_3, C-29), 37.8 (C, C-10), 38.9 (CH_2, C-1), 39.5 (C, C-17), 40.1 (C, C-4), 40.7 (CH_2, C-21), 41.2 (C, C-8), 43.5 (C, C-14), 44.3 (CH_2, C-19), 46.2 (C, C-20), 48.9 (CH, C-9), 49.9 (CH, C-18), 56.5 (CH, C-5), 78.8 (CH, C-22), 79.6 (CH, C-3), 123.5 (CH, C-12), 144.5 (C, C-13), 181.1 (C, C-30).
ESI-MS: 473 [M + H^+] , 495 [M + Na^+] .
HRMS-MALDI: *m/z* [M + Na^+] calcd for $C_{30}H_{48}NaO_4$ 495.3450, found 495.3458.

Macrocarpoic acid B (**2**)

MP: 264-266ºC.
$[\alpha]_D$: +76.2º (*c* 0.1, $CHCl_3$).
Rf : 0.7 ($CHCl_3$-MeOH, 9:1).
IR (KBr): 3450, 2980-2840, 1730, 1710, 1450, 1380, 1230, 1120 cm^{-1}.
^1H NMR (600 MHz, MeOH): 0.95 (3H, s, Me-28), 1.09 (3H, s, Me-24), 1.11 (6H, s, Me-23, Me-26), 1.13 (3H, s, Me-25), 1.26 (3H, s, Me-29)1.33 (3H, s, Me-27), 3.63 (1H, dd, *J* = 3.0 and 11.0 Hz), 5.25 (1H, t, *J* = 3.5).
^{13}C NMR (150 MHz MeOH): 15.5 (CH_3, C-25), 17.2 (CH_3, C-26), 20.3 (CH_2, C-6), 21.7 (CH_3, C-24), 24.0 (CH_2, C-11), 25.8 (CH_3, C-28), 26.2 (CH_3, C-27), 26.3 (CH_2, C-15), 26.6 (CH_3, C-23), 26.7 (CH_2, C-7), 29.0 (CH_2, C-16), 34.3 (CH_3, C-29), 35.0 (CH_2, C-2), 37.8 (C, C-10), 39.5 (C, C-17), 40.1 (CH_2, C-1), 40.6 (CH_2, C-21), 40.5 (C, C-8), 43.4 (C, C-14), 44.2 (CH_2, C-19), 46.0 (C, C-20), 48.0 (C, C-4), 47.5 (CH, C-9), 49.8 (CH, C-18), 56.0 (CH, C-5), 78.7 (CH, C-22), 123.4 (CH, C-12), 143.5 (C, C-13), 181.0 (C, C-30), 219.0 (C, C-3).
ESI-MS: 471 [M + H^+] , 493 [M + Na^+] .
HRMS-MALDI: *m/z* [M + Na^+] calcd for $C_{30}H_{46}NaO_4$ 493.3294, found 493.3301.

Macrocarpol A (**7**)

MP: 310-312ºC.
$[\alpha]_D$: +42.0º (*c* 0.1, $CHCl_3$).
Rf : 0.7 ($CHCl_3$-MeOH, 9:1).
IR (KBr): 3440, 2930, 1680, 1600, 1188 cm^{-1}.
^1H NMR (600 MHz, MeOH): 0.88 (3H, s, Me-29), 0.96 (3H, s, Me-23), 0.97 (3H, s, Me-30), 1.01 (3H, s, Me-24), 1.08 (3H, s, Me-25), 1.09 (3H, s, Me-26), 1.17 (3H, s, Me-27), 3.08 (1H, d, *J* = 11.0 Hz, H-28a), 3.59 (1H, d, *J* = 11.0 Hz, H-28b), 4.58 (1H, dd, *J* = 3.5, 12.0 Hz), 5.21 (1H, t, *J* = 3.5, H-12), 6.32 (1H, d, *J* = 15.9), 6.83 (1H, d, *J* = 8.0, H-3', H-5'), 7.50 (1H, d, *J* = 8.0, H-2', H-6'), 7.64 (1H, d, *J* = 15.9),
^{13}C NMR (150 MHz MeOH): 16.3 (CH_3, C-25), 17.3 (CH_3, C-26), 17.5 (CH_3, C-24), 17.8 (CH_3, C-29), 18.8 (CH_2, C-6), 21.6 (C, C-30), 23.9 (CH_3, C-27), 24.1 (CH_2, C-16), 24.2 (CH_2, C-11), 25.9 (CH_2, C-2), 26.7 (CH_2, C-15), 28.3 (CH_3, C-23), 32.2 (CH_2, C-21), 34.3 (CH_2, C-7), 36.5 (CH, C-22), 37.7 (C, C-17), 38.0 (C, C-10), 38.7 (C, C-4), 39.8 (CH_2, C-1), 40.7 (CH, C-20), 41.3 (CH, C-8), 41.4 (CH, C-19), 43.3 (C, C-14), 49.4 (CH, C-9), 55.3 (CH, C-18), 56.7 (CH, C-5), 70.5 (CH_3, C-28), 115.8 (CH, C-3', C-5'), 116.4 (CH, C-8'), 125.1 (CH, C-12), 127.3 (C, C-1'), 129.5 (CH, C-2', C-6'), 140.8 (C, C-13), 143.8 (CH, C-7'), 157.4 (C, C-4') 167.2 (C, C-9').
ESI-MS: 589 [M + H^+] , 611 [M + Na^+] .
HRMS-MALDI: *m/z* [M + Na^+] calcd for $C_{39}H_{56}NaO_4$ 611.4076, found 611.4082.

Acknowledgments - The authors thanks Dr Juan de Dioz Zuniga Quiroz of Agro Selva Zuniga, Parque Caceres Dorregaray 86-C Pueblo Libre, Lima 21, Peru, for providing the plant material.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1079 - 1084

New Oxidized 4-Oxo Fatty Acids from *Hygrophorus discoxanthus*

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Received: July 24th, 2006; Accepted: August 28th, 2006

Dedicated to the memory of Professor Ivano Morelli.

The results are reported from the first investigation of the secondary metabolites of the basidiomycete *Hygrophorus discoxanthus* (Fr.) Rea. Five new oxidized 4-oxo fatty acids (C_{16}, C_{18}) were isolated from the fruiting bodies and their structures established on the basis of their spectroscopic data and an ozonolysis experiment. Preliminary data indicate a moderate fungicidal activity, suggesting a possible function of these acids as chemical deterrents against mushroom parasites and predators.

Keywords: *Hygrophorus discoxanthus*, Basidiomycetes, 4-oxo-fatty acids, fungicidal activities.

In a search for new prototype (bioactive) agents from higher mushrooms (Basidiomycetes) [1], we were attracted by the species *Hygrophorus discoxanthus* (Fr.) Rea (fam. Hygrophoraceae) [2]. This is a mycorrhizal fungal species, growing solitary, scattered to gregarious in hardwood forests, particularly in the presence of *Fagus* trees, and fruiting in the fall. It is easily recognized by a whitish, viscid cap, with an ochreous-brown border, hence the name, and by the widely spaced, cream colored decurrent gills, turning rust-colored on rubbing. Our own field observations revealed that the fruiting bodies of *H. discoxanthus* are hardly ever attacked by either insects or parasitic fungi.

Fungicidal 4-oxo-2-alkenoic fatty acids were recently isolated from *H. eburneus* (Bull.: Fr.) Fr. [3], and related cyclopentenone and cyclopentenedione derivatives were found in the extracts of various *Hygrophorus* species [4,5]. In addition to the common fungal sterol ergosterol and derivatives, the aroma components of various *Hygrophorus* species were investigated by GC-MS [6]; a ceramide was reported from a Chinese *Hygrophorus* species [7], malodorous indole derivatives were isolated from *H. paupertinus* A. H. Smith & Hesler [8], while muscaflavine and hygrophoric acid were identified as pigments of some *Hygrophorus* fruiting bodies [9]. No investigation of the secondary metabolites of *H. discoxanthus* has yet appeared in the literature. Along with the ecological observations, this prompted a study of the chemical constituents of this mushroom.

To prevent undesired enzymatic reactions, the fresh fruiting bodies were frozen after collection and extracted with EtOAc at –20°C. The crude extract was subsequently partitioned between *n*-hexane and MeCN, and the residue from the more polar layer was separated by chromatography on multiple reverse-phase C-18 columns to give acids **1**-**5**. Remarkably, these compounds exhibit brown-ochreous spots on C-18 TLC-plates sprayed with a sulfovanillin solution, followed by heating, and are thus well differentiable from the fungal ubiquitous oleic and linoleic acids, and methyl linoleate, of similar chromatographic polarity, which are detected as purple spots with the same reagent. In addition, TLC-spots of compounds **1** and **2** respond to UV light (fluorescence quenching at 254 nm).

Acids **1-5** (C_{16} or C_{18}) can be divided between those (**1-2**) presenting an *δ*,*ε*-unsaturated *γ*-oxocrotonate partial structure and those (**3-5**) containing a chetol system (Figure 1). Additionally, some compounds possess either an internal *Z*-configured double bond or a terminal one. Compounds **3-5** are optically active. Acid **1** was obtained as a whitish sticky solid. The UV spectrum showed an intense absorption band at λ_{max} = 234 nm (Log ε = 4.34) attributable to a $\pi \rightarrow \pi^*$ transition of a conjugated keto group, which was corroborated by an intense absorption peak at about 1666 cm^{-1} in the IR spectrum. On the other hand, an IR broad band extending from 3600 to 2800 cm^{-1}, along with a strong band at 1693 cm^{-1} revealed the presence of an unsaturated carboxylic acid. These attributions were firmly confirmed by the signals at δ 170.2 and δ 188.1 in the ^{13}C NMR spectrum of **1**, belonging to an unsaturated carboxylic group and an unsaturated carbonyl group, respectively. The negative ion ESI mass spectrum showed an ion at *m/z* 291 $[M-H]^-$ which, in accordance with data obtained from the NMR spectra, corresponded to the molecular formula $C_{18}H_{28}O_3$.

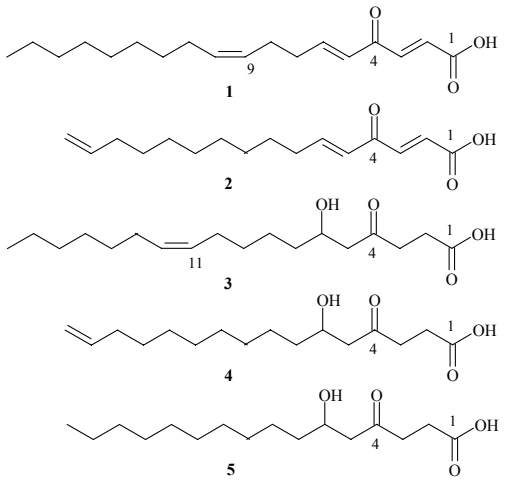

Figure 1: Acids **1-5** isolated from *Hygrophorus discoxanthus*.

The upfield portion of the ^1H NMR spectrum of compound **1** was typical of a long chain unsaturated fatty acid, as indicated by the distorted triplet at δ 0.88, integrating for 3H, attributable to the ω1 methyl group, a broad signal at *δ* 1.10–1.45, integrating for 12H, assignable to the ω2–ω7 methylene protons, and a distorted quartet at *δ* 2.05 typical of an allylic methylene group (C-11). COSY and HMBC (Figure 2) correlations proved that this group was linked to a 1,2-disubstituted double bond, whose carbon signals were found at *δ* 127.1 and 131.6, respectively. The corresponding protons resonated as well separated doublets of triplets at *δ* 5.30 and *δ* 5.42, respectively, and showed a mutual vicinal coupling constant of 10.3 Hz, indicative of a *Z*-configured double bond. Comparison of these data with the literature [10] showed that the structure of compound **1** corresponds, from C-9 to C-18, to that of oleic acid. The remaining eight carbons were assembled as a *δ*,*ε*-unsaturated γ-oxocrotonate unit, attached to C-9 by a C_2 linker, on the basis of the following NMR information. The proton doublets at *δ* 6.75 and *δ* 7.48 (1H each, J_{AB} = 15.7 Hz), which showed HSQC correlations with the carbon signals at *δ* 129.8 and *δ* 139.7, respectively, and HMBC correlations (Figure 2) with the signals at *δ* 170.2 and *δ* 188.1, indicated an *E*-configured double bond positioned between the carboxylic and the carbonyl group. The carbon signal of the ketone displayed additional HMBC cross peaks with two other olefinic methine resonances at *δ* 6.39 and *δ* 7.06 (1H each, vicinal coupling J_{AB} = 15.9 Hz) constituting an *E*-configured double bond, which was joined to C-9 through a CH_2CH_2 group. These two methylenes gave rise to two, well-resolved distorted quartets at *δ* 2.27 (H_2-8) and *δ* 2.38 (H_2-7), respectively, which showed two and three bond HMBC correlations with both C-6 and C-9 (Figure 2).

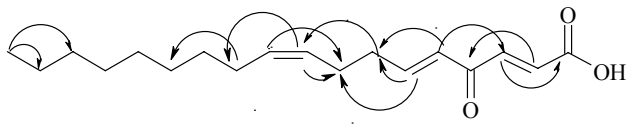

Figure 2: Selected HMBC correlations of compound **1**.

The spectral data of compound **2** were closely related to **1**, the most significant difference being the lack of evidence for an internal non-conjugated double bond. In fact, the UV absorption band at λ_{max} = 235 nm, along with the IR peaks at 1690 and 1664 cm^{-1}, and the almost superimposable patterns of the ^1H- and ^{13}C NMR signals for the C(1)-C(6) moiety clearly proved that acid **2** contains the same *E*,*E*-configured *δ*,*ε*-unsaturated *γ*-oxocrotonate unit as compound **1**. From the mass spectral data, the length of the fatty acid chain in compound **2** could be determined as C_{16}, while the terminal double bond was identified by the signals from the three spin system at *δ* 5.83 (1H, *ddt*, *J* = 17.0, 10.3, 6.7 Hz), *δ* 4.95 (1H, *dtd*, *J* = 10.3, 1.8, 1.5 Hz), and *δ* 5.02 (1H, *dtd*, *J* = 17.0, 1.8, 1.5 Hz).

The molecular formula $C_{18}H_{32}O_4$ of compound **3** was deduced from the ion at *m/z* 311 $[M-H]^-$ in the negative ion ESI spectrum, combined with the

protons and carbons counted from the NMR spectra. Remarkably, the ^{1}H NMR spectrum of **3** did not contain the characteristic signals of the cross conjugated dienone system of **1** and **2**; instead, three overlapping multiplets, each integrating for 2H, were found between δ 2.60-2.82, and were attributed to three different methylene groups adjacent to either saturated carbonyl or carboxylic groups. This assignment was confirmed by the resonances at δ 177.4 and 209.7 in the ^{13}C NMR spectrum, attributed to the carbons of a carboxylic acid and a saturated ketone, respectively. In addition, a broad multiplet at δ 4.10 (1H), which was correlated to a carbon at δ 67.7 in the HSQC spectrum, was firmly assigned to a secondary alcohol. The presence of an internal, non-conjugated, disubstituted olefin was demonstrated by an end absorption band at λ_{max} = 218 nm in the UV spectrum, along with the ^{13}C NMR signals of two methines at δ 129.3 and 130.1, which were correlated to an NMR signal at δ 5.25-5.45 in the HSQC spectrum. The AB coupling constant of 10.5 Hz of these two protons proved the *Z*-configuration of the double bond. A homonuclear COSY experiment, and two and three bonds HMBC correlations (Figure 3) allowed establishment of the 1,4-relationship of the carboxylic group with the ketone, and the 1,3-relationship of the hydroxyl and carbonyl groups.

NMR data alone left the position of the internal double bond undetermined. Therefore, compound **3** was exposed to ozone and, after work-up, the crude reaction mixture was directly subjected to GC analysis. Comparison with an authentic sample revealed heptanal to be formed by ozonolysis of olefin **3**. From all results, the structure of compound **3** was established as (*Z*)-6-hydroxy-4-oxo-octadec-11-enoic acid.

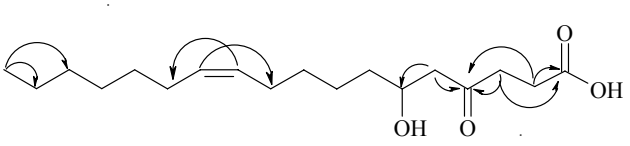

Figure 3: Selected HMBC correlations of compound **3**.

The NMR data of compounds **4** and **5** were closely related to **3** as regards to the 6-hydroxy-4-oxo-carboxylic acid [C(1)–C(6)] unit. In contrast, other than compound **3**, the acids **4** and **5** did not show the signals of an internal double bond. Instead, in the ^{1}H NMR spectrum of compound **4**, the pattern of signals from a three spin system at δ 4.95, 4.99, and 5.83, almost identical to that of acid **2** (see above) were due to a terminal double bond. On the other hand, compound **5** contains a fully saturated fatty acid-like chain, as indicated, in the ^{1}H NMR spectrum, by the characteristic distorted triplet (J = 6.8 Hz) at δ 0.88, assigned to the terminal methyl group, and by a broad peak at δ 1.20-1.60, assigned to the methylenes in the chain. From the mass spectral data, the length of the chain in both compounds **4** and **5** could be determined as C_{16}, thus permitting assignment of the structure of 6-hydroxy-4-oxo-hexadec-15-enoic acid to **4**, and of 6-hydroxy-4-oxo-hexadecanoic acid to **5**.

The absolute configuration of carbinols **3**-**5** has yet to be determined. Compounds **1**-**5** have never been isolated from a natural source; acid **5** was obtained previously as a racemate by synthesis [11].

A preliminary qualitative test indicated that acids **1** and **2** are moderately fungicidal against the phytopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth..

The structures **1**-**5** are closely related to other oxidized C_{16}-C_{22} fatty acids and their derivatives recently isolated from a few *Hygrophorus* species [3-5], for which hypothetical biogenetic relationships have been proposed [3,5]. A rare feature of all these structures is the oxidation to a ketone of the C-4 of the parent fatty acid; a few compounds show an additional site-specific oxidation at C-6, which the optically active alcohols **3**-**5** indicate to occur under enzyme control. Indeed, 6-hydroxy-4-oxo-carboxylic acids like **3**-**5** are, to our knowledge, unprecedented in nature. They can be considered advanced biogenetic precursors of hygrophorones F[12] and G[12] [4a]. Examining the literature data, it was concluded that each *Hygrophorus* species is characterized by its own pattern of oxidized C_{16}-C_{22} fatty acid derivatives, which may thus be considered a significant chemotaxonomic marker. Moreover, due to the fungicidal and bactericidal properties [3-5], these metabolites likely function as "chemical deterrents", protecting *Hygrophorus* fruiting bodies against the attack of parasites and predators.

Experimental

General experimental procedures: Optical rotations were determined on a Perkin-Elmer 241 polarimeter; IR spectra were recorded on an FT-IR Perkin Elmer Paragon 1000 PC spectrometer as neat films on NaCl

discs. UV spectra were obtained in spectrometer grade $CHCl_3$ from a Jasco V-550 spectrophotometer. ^1H and ^{13}C NMR spectra were determined in $CDCl_3$ on a Bruker CXP 300 spectrometer operating at 300 MHz (^1H) and 75 MHz (^{13}C), respectively. ^1H and ^{13}C chemical shifts (δ, ppm) are relative to residual $CHCl_3$ signals [δ_H 7.26; δ_C (central line of t) 77.1, respectively]. 2D NMR spectra (COSY, HSQC, HMBC) were recorded by using standard pulse sequences. The abbreviation s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad are used throughout; coupling constants (*J*) are reported in Hz. ESIMS experiments were carried out using a Finnigan LCQ Advantage MS 1.4 spectrometer, equipped with the Xcalibur 1.4 software. High-resolution ESI mass spectra were determined on a Bruker Apex II FT-ICR mass spectrometer. TLC was performed on sheets precoated with either silica gel F_{254} (Polygram) or with RP-18 F_{254} (Merck, Germany). Compounds were visualized under UV light (254 and 366 nm) and by spraying with a 0.5% solution of vanillin in H_2SO_4-EtOH (4:1), followed by heating. Preparative column chromatography was carried out on LiChroprep RP-18 (25-40 μm, Merck). Reagent grade solvents, redistilled just before use, were employed for extraction; HPLC grade solvents were used for chromatographic separations. GC analysis was performed with a Perkin Elmer Autosystem gaschromatograph.

Fungal material: Fresh fruiting bodies of *Hygrophorus discoxanthus* (Batsch.: Fr.) Fr. were collected on 16 October 2005 in a mixed conifer and beech wood near Brallo, in the province of Pavia, Italy, at an altitude of 1050 m. The mushroom was identified by one of the authors (M.C.) and a frozen voucher specimen has been deposited at the Dipartimento di Chimica Organica, University of Pavia, Italy.

Extraction and isolation: Fruiting bodies (750 g) were frozen at –20 °C, minced, and extracted at –20 °C with EtOAc (3 x 1.5 L), followed by MeOH–H_2O (4:1, 1 L), and H_2O (1 L) at 0°C. The light yellow EtOAc solution was concentrated to dryness *in vacuo* at <30 °C to produce an oily residue (2.1 g), which was partitioned between MeCN (0.5 L) and *n*-hexane (0.5 L). Evaporation of the two layers gave crude residues of 1.02 g and 1.08 g, respectively. Acids **1-5** were contained in the MeCN extract (TLC: R_f = 0.55-0.70; RP-18 F_{254}, solvent system: MeCN-H_2O, 7:1 v/v), which was subjected to column chromatography on a LiChroprep RP-18 column (100 g). Elution was performed with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 100 mL, until a final mixture of MeCN-H_2O, 10:1, v/v. The column was then washed with MeCN (100 mL), followed by Me_2CO (100 mL). Thirty-four fractions (A1-A34), of 35 mL each, were collected. Fraction A9 gave acid **4** (12 mg, 1.6 10^{-3} % of fresh fruiting bodies), fraction A13 gave acid **5** (25 mg, 3.2 10^{-3} %), and fraction A14 afforded acid **3** (34 mg, 4.6 10^{-3} %). Linoleic acid (36 mg, 4.8 10^{-3} %), oleic acid (107 mg, 14 10^{-3} %), and methyl linoleate (37 mg, 4.9 10^{-3} %) were obtained by evaporation of fractions A23, A25, and A27, respectively. Fraction A15 (76 mg) was further separated on a LiChroprep RP-18 column (20 g) eluted with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 50 mL, until a final mixture of MeCN-H_2O, 10:1, v/v. Thirteen fractions (B1-B13), of 40 mL each, were collected. Acid **2** (10 mg, 1.4 10^{-3} %) was isolated by evaporation of fraction B7. Fractions A19 and A20 were pooled together and the residue (110 mg) was further separated on a LiChroprep RP-18 column (20 g) eluted with a gradient of MeCN-H_2O, starting from a mixture 1:1, v/v, and increasing MeCN regularly every 50 mL, until a final mixture of MeCN-H_2O, 10:1, v/v; 14 fractions (C1-C14), each of 35 mL, were collected. Fraction C5 (61 mg) afforded compound **1** (10 mg, 1.4 10^{-3} %) on successive separation on a LiChroprep RP-18 column (15 g) eluted with MeOH-H_2O, 4:1, v/v.

(2*E*, 5*E*, 9*Z*)-4-Oxo-octadeca-2,5,9-trienoic acid (1)

Whitish sticky solid.
Rf: 0.45 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-2800, 3090, 3050, 2920, 2852, 1693, 1666, 1613, 1278, 1216, 1000, 975, 950 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 234 (4.34).
^1H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.10-1.45 (12H, brs H_2-12–H_2-17), 2.05 (2H, q, *J* = 6.8 Hz, H_2-11), 2.27 (2H, distorted q, *J* = 7.0 Hz, H_2-8), 2.38 (2H, distorted q, *J* = 7.0 Hz, H_2-7), 5.30 (1H, dd, *J* = 10.3, 6.5 Hz, H-9), 5.42 (1H, dd, *J* = 10.3, 6.5 Hz, H-10), 6.39 (1H, d, *J* = 15.9 Hz, H-5), 6.75 (1H, d, *J* = 15.7 Hz, H-3), 7.06 (1H, dt, *J* = 15.9, 6.7 Hz, H-6), 7.48 (1H, d, *J* = 15.7 Hz, H-2).
^{13}C NMR: 13.9 (CH_3, C-18), 22.5 (CH_2, C-17), 25.5 (CH_2, C-8), 27.2 (CH_2, C-11), 29.2, 29.3, 29.4, 29.5 ($4CH_2$, C-12, C-13, C-14, C-15), 31.6 (CH_2, C-16), 32.8 (CH_2, C-7), 127.1 (CH, C-9), 129.4 (CH, C-5),

129.8 (CH, C-3), 131.6 (CH, C-10), 139.7 (CH, C-2), 150.8 (CH, C-6), 170.2 (C, C-1), 188.1 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{18}H_{27}O_3$ 291.1960, found 291.1962.

(2*E*, 5*E*)-4-Oxo-hexadeca-2,5,15-trienoic acid (**2**)

Whitish sticky solid.
Rf : 0.5 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3050, 2923, 2851, 1690, 1664, 1625, 1279, 1215, 1000, 915 cm^{-1}.
UV/Vis λ_{max} ($CHCl_3$) nm (log ε): 235 (4.19).
^{1}H NMR: 1.30-1.65 (12H, brs H_2-8–H_2-13), 2.06 (2H, q, *J* = 6.8 Hz, H_2-14), 2.31 (2H, q, *J* = 7.2 Hz, H_2-7), 4.95 (1H, dtd, *J* = 10.3, 1.8, 1.5 Hz, H-16*E*), 5.02 (1H, dtd, *J* = 17.0, 1.8, 1.5 Hz, H-16*Z*), 5.83 (1H, ddt, *J* = 17.0, 10.3, 6.7 Hz, H-15), 6.39 (1H, d, *J* = 15.9 Hz, H-5), 6.75 (1H, d, *J* = 15.7 Hz, H-3), 7.06 (1H, dt, *J* = 15.9, 6.7 Hz, H-6), 7.48 (1H, d, *J* = 15.7 Hz, H-2).
^{13}C NMR: 27.8, 28.7, 28.9, 29.2, 29.3, 29.4 (6 x CH_2, C-8, C-9, C-10, C-11, C-12, C-13), 32.8 (CH_2, C-7), 33.7 (CH_2, C-14), 114.0 (CH_2, C-16), 129.2 (CH, C-5), 129.7 (CH, C-3), 139.0 (CH, C-15), 139.7 (CH, C-2), 151.7 (CH, C-6), 169.5 (C, C-1), 188.2 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{23}O_3$: 263.1647; found: 263.1649.

(*Z*)-6-Hydroxy-4-oxo-octadec-11-enoic acid (**3**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -340º (*c* = 10 mg/mL, $CHCl_3$).
Rf: 0.6 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3010, 2928, 2856, 1713, 1406, 1260, 1201, 1100 cm^{-1}.
^{1}H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.20-1.65 (14H, brs, H_2-14–H_2-17, H_2-7– H_2-9), 2.05 (4H, q, *J* = 7.0 Hz, H_2-10, H_2-13), 2.60-2.82 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6), 5.25-5.45 (2H, m, H-11, H-12).
^{13}C NMR: 14.0 (CH_3, C-18), 22.5 (CH_2, C-17), 25.0, 27.0, 27.1, 28.9, 29.5, 29.6 (6 x CH_2, C-8, C-9, C-10, C-13, C-14, C-15), 27.4 (CH_2, C-2), 31.7 (CH_2, C-16), 36.3 (CH_2, C-7), 37.5 (CH_2, C-3), 49.1 (CH_2, C-5), 67.7 (CH, C-6), 129.3, 130.1 (2 x CH, C-11, C-12), 177.4 (C, C-1), 209.7 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{18}H_{31}O_4$ 311.2222, found 311.2225.

6-Hydroxy-4-oxo-hexadec-15-enoic acid (**4**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -109º (*c* = 11 mg/mL, $CHCl_3$).
Rf: 0.7 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 3010, 2917, 2850, 1702, 1412, 1250, 1080, 1000, 913 cm^{-1}.
^{1}H NMR: 1.25-1.65 (14H, brs, H_2-7–H_2-13), 2.04 (2H, q, *J* = 6.7 Hz, H_2-14), 2.60-2.80 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6), 4.95 (1H, dtd, *J* = 10.3, 1.8, 1.5 Hz, H-16*E*), 4.99 (1H, dtd, *J* = 17.0, 1.8, 1.5 Hz, H-16*Z*), 5.83 (1H, ddt, *J* = 17.0, 10.3, 6.7 Hz, H-15).
^{13}C NMR: 27.4 (CH_2, C-2), 25.3, 28.8, 29.0, 29.2, 29.3, 29.4 (6 x CH_2 C-8, C-9, C-10, C-11, C-12, C-13), 33.7 (CH_2, C-14), 36.3 (CH_2, C-7), 37.6 (CH_2, C-3), 49.1 (CH_2, C-5), 67.8 (CH, C-6), 114.0 (CH_2, C-16), 139.1 (CH, C-15), 177.1 (C, C-1), 209.7 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{27}O_4$ 283.1909, found 283.1911.

6-Hydroxy-4-oxo-hexadecanoic acid (**5**)

Whitish sticky solid.
$[\alpha]_D^{25}$: -95º (*c* = 10 mg/mL, $CHCl_3$).
Rf: 0.65 (RP18, MeCN-H_2O, 7:1).
IR (film): 3600-3200, 2920, 2855, 1710, 1415, 1255 cm^{-1}.
^{1}H NMR: 0.88 (3H, t, *J* = 6.8 Hz, Me), 1.20-1.60 (18H, brs, H_2-7–H_2-15), 2.60-2.80 (6H, m, H_2-2, H_2-3, H_2-5), 4.10 (1H, brm, H-6).
^{13}C NMR: 14.0 (CH_3, C-16), 27.6 (CH_2, C-2), 22.8-29.6 (8 x CH_2, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15), 36.3 (CH_2, C-7), 37.6 (CH_2, C-3), 49.1 (CH_2, C-5), 67.7 (CH, C-6), 177.2 (C, C-1), 209.5 (C, C-4).
Negative ion ESI-FT-ICR-MS: m/z [M – H$^-$] calcd for $C_{16}H_{29}O_4$ 285.2066, found 285.2064.

Ozonolysis of acid 3: A saturated solution of O_3 in CH_2Cl_2-MeOH, 4:1 v/v, was added to compound **3** (3 mg) dissolved in CH_2Cl_2, (0.5 mL) at –78°C. The reaction was quenched after 3 h by adding excess Me_2S and the mixture was left at –20°C overnight. A sample was directly analyzed by GC under the following conditions: column HP-5 (25 m×0.25 mm, 0.33 μm film thickness), injection temperature 250°C, detector (FID) temperature 280°C, carrier gas nitrogen, flow rate 1.27 mL/min, constant flow mode, split splitless injection, ratio 1:35, column temperature program: 40°C for 5 min, then raised to 100°C at a rate of 2°C/min, then raised to 280°C at a rate of 10°C/min, then isothermal at 280°C for 5 min. Enrichment of the peak eluted at 9.69 min with an authentic sample of heptanal, confirmed its identity.

Fungicidal activity: A simple test, adapted from the literature [4a, 12], was carried out to reveal the

possible fungicidal activity of compounds **1**-**5**. Five solutions of compounds **1**-**5** in MeOH, each containing approximately 20 μg of substance, were spotted on F_{254} Merck silica gel plastic sheets, which were sprayed with a conidal suspension of *Cladosporium cucumerinum* Ell. et Arth spores in a glucose mineral medium (Czapek broth). The plates were then incubated at 25°C in the dark in a wet chamber (> 95% humidity) for 5 days, when they were overgrown with a dark gray colored mycelium. White spots (inhibition zones), signaling fungicidal activity, were found, in particular in correspondence with compounds **1** and **2**; they were about eight times smaller than the inhibition area of the reference compound pseudomycin A (20 μg).

Acknowledgments - The authors thank Prof. Mariella Mella and Prof. Giorgio Mellerio for NMR and MS spectra measurements, respectively. Fungicidal tests were carried out by Dr Solveig Tosi. Financial support by the Italian MIUR (Grants COFIN and FIRB) and the University of Pavia (Grant FAR) is acknowledged.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1085 - 1088

Kenyaloside, a Novel *O*,*O*,*O*-Triglycosylated Naphthalene Derivative from the Exudate of Kenyan *Aloe* Species[¶*]

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Received: July 11th, 2006; Accepted: September 2nd, 2006

Dedicated to the memory of Professor Ivano Morelli.

A new naphthalene *O*,*O*,*O*-triglycoside, kenyaloside (**1**), was isolated from the dried exudate of Kenyan *Aloe* species, a bittering and laxative agent. Its structure was established by combined spectral and chemical methods as 1-(β-D-glucopyranosyloxy)-8-(α-L-rhamnopyranosyloxy)-3-(β-D-xylopyranosyloxymethyl)naphthalene.

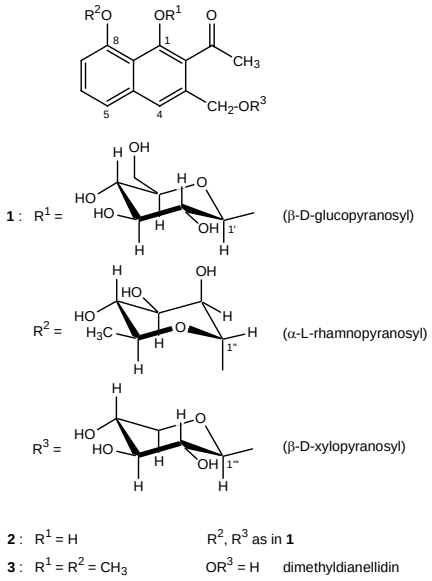
Keywords: aloes, *Aloe ferox*, naphthalene *O*,*O*,*O*-triglycoside, kenyaloside.

As part of a systematic chemical investigation into *Aloe* exudates (bitter aloes) [1], the structural elucidation of a new water-soluble constituent of the exudate of Kenyan *Aloe* species is reported here. This exudate, flowing from the cut leaves of *Aloe ferox* Miller and of its hybrids with *A. spicata* and *A. africana* growing in Kenya [2, 3], when dried, is used as a bittering agent and as a purgative, similarly to Cape aloes [4, 5]. The drug has been reported to contain a number of polyketide metabolites (such as *O*- and/or *C*-glucosides) belonging to the families of 6-phenyl-2-pyrones, 5-methyl-7-hydroxychromones, and 1,8-dihydroxyanthrones (see Ref. 3 for a complete list of such compounds).

The structure of the new product, named kenyaloside (**1**), was determined by spectral and chemical methods. To our knowledge, it represents the first example of a naphthalene glycoside both occurring in *Aloe* species and bearing three different *O*-glycosyl residues [4, 5].

The aqueous extract of the dried exudate of Kenyan *Aloe* species, after partitioning with ethyl acetate, was lyophilized to afford a residue that was chromatographed successively on silica gel and Sephadex LH-20 columns. Kenyaloside (**1**) was obtained in *ca.* 0.1% yield (based on the starting drug). Its molecular formula, $C_{30}H_{40}O_{17}$, was derived from ESI-HRMS (found: m/z 695.21326, calcd for $[M+Na^+]$ m/z: 695.21577). The presence of three *O*-glycosyl residues was suggested by inspection of chemical shifts and coupling constants in the ^1H and ^{13}C NMR spectra of **1** (Table 1); in addition, the NOESY spectrum revealed two significant associations between the anomeric proton at δ 5.77 and the upfield aromatic proton, and between another anomeric proton (at δ 4.31) and both the aromatic proton at δ 7.48 and an Ar-CH_2 group (AB system: δ 4.73, 4.94, J = 12.4 Hz). ^1H and ^{13}C signals due to four aromatic C-H groups, together with the values of ^1H-^1H coupling constants and mutual NOEs, were indicative of a 1,2,3,8-tetrasubstituted naphthalene nucleus.

[¶]Part 19 in the series "Studies on Aloe". For Part 18, see Ref. 1



The existence of an Ar-CO-CH_3 (δ_H 1.95, δ_C 30.84, 206.70) and of an Ar-glycosyloxymethyl group in 2- and 3-positions, respectively, could be established on the basis of the NOE association between H-4 and Ar-CH_2-O- protons and of the correlation observed in the HMBC spectrum between the glycosyloxymethyl protons and the aromatic carbon linked to the acetyl group (C-2). Such an assumption was in agreement with the strong similarity in the UV and IR spectra of **1** with those of dianellidin dimethyl ether (**3**) [6]. In addition, it can be noted that the carbon skeleton and the oxygenation pattern of the kenyaloside aglycone (**1**, where $R^1 = R^2 = R^3 = H$) are both consistent with biogenetic considerations suggesting the cyclization of a decarboxylated polyketide (heptaketide) chain as the key step in the formation of the naphthalene nucleus [7]. The unambiguous identification of the three monosaccharides, including their absolute configuration, the orientation of the glycosidic bond, and the location of each of them on the aglycone moiety, resulted from combined NMR spectral data and hydrolysis experiments, as follows.

When **1** was submitted to β-glucosidase-catalyzed hydrolysis, a diglycoside (**2**) was obtained showing in its NMR spectrum the loss of the β-glucopyranosyl residue from the 1-position of the naphthalene nucleus (Table 1). In fact, both chemical shifts and NOE correlations of the upfield aromatic proton (H-7) and of the methylene protons at 3-position remain unchanged. The expected, but not axiomatic [8], D-configuration of glucose was proven by application of the exciton chirality method developed by Nakanishi to characterize methyl glycosides at the nano-gram level [9, 10]. The monosaccharide isolated from the enzymatic hydrolysis of **1** was converted into the mixture of methyl α- and β-glucopyranosides, which were per-*p*-bromobenzoylated: the CD spectrum of the resulting mixture of the tetra-esters was found to be coincident with that of the corresponding mixture of esters prepared from an authentic sample of D-glucose and in agreement with data reported for α- and β-D-glucopyranosides tetra-*p*-bromobenzoates [9, 10].

Treatment of the diglycoside **2** with α-rhamnosidase from *Fusarium oxysporum* [11] gave L-rhamnose, as demonstrated by derivatization of the sugar and CD spectra comparison, as described above. [9, 10]. That L-rhamnose is involved in an α–glycosidic linkage at the 8-*O*-position of the naphthalene nucleus in **1** and **2** stems from NOE correlations and coupling constants of the anomeric proton of this hexopyranose (Table 1). Finally, the crude monoglycoside isolated from the rhamnosidase-catalyzed hydrolysis of **2**, after separation of L-rhamnose, was heated in HCl solution and the released sugar processed and analyzed according to Nakanishi's method [9, 10]. This pentose was identified as D-xylose in agreement with NMR data of **1** and **2** (Table 1), indicating the presence of a β-xylopyranoside residue [12]. Therefore, the structure of kenyaloside was concluded to be 1-(β-D-glucopyranosyloxy)-8-(α-L-rhamnopyranosyloxy)-3-(β-D-xylopyranosyl-oxmethyl)naphthalene (**1**).

Experimental

General experimental techniques: Optical rotations were measured on a Jasco P-1030 polarimeter, UV spectra on a Hewlett Packard 8452A Diode Array Spectrophotometer, CD spectra on a Jasco J-500 instrument, and IR spectra on a Perkin-Elmer FT-IR 1725 X spectrometer. NMR spectra were recorded on a Bruker AVANCE 400 Spectrometer using a XWIN-NMR software package; chemical shifts (δ) are given in ppm and were referenced to the CD_3OD signals (δ_H 3.30, δ_C 49.0). ESI-HRMS spectra were acquired on a Bruker Daltonics FT-ICR APEX-II mass spectrometer and ESI MS spectra on a ThermoFinnigan LCQ Advantage instrument. Analytical TLC was performed on silica gel 60 F_{254} aluminum sheets (Merck) using the following eluents: *A*, EtOAc-EtOH-H_2O, 100:20:13; *B*, *n*-BuOH-AcOEt-H_2O, 7:2:1; components were detected under an UV lamp and by spraying with either 0.5% Fast Blue B salt (phenols) or with 4% ceric sulfate/ ammonium molybdate solution (sugars), followed by

Table 1: NMR data of compounds **1** and **2** in CD_3OD at 400 MHz (^1H) and 100 MHz (^{13}C).[a, b]

	kenyaloside (**1**)			Compound **2**		
C/H position	δ_H (J, Hz)	δ_C	Selected ^1H-^1H NOEs	δ_H (*J*, Hz)	δ_C	Selected ^1H-^1H NOEs
1		152.69			152.56	
2		123.06			122.96	
3		134.10			134.12	
4	7.48 s	119.77	7.52; 4.73, 4.94; 4.31	7.47 s	119.73	7.51; 4.71, 4.93
4a		137.03			137.05	
5	7.52 d (8.0)	122.94	7.48; 7.45	7.51 dd (8.3, 1.1)	122.83	7.47; 7.45
6	7.45 dd (7.6, 8.0)	127.97	7.52; 7.33	7.45 dd (7.8, 8.3)	127.97	7.51; 7.32
7	7.33 d (7.6)	109.92	7.45; 5.77	7.32 dd (7.8, 1.1)	109.91	7.45; 5.75
8		153.68			153.66	
8a		114.74			114.71	
COCH_3		206.70			206.59	
CO*CH_3*	1.95 s	30.84		1.89	31.62	
CH_2O	4.73 d (12.4)	68.80	4.31, 7.48	4.71 d (12.4)	68.78	4.27, 7.47
	4.94 d (12.4)			4.93 d (12.4)		
1'	4.39 d (8.0)	102.46				
2'	3.23 dd (8.0, 8.8)	73.67				
3'	3.33 m[e]	76.85[c]				
4'	3.33 m[e]	70.67[d]				
5'	3.33 m[e]	77.10[c]				
6'	3.67 dd (5.2, 12.0)	61.64				
	3.89 dd (1.6, 12.0)					
1"	5.77 d (1.8)	100.85	7.33	5.75 d (1.9)	100.83	7.32
2"	4.21 dd (1.8, 3.4)	70.56		4.20 dd (1.9, 3.5)	70.66	
3"	3.85 dd (3.4, 9.2)	71.58		3.84 dd (3.5, 9.3)	71.55	
4"	3.57 t (9.2)	72.42		3.56 t (9.3)	72.40	
5"	3.71 m	70.72[d]		3.70 m	70.71	
CH_3(5")	1.31 d (6.4)	17.02		1.29 d (6.1)	17.02	
1"'	4.31 d (7.2)	103.09	4.73, 4.94, 7.48	4.27 d (7.3)	103.30	4.71, 4.93
2"'	3.29 dd (7.2, 8.8)	73.82		3.23 dd (7.3, 9.0)	73.97	
3"'	3.50 t (8.8)	75.11		3.35 t (9.0)	76.85	
4"'	3.71 m	77.65		3.50 m	70.23	
5"'	3.33 m[e]	63.58		3.19 dd (10.1, 11.5)	65.94	
	4.06 dd (5.2, 12.0)			3.88 dd (5.4, 11.5)		

[a] Spectra recorded at 40°C; [b] all assignments were based on extensive 1D and 2D NMR measurements (COSY, TOCSY, NOESY, APT, HMQC and HMBC); [c,d] signals with the same superscript are interchangeable; [e] covered by the CH_3OH signal.

heating at 150°C. Silica gel 60, 63-200 μm and 40-63 μm (Merck) was used for column and flash chromatography, respectively.

Plant material: The commercial exudate of Kenyan *Aloe* species used in this investigation was purchased from Sessa Carlo spa (Sesto S. Giovanni, Italy). A voucher specimen is kept at the Dipartimento di Chimica Organica e Industriale, Università di Milano

Extraction and isolation: The dried exudate of Kenyan *Aloe* species (250 g) was finely powdered and extracted with water (750 mL) with vigorous mechanical stirring for 24 h at room temperature. After filtration of the insoluble material, the aqueous solution was partitioned with ethyl acetate (2 x 1 L) and lyophilized to give a brown residue (120 g). Of this residue, 40 g was adsorbed onto sea sand and fractioned by flash chromatography (silica gel, 1.5 Kg) eluting with EtOAc containing increasing amounts of MeOH. Separation was monitored by TLC (eluent *A*) and fractions containing **1** (Rf 0.38) were combined, concentrated (3.5 g) and further purified by flash chromatography (silica gel, 500 g) eluting with EtOAc-EtOH-H_2O, 100:20:10. Fractions were combined on the basis of TLC analysis (eluent *A*) and evaporated to dryness. The residue (*ca.* 400 mg) was chromatographed over a Sephadex LH-20 column eluted with MeOH-H_2O (1:1) to give kenyaloside (**1**) (200 mg, 0.08% overall yield) as an amorphous powder, pure by TLC (eluent *A*).

Kenyaloside [1-(β-D-glucopyranosyloxy)-8-(α-L-rhamnopyranosyloxy)-3-(β-D-xylopyranosyl-oxymethyl)naphthalene (1)]

$[\alpha]_D$: - 84.4° (*c* 0.25, MeOH).
Rf : 0.38 (AcOEt-EtOH-H_2O, 100:20:13).
IR (KBr): 1695, 1652,1615 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 226 (4.72), 260 (4.36), 290sh (4.30), 338 (3.94) [for dimethyl dianellidin (**3**) [6]: 223 (4.68), 253 (4.04), 331 (3.61)].
^1H NMR (400 MHz, CD_3OD): Table 1.
^{13}C NMR (100 MHz, CD_3OD): Table 1.
ESI-HRMS: m/z [M + Na^+] calcd for $C_{30}H_{40}NaO_{17}$ 695.21577, found 695.21326.
ESI MS: m/z 695 [M + Na^+], 549 [M-146+Na^+].

Enzymatic hydrolyses: β-Glucosidase (almond emulsin, Sigma, 30 mg) was added to a solution of

kenyaloside (**1**, 50 mg) in H_2O (25 mL), and the mixture was incubated at 37° for 3 h under nitrogen. After adding MeOH, the solution was filtered and concentrated under reduced pressure. Column chromatography of the aqueous residue (eluent: EtOAc-EtOH-H_2O, 100:20:13) gave two fractions. The less polar fraction, after further purification by column chromatography eluting with AcOEt-EtOH (from 5:1 to 1:1), furnished the diglycoside **2** (30 mg, 79%) as a pale yellow powder. The more polar fraction was submitted to column chromatography (eluent AcOEt-EtOH, from 3:1 to 1:1) to give glucose (9 mg, 67%), identified by TLC comparison with an authentic sample (eluent *B*, Rf 0.31).

Compound **2** (20 mg), dissolved in 50 mM phosphate buffer pH 6 (5 mL), was incubated with α-rhamnosidase from *Fusarium oxyporum* CCF 906 (0.2 U) [11] at 35°C for 24 h. After concentration, MeOH was added, the precipitate removed by filtration and the solvent evaporated under reduced pressure. Repeated column chromatographic purification (eluent AcOEt-EtOH, from 5:1 to 1:1 and from 3:1 to 1:1) furnished rhamnose (5 mg, Rf 0.62, eluent *B*, co-TLC with an authentic sample) and a yellow product (10 mg, Rf 0.73, eluent *A*). This was hydrolyzed without further purification (0.1 N HCl, dioxane-H_2O, 1:1, 5 mL; 70°C, 5 h, under nitrogen) to give xylose (Rf 0.50, eluent *B*, co-TLC with an authentic sample), which was purified (1.5 mg) by column chromatography (eluent AcOEt-EtOH, from 3:1 to 1:1).

8-(α-L-Rhamnopyranosyloxy)-3-(β-D-xylopyranosyloxymethyl)naphthalen-ol (**2**)

$[\alpha]_D$: - 36.2º (*c* 0.07, MeOH).
Rf : 0.54(AcOEt-EtOH-H_2O, 100:20:13).
IR (KBr): 1635 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 225 (4.49), 258sh (4.09), 297 (3.99), 334 (3.80).
^1H NMR (400 MHz, CD_3OD): Table 1.
^{13}C NMR (100 MHz, CD_3OD): Table 1.
ESI MS: m/z 533 [M + Na^+], 387 [M-146+Na^+].

Determination of the absolute configuration of the isolated sugars: The isolated monosaccharides were converted into methyl glycopyranosides followed by treatment with excess *p*-bromobenzoyl chloride, as in ref. 9. Comparison of the CD spectra of the resulting per-*p*-bromobenzoates with those of the analogous derivatives prepared from authentic samples allowed the D-configuration for glucose and xylose and the L-configuration for rhamnose to be established.

Acknowledgments – Thanks are due to Dr Lavinia Durì for technical assistance and to MIUR for financial support.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1089 - 1095

New Flavonoid Glycosides from *Chrozophora senegalensis* and Their Antioxidant Activity

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Received: June 27th, 2006; Accepted: September 27th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Bioassay-directed fractionation of an antioxidant methanol extract of the leaves of *Chrozophora senegalensis* using DPPH assay led to the isolation of three new flavonoid glycosides, quercetin 3-*O*-(6"-caffeoyl)-β-D-glucopyranoside-3'-*O*-β-D-glucopyranoside (**1**), quercetin 3-methyl ether-7-*O*-α-L-rhamnopyranosyl-(1→6)-(2"-*p*-coumaroyl)-β-D-glucopyranoside (**2**), acacetin 7-*O*-(6"-*p*-coumaroyl)-β-D-glucopyranoside (**3**), along with five known flavonoids, one phenolic derivative, and three megastigmane glycosides. Their structures were established on the basis of detailed spectral analysis. All isolated compounds were tested for their antioxidant activity on DPPH stable radical, superoxide anion, metal chelating activity, and DNA cleavage induced by the photolysis of H_2O_2. Quercetin 3-*O*-(6"-caffeoyl)-β-D-glucopyranoside-3'-*O*-β-D-glucopyranoside (**1**), quercetin 3'-methyl ether-3-*O*-α-L-rhamnopyranoside (**4**), and 4'''-methyl ether amenthoflavone (**9**) exhibited the highest antioxidant capacity being also able to modulate hydroxyl radical formation more efficiently than other compounds acting as direct hydroxyl radical scavengers and chelating iron.

Keywords: *Chrozophora senegalensis*, Euphorbiaceae, flavonoids, antioxidant activity.

In recent years, a global trend toward the use of natural phytochemicals present in herbs and functional foods as antioxidants was further increased after that it had been reported that some commonly used synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole, have long-term toxicological effects, including carcinogenicity [1]. Of particular interest as possible sources of natural antioxidants are medicinal plants traditionally used to treat conditions related to oxidative stress, such as rheumatism and inflammation. In this regard, many phytochemicals with diversified biological properties have shown promise for the prevention and/or treatment of all diseases in which oxidative stress plays a key role [2]. *Chrozophora senegalensis* (Lam) A Juss. ex Spreng, syn. *Croton senegalensis* (Euphorbiaceae family) is a small tree widely distributed in Mali where it grows wild and is used in folk medicine for the treatment of diarrhea, rheumatism, teniasis, stomachache, rachitis, and venereal diseases. The leaf and root decoctions are also drunk for hairloss [3, 4]. To confirm the use of *C. senegalensis* in Malian traditional medicine, the extracts of the leaves were evaluated for *in vitro* antioxidant activity. A bioassay-guided fractionation procedure showed that

the methanol extract was the active one, while all the other residues were inactive (data not shown). Subsequent fractionation and analysis of the methanol extract led to the isolation and structural characterization of three new flavonoids (**1-3**), together with some known compounds, including five flavonoids (**4-7** and **9**), one phenolic derivative (**8**), and three megastigmane glycosides (**10-12**).

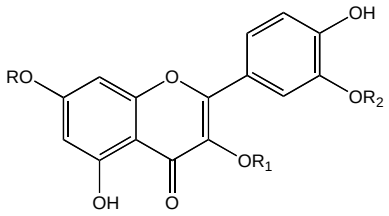
Compound **1** was isolated as a yellow amorphous powder. Its molecular formula was established as $C_{36}H_{36}O_{20}$ by means of ESI-MS ([M-H]$^-$ peak at *m/z* 787), ^{13}C, ^{13}C-DEPT NMR, and elemental analysis. Analysis of 600 MHz NMR spectra suggested a flavonoid skeleton for compound **1**. The ^{1}H-NMR spectrum (Table 1) indicated a 5,7-dihydroxylated pattern for ring A (two *meta*-coupled doublets at δ 6.16 and 6.33, *J* = 1.5 Hz) and a 3',4'-dihydroxylation pattern for ring B (ABX system signals at δ 6.80, d, *J* = 8.5 Hz; 7.58, dd, *J* = 8.5, 2.5 Hz; 7.67, d, *J* = 2.5 Hz), allowing the aglycon to be recognized as quercetin [5]. The ^{1}H-NMR spectrum of **1** also showed signals ascribable to sugar moieties and a caffeoyl residue (Table 1). Two anomeric protons arising from the sugar moieties appeared at δ 5.26 and 4.88 each (1H, d, *J* = 7.5 Hz), which correlated respectively with signals at δ 103.4 and 104.7 ppm in the HSQC spectrum. All the ^{1}H- and ^{13}C-NMR signals of **1** were assigned using 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments. Complete assignments of proton and carbon chemical shifts of the sugar portion were accomplished by DQF-COSY and 1D-TOCSY experiments and allowed the identification of the sugars as two terminal β-D-glucopyranosyl units. The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilylated, and GC retention times compared with those of authentic sugar samples prepared in the same manner. The lower field shifts of H_2-6''' (δ 4.32 and 4.23) of one glucosyl unit suggested the substitution site of the caffeoyl moiety. Unequivocal information could be obtained by 2D-NMR spectra; the HMBC experiment indicated correlations between δ 5.26 (H-1''') and 135.6 (C-3), δ 4.88 (H-1'') and 149.0 (C-3'), δ 4.32 and 4.23 (H_2-6''') and 170.0 (COO). Thus, the structure of **1** was determined as quercetin 3-*O*-(6''-caffeoyl)-β-D-glucopyranoside-3'-*O*-β-D-glucopyranoside.

The molecular formula $C_{37}H_{38}O_{18}$ for compound **2** was determined by ESI-MS ([M-H]$^-$ at *m/z* 769), ^{13}C, ^{13}C-DEPT NMR analyses and was supported also by elemental analysis. Its ^{1}H- and ^{13}C-NMR spectra (see Table 2) indicated that it was a quercetin 3-methyl ether derivative [5]. Its ^{1}H-NMR spectrum further displayed signals for two sugar residues that were easily clarified with the help of 1D-TOCSY and DQF-COSY experiments, leading to the identification of one β-D-glucopyranosyl and one α-L-rhamnopyranosyl residue. The configuration of sugar units was determined as reported for compound **1**. The presence of one *p*-coumaroyl moiety was shown in the ^{1}H-NMR spectrum by the signals at δ 7.45 and 6.73 each (2H, d, *J* = 8.5 Hz) and δ 7.41 and 6.38 each (1H, d, *J* = 16.0 Hz). The HSQC spectrum showed glycosidation shifts for C-6'' (δ 67.5) and acylation shift for H-2'' (δ 4.74) and C-2'' (δ 74.5) of the β-D-glucopyranosyl unit. An unambiguous determination of the sequence and linkage sites was obtained from an HMBC experiment, showing cross peak correlations between δ 5.06 (H-1'') and 164.5

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

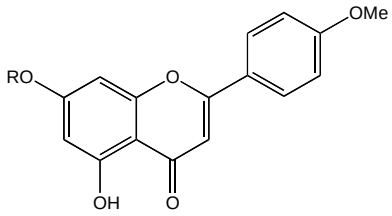
position	δ_H	δ_C
2		159.0
3		135.6
4		179.0
5		163.5
6	6.16 d (1.5)	100.0
7		166.3
8	6.33 d (1.5)	94.2
9		159.0
10		105.8
1'		123.1
2'	7.67 d (2.5)	117.2
3'		149.0
4'		146.4
5'	6.80 d (8.5)	116.0
6'	7.58 dd (2.5, 8.5)	123.5
3'-*O*-Glc 1''	4.88 d (7.5)	104.7
2''	3.58 dd (7.5, 9.0)	74.8
3''	3.52 t (9.0)	77.3
4''	3.42 t (9.0)	71.2
5''	3.53 m	78.4
6''a	3.95 dd (5.0, 12.0)	62.4
3-*O*-Glc1'''	5.26 d (7.5)	103.4
2'''	3.56 dd (7.5, 9.0)	73.6
3'''	3.49 t (9.0)	77.7
4'''	3.40 t (9.0)	71.8
5'''	3.59 m	75.6
6'''a	4.32 dd (5.0, 12.0)	64.2
trans-caffeoyl 1		128.4
2	7.00 d (1.5)	115.4
3		147.5
4		150.1
5	6.81 d (8.8)	116.2
6	6.82 dd (1.5, 8.8)	123.1
α	6.07 d (16.0)	114.6
β	7.39 d (16.0)	147.4
COO		170.0

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



1 R = H
R_1 = (6"-caffeoyl)glc
R_2 = glc

2 R = (2"-*p*-coumaroyl)-glc-(6-1)rha
R_1 = Me
R_2 = H



3 R= (6"-*p*-coumaroyl)glc

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

Table 2: ^{1}H- and ^{13}C-NMR data of compounds **2**-**3** (CD_3OD, 600 MHz)[a].

position	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C
2		157.9		164.5
3		139.9	6.70 s	103.7
4		180.0		184.0
5		164.3		164.0
6	6.54 d (2.0)	101.2	6.56 d (2.0)	100.2
7		164.5		165.3
8	6.73 d (2.0)	95.8	6.77 d (2.0)	95.6
9		158.8		158.6
10		107.1		106.0
1'		123.6		122.8
2'	7.71 d (1.5)	116.2	7.94 d (8.5)	129.4
3'		145.0	7.06 d (8.5)	116.3
4'		149.6		159.0
5'	6.90 d (8.0)	117.3	7.06 d (8.5)	116.3
6'	7.65 dd (1.5, 8.0)	123.3	7.94 d (8.5)	129.4
OMe	3.90 s	56.1	3.92 s	56.3
7-*O*-Glc 1"	5.06 d (7.5)	100.0	5.05 d (7.5)	100.3
2"	4.74 dd (7.5, 9.0)	74.5	3.55 dd (7.5, 9.0)	74.0
3"	3.47 t (9.0)	77.0	3.47 t (9.0)	77.5
4"	3.45 t (9.0)	71.0	3.43 t (9.0)	71.5
5"	3.30 m	77.7	3.61 m	75.8
6"a	4.00 dd (5.0, 12.0)	67.5	4.64 dd (4.5, 12.0)	64.3
6"b	3.60 dd (3.0, 12.0)		4.25 dd (2.5, 12.0)	
Rha 1'"	4.80 d (1.5)	101.9		
2'"	3.94 dd (1.5, 3.4)	72.2		
3'"	3.88 dd (3.4, 9.5)	71.8		
4'"	3.55 t (9.0)	74.5		
5'"	4.20 m	69.6		
6'"	1.12 d (6.5)	17.6		
p-coumaroyl 1		124.9		127.0
2,6	7.45 d (8.5)	130.2	7.45 d (8.5)	129.5
3,5	6.73 d (8.5)	116.5	6.75 d (8.5)	116.0
4		161.0		150.1
α	6.38 d (16.0)	118.0	6.38 d (16.0)	118.0
β	7.41 d (16.0)	146.8	7.43 d (16.0)	147.0
COO		168.7		168.8

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

(C-7), δ 4.74 (H-2") and 168.7 (COO), and δ 4.80 (H-1'") and 67.5 (C-6"). Therefore, the structure quercetin 3-methyl ether-7-*O*-α-L-rhamnopyranosyl-(1→6)-(2"-*p*-coumaroyl)-β-D-glucopyranoside was assigned to compound **2.**

Compound **3** was obtained as a yellow amorphous powder and its ESI-MS showed an $[M-H]^-$ ion peak at *m/z* 591. The molecular formula $C_{31}H_{28}O_{12}$ was confirmed by elemental analysis. In the ^{1}H-NMR spectrum (Table 2) two singlets at δ 6.70 and 3.92, two doublets at δ 6.77 and 6.56 each (1H, d, *J* = 2.0 Hz), and two *o*-coupled protons at δ 7.94 and 7.06 each (2H, d, *J* = 8.5 Hz) were present permitting the identification of the aglycon as apigenin 4'-methyl ether or acacetin [5]. Additionally for **3**, resonances of one anomeric proton and one *p*-coumaroyl residue were observed in the ^{1}H-NMR spectrum at δ 5.05

(1H, d, *J* = 7.5 Hz), 7.45 and 6.75 each (2H, d, *J* = 8.5 Hz) and δ 7.43 and 6.38 each (1H, d, *J* = 16.0 Hz), respectively. 1D-TOCSY, DQF-COSY, and HSQC NMR experiments showed the presence of one β-D-glucopyranosyl unit characterized by an acylation shift at H_2-6 (δ 4.64 and 4.25). The configuration of the glucose unit was determined as reported for compound **1**. HMBC correlations confirmed the substitution sites of each residue allowing compound **3** to be identified as acacetin 7-*O*-(6"-*p*-coumaroyl)-β-D-glucopyranoside.

Compounds **4-12** were identified by 1D- and 2D-NMR spectroscopy and ESI-MS analysis and by comparison of their data with those reported in the literature [9-14] as quercetin 3'-methyl ether-3-*O*-α-L-rhamnopyranoside (**4**), quercetin 3'-methyl ether-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**), apigenin 7-*O*-(6"-*p*-coumaroyl)-β-D-glucopyranoside (**6**), quercetin 3-methyl ether-7-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**7**), 4-hydroxyphenyl-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**8**), 4'''-methyl ether amenthoflavone (**9**), roseoside (**10**), icariside B5 (**11**), and ampelopsisionoside (**12**).

Table 3: Scavenger effect on DPPH stable radical and superoxide anion of methanol fractions and compounds **1-12** isolated from *C. senegalensis.*

Fracts or Compds	DPPH Test $^{a}IC_{50}$ (μg/ml) ± ^{b}SD	Effect on $O_2^{\cdot-}$ $^{a}IC_{50}$ (μg/ml) ± ^{b}SD
A	178 ± 6.7	0.61 ± 0.04
B	14.22 ± 1.1	2.6 ± 0.35
C	7.01 ± 0.6	0.37 ± 0.03
D	6.47 ± 1.5	0.19 ± 0.05
E	4.56 ± 0.8	0.36 ± 0.02
F	25.65 ± 3.6	0.47 ± 0.03
1	9.75 ± 0.9	0.085 ± 0.002
2	1.08 ± 0.4	0.025 ± 0.003
3	61.59 ± 2.5	2.5 ± 0.4
4	6.69 ± 0.7	0.20 ± 0.01
5	52 ± 0.5	0.85 ± 0.06
6	110 ± 24	1.35 ± 0.09
7	94.33 ± 0.7	0.42 ± 0.05
8	-	-
9	4.31 ± 1.1	2.76 ± 0.01
10	527 ± 0.4	50 ± 0.4
11	32 ± 0.5	0.5 ± 0.01
12	25 ± 0.9	0.015 ± 0.03
^{c}Trolox	96 ± 1.7	-
^{d}SOD	-	89 ± 1.5

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

The preliminary *in vitro* biological analysis indicated that compounds **1-7** and **9-12** were able to quench DPPH radicals and exhibited a direct scavenging activity on superoxide anion; this radical was in fact produced by the reduction of β-mercaptoethanol, excluding the Fenton-type reaction and the xanthine/xanthine oxidase system (Table 3). Quercetin 3-*O*-(6"-caffeoyl)-β-D-glucopyranoside-3'-*O*-β-D-glucopyran-oside (**1**), quercetin 3'-methyl ether-3-*O*-α-L-rhamno-pyranoside (**4**), and 4'''-methyl ether amenthoflavone (**9**) exhibited the highest antioxidant capacity. On the other hand, the potent biological activity of quercetin is largely reported in literature [15].

Although both $O_2^{\cdot-}$ and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by the $^{\cdot}$OH radical, which is generated by the reaction between $O_2^{\cdot-}$ and H_2O_2 in the presence of transition metal ions [2]. In fact, the $^{\cdot}$OH radical can react with a number of target molecules including proteins, membrane lipids, and DNA.

Table 4: Effect of methanol fractions and compounds **1-12** isolated from *C. senegalensis* (100 μg/mL) on DNA cleavage induced by the photolysis of H_2O_2 and metal chelating activity.

	UD of supercoiled DNA (% of native DNA)	Ferrozine assay $^{a}IC_{50}$ (μg/mL) ± ^{b}SD
scDNA	100	
A	11 ± 2.4*	-
B	62.7 ± 3.7*	32 ± 4.5
C	78 ± 4.5*	47.6 ± 3.6
D	76.7 ± 2.6*	16.83 ± 2.5
E	95 ± 4.7*	19.74 ± 3.2
F	65 ± 4.6*	28.41 ± 0.9
1	36 ± 1.2*	13.65 ± 2.8
2	7 ± 1.6*	92 ± 1.9
3	9 ± 2.4*	25 ± 2.5
4	70 ± 2.7*	6.19 ± 0.19
5	10 ± 0.8*	44.64 ± 3.6
6	5 ± 0.9*	-
7	37 ± 0.6*	630 ± 67
8	3.4 ± 0.4*	625 ± 50
9	73 ± 4.7*	18.31 ± 2.4
10	2.6 ± 0.6*	-
11	15.3 ± 1.1*	222 ± 32
12	11.3 ± 3.1*	-
DMSO	75.3 ± 3.1*	-
^{c}DTPA	-	77.5 ± 2.3

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

Based on the data obtained from this study, compounds **1**, **4**, and **9** might also be able to modulate hydroxyl radical formation more efficiently than other compounds acting as direct scavengers and chelating iron. In fact, these natural compounds exhibited a more efficient protection against DNA strand scission induced by $^{\cdot}$OH radicals generated by UV-photolysis of H_2O_2 (Table 4), and showed metal

chelating activity capturing ferrous ions before ferrozine, with an IC_{50} value (concentration that inhibited the ferrozine-Fe^{2+} by 50%) of 13.65, 6.19 and 18.31 μg/mL, respectively (Table 4).

These data also suggest that the biological effect of *C. senegalensis* observed from ethnopharmacological studies is due in part to the anti-oxidant action of its active components.

Experimental

General: Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 10 cm microcell. Elemental analysis was obtained from a Carlo Erba 1106 elemental analyzer. UV spectra were recorded on a Perkin-Elmer-Lambda 12 spectrophotometer. A Bruker DRX-600 NMR spectrometer using the UXNMR software package was used for NMR experiments. ESIMS (negative mode) were obtained using a Finnigan LC-Q Advantage Termoquest spectrometer, equipped with Xcalibur software. TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by spraying with $Ce(SO_4)_2/H_2SO_4$ (Sigma-Aldrich, St. Louis, Mo, USA) and NTS (Naturstoffe reagent)-PEG (Polyethylene glycol 4000) solutions. Column chromatography was performed over Sephadex LH-20 (Pharmacia); reversed-phase (RP) HPLC separations were conducted on a Waters 515 pumping system equipped with a Waters R401 refractive index detector and Waters U6K injector, using a C_{18} μ-Bondapak column (30 cm x 7.8 mm) and a mobile phase consisting of MeOH-H_2O mixtures at a flow rate of 2 mL/min. GC analyses were performed using a Dani GC 1000 instrument. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used for all antioxidant assays.

Plant material and chemicals: The leaves of *Chrozophora senegalensis* were collected in Bandiagara, Mali, in 1999 and identified by Prof. N'Golo Diarra of the Departement Medicine Traditionelle (DMT), Bamako, Mali where a voucher specimen (DMT n. 0074) is deposited. pBR322 plasmid DNA, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), diethylenetriaminepentaacetic acid (DTPA) and 3-(2-pyridyl)-5,6-*bis* (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were obtained from Sigma Aldrich Co (St. Louis, USA); β-nicotinamide-adenine dinucleotide (NADH) was obtained from Boehringer Mannheim GmbH (Germany). All other chemicals were purchased from GIBCO BRL Life Technologies (Grand Island, NY, USA).

Extraction and isolation: The air-dried powdered leaves of *C. senegalensis* (600 g) were defatted with *n*-hexane and extracted successively by exhaustive maceration (3 x 1 L, for 48 h) with $CHCl_3$, $CHCl_3$-MeOH 9:1, and MeOH. The extracts were concentrated under reduced pressure to afford 13.4, 14.0, 13.8, and 62.4 g of dried residues, respectively. A portion of the MeOH extract (27.0 g) was partitioned between *n*-BuOH and H_2O to give a *n*-BuOH soluble portion (9.0 g); 5.0 g of this residue were chromatographed over a Sephadex LH-20 column (100 cm x 5 cm) with MeOH as the eluent. A total of 115 fractions were collected (10 mL each). These were combined according to TLC analysis [silica 60 F_{254} gel-coated glass sheets with *n*-BuOH-AcOH-H_2O (60:15:25) and $CHCl_3$-MeOH-H_2O (40:9:1)] to give nine pooled fractions (A-I). Fractions G, H, and I yielded compounds **3** (19.2 mg), **4** (40 mg), and **9** (30 mg), respectively. Fraction A (90 mg) was purified by RP-HPLC using MeOH-H_2O (45:55) to give compounds **10** (6 mg, t_R= 10 min) and **12** (5 mg, t_R= 20 min). Fraction B (36 mg) was purified by RP-HPLC using MeOH-H_2O (1:1) to give compounds **2** (8 mg, t_R= 10 min) and **11** (12 mg, t_R= 20 min). Fraction C (50.5 mg) was purified by RP-HPLC using MeOH-H_2O (45:55) to give compounds **5** (28 mg, t_R= 10 min) and **7** (10.8 mg, t_R= 20 min). Fraction D (100 mg) was purified by RP-HPLC using MeOH-H_2O (45:55) to give compounds **1** (14.5 mg, t_R= 10 min) and **6** (6.5 mg, t_R= 20 min), while fraction E (70 mg) was purified by RP-HPLC using MeOH-H_2O (55:45) to yield compound **3** (11 mg, t_R= 10 min). Finally, fraction F (85 mg) was chromatographed on a RP-HPLC using MeOH-H_2O (1:1) as the eluent to afford compounds **8** (5 mg, t_R= 28 min) and **4** (6.3 mg, t_R= 46 min).

Quercetin 3-*O*-(6''-caffeoyl)-β-D-glucopyranoside-3'-*O*-β-D-glucopyranoside (**1**)

Yellow amorphous powder.
$[\alpha]_D$: -27° (*c* 0.1, MeOH).
UV/Vis λ_{max} (MeOH) nm (log ε): 267 (3.99), 344 (4.32)
^{1}H NMR (600 MHz, CD_3OD): Table 1.
^{13}C NMR (600 MHz, CD_3OD): Table 1.
ESIMS: *m/z* 787 $[M - H]^-$.
Anal. Calcd for $C_{36}H_{36}O_{20}$: C, 54.83; H, 4.60. Found C, 54.79; H 4.62.

Quercetin 3-methyl ether-7-*O*-α-L-rhamnopyranosyl-(1→6)-(2''-*p*-coumaroyl)-β-D-glucopyranoside (2)

Yellow amorphous powder.
$[\alpha]_D$: +18° (*c* 0.1, MeOH).
UV/Vis λ_{max} (MeOH) nm (log ε): 265 (3.92), 356 (4.05).
^{1}H NMR (600 MHz, CD_3OD): Table 2.
^{13}C NMR (600 MHz, CD_3OD): Table 2.
ESIMS: *m/z* 769 $[M - H]^-$.
Anal. Calcd for $C_{37}H_{38}O_{18}$: C, 57.66; H, 4.97. Found C, 57.68; H 5.00.

Acacetin 7-*O*-(6''-*p*-coumaroyl)-β-D-glucopyranoside (3)

Yellow amorphous powder.
$[\alpha]_D$: +11° (*c* 0.1, MeOH).
UV/Vis λ_{max} (MeOH) nm (log ε): 269 (3.99), 321 (3.76).
^{1}H NMR (600 MHz, CD_3OD): Table 2.
^{13}C NMR (600 MHz, CD_3OD): Table 2.
ESIMS: *m/z* 591 $[M - H]^-$.
Anal. Calcd for $C_{31}H_{28}O_{12}$: C, 62.84; H, 4.76. Found C, 62.80; H 4.80.

Acid hydrolysis of compounds 1-3: A solution of each compound (**1-3**, 2.0 mg each) in 1 N HCl (1 mL) was stirred at 80°C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60°C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using an L-CP-Chirasil-Val column (0.32 mm x 25 m). Temperatures of the injector and detector were 200°C for both. A temperature gradient system was used for the oven, starting at 100°C for 1 min and increasing up to 180°C at a rate of 5°C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of L-rhamnose and D-glucose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Antioxidant activity in cell-free systems

Quenching of DPPH: The free radical-scavenging capacity of extracts, fractions and pure compounds was tested by their ability to bleach the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) [16]. The reaction mixture contained 86 μM DPPH and different concentrations of the natural compounds in 1 mL of ethanol. After 10 min at room temperature the absorbance at λ = 517 nm was recorded. Trolox (50 μM), a water-soluble derivative of vitamin E, was used as a standard.

Scavenger effect on superoxide anion: Superoxide anion was generated *in vitro* as described by Paoletti et al. [17]. The assay mixture contained in a total volume of 1 mL, 100 mM triethanolamine-diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, 10 mM β-mercapto-ethanol; some samples contained the natural compounds at different concentrations. After 20 min incubation at 25°C, the decrease in absorbance was measured at λ = 340 nm. Superoxide dismutase (SOD) (80 mU/mL) was used as a standard.

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

Metal chelating activity: The chelating of ferrous ions by fractions and pure compounds was estimated by the ferrozine assay [19]. Briefly, natural compounds were added to a solution of 0.15 mM $FeSO_4$. The reaction was initiated by the addition of 0.5 mM ferrozine and the mixture was shaken

vigorously and left standing at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. DTPA (5 μM) was used as a standard.

Supplementary data: NMR spectral data for quercetin 3'-methyl ether-3-*O*-α-L-rhamno-pyranoside (**4**), quercetin 3'-methyl ether-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**5**), apigenin 7-*O*-(6"-*p*-coumaroyl)-β-D-glucopyranoside (**6**), quercetin 3-methyl ether-7-*O*-α-L-rhamno-pyranosyl-(1→6)-β-D-glucopyranoside (**7**), 4-hydroxyphenyl-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**8**), 4'''-methyl ether amenthoflavone (**9**), roseoside (**10**), icariside B5 (**11**), and ampelopsisionoside (**12**).

Acknowledgments - This work was supported by the "Bioactive Compounds from Medicinal and Food Plants of Developing Countries" project of the Italian Ministry for University and Research (Ministero dell'Università e della Ricerca, MIUR).

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1097 - 1100

N1,*N2*,*N3*-Trisisopentenyl Guanidine and *N1*,*N2*-Diisopentenyl Guanidine, Two Cytotoxic Alkaloids from *Alchornea cordifolia* (Schumach.& Thonn.) Müll. Arg. (Euphorbiaceae) Root Barks

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Received: June 24th, 2006; Accepted: September 5th, 2006

Dedicated to the memory of Professor Ivano Morelli.

This paper describes the purification of two guanidine alkaloids: *N1, N2*-diisopentenyl guanidine (DIPG) **1** and *N1*,*N2*,*N3*-triisopentenyl guanidine (TIPG) **2** from *Alchornea cordifolia* root bark and reports their cytotoxic properties on cancer (HeLa, Mel-5, J774) and non cancer (WI 38) cells. TIPG showed the highest cytotoxicity with IC_{50} values from 0.7 to 14.3 µg/mL (2.6 to 54.3 µM) on the four cell lines while DIPG was much less active: IC_{50} 45.8 and 97.6 µg/mL (234.8 and 500.5 µM) on Mel-5 and HeLa and > 512.8 µM on J774 and WI 38. The results indicate that the cytotoxicity notably decreased with the loss of one isopentenyl substituent.

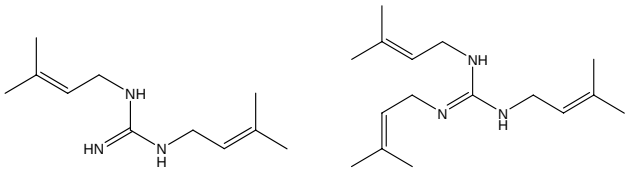
Keywords: *Alchornea cordifolia*, guanidine alkaloids, triisopentenyl guanidine (TIPG), diisopentenyl guanidine (DIPG), cytotoxicity.

A. cordifolia roots are widely used externally and internally to treat different illnesses [1-5] throughout tropical Africa. There have been several phytochemical studies on leaves and stem bark, but only a few old ones have dealt with the roots. The only compounds isolated were gentisic and anthranilic acids, tannins and a small quantity of alkaloids, one of which could be yohimbine, tannins were also found [3, 6].

In 1995, the antitumor activity of methanolic leaf extracts of *A. cordifolia* as well as fractions of different polarity were evaluated at a single high dose of 100 µg/mL against a panel of 60 human tumor cell lines and showed no significant activity [7].

This work reports the isolation and cytotoxic activity of *N1*,*N2*-diisopentenyl guanidine (DIPG) **1** and *N1*,*N2*,*N3*-triisopentenyl guanidine (TIPG) **2** from the root bark of *A. cordifolia*. *N1*,*N2*,*N3*-triisopentenyl guanidine was previously reported to be present in *A. cordifolia* leaves [8] but the reference cited to support the presence of this compound [9] does not mention it. While the crude extract (F) showed no cytotoxic activity on the cell lines tested, the partition of F between hexane and methanol-water gave a polar cytotoxic fraction (F_{MeOH}) while the non-polar fraction (F_{Hex}) showed a lower or no cytotoxicity (Table 1). The active F_{MeOH} fraction contained two major spots that were positive with Dragendorff and anisaldehyde/H_2SO_4 reagents and were purified by

HSCCC. Structures were determined by comparing the ESI-MS, ^{13}C and ^{1}H NMR spectra with literature data [10-12]. NMR chemical shifts of **1** and **2** are similar because of the electron delocalisation over the guanidine structure. Thus each isopentenyl substituent is nearly identical in NMR spectroscopy.



N1,N2-Diisopentenyl guanidine (DIPG) **1** *N1,N2,N3*-Triisopentenyl guanidine (TIPG) **2**

Table 1: *In vitro* cytotoxicity of *A. cordifolia* root bark extracts and isolated alkaloids (IC_{50} in µg/mL ± SEM).

Fractions/ compounds	cancer cell lines			non cancer cell line
	HeLa[a]	Mel 5[a]	J774[b]	WI 38[a]
F	>100	91.2 ± 6.1	ND	>100
F_{Hex}	49.2 ± 3.9	80.7± 19.5	ND	>100
F_{MeOH}	22.2 ± 2.2	20.2 ± 3.5	ND	41.4 ± 1.9
TIPG	11.1 ± 2.1	8.5 ± 1.3	0.7 ± 0.5	14.3 ± 1.6
DIPG	97.6 ± 4.6	45.8 ± 2.6	> 100	>100
Campto.	0.1 ± 0.2	0.7 ± 1.0	ND	2.4 ± 1.9
Colchicine	ND	ND	0.08± 0.02	ND

Campto. = camptothecin;[a] MTT assay; [b] Alamar Blue™ assay; ND = not determined.

Cytotoxicity was assessed on three human cell lines: two cancer (HeLa, Mel5) and one non cancer (WI 38) and one murine cancer cell line (J774) to detect an eventual selectivity. Both compounds showed a dose dependent cytotoxicity on the tested cell lines. In the first set of tests using MTT, we observed that for both isolated compounds Mel-5 cells proved to be the most sensitive while they were less toxic for WI-38. DIPG was less active and showed even no toxicity for WI-38 (Table 1). We also analysed the cytotoxicity on J774 using the Alamar Blue™ test and found that TIPG was very active (IC_{50} = 0.7 µg/mL, 2.6 µM) while DIPG could be considered as not toxic (IC_{50} >100 µg/mL, >512.8 µM). This stresses the importance of the three isopentenyl residues for the cytotoxic activity. Nevertheless, both compounds are much less effective than controls, which are highly cytotoxic compounds.

Up to now *N1*,*N2*,*N3*-TIPG was only reported in *Alchornea* species: *A. javanenesis* [10, 13] and *A. glandulosa* [12]. This last team reported that a crude MeOH leaf extract of *A. glandulosa* and fractions containing **2** exhibited an antiproliferative activity on cancer cells and antimicrobial activities on *Bacillus subtilis* and *Candida tropicalis.* Nevertheless they did not test the activity of the purified compound.

N1,*N2*-DIPG **2** was isolated previously from *Pterogyne nitens* leaves, under the name of pterogynidine [11, 14]. It was reported to inhibit the growth of a mutant yeast strain lacking a DNA repair mechanism but was not evaluated for its cytotoxicity [11]. This plant also contains an isomer *N1*,*N1*-DIPG (pterogynine) which was not tested on yeast [14].

Another monosubstituted guanidine alkaloid (galegine) was isolated from different species of *Galega, Verbesina* and *Schoenus* [15, 16]. Although it was considered as toxic by its direct effect on pulmonary vascular permeability on some animals [17, 18], there is no *in vitro* toxicity study reported on human cell lines. It has also to be noted that aliphatic guanidine alkaloids have been shown to possess different biological properties: agmatine as hypotensive [19] or aplysillamides A and B as antimicrobials [20], an activity which may also be shared by the guanidines from *A. cordifolia.* In fact, *A. cordifolia* root bark extracts possess antimicrobial properties which may at least be due to alkaloids but also probably to tannins [21, 22].

In conclusion, we isolated, for the first time, two isopentenyl guanidine derivatives from *A. cordifolia* root bark. TIPG was shown to possess cytotoxic activity on different cell lines while DIPG was much less active. This emphasizes the importance of the three isopentenyl substituents but TIPG is at least 10 times less cytotoxic than camptothecin or colchicine. Furthermore, the presence of these compounds could partially explain some uses of *A. cordifolia* in African folk medicine.

Experimental

General: ^{13}C NMR (125.7 MHz) and ^{1}H NMR (500 MHz) experiments were carried out in CD_3OD with a Bruker Avance 500 spectrometer. Chemical shifts are reported in part per million (ppm). UV spectra were recorded in MeOH with an UVIKON 933 (Kontron Instrument). A Perkin Elmer spectrometer was used for IR spectra. Mass spectra were obtained by direct injection in ESI positive mode using a LCQ Advantage (Thermo Finnigan) mass spectrometer.

Plant material: The fresh leaves and root barks of *A. cordifolia* were collected in Kinshasa and identified at INERA (Institut National pour l'Etude et la Recherche Agronomique, University of Kinshasa). A voucher specimen is deposited at the Belgian National Botanic Garden (BR) bearing the number SP 848103.

Extraction and isolation: Dried root bark powder (250 g) was moistened with a 500 mL of 10% Na_2CO_3 aqueous solution overnight and then extracted in a Soxhlet successively with EtOAc (1.5 l) and $CHCl_3$ (1.5 l). These extracts were combined and evaporated under reduced pressure. The residue (F: 0.87%) was partitioned between hexane and MeOH-H_2O (8:2) and 500 mg of the polar fraction (F_{MeOH}) purified by HSCCC (High Speed Counter Current Chromatography) (Kromaton III, SEAB, France) with the solvent system hexane/EtOAc/ *n*-BuOH/H_2O/AcOH 1: 1: 2: 5: 0.2 in the head to tail elution mode (the lower phase was the mobile phase) and a flow rate of 2 mL/min to give DIPG **1** (10.5 mg) and TIPG **2** (29.8 mg). T_R of **1:** 450–630 mL, **2**: 906–1200 mL. Due to the presence of AcOH in the solvent system, DIPG and DIPG were obtained as the acetates. Detection of eluate was performed by TLC: Merck silica gel 60 F_{254} plate, toluene/EtOAc/MeOH/ HCOOH 58:15:20:7; Dragendorff and anisaldehyde/ H_2SO_4 reagents.

***N1,N2*-diisopentenyl guanidine** (**1**)

IR (NaCl) ν_{max}: 3186, 1625, 1448 cm^{-1}.
UV (MeOH) λ_{max} nm: 203, 273.
^1H NMR (CD_3OD): 5.25 (tm, 1H, CH, J = 7.0 Hz), 3.78 (d, 2H, N-CH_2, J = 7.0 Hz), 1.90 (s, 3H, CH_3 acetate), 1.77 (s, 3H, CH_3), 1.72 (s, 3H, CH_3).
^{13}C NMR-APT (CD_3OD): 180.1 (C=O, acetate), 157.3 (C=N), 139.0 (C=CH), 119.5 (CH=C), 40.5 (-CH_2-NH-), 25.6 (CH_3), 24.0 (CH_3, acetate), 18.0 (CH_3).
ESI-MS-MS: *m/z* (rel. int.) = 196 $[M]^+$ (100), 127 (8).
Colourless oil, acetate salt, $C_{11}H_{21}N_3$.
Yield: 4.2%

***N1,N2,N3*-triisopentenyl guanidine** (**2**)

IR and UV spectra were identical to *N1,N2*-diisiopentenyl guanidine.
^1H NMR (CD_3OD): 5.24 (tm, 1H, CH, J = 6.8 Hz), 3.82 (d, 2H, N-CH_2, J = 6.8 Hz), 1.89 (s, 3H, CH_3 acetate), 1.77 (s, 3H, CH_3), 1.71 (s, 3H, CH_3).
^{13}C NMR-APT (CD_3OD): 180.1 (C=O, acetate), 156.1 (C=N), 138.6 (C=CH), 120.0 (CH=C), 40.7 (CH_2-NH-), 25.8 (CH_3), 24.2 (CH_3, acetate), 18.0 (CH_3).
ESI-MS-MS: *m/z* (rel. int.) = 264 $[M]^+$ (100), 310 (10), 195 (8).
Colorless oil, acetate salt, $C_{16}H_{29}N_3$.
Yield: 11.92%

Cytotoxicity assay: Two methods were used to assess cytotoxicity depending on availability. They both rely on the same properties of living cells whose enzymes transform substrates introduced in the medium into coloured or fluorescent derivatives. The concentrations of these derivatives have been shown to be proportional to the number of living cells in most cases [23, 24].

MTT (Methylthiazoletetrazolium) assay: Cytotoxicity was determined on HeLa (human cervix carcinoma cells), Mel 5 (human node metastasis derived human melanoma cells clone 32 [25]) and WI38 (human lung fibroblasts) cell lines as described previously [26] with the following modifications: the Dulbecco's Modified Eagle Medium was supplemented with 5% heat-inactivated fetal bovine serum, L-glutamine 0.33%, non-essential amino acids 1%, penicillin/streptomycin 1% and sodium pyruvate 1%. Each extract and alkaloids were tested in a concentration range from 3.1 µg/mL to 50 µg/mL. Camptothecine (Sigma) was used as a positive control.

The results are expressed by IC_{50} values (concentration of compound causing 50% inhibition of cell growth) calculated from graphs using at least five different concentrations of each alkaloid. All experiments were made at least in triplicate.

Alamar Blue™ assay: The assay was run as described previously by Hoet et al. on J774 cells (murine macrophages). Colchicine (Sigma) was used as a positive control [27].

Fluorescence development was calculated as percentage of the control culture, considered as 100%, and IC_{50} values (concentration of extract that reduced fluorescence intensity by 50%) were calculated by linear interpolation according to Hills [28].

Acknowledgments - The authors wish to thank V. Derrider for SM measurements and G. Muccioli for the 1D-NMR spectra.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1101- 1106

Indole Monoterpenes with Antichemotactic Activity from *Psychotria myriantha*: Chemotaxonomic Significance

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Received: June 6th, 2006; Accepted: August 8th, 2006

Dedicated to the memory of Professor Ivano Morelli.

The alkaloid extract of the aerial parts of *Psychotria myriantha* (Rubiaceae) displayed antichemotactic activity on polymorphonuclear leukocytes (PMN) assessed by the Boyden chamber assay. On analysis of the crude extract by LC/APCI/MS and LC/UV/DAD, two major constituents could be detected. In order to rapidly identify the active compounds, a microfractionation was conducted during LC/UV/DAD analysis. By this means, both the collected compounds could be assayed separately in the Boyden chamber and were shown to inhibit PMN chemotaxis. Their isolation was performed by semi-preparative HPLC and their structures elucidated by classical spectroscopic methods, including UV, NMR, MS and HRMS. Both compounds showed characteristics of monoterpene indole glucoside alkaloids; one of them was identified as strictosidinic acid and the other was a new natural product, myrianthosine. The antichemotactic activity of the compounds may be related to an antiacute inflammation activity.

Keywords: *Psychotria myriantha*, Rubiaceae, antichemotactic activity, glucoside indole monoterpene alkaloids, myrianthosine, strictosidinic acid.

Psychotria myriantha Mull. Arg. is a shrub (up to 2 meters high) occurring in southern Brazil [1]. Up to now, no phytochemical work has been reported on the species. As part of our continuing work on the Rubiaceae [2-5], the alkaloid extract from *P. myriantha* was tested for antichemotactic activity. From this extract, two active indole monoterpene alkaloid glucosides were isolated.

A polar alkaloid-rich extract of *P. myriantha* aerial parts was prepared as described in the experimental section. In order to identify the constituents, the alkaloid extract was analyzed by LC/UV/MS, with an atmospheric pressure chemical ionization (APCI) interface. Compound **1** gave an ion at *m/z* 517.1 $[M+H]^+$, while compound **2** gave a molecular ion at *m/z* 531.0 $[M+H]^+$ (Figure 1). The UV spectrum of compound **1** showed absorptions of an indole chromophore (226 and 280 nm), while compound **2** showed three absorptions due to an extended chromophore at 240 (sh), 290 and 350 nm (Figure 1) [4]. LC microfractionation was performed on the extract and the inhibition of polymorphonuclear leukocyte (PMN) chemotaxis by an antichemotactic assay in a modified Boyden chamber was assessed in collected fractions. By this means, inhibition properties could be rapidly linked to two of the LC peaks at retention times of 11.0 and 12.5 min. The migration (μm) of PMN control cells and PMN treated with *P. myriantha* extract, **1** and **2** are shown in Table 1 as mean ± S.D.

For full structure determination, **1** and **2** (Figure 2) were isolated by semi-preparative HPLC. Compound

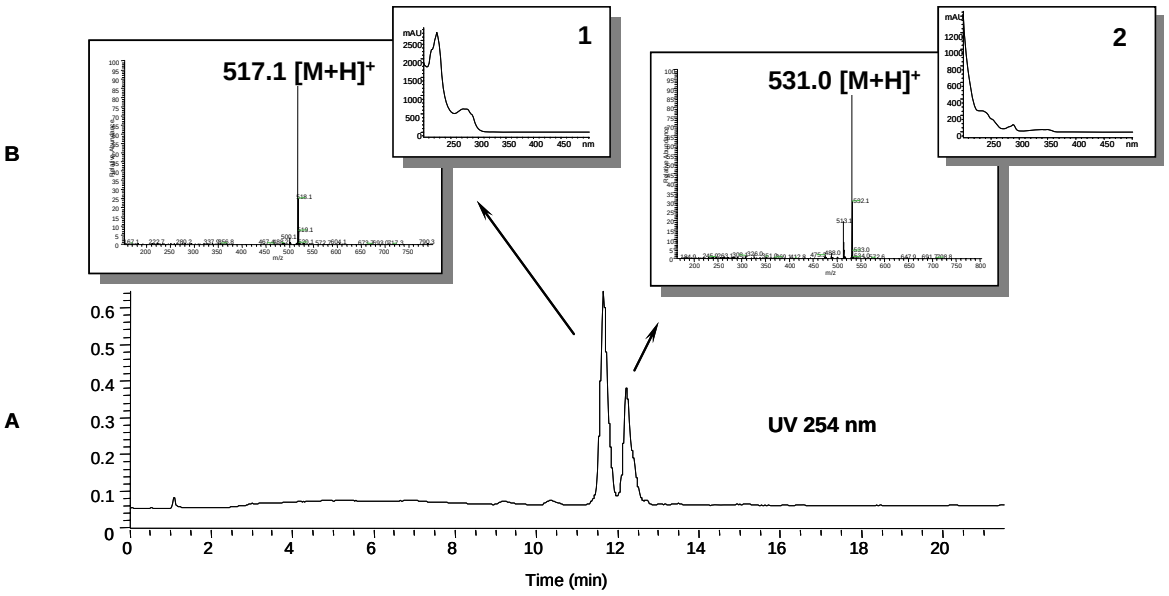

Figure 1: (A) LC-UV-APCI/MS analysis of the alkaloid extract of the aerial parts of *Psychotria myriantha.* (B) UV and mass spectra of compounds **1** and **2**. (LC/UV/MS conditions: see Experimental section).

1 was identified as strictosidinic acid by comparing its data with those of published values [6]. Compound **2** was isolated as an amorphous solid. The high-resolution electrospray mass spectrum (HRESI/MS) showed a $[M+H]^+$ peak at an exact mass of *m/z* 531.2344 corresponding to the formula $C_{27}H_{35}N_2O_9$, suggesting the presence of an additional methyl group when compared to compound **1**. A careful analysis of ^1H, ^{13}C and 2D NMR data indicated the presence of an open secologanin unit. As observed for compound **1**, the ^1H NMR spectrum showed the presence of four signals at δ_H 8.17 (H-9, J = 7.81 Hz), 7.20 (H-10, J = 7.33 and 7.81 Hz), 7.50 (H-11, 7.33 and 7.81 Hz), 7.56 (H-12, J = 7.82 Hz), attributed to the aromatic protons of the indole moiety. However, the presence of two doublets at δ_H 7.93 (H-6, J = 4.8 Hz) and 8.23 (H-5, J = 4.8 Hz) indicated the presence of a double bond between the carbons H-6 and H-5. Analysis of the COSY and the HMBC spectra for the remainder of the molecule suggested the presence of a secologanin unit. All the same, careful analysis of these data revealed some differences when compared with **1**. The HMBC spectrum showed correlations between the signal at δ_H 1.00 and δ_C 119 (C-18), 135 (C-19) and 96 (C-21) suggesting the presence of a methyl group in position C-20. These data indicated that the ring of the secologanin was open. The attachment of the open secologanin unit to the β-carboline skeleton was ascertained from the HMBC spectrum. The correlations between the methine proton at C-3 (δ_H 3.11), and the carbon at C-15 (δ_C 30.5) suggested the attachment of the unit at C-3. These data led to the structure of compound **2**. The HMBC spectrum of compound **2** showed correlations between the anomeric proton at δ_H 4.50 and the carbon at δ_C 95.4, suggesting the presence of a sugar moiety attached to C-21. The 2D NMR experiments (COSY, HMBC and HSQC) allowed the identification of the sugar as β-glucose. Acid and enzymatic hydrolysis confirmed the presence of β-D-glucose. These results pointed to a new natural product named myrianthosine.

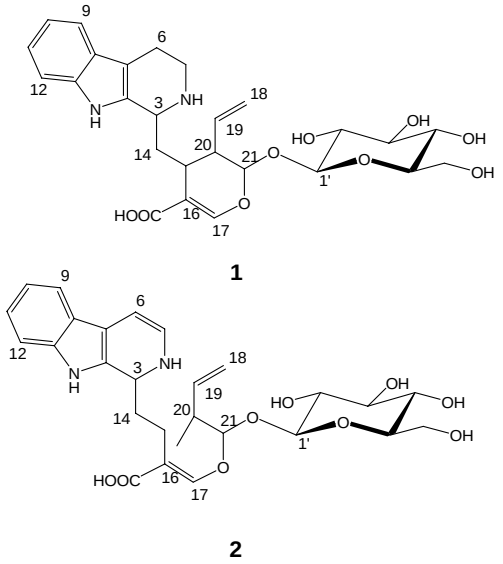

Figure 2: Structures of compounds **1** and **2**.

Psychotria, one of the largest genera of angiosperms, is taxonomically complex and has been the object of investigation by several authors. Due to classification uncertainties, this genus has been placed close to *Cephaelis* and *Palicourea* [7,8]. Based on morphological parameters and geographical distribution, different authors suggested the division of *Psychotria* into three subgenera: *Psychotria* (pantropical), *Tetramera* (species from Africa and Madagascar) and *Heteropsychotria* (including the remainder of the neotropical species) [9-11].

Polyindoline alkaloids are the main metabolites found in the *Psychotria* genus, resulting from condensation of *N*(b)-methyltryptamine moieties [12-16]. These alkaloids have been found particularly in species classified into the subgenus *Psychotria*.

On the other hand, the subgenus *Heteropsychotria* has been characterized by the presence of monoterpene indole alkaloids. Isodolichantoside, correantoside, correantines A, B and C, 20-epi-correantine B, correantine and 10-hydroxycorreantoside were isolated from leaves of *P. correae* growing in Panama [17].

From *Heteropsychotria* species found in the forests of Southern Brazil, glycosylated indole alkaloids have also been identified, such as lyaloside, strictosamide and naucletine from *P. suterella* [5]; umbellatine from *P. umbellate* [18]; brachycerine from *P. brachyceras* [3,4]; and *N*-β-D-glucopyranosyl vincosamide from *P. leiocarpa* [2]. Some *Psychotria* species were formerly included in the genus *Cephaelis*, which is considered by some authors as a synonym of the subgenus *Heteropsychotria* [8,11,16]. However, *Cephaelis* alkaloids are usually tyrosine derivatives, whereas monoterpene indole alkaloids found in *Heteropsychotria* species are tryptophan derivatives [2]. The observation of different alkaloid profiles in different subgenera illustrates the importance of chemical analysis of *Psychotria* species, which may help to establish new groupings within this genus. Alkaloid type segregation among *Psychotria* species may also be related to their geographical distribution. It is remarkable that indole monoterpene alkaloids seem to be a constant feature in neotropical *Psychotria*, as observed for species occurring in Southern Brazil [2-5] as well as in Panama [17,19]. The presence of compounds **1** and **2** in *P. myriantha* corroborates this observation. From the biogenetic point of view, indole monoterpene alkaloids are synthesized from tryptamine and the iridoid secologanin. Different secologanin derivatives have been reported to condense with tryptamine, such as the 10-oxo-1-*epi*-loganin precursor in brachycerine isolated from *P. brachyceras* [3]. The present work is the first report of an indole monoterpene alkaloid (**2**) incorporating an open ring of a secologanin derivative with an additional methyl group. Despite work done on the intermediates in the biosynthesis of secologanin [20], there is no precedent for the monoterpene moiety in the literature, and consequently a biosynthetic scheme for myrianthosine has not been attempted. The fact that strictosidinic acid (**1**) and myrianthosine (**2**) are genuine alkaloids of *P. myriantha* was confirmed by LC analysis of rapidly prepared ethanolic extracts of fresh leaves without acid/base extraction.

Strictosidinic acid (**1**) has previously been isolated from *Hunteria zeylanica* (Retz.) Gardner ex Thwaites (Apocynaceae) and has shown analgesic and antipyretic activities in mice after oral administration [21]. In the present work, it has been demonstrated that this alkaloid, together with myrianthosine (**2**), are responsible for the activity of the alkaloid extract of *P. myriantha* and were able to inhibit *in vitro* polymorphonuclear leukocytes (PMN) chemotaxis (Table 1). This activity has been demonstrated for some second-line anti-inflammatory drugs, such as D-penicillamine [22].

Table 1: Antichemotactic activity of *P. myriantha* alkaloid extract and isolated compounds.

Sample	PMN migration (μm)[a]
Control cells	129.2 ± 1.68
P. myriantha alkaloid extract	12.4 ± 0.84*
myrianthosine	14.6 ± 0.96*
strictosidinic acid	12.6 ± 0.96*
genistein[b]	9.9 ± 1.00*

[a]Values given as mean ± S.D of 10 measurements
[b]Positive control
*Statistically significant - $p<0.005$ (Student's *t*-test)

The inhibition of PMN chemotaxis reported here may be involved in the mechanism of the previously published antipyretic and analgesic activities of strictosidinic acid (**1**) [21] and the analgesic activity of *P. myriantha* extract [23].

Experimental

General: Optical rotation $[\alpha]_D$ was determined using a Perkin-Elmer 241 polarimeter (MeOH, *c* in g/100 mL). ^{1}H- and ^{13}C NMR were recorded on a Varian Unity Inova 500 spectrometer (500 MHz and 125 MHz, respectively) in DMSO-d_6; chemical shifts

in ppm as δ rel. to Me_4Si (int. std.). LC/MS was performed directly after UV-DAD measurements. A Finningan LCQ ion trap (Finningan MAT, San Jose, CA, USA) with APCI interface was used with the following conditions: capillary temp. 150°C; vaporizer temp. 370°C; positive mode; sheath gas flow: 60 arb, corona needle current 5 µA; spectra (150-900 mu). HRESIMS was performed using a Bruker FTMS 4.7T. TLC: silica gel 60 F_{254} Al sheets (Merck), detection at 254 nm and with vanillin-sulfuric acid reagent. LC/UV-DAD analysis of the alkaloid extract was performed on a Hewlett-Packard (Waldbronn, Germany) Series 1100 photodiode array detector (DAD) liquid chromatograph system. The separation was achieved on a Nucleosil 100-5 C_{18} AB column (125 x 4.6 mm i.d., 5 µm; Macherey-Nagel) with MeOH/H_2O (containing Et_3N 2 mM) in the gradient mode (10% of MeOH to 100% in 40 min). The flow rate was 1 mL/min; the UV traces were measured at 210 and 254 nm and UV spectra (DAD) were recorded between 200 and 500 nm. HPLC microfractionation: Fractions were collected, after the LC/UV analysis, every 1 min (1 mL) in Eppendorf tubes by a Gilson collector (FC204). After collection, all fractions were evaporated to dryness on a Speedvac system (RCT 90, Jouan). The content of each fraction was suspended in 1 mL of PMN suspension and then used for the antichemotactic assay. Semi-preparative HPLC was carried out with a Shimadzu LC-8A pump equipped with a Knauer UV detector using a Symmetry-Prep column (7 µm, 19x150 mm, Waters).

Plant material: *P. myriantha* was collected in Reserva Estadual do Turvo, Derrubadas Rio Grande do Sul, Brazil and identified by M. Sobral. A voucher specimen (M. Sobral *et al.*, 8913) was deposited in the ICN Herbarium (Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil).

Extraction and isolation: Dried leaves (823 g) were extracted with EtOH (3.5 l) at room temperature. The extract was concentrated under vacuum at 40 °C and an alkaloid extract was obtained by classical acid/base extraction. In addition to the alkaloid rich CH_2Cl_2 extract, it was noticed that the aqueous fraction was also positive for alkaloids with Dragendorff reagent. In order to extract these alkaloids, a second partition was conducted between the residual aqueous fraction and *n*-BuOH. The butanolic extract (1 g) was purified by semi-preparative HPLC using Symmetry-Prep column (7 µm, 19 x 150 mm, Waters), MeOH-H_2O (30:70) with Et_3N 2 mM, flow rate 10 mL/min, UV 254 nm) providing alkaloids **1** (33 mg) and **2** (12 mg).

Acid hydrolysis: Compounds **1** and **2** were submitted to hydrolysis with 20 mL of HCl 0.05 N at 65°C during 24h. Sugars were extracted by partition with *n*-BuOH and were compared by TLC (solvent: AcOEt/Formic acid/H_2O 100:20:30; detected with *p*-anisaldehyde-H_2SO_4 reagent) with reference compounds: glucose (R_f 0.40), arabinose (R_f 0.45), fructose (R_f 0.41), rhamnose (R_f 0.64), and galactose (R_f 0.33), all 1 mg/mL in H_2O. Compounds **1** and **2** gave glucose (R_f 0.40).

Enzymatic hydrolysis: Compounds **1** and **2** were treated with β-D-glucosidase in 1 mL NaOAc buffer (pH 5.0) for three days at 40°C. The aglycones were extracted by partition with *n*-BuOH, and submitted to LC/UV-DAD analysis hydrolysis in order to confirm hydrolysis.

Antichemotactic assay: Chemotaxis was measured in a Boyden chamber by the method previously described [24]. Prior to the chemotactic assay, rat leukocytes were treated with 100 µg/mL of each sample (alkaloids and extract), at 37°C for 1 h. Plasma collected from rats was incubated at 37°C for 30 min with 65 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli*, and then diluted in Hanks buffer 1:5 (v/v). Chemotactic migration of leukocytes through an 8.0-µm cellulose nitrate filter, towards the chemotactic stimulant (LPS treated plasma) was measured after incubation for 1 h at 37°C using the micrometer on the fine-focus knob of a Nikon Alphaphot-2 YS2 microscope. The distance from the upper surface of the filter to the lower surface of focus still containing two cells allowed the evaluation of leukocyte migration in five microscopic fields per filter. The assay was carried out in duplicate and measurements were statistically analyzed by Student's *t*-test, using genistein as positive control.

Strictosidinic acid (**1**)

Amorphous powder.
$[\alpha]^{25}_D$: +143.14° (*c* 0.1, MeOH).
Rf: 0.30: TLC system: ethyl acetate / acetic acid / formic acid / water (100:11:11:10).
^{1}H NMR (500 MHz, DMSO-d_6): 4.10 (1H, d, J = 10.4 Hz, H-3), 2.92-3.47 (2H, m, H-5), 2.73-2.92 (2H, m, H-6), 7.41 (1H, d, J = 7.81 Hz, H-9), 6.97 (1H, dd, J = 7.33 and 7.81 Hz, H-10), 7.05 (1H, dd,

J = 7.33 and 7.81 Hz, H-11), 7.29 (1H, d, *J* = 7.81 Hz, H-12), 1.95-2.20 (2H, m, H-14), 2.90 (1H, m, H-15), 7.38 (1H, s, H-17), 5.13 (1H, d, *J* = 11.23 Hz, H-18a), 5.30 (1H, d, *J* = 17.09 Hz, H-18b), 5.76 (1H, m, H-19), 2.60 (1H, m, H-20), 5.62 (1H, d, *J* = 9.70 Hz; H-21), 4.63 (1H, d, *J* = 7.81 Hz, H-1'), 3.10 (1H, m, H-2'), 2.90 (1H, m, H-3'), 3.20 (1H, m, H-4'), 4.12 (1H, m, H-5'), 3.50 (1H, m, H-6'α), 3.70 (1H, m, H-6'β).
^{13}C NMR (125 MHz, DMSO-d_6): 132.3 (C), 49.6 (CH), 40.0 (CH_2), 19.2 (CH_2), 106.0 (C), 126.1 (C), 117.8 (CH), 118.7 (CH), 121.2 (CH), 111.5 (CH), 135.8 (C), 33.7 (CH_2), 31.8 (CH), 113.4 (C), 150.0 (CH), 117.8 (CH_2), 135.6 (CH), 44.3 (CH), 95.1 (CH), 170.0 (C), 98.9 (CH), 69.8 (CH), 73.1 (CH), 77.2 (CH), 76.5 (CH), 61.0 (CH_2).
APCIMS *m/z* 517.4 $[M+H]^+$. HRESIMS *m/z* 517.2172 $[M+H]^+$, (calculated for $C_{26}H_{33}N_2O_9$, 517.2186).

Myrianthosine (**2**)

Amorphous powder.
$[\alpha]^{25}_D$: +80.8° (*c* 0.1, MeOH).
Rf: 0.27; TLC system: ethyl acetate / acetic acid / formic acid / water (100:11:11:10).
^{1}H NMR (500 MHz, DMSO-d_6): 3.11 (1H, m, H-3), 8.23 (1H, *J* = 4.80 Hz, H-5), 7.93 (1H, *J* = 4.80 Hz, H-6), 8.17 (1H, d, *J* = 7.81 Hz, H-9), 7.20 (1H, dd, *J* = 7.33 and 7.81 Hz, H-10), 7.50 (1H, dd, *J* = 7.33 and 7.81 Hz, H-11), 7.56 (1H, d, *J* = 7.81 Hz, H-12), 2.70 (2H, m, H-14), 1.25 (2H, m, H-15), 7.40 (1H, s, H-17), 4.60 (1H, d, *J* = 17.1 Hz, H-18a), 4.80 (1H, d, *J* = 10.2 Hz, H-18b), 5.70 (1H, ddd, *J* = 17.10, 10.20 and 2.00 Hz, H-19), 2.60 (1H, m, H-20), 1.00 (3H, d, *J* = 7.8 Hz, CH_3-20), 5.47 (1H, d, *J* = 5.3 Hz; H-21), 4.50 (1H, d, *J* = 7.8 Hz, H-1'), 3.00 (1H, m, H-2'), 3.10 (1H, m, H-3'), 3.20 (1H, m, H-4'), 4.10 (1H, m, H-5'), 3.41 (1H, m, H-6'α), 3.70 (1H, m, H-6'β).
^{13}C NMR (125 MHz, DMSO-d_6): 134.8 (C), 48.5 (CH), 137.0 (CH), 111.8 (CH), 121.0 (C), 121.5 (C), 126.6 (CH), 118.9 (CH), 127.5 (CH), 112.5 (CH), 140.2 (C), 45.6 (CH_2), 30.0 (CH_2), 112.0 (C), 151.0 (CH), 118.9 (CH_2), 134.5 (CH), 45.5 (CH), 95.4 (CH), 170.0 (C), 98.6 (CH), 73.0 (CH), 69.9 (CH), 77.3 (CH), 76.8 (CH), 61.0 (CH_2), 10.4 (CH_3).
APCIMS m/z positive: 531.2 $[M+H]^+$.
HRESIMS *m/z* $[M+H]^+$ calcd for $C_{27}H_{35}N_2O_9$: 531.2337; found: 531.2344.

Acknowledgments - The Swiss National Science Foundation (grant n° 200020-100083 to K. Hostettmann) and CNPq (Brazil) are gratefully acknowledged for supporting this work.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1107 - 1110

HPLC Based Activity Profiling for Inhibitors of Human Neutrophil Elastase in *Isatis tinctoria* Leaf Extracts

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Received: June 7th, 2006; Accepted: August 5th, 2006

Dedicated to the memory of Professor Ivano Morelli.

In continuation of our search for anti-inflammatory constituents in lipophilic extracts of *Isatis tinctoria*, an HPLC-based profiling for inhibitors of human neutrophil elastase was performed. Using a linear gradient profile, fractions with significant activity (>50%) appeared in the second half of the chromatogram and corresponded to moderately to highly lipophilic constituents. The active fractions of intermediate polarity were further analyzed by HPLC, TLC and GC. They contained mainly α-linolenic, linoleic, *cis*-11-octadecenoic acid, oleic and palmitic acids.

Keywords: *Isatis tinctoria*, anti-inflammatory, human neutrophil elastase, α-linolenic acid, linoleic acid, activity profiling.

The woad plant (*Isatis tinctoria* L, Brassicaceae) is an old indigo dye and medicinal plant of temperate climate zones. The plant was used for the treatment of wounds, ulcers, snakebites, and various inflammatory afflictions, but fell into oblivion with the disappearance of woad cultivation [1]. In contrast, the related *I. indigotica* is a widely used plant in traditional Chinese Medicine up to now [2, 3].

Our interest in woad was spurred by its purported anti-inflammatory properties for which we obtained first, *in vitro* evidence in a broad-based pharmacological screening involving more than 20 clinically relevant targets [4]. Subsequently, we identified the alkaloid tryptanthrin as a potent inhibitor of COX-2 and 5-LOX catalyzed eicosanoid synthesis [5, 6], and an indolin-2-one derivative as inhibitor of histamine release from mast cells [7]. We found that the major inhibitor of 5-LOX in woad extracts was γ-linoleic acid [8]. Further anti-inflammatory activities of *Isatis* constituents have been reported by other groups, for example, inhibition of the expression of inducible NO synthase [9] by tryptanthrin, and inhibition of IL-6 release and TNFα by indirubin [10]. Lipophilic *Isatis* extracts displayed activity in various *in vivo* models of inflammation, cutaneous allergy and arthritis [11, 12]. In a clinical pilot study, the same extracts reduced inflammation and water loss in a cutaneous irritation model [13].

A significant inhibition of leucocytic elastase from neutrophils had been observed in our initial *in vitro* screening [4]. This enzyme is one of two main proteinases of neutrophils released in connection with inflammation. It cleaves fibrous elastin, collagens, cartilage proteoglycans, and other matrix proteins. Elevated plasma levels of neutrophil elastase are a characteristic feature of an active inflammation [14, 15]. We, therefore, decided to identify the inhibitors of human neutrophil elastase in woad extracts with the aid of HPLC-based activity profiling using an established bioassay [16]. We had previously used the approach of HPLC profiling for

the identification of a variety of bioactive compounds from *I. tinctoria* [5, 7, 8] and other plants [17].

For HPLC profiling, the SFE extract was dissolved in dichloromethane (10 mg/mL) and separated on an analytical C-18 column. Aliquots of 200 µg extract per injection were repeatedly fractionated. A linear gradient from 10% to 100% acetonitrile over 25 min was followed by isocratic elution for an additional 20 min. In a first profiling step, 15 fractions of 3 min each (Fr. 1-15) were collected. The solvent was removed in an evaporator centrifuge and the dry films were redissolved in 180 µL DMSO for the elastase assay, which was carried out according to a published protocol [16], whereby *p*-nitro-aniline release was quantified by measurements of absorbancy at 405 nm. Representative HPLC chromatograms recorded at 254 and 220 nm and the activity profile of fractions 1 to 15 in the elastase assay are shown in Figure 1.

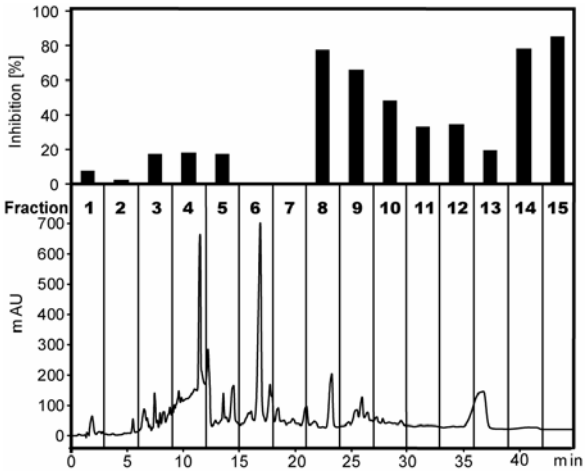

Figure 1: HPLC chromatogram of *Isatis tinctoria* extract recorded at 220 nm (bottom). Vertical lines indicate time windows for fractions 1 to 15. Inhibitory activity of fractions is shown above (mean of 3 independent experiments in two parallels).

Inhibitory activities > 50% were found in fractions 8 (77%), 9 (66%), 14 (78%) and 15 (85%). In a second step, fractions of 0.5 min were collected in the time window 21 to 30 mins, which corresponded to fractions 8 to 10. The HPLC profile of this time window and fractionation steps (8-1 to 10-6) are shown in Figure 2.

Fractions were analyzed by HPTLC (RP-18, HOAc/H_2O (95:5)). Upon staining with Godin's reagent, fractions 8-5, 9-2, 9-5 and 9-6 each showed one single violet spot in the R_f range 0.4 to 0.5. The

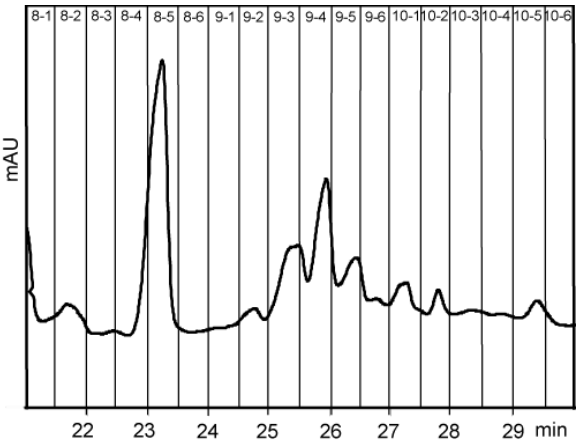

Figure 2: HPLC chromatogram recorded at 220 nm of the time window from 21 to 30 min, corresponding to fractions 8-1 to 10-6. Vertical lines indicate time windows for fractionation.

R_f values were comparable with α-linolenic, linoleic and oleic acids. For further analysis, fractions 8-1 to 10-6 were submitted to GC after derivatization with TMSH. The results of the fatty acid analysis of the major fractions 8-5, 9-2, 9-5 and 9-6 are shown in Figure 3. Identification was carried out by an overlay of GC chromatograms with a standard reference mix (C-18 FAME Isomer Mix, Supelco). Fraction 8-5 consisted of α-linolenic acid, fraction 9-2 of linoleic acid, whereas fraction 9-5 contained oleic acid and *cis*-11-octadecenoic acid in equal proportions. Fraction 9-6 consisted of palmitic acid.

Inhibition of human neutrophil elastase by saturated and unsaturated fatty acids has been recently reported. The IC_{50} of α-linolenic, linoleic, oleic and palmitic acids were in the range of 5 to 15 µM [18]. Hence, inhibitory activity in fractions 8 and 9 can be attributed to these fatty acids. HPTLC and GC analysis of fractions 14 and 15, however, showed that they contained only traces of fatty acids. Elastase inhibition in these fractions seems thus due to highly lipophilic compounds, which remain to be identified.

Experimental

Organic solvents for separations were HPLC grade (Roth, Karlsruhe, Germany). HPLC grade water was obtained from a Milli-Q RG water purification system (Millipore, Schwalbach, Germany).

Leaf material of *I. tinctoria* was harvested in 2001 from first year plants grown on experimental plots of the Agricultural Research Station of Thuringia (TLL), Dornburg, Germany. Fresh leaves were dried on a band drier operating at 60ºC.

Extracts were prepared by supercritical fluid extraction (SFE) on a pilot plant extractor, with the following conditions: CO_2 + 2000 ppm EtOH as modifier, 700 bar, 50ºC. Tryptanthrin concentration in the SFE extract was 0.23%, as determined by ESI-LC-MS. The extract was stored at –32°C. Fractionations were carried out with a HP 1100 HPLC system consisting of autosampler, high-pressure mixing pump, column oven, diode array detector, and HP workstation (Agilent, Waldbronn, Germany) connected to a Gilson FC 2004 fraction collector (Gilson, Middleton, USA). GC analysis of fatty acids was carried out on a Shimadzu 17A system (Shimadzu, Duisburg, Germany) with an AOC 5000 autosampler. TLC analysis was performed on RP-18 F254 HPTLC glass plates (Merck, Darmstadt, Germany).

HPLC separations were performed on a LiChrospher 100 RP-18 cartridge (5 µm, 125 x 4 mm i.d.; Merck, Darmstadt, Germany) using acetonitrile (A) and water (B) as eluents, with the following gradient profile: 10% A (0-2 min), 10% → 100% A (2-25 min), 100% A (25-45 min). The flow rate was set at 1.0 mL/min, and the column oven was at 25°C. HPLC traces were recorded at 220 and 254 nm, and UV-vis spectra from 190-700 nm.

For activity profiling, *Isatis* extract was dissolved in dichloromethane at a concentration of 10 mg/mL. Aliquots of 30 µL of the solution, corresponding to 300 µg extract, were injected for HPLC separation. The column effluent was fractionated into 5 mL vials. Fifteen fractions of 3 min each were collected. The solvent was removed at 50°C in a centrifugal evaporator (Evaporator centrifuge RC 10.22, Jouan GmbH, Unterhaching, Germany). The separation was repeated 4 times. The dried films were redissolved in MeOH (500 µL), transferred into 1.5 mL Eppendorff tubes, and dried again in a centrifugal evaporator prior to shipment for bioassay. In the second round of profiling, the time window from 21 to 30 min was fractionated at higher resolution. A total of 18 fractions of 0.5 min each (8-1 to 10-6) were collected. The fractions were processed as described above.

GC analysis was carried out after derivatization with trimethylsulfoniumhydroxide (TMSH) in MeOH (Macherey-Nagel, Düren, Germany). A 2 µL volume was injected onto a capillary column, DB 225 ms (length 60 m, 0.25 mm i.d., film thickness 0.25 µm) under the following conditions: split (1:20), injector temperature 260°C, mobile phase: H_2, (42 cm /s), detection: FID, detector temperature 270°C, temperature program: start 70 °C, hold for 2 min, 70 °C → 180°C (heating rate 10°C / min), 180°C → 220°C (heating rate 2 °C / min), hold time 5 min at 220°C, 220°C→ 230°C (heating rate 2°C / min), hold 15 min. The identification was carried out in comparison to a standard reference mix (C-18 FAME Isomer Mix, Supelco, Taufkirchen, Germany).

Plates were developed with AcOH/H_2O (95:5) as mobile phase. Compounds were stained with Godin's reagent (equal volumes of solution A and B, freshly mixed; solution A: 1% vanillin in EtOH + 3% $HClO_4$, solution B: 10% H_2SO_4 in EtOH) followed by heating at 105°C for 3 min (Thermoplate S, Desaga, Heidelberg, Germany). The compound in fraction 8-5 showed the same chromatographic mobility and staining as the reference α-linolenic acid (violet zone, R_f = 0.5), the compound in fraction 9-2 showed the same behaviour as linoleic acid (violet spot, R_f = 0.45), and the spot in fractions 9-5 and 9-6 showed the same behaviour as oleic acid (pale violet staining, R_f = 0.4).

Inhibition of neutrophil elastase was determined with human leucocyte elastase, according to a published procedure [16]. Briefly, 125 µL substrate solution (10 mM MeO-Suc-Ala-Ala-Pro-Val-pNA in Tris-HCl-buffer, 60 mM, pH 7.5) were mixed with 10 µL test solution (test substances solubilized in DMSO) + 445 µL Tris-HCL-buffer, pH 7.5 and vortexed. After the addition of 20 µL enzyme solution (approximately 1.05 mU) the samples were incubated for 1 h at 37°C. The reaction was stopped by addition of 500 µL soybean trypsin inhibitor solution (2 mg/mL Tris-HCl-buffer, pH 7.5) and placed in an ice bath. After vortexing, the absorbance was read at 405 nm. The assays were performed three times with duplicate samples and DMSO controls. Inhibition rates were calculated in percent to DMSO controls, and IC_{50} values calculated from the dose-inhibition curves by linear regression. As positive control for the elastase assay, the inhibitor GW311616A [19], with an IC_{50} of 90 nM, was used.

For HPTLC analysis, MeOH solutions (20 µL) of fractions 8-1 to 10-6 were sprayed onto a RP-18 plate with the aid of an AS 30 TLC applicator (Desaga, Heidelberg, Germany) along with reference solutions of α-linolenic, linoleic and oleic acids (1 mg/mL).

Acknowledgments - We thank Dr A. Vetter and Mrs A. Biertümpfel, TLL Jena and Dornburg, for the woad samples, Mrs M. Janka, FU Berlin, for technical assistance, Dr B. Weinreich, Adalbert-Raps-Forschungszentrum, TU München-Weihenstephan, for preparation of the SFE extract, Prof. G. Jahreis and P. Moeckel, Institute of Food Sciences, University of Jena, for fatty acid analysis, and T. Mohn, University of Basel, for assistance in preparation of figures.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1111- 1115

Variation in Artemisinin and Flavonoid Content in Different Extracts of *Artemisia annua* L.

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Received: June 30th, 2006; Accepted: October 24th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Artemisia annua L. is a promising and potent antimalarial drug. This activity has been ascribed to its content of artemisinin, a sesquiterpene lactone that is stage specific and very effective against drug-resistant *Plasmodium* species and which has low toxicity. The *in vitro* antiplasmodial activity of artemisinin is enhanced by the flavonoids of the extract, as recently proposed by the authors. Different extracts (tinctures, infusions and decoctions), obtained from a cultivar selected by the University of Campinas (0.52% artemisinin), were analyzed in order to prove the selectivity of the solvents to obtain high yields of both artemisinin and flavonoids. Tinctures 40 and 60% v/v showed a greater power of extraction in comparison with infusions and decoctions. The best performance was obtained using 60% v/v tincture. The extraction efficiency for artemisinin was 40% and for flavonoids was 29.5%. Among aqueous extracts, the best results were obtained by preparing an infusion with boiling water, left to cool for 15 minutes before filtration. The extraction efficiency for artemisinin was 57.5% and for flavonoids was 8.2%. If leaves are boiled for several minutes the artemisinin concentration is decreased, probably due to the heat instability of this constituent. Also microwave could represent a valid alternative method to extract the phytocomplex, the extraction efficiency for artemisinin was 41.0% and that for flavonoids was 7.7%.

Keywords: *Artemisia annua* L., extracts, artemisinin, flavonoids, HPLC/DAD/MS.

Artemisia annua L. (sweet or annual wormwood) is an annual herb endemic to the northern parts of Chahar and Suiyuan provinces in China, where it is known as 'quinghao' and has been used as a remedy for chills and fevers for more than 2000 years [1, 2]. Traditionally, the plant is used to prepare a drink, as indicated in the Compendium of Treatments (Ben Cao Gang Mu), written in 1596 AD by Li Shizhen: "take a handful of qinghao, soak it in a sheng (liter) of water, and squeeze out the juice and drink it all" [3, 4]. Nowadays the (daily) dose of *A*. *annua* given in the Chinese Pharmacopoeia for the treatment of various fevers, including malaria, is 4.5–9 g of dried plant material, extracted by heating with water [5, 6].

Surprisingly, only a few clinical studies based on the use of either extracts of the plant or traditional preparations are available. The first was carried out in 1992 in China: 144 malaria patients were treated with tablets containing either an ethanolic dried extract of *A. annua* or capsules using the same extract, but formulated in oil [7, 8]. Both treatments were found to be effective in reducing parasitaemia and fever at doses equivalent to 80.8 g (tablets) and 73.6 g (capsules) raw herb, given over a three-day period.

In the most recent literature, after development of high artemisinin-yielding plants (>0.5% per dried weight), clinical trials using either teas or decoctions have also been reported. The principal aim of such

investigations is related to the possibility for populations in endemic areas with either scarce or no access to modern medicines or medical services to cultivate selected cultivars of *A. annua* and prepare either teas or decoctions from the plant material, achieving a positive effect in the treatment of malaria.

An herbal tea prepared from a selected cultivar of *A. annua* with a high content of artemisinin (0.58% w/w dried herb) was evaluated in malaria patients in the Republic of Congo [9]. The dose was according to the recommendations of the Chinese Pharmacopoeia (5 g herbal drug/day for five days) and the extraction process was investigated in order to have the maximum extraction efficiency. It was proved that it is better to make an infusion rather than a short decoction (kept boiling for five minutes) in order to obtain the maximum extraction efficiency of more than 40%. In the study, about 90.9% of patients reported complete disappearance of malaria symptoms within the course of the treatment and it was proposed that the bioavailability of artemisinin from the tea preparations may exceed that from pure artemisinin tablets [9].

Two additional interesting papers regarding clinical studies using traditional preparations appeared almost contemporarily in 2004. In the first investigation [10], the patients received 1 L of preparation from 9 g leaves of a special cultivar of *A. annua* containing 1.39% artemisinin, which resulted in a content of 94.5 mg/L artemisinin, administered in five doses of 200 mL each per day. Even if the given dose of artemisinin was only 19% of the usual daily dose of artemisinin in adults [11], peak plasma levels were 240±75ng/mL artemisinin, approximately 40% of the peak concentrations reported after intake of 500 mg artemisinin in the form of capsules. The data indicated that artemisinin was absorbed faster from herbal tea preparations than from oral solid dosage forms, but the bioavailability was similar [10]. The other study that appeared in 2004 used an herbal drug containing 1.4% artemisinin and the infusions (5 or 9 g/herb /L; artemisinin content 47 and 94 mg/L, respectively) were administered divided into four doses of 250 mL each. Even if there were a higher rate of recrudescence, most of the reported malaria symptoms either improved or resolved within three days after initiation of therapy, as expected for either an artemisinin or quinine treatment [12]. All these studies have pointed out that the presence of flavonoids in the phytocomplex can enhance either the bioavailability or the activity of artemisinin. Thus, experimental evidence from *in vitro* studies suggests that some flavonoids may enhance the action of artemisinin against *P. falciparum* [13]. In an attempt to find an optimal extraction method for both artemisinin and flavonoids, we have reported the best recovery with *n*-hexane to obtain complete extraction of artemisinin and most of the flavonoids [14].

The aim of the present study was to analyze the qualitative and quantitative composition of different extracts of the aerial parts of a cultivar of *A. annua* (0.52% artemisinin) selected by the University of Campinas. The investigated extraction methods were several techniques of infusion and decoction

Table 1: Artemisinin and flavonoid contents of tincture preparations (T40: 40% v/v; T60: 60% v/v).

Sample	artemisinin %	artemisinin % in lyophilized material	extraction efficiency (%)	flavonoids %	flavonoids % in lyophilized material	extraction efficiency (%)
T40	27	0.75	26	41.1	2.64	15.7
T60	41	1.08	40	83.8	3.52	29.5

Table 2: Artemisinin and flavonoid contents of infusion and decoction preparations.

Sample	artemisinin % in lyophilized material	extraction efficiency (%)	flavonoids % in lyophilized material	extraction efficiency (%)
I1	0.72	30.4	5.38	5.61
I2	0.68	27.4	3.24	5.41
I3	0.80	57.5	5.18	8.17
D1	0.68	30.2	3.28	5.34
D2	0.81	35.9	6.18	9.93
M	0.61	41.0	4.89	7.66

I1: sample extracted with 1 L of boiling water, left to cool, filtered and lyophilized; I2: sample extracted with 1 L of boiling water, covered, left to cool, filtered and lyophilized; I3: sample extracted with 1 L of boiling water, left to cool for 15 min, filtered and lyophilized; D1: sample extracted with 1 L of boiling water, kept boiling for 5 min, left to cool, filtered and lyophilized;D2: sample extracted with 1 L of boiling water, kept boiling for 5 min, immediately filtered and lyophilized; M: sample treated with 1 L of water, kept boiling for 5 min with a microwave oven, left to cool, filtered and lyophilized.

(see experimental part) and two tinctures (40% v/v and 60% v/v), prepared according to the European Pharmacopoeia [14].

The flavonoid structures were determined by combining the HPLC/DAD/MS data with those previously reported [15]. Artemisinin was identified by mass spectrometry as the peak at 17.90 min. *n*-Hexane can selectively and exhaustively extract both artemisinin and flavonoids and, for this reason, was considered as a standard for comparison with other preparations.

Very different yields and contents of artemisinin and flavonoids were found in the tested extracts, as reported in Tables 1 and 2. Concerning the percentage of constituents in the *n*-hexane extract, artemisinin made up 12.8% and the total flavonoids 62.8%. In particular, the respective percentages of different flavonoids were 34.3% for casticin and chrysoplenetin, 9.4% for artemetin and 19.1% for eupatin. An additional peak was found in the tinctures and in aqueous preparations and identified as chrysosplenol-D by combining the HPLC/DAD/MS data with those previously reported [16].

The qualitative profile of the two tinctures was similar, while both the content of flavonoids and artemisinin was highest in the 60% v/v tincture, as reported in Table 1. The greater amount of ethanol in T60, compared with T40, increased the efficiency of the extraction. The extraction efficiency for artemisinin was 40% and for flavonoids 29.5%.

However, for all constituents of *A. annua*, the tinctures showed a greater power of extraction in comparison to infusions and decoctions, when the amount of solvent used was greater. In addition, after the freeze-drying process of the tinctures, the obtained dried powder of crude extract showed good technological properties and could be very useful in the formulation of either capsules or extemporaneous preparations.

In the case of infusions and decoctions, the highest contents of artemisinin and flavonoids were obtained from samples I3, D2 and M with 57.5%, 35.9% and 41.0% for artemisinin, respectively, and 8.2%, 9.9% and 7.7% for flavonoids. Artemisinin is known to be heat-unstable and Table 2 shows that tea prepared by adding boiling water to the leaves without further heating (I3) yields higher artemisinin concentrations than if the leaves are boiled for several minutes (D2), according to literature data [9, 10]. No great differences were evidenced between infusion and decoction methods for flavonoid content, but an increase of extraction efficiency was obtained with filtration of the hot solution (I3 and D2). Also a microwave oven can represent a valid alternative method to extract the constituents of *A. annua*, in particular artemisinin.

Experimental

Chemicals: A sample of a selected high-yield cultivar of *A. annua* was provided by P.M.M. of the Universidade Estadual de Campinas (Brazil). The Brazilian hybrid plant was obtained according to the procedure carried out by MEDIPLANT [17]. The percentages of constituents of the herbal drug were artemisinin 0.52% and total flavonoids 2.6%.

Artemisinin was purchased from Sigma (Sigma-Aldrich S.r.l., Milan, Italy). Indena Research Laboratories (Settala, Milan, Italy) kindly provided the reference rutin trihydrate (batch no. K12408717, standard purity 88.17%, considering the content of residual solvents, moisture and amount of impurities), which was used for the calibration of polymethoxylated flavonoids.

All the solvents used for the extraction and HPLC analysis (MeOH, *n*-hexane, dichloromethane, and acetonitrile) were HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA).

Preparation of the n-hexane extract: The dried aerial parts of a sweet wormwood sample were cut into small pieces with an Osterizer. Samples of 200 g material was exhaustively extracted at room temperature by maceration with 2 L of *n*-hexane for 72 h. The eluates were subsequently taken to dryness under reduced pressure to obtain the crude extract.

Preparation of the tinctures: The dried aerial parts of a sweet wormwood sample were cut into small pieces with an Osterizer. Samples of 10 g of material were extracted at room temperature by maceration with 100 g of ethanol [either 40 or 60% v/v (samples T40 and T60)].

Preparation of the decoctions: Sample D1: A 9 g sample of dried aerial parts of sweet wormwood was extracted with 1 L of boiling water, kept boiling for 5 min, then left to cool and filtered. Sample D2: A 9 g sample of dried aerial parts of sweet wormwood was extracted with 1 L of boiling water, kept boiling for 5 min and immediately filtered. Sample M: A 9 g sample of pieces of sweet wormwood was treated with 1 L of water, kept boiling for 5 min in a microwave oven, then left to cool and filtered. For analytical purposes, all the filtrates were lyophilized and provided 2.15 g, 2.09 g, and 3.16 g of dried product, respectively.

Preparation of the infusions: Sample 11: A 9 g sample of dried sweet wormwood was extracted with 1 L of boiling water, then left to cool and filtered. Sample 12: A 9 g sample of dried aerial parts of sweet wormwood was extracted with 1 L of boiling water, covered, then left to cool and filtered. Sample 13: A 9 g sample of dried aerial parts of sweet wormwood was extracted with 1 L of boiling water, then left to cool for 15 min and filtered. For analytical purposes, the filtrates were lyophilized, providing 2.04, 1.89 and 3.44 g of dried product, respectively.

Table 3: Mobile phases used for HPLC analysis.

Time(min)	A %	B %	C %	Flow (mL/min)
0.00	50	50	0	1.000
15.00	50	50	0	1.000
20.00	0	0	100	1.000
23.00	0	0	100	1.000
28.00	50	50	0	1.000

HPLC-DAD and HPLC-MS systems: The HPLC analyses were performed using a HP 1100 Liquid Chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a HP 1040 Diode Array Detector (DAD), an automatic injector, an auto sampler, a column oven and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA, USA).

Separations were performed on a reversed phase column Purospher®Star RP-18, namely Hibar®. The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. Mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values: gas temperature 350°C at a flow rate of 10 L/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30°C, and capillary voltage 3500 V. Full scan spectra from *m/z* 100 to 800 in the positive ion mode were obtained (scan time 1 s).

A prepacked column RP (250 x 4.6 mm) with particle size 5 µm (Merck, Darmstadt, Germany) was employed. The eluents were A: water adjusted to pH 3.2 with formic acid; B: acetonitrile; C: methanol. The mobile phase is reported in Table 3. The system was operated with oven temperature at 26°C. Before HPLC analysis, each sample was filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane (d = 13 mm, porosity 0.45 µm, (Lida Manufacturing Corp.) and immediately injected.

Chromatograms were recorded between 200 and 450 nm. DAD spectra were stored for all peaks exceeding a threshold of 0.1 mAu.

Calibration curves: A calibration curve, obtained from a methanolic solution of artemisinin (1 mg/mL), was used to quantify artemisinin in *n*-hexane extracts and tinctures, while a methanolic solution of artemisinin (0.5 mg/mL) was employed to determine the artemisinin content of infusions and decoctions. The flavonoid amounts were quantified by a methanolic solution of rutin international standard 0.03 mg/mL.

Sample analysis: Samples of 5 mg of the different extracts were accurately weighed and suspended in methanol (1.0 mL). The suspensions were sonicated for 10 min and filtered through a cartridge-type sample filtration unit before HPLC analysis. The tinctures were injected as prepared.

Acknowledgments - The financial support of MIUR (PRIN 2004) and Ente Cassa di Risparmio di Firenze are gratefully acknowledged.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1117 - 1122

Antifungal Evaluation of *Hypericum triquetrifolium* Polar Extracts Against *Fusarium* spp.

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Received: July 28th, 2006; Accepted: November 8th, 2006

Dedicated to the memory of Professor Ivano Morelli.

The chemical composition of different kinds of *Hypericum triquetrifolium* extracts was analyzed by LC-DAD-ESI-MS. Hyperoside, rutin, isoquercitrin and biapigenin were the main constituents. As these natural compounds were reported in the literature for their antifungal activity, the total extracts were tested for their antifungal activity against eight phytopathogenic strains of *Fusarium* species.

Keywords: *Hypericum triquetrifolium*, flavonoids, *Fusarium* ssp., antifungal activity.

The genus *Hypericum* comprises more than 400 species, but only 20 can be found in Italy [1]. The species are herbaceous plants and are widely used in phytotherapy in many countries. Several phytochemical investigations of this genus have led to the isolation of many secondary metabolites, some with antidepressant, antimicrobial and antifungal activities [2-4]. Recently, plant extracts and phytochemicals with either antibacterial or antifungal properties have been investigated actively as alternatives to synthetic pesticides due to their perceived increased level of safety and minimal environmental impact [5]. Plant diseases often reduce quality and quantity of agricultural commodities. In fact, plant pathogens are estimated to cause yield reductions in crops of almost 20% worldwide [6,7]. Infestation by micro-organisms in post-harvest storage can effect the health of humans and livestock, especially if the contaminating organism produces toxic residues either in or on consumable products [8-10]. The fungicides made by synthesis provide the primary means for controlling post-harvest fungal decay of cereals, fruits and vegetables. [8-10]. On the other hand, the extensive use of these synthetic fungicides causes uncontrolled residues and proliferation of resistance in the pathogen populations [11]. Therefore, studies concerning the possible use of biologically active natural products to control decay and prolong storage life of crops have received more and more attention [12,13].

The volatile compounds extracted from different species of *Hypericum* have been tested for their fungicidal activities on *Candida albicans* and *Saccaromyces cerevisiae* by several authors [14,15]. Some compounds from *Hypericum* species, such as xanthones isolated from *H. roeperanum*, exhibited antifungal activity against *Candida albicans* [16], while xanthones, a new γ-pyrone and betulinic acid from *H. brasiliense* showed similar activity against the plant pathogenic fungus *Cladosporium cucumerinum* [17]. Also a phloroglucinol derivative from the aerial parts of *H. calycinum* showed a fungicidal activity on the same phytopathogen [18]. Interesting activity of methanolic extracts of

Table 1: Percentage composition of the reference compounds in the analysed *H. triquetrifolium* extracts.

Extracts[b]	Compounds								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
	0.523[c]	1.000	1.294	1.287	1.563	1.876	2.148	1.678	0.764
	Percentage composition (%)								
MM	6.93	29.8	26.3	21.7	3.12	2.72	4.90	0.3	-
RMMA	3.32	18.5	22.6	11.4	8.5	12.7	21.2	0.6	-
RMMB	5.53	16.8	70.2	-	-	-	-	-	-
SM	1.11	17.6	10.6	2.85	1.92	-	40.2	3.82	-

[a]**1** = chlorogenic acid; **2** = rutin; **3** = hyperoside; **4** = isoquercitrin; **5** = quercitrin; **6**= quercetin; **7** = I,II biapigenin; **8** = hypericin; **9** = hyperforin [b]MM = methanolic macerate; RMMA= ethyl acetate extract by the repartition of methanolic macerate; RMMB = butanolic extract by the repartition of methanolic macerate; SM = methanolic extract by Soxhlet extraction.[c]RRF= response factor of the constituent relative to rutin area/conc. (mg/mL) x purity/100

Table 2: % Inhibition of fungal strains of various extracts of *H. triquetrifolium*.

Fungal strain	Nystatin	MM			RMMA		
	100 ppm	**3200**	**6400**	**10000**	**3200**	**6400**	**10000**
I	100	0	20.2	50.0	66.6	70.0	70.0
II	100	0	25.3	50.0	66.6	70.0	70.5
III	100	0	24.3	50.4	71.4	70.0	70.0
IV	100	0	15.5	51.2	64.2	70.0	70.0
V	100	0	21.3	55.5	60.0	71.5	72.0
VI	100	0	24.6	50.5	57.1	65.6	67.0
VII	100	0	22.7	53.4	52.0	62.0	70.0
VIII	100	0	26.4	55.5	54.0	55.4	70.0

Fungal strain: *F. culmorum* (**I**), *F.graminearum* (**II**), *F. poae* (**III**), *F.avenaceum* (**IV**), *F.equiseti* (**V**), *F. semitectum* (**VI**), *F. sporotrichoides* (**VII**), *F. oxysporum* (**VIII**).
Extracts: MM = methanolic macerate; RMMA= ethyl acetate extract by the repartition of methanolic macerate.
The values are the average of three determinations.

Table 3: % Inhibition of fungal strains of various extracts of *H. triquetrifolium.*

Fungal strain	Nystatin	RMMB			SM		
	100 ppm	**3200**	**6400**	**10000**	**3200**	**6400**	**10000**
I	100	30.0	43.6	50.3	0	13.3	15.0
II	100	30.0	40.2	52.5	0	13.3	15.0
III	100	35.0	44.5	55.5	31.4	45.0	53.0
IV	100	28.0	34.7	50.3	10.7	32.1	40.0
V	100	25.0	36.2	54.5	0	16.0	20.0
VI	100	35.0	47.2	53.1	25.8	42.8	50.1
VII	100	25.0	33.9	50.0	0	8	13.0
VIII	100	25.0	41.5	54.3	0	8	15.0

Fungal strain: *F. culmorum* (**I**), *F.graminearum* (**II**), *F. poae* (**III**), *F.avenaceum* (**IV**), *F.equiseti* (**V**), *F. semitectum* (**VI**), *F. sporotrichoides* (**VII**), *F. oxysporum* (**VIII**); Extracts: RMMB = butanolic extract by the repartition of methanolic macerate; SM = methanolic extract by Soxhlet extraction.
The values are the average of three determinations

H perforatum from Calabria, Italy against phytopathogenic fungi was also reported by Conforti et al. [19]. Other species of *Hypericum* were investigated for their biological activity, such as *H. triquetrifolium* Turra, native to Eastern Europe and the Mediterranean area. This species has been used for its sedative, anthelminthic, anti-inflammatory and antiseptic effects in folk medicine [5]. Extracts of *H. triquetrifolium* showed antimicrobial activity against *Staphylococcus aureus* and *Mycobacterium smegmatis*. In this work, the fungitoxic property of different polar extracts of *H. triquetrifolium* collected in Calabria was evaluated for the first time against eight phytopathogenic strains of *Fusarium* species[20]. We tested the methanolic extracts, rich in hyperoside, rutin, isoquercitrin and biapigenin since some studies are reported in the literature on these main constituents of *Hypericum* and other related species [21]. Furthermore, the post-infection production of flavonoids and polyphenols in plant species suggests that these compounds might either function as phytoalexins [22] or have a protector role against fungal infection [23].

Some plants do not produce phytoalexins when challenged by pathogens, but release toxins that are normally stored as toxic glycosides in the vacuoles of their cells, for example phenolic and iridoid glycosides, glucosinolates and saponins [24]. If the integrity of the cells is broken when they are penetrated by fungal hyphae, the glycoside comes in contact with hydrolysing enzymes, present in other compartments of the same cell, releasing the toxic aglycone. Although this aglycone is not present *in situ* in the intact plant, it is not strictly a phytoalexin, because the involved enzymes (glycosidases) were already present in the healthy plant and not *de novo* formed [25]. The genus *Fusarium* contains a number of soil borne species with worldwide distribution, which have been known for a long time as plant pathogens and produce secondary metabolites toxic to plants (phytotoxins) and animals (mycotoxins), such as fusaric acid, trichothecenes, fumosins and enniatins [26]. Fusaric acid, a compound with moderate toxicity to plants and animals produced by many *Fusarium* species, was one of the first fungal metabolites implicated in plant pathogenesis. Furthermore the potential of *Fusarium* to serve as a model system for soil borne fungal pathogens is outlined [26].

A majority of known fungal secondary metabolites are not classified as mycotoxins. They may have toxic effects on insects (insecticides), plants (herbicides) and microorganisms (antibiotics) or they may have pharmacological effects on vertebrates or act synergistically with known mycotoxins on vertebrates. *Fusarium* spp produce a series of toxins, such as trichothecenes and zearalenones (responsible for several diseases in plants and animals) [27]. Cereals are often invaded by *Fusarium* species, before and after harvest, and the risk of trichothecene contamination of cereals is therefore of great concern [27]. Other kinds of *Fusarium* toxins, fusarins and fumonisins, have been

proposed to be involved in equine diseases [28]. Moreover, fumonisins are considered cancerogenic and they have been found to occur naturally [29]. Plant extracts rich in chlorogenic acid and isolated derivatives of chlorogenic acid showed antifungal activity on *Fusarium oxysporum*, as reported by Lattanzio [30] and Naidu [31], while other authors [32] demonstrated that the antifungal activity of caffeine in coffee beans was antagonized by chlorogenic acid. In tables 2 and 3 are reported data obtained by the agar diffusion test regarding the per cent inhibition of the growth of *Fusarium* strains induced by our extracts. The *H. triquetrifolium* methanolic macerate (MM) tested in these experiments, which contained the higher amount of chlorogenic acid (6.93%) in comparison with the other extracts, exhibited a very weak antifungal activity on the selected eight *Fusarium* strains.

Hyperoside, rutin, and isoquercitrin were the main constituents of *H. triquetrifolium* in the most polar extracts. Hyperoside commonly occurs in a wide range of plants and has shown bactericidal activity [33]. However, several anomalous reports lead to uncertainty as to the antifungal activities of hyperoside. Previous studies demonstrated that it was inactive in *in vitro* bioassays against *Fusarium* spp. and other fungi at >100 μg/mL, while Dall'Agnol et al. reported that crude extracts of *Hypericum* including hyperoside showed no activity against yeast [34]. Hyperoside was tested for antifungal activity on several kinds of *Fusarium* and was considered more potent than some recently discovered natural antifungal products, including some fungicides on the market [35]. It is reported as an important secondary metabolite involved in the control of fungal pathogens *in vitro*, including *Fusarium* species, although the antifungal activity of these compounds in the plant is limited [21]. Hyperoside may serve as a lead compound for the development of fungicides [21]. Although its action against fungi is unknown, its effectiveness, resource availability at low cost, and probable low toxicity to humans make flavonoids potential prototypes for fungicides. Several studies have been carried out on the antifungal activities of these natural compounds. A recent report on the antifungal activity of flavonoids from *Pelargonium radula* showed that *Fusarium graminearum* was strongly inhibited only by the fraction rich in rutin, while the fraction with isoquercitrin as its main constituent inhibited *Candida tropicalis, C. lusitaniae* and *Microsporum gypseum* [36].

The extracts tested in this work (Table 1), containing good amounts of hyperoside, rutin and isoquercitrin [MM, RMMA and RMMB (where isoquercitrin was not detected)], did not exhibit major antifungal activity, with the exception of RMMA, which inhibits the growth of *F. poae* (71.4%) and all the other *Fusarium* strains, with values ranging from 52% to 67% at 3200 ppm. Although this was the most active extract, it was not comparable to nystatin (positive control). Only in RMMA are quercetin and quercitrin present in significant amounts (12.7% and 8.5%, respectively) and consequently the detected activity should be due to the presence of these compounds. The inhibitory action of quercitrin and quercetin, as pure compounds, on the mycelial growth of the crop pathogen, *Verticillium albo-atrum*, has been reported [37]**.** More recently, Conforti et al. [19] demonstrated an antifungal activity, particularly on the phytopathogenic fungus *Pythium ultimum*, of *Hypericum perforatum* extracts and its component, quercetin.

To the best of our knowledge, no data on the antifungal activity against *Fusarium* spp. are reported in the literature for biapigenin, which was the main constituent of the methanolic extract obtained by Soxhlet extraction, even if it was present also in the methanolic macerate. Although the compounds detected in the analyzed extracts as the main constituents have been reported in the literature for their antifungal activity against several microorganisms, such as *Fusarium* spp., these experiments did not show significant antifungal activity of the extracts that contained them.

Experimental

Plant material: The aerial parts of *Hypericum triquetrifolium* were collected on June 2004 at Isola Capo Rizzuto (Calabria, Italy). A voucher specimen (code CLU) was deposited in the herbarium of the Dipartimento di Botanica, University of Calabria, Italy.

Extraction and Purification of standard compounds: The pulverized dried material (400 g) was extracted at room temperature with methanol (MM 12.7 g). The MeOH residue was suspended in H_2O and then partitioned into EtOAc (RMMA 20.2 g) and *n*-BuOH (RMMB 7.6 g), successively. A portion of the EtOAc extract (10 g) was fractionated by gel-permeation and low pressure chromatography (eluted with MeOH) in order to isolate rutin (**2**)

130.6 mg), quercetin (**6**) (99.1 mg), quercitrin (**5**) (15.3 mg), isoquercitrin (**4**) (120.6 mg), hyperoside (**3**) (380 mg) and I3,II8 biapigenin (**7**) (33.1 mg), which were identified by comparison of their spectral data (^{1}H-NMR, ^{13}C-NMR and MS) with those reported in the literature [38-41]. Moreover, a portion of the powdered air-dried vegetable material (118 g) was defatted with light petrol in a Soxhlet apparatus and then extracted with methanol (SM 4.5 g). A preliminary screening by TLC [SiO_2, BAW (60:15:10); RP-18, M-W (7:3); UV 254 nm and 366nm; NTS-PEG] was carried out on each extract in order to show the presence of the marker compounds (**1-9**) for the *Hypericum* genus. The isolated compounds (**2-7**) and the commercial standards of chlorogenic acid (**1**) (Extrasyntheses, Lot.01021203), hypericin (**8**) (Extrasyntheses, Lot: 02072309), and hyperforin (**9**) (Sigma, Lot. 092K1015), containing small quantities of impurities, were analyzed by HPLC-PDA in the same gradient conditions used for the extract samples in order to verify their purity (>98%) before using them as reference compounds.

Sample preparation and LC-DAD-ESI-MS-analyses: Three samples of each extract of *H. triquetrifolium* were dissolved in methanol (2 mg/mL) and filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene membrane before HPLC analyses (PTFE, 0.45 μm, 25 mm). All the extracts were analysed by the previously described method [42], slightly modified for our analytical equipment. The HPLC system consisted of a Waters W600E liquid chromatography pump equipped with an analytical Lichrosorb RP-18 column (250 x 4.6 mm i.d., 5μm, Merck), a Rheodyne injection loop, and a Waters 996 photodiode array detector. The optimum efficiencies of separation were obtained using a linear gradient of a mobile phase of water with 0.1% HCOOH (solvent A), CH_3CN (solvent B) and MeOH (solvent C) at a flow rate of 1.0 mL/min. Gradient elution was carried out starting with a mixture of A-B-C (5:95:0) to (85:15:0) in 10 min, then to (50: 40:10) in 20 min, to (10:75:15) in 10 min, to B-A-C (5: 80:15) in 15 min, and then back to the initial condition in 10 min. Prior to running the gradient, the column was equilibrated for 10 min with solvents A and B (5:95 v/v). The total analytical run time for each sample was 65 min. The spectral data from the PDA detector were collected during the whole run in the range 210-600 nm and the peaks were detected at 270 nm and 590 nm. Chromatographic procedures were performed at room temperature. An aliquot (20 μL) of each sample was analysed in triplicate. The same chromatographic conditions were used for LC-MS analyses performed using these ESI values: sheath gas flow-rate 62 psi, auxilary gas flow 10 psi, capillary voltage –16 V and capillary temperature 200°C. Full scan spectra from m/z 200 to 700 u in the negative ion mode were obtained. The injected volume of the *Hypericum* extracts was 20 μL of a 1.5 mg/mL solution (methanol).

Identification of each constituent was achieved by comparison of the peak retention times, and UV and mass spectra of the extract sample with those of authentic samples (**1-9**). The standard solutions for the authentic samples (10 mg) (**1-7**) were prepared in methanol. Hypericin (**8**) (4.4 mg) was dissolved in pyridine (2 mL) before adding methanol (8 mL), while hyperforin (11.7 mg) was dissolved in a mixture MeOH-ascorbic acid 0.1% (10 mL). All standard and extract samples were injected alternatively. The linearity of the responses for the rutin reference standard (**2**) and for the constituents (**1**, **3-9**) was determined at six levels of concentration with three injections for each level. Rutin was linear from 2.64 ppm to 264 ppm and all the curves had coefficients of linear correlation $r \geq 0.999$. The reproducibility of the injection integration procedure was determined for the constituents (**1-9**) The solutions were injected ten times and the relative standard deviation (R.S.D.) values were calculated (chlorogenic acid 1.56%, rutin 1.02%, hyperoside 0.78%, isoquercitrin 0.71%, quercitrin 0.50%, quercetin 0.56%, I3,II8-biapigenin 0.32%, hypericin 0.98%, hyperforin 1.54%). The repeatability of the method was evaluated by injection of three *H. triquetrifolium* extract solutions of different concentrations (0.5 mg/mL, 1.0 mg/mL, 1.5 mg/mL), each three times. The contents of constituents (**1-9**) were estimated by the following equation:

$$\text{Contents (\%)} = A_{sample}/RF_{std} \times C_{sample} \times \frac{}{100} \times 1/RRF \times$$

where A_{sample} is the peak area of the considered constituent in the test solution, RF_{std} is the mean response factor of rutin in the reference solutions, C_{sample} is the concentration of the test solution (mg/mL) and RRF is the response factor of the considered constituent, relative to rutin (Table 1).

Biological screening: Fungal plant pathogens used in these tests were *Fusarium culmorum* (Smith)

Saccardo (I), *F. graminearum* Schwabe (II), *F. poae* (Peck) Wollenweber (III), *F. avenaceum* (Corda: Fries) (IV), *F. equiseti* (Corda) Saccardo (V), *F. semitectum* Berkeley et Ravenel (VI), *F. sporotrichoides* Sherbakoff (VII) and *F. oxysporum* Schl. (VIII), kindly supplied by the DI.PRO.VAL. (Dipartimento di Protezione e Valorizzazione Agro-alimentare, Università degli Studi di Bologna). All of the used microorganisms were maintained in potato dextrose agar (PDA, Sigma) and subcultured every 30 days.

The phytopathogenic fungi were tested by an agar dilution method. The extracts were dissolved in DMSO (Sigma) and added to the culture medium at a temperature of 40°-45°C, then poured into Petri dishes (∅3 cm). Concentrations of 100, 200, 400, 800, 1600, 3200, 6400 and 10000 ppm were tested. The fungi were inoculated as soon as the medium had solidified. A disc (∅ 0.5 cm) of mycelial material, taken from the edge of seven-day old fungal cultures, was placed at the centre of each Petri dish. The control consisted of a fungal disc placed in PDA, *Hypericum* extract free, + DMSO 1% v/v [43]. The Petri dishes with the inoculum were placed in the dark under controlled temperature conditions of 22 ± 1°C. The efficacy of treatment was evaluated after seven days by measuring the diameter of the fungal colonies when all the free surface of the medium in the control Petri dishes had been covered. The values were expressed in terms of percent inhibition of growth compared to control = 100. The fungicidal activity of the extracts was determined using the technique of Thompson [44] and Carta and Arras [45]: the mycelial discs were transferred from Petri dishes in which no growth was observed (total inhibition = 100) onto fresh plates of PDA, in order to verify, after three days, either the fungistatic or fungicidal activity of such inhibition. All tests were repeated three times.

Acknowledgments - The authors would like to acknowledge the financial support from Regione Marche, Progetto CIPE 17/2003.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1123 - 1128

Antioxidant Activity Analysis for the Selection of *Rosmarinus officinalis* L.

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Received: June 23rd; Accepted: August 25th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Rosmarinus officinalis L. presents a high genetic variability, which is reflected in the chemical composition of the different individuals, and consequently in its biological activity, including antioxidant capacity. The aim of the present research was to correlate the chemical composition of methanolic extracts of the dried leaves of eight rosemary accessions with their antioxidant activity for the selection of plants to optimize the use of rosemary. The eight samples examined, starting from a collection of more than 160 individuals selected by BOTANE Ltd, were cultivated at Illapel, north central Chile, using the same cultivation techniques. The free radical-scavenging capacity was tested by the ability of extracts to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) and to inhibit superoxide anion ($O_2^{\cdot-}$) and hydroxyl radical ($^{\cdot}OH$) production. The metal chelating activity was estimated by the ferrozine assay. All extracts (1-8) contained high concentrations of carnosic acid, and to a minor extent rosmarinic acid, and exhibited antioxidant activity. However, extracts 7 and 8, containing 31.7 and 26.1% of carnosic acid, respectively, have shown a higher biological effect, confirming that the antioxidant activity of *R. officinalis* leaves is primarily related to this phenolic diterpene and suggesting that the measure of antioxidant activity could be considered a good method in the selection of this plant for its optimization. Interestingly, our experimental evidence also suggests that air pollution negatively influences the carnosic acid content. In fact, samples 3 and 4, with a low carnosic acid content, originated from a highly polluted metropolitan area of Santiago city.

Keywords: *Rosmarinus officinalis* L., leaf extract, carnosic acid, free radicals, antioxidant activity.

There is abundant evidence that reactive oxygen and nitrogen species (ROS and RNS) are implicated in several physiological processes, such as in host defence against invading pathogens and signal transduction. An overproduction of such reactive species, however plays a major role in several pathophysiological conditions. The ROS and RNS formed may cause cellular and subcellular damage by peroxidation of membrane lipids, by denaturing cellular proteins and by the breaking of DNA strands, disrupting cellular functions [1]. Lipid oxidation may also reduce the flavor and nutritive value of fats, oils and lipid-containing products. Unsaturated fatty acids are sensitive to oxidation because of their chemical structure, and protein cross-linking, denaturation, polypeptide chain scission, enzyme inactivation and amino acid destruction in the presence of oxidizing lipids have been reported [1]. In this regard, many nutritive and non-nutritive phytochemicals, containing principally polyphenolic compounds and with diverse biological properties, have shown promising responses for the prevention and/or intervention of all diseases in which oxidative stress plays a key role [2]. A large number of polyphenolic compounds with antioxidant activity have been identified in the Labiatae plant

Rosmarinus officinalis L., including phenolic diterpenes such as carnosic acid, carnosol, rosmanol, epirosmanol, 7-methylepirosmanol, and methyl carnosate. In addition, several flavonoids, such as genkwanin, hispidulin 7-*O*-glucoside, cirsimaritin, luteolin, and isoscutellarein 7-*O*-glucoside, are found in Labiatae plants; the phenolic compounds rosmarinic and caffeic acids are also present [3-8]. *R. officinalis* (rosemary) is a typical Mediterranean species, but now is cultivated all over the world. Usually the plant is clonally propagated because of the poor germinability of its seeds and the genetic diversity of the seedlings [9]. *R. officinalis* presents, in fact, a high genetic variability, which is reflected in the chemical composition of the different individuals, and probably in its biological activity.

This plant is used as a spice and folk medicine around the world, as well as in cosmetics. The leaves are used in the preparation of alcoholic beverages, herbal soft drinks and in food preservation. In medicine, the extract is receiving increasing attention due to its antimicrobial, anti-inflammatory and antioxidative constituents [9].

The antioxidant properties of rosemary have been well documented, and there are several reports that have established carnosic acid as the major phenolic diterpenoid present in rosemary leaves with antioxidant activity [10]. Recently this phenolic compound has attracted wide interest as a potential therapeutic agent against several diseases, and research was started to investigate new biological activities. Studies showed that it has chemopreventive, anti-neoplastic [11-13] and radioprotective-antimutagenic [14] effects.

Therefore, the aim of the present research was to correlate the chemical composition of the methanolic extracts of leaves from eight different rosemary accessions, cultivated in Chile using the same cultivation techniques, with their antioxidant activity for the selection of *R. officinalis*, with the view to optimize its use.

Table 1: Origin of plant materials.

Accessions	Country
1	Santiago (Illapel), Chile
2	Santiago (Las Condes), Chile
3	Santiago (Renca), Chile
4	Santiago (Renca), Chile
5	Goodwood, Canada
6	Goodwood, Canada
7	Goodwood, Canada
8	Goodwood, Canada

The plant accessions were collected throughout Chile and other countries (Table 1), and were clonally propagated in order to maintain genetic uniformity. The fresh rosemary samples were dried, and double extraction for 159 hours was conducted for total extraction of carnosic and rosmarinic acids, which was confirmed by chromatographic analysis (data not shown). The yields of extraction of the samples are given in Table 2.

Table 2: Yield of extraction.

	Dry leaves g	First Extraction g	Second Extraction g	Total extract g	Yield %
1	58.0	5.4	4.8	10.2	17.6
2	50.3	5.3	3.1	8.4	16.7
3	52.1	5.6	5.0	10.6	20.3
4	51.7	4.9	3.3	8.2	15.9
5	51.3	3.7	3.5	7.2	14.0
6	50.1	5.0	4.0	9.0	18.0
7	55.4	6.6	6.1	12.7	22.9
8	65.9	6.1	5.9	12.0	18.2

Table 3: Content of carnosic acid and rosmarinic acid of methanol extracts of leaves from different accessions of *Rosmarinus officinalis*.

Extracts	Carnosic acid (%)	Rosmarinic acid (%)
1	13.8±2.6	1.14±0.11
2	19.3±2.2	0.46±0.13
3	14.3±2.6	0.79±0.08
4	10.8±3.2	1.17±0.11
5	11.7±3.0	0.84±0.08
6	12.8±2.8	1.32±0.15
7	31.7±4.9	0.41±0.14
8	26.1±3.1	0.43±0.08

n=6

The biological effects exhibited by these rosemary samples, under our experimental conditions, could be related to an overall effect of the phenolic compounds present in the extracts, but carnosic acid, as previously reported [10], seems to play a key role in the antioxidant activity. All extracts (1-8) containing high concentrations of carnosic acid (Table 3), and, to a minor extent, rosmarinic acid, exhibited antioxidant properties (Tables 4, 5). However, extracts 7 and 8 containing 31.7 and 26.1% of carnosic acid, respectively (Table 3), have shown a higher antioxidant capacity. In fact, the results, summarized in Table 4, showed that all extracts exhibited DPPH free radical scavenging activity, but samples 7 and 8 exhibited higher capacity with IC_{50} values (concentration that inhibited radicals by 50%) of 9.2 and 8.6%, respectively. As DPPH is a synthetic radical, we also investigated the superoxide anion scavenging capacity of these extracts using the method of Paoletti [15], which excludes the Fenton-type reaction and the xanthine/xanthine oxidase system. Also in this assay, samples 7 and 8 showed a major superoxide scavenging effect (Table 4).

Table 4: Scavenger effect of leaf methanol extracts of different accessions of *Rosmarinus officinalis* on DPPH stable radical and superoxide anion.

	DPPH Test	Superoxide radicals
	[a]IC_{50} (μg/mL)	
1	14.8±1.1	18.3±0.9
2	16.1±1.1	22.9±0.8
3	16.8±0.9	24.3±0.7
4	17.9±1.2	24.0±0.4
5	19.4±1.5	26.9±1.1
6	15.8±0.7	20.8±1.1
7	9.2±1.2	13.0±1.1
8	8.6±0.5	12.0±0.8
[b]**Trolox**	95±1.4	-
[c]**SOD**	-	87±3.4

[a]concentration that inhibited radicals by 50%. Values represent the mean ± SD of three experiments, performed in duplicate.
[b]Trolox (50 μM) and [c]superoxide dismutase (SOD) (80 mU/mL) were used as a standard; the results are expressed as % of inhibition.

Although both $O_2^{\cdot -}$ and H_2O_2 are potentially cytotoxic, most of the oxidative damage in biological systems is caused by the $^{\cdot}OH$ radical, which is generated by the reaction between $O_2^{\cdot -}$ and H_2O_2 in the presence of transition metal ions [1]. Based on the data obtained from this study, rosemary extract might also be able to modulate hydroxyl radical formation, acting as a direct scavenger and chelating ion. In fact, all extracts examined exhibited protection against DNA strand scission induced by $^{\cdot}OH$ radicals, generated by UV-photolysis of H_2O_2 (Table 5), and showed metal chelating activity capturing ferrous ions before ferrozine (Table 5). Also in these assays, samples 7 and 8 exhibited a higher effect (Table 5).

Table 5: Effect of methanol extracts of leaves from different accessions of *Rosmarinus officinalis* (100 μg/mL) on DNA cleavage induced by the photolysis of H_2O_2 and metal chelating activity.

	UD of supercoiled DNA	Ferrozine assay
	[a]% of native DNA	[b]IC_{50} (μg/mL)
scDNA	100	
1	46±1.4*	83.2±1.4
2	51±1.7*	89.4±1.8
3	53±2.1*	94.5±0.9
4	50±1.9*	96.7±1.2
5	55±1.8*	106.3±1.5
6	45±0.9*	86.3±0.7
7	83±1.6*	62.5±1.2
8	81±1.5*	57.5±0.8
[c]**DTPA**	-	77±2.7

[a]The hydroxyl radicals generated by the photolysis of H_2O_2 reduced the supercoiled DNA (SCDNA).
[b]concentration that inhibited the ferrozine-Fe^{2+} formation by 50%. [c]DTPA (5 μM) was used as a standard; the results are expressed as % of inhibition.
Values represent the mean ± SD of three experiments, performed in duplicate. *significant vs. supercoiled DNA (p<0.001).

Interestingly, our results, similar to other works reporting that environmental elements affect carnosic acid concentrations [16], also reveal a correlation between carnosic acid concentration and air pollution. In fact, samples 3 and 4, with low carnosic acid contents, 14.3 and 10.8%, respectively, correlating with a low antioxidant activity, originated from a heavily polluted metropolitan area of Santiago city, which ranks as one of the most polluted cities in the world. The main ambient pollutants of concern include carbon monoxide (CO), nitrogen oxides (NO), sulphur dioxide (SO_2), tropospheric ozone (O_3), and particulate matter (PM) [16-18].

In summary, our results suggest that the measure of antioxidant activity could be considered a good method for the selection of this plant for its optimization. Interestingly, our experimental evidence also suggests that air pollution negatively influences the carnosic acid content, justifying further studies to explain the mechanisms involved in this effect.

Experimental

Materials: Rosmarinic acid and carnosic acid, used as standards, were obtained from Addipharma and Sigma, respectively. The water used was Milli-Q quality, methanol and acetonitrile (MeCN) were from Merck and HPLC grade. Inorganic reagents from Merck were PA quality.

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

Plant materials: Plant accessions were kindly borrowed from the special collection of more than 160 individuals used by BOTANE Ltd. to establish its crops and were collected throughout Chile and other countries (Table 1). Accessions were clonally propagated in order to maintain genetic uniformity. Each accession was propagated and established in a greenhouse and then transplanted to square-shaped blocks. Accessions were planted in November 2001 at Illapel, in north central Chile, about 250 Km north of Santiago (31°43'S; 71°07'W; 391 m.a.s.l.). Sampling was performed in a random pattern in the square shaped blocks and on freshly grown material.

Sample preparation: The fresh rosemary samples were dried at 40◦C in a forced air circulation oven (MemmertULM500). Leaves were manually separated and ground in a vertical hammer mill (Peruzzo Milly model 35.010) at 12,000 rpm and 0.8 mm mesh. Sample humidity was determined employing a Sartorius MA30 infrared system. Samples were mixed with 500 mL of methanol and stirred for 15 h at 20 °C in the dark. After stirring and filtering under vacuum, the filtrate was evaporated to dryness in a Rotavapor. The samples were extracted again for 144 hours, as previously described, and the filtrate was evaporated to dryness in a Rotavapor. Double extraction for 159 hours was conducted for total extraction of carnosic and rosmarinic acids, which was confirmed by chromatographic analysis. The extraction yields from the rosemary samples are given in Table 2.

Chromatographic conditions: A binary MeCN-H_2O acidified gradient was used for elution, as previously reported [19]. Two different procedures were developed. Method I, for simultaneous resolution of the three compounds of interest (CA, C, RA), the solvents A and B were MeCN–H_2O–H_3PO_4 (65.1%:34.9%:0.02%) and MeCN–H_2O–H_3PO_4 (22%:78%:0.25%), respectively. At a flow of 1.5 mL/min, the eluent consisted of 100% B during the initial 2 min, then the percentage of solvent A was increased to 100% at 2.1 min and remained at this level for the next 6 min. At 8.1 min the percentage of solvent B was again increased to 100%, where it remained for the last 2 min of the run time. With this method, the retention times were for RA *t*r = 2.7 min, for C *t*r = 5.7 min, and for CA *t*r = 6.6 min. This method requires sample extraction with methanol: water (2:1) in order to extract all lipo-soluble and hydro-soluble antioxidants.

Method II: This chromatographic procedure is isocratic with solvent A as eluent for 6 min. The retention times were for C *t*r = 1.8 min, and for CA *t*r = 2.5 min. With this procedure, hydro-soluble compounds are not resolved, so sample extraction was simply performed with methanol. The detection wavelengths selected to quantify carnosic acid and rosmarinic acid were 230 and 330 nm, respectively, in order to avoid mobile phase absorption.

Antioxidant activity

Quenching of DPPH: Since the DPPH test can accommodate a large number of samples in a short period and is sensitive enough to detect natural compounds at low concentrations, it was used in the present study for a primary screening of the methanolic extracts of *R. officinalis* free radical–scavenging activity. The assay provides information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The reaction mixture contained 86 μM DPPH, and different concentrations of the extracts (5-100 μg/mL) in 1 mL of ethanol. After 10 min at room temperature, the absorbance at λ = 517 nm was recorded [20]. Trolox (50 □M), a water-soluble derivative of vitamin E, was used as a standard. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Scavenger effect on superoxide anion: Superoxide anion was generated *in vitro* during the autoxidation of β-mercapto-ethanol, as described by Paoletti et al. [15]. The assay mixture contained, in a total volume of 1 mL, 100 mM triethanolamine-diethanolamine buffer, pH 7.4, 3 mM NADH, 25 mM/12.5 mM EDTA/$MnCl_2$, 10 mM β-mercapto-ethanol; some samples contained methanolic extracts of the samples examined of *R. officinalis*, at different concentrations (5-100 μg/mL). After 20 min incubation at 25°C, the decrease in absorbance was measured at λ = 340 nm. Superoxide dismutase (SOD) (80 mU/mL) was used as a standard. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

DNA cleavage induced by hydrogen peroxide UV-photolysis: The experiments were performed as previously reported [21], in a volume of 20 μL containing 33 μM in bp of pBR322 plasmid DNA in 5 mM phosphate saline buffer (pH 7.4), and the extracts. Immediately prior to irradiating the samples with UV light, H_2O_2 was added to a final concentration of 2.5 mM. The reaction volumes were held in caps of polyethylene microcentrifuge tubes, placed directly on the surface of a transilluminator (8000 μW cm^{-1}) at 300 nm. The samples were irradiated for 5 min at room temperature. After irradiation, 4.5 μL of a mixture containing 0.25% bromophenol blue, 0.25% xylen cyanol FF, and 30% glycerol were added to the irradiated solution. The samples were then analyzed by electrophoresis on a 1% agarose horizontal slab gel in Tris-borate buffer

(45 mM Tris-borate, 1 mM EDTA). Untreated pBR322 plasmid was included as a control in each run of gel electrophoresis, conducted at 1.5 V/cm for 15 hours. Gel was stained in ethidium bromide (1 μg/mL; 30 min), and photographed on Polaroid-Type 667 positive land film. The intensity of each scDNA band was quantified by means of densitometry.

Metal chelating activity: The chelating of ferrous ions by the methanolic extracts from the 8 samples examined of *R. officinalis* were estimated by the ferrozine assay [22]. Briefly, the extracts (5-200 μg/mL) were added to a solution of 0.15 mM $FeSO_4$. The reaction was initiated by the addition of 0.5 mM ferrozine and the mixture was shaken vigorously and left standing at room temperature for ten minutes. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. DTPA (5 μM) was used as a standard. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Statistical analysis: Statistical analyses were performed using the statistical software package SYSTAT, version 9 (Systat Inc., Evanston IL, USA).

Acknowledgements - The authors gratefully thank CORFO for its financial support through the FDI AT-11 grant and Dr C. Wright for proof reading the manuscript.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1129 - 1132

Hypericum perforatum L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L.: Phytochemical and Morphological Studies

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Received: June 22nd, 2006; Accepted: July 12th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Four species of *Hypericum* growing in Italy were characterized morphologically and chemically: *Hypericum perforatum* L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L. The composition of secondary metabolites (phloroglucinols, naphthodianthrones, flavonoids) in the aerial parts of plants collected in different habitats was analysed. The four species show different compositions of phloroglucinols and naphthodianthrones, but there was no qualitative difference in flavonoid content of the species analysed. Study of main-constituent variation during the ontogenetic cycle showed that hypericin decreases and hyperforin increases during the reproductive phase. In St. John's Wort, hypericin and hyperforin are thought to be localised in black nodules. Our investigation shows no clear correlation between either the presence or absence of nodules and hypericin or hyperforin content.

Keywords: flavonoids, *Hypericum,* naphtodianthrones, ontogenetic cycle, phloroglucinols.

The genus *Hypericum* (Guttiferae) comprises herbs and shrubs, distributed all over the world, with long, opposite leaves and flowers usually organised in a terminal inflorescence.

Many ancient writers wrote about the medical properties of this genus and in particular of St. John's Wort, noting its use as a vulnerary and as a balm for wounds, burns, ulcers, and bites [1-2]. In recent years *Hypericum perforatum* has received increasing attention for the treatment of mild and moderate depression [3-4].

The great interest on *H. perforatum* and its potential for human health have encouraged us to investigate the productivity of some *Hypericum* species growing in Italy. In this work four species were studied: *H. perforatum* L., *H. maculatum* Crantz., *H. calycinum* L. and *H. pulchrum* L. All these species are herbaceous plants. *H. perforatum* is characterized by a two – winged stem and black nodules over the whole plant; *H. maculatum* is different only for the four – edged stem. *H. pulchrum* is characterized by sessile leaves, small flowers, hirsute sepals with black nodules, and petals, stems and sepals with translucent glands. *H. calycinum* has typical inflorescences, but the black nodules and translucent glands are absent [5]. We have characterized each species chemically and morphologically. The chemical study has been concerned with the composition of flavonoids, phloroglucinols and naphtodianthrones; the morphological analysis has regarded the presence and distribution of secretory structures.

In this study we have analysed the secondary metabolites with interesting and demonstrated biological activity (**a**, chlorogenic acid; **b,** rutin; **c**, hyperoside; **d**, isoquercitrin; **e**, quercitrin; **f**, quercetin; **g**, hypericin; **h,** hyperforin) [3-4,6-8]. The MeOH extracts were analysed by RP-HPLC. The identification of peaks was effected on the basis of

the comparison of retention times and the use of a spectral library based on pure compounds previously described.

The qualitative analysis has shown four different profiles. The first profile, belonging to *H. perforatum* is characterized by the presence of all compounds under study: **a** (λ = 270 nm; Rt = 13.5), **b** (λ = 270 nm, Rt = 25.2), **c** (λ = 270 nm, Rt = 25.8), **d** (λ = 270 nm, Rt =26.3), **e** (λ = 270 nm, Rt = 30.2), **f** (λ = 270 nm, Rt = 37.4), **g** (λ = 590 nm, Rt = 42.9), **h** (λ = 270 nm, Rt = 51.3)**.** The second profile, typical of *H. maculatum* is characterized by the absence of hyperforin. The third profile is characterized by the absence of hypericin (*H. calycinum*). The fourth profile, where hypericin and hyperforin are absent, characterises *H. pulchrum*.

For quantitative analysis we produced eight calibration curves, as described in "experimental". For all compounds, a linear relationship between peak area and concentration was observed, with a correlation coefficient always better than $r = 0.997$. Analysis of the four species under study was performed during the flowering phase (Table 1). In all species, the content of **a** was highly variable from 0.16 to 4.98%. Rutin (**b**) and hyperoside (**c**) (not always detectable separately) were the more abundant flavonoids, except in *H. calycinum* where quercitrin (**e**) and quercetin (**f**) were more important. *H. perforatum* was characterized by the presence of hypericin (**g**) (0.13-0.18 %) and hyperforin (**h**) (up to 10%). A considerable amount of **g** was also detected in *H. maculatum* and of **h** in *H. calycinum* (0.54%). The analysis of samples of *H. perforatum* collected at different altitudes showed that the contents of chlorogenic acid, flavonoids and hypericin seem not to be affected by the altitude; on the contrary very significant decreases were found in the **h** content in the sites at higher altitudes (from 10% to 3%) (Table 1).

Furthermore in *H. perforatum*, the analysis of metabolite content was performed during the reproductive phase (pre-flowering I, flowering II and fruiting phase III) with the following results. **a**: the content was variable in the different populations studied; **b, c, d, e**: there was a gradual decrease from the I to III phase; **f**: the content of this compound reached the maximum level during the II phase; **g**: the content decreased with values in a range of less than 10%; **h**: the content was low in the I phase and

Table 1: Secondary metabolites content during the flowering phase (% dry wt.).

Samples (Altitude)	a	b	c	d	e	f	g	h
				H. perforatum				
4 (176 m)	3.45	6.65		2.27	2.97	0.14	0.13	10.05
8 (180 m)	4.98	7.24		7.10	1.50	0.47	0.13	10.76
9 (186 m)	4.70	6.12		3.20	4.10	0.10	0.13	10.10
5 (200 m)	4.62	9.35	2.08	1.30	5.44	0.10	0.15	10.65
12 (200 m)	0.16	7.24		5.89	3.04	0.45	0.14	10.20
7 (470 m)	2.27	8.24		3.70	2.18	0.23	0.13	7.50
11 (500m)	1.27	7.98	2.98	2.59	0.86	0.58	0.18	8.25
6 (800 m)	0.99	5.16	2.88	2.29	0.94	0.23	0.16	5.10
10 (900 m)	3.26	11.78	2.09	2.35	1.50	0.10	0.17	5.40
1 (1090 m)	0.63	4.08	1.89	1.71	1.72	0.57	0.14	3.54
3 (1400 m)	1.73	8.42	1.92	1.36	0.36	1.55	0.15	3.09
2 (1600 m)	0.47	11.13	2.07	1.56	0.47	0.70	0.13	3.74
				H. maculatum				
13 (560 m)	0.64	10.72		3.16	0.19	2.13	0.12	-
				H. calycinum				
14 (180 m)	-	0.30	0.63	0.37	2.28	1.54	-	0.54
				H. pulchrum				
15 (560 m)	0.24	4.30	0.61	2.55	3.52	0.30	-	-

(**a**) chlorogenic acid, (**b**) rutin, (**c**) hyperoside, (**d**) isoquercitrin, (**e**) quercitrin, (**f**) quercetin, (**g**) hypericin and (**h**) hyperforin.

Table 2: Secondary metabolite content (%, dry wt.) during the reproductive phase of *H. perforatum* collected in Comabbio.

	a	b	c	d	e	f	g	h
Phase I	3.69	12.95	3.56	3.48	2.09	0.12	0.13	4.80
Phase II	2.27	8.24		3.70	2.18	0.23	0.13	7.50
Phase III	0.56	3.75	1.26	1.07	1.51	0.15	0.11	8.00

Table 3: Localities and identification numbers of samples.

Samples	Locality	Herbarium No.
H. perforatum		
	Valle d'Aosta	
1	Anthey-St. Andrè (AO - 1090 m)	Hy-101
2	Colle de Joux (AO - 1600 m)	Hy-102
3	Crest (AO - 1400 m)	Hy-103
	Lombardia	
4	Canegrate (MI - 176 m)	Hy-104
5	Collebeato (BS - 200 m)	Hy-105
6	Colle Brianza (LC – 800 m)	Hy-106
7	Comabbio (VA – 470 m)	Hy-107
8	Parabiago Canale Villoresi (MI - 180 m)	Hy-108
9	Parabiago Santa Maria (MI - 186 m)	Hy-109
10	Pezzaze (BS - 900 m)	Hy-110
	Friuli Venezia Giulia	
11	Costa (UD – 500 m)	Hy-111
12	Monte Spaccato (TS – 200 m)	Hy-112
H. maculatum		
	Friuli Venezia Giulia	
13	Ampezzo Carnico (UD - 560 m)	Hm-101
H. calycinum		
	Lombardia	
14	Brescia (BS - 180 m)	Hc-101
H. pulchrum		
	Piemonte	
15	Mondovì (CN - 560 m)	Hp-101

reached the maximum in the III phase, with an increase of more than 50%. Table 2 shows an example (sample collected in Comabbio).

Morphological characteristics

H. perforatum: Leaves: presence, distribution and density of black nodules (b.n.) is variable among populations: sometimes only on the upper side of the lamina, and sometimes on both sides. Along with b.n., it is possible to find translucent glands, which confer the typical aspect at the leaves. Stems: b.n. are always present; there are only differences in the density of these structures. Petals and sepals: b.n. are always present on the borders. In some populations there are also secretory canals on all surfaces. Ovary: the surface is rich in translucent glands. B.n. are only present on the placenta. Stamen: one b.n. is always present between the thecae of anthers.

H. maculatum: This species shows, in particular, red glands on the stems.

H. calycinum: B.n. are completely absent.

H. pulchrum: B.n. are only on the sepals. From the above results it appears that *H. maculatum* and *H. calycinum* may be considered a good source of phloroglucinols and naphthodianthrones, respectively. It is worthwhile to notice that the production of **g** in *H. maculatum* is comparable, in quantity, to that of the well-known *H. perforatum.* It is also important to note the significant influence of altitude on the productivity of **h** in *H. perforatum.* Finally, even if many authors report that **g** and **h** are localised in b.n. [9-10], our investigation shows no clear correlation between the presence/absence of nodules and either hypericin or hyperforin content.

Experimental

Plant material: Fifteen populations of *Hypericum* belonging to the species *H. perforatum*, *H. maculatum*, *H. calycinum* and *H. pulchrum* were collected in different localities of Valle d'Aosta, Piemonte, Lombardia and Friuli Venezia Giulia, in Northern Italy during the summer of 2000 and determined according to Pignatti [5]. Voucher specimens are deposited in the Dipartimento di Biologia, Università di Milano. Table 3 shows localities, altitude and identification numbers of samples.

***Extraction and separation*:** Dried powdered aerial parts (1 g), taken 20-25 cm from the apex, as described in the Italian F.U., were extracted in a Soxhlet apparatus with 200 mL of MeOH for six hours. From the extract solution, 4 mL was diluted to 10 mL and submitted to RP-HPLC on a Merck LiChrospher 100 RP-18 column (5 μm, 250 x 4 mm, flow rate 1mL min^{-1}) with ternary gradient elution [A: H_2O (acidified at 0.3% with H_3PO_4); B: ACN; C: MeOH; gradient: 0 min 100% A; 10 min 85% A, 15% B; 30 min 70% A, 20% B; 40 min 10% A, 75% B; 55 min 5% A, 80% B; minimum re-equilibration time between two injections: 10 min]. The detection range was 270-590 nm.

Chlorogenic acid (**a**), rutin (**b**), hyperoside (**c**), isoquercitrin (**d**), quercitrin (**e**), quercetin (**f**), hypericin (**g**) and hyperforin (**h**) were obtained commercially, **a-g** from Extrasynthese, Genay, France, and **h** from PhytoLab GmbH e Co. KG, Labor Addipharma, Hamburg, Germany. These compounds were used to produce a spectral library in order to identify chromatographic peaks. The concentration of pure compounds was 0.4 mg mL^{-1} and the injection volume was 15 μL. The analytical chromatographic analyses were performed with a Merck-Hitachi L 6200 system with a Hewlett Packard 1040 photo diode array detector, controlled by HP-Chemstation (Hewlett Packard) software. Calibration curves for **a**-**h** were realized with solutions of known concentrations (0.4, 0.2, 0.1, 0.05, 0.025 mg/mL).

Morphological analyses: The morphological analyses were performed using a stereomicroscope, model MZ 6, Leica Mycrosystems S.p.A, Milano, Italy.

Acknowledgements - This research was carried out with the financial support of MIUR, Italy.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1133 - 1136

Chemical Composition and Antimicrobial Activities of Essential Oil of *Stachys glutinosa* L. from Sardinia

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Received: July 13th, 2006; Accepted: September 12th, 2006

Dedicated to the memory of Professor Ivano Morelli.

The oil composition of *Stachys glutinosa* L. from two different areas of Sardinia was analyzed by GC/MS. The oil from Gallura plants was characterized by the four main constituents: terpinen-4-ol (12.7%), α-terpinyl acetate (10.6%), *trans*-cadina-1(6),4-diene (8.5%), and α-terpineol (8.4%) whilst α-cedrene (19.2%), α-terpineol (18.5%), terpinen-4-ol (12.6%), and α-terpinyl acetate (8.6%) were the main compounds in the oil from Ulassai plants. The oils showed good bacteriostatic activities against *Vibrio cholerae* (MIC 0.6%), all the *Candida* tested (1.25%) and *Rodotorula rubra* (2.5%). There were also bactericidal activities against *Candida glabrata* (1.25 %) and *Rodotorula rubra* (2.5%).

Keywords: *Stachys glutinosa* L., Lamiaceae, Sardinia, essential oil, terpinen-4-ol, α-cedrene, antimicrobial.

Stachys glutinosa L. is a fruticose dwarf shrub, widespread in Sardinia, Corsica and the Capraia Islands [1], and is very common on different substrata from sea level to the mountains. The plant is covered by weak thorny stems that emanate an unpleasant smell [2]. The plant is used for medicinal purposes (mostly as an antispasmodic and antiseptic) in folk medicine [3]. A few studies refer to the composition of the essential oil of *S. glutinosa* from Corsica [4,5] and one about the principal compounds of essential oils from Sardinian plants [6].

The aim of this research was to determine the composition of the essential oil of wild *S. glutinosa* in different pedological soils and on the antimicrobial activities of the oils against soil-borne pathogens, mycotoxic species, phytopathogens and opportunistic human pathogens.

Table 1 shows the composition of the essential oils obtained from *S. glutinosa* L. plants harvested in the Gallura and Ulassai areas. Compounds are listed in order of their elution from an HP-5 column. In the oil from the Gallura plants, fifty-nine compounds were identified representing 95.7% of the oil, while in the oil from the Ulassai plants forty-seven compounds were identified representing 97.1% of the oil.

The oil from the Gallura plants was characterized by the four main constituents: terpinen-4-ol (12.7%), α-terpinyl acetate (10.6%), *trans*-cadina-1(6), 4-diene (8.5%), and α-terpineol (8.4%). α-Cedrene (19.2%), α-terpineol (18.5%), terpinen-4-ol (12.6%), and α-terpinyl acetate (8.6%) were the main compounds in the oil from the Ulassai plants.

Table 1: Percentage composition of the essential oils of *S. glutinosa*.

Compounds	KI	Gallura (%)	Ullassai (%)
α-pinene	937	0.4	3.1
β-pinene	978	0.1	0.8
myrcene	996	n.d	0.4
α -phellandrene	1008	0.2	0.2
α-terpinene	1019	0.4	2.3
β-phellandrene	1029	3.5	4.7
γ-terpinene	1060	3.3	4.1
terpinolene	1089	0.3	1.1
trans-sabinene hydrate	1099	n.d	1.2
linalool	1106	1.6	n.d.
cis-p-menth-2-en-1-ol	1126	n.d.	0.5
trans-pinocarveol	1143	n.d.	0.1
neo-isopulegol	1147	n.d.	0.3
isopulegol	1148	0.1	n.d.
terpinen-4-ol	1177	12.7	12.6
thuj-3-en-10-al	1188	n.d.	0.1
α-terpineol	1189	8.4	18.5
Myrtenol	1198	n.d.	0.3
trans-piperitol	1211	n.d.	0.3
linalyl acetate	1260	4	0.5
terpinen-4-ol acetate	1301	0.4	n.d.
Carvacrol	1315	0.1	n.d.
trans-sabinyl acetate	1323	n.d.	1.1
δ-elemene	1329	1	n.d.
α-terpinyl acetate	1348	10.6	8.6
α-copaene	1366	0.2	n.d.
β-bourbonene	1373	0.9	0.8
β-cubebene	1381	0.1	0.1
β-elemene	1383	0.5	n.d.
geranyl acetate	1386	2.9	n.d.
(*Z*)-β-damascone	1399	n.d.	0.3
(*Z*)-caryophyllene	1404	2.9	n.d.
α-cedrene	1414	n.d.	19.2
β-cedrene	1416	0.6	0.3
β-copaene	1426	0.1	n.d.
β-gurjunene	1432	0.1	n.d.
(*Z*)-β-farnesene	1441	0.2	0.5
α-himachalene	1447	0.8	n.d.
Alloaromadendrene	1451	n.d.	0.7
cis-muurola-3,5-diene	1452	0.1	n.d.
trans-muurola-3,5-diene	1454	0.1	n.d.
(*E*)-β-farnesene	1456	0.8	n.d.
cis-muurola-4(14),5-diene	1465	0.1	n.d.
trans-cadina-1(6),4-diene	1470	8.5	0.9
γ-curcumene	1474	0.3	n.d.
γ-himachalene	1479	0.1	n.d.
germacrene D	1487	n.d.	0.4
cis-β-guaiene	1493	n.d.	0.1
Bicyclogermacrene	1495	2.9	n.d.
α-muurolene	1497	0.5	n.d.
trans-muurola-4(14),5-diene	1499	0.1	n.d.
trans-β-guaiene	1501	1.1	0.2
germacrene A	1508	1.5	0.2
δ-amorphene	1514	3.2	0.9
δ-cadinene	1522	0.1	n.d.
trans-calamenene	1529	0.2	0.2
Elemol	1542	1.8	n.d.
geranyl butanoate	1566	n.d.	0.3
Spathulenol	1570	2.2	n.d.
caryophyllene oxide	1573	n.d.	6.8
Globulol	1579	0.7	0.3
Viridiflorol	1589	0.9	0.2
Guaiol	1602	0.4	n.d.
10-*epi*-γ-eudesmol	1621	0.3	n.d.
10-*epi*-α-eudesmol	1625	n.d.	0.1
caryophylla-4(14),8(15)-dien-5-ol *	1630	n.d.	0.6
γ-eudesmol	1631	1.1	n.d.
α-muurolol	1637	n.d.	0.2
epi-α-cadinol	1638	0.7	1.2
epi-α-muurolol	1642	0.4	n.d.
Cubenol	1645	2.6	0.6
Valerianol	1658	0.3	n.d.
14-hydroxy-9-epi-(*E*)-caryophyllene	1667	n.d.	0.4
helifolenol A	1674	0.4	n.d.
epi-α -bisabolol	1677	5.7	n.d.
eudesma-4(15),7-dien-1-β-ol	1683	0.9	0.4
epi-laurenene	1890	0.7	n.d.
Isopimara-9(11),15-diene	1894	n.d.	0.4
Sclarene	1986	0.4	n.d.

Table 1 (*contd*.)

* = correct isomer not identified
n.d. = not detected

The sesquiterpene hydrocarbons contributed the highest percentage (27.0%) of the oil from the Gallura plants. This fraction was dominated by *trans*-cadina-1(6),4-diene (8.5%). The oxygenated monoterpene fraction represented the 22.9% of the total oil, terpinen-4-ol (12.7%) being the most abundant compound. The oxygenated sesquiterpenoids and esters were also relatively high representing 18.4% and 17.9% of the total oil respectively, whereas in the oil from the Ulassai plants the oxygenated monoterpenes constituted the highest portion (33.9%). This fraction was also dominated by α-terpineol (18.5%). The sesquiterpene hydrocarbons represented 24.5% of the total oil, α-cedrene (19.2%) being the major compound. The monoterpene hydrocarbons, oxygenated sesquiterpenes and esters were also relatively high representing 16.7%, 10.8% and 10.2%, respectively, of the total oil.

If we consider the principal components, α-cedrene distinguishes the Ulassai oil from the Gallura oil. Epi-α-bisabolol, on the contrary, distinguishes the Gallura oil from the Ulassai oil, with 5.7% in the former and none detected in the latter. Other minor components present only in Gallura oil were (*Z*)-caryophyllene (2.9%), bicyclogermacrene (2.9%), elemol (1.8%), γ-eudesmol (1.1%), and δ-elemene (1%), whereas, caryophyllene oxide (6.8%), *trans*-sabinene hydrate (1.2%) and *trans*-sabinyl acetate (1.1%), were the minor compounds present only in the Ulassai oil. Previous studies on *S. glutinosa* harvested in Corsica and Sardinia have proposed the three chemiotypes A, B and C, characterized by the presence of α-terpineol-trans-caryophyllene, β-phellandrene, and terpinen-4-ol [5]. According to these proposed division, both the oils might belong to chemiotype C.

Antimicrobial activity: The two tested oils of *S. glutinosa* presented similar antimicrobial activities the mean values of Minimal Inhibitory Concentration (MICs) and Minimal Bactericidal Concentration

(MBC) as summarized in Table 2. The oil of *S. glutinosa* exhibited good bacteriostatic effects against yeast clinical strains, particularly *Candida glabrata*, with the same value of MIC and MBC (1.25%) (*Rodotorula rubra* (MIC and MBC = 2.5%) and against *V. cholerae* 01 (MIC = 0.6%). The oils exhibited moderate bacteriostatic and bactericidal activities in general, but had a good bacteriostatic activities against *Vibrio cholerae* (0.6%). Other interesting bacteriostatic activities were against all the *Candida* tested (1.25%) and *Rodotorula rubra* (1.25%). Bactericidal activities at the tested concentration were against *Candida glabrata* (1.25%) and *Rodotorula rubra* (1.25%). Significantly, all the clinical and environmental strains to have multi-drug resistance, for example: *A. hydrophila* to ampicillin (192 μg/mL), ceftazidime (125 μg/mL), and gentamicin (125 μg/mL), *S. epidermidis* to ampicillin/sulbactam (64 μg/mL), norfloxacin (125 μg/mL), and gentamicin (125 μg/mL); *V. cholerae* to ampicillin (256 μg/mL), ceftazidime (125 μg/mL), cefotaxime (256 μg/mL), doxycyclin (32 μg/mL), and amoxycillin-clavulanate (256 μg/mL). All the bacteria are susceptible to streptomycine (range 0.010 – 0.06 mg/mL) and the yeasts are susceptible to bifonazole (0.02 – 0.05 mg/mL). Other studies [7] of the antimicrobial activity of *Stachys* essential oil was of different species and subspecies.

Table 2: Antimicrobic activity of the essential oil of *Stachys glutinosa*.

Microorganism	MIC(%)	MCB(%)
Aeromonas sobria	1.25	2.5
Candida albicans	1.25	>2.5
Candida glabrata	1.25	1.25
Candida kruseii	1.25	1.25
Candida parapsilosis	1.25	>2.5
Enterococcus faecalis	>2.5	>2.5
Escherichia coli	>2.5	>2.5
Klebsiella pneumonie	>2.5	>2.5
Aeromonas hydrophyla	1.25	>2.5
Rodotorula rubra	1.25	1.25
Staphylococcus aureus (ATCC)	>2.5	>2.5
Staphylococcus aureus	>2.5	>2.5
Staphylococcus epidermidis	2.5	2.5
Streptococcus group D	2.5	2.5
Vibrio alginolyticus	>2.5	>2.5
Vibrio cholerae	0.6	2.5

Experimental

Collection of Plant Material: Plants of *S. glutinosa* L. growing in Ulassai (central Sardinia) on limestone, and Gallura (northern Sardinia) on granite were collected during flowering, in June 2004. Voucher specimens were deposited in the Herbarium SASSA [Dipartimento di Scienze del Farmaco, Università di Sassari], under Acquisition No. 1099

Isolation of the Essential Oil: Fresh plant material was subjected to hydrodistillation using a Clevenger-type apparatus for 2 h yielding 0.25% of yellowish oil. The oil was dried over anhydrous sodium sulfate and stored in sealed vials under refrigeration prior to analysis.

Gas Chromatography: The GC analyses were carried out using a Hewlett Packard 5890 Series II dual FID instrument equipped with HP-WAX and HP-5 capillary columns (30 m x 0.25 mm, 0.25 μm film thickness), working with the following temperature programmed: 10 min at 60°C, and subsequently at 5°C/min up to 220°C; injector and detector temperatures, 250°C; carrier gas, helium (1 mL/min); split ratio, 1 : 20.

Gas Chromatography-Mass Spectrometry: GC-MS analyses were carried out using a Hewlett Packard 6890-5973 GC-MS system operating in the EI mode at 70 eV, using two different columns, a HP Innowax (30 m x 0.25 mm, film thickness 0.50 μm) capillary column and a DB-5 (30 m x 0.25 mm, film thickness 0.25 μm) capillary column. The temperature programmed for HP Innowax was 60-260°C at a rate of 3°C/min, held for 10 min, and for the HP 5 it was 60-300°C at a rate of 3°C/min. Injector and transfer line temperatures were 220°C and 280°C, respectively. Helium was used as the carrier gas, flow rate 1 mL/min. Split ratio, 1 : 10.

Identification of the Components: The identification of the components was made for both the columns, by comparison of their retention time with respect to n-paraffin (C6-C22) internal standards. The mass spectra and retention indices (RI) were compared with those of commercial (NIST 98 and WILEY) and home-made library mass spectra built up from pure compounds and MS literature data [8,9,10,11,12,13].

Area percentages were obtained electronically from the GC-FID response without the use of an internal standard or correction factors.

Microorganisms: A total of 17 strains of bacteria and yeast were investigated, ten were isolated from patients, three from environmental sources and four ATCC (American Type Culture Collection) strains were used as quality control strains. The isolates were identified to the species level by standard procedures, and some clinical and environmental ones were tested for virulence phenotype. Antimicrobial susceptibility to 14 different antibiotics (ampicillin, amoxycillin

clavulanate, amikacin, piperacillin, cefotaxime, ceftazidime, ceftriaxone, ciprofloxacin, ofloxacin, gentamicin, doxycycline, imipenem, meropenem, trimethoprim-sulphamethoxazole) was determined by the Kirby Bauer method, according to the general qualitative assay described by Barry (1986) [14]. Protease activity was tested on Nutrient agar containing 1.5% skim milk, production of protease was shown by the formation of a clear zone caused by casein degradation; hemolysis test was assayed by culturing each strain on agar plates containing rabbit erythrocytes 5%; Hep-2 cells (human laryngeal carcinoma) were used for the adhesion assay. The bacteriostatic and bactericide activities were determined by measuring the Minimal Inhibitory Concentration (MICs) and the Minimal Bactericidal Concentration (MBC) of *Stachys glutinosa* oil performed in microtiter plates using a bacterial inoculum (taken from Luria Berani broth after overnight culture) with a turbidity equivalent to 0.5 MacFarland standard. The essential oils were suspended in the medium with a 0.5% Tween 80 as emulsifier and tested at different concentrations; values of MICs and MBCs are expressed as percent vol/vol of total oil and culture medium used as diluent. Bacterial strains were as follows: *Aeromonas hydrophyla* (isolated from patients), *Aeromonas sobria* (isolated from patients), *Candida albicans* (isolated from patients), *Candida glabrata* (isolated from patients), *Candida kruseii* (isolated from patients), *Candida parapsilosis* (isolated from patients), *Enterococcus faecalis* (ATCC 24212), *Escherichia coli* (ATCC 35218), *Klebsiella pneumonie* (ATCC 700603), *Rodotorula rubra* (isolated from patients). *Staphylococcus aureus* (isolated from patients), *Staphylococcus aureus* (ATCC 43300), *Staphylococcus epidermidis* (isolated from patients), *Streptococcus group D* (isolated from patients), *Vibrio alginolyticus* (soil-borne pathogens), and *Vibrio cholerae* (soil-borne pathogens). All micro-organism species were tested in triplicate.

Acknowledgments - The work was financially supported by INTERRREG III and ex 60% MURST.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1137 - 1140

Molecular Identification of *Panax ginseng* C.A. Meyer in Ginseng Commercial Products[+]

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Received: May 13th, 2006; Accepted: May 27th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Molecular techniques (PCR and RFLP) were used to verify the presence of *Panax ginseng* C.A. Meyer in commercial products containing ginseng. DNA, extracted from four vegetable forms present in marketed products, was amplified with 18df/28ccr primers. The RFLP of the DNA amplified products, obtained using *Inf* I, *Sau* 3*A*1 and *Taq* I endonucleases, allowed the identification of *P. ginseng* and its differentiation from *P. quinquefolium*. *P. ginseng* was detected in 9 out 16 samples tested which, according to the declaration on the labels, contained the drug. Negative results were obtained for products containing the dried extract of the drug. A comparison of the results acquired using the molecular techniques with those using HPLC is also reported.

Keywords: *Panax ginseng*, *P. quinquefolium*, Molecular identification technique, Ginseng commercial products.

Molecular biology constitutes a new frontier for phytochemical analysis, allowing the improvement of previous knowledge, as well as the acquisition of new data. Recently, molecular techniques have been successfully utilised in order to validate plant drugs, overcoming the limitations of traditional analyses [1-4]. In the present study, PCR and RFLP were used in order to authenticate *Panax ginseng* C.A. Meyer (Korean ginseng) in different vegetable forms of ginseng commercial products and to differentiate it from other *Panax* species and from some of their adulterants. A comparison with HPLC identification results was also made.

There is an ongoing question over the labelling of herbal products as "Ginseng". Currently the word "Ginseng" is used to sell a variety of herbs associated with certain claimed therapeutic properties (Table 1). This can be confusing as they neither contain the same constituents nor display the same biochemical properties.

The quality of ginseng commercial products influences their effectiveness and safety of use and depends on the employed raw materials. The most active constituents of *P. ginseng* are steroidal saponins, called ginsenosides. So far 22 ginsenosides have been isolated and characterised, based on triterpene aglycone moieties with dammarane and oleanane structures and on the sugar unit sequences. In the monograph entitled "Ginseng" [5] the European Pharmacopoeia (Ph. Eur.) reports only the whole or cut dried root of *P. ginseng*, that must contain ginsenoside Rf and not less than 0.40% of combined ginsenosides Rg1 and Rb1, calculated with reference to the dried drug. This is, therefore, the only true ginseng (Korean ginseng).

Since the 1990s differentiation and research on the various species of *Panax* have been reported and the

[+]Part 1 in the Series: "Molecular identification of herbal drugs"

use of TLC, GLC and HPLC, as well as chemical techniques have allowed efficient separation and isolation of ginsenosides [6-9]. However, as reported in the Ph. Eur. [5], TLC and HPLC are the official tools to detect the presence of ginsenosides in root commercial samples. These methods need time (two working days), and a large quantity of plant material, as well as reference standards, that are often difficult to obtain.

Table 1: Main recoverable species in ginseng commercial products other than the European Pharmacopoeia species.

Botanical Name	Common names
Panax ginseng C.A. Meyer (Araliaceae) Ph. Eur. species	Korean ginseng Asian ginseng Chinese ginseng Ren shen Panax schinseng Jiln ginseng
Panax quinquefolium Linn	American ginseng
Panax notoginseng Burkill	San-chi ginseng
Panax pseudoginseng N. Wallich	Himalayan ginseng, Tien-chi ginseng
Panax japonicus or *P. japonicum* C.A. Meyer	Japanese ginseng, Ginseng bamboo
Panax trifolium Linn	Dwarf ginseng
Panax zingiberensis C.Y. Wu & Feng	Ginger ginseng
Panax stipuleanatus Tsai & Feng	Pingbiann ginseng
Panax vietnamensis Ha Thi Dung & I.V. Grushvitskii	Vietnamese ginseng
Other *genera* and families	
Eleutherococcus senticosus Maxim (Araliaceae)	Siberian ginseng, Wujia
Echinopanax horridus Decne & Planch. (Araliaceae)	Alaskan ginseng, Devil's club
Aralia nudicaulis Blume (Araliaceae)	Wild ginseng, Salsaparilla
Rumex hymenosepalus J. Torrey (Polygonaceae)	Red Desert ginseng
Pfaffia paniculata Kuntze (Amaranthaceae)	Brazilian ginseng, Suma
Pseudostellaria heterophylla Pax (Caryophyllaceae)	Sometimes used as a ginseng substitute
Caulophyllum thalictroides Regel (Berberidaceae)	Yellow or Blue ginseng, Blue cohosh
Triosteum perfoliatum Linn. (Caprifoliaceae)	Fever root, sometimes called Ginseng
Codonopsis tangshen Oliver (Campanulaceae)	Sometimes used as a ginseng substitute
Lepidium meyenii Walp. (Cruciferae)	Maca, Peruvian ginseng Andean ginseng
Withania somnifera Dun. (Solanaceae)	Indian ginseng Ashwaganda

The objective of the present study was to develop and standardise a reliable and easy molecular method for authentication of *P. ginseng* in the different forms of commercial ginseng products and to compare these results with HPLC identification made on the same samples [10].

Useful amounts of DNA were extracted from all the considered samples (**1-19**). PCR amplification was made using 18df/28ccr primers. The DNA amplified products, digested with the endonucleases *Hinf* I, *Taq* I and *Sau* 3A1, gave fragments of 170 bp; 230 bp and 280 bp; and 120 bp and 580 bp, respectively, characteristic for *P. ginseng*. Similarly, fragments of 60 bp and 100 bp; and 106 bp, 170 bp and 260 bp, were obtained for *P. quinquefolium* (American ginseng) with the use of *Hinf* I and *Taq* I, respectively (Figure 1).

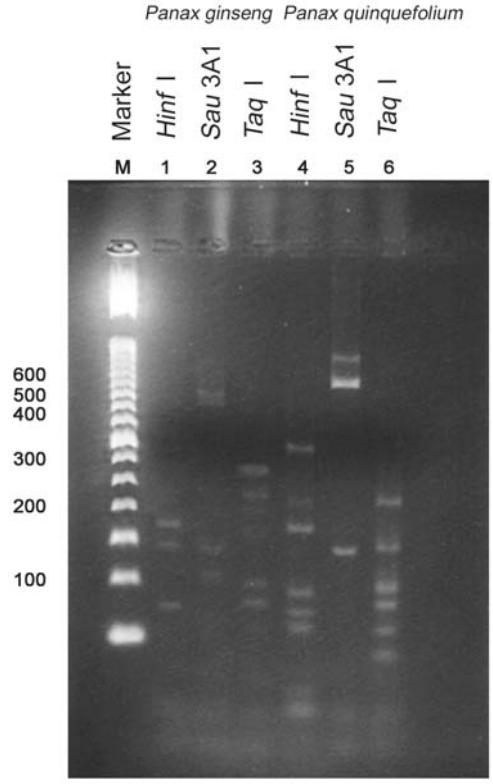

Figure 1: Agarose gel electrophoresis of *Hinf* I, *Sau* 3A1 and *Taq* I restriction fragments obtained from amplified amplicons using the 18df/28ccr primer pair, specific for conserved region 18S-28S of *Panax* species.

For a total of 486 determinations (19 samples, 3 repetitions, 3 extraction protocols, following PCR and restriction with 3 endonucleases), the molecular analysis confirmed the presence of *Panax* species in 12 out of 19 samples tested, as shown in Table 2. Three of the positive samples, named PQ, PQT, PQP (**6**, **11**, **12**), were commercial ginseng products containing *P. quinquefolium* in the form of dried body root, dried root tails and dried root prongs, respectively. Among the samples labelled PG (**1-5**, **7-10**, **13-19**), the presence of *P. ginseng* was confirmed, as reported on the label, in 9 out of 16 samples, but not in three dried body root products (**4**, **5**, **18**) or in four dried extract samples (**15-17**, **19**). The presence of adulterants such as *Mirabilis jalapa* L. and *Phytolacca acinosa* Roxb could be excluded in the considered samples since no specific *Sau* 3A1 digestion fragments [11] for either plant were visualised in agarose gel.

Table 2: DNA identification (PCR and RFLP) of *Panax ginseng* and HPLC detection of ginsenoside Rf in ginseng commercial products.

Sample		DNA identification				HPLC
		Body root	Root tails	Root prongs	Dried extracts	Ginsenoside Rf
1	PGB	+				+
2	PGR	+				+
3	PGBO2	+				+
4	PGBH1	–				–
5	PGBH2	–				–
6	PQ	+				–
7	PGBR		+			+
8	PGT99		+			–
9	PGTO3		+			+
10	PGTO4		+			+
11	PQT		+			–
12	PQP			+		–
13	PGRB	+				–
14	PGBDSPR	+				+
15	PGPHRB				–	–
16	PGNGLC				–	–
17	PGRKPS				–	+
18	PGGNST	–				–
19	PGext				–	+

PG: samples labelled as *P. ginseng.*
PQ: samples labelled as *P. quinquefolium*.
1-12: raw materials; **13-18**: commercial preparations as capsules or tablets; **19**: laboratory hydromethanoholic extract.

HPLC analysis [10], conducted as reported in the Ph. Eur. [5], revealed the presence of all ginsenosides used as reference standards. As required by the Ph. Eur. monograph [5], ginsenosides Rg1 and Rb1 were identified in all tested samples and Rf, characteristic of *P. ginseng*, was detected in only 9 out of the 16 PG samples, which claimed to be based on *P. ginseng*. In particular, ginsenoside Rf was absent from the three PQ samples (**6**, **11**, **12**), as expected for *P. quinquefolium* products, and from seven PG samples (**4**, **5**, **8**, **13**, **15**, **16**, **18**).

The PCR and RFLP results were in accordance with the HPLC data (presence of ginsenoside Rf, characteristic of *P. ginseng*) for the majority of the tested commercial products and in accordance with the species, *P. ginseng*, declared on their labels. PCR and RFLP/HPLC afforded negative results for five products, in the form of dried body root (**4**, **5**, **18**) and of dried extract (**15**, **16**), which excluded the presence of *P. ginseng*, although this was declared on the label. On the other hand, discordance between the molecular results and HPLC data was noted when the preparation was based on dried extracts (**17**, **19**).

Molecular methods have been used to unequivocally allow the authentication to species level of the genus *Panax* and the results were not affected by the nature of the drug. Compared with other methods that detect genome-wise polymorphism simultaneously, such RAPD [12], AP-PCR and AFLP [13], the method applied in this research, based on PCR followed by RFLP, is more reliable for large scale screening of commercial products, is rapid (one working day), and the results are easily readable. However, the procedure failed when the commercial products were dried extracts. A sample of reference *P. ginseng* was processed as described in the European Pharmacopoeia monograph [5], by boiling the root powder in 50% (v/v) aqueous methanol for 1h, to obtain a dried extract (**19**). This prepared extract resulted in a negative result in the molecular analytical procedure, as expected, because DNA molecules are not soluble in the hydromethanolic solvent, whereas the extract gave a positive for Rf on HPLC examination.

Experimental

The analysed commercial ginseng products as raw materials in the form of body root, root tails and root prongs, and as capsules and tablets containing also dried extract were obtained from national health care stores. To protect the Manufacturers' identities the sample sources were labelled as reported in Table 2.

An AB GeneAmp PCR System 9700 thermal cycler was used for the PCR analysis. *P. ginseng* and *P. quinquefolium* dried roots, used as references for the molecular analysis, were kindly provided by the Department of Plant Biology of the University of La Sapienza, Rome, Italy. Chromatography was performed on a Waters chromatographic system equipped with a Waters 600 MS multisolvent delivery system and a Waters 717 Auto sampler. A Waters 996 Photodiode Array Detector was used to monitor the eluates at 203 nm. The chromatographic data were analysed using a Waters Millennium Software version 3.2. Chromatography was performed at room temperature (25° C). Ginsenosides Rg1, Rb1, Rb2, and Rc–Rf, purchased from Extrasynthese, France, were used as reference standards.

Molecular analysis: DNA was extracted from 50-100 mg of each sample using an Invitrogen Easy-DNA Kit [14] and two other molecular protocols [15, 16] in order to compare their effectiveness in the extraction of useful amounts of DNA for molecular analysis. Body roots were previously treated with liquid nitrogen, while the other samples were used directly in the DNA procedures following the Manufacturers' instructions. Sometimes, it was necessary to precipitate with isopropyl alcohol, rinse with 70% ethanol, resuspend in 10 mM TE (Tris-HCl, pH 8.0, 1 mM EDTA) and precipitate a second

time in the presence of 0.3 M sodium acetate and 2 volumes of ethanol. The final pellet, after a second rinse in 70% ethanol, was resuspended in sterile distilled water (50 µL). The PCR amplification was performed on all ginseng DNA samples using oligonucleotide primers 18df/28ccr. This primer pair amplifies the conserved region, 18S-28S, including ITS1 and ITS2, highly variable regions for *Panax* species [17]. Amplification reactions were performed with reaction mixtures and with reaction conditions previously reported [11], using DNA *Taq* polymerase W1 (Invitrogen, Italy). For the RFLP analysis, amplified 18S rDNA fragments (11 µL aliquots) were separately digested in a final volume of 20 µL at 37°C for 3h and 65°C for 16h with 1.5-2 Units for each of the following endonucleases: *Hinf* I, *Sau* 3*A*1, *Taq* I (New England BioLabs, UK). Restriction fragments were analysed by electrophoresis in 1% agarose gels buffered in 0.5 X TBE [TBE buffer: 90 mM Tris (hydroxymethyl)-aminomethane, 90 mM boric acid, 3 mM ethylene-diaminetetraacetate Na salt, pH 8.3] and visualised by UV light after staining with ethidium bromide. The size marker was 50 bp ladder (Invitrogen, Italy).

Chemical analysis: Analysis of the principal active constituents was performed on all available samples by the HPLC method described in the "Ginseng" monograph of the Ph. Eur. [5], using a 5 µm (25 cm x 4.6 mm) Kromasil KR100-5NH2 E6170 column, a mobile phase filtered on an Alltech nylon membrane 47 mm, 0.45 µm, and degassed by a Waters in line degasser, at a flow rate of 1 mL/min.

Acknowledgments - The authors thank Professor Marcello Nicoletti (University "La Sapienza", Rome, Italy) for his suggestions to improve the paper.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1141 - 1145

Lipoxygenase Inhibitory Activity of Boropinic Acid, Active Principle of *Boronia pinnata*

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Received: March 21st, 2006; Accepted: April 24th, 2006

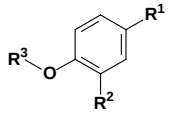
Dedicated to the memory of Professor Ivano Morelli.

Boropinic acid and other natural prenyloxycinnamic and benzoic acids were easily synthesized in high yield by a two-step sequence from the corresponding *p*-hydroxy aromatic acids and were assayed for radical scavenging activity using the DPPH test and for inhibition of enzymatic lipid peroxidation mediated by soybean 5-lipoxygenase. Compared to other acids and to known antioxidant compounds like BHT, Trolox and ascorbic acid, boropinic acid was far more active in the lipoxygenase test (IC_{50} = 7.6 ng/mL, $p < 0.05$). The recorded inhibition value suggested that boropinic acid acted as an enzyme inhibitor rather than a mere radical or peroxide scavenger. This hypothesis was confirmed by studying the interaction between boropinic acid and soybean 5-lipoxygenase by molecular modelling techniques.

Keywords: anti-inflammatory activity, antioxidant activity, boropinic acid, lipoxygenase, prenyloxy acids.

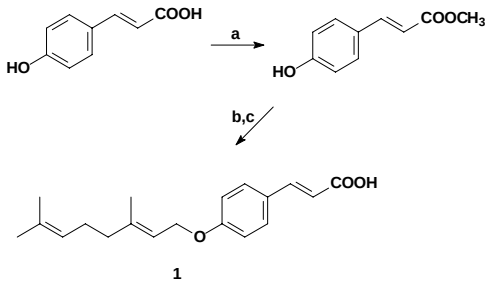
Secondary metabolites of phenylpropanoic acid biosynthetic origin containing sesquiterpenyl, monoterpenyl and isopentenyl chains attached to a phenol group represent quite a rare group of natural products. Some of these compounds, including coumarins [1], anthraquinones [2], xanthones [3], flavonoids [4] and carboxylic acids, have been recently studied chemically and pharmacologically. Among the latter, cinnamic and benzoic acids have been shown recently to have valuable biological properties [5].

To the best of our knowledge, only five prenyloxy-phenylpropenoic acids have been reported from natural sources: 3-(4'-geranyloxyphenyl)-2-*trans* propenoic acid (**1**), 3-(4'-geranyloxy-3'-methoxy-phenyl)-2-*trans* propenoic acid (**2**), isolated from *Acronychia baueri* Schott [6], boropinic acid (**3**), extracted from *Boronia pinnata* Sm. [7], valencic acid (**4**),isolated from *Citrus sinensis* L. and *Aegle marmelos* [8], and 4-isopentenyloxy-3-methoxy benzoic acid (**5**), isolated as a methyl ester from the liverwort *Trichocolea lanata* (Ehrh.) Dumm. [9]. The aim of this study was to synthesize these natural prenyloxy-carboxylic acids and to test their antioxidant activity.



1 R^1 = -CH=CH-COOH, R^2 = -H, R^3 = geranyl
2 R^1 = -CH=CH-COOH, R^2 = $-OCH_3$, R^3 = geranyl
3 R^1 = -CH=CH-COOH, R^2 = $-OCH_3$, R^3 = isopentenyl
4 R^1 = -COOH, R^2 = -H, R^3 = isopentenyl
5 R^1 = -COOH, R^2 = $-OCH_3$, R^3 = isopentenyl

The synthesis of compounds **1**, **3**, **4** and **5** was accomplished following an environmentally friendly route similar to that reported for the synthesis of compound **2** [5]. Compound **1** was obtained in 97% overall yield starting from commercially available *p*-coumaric acid that was first converted into its methyl ester by refluxing in MeOH catalyzed by concentrated H_2SO_4, then alkylated with geranyl bromide and hydrolyzed in a basic medium (Scheme 1).



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Scheme 1: Reagents and conditions: a) MeOH, conc. H_2SO_4 (cat.), reflux, 12 h; b) geranyl bromide, K_2CO_3, acetone, reflux, 2h; c) NaOH 2N, 70°C, 1h

Compounds **3** and **5** were obtained, using the same reaction conditions as above, in 96% and 98% yield from ferulic acid and vanillic acid, respectively, while compound **4** was synthesized in 99% yield by a one-pot alkylation-basic hydrolysis from commercially available methyl *p*-hydroxy benzoate and employing, in all cases, 4-bromo-2-methyl-2-butene as alkylating agent.

Table 1: DPPH radical scavenging activity of prenyloxy-carboxylic acid.

Compound	IC_{50}, μmol/mL[a]
1	0.065 ± 0.0060
2	0.011 ± 0.0011
3	0.011 ± 0.0008
4	0.011 ± 0.0008
5	0.052 ± 0.0048
Ascorbic acid	$6.24 \times 10^{-4} \pm 3.97 \times 10^{-5}$
BHT	$5.24 \times 10^{-4} \pm 4.87 \times 10^{-5}$
Trolox	$0.30 \times 10^{-4} \pm 0.39 \times 10^{-5}$

[a] $p < 0.05$ using Student's *t* test

Compounds **1-5** were first assayed to evaluate their radical scavenging activity by the DPPH test [10], using Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), BHT (butyl hydroxy toluene) and ascorbic acid as positive controls. Results are reported in Table 1.

As shown in Table 1, no acid exhibited an appreciable radical scavenging activity compared to the controls, particularly those having a free phenolic hydroxyl group like Trolox and BHT. So, the lack of any significant radical scavenging ability of compounds **1-5** may be due to the alkylation of the phenol moiety with either a geranyl or isopentenyl group.

We then evaluated the inhibition of polyunsaturated fatty acid (PUFA) peroxidation catalyzed by soybean 5-lipoxygenase (5-LOX) [11]. Assessing the inhibitory effect of a chemical on this enzyme is noteworthy, as lipoxygenases are nowadays recognized as playing a major role in cancer cell growth, metastasis, invasiveness, cell survival and induction of tumor necrosis factor (TNF) [12,13]. More particularly, it has been observed that the inhibition of the 5-LOX pathway has a chemopreventative effect in lung carcinogenesis, prevents the biological activation of different types of carcinogens, decreases cell proliferation, and induces apoptosis [14-16]. We used soybean 5-LOX in our study as, despite differences in the number of aminoacids between plant and mammalian LOXs, it has been reported that these proteins are similar in topology, with high similarities in the respective active sites and mechanism of catalysis [17].

Table 2: Inhibition of 5-LOX-mediated PUFA peroxidation by prenyloxy-carboxylic acid.

Compound	IC_{50}, μmol/mL[a]
1	0.006 ± 0.0005
2	0.262 ± 0.0220
3	$2.89 \times 10^{-5} \pm 2.62 \times 10^{-6}$
4	> 100
5	> 100
Ascorbic acid	0.105 ± 0.0072
BHT	0.023 ± 0.0052
Trolox	0.047 ± 0.0048

[a] $p < 0.05$ using Student's *t* test

Results on the inhibition of lipoxygenase mediated lipid peroxidation are reported in Table 2. Trolox, BHT and ascorbic acid were used as positive controls. As reported in Table 2, the pattern of antioxidative activity of **1-5** is similar to that recorded for the radical scavenging activity, with the notable exception of boropinic acid (**3**). This cinnamic acid derivative is far more active, not only in respect to compounds **1**, **2**, **4** and **5**, but also when compared to the positive controls. The value recorded for boropinic acid suggested that it did not act as a mere reducing agent like Trolox, BHT and ascorbic acid, but more likely as an effective 5-LOX inhibitor. To rationalize tentatively the inhibitory mechanism observed for boropinic acid and the lack of efficacy

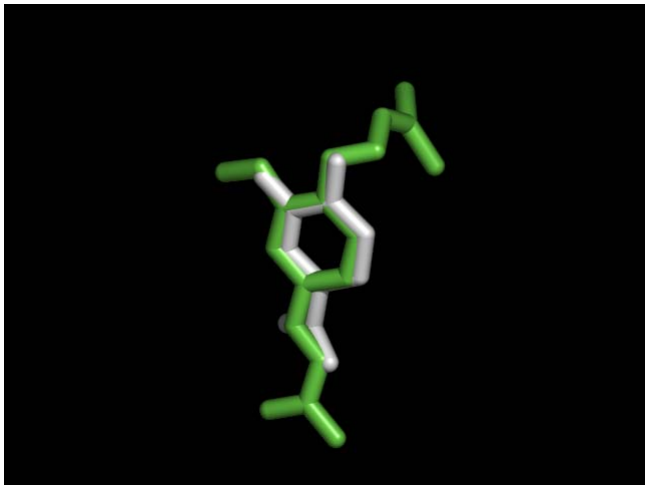

Figure 1: Comparative positions of boropinic acid (green sticks) and DHB (white sticks) in their complexes with LOX after protein backbone alignment (for simplicity, bond orders, hydrogen atoms and the protein are not shown).

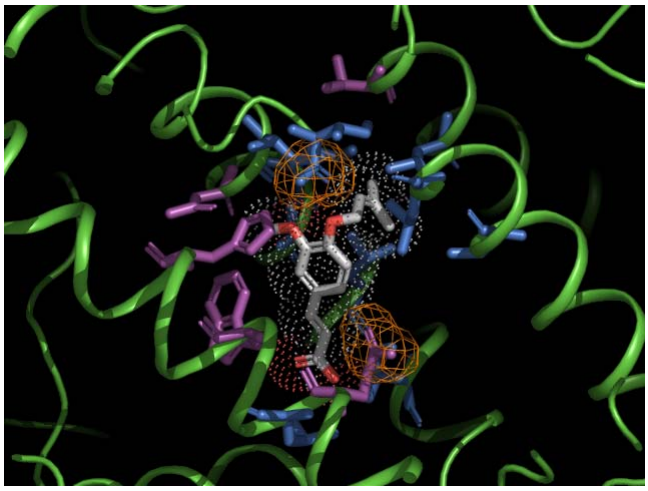

Figure 2: Modelled lipoxygenase / boropinic acid complex. The protein backbone is represented as green ribbons. Boropinic acid is represented as stick and dots. Amino acid residues with atoms within 5Å from boropinic acid atoms are represented as sticks (amino acids with hydrophobic side chains are in azure and the remaining amino acids are in magenta). For simplicity, hydrogen atoms of the ligand and the displayed residues are not shown. It can be noted that the prenyloxy group can be inserted in a cluster of hydrophobic amino acids. In this position, boropinic acid is potentially involved in interactions with two polar groups similar to the case of the DHB ligand (observed in the PDB structure 1n8q). The groups of the protein implied in the polar interactions are the carboxy-terminal group, Ile 857, and the amide side chain of Gln 514 (both groups are highlighted by the orange mesh).

of the other four natural carboxylic acids we have inferred a possible lipoxygenase/ligand docking by comparative modelling.

Among ligands reported to have an inhibitory effect on soybean lipoxygenase [17], 3,4-dihydroxybenzoic acid (DHB) showed the most significant structural similarity with boropinic acid and we have adopted its geometry of binding to the same cavity in the lipoxygenase protein as a model for the docking of boropinic acid. We have initially assigned to the phenyl ring of boropinic acid the same position and orientation known for the phenyl ring of DHB in the lipoxygenase/DHB complex (PDB structure 1n8q). In order to minimize collisions with the protein atoms, boropinic acid was subjected to structure optimization. The final docking of boropinic acid (**3**) required minor adjustments with respect to its initial position of insertion in the protein cavity. In Figure 1 are shown the mutual positions of boropinic acid and the DHB, as viewable if the two respective theoretical and experimental complexes with lipoxygenase were superimposed by aligning the protein backbones. The interactions of **3** and DHB should involve at least some common amino acid residues in the protein cavity. A peculiar feature of the modelled lipoxygenase/boropinic acid complex (Figure 2) is the possibility for the apolar atoms of the isopentenyloxy group to be oriented and enter in van der Waal's contact with a cluster of hydrophobic amino acids. Since this additional interaction might contribute to the enhancement of the complex stability, it seems that the loss of activity of lipoxygenase in the presence of boropinic acid could be the result of enzyme inhibition as a consequence of stable ligand docking in the active site, while similar experiments performed for all the other acids showed that the geranyloxycinnamic ones are too big to fit the active site while benzoic acids are too small to get more stable interaction with the enzyme.

In summary, the results described herein provide a new high yielding and environmentally friendly synthetic route to prenyloxy aromatic acids and indicate boropinic acid as a lead compound of a novel class of selective LOXs inhibitors.

Experimental

***General experimental*:** For the synthesis of compounds **1-5** the same general procedure as that reported previously was followed [5].

3-(4'-Geranyloxyphenyl)-2-*trans* propenoic acid (**1**)

Yield: 97%.
MP: 156-157ºC.
IR (KBr): 3550, 1690 cm^{-1}.
^{1}H NMR: [6]
^{13}C NMR (100 MHz $CDCl_3$): 16.1 (CH_3), 17.5 (CH_3), 25.6 (CH_3), 26.2 (CH_2), 39.4 (CH_2), 64.9 (CH_2), 115.3 (CH), 117.6 (CH), 119.8 (CH), 123.8 (CH),

128.3 (C), 129.3 (CH), 131.4 (C), 141.6 (C), 144.2 (CH), 157.7 (C), and 168.9 (C).
Anal. Calcd for $C_{19}H_{24}O_3$: C, 75.97; H, 8.05; O, 15.98. Found C, 75.96; H, 8.07, O, 15.99.

3-(4'-Geranyloxy-3'-methoxyphenyl)-2-*trans* propenoic acid (2)

Yield: 96%.
Analytical data are in full agreement with those reported in the literature [5]

Boropinic acid (3)

Yield: 96%.
Analytical data are in full agreement with those reported in the literature [7]

Valencic acid (4)

Yield: 99%.
MP: 131-132ºC.
IR: [21]
^{1}H NMR: [19]
^{13}C NMR (100 MHz $CDCl_3$): 18.7 (CH_3), 26.2 (CH_3), 66.5 (CH_2), 116.0 (CH), 120.9 (CH), 122.1 (C), 132.2 (CH), 139.1 (C), 162.7 (C), 170.5 (C).
Anal. Calcd for $C_{12}H_{14}O_3$: C, 69.89; H, 6.84; O, 23.27. Found C, 69.88; H, 6.82, O, 23.26.

4-Isopentenyloxy-3-methoxy benzoic acid (5)

Yield: 98%.
MP: 141-142ºC.
IR (KBr): 3600, 1695 cm^{-1}.
^{1}H NMR (400 MHz $CDCl_3$): 1.70 (3H, s, CH_3), 1.74 (3H, s, CH_3), 3.83 (3H, s, OCH_3), 4.55-4.61 (2H, m, OCH_2), 5.72-5.77 (1H, m, olefinic proton), and 7.01-7.72 (3H, m, aromatic protons)
^{13}C NMR (100 MHz $CDCl_3$): 18.0 (CH_3), 27.0 (CH_3), 55.9 (CH_3), 66.2 (CH_2), 115.4 (CH), 116.4 (CH), 119.9 (CH), 124.6 (CH), 125.8 (C), 138.0 (C), 147.3 (C), 150.7 (C), and 168.8 (C).
Anal. Calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83; O, 27.09. Found C, 66.08; H, 6.82; O, 27.07.

DPPH test: Radical scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) Tests were carried out in triplicate. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), BHT (butylated hydroxytoluene) and ascorbic acid were used as positive controls and purchased from SIGMA.

Inhibition of lipid peroxide formation test: Lipid peroxidation inhibitory activity was evaluated using the 5-lipoxygenase test in conjunction with positive controls. The activity of the enzyme was assayed spectrophotometrically according to the method of Holman, as modified by Sud'ina et al. [11]. The assay mixture (1 mL) contained: 10 mM linoleic acid, the sample (or the same quantity of solvent as reference) and 50 mM sodium phosphate, pH 6.8. This mixture was maintained at 20°C for 20 min. Subsequently, 0.18 μg mL^{-1} commercial 5-lipoxygenase was added to the mixture and the formation of hydroperoxides from linoleic acid was observed spectrophotometrically at 235 nm at 20 °C.

Docking experiments: Molecular Dynamics (MD) of lipoxygenase (PDB structure 1n8q) was performed with the program NAMD (v2.5) [18] after having removed all heteroatoms from the structure (ligands and crystallization water molecules). The simulation was carried out in explicit solvent under periodic boundary conditions using the CHARMM 27 parameter set. The system was minimized for 500 steps (1 fs per step) and the MD simulation was carried out for 500000 steps (500 ps) at a temperature of 310 K. Molecular representations were made with the program PyMol.

Acknowledgments - Authors from Perugia and Chieti would like to acknowledge financial support from MIUR National Project "Sviluppo di processi sintetici ecocompatibili nella sintesi organica" COFIN 2004. Authors from Urbino would like to acknowledge financial support from Regione Marche, Progetto CIPE 17/2003.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1147 - 1150

A Convenient Synthesis of 5′-Iodoresiniferatoxin (I-RTX)

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Received: June 16th, 2006; Accepted: July 5th, 2006

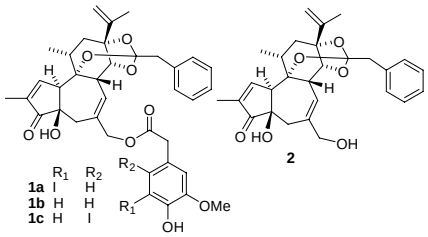
Dedicated to the memory of Professor Ivano Morelli.

Starting from resiniferonol orthophenylacetate (ROPA, **2**) and commercial 5-iodovanillin (**5a**), a convenient synthesis of the ultrapotent vanilloid antagonist 5′-iodoresiniferatoxin (**1a**) was achieved, overcoming the problems involved in the direct iodination of either resiniferatoxin (**1b**) or homovanillic acid (**3a**).

Keywords: Vanilloid antagonists, 5'-iodoresiniferatoxin, resiniferatoxin, TRV1, resiniferonol orthophenylacetate.

The study of ion channels strongly depends on the availability of compounds that can either activate or inhibit their function with high selectivity and potency[1]. While there is no shortage of ligands for sodium-, potassium-, and calcium-channels [1], most channels of the TRP type still await de-orphanization in terms of small molecule activators and/or inhibitors [2]. A remarkable exception is TRPV1, the capsaicin receptor, for which a large number of ligands (vanilloids) are available [3]. Most TRPV1 activators are either natural products or compounds derived from (or inspired by) natural products. Conversely, vanilloid antagonists are mainly synthetic compounds that have emerged from the random screening of chemical libraries. Nevertheless, none of them approaches the potency of 5'-iodoresiniferatoxin (I-RTX, **1a**), a natural product - derived ligand. I-RTX inhibits TRPV1 activation with a one-digit nanomolar $K_{(i)}$, [4]. While impressive, this value probably even underestimates the actual potency of I-RTX, whose intracellular penetration is slow compared to the time frame of most assays for vanilloid activity [5]. I-RTX is not only important as a molecular probe, but also as a potential drug, and has been investigated, with impressive results, in animal models of antitussive [6] and analgesic [7] activity.

I-RTX was serendipitously discovered by Wahl while attempting to prepare a radioactive derivative of the ultrapotent vanilloid agonist resiniferatoxin (RTX, **1b**) [4]. The molecular bases for the reversal of activity induced by aromatic iodination *ortho* to the phenolic hydroxyl are unknown, but a similar observation was made with capsaicinoids for iodination at the carbons *ortho*- and *meta*- to the phenolic hydroxyl [8]. Remarkably, iodination of RTX *meta* to the phenolic hydroxyl generated instead a partial agonist (**1c**) [9].

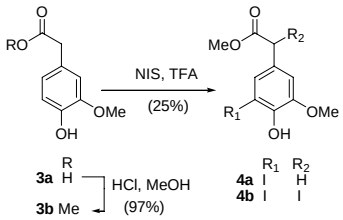


Despite the relevance of I-RTX for pharmacological research, a convenient synthesis of this compound has not yet been reported. The original synthesis by Wahl [4] was improved by a Merck group [10], and is based on the iodination of RTX with the sodium iodide/chloramine T system. Since RTX is labile in these conditions, the reaction requires careful control, and must be quenched at incomplete conversion. [10]. After HPLC purification, I-RTX was eventually

obtained in *ca* 22% yield. Given the low yield of the reaction, its problematic scale-up, and the exorbitant price of RTX [11], this synthesis is unsuitable to produce the amounts of I-RTX needed to profile its bioactivity in *in vivo* experiments.

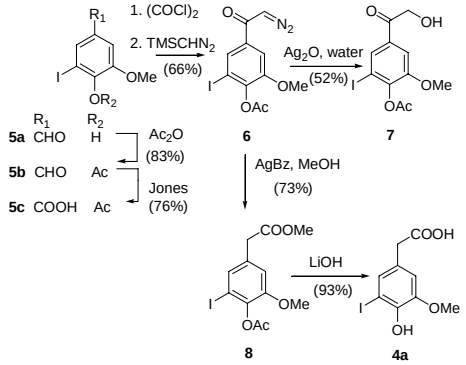
We reasoned that resinifernol orthophenylacetate (ROPA, **2**), the terpenoid core of RTX, would be a more convenient starting point for the synthesis of I-RTX, both in terms of availability of the starting material and purification of the final product. Thus, while RTX is a highly offensive compound, ROPA can be manipulated under normal laboratory conditions, and can be obtained relatively easily from the partially hydrolyzed latex of *Euphorbia resinifera* Berg., a household plant [12]. Conversely, the isolation of RTX from the native latex is difficult and hazardous due to its obnoxious properties and to the occurrence of irritant and tumor-promoting ingenol and deoxyphorbol esters that share the polarity and chromatographic behavior of RTX [12]. Finally, carrying out the iodination at the stage of a simple vanillyl derivative will also solve the problem of the instability of the terpenoid core of ROPA in the iodinating conditions. A similar strategy has been reported for the preparation of 6'-iodoresiniferatoxin (**1c**) [9], but, surprisingly, no attempt has been made so far to extend this strategy to its more important 5'-isomer.

The iodination of homovanilllic acid (**3a**) was first investigated (Scheme 1). This compound and its esters have been reported to be poor substrates for aromatic iodination [9], and also, in our hands, complex mixtures were obtained with a variety of iodinating conditions. However, methyl homovanillate (**3b**) could be iodinated, albeit in poor yield, with the *N*-iodosuccinimide (NIS) - trifluoroacetic acid (TFA) protocol [13]. The compound obtained (**4a**) contained *ca*. 5-10% of an impurity, tentatively identified as the product of α, 5′-bis-iodination (**4b**) on the basis of MS and ^{1}H NMR spectroscopic evidence. Thus, a peak corresponding to the incorporation of two iodine atoms was observed in the MS, while the ^{1}H NMR spectrum showed two additional *meta*-coupled aromatic protons. This by-product could not be removed by either chromatography or crystallization. After hydrolysis and Mitsunobu esterification [14] with ROPA, I-RTX (**1a**) was obtained, still contaminated, however, with the corresponding bis-iodinated impurity. Since preparative HPLC could not afford a completely pure material, this approach, though simple, was abandoned, and an alternative strategy based on the homologation of 5-iodovanillic acid was explored (Scheme 2).



Scheme 1: Iodination of methyl homovanillate (**3b**).

While homovanillic acid (**3a**) is expensive, 5-iodovanillin (**5a**) is cheap and commercially available in high purity [15]. After acetylation and oxidation, an acetylated carboxylic acid precursor for the one-carbon Arndt-Eisert homologation was obtained (**5c**). Reaction with oxalyl chloride and next with trimethylsilyldiazomethane [16] afforded the stable diazoketone **6**. The Wolf rearrangement of **6** in water with silver oxide gave mainly the corresponding acyloin **7**, while the reaction was successful after switching to the methanol-silver benzoate system. [17]. The resulting acetylated methyl ester was next hydrolyzed (LiOH, THF-water), affording 5'-iodohomovanillic acid (**4a**) as a crystalline compound in 43% yield overall from **5a** (Scheme 2).



Scheme 2: Synthesis of 5'-iodohomovanillic acid (**4a**) from commercial 5-iodovanilline (**5a**).

The final Mitsunobu coupling of **4a** and ROPA (**2**) could be carried out with crude ROPA (*ca*. 80%, HPLC) and the DIAD-TPP redox couple. After solvent removal, the residue was dissolved in toluene and cooled to remove the hydrazodicarboxylate-triphenylphosphine oxide crystalline adduct [18] Further purification by gravity column chromatography on neutral alumina affording I-RTX (**1a**) as a colorless foam in a reproducible yield of 52% and a HPLC purity of *ca* 95%.

In conclusion, a convenient synthesis of I-RTX, an ultrapotent vanilloid antagonist, has been reported,

overcoming the problems posed by the iodination of RTX and homovanillic acid and filling an important gap in vanilloid research.

Experimental

Acetyl-5-iodovanillic acid (**5c**)**:** To a solution of 5-iodovanillin acetate (**5b**, 1g, 3.12 mmol, prepared from commercial 5-iodovanillin (**5a**) by reaction with Ac_2O-pyridine) in acetone (10 mL), freshly prepared Jones reagent [19] was added (3 mL). After stirring overnight at room temperature, the reaction was worked up by concentration and filtration over Celite. The filtrate was then extracted with diethylether, and the organic phase was washed with brine. After drying and removal of the solvent, the residue was crystallized from diethylether to afford 800 mg (76%) of **5c** as a white powder.

MP: 199ºC.
Rf: 0.37 (light petroleum -EtOAc 7:3).
IR (KBr): 3072, 1764, 1683, 1572, 1409, 1294, 1193, 1168, 1037 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 2.39 (3H, s, Ac), 3.89 (3H, s, OMe), 7.64 (1H, d, J = 1.5 Hz, Ar-H), 8.16 (1H, d, J = 1.5 Hz, Ar-H).
^{13}C NMR (75 MHz, $CDCl_3$): 20.9 (CH_3), 56.5 (CH_3), 91.7 (C), 113.8 (CH), 129.0 (CH), 132.8 (C), 145.4 (C), 151.6 (C), 167.5 (C), 170.3 (C).
CI-EIMS: m/z $[M+H]^+$ 321 $[C_{10}H_9IO_5 + H]^+$

α-Diazo-5-iodoacetovanillone acetate (**6**)**:** To a cooled solution of **5c** (700 mg, 2.1 mmol) in dry CH_2Cl_2 (4 mL), oxalyl chloride (0.73 mL, 8.4 mmol, 4 mol. equiv.) and cat. DMF (0.20 mL) were added. After stirring for 1 h at 0°C and 90 min at room temperature, the reaction was worked up by evaporation, and the residue dissolved in THF-acetonitrile (1:1, 10 mL). After cooling to 0°C, $TMSCHN_2$ (2M in diethylether, 1.84 mL, 3.94 mmol, 1. 9 mol. equiv.) was added. The brownish-colored reaction was stirred at 0°C for 30 h, and then quenched by the addition of 0.5 N acetic acid. The reaction was then worked up by the addition of satd $NaHCO_3$, and the organic phase was separated, washed with brine and evaporated. The residue was purified by gravity column chromatography on silica gel (25 g, light petroleum -EtOAc 8:2 as eluant) to afford 471 mg (66%) **6** as a yellowish powder.

MP: 143ºC.
Rf : 0.32 (light petroleum -EtOAc 7:3).
IR (KBr): 3113, 3027, 2412, 2117, 1768, 1566, 1407, 1282, 1196, 1027, 903 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 2.23 (3H, s, Ac), 3.73 (3H, s, OMe), 5.71 (s, 1H), 7.29 (1H, d, J = 1.5 Hz, Ar-H), 7.49 (1H, d, J = 1.5 Hz, Ar-H).
^{13}C NMR (75 MHz, $CDCl_3$): 20.9 (CH_3), 56.5 (CH_3), 91.7 (C), 110.8 (CH), 128.7 (CH), 136.6 (C), 144.3 (C), 151.9 (C), 167.6 (C), 184.0 (C), 225.1 (C).
CI-EIMS: m/z $[M+H]^+$ 361 $[C_{11}H_9IN_2O_4 + H]^+$

Methyl 5′-iodohomovanillate (**8**)**:** To a refluxing solution of **6** (350 mg, 0.97 mmol) in methanol (4 mL), freshly prepared silver benzoate [17] (140 mg) and triethylamine (2 mL) were added. After refluxing for 1 h, the reaction was worked up by filtration over silica gel and evaporation, and the residue was crystallized from diethylether to afford 260 mg (73%) **8** as an amorphous brownish powder.

Rf: 0.45 (light petroleum -EtOAc 7:3).
IR (KBr): 3644, 1767, 1737, 1463, 1415, 1279, 1189, 1042, 1010, 901 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 2.35 (3H, s, Ac), 3.54 (2H), s), 3.69 (3H, OMe), 3.80 (3H, s, OMe), 6.86 (1H, d, J = 1.5 Hz, Ar-H), 7.29 (1H, d, J = 1.5 Hz, Ar-H).
^{13}C NMR (75 MHz, $CDCl_3$): 20.9 (CH_3), 40.5 (CH_2), 52.4 (CH_3), 56.2 (CH_3), 91.7 (C), 113.8 (CH), 130.9 (CH), 134.2 (C), 141.9 (C), 151.4 (C), 168.1 (C), 171.3 (C).
CI-EIMS: m/z $[M+H]^+$ 365 $[C_{12}H_{13}IO_5 + H]^+$

5′-Iodohomovanillic acid (**4a**)**:** To a solution of **8** (240 mg, 0.66 mmol) in water-THF 2:1 (3 mL), LiOH (194 mg, 4.6 mmol, 7 mol. equiv.) was added. After stirring at room temperature overnight, the reaction was diluted with water, extracted with EtOAc, sequentially washed with 2 N H_2SO_4 and brine, and then evaporated. The residue was purified by crystallization from CH_2Cl_2, affording 190 mg of a white powder.

MP: 178ºC.
Rf: 0.15 (light petroleum -EtOAc 6:4).
IR (KBr): 3412, 1710, 1506, 1273, 1222, 1161, 1025, 884, 824 cm^{-1}.
^1H NMR (300 MHz, $CDCl_3$): 3.54 (2H, s), 3.88 (3H, s, OMe), 6.10 (1H, -OH, s), 6.76 (1H, d, J = 1.5 Hz, Ar-H), 7.21 (1H, d, J = 1.5 Hz, Ar-H).
^{13}C NMR (75 MHz, acetone-d_6): 44.7 (CH_2), 55.5 (CH_3), 88.9 (C), 114.2 (CH), 124.9 (CH), 129.6 (C), 148.0 (C), 148.1 (C), 172.0 (C).
CI-EIMS: m/z $[M+H]^+$ 309 $[C_9H_9IO_4 + H]^+$

5′-IodoRTX (**1a**)**:** To a cooled (0°C) stirred solution of ROPA (**2**, 220 mg, 0.47 mmol) and 5′-iodohomovanillic acid (**4a**, 145 mg, 0.47 mmol, 1

mol. equiv.) in dry THF (2 mL), triphenylphosphine (TPP, 147 mg, 0.56 mmol, 1.2 mol. equiv.) and diisopropylazodicarboxylate (DIAD, 0.101 mL, 0.56 mmol,. 1.2 mol. equiv.) were added. After stirring at room temperature for 2 h, the reaction was worked up by evaporation, and the residue was dissolved in toluene (*ca*. 5 mL) and cooled to 4°C overnight. After filtration of the copious white precipitate, the filtrate was purified by gravity column chromatography on alumina (25 mL, light petroleum-EtOAc 8:2 as eluant) to afford 184 mg (52%) of **1a**, having physical and spectroscopic (^{1}H NMR) properties identical to those reported in ref. 10.

^{13}C NMR (75 MHz, $CDCl_3$): 10.4 (CH_3), 18.9 (CH_3), 19.9 (CH_3), 33.1 (CH), 35.8 (CH_2), 39.2 (CH), 39.4 (CH_2), 40.5 (CH_2), 41.1 (CH_2), 55.4 (CH_3), 56.4 (CH), 72.0 (C), 73.5 (C), 80.6 (CH), 81.2 (C), 84.5 (C), 110.8 (CH_2), 112.0 (CH), 117.9 (C), 126.7 (CH), 127.3 (C), 127.4 (CH), 128.9 (CH), 131.2 (CH), 131.4 (CH), 134.0 (C), 135.0 (C), 136.7 (C), 145.1 (C), 146.1 (C), 146.5 (C), 158.5 (CH), 171.1 (C), 208.4 (C).

Acknowledgments - We are grateful to Università del Piemonte Orientale for financial support to A.E.-C, and to Dr Sara Bacchiega for preliminary work on the iodination of vanillic derivatives.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1151 - 1158

Acaricides of Natural Origin. Part 2. Review of the Literature (2002-2006)[¶]

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Received: June 14th, 2006; Accepted: November 7th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Acari are responsible for millions of dollars worth of damage each year as a result of infestations of animals, plants and man. They directly affect our health and prosperity as animal and plant parasites, vectors of disease, and producers of allergens. The indiscriminate use of pesticides has quickly induced resistance in many parasites. At present, the control of acarid parasitic diseases in agriculture, human and veterinary medicine is mainly based on the use of drugs; and for this reason the lack of effective drugs often prevents the control of some parasitic diseases, making them more serious and important. The use of commercial drugs involves many problems, besides the drug-resistance shown by the most important parasites. Environmental damage and the toxicity of many synthetic drugs, represent the main problems that strongly limit drug use. In addition, drug residues in plant and animal food products are important reasons for further economic losses for farmers and must be regarded as potentially hazardous to man and the environment. Plant-derived compounds are generally more easily degradable and could show a smaller negative environmental impact with respect to synthetic drugs. For these reasons, the evaluation of the antiacarid activity of plant extracts is increasingly being investigated in order to obtain new leads, as demonstrated by recent studies that have evaluated and confirmed the effectiveness of many plant compounds on bacteria, fungi, protozoa, helminths and arthropods. This review will be limited to the class Arachnida, sub-class Acaridi, particularly to their control in agriculture, veterinary and human medicine using natural methods.

Keywords: Acaricides, natural, human and veterinary medicine.

Mites and ticks, collectively known as the Acari, are of interest to humans for a variety of reasons. They affect our health and well being directly as plant, animal and human parasites, vectors of disease, and producers of allergens. The Class Arachnida, to which the order Acari belongs, together with the Classes Insecta, Crustacea and others, constitute the Phylum Arthropoda. All the classes contain species useful to man, but also many pests that are responsible for millions of dollars worth of economic losses each year as a result of infestations of animals, man and plants. This review will be limited to the order Acari, particularly to their control with natural methods in agriculture, veterinary and human medicine. This report follows and upgrades the previous one [1].

The indiscriminate use of inorganic pesticides destroyed many harmless species, including natural enemies of these mites and ticks [2]. After organochlorine and organophosphate pesticides were introduced, resistance was quickly acquired by many arthropod parasites, including acari; fortunately many useful predatory mites became resistant too. The emergence of resistance to parasiticides is one of the most serious challenges faced by man. Perhaps it is the simplicity of treating parasite attacks with either very effective drugs or pesticides on a routine basis, and the proven cost-effective gains in productivity that accrue in the short term, that has led to the predominance of synthetic pesticides [3]. Broadly speaking, resistance is the ability of the parasites to survive doses of drugs that would normally kill them at the same stage of development. The resistance is inherited and selected because the survivors of the

[¶]For Part 1 see Ref. 1

pesticide treatment pass the genes for resistance on to their offspring. Drug susceptibility is a resource that needs to be preserved, using appropriate techniques of parasite management. The application of synthetic chemical substances is still the common method to either control or eradicate parasites of plant and animals, but many acaricides have non-specific properties, affecting other organisms (crops, non-vertebrates and vertebrates). Plants are the richest source of organic compounds on Earth, many of which are endowed with pesticide properties.

In veterinary medicine, the control of ectoparasites is of great importance due to their effects on livestock profitability and the health status of animals. Infestations of livestock can cause intense irritation, leading to poor condition, weight loss, reduced milk yield, and hide and fleece damage. Furthermore, many species of acari are responsible for transmission of diseases either to the host animals themselves or act as vectors of a number of diseases to humans [4].

Apiculture

During the 90s, several cases of resistance of *Varroa* mites (Acari: Varroidae) to common acaricides employed in beekeeping were reported from different countries [5–8]. In Italy, the consequences of the resistance led to disastrous colony losses. Available statistics show that in certain districts, losses often exceeded 70% and, in some locations, even reached 90% [9]. *Varroa* mites suck the body fluids from adults and brood, preferring the latter, especially the drone brood. The problem of developing suitable treatments was difficult in the case of the *Varroa* mites because most substances effective against the parasites have unacceptable side effects on bees. Since the creation of the EU *Varroa* experts' group, several lines of research in alternative control measures have been explored: apicultural techniques for reducing the number of mites, increasing bee resistance, and searching for acaricidal products that are generally recognized as safe for humans, such as some natural derivatives [10]. Many natural compounds have been evaluated for their effectiveness against *Varroa* mites [1]. During the five-year period employed in this review, some papers have described the use of simple carboxylic acids, such as formic [11–16] and oxalic [17–19] acids. Their efficacy has been known since 1980 [20]. Furthermore, a paper has appeared in the literature [21] about two different formulations of thymol, a well-known varroacidal agent, The main goals of this study were to determine their effectiveness against *V. destructor* in an apiary in Sardinia (Italy), taking into account natural mite mortality in control hives and, simultaneously, to determine the persistence of both formulations and residues in honey and wax. Both thymol formulations, after the treatments, reduced significantly the levels of mite infestations of adult bees and sealed brood, but their efficacy, expressed as percentage of mortality, was lower for both products than the efficacy previously obtained with the same products under other experimental conditions. The residues were relatively higher in wax than in honey, because of the lipophilicity of thymol.

Other monoterpenes seem to be valuable as control agents for this mite, both in laboratory assays and in field treatments. The most active ones were linalyl acetate, myrtenyl acetate, perillyl acetate and thymyl acetate. In the field trials, all four monoterpenoid-treated groups were statistically significantly different from the control group in reduction of *V. destructor* infestation, yielding a 51-64% reduction of the mite when compared with the control group [22]. In an Argentinean study, the repellent and acaricidal effects of some essential oils from the most typical wild plant species from the northern part of the country were evaluated against *V. destructor*, using a complete exposure test. The lowest LD_{50} values for mites were registered for *Acantholippia seriphioides* (1.27 μL per cage) and *Schinus molle* (2.65 μL per cage) after 24 hours and for *Wedelia glauca* (0.59 μL per cage) and *A. seriphioides* (1.09 μL per cage) after 72 hours of treatment [23].

Veterinary and Human Medicine

(a) Ticks: Particularly in the tropics, but also in many other countries, arthropod-borne diseases are among the major limiting factors to the efficient production of livestock and poultry. These diseases cause weakening, lameness, blindness, wasting, congenital defects, abortions, sterility, and death of the infested animals. Some exotic arthropod-borne diseases of livestock are zoonotic and affect humans as well as animals. Some of the most devastating of all animal diseases caused by arthropod-borne blood protozoa, include babesiosis of cattle, sheep, goats, horses, and swine; theileriosis, the East Coast fever syndrome, and Mediterranean fever; the trypanosomiases causing illness in cattle, sheep, goats, camels, pigs, dogs, and many wild game

species; as well as several arthropod-borne protozoa that cause diseases of birds. The most prominent groups of arthropods that transmit etiological agents pathogenic to livestock are those that are hematophagous, such as ticks. The tick-borne diseases they transmit are among the most significant animal health deterrents to efficient livestock production. Ticks are obligate ectoparasites of vertebrates and they parasitize all vertebrate groups, except fishes.

The family Ixodidae comprises approximately 80% of all tick species, with the most economically important ixodid ticks that attack livestock in tropical regions belonging to the genera *Amblyomma, Boophilus, Rhipicephalus* and *Hyalomma. Ixodes scapularis* L., the black-legged tick, is the primary vector of disease-causing agents in humans in North America, especially Lyme disease, human granulocytic ehrlichiosis, and human babesiosis. Among the various strategies that have been considered for their control, one is the use of tick pheromones to facilitate targeted delivery of acaricides in the ticks natural habitat. Ticks use many different pheromones to regulate their behavior, especially for sexual activity. Some cause assembly, a type of behavior known as arrestment, defined as the cessation of kinetic activity. The chemical composition of the arrestment pheromone of *I. scapularis* has been identified so that it can be used as an aid in tick control [24]. The isolates that caused the arrestment were characterized as guanine and xanthine. The strongest responses were found when hematin was also present. These substances were used to formulate a preparation containing a mixture of pheromones and acaricidal substances, such as DEET (*N*,*N*-diethyl-*m*-toluamide) and permetrin. In this way, the efficacy of the acaricides was enhanced.

In further searching for alternative methods of reducing Lyme disease, the activity against *I. scapularis* nymphs was determined of 15 natural products isolated from the essential oil components extracted from the heartwood of *Chamaecyparis nootkatensis*. Nootkatone was the most effective eremophilane sesquiterpene, with an LC_{50} value of 0.029 mg/mL. Residual LC_{50} values for nootkatone did not differ significantly at 4 weeks post-treatment from the observations made after the initial 24 hours treatment. Among nymphal tick repellents, the most active compound was valencene-13-ol, with a repellent dose (RD_{50}) of 0.03 mg/mL at 4 hours, compared to 3.8 mg/mL for DEET. The ability of these natural products to kill and repel ticks at relatively low concentrations may represent a future alternative to the use of synthetic pesticides [25].

Boophilus species are one-host ticks, which occur in all tropical and sub-tropical regions of the world, where they feed preferably on cattle. They are the main vectors of *Babesia* species, *B. bovis* and *B. bigemina*, causing babesiosis in cattle. *Boophilus* ticks, together with many other tick species, also transmit *Anaplasma marginale*, the rickettsia that causes anaplasmosis of cattle on all continents. The naturally occurring avermectins and milbemycins are fermentation products of actinomycetes in the genus *Streptomyces*. They are 16-membered, macrocyclic lactones, which have structural similarities to antibacterial macrolides and antifungal polyenes, but lack their antifungal and antibacterial activities and do not inhibit either protein or chitin synthesis [26]. Milbemycins, first described from a culture of *S. hygroscopicus*, are structurally similar to the avermectins, but lack the disaccharide substituent at C13 [27]. Mishima et al. [28] first reported the acaricidal activity of milbemycins. Moxidectin, a synthetically modified milbemycin derived from the fermentation product nemadectin [29], is used for insect and helminth control in animal health applications. Since 2001, only one paper about the use of moxidectin against this tick has been published [30]. It works like other macrocyclic lactones opening chloride channels in the nerve cells, causing paralysis. The macrocyclic lactone, moxidectin has a broad-spectrum activity against important internal and external parasites of cattle, including ticks [31–33]. The efficacy of a 1% injectable formulation of moxidectin at the dose of 0.20 mg/kg body weight by subcutaneous injection was greater than 95%. Furthermore, there was no evidence of either any local or systemic adverse reaction in treated animals and all cattle remained healthy throughout the trial period. For rapidly screening many compounds, a larval immersion microassay that offers superior sensitivity and flexibility to accommodate multiple formulations has been developed using the tick *Amblyomma americanum* (L.). This assay proved suitable for the identification and characterization of active molecules from natural product libraries, and it can be a useful tool to prioritize molecules for further *in vivo* testing in animal models [34].

(b) House dust mites: The term "house dust mites" is applied to a large number of mites found in association with dust in dwellings. Unlike some other

kinds of mites, house dust mites are not parasites of living plants, animals, or humans. House dust mites primarily live on dead skin cells regularly shed by humans and their animal pets. Skin cells and squames, commonly called dandruff, are often concentrated in parlor and sitting rooms, mattresses, frequently used furniture and associated carpeted areas, and may harbor large numbers of these microscopic mites. For most people, house dust mites are not harmful. The medical significance of house dust mites arises because their microscopic molted skins and feces, being major constituents of house dust, induces allergic reactions in some individuals. For those individuals, inhaling the house dust allergen triggers either rhinitis or bronchial asthma. Expert panel reports and position statements from the European Union, the US National Heart, Lung and Blood Institute (NHLBI), and the American Academy of Allergy, Asthma and Immunology (AAAAI) have recommended dust mite allergen avoidance as an integral part of asthma management [35–38]. House dust mites belong to different genera and species; the main ones are *Dermatophagoides pteronyssinus, D. farinae* and *Euroglyphus maynei* (Acari: Pyroglyphidae). However, there is great variation in the acarid fauna of the different regions of the world. *Dermatophagoides pteronyssinus* (literally "skin-eating mites") is considered as the true house dust mite and has a cosmopolitan distribution. Together with *D. farinae* (=flour, also infests stored food), it accounts for 80-90% of the total mite population generally found in houses. No pesticides are currently labeled for house dust mites. However, some commercial products are available for treatment of house dust mites and their allergens. The active ingredients are benzyl benzoate and tannic acid.

Recently, a new daphnane diterpenoid, rediocide F, was isolated together with the known rediocides A, C and E, from the *n*-hexane extract of *Trigonostemon reidioides* roots by bioassay-guided fractionation using acaricidal activity on *Dermatophagoides pteronyssinus*. The structure of rediocide F was established as the demethyl analog of rediocide C. All the compounds exhibited potent activity against *D. pteronyssinus* with LC_{50} values of 2.53, 0.78, 5.59 and 0.92 μg/cm^2, respectively [39]. Three *Uvaria* species, namely *U. klaineana, U. mocoli* and *U. versicolor* were tested *in vitro* against *D. pteronyssinus*. The most active extracts were the crude methanol and *n*-hexane extracts of *U. versicolor* stems, with EC_{50} values of 0.095 g/m^2 and 0.12 g/m^2, respectively. The successive bioassay-guided fractionation of the *n*-hexane extract led to the isolation of benzyl benzoate, which exhibited an EC_{50} value of 0.045 g/m^2. A new flavanone, versuvanone, and the known oxoaporphine liriodenine were also isolated from this species and showed EC_{50} values > 1.5 g/m^2. A weak acaricidal activity (0.85 g/m^2) was observed for the dichloromethane extract of *U. klaineana*, due again to the presence of benzyl benzoate. *U. mocoli* extracts were inactive [40]. Other researchers tested the acaricidal activity of materials derived from the rhizome of *Cnidium officinale* Makino against *T. putrescentiae* adults using direct contact application and fumigation methods. The biologically active constituent was identified as butylidenephthalide by spectroscopic analyses. On the basis of 24-hours LD_{50} values, the acaricidal activity of butylidenephthalide (5.80 μg/cm^2) was more pronounced than that of the standard drugs benzyl benzoate (9.75 μg/cm^2) and DEET (16.26 μg /cm^2). Butylidenephthalide caused lethargy in the treated mites, leading to death without knockdown, whereas benzyl benzoate and DEET caused death associated with uncoordinated behavior. In a fumigation test with *T. putrescentiae* adults, butylidenephthalide was much more effective in closed containers than in open ones, indicating that the effects of this compound was largely due to action in the vapor phase [41]. Among essential oils, those obtained from *Pinus* species revealed promising activity against *T. putrescentiae*. The oils obtained from *P. pinea, P. halepensis, P. pinaster* and *P. nigra* have been evaluated for their acaricidal activity by aerial diffusion. Among them, the oil from *P. pinea* showed the best activity (100% deaths, while those from *P. halepensis* and *P. pinaster* were partially effective only at the higher dose); and the dose of 8 μL showed a percentage of dead mites statistically higher than that of the lower dose. The main constituents of the essential oil of *P. pinea* branches were α-pinene, β-caryophyllene, myrcene, 1,8-cineole, and limonene. Of these, α-pinene, β-caryophyllene, and myrcene were ineffective, whereas 1,8-cineole and limonene showed 100% acaricidal activity at 8 μL. Only 1,8-cineole maintained 100% acaricidal activity, also at the lower concentration of 6 μL [57].

Agriculture

Plant-feeding mites play important roles as agricultural pests of timber, fruits, vegetables, forage crops, and ornamentals. In many instances, lack of information about the correct identity of the mites, as

well as inadequate knowledge regarding their biology and ecology, have hampered our ability to combat effectively these mite pests. Their small size and cryptic appearance make mites difficult to detect, and thus infestations are often overlooked. Once established in a new area, certain biological characteristics allow rapid escalation to pest status. Miticidal compounds, as in veterinary and human medicine, cannot be toxic to the plant host and no harmful residues must be found in foods. Furthermore, in agriculture, an additional feature is requested: they must be devoid of undesirable effects on useful non-target organisms, like pollinators and predator arthropods The main species are *Tetranychus* sps, *Oligonychus* sps (Acari: Tetranychidae), *Phyllocoptruta oleivora*, and *Tegolophus australis* (Acari: Eriophyidae). Among these, the two-spotted spider mite, *Tetranychus urticae*, a polyphagous pest, is probably one of the most dangerous for crops and ornamentals, particularly in glasshouses. Its high reproductive capacity enables it to cause serious damage in a short period. Furthermore, this parasite has developed resistance to many synthetic acaricides (see i.e. [42–45]), apart from the fact that many of these substances are toxic to useful non-target arthropods [46–48].

Among promising plant species, *Chenopodium ambrosioides* var. *ambrosioides* has been evaluated. An emulsifiable concentrate (UDA-245), obtained from the essential oil was compared with commercially available pesticides for their effectiveness to control the adult stage and egg hatch of the twospotted spider mite, *Tetranychus urticae* and the European red mite, *Panonychus ulmi*. A 0.5% UDA-245 was more effective than 0.7% neem oil on adult twospotted spider mites. In the case of the European red mite, UDA-245 was as effective as 0.006% (AI) abamectin. Furthermore, UDA-245 at 0.5% significantly reduced egg hatch of the twospotted spider mite, 5 and 9 days after treatment, and of the European red mite 6 days after treatment. Egg hatch was significantly lower using 0.006% abamectin, 0.7% neem oil, and 1.0% insecticidal soap than UDA-245. Residual tests indicated that UDA-245 may be persistent in the environment only for a few hours. Only 23% mortality was noted when mites were introduced on bean leaves 1 hour after treatment with 2% UDA-245. At the recommended dose of 0.5%, UDA-245 was not considered to be phytotoxic to most plants tested, i.e., lettuce, roses, and tomatoes. Results suggest that a greenhouse integrated pest management program using UDA-245 could effectively and selectively control mite infestations by treating "hot spots", with negligible effect on biological control agents when treating before introduction or when natural enemies are absent [49].

Other natural derivatives effective against *Tetranychus urticae* can be obtained from leaves of wild tomato species, *Lycopersicon hirsutum*, *L. pennellii* and *L. pimpinellifolium*. Crude chloroform, ethanol and *n*-hexane extracts of the leaves were tested for their antibiosis and for their repellency. The antibiosis was assayed as a 6-hours no-choice test. The method for repellency utilized a ring bioassay. Chloroform leaf extracts of *L. hirsutum* exhibited the greatest antibiotic activity, and the *n*-hexane extracts exhibited the greatest repellency. Among the major chemical compounds of the extracts, α-curcumene, α-zingiberene, β-caryophyllene, 2-undecanone, and 2-tridecanone were detected. Lethality of the extracts was mainly associated with the presence of high concentrations of 2-tridecanone, while repellency of extracts was mainly associated with the presence of β-caryophyllene [50]. Another useful plant genus that can control *Tetranychus urticae* is *Taxus*. *T. cuspidata* and *T. media* var. *Hicksii* contain paclitaxel, among other taxoids, on the surface of the needles. These compounds were extracted by 5 seconds dipping of the needles in water at 96°C, 60°C and 40°C. The extracts with the higher concentration of paclitaxel were more harmful to the mites, increasing their mortality, prolonging development and lowering the average fecundity [51]. The citrus red mite, *Panonychus citri*, feeds on leaves, fruit and green twigs of all *Citrus* species. The infestation can result in heavy leaf drop, twig die-back and even death of large limbs. Various natural derivatives have been evaluated against this pest. A recent investigation has suggested that *Panonychus citri* is the most common causative allergen in citrus-cultivating farmers with either asthma or allergic rhinitis. Citrus red mite is a common sensitizing allergen among children living around citrus orchards [52].

It has been observed that spraying a *Mikania micrantha* alcohol extract on *Panonychus citri* could significantly decrease the survival rates of its eggs, larvae and nymphs. Furthermore, both the fecundity and the longevity of female *P. citri* fed on the leaves treated with *M. micrantha* alcohol extracts were significantly reduced. In a field experiment, the

efficacy of *M. micrantha* alcohol extracts was compared with water, alcohol, and pyridaben, a widely used acaricide in commercial control of red mite in sweet orange orchards. The survival rates of *P. citri* eggs, larvae and nymphs in the treatments were lower than the control, and were better than the pyridaben treatment [53].

Another study evaluated the acaricidal activity against this mite of a ginkgolic acid, 6-[(*Z*)-10-heptadecenyl]-2-hydroxybenzoic acid, isolated from the external seed coat of *Ginkgo biloba* [54]. Laboratory bioassays showed that this compound possessed powerful contact toxicity, similar to that of pyridaben and significantly superior to that of omethoate. Furthermore, it showed a quick-acting acaricidal activity, and was much faster-acting than either pyridaben or omethoate.

It has been observed that in the *Ageratum conyzoides* intercropped *Citrus* orchards, the populations of *Panonychus citri* were reduced. This could be explained by increases in the population of the predatory mite *Amblyseius newsami*, an effective natural enemy of citrus red mite. In fact the study showed that *A. conyzoides* produced and released volatile allelochemicals into the air in the intercropped citrus orchard, and these volatiles influenced the olfactory responses of *A. newsami* and *P. citri*. It has been observed that *A. conyzoides* fresh leaves, its essential oil, and major constituents, demethoxy-ageratochromene, β-caryophyllene, α-bisabolene, and *(E)*-β-farnesene, attracted *A. newsami* and slightly repelled *P. citri* [55]. To evaluate if some natural miticides were less toxic to useful arthropods than to phytophagous mites, some trials were conducted in apple orchards [56]. The European red mite, *Panonychus ulmi*, was the dominant and more harmful phytophagous species, followed by the apple rust mite, *Aculus schlechtendali*. Two predacious mites, *Typhlodromus pyri*, and *Zetzellia mali*, were often found in the orchards. Abamectin had favorable selectivity, being more toxic to the two phytophagous mites than to the useful ones.

Conclusions

The control of parasitic diseases is mainly based on the use of effective drugs, both in agriculture and human and veterinary medicine; for this reason the lack of effective drugs often prevents the control of some parasitic diseases, making them more serious and important. At present, however, the use of commercial drugs involves many problems that strongly limit their use: foremost, the drug-resistance problem shown by the most important parasites, the environmental damage and the toxicity of many synthetic drugs.

Since plant-derived compounds are generally more easily degradable and could show reduced environmental damage with respect to synthetic drugs, at present the evaluation of the antiparasite activity of plant extracts is being increasingly investigated, as demonstrated by recent studies that have evaluated and confirmed the effectiveness of many plant compounds on bacteria, fungi, protozoa, helminths and arthropods.

Perhaps human and veterinary medicine are the most suitable fields for a real application of natural drugs. Treatment of these pathologies is mostly topical, and particular drug-formulations are not required. Furthermore, generally only a few treatments are necessary to kill all the parasites. In agriculture, in spite of the studies performed to date, these substances are perhaps still far from their most effective use: their main useful feature, that is their biodegradability, is also their weakness. Often, many products are not able to persist in the environment for a period of time sufficient for pest control. Further studies are necessary to prepare better formulations that allow us to solve this problem. Other important future research topics should concentrate on the evaluation of the toxicity of these compounds, an unknown feature for many natural compounds.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1159 - 1180

Chemical and Biological Activity of Triterpene Saponins from *Medicago* Species

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Received: June 1st, 2006; Accepted: September 7th, 2006

Dedicated to the memory of Professor Ivano Morelli.

Naturally occurring saponins are a large group of triterpene and steroid glycosides characterized by several biological and pharmacological properties. The *Medicago* genus represents a valuable source of saponins which have been extensively investigated. This review summarizes the chemical features of saponins from *Medicago* species and their biological activity, with particular attention to their antimicrobial, insecticidal, allelopathic and cytotoxic effects. Influence of saponins on animal metabolism is also reported.

Keywords: *Medicago*, *M. sativa*, saponins, chemical structure, biological activity.

Saponins are a large group of plant metabolites including triterpenoids, steroids and steroidal alkaloids glycosylated with one or more sugar chains [1, 2]. They are commonly distinguished by their surfactant and hemolytic activities. Naturally occurring saponins display a broad spectrum of biological and pharmacological properties such as fungicidal, molluscicidal, antibacterial, antiviral and antitumor activities [2-6]. Due to their chemical, physical and physiological characteristics, commercial products containing plant saponins are available and used in the pharmaceutical, cosmetic and food industries [7-8]. Some saponins are the starting material for the semisynthesis of drugs and some are used as emulsifiers and foaming agents in food. Plant extracts rich in saponins have been used as folk detergents and are ingredients of cosmetic preparations such as lipsticks, shampoos and toothpaste.

Saponins are produced by many plant species and their distribution in the plant kingdom seems to be correlated with the structural type. That is, steroidal saponins have been found almost exclusively in the Monocotyledons, while triterpenoid saponins mainly occur in the Dicotyledons and are practically absent in the Gymnosperms [6, 9]. The *Leguminosae* have been extensively investigated for their saponin content and within this family of plants, the *Medicago* genus represents a particularly rich source of bioactive saponins [10-15].

The genus *Medicago* includes 83 different species, the most known represented by *M. sativa* L. (syn. *M. media* Pers.), or alfalfa, a highly valued forage crop [16, 17]. The chemical structure of saponins from several species within the genus has been determined [18-46]. Generally they are complex mixtures of high-molecular weight triterpene glycosides with medicagenic acid, hederagenin, zanhic acid, bayogenin and soyasapogenols A and B as the dominant aglycones. Recently the 2β,3β-dihydroxy-23-oxo-olean-12-en-28-oic acid has been identified as a new aglycone moiety in the two species *M. arborea* [45] and *M. hybrida* [46]. A summary of the structural types of sapogenins isolated from the various species of *Medicago* is reported in Figure 1.

Sugars or sugar chains are generally linked at the C-3 position of the aglycone (monodesmosides) and

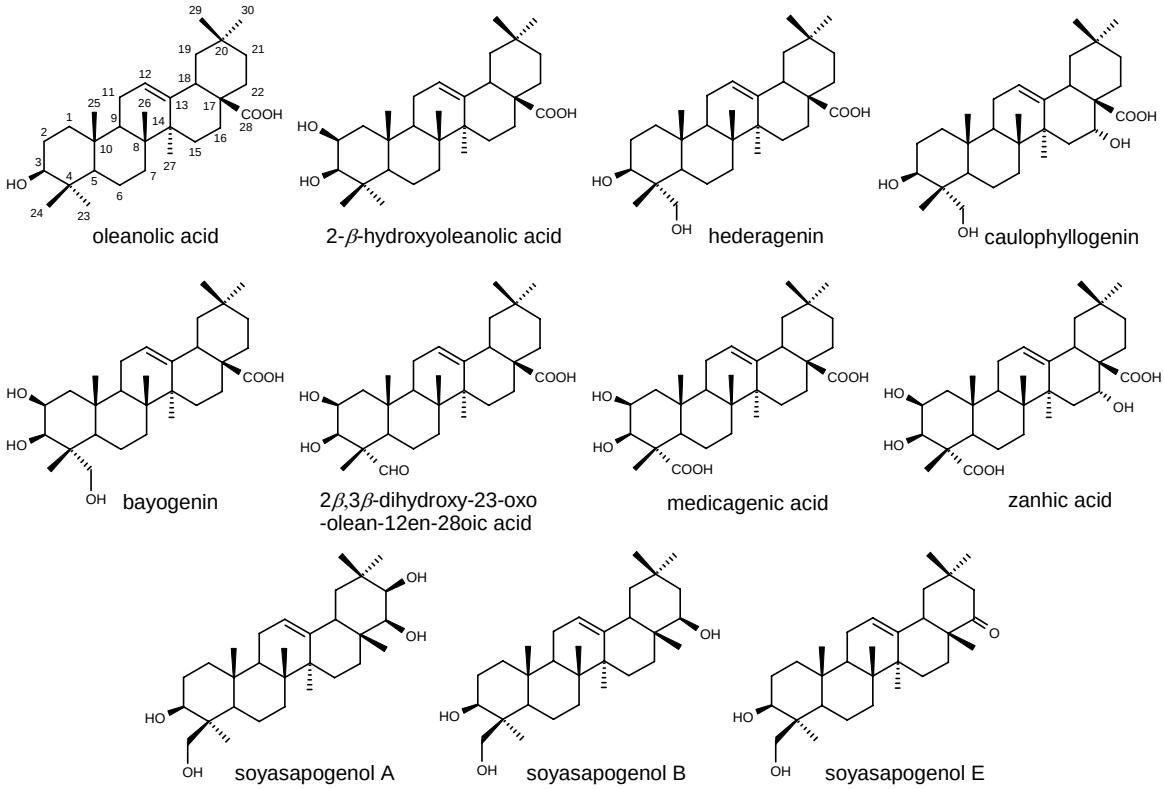

Figure 1: Chemical structure of sapogenins detected in *Medicago* species.

additionally at the C-28 position, giving the corresponding bidesmosides. A tridesmoside saponin (extra sugar at the C-23 position) has only been identified in *M. sativa* [36] and *M. truncatula* [42].

The most abundant monosaccharide units found in the *Medicago* saponins are: arabinose, rhamnose, xylose, glucose, and glucuronic acid. Saponins in the *Medicago* species are produced in all the plant organs: leaves, flowers, roots, seeds and sprouts [10-49]. Their content in the plant material changes as a function of several factors, such as plant organs, genotype, cutting, year and stage of growth, and environmental effects, as reported for *M. sativa*, the most studied species of the genus [50-55]. The chemotaxonomic significance of saponins has also been investigated as their composition can discriminate among *Medicago* species [56-59]. The occurrence of saponins in the *Medicago* genus is long known [60], and their composition has been studied in several species. Structure elucidation of complex saponin mixtures differentiates their aglycone composition [61, 62]. In particular, investigation of several annual and perennial wild and cultivated *Medicago* species, showed variability in the aglycone composition of the saponins from each species. Medicagenic acid was detected in some of them and soyasapogenol B was often present in the form of soyasaponin I, a common saponin of the *Leguminosae* family [10, 63].

Chemical analysis of saponins is not simple due to their 'soapy' properties due to sugars in the molecules. Their presence can be evaluated by biological tests involving their toxic haemolytic [64], fungicidal [65] and insecticidal [66] properties. Chemical methods also have been used, such as TLC [10], HPLC [67], GC and GC/MS [52, 68, 69], the last technique being used to analyze and quantify only the aglycone moieties. Capillary electrophoresis [70] and LC/MS methods [40, 42, 43] have also been employed for the identification and quantification of saponins in the plant extracts.

Structure investigation of *Medicago* saponins is usually performed by preliminary identification of the sapogenins and sugars released after acid hydrolysis from pure saponins obtained by direct and reverse-phase chromatographic separation of the raw saponin mixtures. Detailed information on the saponin structure, however, could be obtained only by a combination of analytical methods, including

MS [71] and NMR analyses [72, 73], performed on pure compounds. The MS spectra allowed subsequent fragmentation of the sugar chains to give the corresponding aglycones, NMR analyses (^{1}H, ^{13}C and 2D experiments) allowed the determination of all the carbon atoms and the sugar linkage in the molecules, while the absolute configuration of monosaccharides was generally obtained by GC analyses with a chiral capillary column. Detailed investigations on saponin chemical structures have until now been reported for *M. arabica, M. arborea, M. hybrida, M. lupulina*, *M. polymorpha, M. sativa* and *M. truncatula* [19-46]. Characterized saponins from these species of *Medicago* are listed in Tables 1-7.

Table 1: Saponins identified in *M. arabica* leaves [41].

Aglycone	3 OH substituted	28 COOH substituted
2*β*-Hydroxy oleanolic acid	*α*-L-Ara(1→2)-*β*-D-Glc	*β*-D-Glc
Hederagenin	*α*-L-Ara	-
Hederagenin	*α*-L-Ara	*β*-D-Glc
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	-
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	*β*-D-Glc
Hederagenin	*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara	*β*-D-Glc
Bayogenin	*α*-L-Ara	-
Bayogenin	*α*-L-Ara	*β*-D-Glc

M. arabica leaves are characterized by the presence of short sugar chain saponins, including mono and bidesmosides of 2-*β*-hydroxyoleanolic acid, hederagenin and bayogenin (Table 1). *M. arborea* leaves produce saponins containing up to seven sugars, identified as mono and bidesmosides of medicagenic and zanhic acid (Table 2). Saponins from *M. hybrida* roots are characterized by the presence of short sugar chain bidesmosides of hederagenin and medicagenic acid (Table 3). *M. lupulina* leaves contain mono and disaccharide saponins of hederagenin and medicagenic acid (Table 4), while saponins from the leaves of *M. polymorpha* predominantly consist of short sugar chain bidesmosides of hederagenin and caulophyllogenin (Table 5). Saponins from the roots and the aerial parts of *M. sativa* are a complex mixture of both short and long sugar chains of mono and bidesmosidic compounds with hederagenin, medicagenic acid, zanhic acid and soyasapogenols as the most representative aglycones. In this species a tridesmoside saponin containing eight monosaccharide units and a *β*-maltoside derivative, 3-*O*-[*α*-D-glucopyranosyl(1→4)-*β*-D-glucopyranosyl] medicagenic acid, were also identified in the aerial parts (Table 6). *M. truncatula* saponins from both roots and aerial parts (Table 7) are long sugar chain bidesmosides of medicagenic and zanhic acid.

Table 2: Saponins identified in *M. arborea* leaves [45].

Aglycone	3 OH substituted	28 COOH substituted
2*β*-Hydroxy oleanolic acid	*α*-L-Rha(1→2)-*α*-L-Ara(1→2)-*β*-D-Glc	-
Bayogenin	*β*-D-GlcA	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
2*β*,3*β*-Dihydroxy-23-oxo-olean-28-oic acid	*β*-D-GlcA	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-Glc	*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-GlcA	*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-GlcA	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
Medicagenic acid	*β*-D-GlcA	*β*-D-Api(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-Glc	*α*-L-Ara(1→3)-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-GlcA	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-Glc	*α*-L-Ara(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-GlcA	*β*-D-Api(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-GlcA	*α*-L-Ara(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*β*-D-Glc(1→2)-*β*-D-Glc	*α*-L-Ara(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Zanhic acid	*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Api(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara
Soyasapogenol A	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	*α*-L-Rha
Soyasapogenol B	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	-

Table 3: Saponins identified in *M. hybrida* roots [46].

Aglycone	3 OH substituted	28 COOH substituted
Oleanolic acid	*β*-D-Gal(1→2)-*β*-D-GlcA	*β*-D-Glc
Oleanolic acid	*β*-D-Gal(1→2)-*β*-D-GlcA	*α*-L-Rha(1→4)-*β*-D-Glc
Hederagenin	*β*-D-Glc	-
Hederagenin	*β*-D-GlcAMe	-
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	-
Hederagenin	*β*-D-GlcA	*β*-D-Glc
Hederagenin	*β*-D-GlcAMe	*β*-D-Glc
Hederagenin	*α*-L-Rha(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Glc
Bayogenin	*β*-D-Glc	*β*-D-Glc
2*β*,3*β*-Dihydroxy-23-oxo-olean-12-en-28-oic acid	*β*-D-GlcA	*β*-D-Glc
Medicagenic acid	*β*-D-Glc	-
Medicagenic acid	*β*-D-Glc	*β*-D-Glc
Medicagenic acid	*β*-D-GlcA	*β*-D-Glc
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Glc

Table 4: Saponins identified in *M. lupulina* [28].

Aglycone	3 OH substituted	28 COOH substituted
Hederagenin	*β*-D-Glc	-
Medicagenic acid	*β*-D-Glc	-
Medicagenic acid	*β*-D-Glc	*β*-D-Glc
Soyasapogenol B	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	-

Table 5: Saponins identified in *M. polymorpha.*

Aglycone	3 OH substituted	28 COOH substituted	Ref.
Oleanolic acid	*α*-L-Rha(1→2)-*α*-L-Ara	*β*-D-Glc-(1→6)-*β*-D-Glc	[38]
Hederagenin	*α*-L-Rha(1→2)-*α*-L-Ara	-	[38]
Hederagenin	*α*-L-Ara	*β*-D-Glc-(1→6)-*β*-D-Glc	[38]
Hederagenin	*α*-L-Rha(1→2)-*α*-L-Ara	*β*-D-Glc	[38]
Hederagenin	*α*-L-Rha(1→2)-*α*-L-Ara	*β*-D-Glc-(1→6)-*β*-D-Glc	[38]
Caulophyllogenin	*α*-L-Rha(1→2)-*α*-L-Ara	*β*-D-Glc	[38]
Caulophyllogenin	*α*-L-Rha(1→2)-*α*-L-Ara	*β*-D-Glc-(1→6)-*β*-D-Glc	[38]
Soyasapogenol B	*α*-L-Rha(1→2)-*β*-D-Glc(1→2)-*β*-D-GlcA	-	[33]

Branched sugar chain saponins were identified in this species, as in *M. arborea* and *M. sativa.* Methyl ester derivative of saponins were also found in *M. hybrida* and *M. sativa*, but these were recognized as artifacts obtained during the extraction with methanol [74].

The nature of the saccharide units, their position on the molecule and the similarity of the sugar chains on saponins from the different species, have suggested high enzymatic selectivity for the sugar position. Hederagenin often contains an *α*-L-arabinopyranose unit as the first sugar in its 3-*O* position. Alternatively a *β*-D-glucopyranose or the corresponding uronic derivative are present as in *M. hybrida* root saponins. The second monosaccharide unit linked at the C-2 position of *α*-L-arabinopyranose can be *α*-L-rhamnopyranose, as in *M. polymorpha*, or *β*-D-glucopyranose as in *M. arabica* and *M. sativa*.

By contrast, in all the studied species of *Medicago*, saponins of medicagenic and zanhic acids are always characterized by the presence of *β*-D-glucopyranose or *β*-D-glucuronopyranose units as the first sugar in the 3-*O* position. The second monosaccharide *β*-D-glucopyranose, linked predominantly at the C-2 position, as in *M. arborea, M. hybrida* and *M. sativa*, or at the C-3 position, as in *M. truncatula* suggesting the presence of a specific glucosyltransferase in this species. Different sugar linkage positions were also detected in the 3-*O* disaccharide chain of *M. sativa*, in which the 1→3 and 1→4 linkage between the first and the second monosaccharide were found. Trisaccharides are predominantly 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucopyranosyl derivatives.

The C-28 glycosylated saponins showed the presence of the *β*-D-glucopyranose unit esterified at the carboxylic group, the disaccharide chain 28-*O*-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (only found in *M. polymorpha*), and chains with more than two sugars, always characterized by *α*-L-arabinopyranose, directly linked at the C-28, and an *α*-L-rhamnopyranose in the central position, linked (1→4) with a *β*-D-xylopyranose. Branching points are formed by *α*-L-arabinopyranose or *β*-D-apiofuranose linked (1→3) at the *β*-D-xylopyranose unit. These features are typical of saponins extracted from *M. arborea*, *M. sativa* and *M. truncatula* and suggest high enzymatic selectivity for the sugar position independent of the involved genin.

Table 6. Saponins identified in *M. sativa* leaves and roots.

Aglycone	3 OH substituted	28 COOH substituted	Ref.
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	-	[13]
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	*β*-D-Glc	[37]
Hederagenin	*β*-D-Glc(1→2)-*α*-L-Ara	*β*-D-Glc	[27]
Hederagenin	*β*-D-Glc(1→3)-*β*-D-Xyl	*β*-D-Glc	[37]
Hederagenin	*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara	-	[23]
Hederagenin	*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara	*β*-D-Glc	[24]
Bayogenin	*β*-D-Gal(1→2)-*β*-D-GlcA	*β*-D-Glc	[39]
Medicagenic acid	-	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[34]
Medicagenic acid	*β*-D-Glc	-	[19]
Medicagenic acid	*β*-D-GlcA	-	[31]
Medicagenic acid	*β*-D-Glc	*β*-D-Glc	[22]
Medicagenic acid	*β*-D-Glc	*α*-L-Rha(1→2)-*α*-L-Ara	[30]
Medicagenic acid	*β*-D-GlcA	*α*-L-Rha(1→2)-*α*-L-Ara	[36]
Medicagenic acid	*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[25]
Medicagenic acid	*β*-D-GlcA	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[31]
Medicagenic acid	*β*-D-GlcA Me ester	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[39]
Medicagenic acid	*α*-D-Glc(1→4)-*β*-D-Glc	-	[29]
Medicagenic acid	*β*-D-Glc(1→3)-*β*-D-Glc	*β*-D-Glc	[39]
Medicagenic acid	*β*-D-Gal(1→2)-*β*-D-Glc	*β*-D-Glc	[27]
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[27]
Medicagenic acid	*α*-L-Rha(1→6)-*β*-D-GlcA(1→2)-*β*-D-Glc	-	[20]
Medicagenic acid	*β*-D-Glc(1→6)-*β*-D-Glc(1→3)-*β*-D-Glc	-	[21]
Medicagenic acid	*α*-L-Rha(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	-	[39]
Medicagenic acid	*α*-L-Rha(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Glc	[27]
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Glc	[39]
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[39]
Medicagenic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Api(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara	[39]
Medicagenic acid	*β*-D-Glc(1→2)-[*α*-L-Rha(1→3)]-*β*-D-Glc (1→2)-*β*-D-Glc	*β*-D-Glc	[32]
Medicagenic acid	Glc-malonyl	-	[40]
Medicagenic acid	Glc-malonyl	Glc	[40]
Zanhic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara	[39]
Zanhic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Api(1→3)-[*β*-D-Xyl(1→4)]-*α*-L-Rha(1→2)-*α*-L-Ara	[39]
Zanhic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Api(1→3)-*β*-D-Xyl(1→4)-*α*-L-Rha 23 COOH substituted: *α*-L-Ara	[36]
Zanhic acid	*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc	*β*-D-Api(1→3)-*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara 23 COOH substituted: *α*-L-Ara	[36]
Soyasapogenol A	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	*α*-L-Rha	[39]
Soyasapogenol B	*β*-D-Glc(1→2)-*β*-D-GlcA	-	[26]
Soyasapogenol B	*α*-L-Rha(1→2)-*β*-D-Glu(1→2)-*β*-D-GlcA	-	[26]
Soyasapogenol B	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	-	[26]
Soyasapogenol E	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	-	[26]
Soyasapogenol E	*α*-L-Rha(1→2)-*β*-D-Gal(1→2)-*β*-D-GlcA	22-*O*-maltol	[35]

A very interesting structural feature of these substances, is the presence of an aldehyde group at the C-23 position in 2*β*,3*β*-dihydroxy-23-oxo-olean-12-en-28-oic acid (Figure 1), a new aglycone of saponins from *M. arborea* and *M. hybrida*. This metabolite might in fact represent an interesting biosynthetic intermediate in the oxidative steps that lead from a methyl group to the corresponding carboxylic acid [1, 75]. That is, if we consider the following genins found in the genus *Medicago*: 2*β*-hydroxyoleanolic acid, bayogenin, 2*β*,3*β*-dihydroxy-23-oxo-olean-12-en-28-oic acid and medicagenic

Table 7: Saponins identified in *M. truncatula* leaves and roots.

Aglycone	3 OH substituted	28 COOH substituted	Ref.
Hederagenin	GlcA	-	[40]
Hederagenin	Glc-Ara	Glc	[40]
Medicagenic acid	Glc	-	[40]
Medicagenic acid	Glc-malonyl	-	[40]
Medicagenic acid	Glc-Glc	-	[40]
Medicagenic acid	Glc	Glc	[40]
Medicagenic acid	Glc-malonyl	Glc	[40]
Medicagenic acid	*β*-GlcA	*β*-Glc	[44]
Medicagenic acid	*β*-Glc	*β*-Xyl(1→4)-*α*-Rha(1→2)-*α*-Ara	[42, 44]
Medicagenic acid	*β*-GlcA	*β*-Xyl(1→4)-*α*-Rha(1→2)-*α*-Ara	[42, 44]
Medicagenic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Rha(1→2)-*α*-Ara	[44]
Medicagenic acid	*β*-Glc(1→3)-*β*-Glc	*β*-Xyl(1→4)-*α*-Rha(1→2)-*α*-Ara	[44]
Medicagenic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Ara(1→3)-[*β*-Xyl(1→4)]-*α*-Rha(1→2)-*α*-Ara	[44]
Medicagenic acid	*β*-Glc(1→3)-*β*-Glc	*β*-Api-(1→3)-[*β*-Xyl(1→4)]-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc	*β*-Xyl(1→4)-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Rha[4-Ac](1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*β*-Xyl(1→4)-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Ara(1→3)-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*β*-Api(1→3)-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*α*-Ara(1→3)-[*β*-Xyl(1→4)]-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	*β*-Glc(1→3)-*β*-Glc	*β*-Api-(1→3)-[*β*-Xyl(1→4)]-*α*-Rha(1→2)-*α*-Ara	[44]
Zanhic acid	Glc-Glc-Glc	Xyl-Rha-Ara, 23 COOH substituted: Ara	[42]
Zanhic acid	Glc-Glc-Glc	Api-Xyl-Rha-Ara, 23 COOH substituted: Ara	[42]
Soyasapogenol B	*α*-Rha(1→2)-*β*-Gal(1→2)-*β*-GlcA	-	[40, 43]
Soyasapogenol B	*α*-Rha(1→2)-*β*-Xyl(1→2)-*β*-GlcA	-	[43]
Soyasapogenol E	*α*-Rha(1→2)-*β*-Gal(1→2)-*β*-GlcA	-	[40, 43]

acid, all the oxidative products at C-23 can be observed. The above genins all possess the same stereochemistry (2β,3β*)* in the hydroxylated triterpene carbons with the different functional groups at the C-23 position. The presence of an aldehyde group in 2*β*,3*β*-dihydroxy-23-oxo-olean-12-en-28-oic acid, identified for the first time in *Medicago* spp, indicates a possible biosynthetic pathway for the sapogenins of this genus. Accordingly, medicagenic acid may originate from bayogenin by subsequent oxidative enzymatic steps involving the formation of 2*β*,3*β*-dihydroxy-23-oxo-olean-12-en-28-oic acid while bayogenin may originate by a selective oxidative demethylation at C-23 from 2*β*-hydroxyoleanolic acid. In a similar way, the two 16*α*-hydroxy triterpenes found in this genus, caulophyllogenin and zanhic acid (Figure 1) probably originate by enzymatic oxidation of hederagenin and medicagenic acid, respectively. The biosynthesis of these compounds in the genus *Medicago* has never been extensively investigated, and only a few papers have been published [76-78].

Saponin extracts as well as purified saponins from selected species of *Medicago* have different biological properties [10-14]. Their antimicrobial, insecticidal, allelopathic and cytotoxic effects are described below. The influence of saponins on animal metabolism is also reviewed.

Antimicrobial activity

Saponins are likely to be implicated in plant defense mechanisms against microbial or fungal infections. In some plants wounding of tissues in response to a pathogenic attack causes the hydrolysis of saponins to derivatives with strong antibiotic activity [79, 80]. On the other hand, resistance to infestation by certain fungi in plants such as oat is associated with the specific presence of saponins (e.g. avenacins). Nevertheless, antifungal efficacy of saponins has been demonstrated *in vitro* for a number of plant species [2, 7, 79, 81] but little data is available on their antibacterial activity [2].

A compilation of microorganisms used to assess antifungal and antibacterial activity of saponins from *Medicago* spp. is reported in Table 8. Data derive from incubation of *Medicago* dry meals, saponin extracts and purified saponins from different species and plant organs.

Antifungal efficacy of *Medicago* has been primarily studied with the model fungus *Trichoderma viride* [10, 29, 31, 63, 65, 82-85] which appeared particularly sensitive to the presence of saponins in the growth medium. A bioassay to determine the content of saponins in the plant was developed [58] based on saponin toxicity towards this fungus. Growth of *T. viride* was in fact found inversely correlated with the amount of *Medicago* saponins in the incubation medium thus representing a useful index to evaluate the total percentage of these metabolites.

As described (Table 8), saponins from *Medicago* have been assayed *in vitro* against phytopathogenic species and their activity well established not only against specific pathogens of *Medicago*, but also against some fungi generally pathogenic to cereals [10, 14, 28, 31, 65, 85-95]. A higher antifungal activity was found for the saponins from the roots than from the aerial parts of *M. sativa* [92, 93, 95]. Furthermore, assays with purified saponins from the same species [93] indicated that the growth of the two pathogens *Botrytis tulipae* and *Phloma narcissi* was mostly affected by the following compounds: medicagenic acid; 3-*O*-*β*-D-glucopyranosyl-medicagenic acid; 3-*O*-*β*-D-glucopyranosyl-28-*O*-[*β*-D-xylopyranosyl(1→4)-*α*-L-rhamopyranosyl(1→2)-*α*-L-arabinopyranoside] medicagenic acid and 3-*O*-*β*-D-glucuronopyranosyl- 28-*O*-[*β*-D-xylopyranosyl (1→4)-*α*-L-rhamopyranosyl (1→2)-*α*-L-arabino-pyranoside]medicagenic acid. The screening of saponin extracts from several *Medicago* spp. has shown that *M. arabica* possesses antifungal efficacy several times higher than that of *M. sativa* [95] and the most sensitive pathogens were *Rhizoctonia solani*, *B. tulipae*, *P. narcissi*, *Fusarium oxysporium* ssp. *tulipae* and *Pestalotia* ssp.

Besides their phytopathogenic potential the antimicrobial activity of saponins from *Medicago* against human pathogens has also been investigated [14, 96-104]. Preliminary studies have concerned the effect against some yeasts and dermatophytes of a gluco derivative of medicagenic acid named G2 and later identified as 3-*O*-*β*-D-glucopyranosyl-medicagenic acid. The compound was found to be particularly effective against *Cryptococcus neoformans* with an MFC of 4 μg/mL [2, 7, 79, 80]. More recent investigations [104] on dermathophytes have shown that *Trichophyton interdigitale* and *Microsporium gypseum* were susceptible to *Medicago* saponins especially to glycosides of medicagenic acid, such as 3-*O*-*β*-glucopyranoside (MIC < 62.5 μg/mL) the most bioactive phytochemical.

The study of the antifungal activity of saponins from *M. sativa*, *M. arborea* and *M. arabica* against a selection of medically important yeasts (*Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae*, *Cryptococcus laurentii* and *Blastomyces capitatus*) [103] has shown that *S. cerevisiae* was the most susceptible, being highly inhibited when treated with the sapogenin mixtures from the aerial parts of the three different species of *Medicago* (MICs of 125, 62.5 and 175 μg/mL for *M. sativa*, *M. arabica* and *M. arborea,* respectively). A very low MIC value (42.5 μg/mL) was observed when the same strain was treated with medicagenic acid, which represents the dominant aglycone found in *M. sativa* (50%) and *M. arborea* (30%) aerial organs. Medicagenic acid also inhibited the two mycetes *C. tropicalis* and *B. capitatus*, with an MIC of 125 μg/mL.

Although strongly antifungal, saponins are reported to have only weak or no growth inhibitorial effects against bacteria [80]. To the best of our knowledge only one investigation has been carried out to evaluate the antibacterial activity of saponins from *Medicago* species and they were found not very active (MICs > 500 μg/mL) against Gram negative bacteria [103]. Nevertheless, they displayed some efficacy against selected Gram positive bacteria [103]. In particular, sapogenins obtained on acid hydrolysis of saponins from *M. arabica* aerial parts and roots were the most effective, showing good growth inhibitorial activity towards three different strains of *S. aureus*, two strains of *E. faecalis*, and against *B. subtilis* and *B. cereus* (Table 8). *In vitro* antibacterial assays with purified aglycones from *Medicago* saponins showed that medicagenic acid had significant activity against *S. aureus* (MIC 52.5 μg/mL) and two strains of *E. faecalis* (MICs 50 and 32.5 μg/mL)

The *in vitro* effects of some saponins from *M. sativa* on rhizosphere bacteria suspension, showed that 3-*O*-*β*-D-glucopyranosylmedicagenic acid sodium salt

Table 8: Overview of antimicrobial studies with saponins from *Medicago* spp.

Saponin source	Microorganisms	Ref.
	a) Phytopathogenic fungi	
M. sativa leaves Total saponins	*Fusarium oxysporum, F. solani, Phytophtora drechsleri* *Phoma sp,. Rhizoctonia solani, Verticillium albo-atrum*	[65]
M. sativa tops *M. sativa* roots Total saponins	*Alternaria solani* *Pytium myriotylum* *P. butleri, P. sp. PRL2142, Sclerotium rolfsii*	[86, 87]
M. sativa roots Total extract Total saponins Saponin sugars Sapogenins	*Rhizoctonia solani*	[88]
M. sativa roots 3-*O*-*β*-D-Glc Medicagenic acid	*Aspergillus niger,* *Fusarium oxysporum* sp. *Lycopersici, Phytophtora cinnamommi, Rhizopus mucco,Sclerotium rolfsii*	[82]
M. sativa roots 3-*O*-[*α*-D-Glc(1→4)-*β*-D-Glc] Medicagenic acid (Medicagenic acid *β*-maltoside)	*Aspergillus niger* *Fusarium oxysporum* sp. *Lycopersici, Phytium aphanidermatum, Rhizoctonia solani, Sclerotium rolfsii*	[29]
M. sativa roots Compound G2 (3-*O*-*β*-D-Glc Medicagenic acid)	*Aspergillus niger* *Fusarium oxysporum, Geotrichum candidum* *Phytium aphanidermatum,Phytophtora cinnamommi* *Rhizoctonia solani, Rhizopus mucco, Sclerotium rolfsii*	[14]
Meal from aerial parts of: *M. arabica, M. doliata*, *M. heyniana*, *M. murex, M. sativa* Total saponins from aerial parts of: *M. arabica, M. heyniana, M. murex, M. polymorpha, M. sativa* *M. sativa* roots Total saponins Total prosapogenins 3-*O*-*β*-D-Glc Medicagenic acid Medicagenic acid	*Cephalosporium gramineum*	[89, 91, 94]
Meal from aerial parts of: *M. arabica, M. doliata, M. heyniana, M. murex, M. sativa* *M. sativa* roots Total saponins Total prosapogenins 3-*O*-*β*-D-Glc Medicagenic acid Medicagenic acid	*Gaeumannomyces graminis* v. *tritici*	[90, 91]
M. sativa aerial parts *M. sativa* roots Total saponins	*Alternaria zinniae,* *Botrytis cinerea, B. tulipae,* *Phoma narcissi, P. poolensis, Rhizoctonia solani*	[92]
M. sativa leaves and roots 3-*O*-[*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara]-28-*O*-*β*-D-Glc Hederagenin 3-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid 3-*O*-*β*-D-GlcAc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid 3-*O*-[*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc]-23-*α*-L-Ara-28-*O*-[*β*-D-Api(1→3)-*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Zanhic acid Soyasaponin I Hederagenin, Medicagenic acid, Soyasapogenol B	*Botrytis tulipae* *Phoma narcissi*	[93]

Table 8 (*Contd.*)

M. arabica shoots Total saponins	*Alternaria tenui, Botrytis cinerea, B. tulipae, Fusarium oxysporium* sp. *Callistephi, F. oxysporium* sp. *Narcissi, F. oxysporium* sp. *Tulipae, Pestalotia ssp., Phoma narcissi P. poolensis, Pythium ultimum, Rhizoctonia solani, Stangospora curtisii*	[95]
	b) Human pathogenic fungi	
M. sativa Compound G2 (3-*O*-*β*-D-Glc Medicagenic acid)	*Candida albicans,C. guilliermondii, C. krusei, C. parapsilopsis, C. pseudotropicalis, C. tropicalis Cryptococcus neoformans, Epidermophyton floccosum Geotrichum candidum, Microsporium canis Rhodotorula glutinis, Torulopsis candida, T. glabrata Trycophyton mentagrophytes, T. mentagrophytes* var. *granulare, Trichopyton rubrum,T. tonsurans*	[14, 96-100]
3-*O*-*β*-D-Glc Medicagenic acid	*Scopulariopsis brevicaulis, Trycophyton mentagrophytes*	[101, 102]
M. arabica tops Total saponins, Sapogenins, Bayogenin	*Blastomyces capitatus, Candida albicans, C. tropicalis Cryptococcus laurentii, Saccharomyces cerevisiae*	[103]
M. arabica roots Total saponins, Sapogenins		
M. arborea tops Total saponins, Prosapogenins, Sapogenins		
M. sativa tops Total saponins, Prosapogenins, Sapogenins Medicagenic acid, Hederagenin		
M. sativa roots Total saponins, Sapogenins		
Medicago sp. 3-*O*-*α*-L-Ara-Hederagenin 3-*O*-[*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara]-Hederagenin 3-*O*-*β*-D-Glc-Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc-Medicagenic acid 3-*O*-*β*-D-GlcAc-28-*O*-*β*-D-Glc-Medicagenic acid 3-*O*-[*β*-D-Glc(1→2)-*β*-D-Glc]-28-*O*-*β*-D-Glu-Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara]-Medicagenic acid 3-*O*-*β*-D-GlcAc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara]-Medicagenic acid 3-*O*-[*β*-D-Glc(1→2)-*β*-D-Glc]-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara]- Medicagenic acid 3-*O*-[*β*-D-Glc(1→2)-*β*-D-Glc(1→2)-*β*-D-Glc]-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara]-Zanhic acid Soyasaponin I Hederagenin, Medicagenic acid	*Micropsorum gypseum, Trichophyton interdigitale*	[104]
	c) Bacteria	
M. arabica tops Total saponins, Sapogenins, Bayogenin	*Acinebacter baumanii*, *Bacillus subtilis*, *B. cereus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*	[103]
M. arabica roots Total saponins, Sapogenins, Hederagenin		
M. sativa roots Total saponins, Sapogenins		
M. arborea tops Total saponins, Prosapogenins, Sapogenins		
M. sativa tops Total saponins, Prosapogenins, Sapogenins, Medicagenic acid		
	d) Soil Bacteria	
M. sativa 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc Medicagenic acid Soyasaponin I 3-*O*-*β*-D-Glc Medicagenic acid Na^+ salt Medicagenic acid Na^+ salt	*Agrobacterium tumefaciens*, *Bacillus thuringensis Curtobacterium flacumafaciens, Pseudomonas fluorescens*	[105]

Table 8 (*Contd.*)

	e) Others	
Total saponins from aerial parts of: *M. aculeata, M. arabica,M. blancheana, M. carstiensis* ***M. ciliaris, M. coerulea, M. coronata , M. disciformis*** *M. doliata, M. falcata,M. glutinosa, M. granadensis* *M. hemicycla, M. heyniana, M. hybrida, M. intertexta* *M. laciniata, M. lupulina, M. minima, M. murex* *M. muricoleptis, M. noeana,M. orbicularis, M. polyceratia* *M. polymorpha, M. praecox, M. radiata, M. rigidula* *M. rotata* , *M. rugosa*, *M. sativa* , *M. sauvagei* , *M. scutellata* *M. soleirolii, M. tornata*, *M. turbinata, M. truncatula*	*Trichoderma viride*	[10, 29, 31, 51, 63, 65, 82-85]
M. sativa roots Total saponins 3-*O*-[*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara] Hederagenin 3-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-GlcAc Medicagenic acid 3-*O*-[*α*-D-Glc-(1→4)-*β*-D-Glc] Medicagenic acid (Medicagenic acid *β*-maltoside) 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc-Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid 3-*O*-*β*-D-GlcAc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid 3-*O*-[*β*-D-Glc(1→2)-*β*-D-Glc]-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid		
M. lupulina roots 3-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc Medicagenic acid Medicagenic acid		

could negatively affect them and, could negatively affect them and, in most cases, its activity corresponded to that of the corresponding aglycone, medicagenic acid disodium salt [105].

Investigations on the structure-activity relationships of *Medicago* saponins have led to contrasting results [11, 86, 106]. The number, kind and sequence of the sugar residues in the molecules have been differently correlated with their antimicrobial effects. A detailed study on the activity of different saponins from alfalfa roots against *T. viride* indicated that the monodesmoside derivatives of medicagenic acid were more active than the related bidesmosides, even though no straight correlation between the number of sugars in the molecule and its bioactivity could be established [31]. Moreover, the antifungal activity of medicagenic acid and its derivatives were reported as dependent on the presence of functional groups, such as carboxy and hydroxy in the molecule. In some studies, reduction of bioactivity was related to the presence of a sugar moiety at the 28-*O*-position of the saponin [11, 86, 106].

Bioassays with saponins from *Medicago* sp. against human pathogenic fungi and bacteria indicated that the sugar moieties are not required for antimicrobial activity [103]. This study proved that sapogenins were more active than the related prosapogenins and saponins.

Insecticidal activity

Toxicity of saponins to insects is known, and it has been suggested that they might also provide plant protection from insect predation [107]. To support this hypothesis, the herbivore-induced response of alfalfa was recently examined through assays with *Spodoptera littoralis* larvae, and it was observed that the levels of total saponins increases in the young foliage of damaged plants [108].

Several works on the insecticidal and antifeedant properties of saponins against several classes of insects have been published [109, 110]. A list of saponin source and related insects and pests on which they have been tested, is reported in Table 9. Saponins from alfalfa roots and shoots were reported to be active against the peach aphid (*Myzus persicae*) [109], and found to be toxic to the larvae of the grass grub (*Costelytra zealandica*) [111]. Several species of locusts have shown increasing mortality when fed

on alfalfa; their larvae developed more slowly and the emerging adults were smaller than when they were fed saponin-free herbage [109]. Alfalfa root saponins, rich in medicagenic acid, are toxic to the flour beetle (*Tribolium castraneum*) and their toxicity increased when some of the sugars were removed by hydrolysis [109]. On the contrary, it has been described that several alfalfa pests, such as alfalfa weevil (*Hypera postica*), spotted aphid (*Tereoaphid maculata*), clover root curculio (*Stona hispidulus*), and seed chalcid (*Bruchophagus roddi*) are hardly affected by a saponin-rich diet, suggesting that they have evolved strategies to overcome the toxicity of the saponins of the plant on which they prey [109].

Crude mixtures and purified saponins from alfalfa leaves were tested against potato leafhopper (*Empoasca fabae* Harris) and pea aphid (*Acyrthosiphon pisum* Harris). Larvae were fed with a diet containing 0.01-5.0% saponins for a few days. An increase of mortality was observed for all the tested organisms, in particular for those fed on saponins containing medicagenic acid [112].

Saponins extracted from the leaves of 41 alfalfa varieties, with a different content of saponins and sapogenins, were assayed *in vitro* against larvae of the yellow mealworm (*Tenebrio molitor* L.). Results showed a good correlation between larvae mortality and saponin concentration so this biological assay was proposed to detect alfalfa saponins in plant material [66, 113].

Alfalfa saponin mixtures also have been tested against the summer fruit tortrix moth (*Adoxophyes orana* F.v.R.), the European grape moth (*Lobesia botrana* Den. & Schiff.) and the European corner borer (*Ostrinia nubilalis* Hb.). The increasing amount of saponins added to the diet (from 1 to 1000 ppm) increased larval mortality from 11.3% at 1 ppm to 46.1% at 1000 ppm. The contact effect accounted for a maximum of 22.7% mortality. No appreciable differences were detected in the insecticidal activity exerted by crude saponins derived from alfalfa leaves and roots [12].

Saponins isolated from the aerial parts of alfalfa were tested against the Colorado potato beetle (*Leptinotarsa decemlineata* Say). Larvae were fed on potato leaves sprayed with 0.5 and 1% saponin solutions; no repellent effects were observed for any of the tested compounds, but insect feeding proved to be less intense on saponin-treated leaves. The larvae fed on saponin treated leaves had the lowest body weight gain, suggesting the antifeedant activity of the compounds. The insect mortality from eating saponin-treated leaves was 100% at both tested concentrations [114]. Other experiments showed that the larvae of Colorado potato beetles reared on potato leaves treated with a 0.5% solution of total saponins from *M. sativa* roots and tops, died after 4-6 days because of fasting. Lower saponin doses (from 0.1 to 0.001%) reduced the insect feeding less causing an inhibition in growth and an extension of the larval stage. Mortality was reached at a level of 76.7-100%. No evident differences have been found in saponin activity from the tops or the roots of alfalfa [115].

The Colorado potato beetle was also used to differentiate insecticidal activity of saponins from *M. arabica, M. hybrida* and *M. murex* roots and tops. Total saponins were included in the insect diet as a solution applied on potato leaves on which larvae were reared. All saponins reduced larval feeding, growth rate and mortality in a dose dependant manner. All the saponins showed a high insecticidal activity at the concentration of 0.5%. Saponins from *M. murex* roots and from *M. arabica* and *M. hybrida* aerial parts were found to be the most active, probably due to the differences in their saponin composition [116].

Crude alfalfa root saponins, their prosapogenins produced by alkaline hydrolysis, and medicagenic acid sodium salt, were tested in field trials against spider mite (*Tetranychus urticae* Koch.) and hop aphid (*Phoron humuli* Schrank). Plants were sprayed with a 0.1 and 0.2% solution of saponin products. Prosapogenins were the most active against both phytophages, while crude saponins and medicagenic acid sodium salt were less active [117].

Dried alfalfa leaf and root tissues incorporated in an artificial diet to give the final saponin concentration of 0.1, 0.5 or 1.6% mg/g fresh weight, a cholesterol-precipitable saponin fraction from the plant leaves and a total saponin mixture from the roots were used to evaluate their toxic potential against the polyphagus insect european corn borer. The growth and development of larvae were significantly inhibited after feeding. Root saponins were somewhat more harmful than saponins from the tops [118].

Total saponins from *M. sativa* roots and leaves and individual saponins and sapogenins were tested on a polyphagous pest, the army-worm *Spodoptera*

Table 9: List of pests and insects used to evaluate the insecticidal activity of saponins from the *Medicago* spp.

Saponin source	Insect	Ref.
M. sativa tops Total saponins	Grass grub (*Costelytra zealandica*)	[111]
	Pea aphid (*Acyrthosiphon pisum* Harris) Potato leafhopper (*Empoasca fabae* Harris)	[112]
	Yellow mealworm (*Tenebrio molitor*)	[66, 113]
	Colorado potato beetle (*Leptinotarsa decemlineata* Say)	[114]
M. sativa tops *M. sativa* roots Total saponins	Alfalfa weevie (*Hypera postica*) Clover root curculio (*Stona hispidulus*) Flour beetle (*Tribolium castraneum*) Peach aphid (*Myrus persicae*) Seed chalacid (*Bruchophagus rodoli*) Spotted aphid (*Thereoaphid maculata*) Several species of locusts	[109]
	European corner borer (*Ostrinia nubilalis* Hb.) European grape moth (*Lobesia botrana* Den. & Schiff.) Summer fruit tortrix moth (*Adoxophyes orana* F.v.R.)	[12]
	Colorado potato beetle (*Leptinotarsa decemlineata* Say)	[115]
M. arabica *tops* M. arabica *roots* *Total saponins* M. hybrida *tops* M. hybrida *roots* Total saponins *M. murex* tops *M. murex* roots Total saponins	Colorado potato beetle (*Leptinotarsa decemlineata* Say)	[116]
M. sativa roots Total saponins Prosapogenins Medicagenic acid Na^+ salt	Hop aphid (*Phoron humuli* Schrank) Spider mite (*Tetranychus urticae* Koch.)	[117]
M. sativa tops Total saponins Cholesterol-precipitable saponins *M. sativa* roots Total saponins	European corner borer (*Ostrinia nubilalis* Hb.)	[118]
M. sativa 3-*O*-*β*-D-Glc-28-*O*-[*α*-L-Ara(1→2)-*β*-D-Glc(1→2)-*α*-L-Ara] Hederagenin 3-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc Medicagenic acid 3-*O*-*β*-D-Glc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid 3-*O*-*β*-D-GlcAc-28-*O*-[*β*-D-Xyl(1→4)-*α*-L-Rha(1→2)-*α*-L-Ara] Medicagenic acid Soyasaponin I, Hederagenin, Medicagenic acid, Soyasapogenol A, Soyasapogenol B, Soyasapogenol E, 3-*O*-*β*-D-Glc Medicagenic acid Na^+ salt 3-*O*-*β*-D-Glc-28-*O*-*β*-D-Glc Medicagenic acid Na^+ salt Soyasaponin I Na^+ salt, Medicagenic acid Na^+ salt	Army-warm (*Spodoptera littoralis* Boisd.)	[119]

littoralis. Total saponins (1, 10 and 100 ppm) and a series of pure saponins (10 ppm) and sapogenins (20 ppm) were given in the food and their effects examined during larval development as well as in the resulting pupae and adults. At 1 ppm, root saponins caused a nearly 70% mortality and the emerged females exhibited about 60% fertility reduction. Total saponins from the aerial parts were less active, although the increase of mortality and the reduction of fecundity were significant. All the pure saponins lowered the food consumption and reduced the larval growth rate although to a different extent. Aglycones

influenced the larval development in a similar way, medicagenic acid was found to be the most active, hederagenin and soyasapogenols A and B exhibited only moderate activity, while soyasapogenol E was inactive. Medicagenic acid sodium salt and its 3-*O*-*β*-D-glucopyranosyl and medicagenic acid 3-*O*-*β*-D-glucopyranosyl-28-*O*-*β*-D-glucopyranoside derivatives were the most active substances. Additionally, all the tested *α*-L-arabinopyranosyl glycosides were inactive, while the corresponding aglycones or glycosides were active. Based on those results it has been suggested that glycosylated saponins are bioactive only when they are hydrolyzed by insect gut glycosidases and release an active aglycone; complex glycosides containing arabinopyranosyl units apparently resist the action of the enzymes [119].

The spraying of winter wheat with different saponin concentrations at various phenological phases had no negative effects on growth parameters, grain yield and quality of wheat flour. Results indicated that saponins (0.01-0.1%) can be applied on a wheat crop as fungicides or insecticides [120].

Allelopathic effects

Alfalfa, as other forage legumes, has the reputation as an important rotation crop to improve nitrogen availability in the soil. In some cases the increase of nitrogen by *Medicago* does not correspond to an increase of grain yield in the succeeding rotated crop, suggesting that some factors might interfere with the utilization of nitrogen. This effect has been experimentally correlated with the presence, in alfalfa plant material, of saponins which display allelopathic activity [11, 13, 121-124].

The role of alfalfa saponins as allelopathic agents was first reported in 1955 by Mishutin and Naumova [125] who observed that growth of cotton was influenced by the use of alfalfa as a rotation crop. Detrimental effects on cotton-seed germination was also shown in *in vitro* assays with alfalfa saponins. Later investigations have shown that saponins from various species of *Medicago* act as allelochemicals, some with a defined specificity towards different plants [126-130]. The allelopathic potential of medicagenic acid glycosides has been noted. Depending on their concentration they may function as plant growth inhibitors (high concentrations) or stimulators (low concentrations) [121, 129, 130]. They also inhibit the growth of several weeds and cereals [11, 13, 121, 128-130]: elongation of roots and shoots of *Bromus secalimus* and *Echinochloa crus-galli* was inhibited by 10 ppm saponin (19-11 and 28-17%, respectively), while growth of wheat roots was 50% reduced at the concentration of 100 ppm compared to the control [129, 130]. Other saponins such as soyasapogenol B and hederagenin glycosides were in general found less active as growth inhibitors than medicagenic acid derivatives [121, 129].

The different allelopathic potential of *Medicago* species was related to their different content of saponins. Thus, for example, saponins (medicagenic and soyasapogenol glycosides) isolated from the seeds of *M. lupulina* were able to inhibit the growth of the cereals oat, barley, wheat and rye, whereas saponins from the seeds of *M. sativa* (containing only soyasapogenol glycosides) had no effects on wheat and rye development, but only on that of barley and oat [121, 129]. The use of plant material of various physiological ages indicated that alfalfa at immature stages is more phytotoxic since it likely contains higher amounts of allelochemicals [126].

Soil texture also was found to influence the inhibitory activity of alfalfa saponins [121, 127]. In a detailed study it has been in fact shown that finely powdered alfalfa roots in sandy soil causes a more pronounced detrimental effect on wheat growth than incorporation in heavy clay soils.

An autotoxic effect of alfalfa also has been reported [131].Despite their allelopathic activity, however, saponins produced by the plant species seem not to be involved in the autoxic effects which instead have been attributed to water-soluble phenolic components [132-135]. In particular the isoflavonoids medicarpin and its methoxy analogue, 4-methoxymedicarpin, and chlorogenic acid purified from alfalfa leaves were assayed in *in vitro* experiments and found to contribute to the plant autoallelopathy which results in a yield decrease, low seed germination and poor growth when alfalfa is sown in soils where the same species was previously cropped.

The physiological mechanism of action of saponins as allelopathic agents is not clear. Inhibition of seed germination has been correlated with a decrease in

oxygen diffusion through the seed coat [121], while seedling growth retardation has not been well studied. Comparison of the allelopathic effects of structurally different saponins from *Medicago* species revealed some structure-activity relationships. As found for antimicrobial activity, monodesmosides were in general more active than the related bi- and tridesmosides while medicagenic acid glycosides having glucose at the C-3 position were more active than similar compounds substituted with glucuronic acid, and zanhic acid glycosides were more effective than the 3-*O*-glucuronides of medicagenic acid [11, 121, 129].

Effects on animals

The significance of natural saponins in animal nutrition has been widely investigated [136, 137]. Studies of the effects of the saponins from *Medicago* species have been carried out only for *M. sativa*, due to the importance of this species as forage and as an industrial source of leaf protein concentrate used in animal diets. An excellent review on this topic is available [138]. Saponins may have significant effects on all the phases of animal metabolism from ingestion to excretion. Alfalfa saponins influence rumen fermentation and affect microbial protein synthesis in the rumen, the site of nutrient digestion. Moreover they suppress fermentation in rumen cultures [139], and *in vivo* investigations [140] have confirmed a general decrease of fermentation associated with a symptomatic decrease of volatile fatty acids and cellulose digestion. A significant reduction of protozoa in rumen of sheep receiving alfalfa saponins was also reported [140]. Moreover, endogenous bacteria appeared morphologically modified when treated with alfalfa saponins [139]. All these effects on animal nutrition have been related to the ability of saponins, or their aglycones, to interact with cell membrane sterols and other metabolites [138, 141]. Saponins are in fact able to complex cholesterol, and their anti-nutritional effects were lowered by addition of cholesterol to the diet. Retardation of growth by alfalfa dietary saponins has been observed in livestock and laboratory animals, probably due to the bitter and astringent sensory characteristics of the processed grain products. One mechanism that might account for the growth depressing effects of saponins is the lowering of feed intake because of unpalatability.

No clear information is available on the lethal dose or minimum inhibition concentration of alfalfa saponins towards livestock. Animal species differ in their susceptibility to saponins, however. Poultry are more sensitive than other farm animals. A variety of alfalfa with 1.47% of saponins caused an average reduction of 11% in weight gain of chicks compared to a low-saponin variety containing 0.59% of the active compounds [142, 143]. No effects were reported when calves were fed with alfalfa hay containing up to 2.62% saponin [144]. Though accurate estimates of detrimental saponin levels are lacking, high- and low-saponin germplasm has been defined in the literature (and set as a goal in breeding programs), mostly based on responses of monogastric animals, or biological assays (e.g. *Trichoderma viride* test and the hemolytic test). Conventionally an average concentration of about 2.0% and 0.8% were considered to be high and low, respectively [143, 145].

Determination of saponins by semi-quantitative methods based on biological assays may give erroneous results. For instance, glycosides of zanhic acid are weakly detectable by biological tests, although they are classified as toxic/moderately toxic compounds, with an LD_{50} value of 562 mg/kg body weight calculated for hamsters [146]. Sensory test trials on human volunteers, using saponins isolated from alfalfa aerial parts, showed that zanhic acid tridesmoside is the most bitter, astringent and throat-irritating compound of all the tested saponins [36]. This compound is also reported to have the highest intestinal membrane depolarizing activities compared to other alfalfa saponins [147]. It also has been described as causing breathing problems and nervous system perturbations to hamsters, followed by death after 24h. Bloat syndromes were observed at necropsy [146].

Rats fed alfalfa saponins at levels of 1% in the diet for up to 26 weeks showed no toxic effects; a potentially beneficial reduction of serum cholesterol and triglycerides was observed instead [148]. No adverse reactions have been detected in the non-human primate, *Macaca fascicularis*, following consumption of a mixture of alfalfa top saponins for up to 78 weeks. The metabolites decreased cholesterolemia without changing the level of high density lipoprotein-cholesterol; hence, they reduced the total cholesterol/high density lipoprotein-

cholesterol ratio. Furthermore, saponins decreased intestinal adsorption of cholesterol, and increased excretion of neutral steroids and bile acids [149]. As these compounds interact with cholesterol and directly interfere with its absorption, a possible application in some human pathologies can be hypothesized, although toxicity of alfalfa saponins for human consumption needs detailed investigation.

Furthermore, *in vitro* studies indicated that saponins from *M. sativa* roots and aerial parts have some effects on pancreatic lipase activity. Results showed that they stimulated lipolytic activity and did not influence the proteolytic and amylolytic activities of Neopancreatium, a mixture of porcine pancreatic enzymes such as trypsin, chimotrypsin, lipase and amylase. An increase of the stimulatory effects of saponins was observed when sodium cholate was added to the medium [150].

Cytotoxic and tumor-promoter inhibitory activities

Although the cytotoxicity of triterpenoid saponins is known [151], saponins from *Medicago* species have never been extensively investigated, although saponins from *M. sativa* leaves showed dose-dependant growth inhibition *in vitro* of human leukemic cell line K562 [12]. No significant effects on clonogenic survival were observed when purified saponins from *M. sativa* roots, leaves and seeds were tested *in vitro* against MCF7 human breast carcinoma cells and HeLa human cervical carcinoma cells, although MCF7 was more sensitive to the treatment. Inhibition of tumoral cell growth was instead observed when saponins were used in association with cis-platin. The growth of MCF7 cells was 18-33% (saponin concentration 25 μg/mL; cis-platin 4 μg/mL), compared to 40% survival when only cis-platin was used. Root and seed saponins were found to be more active than saponins from leaves. All the tested saponins enhanced the cis-platin induced toxicity, although HeLa cells were significantly less affected [152]. As reported [153], saponins seem to act as promotors probably affecting cell membrane permeability cis-platin diffusion in the cells.

The lack of information on the cytotoxicity of saponins from *Medicago* spp. does not allow additional indications of their activity, but their antitumoral, chemopreventive and antimutagenic properties can be extrapolated from those of bioactive saponins from other plants but found in *Medicago* spp. For example, saponins from soybean, including soyasaponin I (soyasapogenol B 3-*O*-*α*-L-rhamopyranosyl(1→2)-*β*-D-glucopyranosyl(1→2)-*β*-D-glucuronopyranosyl) found in almost all the studied *Medicago* species, are known for their chemopreventive properties [154-156]. Orally consumed soybean saponins are not adsorbed in the small intestine and appear to reach the colon [157] where they exert their beneficial effects. They are able to suppress the growth of human colon carcinoma cells *in vitro* [158, 159], and to inhibit the chemically induced colonic aberrant crypt formation in CF1 mice [160]. Soyasaponin I from *W. brachybotrys* has also been shown to strongly inhibit mouse skin tumor promotion [161].

Antimutagenic and antiproliferative [162-164] activity has also been observed for some hederagenin monodesmosides from *Hedera helix*, including 3-*O*-*α*-L-arabinopyranosyl hederagenin and 3-*O*-*α*-L-rhamopyranosyl(1→2)-*α*-L-arabinopyranosyl hederagenin in *M. arabica* and *M. polymorpha*, respectively.

The saponin 3-*O*-*α*-L-rhamopyranosyl(1→2)-*β*-D-glucopyranosyl (1→2) -*β*-D- glucuronopyranosyl soyasapogenol E from *Wistaria brachybotrys* (Leguminosae), named wistaria saponin D, showed antitumor promoting activity [165]. The same sapogenin has also been found in *M. sativa* and *M. truncatula*.

In this context, the mutagenic activity of some saponins and sapogenins from *M. sativa* have been evaluated. Soyasaponin I, in a concentration up to 500 μg, medicagenic acid (up to 200 μg) and its 3-*O*-glucopyranosyl derivative (up to 200 μg), were tested according to the Ames assay against *Salmonella typhimorium* strains TA97, TA98, TA100 and TA102. Results showed that saponins did not increase the number of $\underline{his}^+$ revertants in any of the strains, neither in the absence nor in the presence of metabolic activation (S9 fraction from rat liver) [166].

Conclusion

Saponins from the *Medicago* genus are a complex group of pentacyclic triterpene glycosides which

display antimicrobial, insecticidal, allelopathic and cytotoxic properties, together with antinutritional effects. Particularly studied *M. sativa*, the most important species within the genus from an agronomic point of view.

The biological activities of *Medicago* saponins are related to their chemical structure in that monodesmosides are more active than the corresponding bidesmosides, and the aglycone and the nature and position of the sugar in the molecule might be important factors in determining their efficacy.

Based on their bioactivity, plant saponins are already used commercially. Data summarized here might suggest further applications of saponins from *Medicago*.

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NPC Natural Product Communications

2006
Vol. 1
No. 12
1181 - 1204

Non-nitrogenous Plant-derived Constituents with Antiplasmodial Activity

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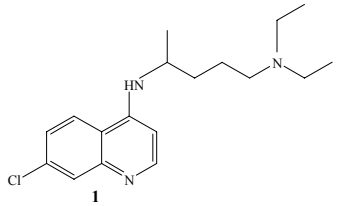
Received: August 2nd, 2006; Accepted: September 27th, 2006

Dedicated to the memory of Professor Ivano Morelli.

The paper is a compilation of the studies reported in the literature concerning non-nitrogenous natural constituents that have shown antiplasmodial activity and aims to provide a basis for further *in vivo* studies as well as for clinical trials to develop new antimalarial agents. Due to the increasingly unsatisfactory outcomes for *N*-heterocyclic drugs, coupled with the rising incidence of the deadly *falciparum* malaria, the advent of non-nitrogenous lead compounds is timely, signaling a new era of antimalarial chemotherapy. Currently a few non-nitrogenous molecules are used in therapy, but many promising molecules of plant origin are under study, such as peroxide sesquiterpenes, quinoid triterpenes, quassinoids, gallic acid derivatives, lignans, flavonoids and biflavonoids, xanthones, naphthoquinones and phenylanthraquinones. Many of these constituents are isolated from plants used traditionally to treat malaria and fever. Ethnopharmacology can still be considered as a rich source of lead molecules.

Keywords: Plant-derived non-nitrogenous, malaria, *in vitro* and *in vivo* studies, terpenoids, polyphenols.

Malaria is one of the oldest life-threatening parasitic diseases diffused in the tropical regions of the world. It causes more than 300 million acute illnesses and at least 1-2.7 million deaths annually (mainly children under the age of five in sub-Saharan Africa). The majority of malaria deaths are due to cerebral malaria and other complications as a result of malaria-related anemia, and the cost in human life, incapacity for work, programs of control and medical treatment are enormous [1,2]. There are four types of human malaria: *Plasmodium vivax, P. falciparum*, *P. malariae,* and *P. ovale,* the first two of which are the most common, and *P. falciparum* is the most deadly type of malaria infection. The malaria situation is aggravated by the appearance of strains of *P. falciparum* resistant to antimalarial drugs as well as by the resistance of vector *Anopheles* mosquitoes to DDT and other insecticides. These are the principal factors that contribute to the difficulty of malaria control and it is unrealistic to think about eradication of this disease by means of destruction of the vector or use of vaccination. Studies in a number of African countries have shown that the emergence of chloroquine-resistant malaria parasites is associated with a two-fold increase in malaria deaths, but in one study in Mlomp, Senegal it was shown that malaria mortality in children under the age of four increased 11-fold within six years of the emergence of chloroquine-resistance [3]. Thus, chloroquine (**1**) represents one of the most effective anti-malarial drugs, but if used as monotherapy its effectiveness is rapidly lost.

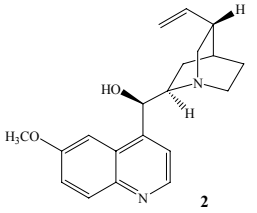


1

Chloroquine is an analog of quinine (**2**), a natural constituent, which is not only considered as the most important lead molecule for the synthesis of the majority of the existing antimalarial drugs but is also currently used in therapy, especially in severe and

complicated cases of malaria caused by chloroquine-resistant strains of *P. falciparum.*

Quinine was isolated in 1820 from *Cinchona* sp. bark, because the antimalarial properties of these plants had been known for several centuries. Jesuit missionaries in Peru around 1630 discovered that the bark of the cinchona tree allayed fever and a few years later exported the bark to Europe, where it was included in pharmacopoeias to treat fever.

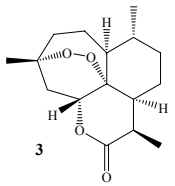


2

From the 1930s, chemically related molecules such as chloroquine (**1**), mefloquine, amodiaquine, mepaquine and pamaquine were developed [1,2]. These molecules are all characterized by the presence of nitrogen heterocycle moieties, and apart from the diminished effectiveness due to resistance by *P. falciparum*, they generally present some disadvantages and risks for the patient or user. Chloroquine is limited in its geographical use, only working in the Middle East, Mexico and Central America. Mefloquine is expensive, 100 times more so than chloroquine, and has resulted in seizures and psychiatric disorders. Halofantrine is equally expensive, unsuitable for prophylaxis, and has led to cases of cardiotoxicity. Even quinine is never totally effective, and its toxic side effects deter its usage [1,2]. Against this disheartening backdrop of the increasingly unsatisfactory performance of the *N*-heterocyclic drugs coupled with the rising incidence of the deadly *falciparum* malaria, the advent of non-nitrogenous lead compounds was not only timely, but also fortuitous and signalled a new era of antimalarial chemotherapy.

Plant-derived non-nitrogenous antimalarials have made, and continue to make, an immense contribution to malaria chemotherapy. In particular, artemisinin (**3**), isolated from the Chinese plant *Artemisia annua* L., has recently been used successfully against malaria resistant to chloroquine and the aim of this review is to consider the potential of plants to provide new antimalarial treatments.

In the last decades many plant extracts, especially those from species with a reputation for use in traditional medicines, have been evaluated in the laboratory for their *in vitro* antiplasmodial activities and some have also been tested *in vivo*, usually in mice infected with *P. berghei* or *P. yoelii* [4,5]. In some cases, the constituent(s) responsible for the observed activities have been isolated by bioassay-guided fractionation and their structures elucidated, many of them being non-nitrogenous derivatives.

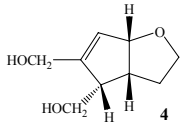


3

This paper reports on the antiplasmodial evaluation of these constituents according to their structures: terpenoids, polyphenols and other constituents.

Terpenoids

In the last three decades all the classes of terpenoids have been investigated to evaluate their antimalarial potency.



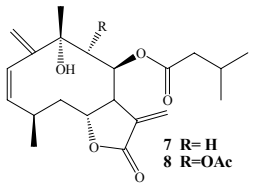
4

Among monoterpenoids, a simple molecule, an iridoid related aglycone, compound **4**, isolated from the roots of *Scrophularia lepidota* Boiss. (Scrophulariaceae), showed a low anti-plasmodial activity (the 50% inhibitory concentration (IC_{50}) was 240 μM). The result was nevertheless interesting because **4** showed a weak FabI enzyme inhibitory activity (IC_{50}=590 μM): FabI is a key enzyme of *Plasmodium falciparum* fatty acid biosynthesis and it can be used as a novel biological target to be used in the search for novel antiplasmodial constituents [6].

Bioassay-guided fractionation of the dichloromethane extract of the fruits of *Renealmia cincinnata* (Zingiberaceae), whose fruits are widely used in Cameroon to treat fevers, led to the isolation of six sesquiterpenoids of which two known ones, **5** and **6**, were the most active; their IC_{50} values were 6.8 and 7.4 μM, respectively, using 3D7 chloroquine-sensitive *P. falciparum* strains [7].

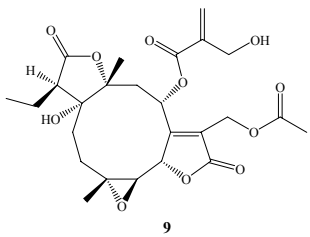
Among sesquiterpenes some lactone derivatives deserve to be mentioned because of the interesting activity [8-11]. A bioassay-guided fractionation of *Neurolaena lobata* (L.) R. Br. (Asteraceae), an

important medicinal plant in Central America and the Caribbean region, where it is used for a variety of diseases including malaria, resulted in the isolation of seven sesquiterpene lactones that showed IC_{50} values ranging from 0.62 to 19.27 μM against the NF54 strain (chloroquine-sensitive) and the clone A1A9 (chloroquine-resistant) of *P. falciparum in vitro* [8]. The most active components were neurolenin A (**7**) with IC_{50} of 0.92 μM and neurolin B (**8**) with IC_{50} of 0.62 μM. It was found that the structural requirements for high antiplasmodial activity *in vitro* is an *α*,β-unsaturated keto function. Additionally, a free hydroxy function at C-8 increased the antiplasmodial activity [8, 12].



7 R= H
8 R=OAc

The sesquiterpene dilactone 16,17-dihydro-brachycalyxolide (**9**) was isolated from *Vernonia brachycalyx* (Asteraceae), a herb growing in East Africa and used by the Maasai, the Kipsigis and other East African tribes as a treatment for parasitic diseases [9]. This compound showed an IC_{50} of 26.9 μM using the 3D7 chloroquine-sensitive *P. falciparum* strain. The IC_{50} values for other tested strains, K39, V1/S and Dd2, were in a similar range, 8.3, 5.9 and 32 μM. This compound also strongly inhibited the proliferation of human lymphocytes at the same concentrations [13].

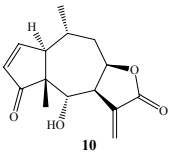


9

Another known sesqiterpene lactone, brevilin A was isolated from *Centipeda minima,* a plant used by the Chinese people to treat colds, nasal allergies, asthma, malaria and amoebiasis [10]. Brevilin A showed an IC_{50} of 9.42 μM against the W2 chloroquine-resistant strain [10].

Recently, the antimalarial activity of lactucin and lactupicrin isolated from *Cichorium intybus* L. (Asteraceae) was also determined against the HB3 clone of strain *Honduras-1* of *Plasmodium* which is chloroquine sensitive and pyrimethamine resistant. The complete inhibitory activity (IC_{100}) for lactucin was 38.5 μM and the value for lactucopicrin was 126 μM [11].

Four sesquiterpene lactones of the pseudo-guaianolide type, the typical constituents of *Arnica montana* L. (Asteraceae), i.e. helenalin (**10**), dihydrohelenalin and their acetates, have shown activities against asexual blood forms of *Plasmodium falciparum in vitro* cultures (NF54, clone A1A9) [14]. The IC_{50} values of the four compounds were in the range from 0.23 to 7.41 μM and the most active constituent was helenalin (**10**), whose potency was comparable to that found for artemisinin (IC_{50} 0.14 μM).

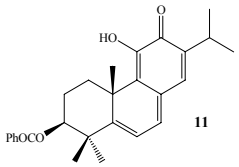


10

Because of the cytotoxic effects of sesquiterpene lactones on various types of cells, the cytotoxic/antiplasmodial ratio was also evaluated as a measure of therapeutic efficiency. Using the cytotoxicity data obtained for helenalin and artemisinin against the human carcinoma cell lines GLC4 and COLO 320, ten times higher toxicity was found for helenanin, which makes its therapeutic usefulness questionable [14].

Several sesquiterpene lactones isolated from *Eupatorium semialatum* Benth. (Asteraceae), a plant used in the traditional medicine of Central America for malaria and dysentery, were assayed *in vitro* for their activities against *Plasmodium falciparum* (K1 strain) using the pLDH-assay [15]. All the compounds were tested and exhibited a moderate activity (IC_{50} 8.9-31.7 μM) if compared to chloroquine (IC_{50} 0.18 μM). Nevertheless these results concerning their *in vitro* activity could justify the traditional use of the plant against malaria [15].

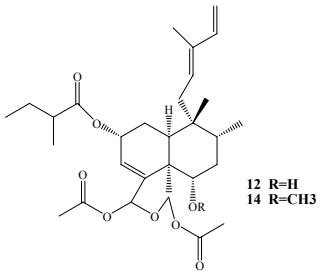
Several diterpenoids with different structures were also reported for their antiplasmodial activity. Among the abietane-type derivatives, 3-*O*-benzoyl-hosloppone (**11**) was isolated from the roots of *Hoslundia opposita* (Lamiaceae) used in East and West Africa to treat malaria [16]. The IC_{50} against the multidrug resistant strain K_1 of *Plasmodium falciparum* was 0.95 μM and the activity of this molecule was attributed to the presence of an α,β-unsaturated carbonyl moiety [16,17].



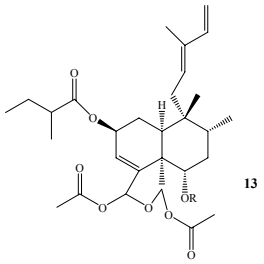
11

Several studies have recently been carried out on labdane and isopimarane diterpenoids, but most of them have shown a modest *in vitro* activity against chloroquine-sensitive *P. falciparum* strains [18-20]. Among the tested constituents only 8(9),15-isopimaradien-3β-ol, isolated from *Platycladus orientalis* (L.) Franco (Cupressaceae), gave interesting IC_{50} values (7.1 μg/mL, 24.6 μM) in the inhibition of the growth of 3D7 *P. falciparum* strain [18].

Clerodane diterpenoids with a mild antiplasmodial activity have recently been isolated from two species of Flacourtiaceae, *Laetia procera* (Poepp.) Eichler a typical species of French Guiana [21] and *Casearia grewiifolia* Vent., growing widely in the northern and northeastern parts of Thailand and used traditionally as a tonic and a febrifuge [22].



12 R=H
14 R=CH3



13

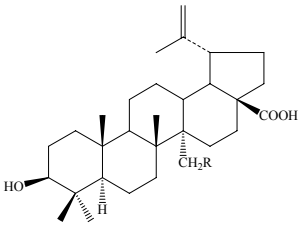
The compounds isolated from *C. grewiifolia* were tested against K1 multidrug resistant strains using artemisinin as positive control, while the derivatives isolated from *L. procera* were tested against F32 Tanzania (a chloroquine-sensitive strain) and FcB1-Columbia (a chloroquine-resistant strain), using chloroquine as a positive control. The most active clerodane diterpenoids were compounds **12**-**14** isolated from *L. procera,* showing activities against *P. falciparum* with an IC_{50} as low as 0.5 μM on both FCb1 and F32 strains. The IC_{50} values were 0.62 and 0.54 μM, respectively, in the two strains for compound **12**, 0.57 and 0.59 μM for **13,** and 0.58 and 0.66 μM for **14** [21]. It was also observed in this study that the hydrolysis of the diacetal moiety lowered their biological activity [21].

In 1997 Bringmann and coworkers [23] first reported on the moderate activity of betulinic acid against *P. falciparum in vitro*, with an IC_{50} of 23.0 μM. This widespread constituent was isolated after a bioassay-guided fractionation from *Triphyophyllum peltatum* (Dioncophyllaceae) and *Ancistrocladus heyneanus* (Anciostrocladaceae). In 1999 Steele et al. [24] confirmed the *in vitro* activity of betulinic acid, but found that it was ineffective in *in vivo* experiments. Betulinic acid was also isolated from an ethanol extract of the root bark of the Tanzanian tree *Uapaca nitida* Mull-Arg. (Euphorbiaceae) used in Tanzania to treat malaria. It showed *in vitro* antiplasmodial IC_{50} values similar to those obtained in the study of Bringmann et al. in 1997 [24]; the IC_{50} values against chloroquine resistant (K1) and sensitive (T9-96) *P. falciparum* strains were 43.0 μM and 63.6 μM, respectively. The *in vitro* activities of several related triterpenes were also evaluated. Betulin was found to be inactive at 1164 μM for both K1 and T9-96. Ursolic acid exhibited IC_{50} values similar to betulinic acid, 80.0 μM and 61.4 μM, respectively. Oleanolic acid exhibited higher IC_{50} values, 194.7 μM and 154.8 μM against K1 and T9-96, respectively. Thus, among the triterpenes, betulinic acid showed the highest activity and for this reason was further tested for *in vivo* activity in a murine malaria model (*P. berghei*). However, the top dosage of 250 mg/kg/day was ineffective in reducing parasitaemia and exhibited some toxicity, and thus not advisable for clinical use [24].

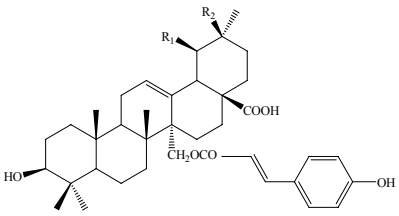
An investigation of *Gardenia saxatilis* Geddes (family Rubiaceae), a plant with folkloric use against malaria and distributed in the northeastern part of Thailand, led to the isolation of several triterpenoids which were assayed for antiplasmodial activity using the K1 multidrug resistant strain [25].

Four compounds, namely messagenic acid A (**15**) and messagenic acid B (**16**), the 27-*O*-*p*-(*Z*)- and 27-*O*-*p*-(*E*)-coumarate esters of betulinic acid, and a mixture of uncarinic acid E (27-*O*-*p*-(*E*)-coumaroyloxy-oleanolic acid) (**17**) and 27-*O*-*p*-(*E*)-coumaroyloxy-ursolic acid (**18**) showed moderate activity with IC_{50} values of 2.43, 6.14 and 4.69 μM, respectively. The results indicated that *p*-coumarate moieties at the

27-position contributed to antiplasmodial activity. As both the *p*-(*Z*)-coumarate ester **15** and the isomeric *p*-(*E*)-coumarate ester **16** were active in the assay, it was noteworthy that the difference in geometry of the double bond in the ester moieties did not significantly effect antiplasmodial activity of the triterpenes, while the introduction of a methoxyl group to the 3-position of *p*-(*E*)-coumarate moiety gave a ferulate moiety which resulted in a loss of activity [25].

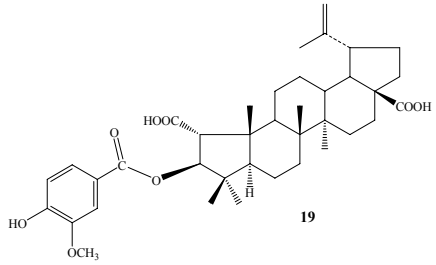


15 R=*p*-(*Z*)-coumaroyl
16 R=*p*-(*E*)-coumaroyl



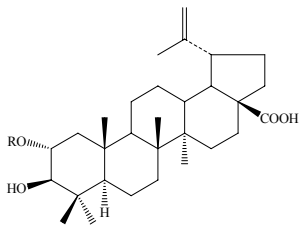
17 R_1=H R_2=CH_3
18 R_1=CH_3 R_2=H

A very recent investigation [26] tested several ceanothane- and lupane-type triterpenes isolated from the root bark of *Ziziphus cambodiana* Pierre (Rhamnaceae) were antiplasmodial activity. 3-*O*-Vanillylceanothic acid (**19**), 2-*O*-*E*-*p*-coumaroyl alphitolic acid (**20**) and zizyberenalic acid (**21**) exhibited significant *in vitro* antiplasmodial activity against the parasite *Plasmodium falciparum* (K1 multidrug resistant strain), with IC_{50} values of 5.81, 1.45 and 6.61 μM, respectively.

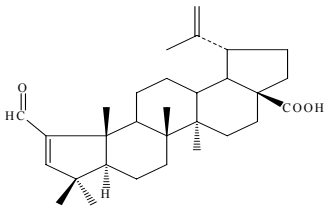


19

A comparison of the structures of the tested compounds indicated that the *p*-coumaroyl moiety in **20** and the vanillyl group of compound **19** were crucial for high antiplasmodial potential. Introduction of a double bond in ring A of the ceanothane-type triterpene **21** greatly increased the inhibitory activity in the antiplasmodial assay [26].



20 R=*p*-(*E*)-coumaroyl



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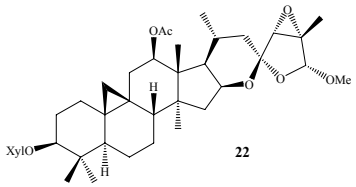
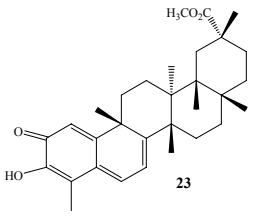


22

Another extensive investigation on the antimalarial effects of triterpenoids isolated from several species of the genus *Cimicifuga* was carried out by Takahara and coworkers [27]. Fifty-nine compounds belonging to five different structural groups were investigated. Almost all the compounds tested showed activity in the 1–56 μM concentration range against *Plasmodium falciparum* FCR-3 strain.

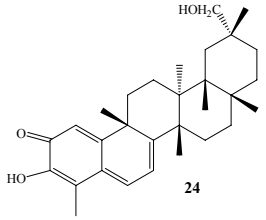
Twenty-five compounds had an IC_{50} 1-3 μM and nineteen of them had a common 16, 23:23, 26:24, 25-triepoxy group in the side-chain moieties. The most active compound was (26*S*)-*O*-methylactein (**22**) [27].

Studies on a species of the Celastraceae family, *Celastrus paniculatus* Willd. from Thailand, known locally as Kra-Thong-Lai and sold in the form of pressed pills for the treatment of malaria, led to the isolation of a moderately active antiplasmodial constituent, a quinonoid triterpene, pristimerin (**23**). The IC_{50} value against K1 strain was 0.42 μM [28].



23

These findings were also confirmed by an investigation on another species of the Celastraceae family, *Salacia krauss,* a small shrub growing in Mozambique and KwaZulu-Natal Province, South Africa and traditionally used to treat bilharzia and dysentery. Thus, a bioassay-guided fractionation of the roots resulted in the isolation of six quinone methides including pristimerin [29]. Each of these compounds was tested *in vitro* against two strains of *P. falciparum*, a chloroquine-resistant strain (K1) and a chloroquine-sensitive reference strain (NF54). The highest activities were found for isoiguesterol (**24**) with an IC_{50} of 22.9 ng/mL (51.1 nM) against K1 and IC_{50} of 54.1 ng/mL (127 nM) against NF54.



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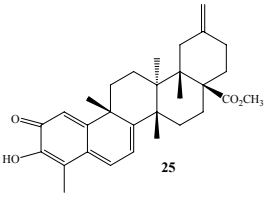


25

Another constituent, 17-(methoxycarbonyl)-28-*nor*isoiguesterin (**25**), displayed an IC_{50} of 27.6 ng/mL (60.9 nM) against K1 and an IC_{50} of 37.1 ng/mL (81.9 nM) against NF54. In addition, all the six isolated quinone methides were found to be cytotoxic against the human adenocarcinoma cell line HT-29 in the range of 1300 ng/mL up to 6060 ng/mL. They displayed, however, a 10-100-fold higher activity against plasmodia than against HT-29 cells, thus indicating some selectivity. Furthermore, compound **25** was also tested *in vivo* against *P. berghei* in mice. However, parenteral administration at 10 mg/kg body weight lead to the death of mice after 1 day, whereas 5 mg/kg and 1mg/kg parenteral as well as 30 mg/kg per oral neither cured mice nor reduced parasitaemia of *Plasmodium berghei* significantly [29].

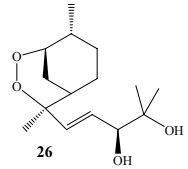
Recently four tanshinones, i.e. 20-norditerpenes with an abietane-type skeleton containing a quinone moiety in the C-ring, were isolated from *Perovskia abrotanoides* Kar. (Lamiaceae) and moderately inhibit growth of cultured malaria parasites (3D7 strain of *Plasmodium falciparum*); the IC_{50} values ranged from 12.5 to 26.9 μM [30].

Artemisinin and other peroxides

Among the terpenoid derivatives, artemisinin (quinghaosu) (**3**) is one of the most well-known antiplasmodial drugs, it has few adverse side effects, making this by far the most useful natural product discovered to date to treat chloroquine-resistant malaria.

Artemisinin is an unusual sesquiterpene trioxane lactone containing an endoperoxide bridge which is essential for its activity. It (**3**) was isolated in 1972 by Chinese scientists from *Artemisia annua* (Asteraceae), a Chinese herb that has been used for over 2,000 years as a remedy for chills and fever. It was quickly observed that this molecule is a rapidly acting antimalarial drug effective against chloroquine and other drug-resistant parasites, and was as good as quinine (but less toxic) for the treatment of cerebral malaria. It is very active *in vitro*, with IC_{50} values between 1-100 nM depending on the *Plasmodium* strain [31,32]. As artemisinin is a non-polar compound, derivatives including ethers (artemether, arteether) and esters (sodium artesunate, sodium artelinate) were prepared to improve its formulation characteristics. These derivatives are now increasingly used as an alternative to quinine [33].

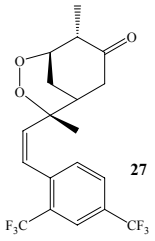
A number of other naturally occurring peroxides, not only from *Artemisia* sp. but also from other members of the Asteraceae (*Achillea millefolium*, *Anthemis nobilis*, *Heterothalamus psiadioides*), have also been tested [34]. It was found that although all of them showed some activity, none was as active as artemisinin (**3**). A weakly active peroxide (1*S*)-1-hydroxy-α-bisabolol oxide A acetate was isolated from *Artemisia abrotanum,* a plant widely cultivated in Europe for its aromatic properties. This compound showed interesting antiplasmodial *in vitro* activity, the IC_{50} being 17.9 μM [35].



26

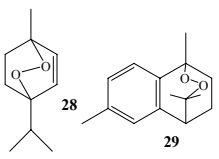
The functional group associated with the activity, namely the endoperoxide, is also present in the structure of another natural antimalarial, yingzhaosu A (**26**) first isolated in 1979 from another Chinese plant, *Artabotrys uncinatus* (Lam.) Merr. (Annonaceae). This constituent is a typical 1,2-dioxane and it occurs as a decomposition product from the stored roots of a sparsely growing vine [36].

Although the evidence of its antimalarial activity is largely anecdotal, **26** is reported to be active against *P. berghei*. However, owing to the limitations imposed by a poor supply of yingzhaosu A, the total synthesis of **26** was proposed in 1991 starting from R-(-)-carvone [37]. Total synthesis proved to be long and tedious, but in 1994 efforts led to semisynthetic first generation derivatives as potential drug candidates. Structurally related but simplified analogues containing the 2,3-dioxabicyclo [3.3.1] nonanes were synthesized. The analogue arteflene (Ro 42-1611, **27**) is a highly active, synthetic antimalarial endoperoxide [38] which can be considered a new lead molecule because of its lower rate of recrudescence, longer lasting therapeutic effects, and a longer half-life than that of artemisinin (**3**) and its commercial derivatives [39]. Later on, a series of endoperoxides containing a sulfide or a sulfone group were synthesized and some members of this class of sulfone endoperoxides have a good *in vivo* therapeutic index (efficacy/toxicity) [40].



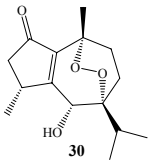
27

Several other endoperoxides have been isolated from plant sources, but most of them did not show high potency against *P. falciparum* strains. The exception to this is ascaridole (**28**), isolated from *Chenopodium ambrosioides* (Chenopodiaceae) and reported to be a potent inhibitor of plasmodial growth; at a concentration of 0.05 μM, development of plasmodium was arrested after 3 days [41]. Zingiberene 3,6-β-endoperoxide and zingiberene 3,6-α-endoperoxide isolated from two Brazilian species, *Eupatorium rufescens* and *Senecio selloi*, were reported to be active with an IC_{50} value of 49 μM against FCH-5 *Plasmodium* strains [42]**.** 10,12-Peroxycalamenene (**29)**, a sesquiterpene with an endoperoxide group similar in structure to artemisinin, was isolated from *Cyperus rotundus*, a Tanzanian plant used traditionally to treat malaria; it showed an IC_{50} value of 2.33 μM against the K1 strain [43].



28 **29**

Two epimers, nardoperoxide (**30**) and isonardoperoxide (**31**) were isolated from *Nardostachys chinensis* (Valerianaceae) and tested for antimalarial activity [44-46]. Their EC_{50} values against *P. falciparum* were 1.5 μM and 0.6 μM, respectively, values comparable with that of quinine (0.11 μM). In addition, studies of cytotoxicity against FM3A and KB cells showed that the selectivity (cytotoxicity/antimalarial activity) of these compounds was comparable to that of quinine. Therefore, these compounds could be considered as promising leads for a new class of antimalarial drugs.



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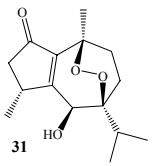
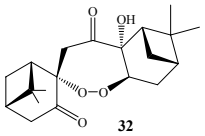


31

Finally, a diterpene peroxide (**32**) isolated from the spice cardamom, *Amomum krevanh* Pierre (Zingiberaceae) showed an antiplasmodial activity about one-tenth that of artemisinin, having an IC_{50} of 0.17 μM [47].



32

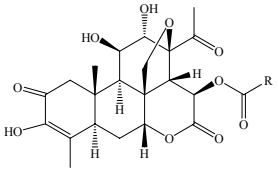
Quassinoids

Quassinoids are terpenoid bitter principles of the *Simaroubaceae* family, including the genera *Ailanthus*, *Brucea*, *Eurycoma* and *Simarouba*. Originally, these bitter substances were termed *quassin*, after a man by the name of "Quassi" who treated fever with the bark of these plants [48, 49]. Chemically they are degraded triterpenes and are categorized into five groups according to their basic skeleton.

Many quassinoids display a wide range of biological activities *in vitro* and/or *in vivo,* and their activity is related to both the position and nature of the ester group and, on the other hand, to the substitution of the A nucleus [48]. Constituents with antiplasmodial activity are mainly represented by the C-20 skeleton. An α,β-unsaturated ketone in ring A and an oxymethylene bridge in ring C are generally considered necessary for antimalarial activity [49].

At the end of the 1940s it was demonstrated that the majority of *Simaroubaceae* have activity on malaria in birds [50] and at the beginning of the 1980s a strong antimalarial activity *in vitro* of many derivatives was demonstrated [51]. Although several quassinoids are cytotoxic, results do indicate that cytotoxicity and antimalarial activity are not correlated, suggesting that the antimalarial activity is not merely cytotoxicity, but that selectivity is present [52]. Therefore, more investigations should be carried out in order to obtain specific information regarding the mechanism of action of these compounds.

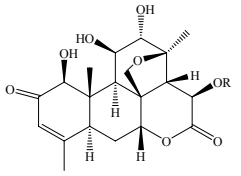
The first molecules tested *in vivo* using *P. berghei*-infected mice were bruceine B (**33**) and brusatol (**34**). They showed some activity, but they were found to be toxic at higher levels than were necessary for antimalarial activity [53].



33 R=CH_3
34 R= $CH{=}C(CH_3)_2$

Active quassinoids have also been isolated from the fruits of *Simarouba amara* of the Republic of Panama [54], *Ailanthus altissima* [55], *Simana cedron* [56], the Brazilian plant *Simaba guianensis* [57]*, Eurycoma longifolia* [58], the Central African *Hannoa chlorantha* and *Hannoa klaineana* [59], the Guinanan *Picrolemma pseudocoffea* [60], and stems of the Indonesian plant *Quassia indica* [61]. All the tested quassinoids showed good activity against chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* and against *P. vinckei petteri* or *P. berghei* in mice. Studies on the structure–activity relationships of the quassinoids [48,62] indicated that the type and presence of an ester group at C-15 was vital for the antiplasmodial activity. Ring A substitution also affected the activity, with a diosphenol moiety in ring A giving the highest activity. The glycosides were found to be generally less active than the corresponding aglycones [48,62].

The most active quassinoids reported in the literature are gutolactone (**35**) and simalikalactone D (**36**) isolated from the bark of *Simaba guianensis* collected near Manaus, Brazil. They were tested against two *Plasmodium falciparum* strains: the W-2 Indochina, a chloroquine-resistant strain, and the D-6 Sierra Leone, a mefloquine-resistant strain. Most notably, the activity was the same for the two different strains, since compound **35** showed IC_{50} values of about 9 nM and compound **36** displayed an IC_{50} of about 3.4 nM. Both compounds presented *in vitro* antimalarial activity similar to or better than that of known antimalarials used as standards (chloroquine, mefloquine, artemisinin, quinine) [57].



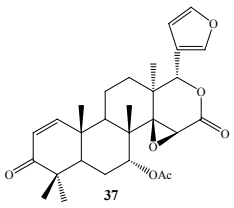
35 R=$COCHC(CH_3)_2$
36 R=$COCH(CH_3)CH_2CH_3$

Limonoids

Limonoids are bitter constituents which have a polyoxygenated triterpenoid skeleton biosynthetically connected to the quassinoids. Limonoids are most often found in the family Meliaceae and less frequently in the families Rutaceae and Cneoraceae. Of the over 300 limonoids known today, about one-third are accounted for by neem (*Azadirachta indica*) and Chinaberry (*Melia azedarach*) [63, 64]. The first limonoid found active against *Plasmodium* was gedunin (**37**) with an IC_{50} of 0.040 μM (0.02μg/mL) isolated from *Melia azedarach* [65]. However, this compound was not active *in vivo* against *Plasmodium berghei* in mice. [66].

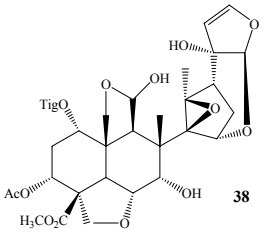
On the other hand, recently it has been found that the combination of gedunin with chloroquine has an additive effect [67]. In addition, a recent *in vivo* reinvestigation [68] of the antimalarial activity of gedunin (**37**) in CD-1 mice infected with *Plasmodium berghei* led to some interesting results. When orally administered at 50 mg kg^{-1} day^{-1} for four days, gedunin (**37**) was able to suppress the parasitaemia level by 44%. However, no clear dose-response effects were observed in the 0-100 mg kg^{-1} day^{-1} dose range. Preliminary pharmacokinetics in Sprague-Dawley rats showed poor absorption, but a binary treatment of 50 mg kg^{-1} day^{-1} gedunin with 25 mg kg^{-1} day^{-1} dillapiol, a cytochrome P450 inhibitor, increased parasitaemia clearance in mice to 75%. A clear dose-response curve was observed in the 0-50 mg kg^{-1} day^{-1} gedunin dose range when administration was combined with 25 mg kg^{-1} day^{-1} dillapiol. In addition, 7-methoxygedunin, a semi-synthetic derivative which is more stable to degradation than gedunin, suppressed the level in

mice by 67% at 50 mg kg $^{-1}$ day^{-1}. When administered at this dose in combination with 25 mg kg^{-1} day^{-1} dillapiol, clearance increased to 80%. These results demonstrate the potential efficacy of gedunin and the value of combination therapy [68].



37

Studies on the leaves of *Azadirachta indica* collected in India resulted in the isolation of four limonoids active against the chloroquine-resistant K1 strain of *P. falciparum* [69]. Further investigations on *A. indica* have been carried out by Jones and coworkers [70] and Dhar and coworkers [71]. Jones and his co-workers looked at azadirachtin (**38**) and a series of 17 semisynthetic derivatives and their effects *in vitro* on male gamete production from malarial microgametocytes.



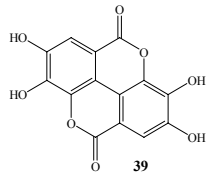
38

Azadirachtin (**38**) and three of the semisynthetic derivatives were found to inhibit the formation of mobile male gametes *in vitro*. This study indicated that the presence of a hemiacetal group at C-11 was vital to the activity. Dhar and coworkers [71] investigated the seeds of *A. indica* and found that the extract was active against all the erythrocytic stages of *P. falciparum*. In addition, the neem extracts also revealed a gametocytocidal effect with inhibition of the asexual stages of the parasite. All stages of maturation of the gametocytes were affected, unlike artemisinin and primaquine which only affect the immature stages [71].

Polyphenols

Over the last three decades studies on polyphenol plant constituents have shown antiplasmodial activity by almost all the classes of polyphenols. Simple galloyl derivatives isolated from *Swintonia forworthyi* Elmer (Anacardiaceae), a large tree of the Philippines, showed activity against two strains of *P. falciparum* (W-2, a chloroquine-resistant one and D-6, a chloroquine-sensitive one). Methyl gallate showed an IC_{50} of 19 μM for the D6 and an IC_{50} of 10.9 μM for the W2 strain. Methyl 3-*O*-galloylgallate showed an IC_{50} of 28.8 μM and for W2 a value of 13.7 μM against D6. Methyl gallate demonstrated a selectivity index of >5 towards the D6 strain and >8 towards the W2 strain when compared with cytotoxicity towards BC1, Lu1, CoI2, KB-V1, and LNCaP cancer cells, while methyldigallate demonstrated a selectivity index of >4 against both strains [72].

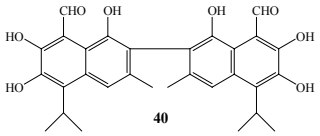
β-Glucogallin, the ester glucoside of gallic acid, and 1-*O*-galloyl-6-*O*-luteoyl-α-D-glucose are constituents of *Phyllanthus niruri* L. (Euphorbiaceae), a medicinal plant widely distributed in Indonesia that is often used in folk medicine to treat malaria and other diseases. They were active against the chloroquine-susceptible *P. falciparum* strain FCR-3 (IC_{50} 14.6 and 2.21 μM, respectively) [73].



39

Ellagic acid (**39**) and 3,4,5-trimethoxyphenyl-(6′-*O*-galloyl)-*O*-β-D-glucopyranoside, isolated from *Tristaniopsis calobuxus* Brongiart & Gris, *T. yatensis* J.W. Dawson and *T. glauca* Brongiart & Gris (Myrtaceae) inhibited the growth of chloroquine-sensitive and resistant clones. Their IC_{50} values were 0.5 and 3.2 μM, respectively [74].

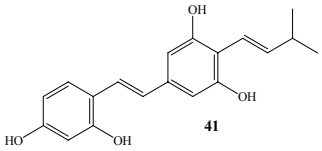
Gossypol (**40**), the most abundant component of cottonseed (cotton=*Gossypium* sp., Malvaceae), is known for a variety of biological activities, including antispermatogenic, anticancer, antiparasitic and antiviral activity. It also demonstrated a weak antimalarial activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum*, with IC_{50} values in the order of 10 μM. [75].



40

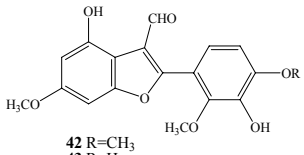
Three prenylated stilbenes, isolated from the edible fruits of *Artocarpus integer* (Moraceae), popular among the people in Thailand, exhibited moderate activity. Their EC_{50} values against the K1 multidrug

resistant strain were 5.66 μM, 26.3 μM and 32.0 μM, respectively, with the novel compound **41**, being the most active [76]. Two other stilbenes, longistylin A and C, isolated from the roots and leaves of *Cajanus cajan* (L.) Millsp. (Fabaceae) showed a moderately high activity *in vitro* against the chloroquine-sensitive *Plasmodium falciparum* strain 3D7 [77].



41

Bioassay-guided fractionation of the leaves from *Andira inermis* led to the isolation of numerous polyphenol constituents including isoflavones, dihydroflavonols and three novel 2-arylbenzofuran-3-carbaldehydes, andinermal A–C. Andinermal A (**42**) exhibited the strongest antiplasmodial activity *in vitro* with IC_{50} values of 6.69 μM against the poW strain (chloroquine-sensitive) and 11.3 μM against the Dd2 strain (chloroquine-resistant). Andirnermal C (**43**) was slightly less active and the values were 17.8 μM (poW) and 19.0 μM (Dd2), respectively [78].



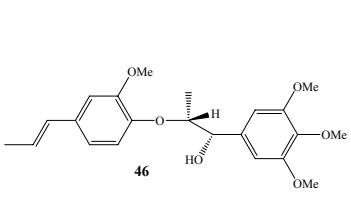
42 R=CH_3
43 R=H

Two 5-methylcoumarin epoxides and several 4-phenylcoumarins have been found to be active against *P. falciparum* strains *in vitro*. The first compounds were isolated from the roots of *Vernonia brachycalyx* Hoffm. (Asteraceae), an herb used by the Maasai, the Kipsigis and other East African tribes as a treatment for parasitic diseases [79]. Their structures were 2′-epicycloisobrachycoumarinone epoxide (**44**) and cycloisobrachycoumarinone (**45**) epoxide, both of which showed antiplasmodial activity against chloroquinesensitive (3D7) and chloroquine-resistant (Dd2) strains of *P. falciparum in vitro*. IC_{50} values for the strain 3D7 were 160 and 111 μM, respectively, while the IC_{50} values for the strain Dd2 were 54 μM for both compounds [79].

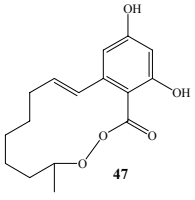
A second group of coumarins active against *Plasmodium* strains was isolated from the stem bark of *Exostema mexicanum* (Rubiaceae), used in Latin American folk medicine as a quinine substitute for malaria treatment. The most lipophilic compound, 4′,5,7,8-tetramethoxy-4-phenylcoumarin (*O*-methyl-exostemin), revealed the strongest antiplasmodial activity with IC_{50} values of 10.5 μM (poW strain *P. falciparum*) and 4.68 μM (Dd2 strain) [80].

Four coumarins, theraphins A-D, isolated from *Kayea assamica* King & Prain (Clusiaceae), an evergreen tree used as a remedy for treating fevers in India, were tested against a panel of human cancer cell lines to assay their cytotoxicity, and tested for antimalarial activity against the D6 (chloroquine-sensitive) and W2 (chloroquine-resistant) clones of *Plasmodium falciparum*. The constituents were characterized by a 1-hydroxypropyl moiety linked to C-4, a 1-oxobutyl moiety linked to C-8 and an isoprenyl chain linked to C-6. They showed modest antiplasmodial activities, with IC_{50} values in the range 9.7–11.1 μM against the D6 clone, and IC_{50} values in the range 5.1–10.4 μM against the W2 clone. However, their Selectivity Indices (SI=KB IC_{50}/*P. falciparum* IC_{50}) were less than 1.0, although the values for theraphin D (i.e. 11(S)-(-)-8,8-dimethyl-5-hydroxy-4-(1-hydroxypropyl)-10-(1-oxobutyl)-2*H*,8*H*-benzo(1,2-b:3,4-b′)di-pyran-2-one) were 4.70 and 5.02 for the D6 and W2 clones, respectively. These observations indicated that the coumarin derivatives possess little potential as antimalarial drugs, although appropriate structure modifications of some of them might improve the SI level leading to derivatives of greater antimalarial potential [81].

Among neolignan derivatives, polysyphorin (**46**), isolated from *Rhaphidophora decursiva* (Araceae), a vine growing in Vietnam, showed antiplasmodial activity. From the same plant was also isolated a new active benzoperoxide, rhaphidecurperoxin (**47**) [82]. Compounds **46** and **47** were tested against the oral epidermoid cancer line KB and cultures of the chloroquine-sensitive clone D6 and chloroquine-resistance clone W2 of *P. falciparum.* The IC_{50} of the neolignan was 0.92 μM (D6 strain) and 0.84 μM (W2 strain) with selectivity indices of 5 and 6, respectively, compared to the KB cell line.



46

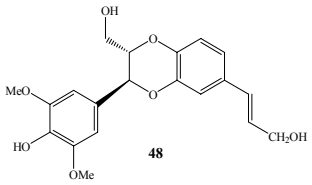


47

Therefore, compound **46** appears promising and further evaluation in *in vivo* antimalarial models should be pursued. It was also interesting to note that

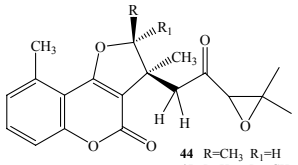
47, which contains a peroxide ester, a moiety similar to the endoperoxide bridge of artemisinin, showed a moderate antimalarial activity; its IC_{50} against the D6 strain was 1.76 μM and against W2 was 1.37 μM. However, due to its poor SI values (0.7 and 1, respectively), this compound is not considered of great interest as an antimalarial agent [82].

Recently the neolignan nitidanin (**48**) has been isolated from *Grewia bilamellata* Gagnep. (Tiliaceae). It displayed weak antimalarial activity in cultures of *P. falciparum* clones D6 and W2 (IC_{50} 21.2 and 18.4 μM, respectively). The same derivative tested against the human oral epidermoid carcinoma cell line (KB) showed a minimal cytotoxicity (ED_{50} >99.0 μM) and thus its selectivity index (SI) expressed as ED_{50} (KB)/IC_{50} (*P. falciparum*) was high against both *Plasmodium* clones (>4.6 and 5.4, respectively). Thus, this molecule could represent a model structure because several neolignans with antimalarial activity were previously reported, but were highly cytotoxic [83].



48

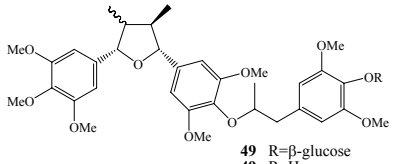
Several other lignans are reported to have antiplasmodial activity. Two of them, termilignan and anolignan B were isolated from *Terminalia bellerica* (Combretaceae), a species extensively used in the Indian system of traditional medicine for the treatment of fever, cough, diarrhea, dysentery and skin conditions [84]. These compounds were tested against the chloroquine-susceptible strain 3D7 of *Plasmodium falciparum* and showed IC_{50} values of 9.6 and 20.5 μM, respectively [84].



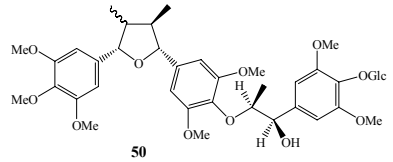
44 R=CH_3 R_1=H
45 R=H R_1=CH_3

Another antiplasmodial lignan was isolated from a palm, *Euterpe precatoria* Mart. (Aracaceae): it was the 8-5′linked lignan dehydrodiconiferyl dibenzoate showing a similar antiplasmodial activity. The IC_{50} value was 12 μM when the compound was tested against the chloroquine-sensitive 3D7 *Plasmodium falciparum*.[85]

Phytochemical investigation of the aerial parts of *Bonamia spectabilis* (Choisy) Hall. (Convolvulaceae) led to the isolation of some active tetrahydrofuran-type sesquilignans (**49-52**). The derivatives were tested for their antiplasmodial activity against a chloroquine-sensitive strain (PoW) and a chloroquine-resistant clone (Dd2) of *Plasmodium falciparum*. Bonaspectin C 4′′-O-glucoside (**49**), its aglycone (**49a**), and bonaspectin D 4′′-O-glucoside (**50**) revealed the highest antiplasmodial activities (IC_{50} values: 1.3, 2.0, 6.5 μM [PoW]; 1.7, 4.6, 3.7 μM [Dd2], respectively.

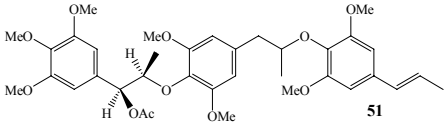


49 R=β-glucose
49a R=H

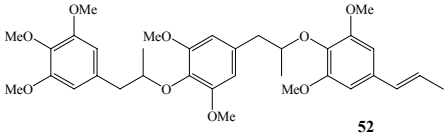


50

The sesquineolignans **51** and **52** revealed antiplasmodial activity with IC_{50} values of 9.9, 3.0 μM (PoW) and 10.9, 8.5 μM (Dd2), respectively.



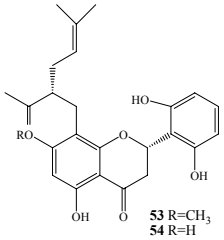
51



52

There was no significant difference of activity between the chloroquine-sensitive strain PoW and the chloroquine-resistant clone Dd2, however the phenylpropanoid dimers showed lower antiparasitic activities than the related trimers [86].

Several flavonoids, including biflavonoids, have been recognised for their antiplasmodial activity. Among them two flavanones, exiguaflavanone A (**53**) and exiguaflavanone B (**54**), were isolated from Artemisia indica from Thailand [87]. The assay was carried out with P. falciparum (K1, multidrug-resistant strain) and the constituents exhibited an IC_{50} of 10.8 μM and 16.0 μM, respectively [87].



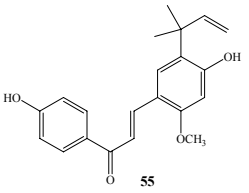
53 R=CH_3
54 R=H

Two flavones, 5,7,4'-trimethoxyflavone and 5,7,3',4'-tetramethoxyflavone isolated from *Kaempferia parviflora* (Zingiberaceae), another plant from Thailand, [88] showed a weak antiplasmodial activity (IC_{50} values were 11.9 and 12.5 μM, respectively).

Three flavonol glycosides (all kaempferol derivatives) isolated from *Hydrangea macrophylla* Seringe var. *thunbergii* Makino (Hydrangeaceae), a Japanese plant, were tested for the antimalarial properties and the cytotoxic activity against KB3-1 cells. The compounds exhibited characteristic anti-malarial activity: in particular, approximately 60% of proliferation of the parasite was inhibited even at the concentration of 0.5 ng/mL. On the other hand, these flavonol glycosides have little influence on the growth of KB 3-1 representing the host cell [89].

Other flavonoids, namely (R)-4''-methoxydalbergione, obtusafuran, 7,4'-dihydroxy-3'-methoxyisoflavone, and isoliquiritigenin, isolated from the heartwood of *Dalbergia louvelii*, inhibit the growth of *P. falciparum in vitro*. Their IC_{50} values ranged from 5.8 to 8.7 μM [90].

Five rotenoids, a chalcone and an isoflavone isolated from the stem bark of *Milletia usaramensis* subsp. *usaramensis* (Fabaceae), a plant of Kenya were tested against chloroquine-resistant (W2) and chloroquine-sensitive (D6) strains of *P. falciparum*. The chalcone 4'-O-geranylisoliquiritigenin was the most potent compound (IC_{50} values were 8.7 and 10.6 μM, respectively). Among the rotenoids, those containing a prenyl or a 2,2-dimethylpyrano substituent were most potent (IC_{50} values were between 19.4 and 70.1 μM) [91].

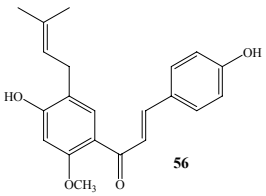


55

Another simple chalcone, licochalcone A (**55**) isolated from *Glycyrrhiza* species in different amounts, is reported as having *in vitro* and *in vivo* antimalarial activity [92, 93].

In *in vivo* tests against *P. yoelii* in mice, oral doses of 1000 mg/kg resulted in the complete eradication of the malaria parasite and no toxicity was noted [92]. *In vitro* the IC_{50} was 1.78 μM (0.6 μg/mL) on the chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) strain of *P. falciparum*. An intraperitoneal injection of 15 mg/kg/ of licochalcone A twice daily for three days led to the survival of mice infected with *P. yoelli* and clearance of parasites. Oral administration (50 mg/kg) of a suspension of this flavonoid to mice infected with *P. yoelli* revealed that after four days, that the animals were no longer infected, thus it is efficient in controlling the infection [93]. A further investigation on licochalcone A showed that it is a potent membrane-active agent that transforms normal erythrocytes into echinocytes in parallel with the inhibition of growth of *Plasmodium falciparum* cultures. Thus, the *in vitro* antiplasmodial effect apparently is an indirect effect on the host cell. This effect could also be transiently observed *in vivo* after intravenous administration of the compound, but the cells returned quickly to the normal shape, presumably as the result of redistribution of licochalcone A in lipophilic compartments of the body or removal of damaged erythrocytes [94]. Recently it has also been demonstrated that licochalcone A can inhibit the bc(1) complex (ubiquinol-cytochrome c reductase) as well as complex II (succinate ubiquinone reductase, SQR) of *Plasmodium falciparum* mitochondria at very low concentrations. Because the property of the *P. falciparum* bc(1) complex is different from that of the mammalian host, chalcones could be promising candidates for a new antimalarial drug [95].

Xanthohumol (**56**), an isomer of licochalcone A, and seven derivatives isolated from *Humulus lupulus* L (Cannabinaceae) were tested for their *in vitro* antiplasmodial activity against the chloroquine-sensitive strain poW and the multiresistant clone Dd2. Of the eight compounds tested, four possessed activity with IC_{50} values <25 μM against at least one of the two strains of *Plasmodium falciparum*. The main hop constituent, the chalcone xanthohumol, was the most active with IC_{50} values of 8.2 μM (poW) and 24.0 μM (Dd2). Three of these compounds were additionally active in the haemin-degradation assay [96].



56

Other prenylated chalcones isolated from *Crotalaria orixensis* L. (Fabaceae) have been tested for *in vitro* antiplasmodial activity against NF-54 chloroquine sensitive strains. The most active compound was 3',5'-diprenyl-4,2',4'-trihydroxy chalcone, which inhibited the parasites 100% at 5.09 μM [97]. Within the same study it was shown that substitution at the 4' and 4-hydroxyl groups decreases the activity. The presence of prenyl moieties can affect the activity positively especially with free 4,4'-dihydroxy systems [97].

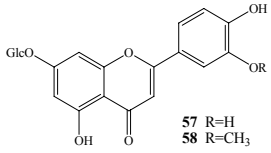
Structure-activity relationship studies of antimalarial chalcones were carried out using a series of forty oxygenated derivatives obtained by synthesis [98]. Good antimalarial activity was found among alkoxylated chalcones with polar A rings, in particular those substituted with electron-withdrawing groups or replaced by quinoline rings. The size characteristics of ring B (large, alkoxylated) and the electronic properties of ring A (electron deficient) are considered as important for antimalarial activity [98].

Two studies reported the antiplasmodial activity of prenylated flavonoids isolated from *Erythrina abyssinica* L. (Leguminose), a species widely used in Africa to treat infectious diseases. Flavonoids were tested against two *Plasmodium* strains: the chloroquine-sensitive D6 and the chloroquine-resistant W2 clones. Chalcones, flavanones and isoflavones with prenyl moieties showed weak activity against both strains with IC_{50} values ranging from 4.9 to 27.7 μM [99,100]. From another *Erythrina* species, *Erythrina sacleuxii*, several flavanones, isoflavones, and isoflavanones with isoprenyl moieties were isolated. These compounds displayed a similar antiplasmodial activity against the chloroquine-sensitive D6 and the chloroquine-resistant W2 *Plasmodium* strains. Their IC_{50} values ranged from 4.9 to 28.0 μM [101].

Three new prenylated flavonoids, namely the two flavanones 5,7,3'-trihydroxy-4',5'-(2''',2'''-dimethyl-pyran)-8,2'-di(3-methyl-2-butenyl)-(2*S*)-flavanone and 5,7,3'-trihydroxy-4'-methoxy-8,2'-di(3-methyl-2-butenyl)-(2*S*)-flavanone and the flavan 7,3', 4'-trihydroxy-6-methoxy-8,2'-di(3-methyl-2-butenyl)-(2*S*)-flavan, were isolated from the roots of *Dendrolobium lanceolatum* (Dunn) Schindl. (Fabaceae) and assayed against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain) and several cancer cell lines. They exhibited antimalarial activity with IC_{50} values of 5.3, 7.1, and 6.9 μM, respectively. However the flavanones were also cytotoxic, in particular the first flavanones showed strong cytotoxicity against the cancer cell lines KB, BC, and NCI-H187 with IC50 values of 2.4, 3.3, and 1.2 μM, respectively, while the latter showed moderate cytotoxicity against the NCI-H187 cell line with an IC_{50} value of 17.5 μM [102].

The dihydrochalcone, 2',4,6'-trihydroxy-4'-methoxydihydrochalcone (asebogenin) isolated from *Piper hispidum* Sw (Piperaceae), a species used by the indigenous population of Central America to treat malaria or fever, exhibited an IC_{50} of 56 μM for poW strains and 35 μM for Dd2 strains [103].

Two common flavone glycosides, luteolin 7-*O*-β-D-glucopyranoside (**57**) and chrysoeriol 7-*O*-β-D-glucopyranoside (**58**), isolated from *Phlomis brunneogaleata* Hub.-Mor. (Lamiaceae), were determined to be the major anti-malarial principles of this plant. Their IC_{50} values were 5 and 13 μM, respectively, using a K1 strain (chloroquine- and pyrimethamine-resistant). The same compounds, tested with skeletal myoblast L6 cells in order to evaluate their cytotoxicity, did not show any activity at the maximum tested dose of 90 μg/mL (about 200 μM). In addition, compound **57** showed a promising FabI-inhibiting effect (the IC_{50} was about 22.2 μM) [104].

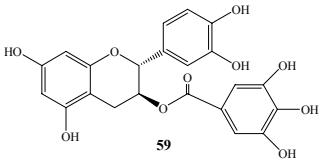


57 R=H
58 R=CH_3

Bioassay-guided fractionation of a *Satureja parvifolia* (Philippi) Epling. (Lamiaceae) MeOH extract led to the isolation, among others, of eriodictyol and luteolin as its active components against *Plasmodium falciparum* K1 strain. The IC_{50} value of luteolin was 22.3 μM while that of eriodictyol was 59.7 μM. Besides their moderate antiplasmodial activity, flavonoids showed a very low toxicity on the mammalian KB cell line and

eriodictyol was the most selective compound as a result of its rather low cytotoxicity (IC_{50} 604.2 μM) [105].

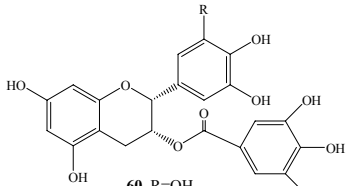
A very recent investigation reported on the inhibition by several flavonoids of different enzymes of *Plasmodium falciparum* fatty acid biosynthesis: α-ketoacyl-ACP-reductase (FabG), α-hydroxacyl-ACP-dehydratase (FabZ), and enoyl-ACP-reductase (FabI). About forty related structures were investigated and several compounds were found to have very good activity against all three enzymes. The flavones and flavonols exhibiting a simple substitution pattern (that is, no hydroxy groups on ring B and one or two hydroxy groups on rings A/C) show moderate inhibition effects toward FabG (10-100 μM), FabZ (20-30 μM), and FabI (10 μM) while flavonoids having more than one hydroxyl substitution on ring B exhibited strong activity toward all three enzymes (IC_{50} 0.5-8 μM). The methylation of any of the hydroxy groups in flavonols generally abolishes almost all activity against all three enzymes. Among the flavanones tested, only 5,7-dimethoxy-8-methylflavanone showed some inhibitory activity against FabZ (40 μM). The isoflavonoids tested showed moderate and selective activity only against FabZ with IC_{50} values in the range of 7-30 μM. The most active compounds were C-3 galloyl acid esters of catechins, which are strong inhibitors of all three enzymes (IC_{50} 0.2-1.1 μM). Catechins and epicatechins, carrying a free hydroxy group at C-3, neither inhibit the enzymes nor have antiplasmodial activity. This study suggests that flavonoids and analogues are promising antimalarial agents, thus adding new targets to the broad spectrum of biological activities demonstrated by these compounds [106].



59

Within the same investigation it was shown for the first time that C-3 galloyl acid esters of catechins had *in vitro* activity against chloroquine-sensitive (NF54) and -resistant (K1) *P. falciparum* strains in the low to submicromolar range. The most active compound was (-)catechin gallate (**59**) with EC_{50} values of 3.2 and 0.4 μM, respectively [106].

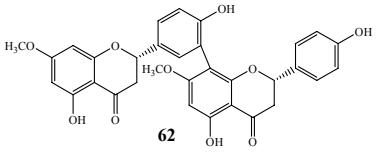
The same finding was also confirmed by another recent investigation [107]. Within this study two *P. falciparum* strains were investigated, namely 3D7, a chloroquine-sensitive one, and F9CR-1/FVO, a chloroquine-resistant one. Remarkably, pronounced plasmodicidal effects on both tested parasite strains were measured for (-)epigallocatechin gallate (**60**, IC_{50} 30 μM for 3D7 and 20 μM for F9CR-1/FVO), and (-)epicatechin gallate (**61**, IC_{50} 7 μM for 3D7 and 5 μM for F9CR-1/FVO).



60 R=OH
61 R=H

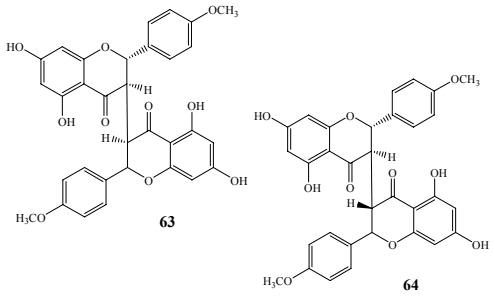
Furthermore a synergism was observed between artemisinin and these two derivatives on the 3D7 drug-sensitive parasite strain using sublethal doses of artemisinin, ranging from 1 to 10 nM, both of them in the presence (and in the absence) of 15 μM (-) epigallocatechin gallate (**60**) or of 5 μM (-) epicatechin gallate (**61**) [107].

Several biflavonoids have shown antiplasmodial activity. Among biflavanones, 7,7'-di-*O*-methyltetrahydromentoflavone (**62**) isolated from *Rhus retinorrhoea* (Anacardiaceae), a tree growing in the southern parts of Saudi Arabia, showed weak antiplasmodial activity but no cytotoxicity [108].



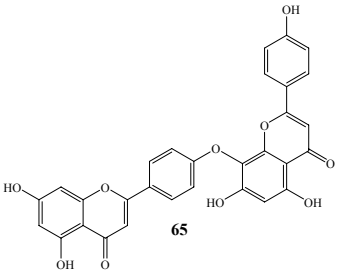
62

The compound exhibited weak antimalarial activity against *Plasmodium falciparum* (W2 clone) with an IC_{50} of 1.6 μM, and activity against *P. falciparum* (D6 clone) with an IC_{50} of 4.6 μM. [108].

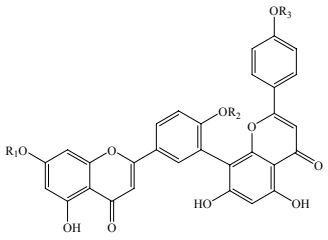


63 **64**

Two biflavanone isomers of **62,** namely sikokianin B (**63**) and sikokianin C (**64**) with moderate activity (IC_{50} values of about 1 μM) against a chloroquine-resistant strain (K1) and a drug-sensitive strain (FCR3) of *Plasmodium falciparum* were also isolated from *Wikstroemia indica* (Linne) C.A. Meyer (Thymelaeceae). Their activity for the K1 strain was nearly the same as chloroquine but they were less than 2% as active as artemisinin [109].



65



66 R_1= H, R_2=CH_3, R_3=H
67 R_1= CH_3, R_2=CH_3, R_3=H
68 R_1= H, R_2=CH_3, R_3=CH_3
69 R_1= CH_3, R_2=CH_3, R_3=CH_3

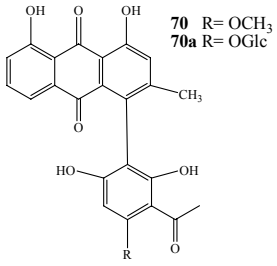
Recently, the antiplasmodial activity of eight other natural biflavones was evaluated [110]. Lanaroflavone (**65**) showed the highest antiplasmodial activity (IC_{50} of 0.48 μM) when studied *in vitro* on a K1 chloroquine-resistant strain of *Plasmodium falciparum*. Other biflavones of the amentoflavone type, namely bilobetin (**66**), ginkgetin (**67**), isoginkgogetin (**68**) and sciadopitysin (**69**) showed medium activity (IC_{50} values were 6.7, 2.0, 3.5, and 1.4 μM, respectively). Lanaroflavone also exhibited a high selectivity index value (SI=159), indicating selective antiplasmodial activity and no significant cytotoxicity [110].

A new biflavanoid, ent-naringeninyl-(I-3α,II-8)-4'-*O*-methylnaringenin, isolated from the root bark of *Garcinia livingstonei* collected in Tanzania, showed moderate activity against *P. falciparum* (chloroquine-sensitive Ghana strain); the IC_{50} was 6.7 μM. Within the same assay the biflavonoids (+)-volkensiflavone and (+)-morelloflavone were also tested and displayed IC_{50} values of 6.0 and 48.0 μM, respectively [111].

Several phenylanthraquinones showed considerable activity with only a little cytotoxicity as well, whereas the individual anthraquinone and phenyl moieties were completely inactive. Knipholone (**70**) and three of its natural derivatives, along with seven structurally-related but simplified compounds, have been examined for their antiplasmodial activity against asexual erythrocytic stages of two strains of *Plasmodium falciparum in vitro* (K1/chloroquine-resistant and NF54/chloroquine-sensitive). All the phenylanthraquinones showed considerable activity with IC_{50} values 0.38-2.37 μM for the K1 strain and 0.42-2.64 for the NF 54 strain. Knipholone (**70**) and its natural derivatives can therefore be considered a new group of potential antimalarials [112].

From another *Bulbine* species, *B. frutescens* (L.) Wild (Asphodelaceae), three novel phenylanthraquinones were isolated, namely 4'-O-demethyl-knipholone-4'-O-beta-D-glucopyranoside (**70a** a glycoside derivative of knipholone), and gaboroquinones A and B. These were tested against the chloroquine- and pyrimethamine-resistant K1 strain and against the strain NF54 of *P. falciparum* which is sensitive to all known drugs.

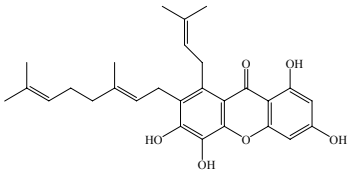
The glycoside **70a** displayed the best activity (IC_{50} 0.7 μM for both strains) and did not exhibit any cytotoxic effects on mammalian cells, at least at concentration below 0.15 mM, the highest concentrations tested [113].



70 R= OCH_3
70a R= OGlc

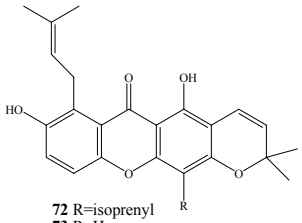
Morinda lucida is widely used in West Africa to treat malaria and other tropical diseases. Anthraquinones isolated from this plant have been tested against chloroquine–susceptible (3D7) and chloroquine-resistant (Dd2) strains. Their activity was moderate in both strains, with IC_{50} values between 21.4 and 87.8 μM. Structure-activity relationships studies showed that an aldehyde group at C-2 and a phenolic hydroxy group at C-3 enhance activity of these anthraquinones against *Plasmodium* strains [114,115]

Xanthones from *Garcinia dulcis* and *G. cowa* (Clusiaceae) have been investigated for antiplasmodial activity [116,117]. *G. cowa* is widely distributed in Thailand where it is used as an antipyretic, while *G. dulcis* is mostly known for its disinfective activity [116,117]. Among the five xanthones isolated from *G. dulcis*, the most active against chloroquine-sensitive strains of *P. falciparum* ((T9/94 line) is garciniaxanthone (**71**) with an IC_{50} of 2.06 μM. The presence of isoprenyl moieties at C-2, C-7 or C-8 enhanced the antiplasmodial activity [116,117].

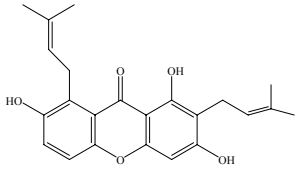


71

Recently, twenty-two xanthones isolated from *Calophyllum caledonicum* and *Garcinia vieillardii*, (Clusiaceae) were tested against chloroquine-resistant strains of *Plasmodium falciparum* (FcB1/colombia) [118]. The most potent xanthones were found to be **72**, **73** and **74** (IC_{50} of c.a. 1.0 μg/mL) which are 1,3,7 trioxygenated and prenylated at the positions 2 and 8. The relationship between antimalarial activity and molecular structure of xanthones has been explored. Firstly, the position of the hydroxyl groups appears to be important, as indicated by the observed differences in activity. Indeed, oxygenation at the positions 1, 3 and 7, seems to improve antimalarial activity. Secondly, substitution with a 1,1-dimethylallyl chain or the presence of an additional pyran ring appear to be activity-enhancing factors, as well as substitution with two isopentenyl chains or combination of one isopentenyl chain and a pyranic ring. Moreover, hydroxylation of the prenyl side chain is not required for activity [118].



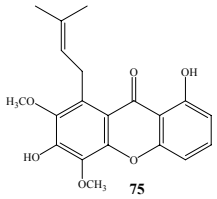
72 R=isoprenyl
73 R=H



74

In addition, the *in vivo* antimalarial activity of some hydroxyxanthones was recently demonstrated for the first time [119].

Another study reported on a series of oxygenated xanthones which were synthesized and evaluated *in vivo*, using four-day suppressive assays against *Plasmodium berghei* ANKA in BALB/c mice. When given at a dose of 20 mg/kg/day for four days, most of the compounds produced significant chemosuppression of parasitaemia. The most active compound was 1,3,6,8-tetrahydroxyxanthone, which reduced the percentage of erythrocytes infected by 70.5%, followed by norlichexanthone (44.3%) and its isomer, 1,3,8-trihydroxy-6-methylxanthone (37.0%). While di-*C*-allyl-dihydroxyxanthone showed lower but still notable activity (33.4%), 1,3-dihydroxyxanthone was much less active (15.1%). This is the first demonstration of the antimalarial activity of some hydroxyxanthones *in vivo* [119]. In a different investigation, four xanthones isolated from the roots of *Andrographis paniculata* Nees (Acanthaceae), namely 1,8-di-hydroxy-3,7-dimethoxy-xanthone, 4,8-dihydroxy-2,7-dimethoxy-xanthone,1,2-dihydroxy-6,8-dimethoxyxanthone and 3,7,8-trimethoxy-1-hydroxy xanthone, were assayed *in vitro* using a chloroquine-sensitive strain FSG. 1,2-Dihydroxy-6,8-dimethoxy-xanthone was the most active (IC_{50} of 4 μg/mL), and it was tested *in vivo* in mice with a *Plasmodium berghei* infection using the Peters' 4-day test. A substantial reduction (62%) of parasitaemia was observed in mice with a 30 mg/kg dose. *In vitro* cytotoxicity against mammalian cells revealed that 1,2-dihydroxy-6,8-dimethoxy-xanthone is non-cytotoxic with an IC_{50}>32μg/mL [120].



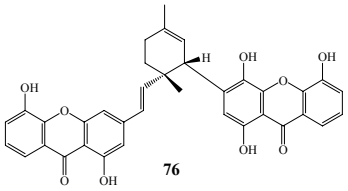
75

Finally, in 2006 several papers reported on the antimalarial activity of some natural xanthones. A new prenylated xanthone, 5-O-methylcelebixanthone (**75**), together with six related constituents from the roots of Cratoxylum cochinchinense (Lour.) Blume (Clusiaceae) have been tested for antiplasmodial and cytotoxic activity. Four derivatives including the new one showed cytotoxic activity against the human lung cancer cell line (NCI-H187) with IC_{50} values ranging from 1.4 μM to 0.011 mM. In the same concentration

ranges they showed antimalarial activity against Plasmodium falciparum with IC_{50} values from 5.62 μM and 0.015 mM [121].

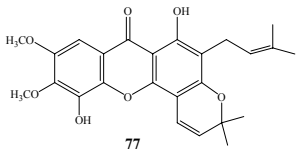
Five other previously known prenylated xanthones isolated from the root bark of *Garcinia livingstonei* collected in Tanzania, were tested against a chloroquine-sensitive Ghana strain of *P. falciparum*. The dimeric xanthone garcilivin A (**76**) showed the highest antiparasitic activity (IC_{50} 6.7 μM) but it was cytotoxic in the same range of concentration (IC_{50} 2.0 μM against MRC-5 cells). Its diastereoisomer garcilivin C and the monomeric xanthones showed IC_{50} values ranging from 10 to 68 μM against *Plasmodium* with remarkable selectivity against MRC-5 cells (IC_{50}>32 μM) [111].

Three polyprenylated structurally related xanthones (gaboxanthone, symphonin and globuliferin) isolated from *Symphonia globulifera* L (Guttiferae), a tree whose bark is used in the Northwestern province of Cameroon to treat malaria, were tested for their anti-plasmodial activity against the W2 strain of *P. falciparum*, which is resistant to chloroquine and other antimalarials.



76

They all exhibited good to moderate activity relative to chloroquine, and symphonin (**77**) had the best potency (IC_{50} was 1.29 μM). From the structure–activity relationship, it appeared that the cyclization of one of the isopentenyl groups (positions 2 and 4) to give a pyran ring increases the potency of xanthones. The best result was obtained when the dimethylpyran ring is attached to positions 3 and 4 of the xanthone nucleus as in symphonin (**77**) [122]. A benzophenone, guttiferone A was also isolated and found to be moderately active (IC_{50} 3.17 μM) [122].

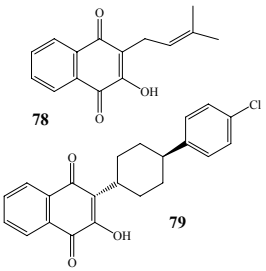


77

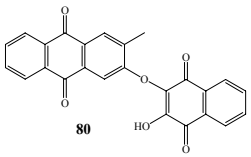
A further study on prenylated xanthones was carried out on a new prenylated xanthenedione, 1,2-dihydro-3,6,8-trihydroxy-1,1,7-tri(3-methylbut-2-enyl) xanth-en-2,9-dione and five known xanthones isolated from the stem bark of *Allanblackia monticola* Staner L.C. The compounds were tested on two strains of *Plasmodium falciparum*, F32 (chloroquine sensitive) and FcM29 (chloroquine resistant). The IC_{50} values obtained ranged from 1.4 to 21 μM. Their cytotoxicity was estimated on human melanoma cells (A375) and the cytotoxicity/antiplasmodial ratio was found to be between 40 and 70 [123].

Other Constituents

Lapachol (**78**), a simple hydroxynaphthoquinone, is known for many pharmacological properties including antimalarial activity. It is present in many members of the Bignoniaceae family and it has been used as a template for the synthesis of the antimalarial drug atovaquone (**79**) [124].



In a very recent paper a naphthoquinone–anthraquinone coupled pigment named newbouldiaquinone A (**80**) together with other naphthoquinones isolated from *Newbouldia laevis* Seem. (Bignoniaceae), a tropical African species widely used for the treatment of several diseases including malaria, were tested *in vitro* against *P. falciparum* NF54 and R strains [125].

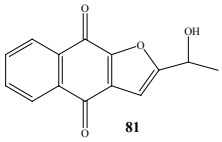


80

The most active compounds were newbouldiaquinone A, lapachol, α-lapachone and β-lapachone which showed a moderate suppression of parasitic growth [125].

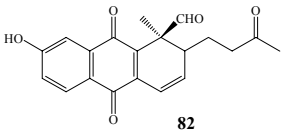
Several papers report the isolation of active naphthoquinones from Bignoniaceae. Five furanonaphthoquinones isolated from *Tabebuia ochracea* ssp. *neochrysantha* (Bignoniaceae), a plant used traditionally in the Amazon to treat malaria, were tested against *P. falciparum* and *P. berghei in vitro*. The most active constituent was represented by a mixture of two compounds that could not be

separated: 5- and 8-hydroxy-2-(1'-hydroxy ethyl)naphtho[2,3-*b*]furan-4,9-dione. The IC_{50} values obtained with this mixture were 0.17 μM (against *P. berghei*) and 0.67 μM (against FcB2 chloroquine-resistant strain of *P. falciparum*). For the former parasite, the IC_{50} value for chloroquine was 0.05 μM, while for *P. falciparum* the IC_{50} value was 0.11 μM. These results indicate that the furanonaphthoquinones isolated from *T. ochracea* are potential antimalarial compounds [126]. Four naphthoquinoids isolated from *Kigelia pinnata* (Bignoniaceae) root bark were assessed *in vitro* against chloroquine-sensitive (T9-96) and chloroquine-resistant (K1) *Plasmodium falciparum* strains and for cytotoxicity using KB cells. The most active one, 2-(1-hydroxyethyl)naphtho[2,3-*b*]furan-4,9-dione (**81**), has good activity against both strains; IC_{50} values were 627 nM for the K1 strain and 718 nM for the T9-96 strain [127].



81

Several novel structurally related, prenylated naphthoquinones (sterekunthals A and B, pyranokunthones A and B) and one novel prenylated anthraquinone (anthrakunthone) isolated from the root bark of *Stereospermum kunthianum* Cham (Bignoniaceae), a plant used in Uganda to treat fever, have been tested against the chloroquine-sensitive strain poW and the chloroquine-resistant clone Dd2. The quinones showed different degrees of activity against the two strains of *P. falciparum* and sterekunthal A (**82**) was the most effective one [IC_{50} values: 3.85 μM (PoW); 1.18 μM (Dd2)].



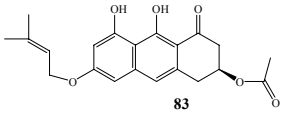
82

It was also shown that the 4-hydroxy group is an important structural feature for the antiplasmodial activity of these compounds, as sterekunthal B is distinctly less active than pinnatal [128]. The IC_{50} values were comparable to those of related naphthoquinones isolated from *Kigelia pinnata* DC [127]. On the other hand, these compounds also exhibited marked toxicity against endothelial ECV-304 cells and hence their antiplasmodial effect seems to be due to general cytotoxicity [129].

A number of isofuranonaphthoquinones isolated from *Bulbine capitata* Poelln. (Asphodelaceae) showed only weak antiplasmodial activity both against the 3D7 (chloroquine-sensitive) and the K1 (chloroquine-resistant) strains. The plant is used in Botswana for its claimed antibiotic and antipyretic properties. The IC_{50} values for both strains were between 23 and 92 μM , suggesting that these compounds are unlikely to have a significant *in vivo* activity when used alone [129].

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), isolated from *Nephenthes thorelii*, a species related to *N. ampullaria* and used to treat malaria in Malaysia, was active against *P. falciparum*, with an IC_{50} value of 0.27 μM. The quinone structure is believed to be essential for the activity whereas the presence of a heteroatom such as oxygen or chlorine in synthetic derivatives at position 3 of the naphthoquinone nucleus causes weakening or loss of activity [130].

Another interesting group of constituents tested for antimalarial activity are the anthranoids. A highly active derivative of this class is vismione H (**83**), isolated from *Vismia guineensis* (Clusiaceae). The IC_{50} against the sexual erythrocytic stages of *P. falciparum* (NF 54, clone A1A9) was 0.23 μM [131].



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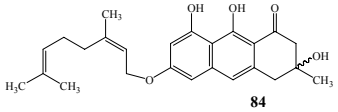

84

From another species of Vismia, *V. orientalis* Engl., a plant used in traditional medicine in Tanzania, vismione D (84) was isolated and exhibited antiprotozoal activity against *Plasmodium falciparum* strain K1 (IC_{50} 2.4 μM). However, it was also found slightly cytotoxic against human L6 cells (IC_{50} 10 μM) [132]

Concluding remarks

The prevalence of malaria in tropical zones worldwide, together with the lack of a vaccine and the appearance of strains of malaria parasite resistant to commercially available anti-malarial drugs based

on quinoline derivatives, makes the search for new effective anti-malarial drugs a global demand.

From the examination of the literature of the last decades it appears that a large number of plants used as antimalarial in the traditional medicine or related species have been investigated. Bioassay-guided fractionation of the extracts was generally used to find the active constituents and a large number of non-nitrogenous molecules have been found to possess a moderate to high *in vitro* antiplasmodial activity. However, only a few compounds have also been tested for *in vivo* antimalarial activities. Based on the literature compilation reported here the following three main conclusions can be drawn.

Firstly, only a few molecules result possessing a moderate to high activity and therefore should be considered for further investigations. They including peroxide sesquiterpenes, quinoid triterpenes, quassinoids, gallic acid derivatives, lignans, flavonoids and biflavonoids, xanthones, naphthoquinones and phenylanthraquinones.

Secondly, cytotoxicity of many of these derivatives has been evaluated in order to obtain the selectivity index, and results indicate that cytotoxicity and antimalarial activity are generally not correlated. It would be highly advantageous to consider these molecules as potential new antimalarial drugs.

Thirdly, although some of the investigated compounds are not particularly active, they are nevertheless interesting because they might strengthen chloroquine activity or restore chloroquine sensitivity in resistant strains of *P. falciparum.* Partially effective treatments might be beneficial in that the course of the disease is shortened, perhaps reducing anaemia and lowering the risk of death or serious illness from other anaemia-related diseases. Other possible benefits could be the alleviation of symptoms such as pain and fever and immunomodulation leading to increased immunity.

Another important aspect, not yet developed, is the search for molecules with little or no antiplasmodial activity which can synergistically act with known antimalarial drugs against *Plasmodium*. Thus, it is known that several flavonoids of *A. annua* can promote and enhance the antiplasmodic activity of artemisinin [133, 134], and recently it has been demonstrated that epigallocatechin gallate, epicatechin gallate and green tea extract not only have moderate antiplasmodial activity but also produce synergism in the presence of sublethal doses of artemisinin [107]. Also these molecules could have an important role in fighting malaria.

Acknowledgments - The financial support of MIUR (PRIN 2004) and Ente Cassa di Risparmio di Firenze is gratefully acknowledged.

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Natural Product Communications 1 (1-12) 1-1204 (2006)

ISSN 1934-578X (print)
ISSN 1555-9475 (online)

NPC Natural Product Communications

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