fractions (fr. 3d-1 to 3d-11). Compounds **3** (5 mg), and **8** (5 mg) were isolated from repeated column chromatography of fraction 3d-3 (16 mg) and 3d-9 (16 mg), respectively. Repeated column chromatography of fraction 3d-8 (381 mg) with CH_2Cl_2–MeOH of increasing polarity, followed by a Sephadex LH20 column (in MeOH), yielded **1** (9.5 mg). A portion of fraction 4 (17.0 g) was fractionated by silica gel chromatography, and eluted with CH_2Cl_2–MeOH of increasing polarity to provide 8 fractions (fr. 4a to 4h), in which **7** (9 mg) and **5** (3.5 mg) were obtained from two individual repeated column chromatographies (silica gel, eluting with $CHCl_3$–MeOH of increasing polarity) of fractions 4d (0.5 g) and 4e (1.6 g), respectively. Fraction 5 (15.4 g) was chromatographed in a similar manner to afford 14 selected fractions (fr. 5a to 5n). **9** (14 mg) and **6** (16 mg) were furnished from two successive column chromatographies (silica gel, $CHCl_3$-MeOH of increasing polarity) of fractions 5e (270 mg) and 5f (279 mg), respectively. All known compounds were identified by comparison of their spectroscopic data (NMR and MS) with those reported in the literature. Complete ^{13}C-NMR data of **5** and **6**, which have not previously been published, are also reported in this paper.

3-O-(4-hydroxy-3-methoxybenzoyl)ceanothic acid (**1**): Colorless solid, mp 183–185 °C. $[\alpha]_D^{27}$ -22.0° (c=0.229, MeOH). UV λ_{max} (MeOH) nm (log ε): 216 (4.44), 263 (4.07), 294 (3.89). IR (KBr) cm^{-1}: 3430, 2943, 1699, 1602, 1512, 1458, 1378, 1281, 1216, 1104, 1032, 985, 884, 765. HR-FAB-MS *m/z*: 635.3592 $[M-H]^-$ (calcd for $C_{38}H_{52}O_8$-H 635.3584). FABMS *m/z* 635 ($[M-H]^-$, 22), 423 (10), 219 (10), 205 (15), 167 (100). ^{1}H- and ^{13}C-NMR (C_5D_5N): Table 1.

2-*O-E-p-Coumaroylalphitolic acid* (**5**): ^{13}C-NMR (C_5H_5N) δ: 178.8 (s, C-28), 167.5 (s, C-9′), 161.4 (s, C-4′), 151.3 (s, C-20), 144.7 (d, C-7′), 130.5 (d, C-2′), 130.5 (d, C-6′), 126.2 (s, C-1′), 116.8 (d, C-3′ and C-5′), 116.0 (d, C-8′), 110.0 (t, C-29), 79.8

(d, C-3), 73.8 (d, C-2), 56.5 (s, C-17), 55.7 (d, C-5), 50.8 (d, C-9), 49.8 (d, C-18), 47.7 (d, C-19), 45.0 (t, C-1), 42.9 (s, C-14), 41.1 (s, C-8), 40.5 (s, C-4), 38.8 (s, C-10), 38.6 (d, C-13), 37.6 (t, C-22), 34.6 (t, C-7), 32.8 (t, C-16), 31.1 (t, C-21), 30.2 (t, C-15), 29.1 (q, C-23), 26.0 (t, C-12), 21.3 (t, C-11), 19.4 (q, C-30), 18.7 (t, C-6), 17.4 (q, C-24 and C-25), 16.3 (q, C-26), 14.8 (q, C-27).

Alphitolic acid (**6**): ^{13}C-NMR (C_5H_5N) δ: 179.1 (s, C-28), 151.3 (s, C-20), 110.0 (t, C-29), 83.3 (d, C-3), 68.9 (d, C-2), 56.7 (s, C-17), 56.1 (d, C-5),[a] 51.0 (d, C-9), 49.8 (d, C-18), 48.2 (t, C-1), 47.8 (d, C-19), 42.9 (s, C-14), 41.2 (s, C-8), 39.9 (s, C-4), 38.7 (s, C-10),[a] 38.6 (d, C-13), 37.6 (t, C-22), 34.8 (t, C-7), 32.9 (t, C-16), 31.2 (t, C-21), 30.2 (t, C-15), 29.2 (q, C-23), 26.1 (t, C-12), 21.4 (t, C-11), 19.5 (q, C-30), 18.8 (t, C-6), 17.7 (q, C-25), 17.5 (q, C-24), 16.5 (q, C-26), 14.9 (q, C-27), ('a' indicates for the assignments may be reversed for signals with the same superscript).

Antimycobacterial assay The antimycobacterial activity was assessed against *M. tuberculosis* H_{37}Ra using the Microplate Alamar Blue Assay.[22] Briefly, initial candidate compound dilutions were prepared in DMSO, and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC medium in the microculture plates. 100 μl of 5×10^4 CFU/ml of *M. tuberculosis* in 7H9GC-Tween was added to each well of 96-well microculture plates containing the test compound. Plates were incubated at 37 °C for 7 d. To three control wells which contained drug and medium, bacteria and medium, or medium only, Alamar Blue dye solution (20 μl of Alamar Blue solution and 12.5 μl of 20% Tween) was added daily until a color change from blue to pink occurred, at which time the dye was added to all remaining wells. Plates were incubated at 37 °C, and results were recorded at 24h post-dye addition. Fluorescence was measured in a Cytofluoro Series 4000 Fluorescence Multi-Well Plate Reader (Per-Septive Biosystems, Framingham, MA, USA) in bottom-reading mode with excitation

at 530 nm and emission at 590 nm. Percent inhibition was defined as (1- test well FU/mean FU of triplicate control wells) × 100. The lowest drug concentration effecting inhibition of ≥ 90% was considered the MIC. Experiments were usually completed within 10 d. Standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.004, 0.06 and 2.5 μg/ml, respectively.

Antiplasmodial assay Antiplasmodial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain) which was cultured continuously according to the method of Trager and Jensen.[23] Quantitative assessment of antiplasmodial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al.*[24] The inhibitory concentration is that which causes 50% reduction in parasite growth, as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng/ml was observed for the standard drug, artemisinin, in the same test system.

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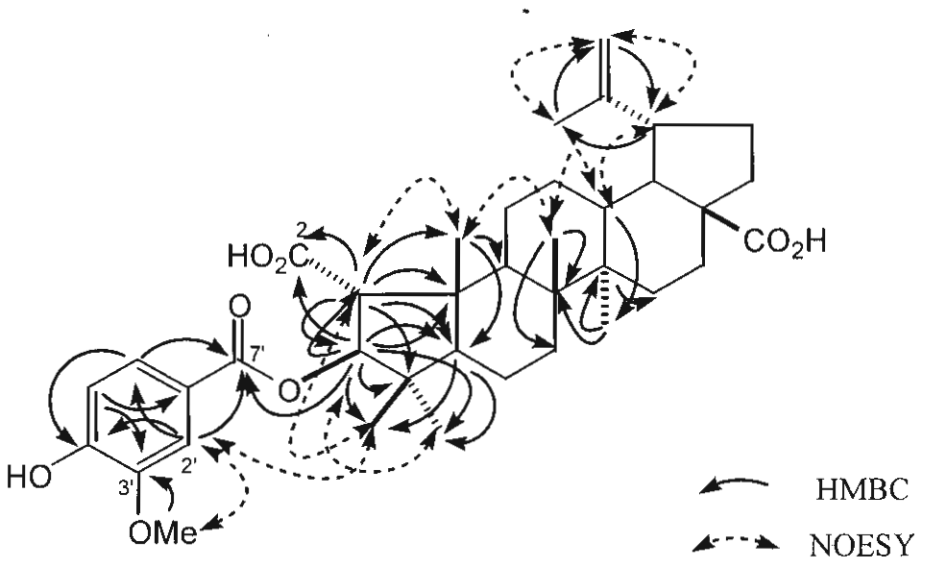
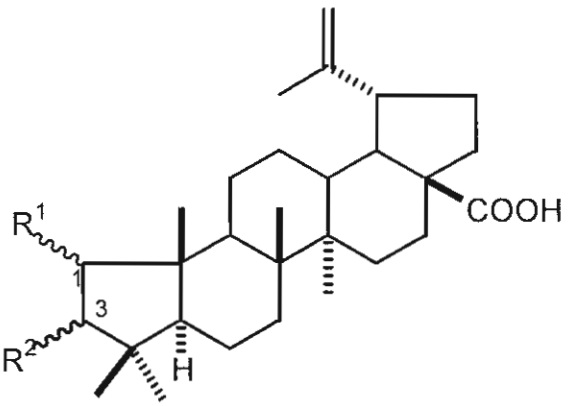
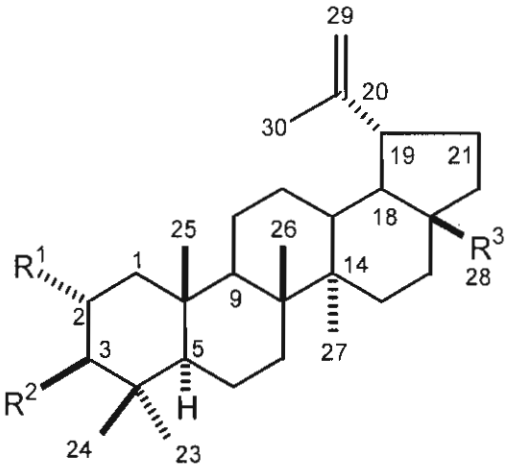
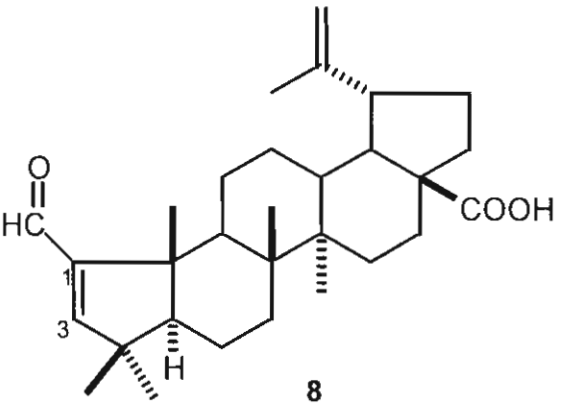


Fig. 1. HMBC and NOESY Correlations of Compound **1**



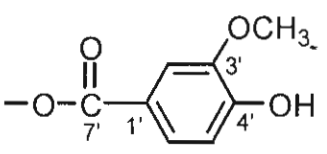
1 $R^1=\alpha$ COOH, $R^2=\beta$ **A**
7 $R^1=\beta$ CHO, $R^2=\alpha$ OH
9 $R^1=\alpha$ COOH, $R^2=\beta$ OH



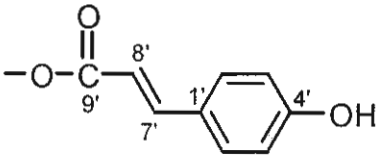


8

2 R^1=H, R^2=OH, R^3=CH_3
3 R^1=H, R^2=OH, R^3=CHO
4 R^1=H, R^2=OH, R^3=COOH
5 R^1=**B**, R^2=OH, R^3=COOH
6 R^1=R^2=OH, R^3=COOH



A



B

Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1** (C_5D_5N)

Position	δ_C	δ_H
1	64.3	3.15 (1H, s)
2	176.8	
3	86.0	5.94 (1H, s)
4	43.8	
5	56.9	2.18 (1H, m)
6	18.8	1.43 (2H, m)
7	34.6	1.38 (2H, m)
8	43.5	
9	45.4	2.12 (1H, m)
10	49.6	
11	24.2[a]	2.15 (1H, m), 1.58 (1H,m)[b]
12	26.3[a]	1.97 (1H, m), 1.23 (1H, m)[b]
13	39.1	2.74 (1H, br t, *J*=10.1 Hz)
14	42.2	
15	30.5	1.86 (1H, m), 1.20 (1H, m)
16	32.9	2.57 (1H, br d, *J*=12.3 Hz), 1.47 (1H, m)
17	56.6	
18	49.6	1.66 (1H, m)
19	47.6	3.47 (1H, m)
20	151.1	
21	31.3	2.17 (1H, m), 1.20 (1H, m)
22	37.6	2.18 (1H, m), 1.51 (1H, m)
23	30.7	1.53 (3H, s)
24	20.3	1.11 (3H, s)
25	18.5	1.23 (3H, s)
26	17.1	1.10 (3H, s)
27	15.0	1.02 (3H, s)
28	179.0	
29	109.8	4.82 (1H, br s), 4.62 (1H, br s)
30	19.7	1.63 (3H, s)
1′	121.9	
2′	116.4	7.86 (1H, br s)
3′	148.5	
4′	153.4	
5′	113.5	7.28 (1H, br d, *J*=8.2 Hz)
6′	124.6	7.90 (1H, br d, *J*=8.2 Hz)
7′	166.3	
OMe	55.8	3.75 (3H, s)

[a, b] Interchangeable within a column.

Table 2. IC_{50} Values for Antiplasmodial and MIC Values for Antimycobacterial Activities of Triterpenes **1**- **9**

Compound	IC_{50} (μg/ml)	MIC (μg/ml)
3-*O*-vanillylceanothic acid (**1**)	3.7	25
lupeol (**2**)	inactive[a]	inactive[b]
betulinaldehyde (**3**)	6.5	25
betulinic acid (**4**)	inactive[a]	25
2-*O*-*E*-*p*-coumaroylalphitolic (**5**)	0.9	12.5
alphitolic acid (**6**)	inactive[a]	50
zizyberanalic acid (**7**)	inactive[a]	50
zizyberenalic acid (**8**)	3.0	100
ceanothic acid (**9**)	inactive[a]	inactive[b]

[a] Inactive at 10 μg/ml.
[b] Inactive at >200 μg/ml.

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Ms. Ref. No.: STEROIDS-D-05-00002

Biotransformations of 20-hydroxyecdysone and analogues by *Curvularia lunata* NRRL 2178

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Abstract

20-Hydroxyecdysone, the arthropod moulting hormone, was biotransformed by the fungus *Curvularia lunata* NRRL 2178 to the rare ecdysteroid, 2-dehydro-3-*epi*-20-hydroxyecdysone, and the novel 3α,9α-cyclo ecdysteroid analogue, (20*R*,22*R*)-3β,14α,20,22,25-pentahydroxy-3α,9α-cyclo-5β-cholest-7-en-2,6-dione in 14 and 44% yields, respectively. Ponasterone A and pterosterone were similarly biotransformed to the corresponding 2-dehydro-3-*epi*- and 3α,9α-cyclo-analogues.

Keywords: Ecdysteroid; 2-Dehydro-3-*epi*-20-hydroxyecdysone; 3α,9α-Cyclo ecdysteroid; Biotransformation; *Curvularia lunata* NRRL 2178

1. Introduction

Ecdysteroids are arthropod moulting hormones found in both invertebrates and plant species [1,2]. 20-Hydroxyecdysone (**1**) is the representative of this class of compound. 2-Dehydro and 3-dehydro ecdysteroids are rare ecdysteroids of interest in

Structures **1**, **7**, **8** ---------->

view of their metabolism, moulting activity and synthesis [3–7]. 2-Dehydro-3-*epi*-20-hydroxyecdysone (**2**) is the 2-dehydro analogue of **1** isolated in only 0.15 mg quantity from the seeds of *Froelichia floridana* [8]. 3-Dehydro-20-hydroxyecdysone (**3**) and its 20-deoxy analogue **4** are ecdysteroids detected in some insects [3,9], whereas 3-dehydro-2-deoxyecdysone (**5**), the 2-deoxy analogue of **4**, was another rare ecdysteroid isolated from a plant [10] and an insect [11] species. As part of our work on the synthesis of 2- and 3-dehydro analogues of ecdysteroids [12,13], we have investigated alternative methods of synthesizing these compounds and microbial transformation of ecdysteroids was among them.

Structures **2**, **9**, **10**, and structures **3–5** ---------->

A number of fungi including *Curvularia lunata* NRRL 2178 have been selected for our screening. *C. lunata* is known to effect hydroxylation, especially 11β-hydroxylation of steroids [14], whereas oxidation of hydroxyl group to the corresponding keto function [15,16] is not a common biotransformation. It has been reported that *C. lunata* degraded the side chains of ecdysteroids to yield poststerone and rubrosterone [17]. We now report on the conversion of **1** by *C. lunata* NRRL 2178 to 2-dehydro-3-*epi*-

20-hydroxyecdysone (**2**) and the unprecedented 3α,9α-cyclo ecdysteroid analogue, (20*R*,22*R*)-3β,14α,20,22,25-pentahydroxy-3α,9α-cyclo-5β-cholest-7-en-2,6-dione (**6**) in 14 and 44% yields, respectively. The biotransformations of ponasterone A (**7**) and pterosterone (**8**) to the corresponding 2-dehydro-3-*epi*-ecdysteroids **9** and **10** (10 and 7% yields), and the 3α,9α-cyclo ecdysteroid analogues **11** and **12** (48 and 35% yields) are also reported.

Structures **6**–**12** ---------->

2. Experimental

2.1. General methods

Melting point was determined using an Electrothermal melting point apparatus and is uncorrected. Optical rotations were recorded on a Jasco DIP-370 digital polarimeter. UV spectra were recorded in MeOH on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were recorded in KBr on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H and ^{13}C NMR were recorded on a Bruker AVANCE 400 spectrometer operating at 400 and 100 MHz, respectively. For the spectra taken in pyridine-d_5, the residual nondeuterated solvent signal at δ 8.71 and the solvent signal at δ 149.90 were used as references for ^{1}H and ^{13}C NMR spectra, respectively. FABMS and ESMS were measured on a Finnigan MAT 90 and a Finnigan LC-Q mass spectrometer. APCI-TOFMS were measured with a Bruker MicroTOF mass spectrometer. Merck silica gel 60 (finer than 0.063 mm) and precoated silica gel 60 F_{254} plates were used for column chromatography

and TLC, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

2.2. Microorganism, media and culture conditions

The stock culture of *Curvularia lunata* NRRL 2178 was maintained on a potato dextrose agar slant. Erlenmeyer flasks (250 ml), each containing 100 ml of liquid medium consisting of 0.1% peptone, 0.1% yeast extract, 0.1% beef extract and 0.5% glucose [16] were inoculated with freshly obtained *C. lunata* cultured from the agar slant on a rotary shaker at 200 rpm. After cultivation at ambient temperature for 72 h, the ecdysteroid solution (10 mg of ecdysteroid dissolved in 100 µl DMSO and 5 µl Tween 80) was added to each flask, and the incubation continued for 14-17 days. Culture control consisted of fermentation blank in which *C. lunata* was grown under identical condition but without ecdysteroid substrate.

2.3. Biotransformation of 20-hydroxyecdysone (1)

20-Hydroxyecdysone (**1**) (10 mg × 28) was fed to *C. lunata* NRRL 2178 in Erlenmeyer flasks (250 ml × 28), each containing 100 ml of the same medium as outlined above. After 17 days the culture was filtered and the mycelium was washed with *n*-BuOH. The broth was extracted with *n*-BuOH, washed with H_2O and the solvent evaporated under reduced pressure by co-distillation with H_2O. The crude products (450 mg) was subjected to column chromatography eluting with CH_2Cl_2–MeOH (92:8) to give compound **6** (48 mg, 44% based on the unrecovered starting material), compound **2** (20 mg) which was contaminated by a minor component, and the starting material **1** (170

mg). The impure **2** was further purified by reversed phase HPLC (column: Hypersil BDS C18, 5 μm, 4.6 × 250 mm, mobile phase: MeOH–H_2O (40:60), flow rate: 0.5 ml/min, detector: 254 nm) to yield 2-dehydro-3-*epi*-20-hydroxyecdysone (**2**) (15 mg, 14%; t_R 27.61 min) and a small quantity (< 1 mg; t_R 31.63 min) of an unstable compound.

Compound **2**: Amorphous; $[\alpha]_D^{25}$ +47.3 (c 0.06, MeOH); UV, λ_{max} (nm) 241 (log ε 4.06); IR, ν_{max} (cm^{-1}): 3402, 2964, 2830, 1718, 1662, 1552, 1452, 1383, 1315, 1208, 1072; ^1H NMR (pyridine-d_5, 400 MHz): δ 1.09 (3H, s, 19-Me), 1.16 (3H, s, 18-Me), 1.39 (2 × 3H, s, 26-Me, 27-Me), 1.54 (3H, s, 21-Me), 2.05 (1H, partially overlapping signal, H-4α), 1.85 (1H, obscured signal, H-24a), 2.28 (1H, obscured signal, H-24b), 2.40 (1H, d, J = 13.6 Hz, H-1α), 2.44 (1H, obscured signal, H-4β), 2.73 (1H, d, J = 13.6 Hz, H-1β), 2.88 (1H, overlapping signal, H-5), 2.92 (1H, overlapping signal, H-17), 3.27 (1H, m, H-9), 3.85 (1H, brd, J = 9.1 Hz, H-22), 4.60 (1H, dd, J = 11.7, 7.2 Hz, $W_{½}$ = 22 Hz, H-3), 6.20 (1H, brs, H-7); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 17.8 (C-18), 20.9 (C-11), 21.4 (C-16), 21.6 (C-21), 23.0 (C-19), 27.5 (C-23), 30.0 (C-26), 30.1 (C-27), 31.5 (C-15), 31.8 (C-12), 36.2 (C-4, C-9), 42.6 (C-24), 43.0 (C-10), 48.1 (C-13), 49.2 (C-1), 50.0 (C-17), 55.8 (C-5), 69.6 (C-25), 74.9 (C-3), 76.8 (C-20), 77.5 (C-22), 84.0 (C-14), 121.2 (C-7), 165.2 (C-8), 200.3 (C-6), 209.9 (C-2); ESMS (–ve): *m/z* (% rel. intensity) 477 $[M–H]^-$ (100); HR-FABMS (–ve), *m/z*: 477.2858 $[M–H]^-$. $C_{27}H_{42}O_7$–H requires 477.2852.

Compound **6**: Mp: 232–234 °C (from CH_2Cl_2–MeOH); $[\alpha]_D^{25}$ +46.6 (c 0.16, MeOH); UV, λ_{max} (nm) 246 (log ε 4.04); IR, ν_{max} (cm^{-1}): 3504, 3397, 2961, 2851, 1762, 1670, 1559, 1457, 1385; ^1H NMR (pyridine-d_5, 400 MHz): δ 1.03 (3H, s, 19-Me), 1.20 (3H, s, 18-Me), 1.38 (2 × 3H, s, 26-Me, 27-Me), 1.50 (3H, s, 21-Me), 1.55 (1H, partially overlapping signal, H-11ax), 1.83 (1H, obscured signal, H-24a), 1.90 (1H, obscured signal, H-11eq), 1.91 (1H, brd, J = 14.1 Hz, H-4α), 2.03 (1H, obscured signal, H-16a),

2.21 (1H, dd, J = 14.1, 8.1 Hz, H-4β), 2.31 (1H, obscured signal, H-24b), 2.33 (2 × 1H, AB quartet, partially obscured signal, H-1α, H-1β), 2.44 (1H, obscured signal, H-16b), 2.68 (1H, brd, J = 8.1 Hz, H-5), 2.81 (1H, t, J = 8.6 Hz, H-17), 3.85 (1H, brd, J = 8.0 Hz, H-22), 6.43 (1H, brs, H-7); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 14.3 (C-19), 18.0 (C-18), 21.4 (C-16), 21.6 (C-21), 22.4 (C-11), 27.4 (C-23), 29.9 (C-26), 30.2 (C-27), 30.9 (C-12), 31.5 (C-15), 33.4 (C-4), 42.5 (C-24), 46.2 (C-1), 47.2 (C-13), 49.7 (C-17), 50.1 (C-10), 54.4 (C-9), 56.7 (C-5), 69.6 (C-25), 76.8 (C-20), 77.5 (C-22), 83.9 (C-14), 90.8 (C-3), 124.9 (C-7), 159.9 (C-8), 201.3 (C-6), 211.7 (C-2); ESMS (+ve), *m/z* (% rel. intensity): 499 $[M+Na]^+$ (100); HR-FABMS (–ve), *m/z*: 475.2698 $[M-H]^-$. $C_{27}H_{40}O_7$–H requires 475.2695. HMBC correlations (pyridine-d_5): H-1 (C-2, C-3, C-5, C-9, C-10), H-4 (C-2, C-3, C-5, C-6, C-9, C-10), H-5 (C-1, C-3, C-6, C-7, C-9, C-10), H-7 (C-5, C-9, C-14), H-11 (C-3, C-8, C-9, C-10, C-13), H-16 (C-17), H-17 (C-13, C-16, C-18, C-21), 18-Me (C-12, C-13, C-14, C-17), 19-Me (C-1, C-5, C-9, C-10), 21-Me (C-17, C-20), H-22 (C-20, C-23, C-24), H-24 (C-25), 26-Me, 27-Me (C-24, C-25).

2.4. Biotransformation of ponasterone A (7)

Ponasterone A (**7**) (10 mg × 5) was subjected to biotransformation in the same manner as for compound **1** for 14 days. The crude extract was chromatographed to yield compound **11** (8.5 mg, 48% based on the unrecovered starting material), compound **9** (3.5 mg) which was contaminated by a minor component, and the starting material **7** (32 mg). The impure **9** was further purified by reversed phase HPLC (column: μBondapak C18, 10 μm, 3.9 × 300 mm, mobile phase: MeOH-H_2O (3:2), flow rate: 0.5 ml/min, detector: 254 nm) to yield 2-dehydro-3-*epi*-ponasterone A (**9**) (1.8 mg, 10%; t_R 18.70 min) and a small quantity (< 0.5 mg; t_R 22.96 min) of an unstable compound.

Compound **9**: $[\alpha]_D^{25}$ +2.5 (c 0.04, MeOH); UV, λ_{max} (nm) 241 (log ε 4.02); ^{1}H NMR (pyridine-d_5, 400 MHz): δ 0.82 (3H, d, J = 6.2 Hz, 26-Me), 0.83 (3H, d, J = 6.2 Hz, 27-Me), 1.08 (3H, s, 19-Me), 1.18 (3H, s, 18-Me), 1.53 (3H, s, 21-Me), 2.05 (1H, partially overlapping signal, H-4α), 2.39 (1H, d, J = 13.4 Hz, H-1α), 2.44 (1H, obscured signal, H-4β), 2.74 (1H, d, J = 13.4 Hz, H-1β), 2.86 (1H, obscured signal, H-17), 2.89 (1H, obscured signal, H-5), 3.29 (1H, m, H-9), 3.78 (1H, brd, J = 10.7 Hz, H-22), 4.59 (1H, m, $W_{½}$ = 24 Hz, H-3), 6.22 (1H, brs, H-7); ESMS (–ve): *m/z* (% rel. intensity) 461 $[M–H]^-$ (100); HR-TOFMS (APCI, +ve), *m/z*: 445.2949 $[M+H–H_2O]^+$. $C_{27}H_{42}O_6+H–H_2O$ requires 445.2949.

Compound **11**: Amorphous; $[\alpha]_D^{25}$ +3.8 (c 0.08, MeOH); UV, λ_{max} (nm) 246 (log ε 4.01); IR, ν_{max} (cm^{-1}): 3420, 2920, 2847, 1761, 1678, 1620, 1461; ^{1}H NMR (pyridine-d_5, 400 MHz): δ 0.81 (3H, d, J = 6.2 Hz, 26-Me), 0.82 (3H, d, J = 6.2 Hz, 27-Me), 1.04 (3H, s, 19-Me), 1.22 (3H, s, 18-Me), 1.49 (3H, s, 21-Me), 1.55 (1H, partially overlapping signal, H-11ax), 1.86 (1H, obscured signal, H-11eq), 1.91 (1H, d, J = 14.1 Hz, H-4α), 2.02 (1H, obscured signal, H-16a), 2.20 (1H, dd, J = 14.1, 8.1 Hz, H-4β), 2.33 (2 × 1H, AB quartet, partially obscured signal, H-1α, H-1β), 2.44 (1H, obscured signal, H-16b), 2.69 (1H, brd, J = 7.9 Hz, H-5), 2.75 (1H, t, J = 8.7 Hz, H-17), 3.79 (1H, brd, J = 10.1 Hz, H-22), 6.44 (1H, brs, H-7); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 14.3 (C-19), 18.0 (C-18), 21.4 (C-16), 21.5 (C-21), 22.4 (C-11, C-26), 23.3 (C-27), 28.1 (C-25), 30.2 (C-23), 30.9 (C-12), 31.5 (C-15), 33.4 (C-4), 37.1 (C-24), 46.1 (C-1), 47.2 (C-13), 49.5 (C-17), 50.1 (C-10), 54.3 (C-9), 56.7 (C-5), 76.6 (C-20), 76.7 (C-22), 83.9 (C-14), 90.7 (C-3), 125.0 (C-7), 159.8 (C-8), 201.3 (C-6), 211.7 (C-2); ESMS (+ve), *m/z* (% rel. intensity): 483 $[M+Na]^+$ (100); HR-FABMS (–ve): *m/z* 459.2748 $[M–H]^-$. $C_{27}H_{40}O_6$–H requires 459.2746.

2.5. Biotransformation of pterosterone (8)

Pterosterone (**8**) (10 mg × 5) was subjected to biotransformation in similar manner as for compound **1** for 14 days. The crude extract was chromatographed to yield compound **12** (7 mg, 35% based on the unrecovered starting material), compound **10** (2.8 mg) which was contaminated by a minor component, and the starting material **8** (30 mg). The impured **10** was further purified by reversed phase HPLC (column: μBondapak C18, 10 μm, 3.9 × 300 mm, mobile phase: MeOH-H_2O (1:1), flow rate: 0.5 ml/min, detector: 254 nm) to yield 2-dehydro-3-*epi*-pterosterone (**10**) (1.4 mg, 7%; t_R 22.41 min) and a small quantity (< 0.3 mg; t_R 25.94 min) of an unstable compound.

Compound **10**: $[\alpha]_D^{25}$ +19.8 (c 0.06, MeOH); UV, λ_{max} (nm) 241 (log ε 4.03); ^{1}H NMR (pyridine-d_5, 400 MHz): δ 1.02 (3H, d, J = 6.3 Hz, 26-Me), 1.03 (3H, d, J = 6.3 Hz, 27-Me), 1.08 (3H, s, 19-Me), 1.16 (3H, s, 18-Me), 1.54 (3H, s, 21-Me), 2.05 (1H, partially overlapping signal, H-4α), 2.39 (1H, d, J = 14.3 Hz, H-1α), 2.44 (1H, obscured signal, H-4β), 2.74 (1H, d, J = 14.3 Hz, H-1β), 2.85 (1H, overlapping signal, H-17), 2.89 (1H, overlapping signal, H-5), 3.94 (1H, m, H-24), 4.11 (1H, brd, J = 12.1 Hz, H-22), 4.60 (1H, dd, J = 11.6, 7.8 Hz, H-3), 6.21 (1H, brs, H-7); ESMS (–ve): *m/z* (% rel. intensity) 477 $[M–H]^-$ (100); HR-TOFMS (APCI, +ve), *m/z*: 461.2910 $[M+H-H_2O]^+$. $C_{27}H_{42}O_7$+H–H_2O requires 461.2898.

Compound **12**: Amorphous; $[\alpha]_D^{25}$ +5.3 (c 0.08, MeOH); UV, λ_{max} (nm) 246 (log ε 4.04); IR, ν_{max} (cm^{-1}): 3413, 2936, 2855, 1761, 1678, 1382; ^{1}H NMR (pyridine-d_5, 400 MHz): δ 1.02 (2 × 3H, d, J = 6.3 Hz, 26-Me, H-27), 1.05 (3H, s, 19-Me), 1.21 (3H, s, 18-Me), 1.51 (3H, s, 21-Me), 1.56 (1H, partially overlapping signal, H-11ax), 1.85 (1H, obscured signal, H-11eq), 1.94 (1H, d, J = 14.1 Hz, H-4α), 2.06 (1H, obscured signal, H-

16a), 2.20 (1H, dd, J = 14.1, 8.1 Hz, H-4β), 2.33 (2 × 1H, AB quartet, partially obscured signal, H-1α, H-1β), 2.46 (1H, obscured signal, H-16b), 2.69 (1H, brd, J = 7.8 Hz, H-5), 2.74 (1H, t, J = 8.4 Hz, H-17), 3.96 (1H, m, H-24), 4.11 (1H, brd, J = 10.4 Hz, H-22), 6.43 (1H, brs, H-7); ^{13}C NMR (pyridine-d_5, 100 MHz): δ 14.3 (C-19), 17.0 (C-26), 18.0 (C-18), 19.5 (C-27), 21.4 (C-16), 21.5 (C-21), 22.4 (C-11), 30.9 (C-12), 31.5 (C-15), 33.4 (C-4), 34.0 (C-25), 35.8 (C-23), 46.1 (C-1), 47.1 (C-13), 49.5 (C-17), 50.1 (C-10), 54.3 (C-9), 56.7 (C-5), 76.6 (C-24), 76.7 (C-20), 77.4 (C-22), 83.9 (C-14), 90.7 (C-3), 125.0 (C-7), 159.8 (C-8), 201.3 (C-6), 211.9 (C-2); ESMS (+ve), *m/z* (% rel. intensity): 499 $[M+Na]^+$ (100); HR-FABMS (–ve): *m/z* 475.2699 $[M-H]^-$. $C_{27}H_{40}O_7$–H requires 475.2695.

3. Results and discussion

3.1. Biotransformation products of 20-hydroxyecdysone (1)

Compound **2** was obtained as the minor biotransformation product of 20-hydroxyecdysone (**1**) by *C. lunata* NRRL 2178. The HR-FAB mass spectrum (negative ion mode) indicated a pseudomolecular ion $[M-H]^-$ at *m/z* 477.2858, corresponding to a molecular formula of $C_{27}H_{42}O_7$. The ^{1}H and ^{13}C NMR values were assigned on the basis of COSY, DEPT, HMQC and HMBC spectra. Compound **2** exhibited two carbonyl resonances of δ 200.3 and 209.9 in the ^{13}C NMR spectrum, indicating the presence of an α,β-unsaturated and a saturated keto functions. The ^{1}H NMR spectrum of **2** showed all the signals arising from the protons on the side-chain, and the B, C and D rings of 20-hydroxyecdysone (**1**). The striking differences, as compared with **1**, were the absence of a carbinolic signal (either H-2 or H-3) and the splitting pattern (dd, J = 11.7 and 7.2 Hz) of

the remaining deshielded carbinolic signal at δ 4.60 in the A ring. The presence of two doublet signals, J = 13.6 Hz, at δ 2.40 and 2.73 suggested that the keto group located at the 2-position. Since the band width at half height ($W_{1/2}$) value of H-3 was large (22 Hz), it followed that the 3-hydroxyl group should assume the α-orientation as shown. Compound **2** was thus concluded to be 2-dehydro-3-*epi*-20-hydroxyecdysone synthesized previously by our group [13].

Compound **6** was obtained as the major product of biotransformation of 20-hydroxyecdysone (**1**) by *C. lunata* NRRL 2178. This compound exhibited a pseudomolecular ion $[M-H]^-$ at *m/z* 475.2698 in the HR-FAB mass spectrum (negative ion mode) compatible with a molecular formula of $C_{27}H_{40}O_7$. The UV spectrum exhibited the absorption band of an α,β-unsaturated keto group at 246 nm. The IR spectrum showed absorption bands at 1762 and 1670 cm^{-1} corresponded respectively to a saturated and an α,β-unsaturated keto groups. The high IR absorption frequency of the saturated keto group was due to the presence of a cyclopentanone ring. Assignments of the ^1H and ^{13}C NMR data were accomplished by COSY, DEPT, HMQC and HMBC techniques. The ^{13}C NMR spectrum showed two resonances of the saturated and unsaturated keto groups at δ 211.7 and 201.3 respectively. The ^1H NMR spectrum of **6** displayed only one carbinolic proton of H-22 as a broad doublet (J = 8.0 Hz) at δ 3.85. The broad singlet nature of H-7 (instead of a doublet signal resulted from the long-range coupling with H-9 for most of ecdysteroids) and the absence of the H-9 signal indicated that the 9-position was substituted. One possible structure was to connect the C-9 to C-3 and the saturated keto group was consequently placed at the 2-position (see Fig. 1). The ^{13}C NMR signals of C-9 and C-3 appeared at δ 54.4 and 90.8 which were consistent with carbon resonances of such environments.

Fig. 1 ---------->

The ^{13}C NMR resonance at C-1 appeared at δ 46.2 whereas the ^{1}H NMR signals of two H-1 appeared as an AB quartet at δ 2.33. The rest of the ^{13}C resonance of **6** were comparable to those of compound **2**, except for the C-10 (δ 50.1) and C-19 (δ 14.3) resonances of the former were respectively unusually downfield and high field than those of the latter (δ 43.0 and 23.0 respectively). This was possibly due to the unusual environment of these two carbons compared with the parent compound **1**.

The most informative evidence for the linkage between C-3 and C-9 was the HMBC correlation of H-4 with C-9 and that of H-11 with C-3 (see Experimental). Since epimerization at the 5-position occurred quite frequently during chemical transformation of ecdysteroids [12,18], it was necessary to investigate whether the *cis* relationship between H-5 and 19-Me was preserved throughout the biotransformation process. The NOESY experiment was performed and correlations of H-1, H-5 and H-11ax with19-Me was observed. A/B ring junction was therefore in the *cis* orientation. The structure of the major biotransformation product was thus concluded to be (20*R*,22*R*)-3β,14α,20,22,25-pentahydroxy-3α,9α-cyclo-5β-cholest-7-en-2,6-dione (**6**), the ecdysteroid analogue with a novel C-3 to C-9 linkage.

Possible mechanisms for the biotransformations of **1** to **2** and **6** is outlined in Fig. 2. Initially, compound **1** might be oxidized at C-2 hydroxyl group to **13** which was then subjected to C-3 epimerization to the more stable epimer **2** [13]. An alternative route involved C-2 oxidation of **1** to the intermediate **14**, followed by generation of the nucleophilic center at C-9 which would then yield **15** by C-3 and C-9 bond formation. Oxidation at C-2 hydroxyl group of **15** would then give rise to **6**. Another possible alternative route for the bioconversion of **1** to **6** was C-3 oxidation of **13** to **16**, followed by C-3 and C-9 bond formation. It should be noted that attempts to isolate the proposed

intermediates **13**–**15** have not yet been successful. Further work is needed to establish the mechanisms for these microbial transformations.

Fig. 2 ---------->

3.2. Biotransformation products of ponasterone A (7)

Compound **9** was the minor product arising from biotransformation of ponasterone A (**7**). This compound showed a pseudomolecular ion $[M-H]^-$ at *m/z* 461 in the ESMS (negative ion mode) and the HR-TOFMS (positive ion mode) exhibited a fragment ion $[M+H-H_2O]^+$ at *m/z* 445.2949 compatible with a molecular formula of $C_{27}H_{42}O_6$. The ^1H NMR spectral pattern of **9** was very similar to that of 2-dehydro-3-*epi*-20-hydroxyecdysone (**2**), except for the signal of 26-Me and 27-Me which appeared as a doublet, $J = 6.2$ Hz, at δ 0.82 and 0.83 instead of a singlet at δ 1.39. Compound **9** was thus concluded to be 2-dehydro-3-*epi*-ponasterone A.

Compound **11** was the major biotransformed product of ponasterone A (**7**). Its molecular formula of $C_{27}H_{40}O_6$ was derived from the pseudomolecular ion $[M-H]^-$ at *m/z* 459.2748 of the negative ion HR-FABMS. The ^1H NMR spectral features of **11**were very similar to those of compound **6**, except for the signals of 26-Me and 27-Me which appeared as two doublets, $J = 6.2$ Hz, at δ 0.81 and 0.82. The ^{13}C NMR chemical shifts of **11** were comparable to those of compound **6**, except for those of C-22 to C-27. This led to a conclusion that the major compound was (20*R*,22*R*)-3β,14α,20,22-tetrahydroxy-3α,9α-cyclo-5β-cholest-7-en-2,6-dione (**11**).

3.3. Biotransformation products of pterosterone (8)

Compound **10** was obtained as the minor product of the biotransformation of pterosterone (**8**) by *C. lunata*. The ESMS and the HR-TOFMS established its molecular formula of $C_{27}H_{42}O_7$. The ^{1}H NMR spectral features indicated the presence of a 2-keto-3α-hydroxyl system as those compounds **2** and **9**. The only different spectral features were those arising from pterosterone side-chain, i.e. the presence of H-22 and H-24 signals as a broad doublet (J = 12.1 Hz) at δ 4.11 and a multiplet at δ 3.94, and the doublet (J = 6.3 Hz) signals of 26-Me and 27-Me δ 1.02 and 1.03. The structure of this compound was concluded as 2-dehydro-3-*epi*-pterosterone (**10**).

Compound **12** was obtained as the major biotransformation product of pterosterone (**8**). Positive ion ESMS at *m/z* 499 $[M+Na]^+$ and negative ion HR-FABMS at *m/z* 475.2699 $[M-H]^-$ established a molecular formula of $C_{27}H_{40}O_7$. As expected, the ^{1}H NMR features of the nucleus of **12** were very similar to those of compounds **6** and **11**. The different spectral features are those of the side-chain which are very similar to those of compound **10**. The ^{13}C NMR chemical shifts of **12** were comparable to those of compound **6**, except for those of C-23 to C-27. The structure of the major product was thus established as (20*R*,22*R*)-3β,14α,20,22,24-pentahydroxy-3α,9α-cyclo-5β-cholest-7-en-2,6-dione (**12**).

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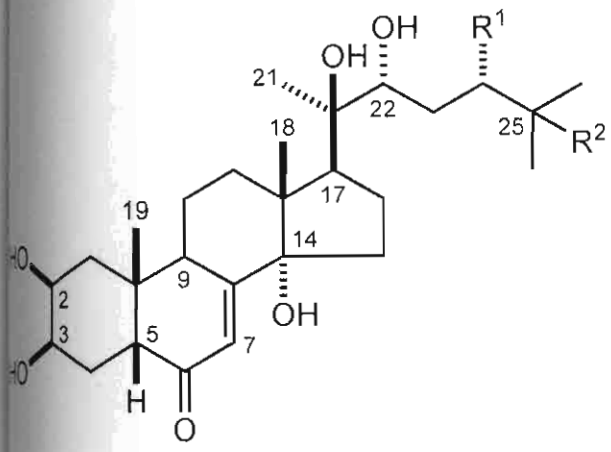
Figure legends:

Fig. 1. Partial three-dimensional structure of compound **6**.

Fig. 2. Possible mechanisms for the biotransformations of compound **1** to compounds **2** and **6**.



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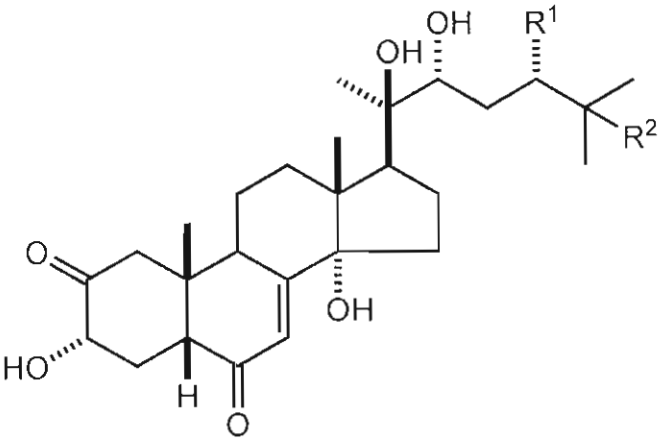
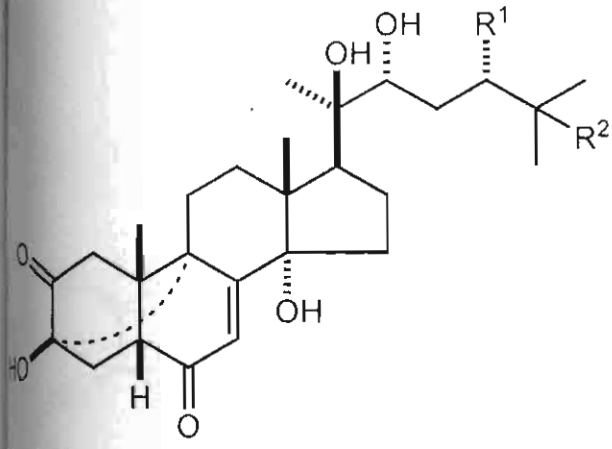
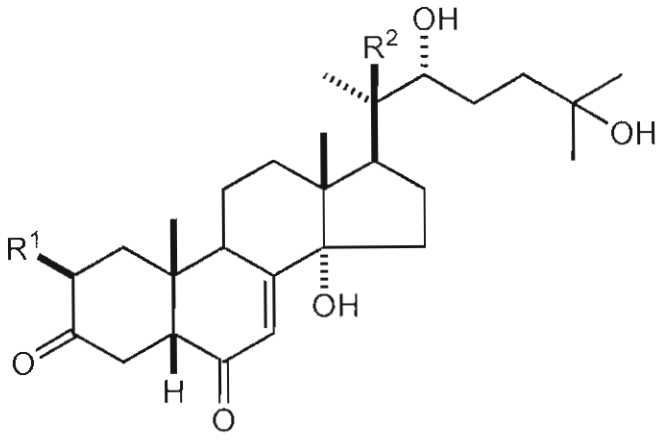
7 R^1 = R^2 = H
8 R^1 = OH, R^2 = H



2 R^1 = H, R^2 = OH
9 R^1 = R^2 = H
10 R^1 = OH, R^2 = H



6 R^1 = H, R^2 = OH
11 R^1 = R^2 = H
12 R^1 = OH, R^2 = H



3 R^1 = R^2 = OH
4 R^1 = OH, R^2 = H
5 R^1 = R^2 = H

Fig. 1 REVISED.cdx

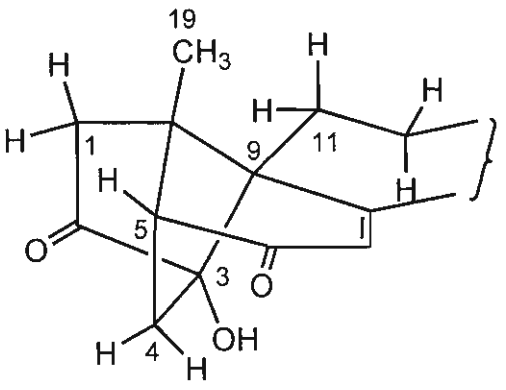


Fig. 1

Fig. 2.cdx

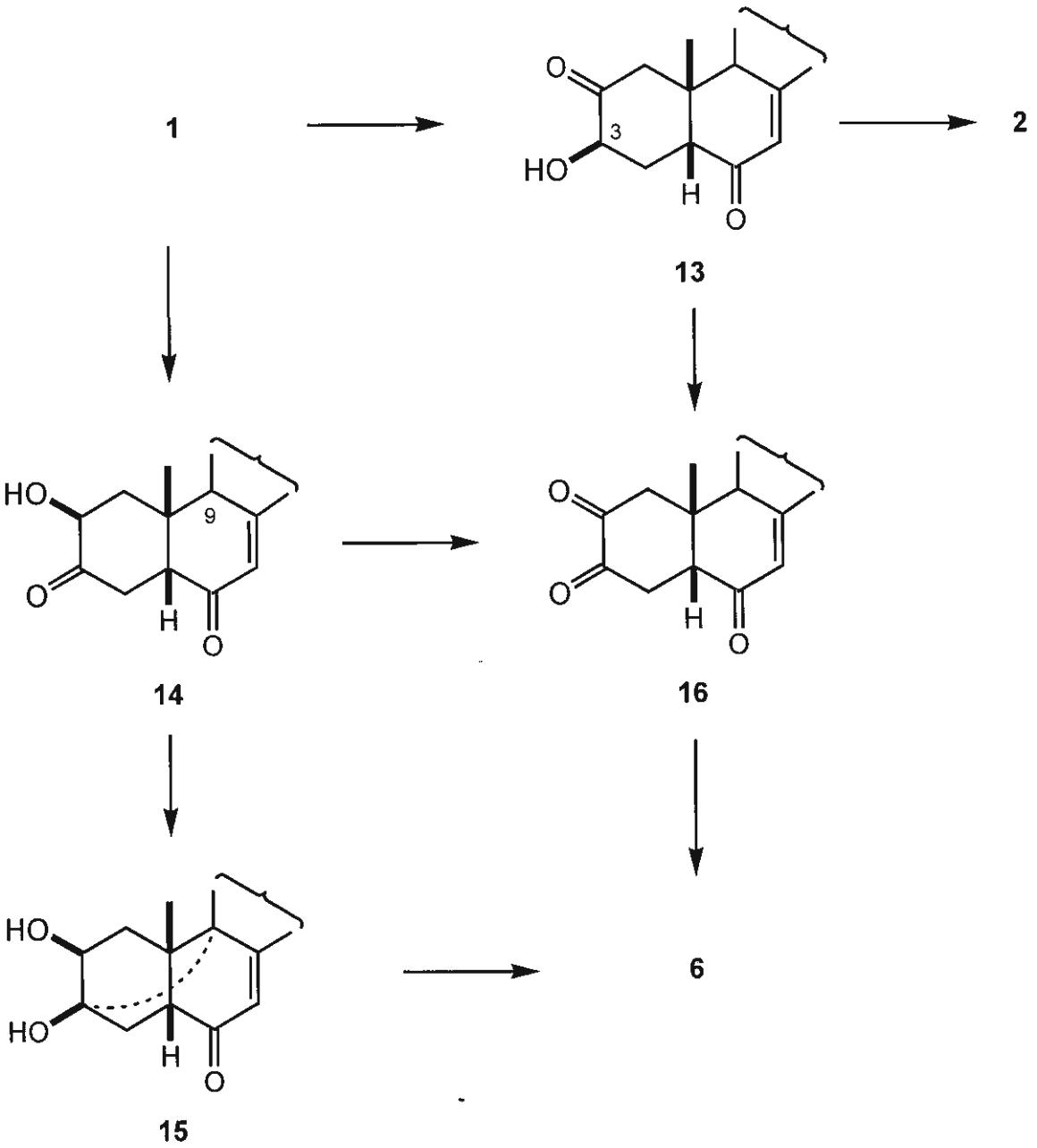


Fig. 2

Antibacterial Pterocarpans from *Erythrina subumbrans*

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Abstract

Seven pterocarpans, erybraedin B (**1**), erybraedin A (**2**), phaseollin (**3**), erythrabyssin II (**4**), erystagallin A (**5**), erythrabissin-1 (**6**) and erycristagallin (**7**), two flavanones, 5-hydroxysophoranone (**8**) and glabrol (**9**), and one isoflavone, erysubin F (**10**), were isolated from the stems of *Erythrina subumbrans* (Leguminosae). Their structures were identified by means of spectroscopy. This is the first report of the isolation of the non-alkaloidal compounds from *E. subumbrans* and the observed dehydration of 6a-hydroxypterocarpans **5** and **6** in $CDCl_3$ to the corresponding pterocarpenes **11** and **12**, respectively. Compoumds **8** and **9** were isolated for the first time from the genus *Erythrina*. Compounds **2** and **4** exhibited the highest degree of activity against *Streptococcus* strains with an MIC range of 0.78-1.56 μg/ml, whereas compound **7** exhibited the highest degree of activity against *Staphylococcus* strains, including drug-resistant strains (MRSA and VRSA), with an MIC range of 0.39-1.56 μg/ml. Interestingly, compounds **2**, **4**, **5** and **7** were more active against several strains of *Streptococcus* and *Staphylococcus* than the standard antibiotics

vancomycin and oxacillin. Compound **7** showed the highest level of activity against all VRSA strains tested, with an MIC range of 0.39-1.56 μg/ml, which were resistant to both antibiotics. These compounds may prove to be a potent phytochemical agent for the antibacterial activity, especially against the MRSA and VRSA strains.

Keywords: *Erythrina subumbrans*; Leguminosae; Pterocarpans; Flavanones; Isoflavone; Antibacterial activity

1. Introduction

The genus *Erythrina* (Leguminosae) is distributed in tropical and subtropical region of the world and has been used in traditional medicine for the treatment of various diseases, especially microbial infections (Mitscher et al., 1987). These plants are known to be a rich source of bioactive alkaloids (Ghosal et al., 1971; Furukawa et al., 1976; Barakat et al., 1977; Cordell, 1981) and flavonoids, mainly isoflavones, pterocarpans, flavanones and isoflavanones (Chacha et al., 2005). Some of these flavonoids have been found to display a variety of biological activities, such as antimicrobial activity (Mitscher et al., 1987, 1988a,b), anti-HIV activity (McKee et al., 1997), antibacterial activity (Tanaka et al., 2002; Sato et al., 2002), anti-inflammatory activity (Njamen et al., 2003) and anti-plasmodial activity (Yenesew et al., 2004).

Erythrina subumbrans Merr. is found in South East Asia and its bark has been used in folk remedies for the treatment of cough, post partum vomiting and as poultices (Mitscher et al., 1987). Previous phytochemical studies of this plant reported four erythrinan alkaloids: erythramine, hypaphorine (Folkers and Koniuszy, 1939), erysodine and erysopine (Folkers et al., 1941). A preliminary screening of crude extracts of *E. subumbrans* in our laboratory indicated that the hexane and dichloromethane extracts from its stems possess antimicrobial activity against Gram-positive bacteria. A bioassay-guided study of both crude extracts led to the isolation and identification of seven pterocarpans, two flavanones and one isoflavone as the constituent responsible for the antibacterial activity that was observed, which is described in this article.

2. Material and methods

2.1 General Experimental Procedures

Melting points were determined on an Electrothermal apparatus and are uncorrected. UV spectra were measured with a Perkin Elmer Lamda 20 spectrophotometer. IR spectra were obtained using a Perkin Elmer Spectrum 2000 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer, operating at 400 and 100 MHz, respectively. Mass spectra were recorded with a Finnigan LCQ Advantage instrument. Quick column chromatography (QCC) and column chromatography (CC) were carried out using Merck silica gel 60 PF_{254} and silica gel 60 (<0.063 mm), respectively. For TLC Merck precoated silica gel 60 F_{254} plates were used. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

2.2 Plant material

The stems of *E. subumbrans* were collected from Kangkajan national park, Kangkajan district, Phetchaburi province, Thailand. A voucher specimen (No. BKF 091954) has been deposited at the herbarium of the Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok.

2.3 Extraction and separation

The air-dried, powdered stems of *E. subumbrans* (1.0 kg) were extracted successively with *n*-hexane and CH_2Cl_2, using a Soxhlet apparatus. The hexane and CH_2Cl_2 extracts were filtered and concentrated until dry in vacuo. The hexane extract (9.8 g) was subjected to a quick CC on silica gel, using a gradient solvent system of hexane, hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH and MeOH in increasing proportions of the polar solvent to afford 11 fractions (H1-11). Fr. H7 (1.3 g) was further fractionated by CC on silica gel, using a gradient solvent system of EtOAc-hexane (8:92) and EtOAc in increasing proportions of the polar solvent to furnish 15 fractions (H12-26). Fr. H14 (575 mg) was chromatographed twice in succession on silica gel, using a gradient of EtOAc-hexane from 5:95 to 15:85 and EtOAc-hexane (6:94) to afford 7 fractions (H27-33). Fr. H32 (155 mg) was fractionated into 5 fractions (H34-38) on a siliga gel column, using acetone-hexane (7:93). Fr. H38 (23 mg) was purified by CC on silica gel, using EtOAc-hexane (10:90) to give 11 fractions (H39-49). Fr. H42 yielded **1** (2 mg). Fr. H15 (222 mg) was rechromatographed on silica gel, using MeOH-$CHCl_3$ (0.2:99.8) to yield 14 fractions (H50-63). Fr. H53 afforded **2** (8 mg). Fr. H10 (3 g) was subjected to a quick CC on silica gel, using a gradient solvent system of hexane, hexane-acetone, acetone, acetone-MeOH and MeOH in increasing proportions of the polar solvent to give 19 fractions (H64-82). Fr. H69 (547 mg) was fractionated twice in succession on silica gel, using a gradient solvent system of $CHCl_3$ and MeOH-$CHCl_3$ (5:95) in increasing polarity and EtOAc-hexane (20:80) to give 11 fractions (H83-93). Fr. H84 (33 mg) was chromatographed on silica gel, using EtOAc-hexane (10:90) to yield **8** (6 mg). Fr. H85 (37 mg) was purified by CC on silica gel, using EtOAc-hexane (10:90) to afford 6 fractions (H94-99). Fr. H96 furnished **3** (3 mg). Fr. H70 (808 mg) was subjected to CC on silica gel, using MeOH-$CHCl_3$ (20:80) to yield 14 fractions (H100-113). Fr. H105 afforded **4** (6 mg). Fr. H110 (146 mg) was separated by CC on silica gel, using EtOAc-hexane (30:70) to give 6 fractions (H114-

119). Fr. H116 provided **5** (12 mg). Fr. H72 (252 mg) was rechromatographed on silica gel, using MeOH-$CHCl_3$ (2:98) to furnish 12 fractions (H120-131). Fr. H130 gave **6** (11 mg).

The CH_2Cl_2 extract (14.5 g) was subjected to a quick CC on silica gel, using a gradient solvent system of $CHCl_3$, $CHCl_3$-MeOH and MeOH in increasing proportions of the polar solvent to give 8 fractions (C1-8). Fr. C2 (11.6 g) was further purified by CC on silica gel, using a gradient solvent system of acetone-hexane (5:95) and acetone in increasing polarity to yield 13 fractions (C9-21). Fr. C17 (1.48 g) was subjected to a quick CC on silica gel, using a gradient solvent system of EtOAc-hexane (30:70), EtOAc, EtOAc-MeOH and MeOH in increasing proportions of the polar solvent to give 15 fractions (C22-36). Fr. C24 (471 mg) was chromatographed on silica gel, using MeOH-$CHCl_3$ (2:98) to furnish 8 fractions (C37-44). Fr. C39 (91 mg) was further purified by CC on silica gel, using EtOAc-hexane (30:70) to give 8 fractions (C45-52). Frs. C41 and C49 afforded **6** (27 mg) and **9** (5 mg), respectively. Fr. C26 (111 mg) was subjected to CC on silica gel, using EtOAc-hexane (3:97) to yield 4 fractions (C53-56). Fr. C56 furnished **10** (8 mg). Fr. C19 (867 mg) was fractionated by CC on silica gel, using EtOAc-hexane (25:75) to give 17 fractions (C57-73). Fr. C72 (206 mg) was rechromatographed on silica gel, using acetone-hexane (30:70) to yield 10 fractions (C74-83). Fr. C76 afforded **7** (7 mg).

2.4 Bioassays

2.4.1 Antibacterial activity

Antibacterial activity was determined by disc diffusion method (Bauer et al, 1966). Sixteen strains of Gram-positive bacteria (listed in Table 1) were tested against ten isolated compounds from *E. subumbrans* stems. The test compounds were dissolved in dimethyl sulfoxide (DMSO) and two-fold serial dilution was made with DMSO. Each 0.1 ml of the

bacterial suspensions [10^5-10^6 CFU (colony forming unit)/ml] was spread on Mueller-Hinton agar plates. Sterile filter paper discs (6 mm in diameter) were impregnated with 10 µl of each serial two-fold dilution of the test material and placed on the inoculated plates. The plates were then incubated at 37 °C for 16-18 h. DMSO was used as negative control, while vancomycin and oxacillin (E-test, AB Biodisk, Sweden) were used as positive control. At the end of the incubation period, the diameter of the inhibition zone was measured. All tests were performed in duplicate. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth.

3. Results and discussion

The finely powdered stems of *E. subumbrans* were extracted successively with *n*-hexane and CH_2Cl_2 in a Soxhlet apparatus. The hexane and CH_2Cl_2 extracts on chromatography over silica gel gave seven pterocarpans, two flavanones and one isoflavone. These compounds were identified as erybraedin B (**1**) (Mitscher et al., 1988a), erybraedin A (**2** (Mitscher et al., 1988a), phaseollin (**3**) (Perrin, 1964), erythrabyssin II (**4**) (Tanaka et al., 1998) erystagallin A (**5**) (Tanaka et al., 1997), erythrabissin-1 (**6**) (Fomum et al., 1986), erycristagallin (**7**) (Mitscher et al., 1984), 5-hydroxysophoranone (**8**) (Baruah et al., 1984; Matsuura et al, 1994), glabrol (**9**) (Asada et al., 2000) and erysubin F (**10**) (Tanaka et al., 2001 by comparison of their physical and spectroscopic data with those reported in the literature. Interestingly, we were also able to obtain two more pterocarpenes, named eryvarin E (**11**) (Tanaka et al., 2001b) and eryvarin D (**12**) (Tanaka et al., 2001b; Yenesew et al., 2003) as artifacts, leaving compounds **5** and **6** respectively in $CDCl_3$ after a few weeks. The structures of compounds **1-12** are presented in Fig. 1.

This is the first report regarding the isolation of the non-alkaloidal compounds from *E. subumbrans* and the observed dehydration of 6a-hydroxypterocarpan **5** and **6** in $CDCl_3$ to form the corresponding pterocarpenes **11** and **12**, respectively. In addition, compoumds **8** and **9** were first isolated from the genus *Erythrina*. A preliminary screening of crude extracts of *E. subumbrans* in the laboratory revealed that only the hexane and CH_2Cl_2 extracts showed moderate to strong activity against several strains of Gram-positive bacteria. None of the extracts showed activity against Gram-negative bacteria and fungi. Therefore, the antimicrobial activity of the pure compounds was evaluated only against the Gram-positive bacteria, the results of which are presented in Table 1. Erybraedin A (**2**) and erythrabyssin II (**4**) showed the highest level of activity against *Streptococcus* strains, with an MIC range of 0.78-1.56 μg/ml, followed by erystagallin A (**5**) and erycristagallin (**7**) (0.78-3.13 μg/ml), while the most active compounds against *Staphylococcus* strains, including drug-resistant strains (MRSA and VRSA), were erycristagallin (**7**) (0.39-1.56 μg/ml), erythrabyssin II (**4**) (0.78-3.13 μg/ml), erybraedin A (**2**) (0.78-6.25 μg/ml) and erystagallin A (**5**) (0.78-12.5 μg/ml) in that order. Among the tested compounds, isoflavone (**10**) showed the lowest level of activity against all strains of *Streptococcus* and *Staphylococcus*. Flavanones **8** and **9** and three pterocarpans **6**, **11** and **12** showed weak acitivity against several strains of *Streptococcus* and *Staphylococcus*. It should be noted that all the pterocarpans that were tested possessed a hydroxyl group at C-3, a prenyl group at C-10 and a hydroxyl or methoxyl group at C-9, whereas the flavanones and isoflavone that were tested possessed hydroxyl groups at C-7 and C-4′ and prenyl groups at C-8 and C-3′ in their molecules. The results suggest that the differences in core structures (pterocarpan, flavanone, isoflavone), as well as the presence and the position of substituent groups in the molecules, play an important role in the antibacterial activity. Futhermore, it appears that, with regard to pterocarpans, the presence of prenyl groups at C-2 or C-4 and C-10, hydroxyl groups at C-3 and C-9 and/or a hydroxyl group at C-6a, enhanced activity. On the

other hand, it seems that replacement of the hydroxyl group at C-9 with the methoxyl group reduced the antibacterial activity. However, with regard to flavanones, the presence of a hydroxyl group at C-5 and more bulky substituents in the B-ring might either enhance or reduce activity depending on *Streptococcus* and *Staphylococcus* strains tested, respectively. Compounds **2**, **3**, **4**, **5**, **6**, **7** and **10** have previously been shown to display antibacterial activity against a number of bacteria (Kamat et al., 1981; Mitscher et al., 1987, 1988a,b; Iinuma et al., 1994; Tanaka et al., 2002; Sato et al., 2002) but their activity against sixteen strains of the bacteria (listed in Table 1) in this study has not been reported previously. Sato et al. (2002) reported that erycristagallin (**7**) showed the highest antibacterial activity against mutans streptococci, other oral streptococci, and *Actinomyces* and *Lactobacillus* species with an MIC range of 1.56-6.25 μg/ml, followed by erystagallin A (**5**) (3.13-12.5 μg/ml), while Tanaka et al (2002) also demonstrated the highest anti-MRSA activity of **7** (3.13-6.25 μg/ml).

In conclusion, compounds **2**, **4**, **5** and **7** isolated from *E. subumbrans* stems would serve as promising candidates for chemotherapeutic agents, especially against MRSA and VRSA strains, since these compounds showed higher activity than those of vancomycin and oxacillin. It should also be noted that compound **7** showed the highest level of activity against all VRSA strains tested, with an MIC range of 0.39-1.56 μg/ml, which were resistant to both antibiotics.

Acknowledgements

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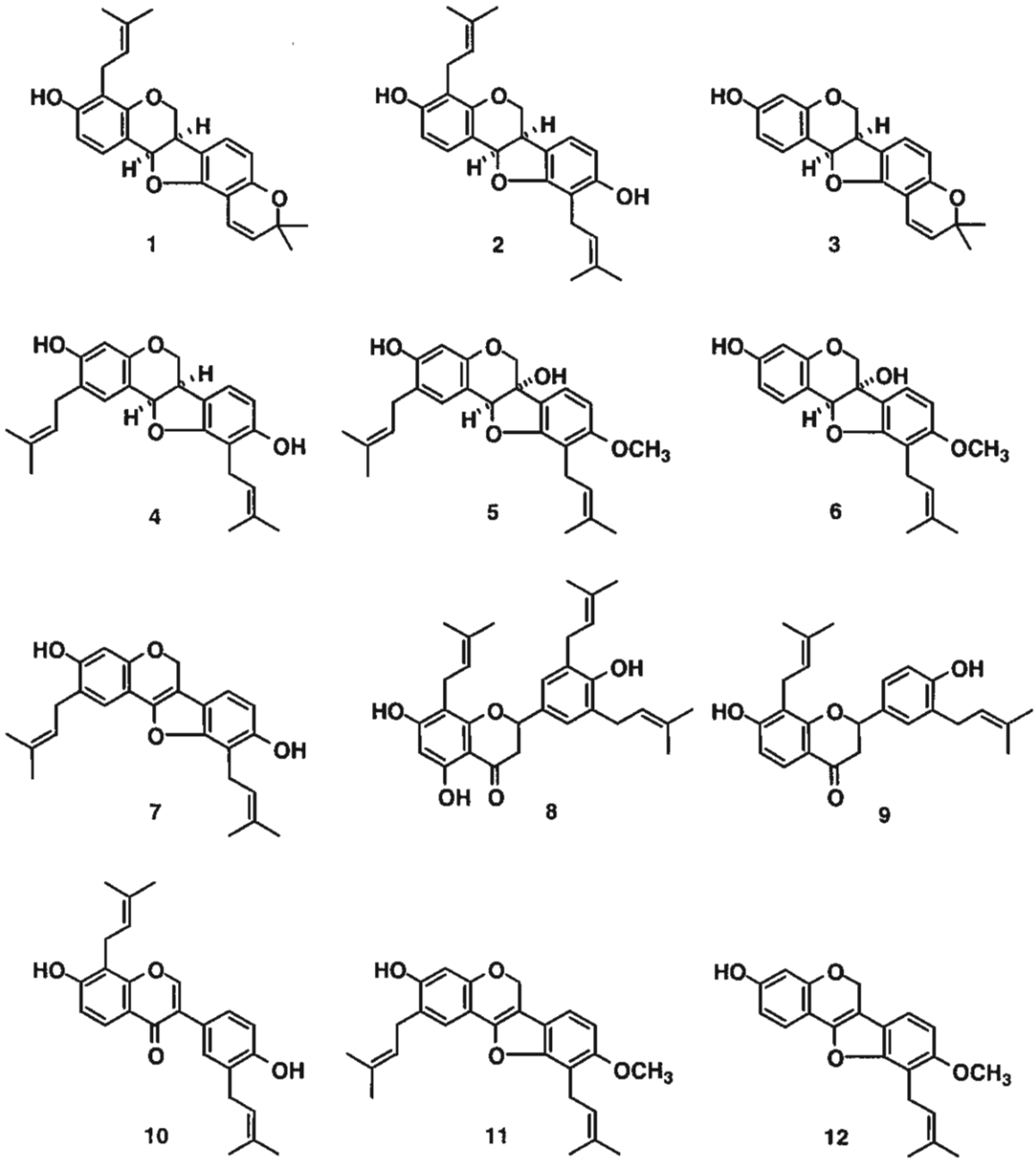

Fig. 1. Structures of isolated compounds from *E. subumbrans*

Table 1. Antibacterial activities (MIC, μg/ml) of some isolated compounds from *E. subumbrans*

Bacteria	Compound										Antibiotics	
	2	4	5	6	7	8	9	10	11	12	Vancomycin	Oxacillin
Genus *Streptococcus*												
S. agalactiae 16442	0.78	1.56	1.56	6.25	3.13	25	>50	>50	>50	>25	2.0	1.5
S. agalactiae 11159	0.78	1.56	0.78	12.5	3.13	0.78	25	>50	>50	25	1.0	3.0
S. constellatus 12428	0.78	1.56	0.78	6.25	0.78	>50	12.5	>50	>50	25	2.0	4.0
S. mutans 27175	0.78	0.78	NT	1.56	1.56	> 50	>50	>50	>50	>25	0.02	<0.02
S. milleri group	0.78	0.78	1.56	6.25	0.78	> 50	12.5	>50	>50	12.5	4.0	0.38
S. sobrinus	1.56	1.56	3.13	6.25	3.13	6.25	50	50	50	25	1.5	0.75
Genus *Staphylococcus*												
Staphylococcus coaggulase negative	1.56	1.56	6.25	6.25	0.78	>50	25	>50	25	50	0.09	2.0
S. aureus ATCC25923	3.13	1.56	3.13	6.25	1.56	>50	50	>50	>50	>25	2.0	0.03
S. aureus ATCC29213	NT	NT	NT	6.25	0.78	NT	25	50	50	>25	4.0	25
Drug-resistant strains												
S. aureus MRSA N1	0.78	0.78	0.78	6.25	1.56	>50	50	>50	>50	>25	2.0	>256
S. aureus MRSA 20625	3.13	0.78	6.25	12.5	1.56	>50	>50	>50	50	>25	1.5	>256
S. aureus MRSA 20626	0.78	0.78	1.56	6.25	1.56	>50	25	>50	>50	>25	2.0	>256
S. aureus MRSA 20627	1.56	1.56	1.56	6.25	0.78	>50	12.5	>50	50	>25	2.0	>256
S. aureus VRSA 20622	3.13	1.56	12.5	12.5	1.56	>50	6.25	>50	50	>25	>256	>256
S. aureus VRSA 20623	6.25	3.13	3.13	6.25	0.39	>50	12.5	>50	25	>25	>256	>256
S. aureus VRSA 20624	6.25	1.56	12.5	6.25	1.56	>50	6.25	50	50	3.13	>256	>256

NT: Not tested.

Bioactive constituents of the root barks of *Artocarpus rigidus* subsp. *rigidus*

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Abstract

Investigation of the chemical constituents of the root barks of *Artocarpus rigidus* Blume subsp. *rigidus* has led to the isolation of six structurally diversified phenolics. These included two new phenolics with modified skeletons, the flavonoid artorigidusin A (**1**) and the chromone artorigidusin B (**2**), together with four known phenolics, the xanthone artonol B (**3**), the flavonoid artonin F (**4**), the flavonoid cycloartobiloxanthone (**5**) and xanthone artoindonesianin C (**6**). Compounds **1**, **4** and **5** exhibited moderate to low antiplasmodial activity against *Plasmodium falciparum*. All compounds showed antimycobacterial activity against *Mycobacterium tuberculosis*, with **4** being the most active compound (MIC 6.25 μg/ml). Compounds **5** and **6** exhibited moderate activity against the KB cells, whereas **2**, **5** and **6** showed varying toxicity to the BC cells. Compounds **1-3**, **5** and **6** were active to the NCI-H187 cytotoxicity assay, with **3** being the most active compound (IC_{50} 1.26 μg/ml).

Keywords: Artocarpus rigidus subsp. *rigidus*; Moraceae; Artorigidusin A; Artorigidusin B; Antiplasmodial activity; Antimycobacterial activity; Cytotoxicity

1. Introduction

The genus *Artocarpus* belongs to the Moraceae family, consisting of about 47 species distributed in the south-eastern Asia and the Pacific and some species are cultivated throughout the tropics (Burkill, 1966). A number of *Artocarpus* species including *A. rigidus* Blume subsp. *rigidus* are found wild in the southern part of Thailand. No phytochemical investigation of this plant species has been reported to date. As part of our ongoing project on bioactive compounds from Thai plants for the treatment of tropical diseases, we have investigated this plant species and it was found that the $CHCl_3$ and MeOH extracts exhibited antiplasmodial and antimycobacterial activities. Both crude extracts also showed cytotoxic activity against human epidermoid carcinoma of the mouth (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) cells. The present report deals with the isolation and antiplasmodial, antimycobacterial and cytotoxic activities of the isolated new compounds **1** and **2**, and the known compounds **3**–**6**.

2. Results and discussion

Investigation of the chemical constituents of the root barks of *A. rigidus* subsp. *rigidus* resulted in the isolation of six phenolics. These included two new phenolics with modified skeletons, the flavonoid artorigidusin A (**1**) and the chromone artorigidusin B (**2**), together with four known structurally modified phenolics, the xanthone artonol B (**3**), the flavonoid artonin F (**4**), the flavonoid cycloartobiloxanthone (**5**) and the xanthone artoindonesianin C (**6**). Compounds **3** and **4** were identified as artonol B and artonin F isolated previously from *A. communis* on the basis of spectroscopic (^1H and ^{13}C NMR

spectral) comparisons (Aida et al., 1997; Hano et al., 1990). On the same basis, compounds **5** and **6** were identified as cycloartobiloxanthone and artoindonesianin C isolated from *A. nobilis* (Sultanbawa and Surendrakumar, 1989) and *A. teysmanii* (Makmur et al., 2000), respectively.

Compound **1** was obtained as pale brownish yellow amorphous, m.p. 224-226 °C. The IR spectrum showed absorptions for hydroxyl (3535, 3434 cm^{-1}) and conjugated keto (1662 cm^{-1}) groups of a flavone. The HR-FABMS (positive ion mode) gave an $[M+H]^+$ ion at *m/z* 435.1446, corresponding to a molecular formula of $C_{25}H_{22}O_7$. The ^1H NMR spectrum of **1** revealed two doublet (J = 1.9 Hz) at δ 6.24 and 6.56 corresponded respectively to the characteristic H-6 and H-8 signals of a flavonoid. The 5,7-dihydroxyl substituted pattern was confirmed by HMBC correlations as shown in Fig. 1. Placement of the 5-hydroxyl group was confirmed by the presence of a chelated signal at δ 13.14 in the ^1H-NMR spectrum of **1**. The ^1H NMR spectrum of **1** revealed an ABX type of signals as two double doublets at δ 2.44 (J = 16.0 and 6.6 Hz) and 3.35 (J = 16.0 and 2.0 Hz) and a broad doublet at δ 3.97 (J = 6.6 Hz) assignable to two H-9 and the H-10 signals, respectively. The presence of an isopropenyl group was evident from the singlet signal at δ 1.75 and two broad singlet signals at δ 4.30 and 4.63. The point of attachment of this moiety is at the 10-position as indicated by the HMBC correlations (see Fig. 1).

The presence of the 2,2-dimethylpyran ring system was evident from two singlet signals at δ 1.44 (17-Me) and 1.46 (18-Me), and two olefinic resonances at δ 5.74 (H-15) and 6.75 (H-14). The orientation of this moiety in the molecule as well as the placement of the phenolic hydroxyl groups at the 2'- and 5'-positions were established by HMBC experiments (Figure 1). Assignments of the ^1H and ^{13}C NMR spectral data of **1** were confirmed by COSY, DEPT, HMQC and HMBC experiments. The ^1H and ^{13}C NMR data

of **1** were similar to those of artonol E (Aida et al., 1997), except for the absence of a methoxyl signal of the former at the 7-position. The structure of this flavonoid was thus established as **1** named artorigidusin A.

Fig. 1. Structure and HMBC correlations of compound **1**.

Compound **2** was a pale brownish orange amorphous, the IR absorption bands of which exhibited hydroxyl (3383 cm^{-1}), conjugated keto (1659 cm^{-1}) and nonconjugated keto (1727 cm^{-1}) functional groups. The HR-FABMS showed the $[M+H]^+$ peak at *m/z* 387.1802, corresponding to a molecular formula of $C_{22}H_{26}O_6$. The broad singlet signal at δ 12.75 in the ^1H NMR spectrum of **2** suggested the presence of the chelated 5-hydroxyl group. The only aromatic resonance appeared as a singlet signal of H-6 at δ 6.22. The broad singlet signal of two methyl groups at δ 1.43 and two doublets (J = 10.0 Hz) of olefinic resonances at δ 5.51 and 6.55 revealed the presence of a 2,2-dimethylpyran ring. HMBC experiments (see Fig. 2) showed long-range correlations consistent with the partial structure of this part of the molecule.

Fig. 2. Structure and HMBC correlations of compound **2**.

The presence of the 1,1-dimethylpropanol moiety at the 3-position was evident from two sets of two-proton multiplets at δ 2.52 (H-12) and 1.60 (H-13), and a singlet of two methyl groups (15-Me and 16-Me) at δ 1.26. The two singlet signals at δ 3.81 (H-9) and 2.30 (H-11) were attributable to the $-CH_2COCH_3$ substituent at the 2-position. The presence of the saturated keto group at C-10 was confirmed by the ^{13}C NMR resonance at δ 201.0. HMBC experiments (see Fig. 2) of these two substituents were in agreement

with the structure. The structure of this chromone was thus concluded to be **2** named artorigidusin B.

Compounds **1**, **4** and **5** exhibited moderate to low antiplasmodial activity against *Plasmodium falciparum,* whereas compounds **2**, **3** and **6** were inactive. All compounds showed antimycobacterial activity against *Mycobacterium tuberculosis*, with **4** being the most active compound (MIC 6.25 μg/ml). For cytotoxic activity, compounds **5** and **6** exhibited moderate activity against the KB cells, whereas **2**, **5** and **6** showed varying toxicity to the BC cells. Compounds **1-3**, **5** and **6** were active against the NCI-H187 cytotoxicity assay, with artonol B (**3**) being the most active compound (IC_{50} 1.26 μg/ml). The high and selective cytotoxic activity of **3** against the NCI cells is worth mentioning. Future work on structural modification of this compound to obtain analogues with higher toxicity against the NCI cells should be pursued.

3. Experimental

3.1. General

Melting points were determined with an Electrothermal melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer Spectrum BX spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer, operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). For spectra taken in acetone-d_6 and $CDCl_3$, the residual nondeuterated solvent signals at δ 2.04 and 7.24, and the solvent signals at δ 29.80 and 77.00 were used as references for ^{1}H and ^{13}C NMR spectra, respectively. FABMS and ESMS spectra were measured with a Finnigan MAT 90 and a Finnigan LC-Q mass spectrometer. Unless indicated otherwise, column

chromatography and TLC were carried out using Merck silica gel 60 (finer than 0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

3.2. Plant material

The root barks of *A. rigidus* subsp. *rigidus* were collected from Jana district, Songkhla province in 2001. A voucher specimen is deposited at the Faculty of Science, Chiang Mai University, Thailand (Wisit Arjchomphu No. 011).

3.3. Extraction and isolation

The dried root barks (1.5 kg) were milled and extracted successively with *n*-hexane, $CHCl_3$ and MeOH in a Soxhlet extraction apparatus. The extracts were evaporated to dryness under reduced pressure at temp. about 40 °C. The hexane extract (brownish syrup, 8.4 g), the $CHCl_3$ extract (dark brownish sticky solid, 16.1 g) and the methanolic extract (dark brownish mass, 45.6 g) were respectively obtained.

The $CHCl_3$ extract (14.9 g) was fractionated by quick column chromatography (Merck silica gel 60 PF_{254}, 250 g), eluting with *n*-hexane–$CHCl_3$, $CHCl_3$ and $CHCl_3$–MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 12 combined fractions (C1–C12) were obtained. Fraction C10 was rechromatographed over silica gel (0.063–0.200 mm, 125 g) with *n*-hexane–CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2–MeOH as eluting solvent to give 11 subfractions. Subfraction 3 was chromatograped using CH_2Cl_2 and CH_2Cl_2–MeOH as eluents, with increasing amount of the more polar solvent,

followed by column chromatography eluted under isocratic conditions (0.9% MeOH in CH_2Cl_2) to yield compound **1** as pale brownish yellow amorphous (7 mg).

Fractions C11 and C12 were combined and chromatographed over silica gel (0.063–0.200 mm, 150 g) using *n*-hexane–$CHCl_3$, $CHCl_3$ and $CHCl_3$–MeOH as eluents, with increasing amount of the more polar solvent to give 15 subfractions. Subfraction 6 was subjected to three repeated column chromatography with similar eluting solvent systems to give artonol B (**3**) as pale orange needles (14 mg), m.p. 267–270 °C.

Subfraction 5 was rechromatographed twice using CH_2Cl_2 and CH_2Cl_2–MeOH as eluents, with increasing amount of the more polar solvent to afford three selected subgroups. Subgroup 1 was similarly chromatographed, followed by column chromatography eluted under isocratic conditions (0.9% MeOH in $CHCl_3$) to yield pale yellow amorphous as artonin F (**4**) (2 mg). Subgroup 2 was similarly chromatographed twice to afford dark yellow needles (15 mg) which was identified to be cycloartobiloxanthone (**5**). The selected subgroup 3 was rechromatographed under isocratic conditions (0.9% MeOH in CH_2Cl_2), followed by another column chromatography under isocratic elution (0.8% MeOH in CH_2Cl_2) to give compound **2** as pale brownish orange amorphous (7 mg).

The MeOH extract (40.3 g) was fractionated by quick column chromatography (Merck silica gel 60 PF_{254}, 250 g) eluting with CH_2Cl_2, CH_2Cl_2–EtOAc, EtOAc, EtOAc–MeOH and MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 17 groups of eluting fractions (fractions M1–M17) were obtained. Fraction M7 was rechromatographed twice eluting with CH_2Cl_2, CH_2Cl_2–MeOH with increasing amount of the more polar solvent to yield pale yellow solid of artoindonesianin C (**6**) (2 mg).

3.4. Compound characterization

3.4.1. Artorigidusin A (1)

Pale brownish yellow amorphous, m.p. 224-226 °C. IR (KBr): ν_{max} cm^{-1} 3535, 3434, 3159, 2964, 2913, 2855, 1662, 1615, 1560, 1515, 1451, 1401, 1370, 1262, 1183, 1095, 1028, 863, 802. For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1. ESMS: *m/z* (rel. int.) 435 $[M+H]^+$ (100); HR-FABMS (positive ion mode): *m/z* 435.1446 $[M+H]^+$ (calcd. for $C_{25}H_{22}O_7$+H, 435.1444).

3.4.2. Artorigidusin B (2)

Pale brownish orange amorphous; IR (KBr): ν_{max} cm^{-1} 3383, 2968, 2924, 2852, 1727, 1659, 1578, 1486, 1435, 1350, 1267, 1176, 1159, 1113, 1085, 822. For ^{1}H and ^{13}C NMR spectroscopic data, see Table 1. ESMS: *m/z* (rel. int.) 409 $[M+Na]^+$ (100); HR-FABMS (positive ion mode): *m/z* 387.1802 $[M+H]^+$ (calcd. for $C_{22}H_{26}O_6$+H, 387.1808).

3.5. Biological evaluations

3.5.1. Antiplasmodial assay

The parasite *Plasmodium falciparum* (K1, multidrug resistent strain) was cultured continuously according to the method of Trager and Jensen (1976). The quantitative assessment of the antiplasmodial activity *in vitro* was performed by means of the microculture radioisotope technique based upon the method described by Desjardins et al. (1979). The inhibitory concentration (IC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of $[^{3}H]$-hypoxanthine

by *P. falciparum*. An IC_{50} value of 1 ng/ml was observed for the standard sample, dihydroartemisinin, in the same test system.

3.5.2. Antimycobacterial assay

The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (Collins and Franzblau, 1997). The lowest drug concentration effecting an inhibition of ≥ 90% was considered the MIC. The standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.004, 0.06 and 2.5 μg/ml, respectively.

3.5.3. Cytotoxicity assays

The cytotoxicity assays against human epidermoid carcinoma of the mouth (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) cells were performed employing colorimetric method (Skehan et al., 1990). The standard drug ellipticine exhibited IC_{50} values against these cell lines at 1.33, 1.46 and 0.39 μg/ml, respectively.

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Table 1

^{1}H (300 MHz) and ^{13}C (100 MHz) NMR chemical shifts for compounds **1** (in acetone-d_6) and **2** (in $CDCl_3$)

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
2		161.0		157.9
3		112.1		122.2
4		180.9		181.8
4a		104.9		104.8
5	13.14, *s*	163.2	12.75, *br s*	161.9
6	6.24, *d* (1.9)	99.8	6.22, *s*	100.1
7	9.80, *br s*	164.5		159.5
8	6.56, *d* (1.9)	94.9		100.7
8a		157.5		152.0
9	2.35, *dd* (16.0, 2.0)	22.3	3.81, *s*	46.7
	2.44, *dd* (16.0, 6.6)			
10	3.97, *br d* (6.6)	37.7		201.0
11		145.4	2.30, *s*	30.0
12	4.30, *br s*	111.7	2.52, *m*	19.1
	4.63, *br s*			
13	1.75, *s*	21.8	1.60, *m*	42.2
14	6.75, *d* (10.0)	117.2		70.5
15	5.74, *d* (10.0)	129.7	1.26, *s*	29.3
16		78.3	1.26, *s*	29.3
17	1.44, *s*	28.0	6.55, *d* (10.0)	114.6
18	1.46, *s*	28.0	5.51, *d* (10.0)	127.2
19				78.0
20			1.43, *s*	28.2
21			1.43, *s*	28.2
1'		107.1		
2'	8.02, *br s*	145.3		
3'		110.4		
4'		145.4		
5'	7.59, *s*	137.3		
6'		128.6		

Assignments were confirmed by COSY, HMQC, HMBC and DEPT experiments.

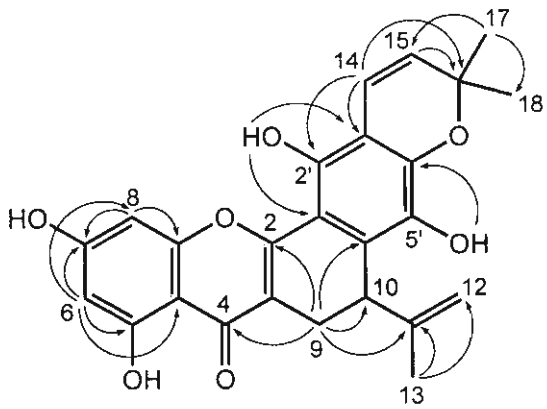
Table 2

Antiplasmodial, antimycobacterial and cytotoxic activities of compounds **1**–**6**

Compound	Antiplasmodial (IC_{50}, μg/ml)	Antimycobacterial (MIC, μg/ml)	Cytotoxicity (IC_{50}, μg/ml)		
			KB	BC	NCI-H187
1	7.9	50	Inactive[b]	Inactive[b]	5.7
2	Inactive[a]	12.5	Inactive[b]	12.10	15.63
3	Inactive[a]	100	Inactive[b]	Inactive[b]	1.26
4	2.4	6.25	Inactive[b]	Inactive[b]	Inactive[b]
5	3.7	25	8.56	4.23	11.83
6	Inactive[a]	12.5	8.4	7.7	7.1

[a] Inactive at ≥ 10 μg/ml; [b] Inactive at ≥ 20 μg/ml.

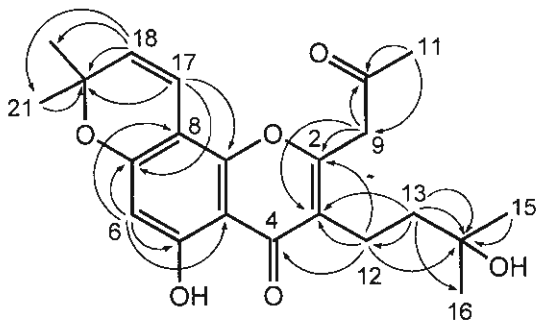
Fig. 1 (A. Suksamram).cdx



1

HMBC correlation

Fig. 2 (A. Suksamrarn).cdx



2

HMBC correlation

Antifungal triterpene glycosides from the fruits of *Sapindus rarak*

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Abstract

Investigation of the fruits of *Sapindus rarak* (Sapindaceae) resulted in the isolation of a triterpene hederagenin (**3**) and five monodesmosidic hederagenin derivatives. These included a new acylated derivative, 23-*O*-acetylhederagenin 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)- α-L-arabinopyranoside (rarakoside) (**5**) and four known derivatives, mukurozi-saponin E_1 (**1**), sapindoside B (**2**), 23-*O*-acetylhederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabin opyranoside (**4**) and α-hederin (**6**). A mixture of two steroid glucosides, β-sitosterol 3-*O*-β-D-glucopyranoside and stigmasterol 3-*O*-β-D-glucopyranoside were also isolated. Compounds **2** and **6** exhibited antifungal activity, the former of which was more active. Compound **2** showed high activity against *Candida albicans* ATCC 10231, *C. krucei*, *Trichophyton mentagrophytes*, *T. rubrum* and *Acremonium* spp., whereas compound **6** was highly active against *T. rubrum*. Compound **2** showed moderate activity against *C. tropicalis*, whereas compound **6** was moderately active against *Acremonium* spp. The results indicated that the presence of the sugar moiety is essential to antifungal activity and that the presence of the acetoxyl group(s), both on the sugar moiety and on the aglycone unit, resulted in loss of antifungal activity.

Keywords: *Sapindus rarak*; Sapindaceae; Triterpene glycoside; Antifungal activity

1. Introduction

Sapindus rarak A. DC. (Sapindaceae) is a medium-sized tree, generally up to 15 m in height. This plant species has been used as a folk medicine for hair care, anti-dandruff [1,2]. An infusion of the fruits is used to remove pimples [1]. The juice of the fruits causes intoxification to fishes [3]. The crude extract of this plant was reported to possess antifungal activity [4]. The molluscicidal saponins hederagenin 3-*O*-(3,4-diacetyl-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside, mukurozi-saponin G, mukurozi-saponin E_1 (**1**) and sapindoside B (**2**) [5], and the uncommon polyol quebrachitol and the sesquiterpene glycoside mukurozioside IIb [6] have been isolated from the pericarps of this plant species. In the course of our investigation for bioactive constituents from the Thai medicinal plants, it was found that the EtOAc and MeOH extracts of the fruits (excluding seeds) of this plant species exhibited antifungal activity, these extracts were therefore subjected to chemical investigation.

2. Experimental

2.1. General experimental procedures

IR spectra were taken as KBr pellets on a Perkin-Elmer Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. FABMS spectra were measured with a Finnigan MAT 90 instrument. Unless indicated otherwise, column chromatography and TLC were carried out using Merck silica gel 60 (finer than 0.063 mm) and precoated silica gel 60 F_{254} plates,

respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating.

2.2. Plant material

The fruits of *S. rarak* were collected from Prachin Buri province, Thailand. The plant species was identified by James F. Maxwell and a voucher specimen is deposited at the Faculty of Science, Ramkhamhaeng University, Thailand (No. RU 040).

2.3. Preparation of the extract

The dry fruits excluding seeds (3.9 kg) were chopped to small pieces and extracted successively with hot *n*-hexane, EtOAc and MeOH (6×3 l for each extract) to give the hexane (orange-yellow wax, 8.9 g, 0.23%), EtOAc (dark brownish viscous syrup, 265.9 g, 6.81%) and MeOH (dark brownish viscous syrup, 919.0 g, 23.56%). The EtOAc and MeOH extracts gave positive antifungal assay and were selected for the isolation of the active components.

2.4. Isolation and identification of triterpene and triterpene glycosides

The EtOAc extract which exhibited antifungal activity was chromatographed (Merck silica gel, 0.063–0.200 mm) using *n*-hexane: EtOAc, EtOAc, EtOAc: MeOH and MeOH as eluting solvents with increasing the quantity of the more polar solvent. The eluates were examined by TLC and 12 combined fractions (E1–E12) were obtained. Fraction E5 was chromatographed, eluting with $CHCl_3$ and $CHCl_3$: MeOH with

increasing the MeOH content, to give 6 subfractions. Subfraction 5 was recrystallized to yield hederagenin (**3**) as colorless needles (82 mg, 0.002%), mp >300 °C. The identity of **3** was made by spectral comparison with those reported previously [7,8]. Compound **3** gave bluish coloration with the anisaldehyde reagent.

Fraction E12 was subjected to quick column chromatography (Merck silica gel 60 PF_{254}) eluting with *n*-hexane: EtOAc, EtOAc and EtOAc: MeOH to give 10 subfractions. Subfraction 7 was rechromatographed twice, with CH_2Cl_2 and CH_2Cl_2: MeOH as eluting solvent, followed by another two repeated column chromatography, with EtOAc and EtOAc: MeOH as eluting solvent to afford compound **4** as white powder (12 mg, 0.0003%). Compound **4** was identical to 23-*O*-acetylhederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside isolated from *S. emarginatus* [9]. Subfraction 8 was subjected to three repeated column chromatography eluting with EtOAc and EtOAc: MeOH, followed by reversed-phase column chromatography (silica gel 60 RP-18 (40–63 μm) eluting with H_2O: MeOH with increasing the MeOH content to yield the new triterpene saponin 23-*O*-acetylhederagenin 3-*O*-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (rarakoside) (**5**) as white powder (12 mg, 0.0003%).

The MeOH extract which also showed antifungal activity was fractionated by quick column chromatography (Merck silica gel 60 PF_{254}), eluting with $CHCl_3$, $CHCl_3$: MeOH and MeOH with increasing the quantity of the more polar solvent, to yield 5 combined fractions (M1–M5). Fraction M4 was chromatographed using $CHCl_3$: MeOH as eluting solvent to give 19 subfractions. The solid obtained from subfraction 8 was washed with $CHCl_3$ to yield a mixture of β-sitosterol 3-*O*-β-D-glucopyranoside and

stigmasterol 3-*O*-β-D-glucopyranoside, the ^{1}H-NMR spectra of which were identical to those of a mixture of authentic compounds. Fraction M5 was chromatographed using $CHCl_3$ and $CHCl_3$: MeOH as eluents and the sixth combined fraction was rechromatographed eluting with CH_2Cl_2 and CH_2Cl_2: MeOH to give 10 subfractions. Subfraction 2 was subjected to column chromatography, eluting with CH_2Cl_2 and CH_2Cl_2: MeOH, followed by column chromatography with EtOAc and EtOAc: MeOH as eluents to give hederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**1**) (18 mg, 0.0005%). The identity of compound **1** was made by spectroscopic comparison with those of mukurozi-saponin E_1 isolated earlier from this plant species [5]. Subfraction 4 was similarly chromatographed, eluting with CH_2Cl_2: MeOH, followed by column chromatography under isocratic condition (CH_2Cl_2: MeOH, 95:5) to yield white solid (14 mg, 0.0004%) which was identified as hederagenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (α-hederin) (**5**) by spectroscopic (^{1}H- and ^{13}C-NMR) comparison with those reported previously [7,10]. Subfraction 6 was chromatographed, with EtOAc and EtOAc: MeOH as eluents to give hederagenin 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (sapindoside B) (**2**) (36 mg, 0.0009%) as colorless crystals, mp 238–240 °C (dec.). The identity of compound **2** was confirmed by spectroscopic comparison with the literature values [5,11]. All of the triterpene glycosides gave green coloration with the anisaldehyde reagent, except compound **5** that exhibited bluish green coloration with this reagent.

Compound 5: White powder, mp 217–220 °C (dec.); $[\alpha]^{30}_{D}$ + 24.0 ° (c 0.10, MeOH); IR bands (KBr): 3421, 2927, 1734, 1647, 1559, 1458, 1386, 1238, 1047, 668 cm^{-1}; ^{1}H-NMR (400 MHz, pyridine-d_5), aglycone protons: δ 0.88 (3H, *s*, 25-Me), 0.92(3H, *s*, 29-Me), 0.99 (3H, *s*, 30-Me), 1.01 (3H, *s*, 26-Me), 1.12 (3H, *s*, 24-Me), 1.31 (3H, *s*, 27-Me), 2.05 (3H, *s*, AcO), 3.36 (1H, *bd*, *J* ca 13 Hz, H-18), 3.93 (1H, overlapping signal, H-3), 4.52, 4.59 (2×1H, overlapping signal, H_2-23), 5.47 (1H, overlapping signal, H-12), sugar protons: δ 1.58 (3H, *d*, *J* 6 Hz, H-6''), 3.92 (1H, overlapping signal, H-5'''a), 4.08 (1H, *t*, *J* 8.0 Hz, H-2'''), 4.20 (3×1H, overlapping signal, H-3', H-3''', H-4'''), 4.22 (1H, overlapping signal, H-4'), 4.37 (1H, overlapping signal, H-5'''b), 4.47 (1H, *t*, obscured signal, H-4''), 4.50 (1H, overlapping signal, H-5'a), 4.59 (2×1H, overlapping signal, H-2', H-5'b), 4.65 (1H, *m*, H-5''), 4.76 (1H, *dd*, obscured signal, H-3''), 4.90 (1H, overlapping signal, H-2''), 4.92 (1H, overlapping signal, H-1'), 5.47 (1H, overlapping signal, H-1'''), 6.31 (1H, *bs*, H-1''); ^{13}C-NMR (100 MHz, pyridine-d_5), aglycone carbons: δ 13.5 (C-24), 16.0 (C-25), 17.4 (C-26), 18.4 (C-6), 20.9 (acetyl CH_3), 23.9 (C-11, C-16, C-30), 26.0 (C-27), 26.2 (C-2), 28.3 (C-15), 31.0 (C-20), 33.0 (C-7), 33.4 (C-22), 34.4 (C-21), 36.9 (C-10), 38.7 (C-1), 39.8 (C-8), 42.1 (C-14)[a], 42.2 (C-18)[a], 42.4 (C-4)[a], 46.7 (C-19)[b], 46.9 (C-17)[b], 48.4 (C-5)[c], 48.6 (C-9)[c], 66.0 (C-23), 82.3 (C-3), 122.2 (C-12), 145.3 (C-13), 170.7 (acetyl CO), 180.1 (C-28), sugar carbons: 18.5 (C-6''), 66.1 (C-5'), 67.4 (C-5'''), 69.6 (C-4')[d], 69.7 (C-5'')[d], 71.3 (C-4'''), 71.8 (C-2''), 72.9 (C-4''), 74.7 (C-3'), 75.3 (C-2'), 75.8 (C-2'''), 78.5 (C-3'''), 83.3 (C-3''), 101.5 (C-1''), 105.2 (C-1'), 107.4 (C-1'''), [a-d]assignments may be reversed for signals with the same superscript; HMBC correlations: H-1' (C-3), H-3 (C-1'), H-1'' (C-2'), H-2' (C-1''), H-1''' (C-3''), H-3'' (C-1'''), H-23 (acetyl CH_3); FABMS (negative ion mode): *m/z* (rel. int.) 923 $[M–H]^-$ (100), 791 $[(M–H) –132]^-$ (20), 749 $[(M–H)–132–42]^-$ (15), 603

[(M–H)–132–42–146]$^-$ (20), HR-FABMS (negative ion mode): *m/z* 923.5019 [M–H]$^-$ (calcd. for $C_{48}H_{76}O_{17}$–H, 923.5004).

2.5 Bioassay

All fungi, except *Candida albican* ATCC 10231, were clinical isolates kindly provided by Ramathibodi Hospital, Mahidol University, Bangkok. Antimicrobial activity was determined by disc diffusion method [12]. Test compounds were dissolved in dimethyl sulfoxide (DMSO) and two-fold serial dilution was made with DMSO. The bacterial and yeast inocula were prepared by suspending in sterile phosphate buffer saline for colonies from 24 h culture in Todd-Hewitt broth and Sabouraud liquid medium respectively. For the filamentous fungi, the inoculum was prepared with spores derived from 5–15 days culture on Sabouraud Dextrose agar. The cell density of each inoculum was adjusted to obtain a final concentration of approximately 10^6 CFU/ml of bacteria, 10^5 CFU/ml of filamentous fungi and 10^4 CFU/ml of yeast. Sterile filter paper discs (6 mm in diameter) were impregnated with 10 µl of each two-fold serial dilution of the test material and placed on the inoculated plates. The plates were then incubated at 37 °C for 16–18 h for bacteria and yeast, and at 28 °C for 24–48 h for filamentous fungi. DMSO was used as the negative control. For the positive controls, vancomycin and oxacillin were used for bacteria whereas amphotericin B was used for yeast and filamentous fungi. At the end of the incubation period the diameter of the inhibition zone was measured. All tests were performed in duplicate. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth. The results of antifungal assay of compounds **2** and **6** are presented in Table 1. Compounds **1** and **3–5** were negative to antifungal evaluation. Compounds **1–6** were resistant against Gram positive and Gram negative bacteria tested.

3. Results and discussion

3.1. Structural elucidation of new triterpene glycoside

Compound **5** was a new triterpene glycoside obtained as a white powder. The HR-FABMS (negative ion mode) showed the $[M-H]^-$ peak at *m/z* 923.5019 compatible with a molecular formula of $C_{48}H_{76}O_{17}$. The ^{1}H-NMR spectral features are a typical spectrum of a triterpene glycoside. The ^{1}H-NMR data of the aglycone part of **5** were similar to those of hederagenin (**3**). The significant differences were the upfield and downfield shifts of the H-3 and H_2-23 signals. The ^{1}H- and ^{13}C-NMR data revealed the presence of the anomeric signal of a rhamnose moiety at δ_H 6.31 and δ_C 101.5. The anomeric signals of the xylose and arabinose moieties were assigned at δ_H 5.47 and at δ_C 107.4, and at δ_H 4.92 and δ_C 105.2, respectively, by the 2D techniques. The fragment ions at *m/z* 791 and 749 corresponded respectively to the loss of the xylose unit, and the xylose unit and acetyl group from the molecular ion, whereas that at *m/z* 603 corresponded to fragment ion with the loss of the xylose unit, acetyl group and rhamnose unit. The mass spectral fragmentation pattern has allowed the attachment sequence of the sugar residues as shown in **5**. The presence of an acetoxyl group was evident from the IR absorption at 1734 cm^{-1}, the ^{1}H-NMR resonance of the methyl group at δ 2.05 and the ^{13}C-NMR resonance of the carbonyl group at δ 170.7. The acetoxyl group should locate at the 23-position, since considerable downfield shifts of H_2-23 were observed in going from compound **1** or **2** (δ 3.93 and 4.28) to compound **5** (δ 4.52 and 4.59). HMBC correlation of H-23 with the acetyl carbonyl carbon also confirmed the placement of the acetyl function. Furthermore, a relatively large upfield shift of the H-3 of **5** (δ 3.93) was observed as compared with those of compounds **1** (δ

4.30) and compound **2** (δ 4.28). The same magnitude of upfield shift was observed for the H-3 of compound **4** [9].

The linkages between the sugar unit and the triterpene, and between the sugar units were established from the HMBC correlations. The HMBC spectrum showed correlation peaks of the anomeric proton of arabinose (H-1') with C-3 and H-3 with C-1' indicating that the arabinose unit attached to the aglycone unit at the 3-position. A pronounced (8.4 ppm) downfield shift of C-3 signal of **5**, as compared with that of compound **3**, was also noted. HMBC correlation of the anomeric proton of rhamnose (H-1") with C-2' of arabinose and of H-2' with C-1" indicated that the 1-position of the rhamnose unit linked to the 2-position of the arabinose unit. Similarly, the correlation between the anomeric proton of the xylose unit (H-1'") and the 3-position of the rhamnose unit (C-3"), and of H-3" and C-1'" revealed that the 1-position of the terminal xylose unit linked to the 3-position of the rhamnose unit. A large (10.7 ppm) downfield shift of C-3" of **5**, as compared with that of compound **6**, was also observed. The structure of **5** was elucidated as 23-*O*-acetylhederagenin 3-*O*-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside named rarakoside.

3.2. Antifungal activity

Sapindoside B (**2**) and α-hederin (**6**) exhibited antifulgal activity, the former of which was more active. Compound **2** showed high activity against *Candida albicans* ATCC 10231, *C. krucei*, *Trichophyton mentagrophytes*, *T. rubrum* and *Acremonium* spp., whereas compound **6** was highly active against *T. rubrum*. Compound **2** showed moderate activity against *C. tropicalis*, whereas compound **6** was moderately active

against *Acremonium* spp. However, compounds **1** and **3–5** were resistant against all fungi. The positive antifungal activity of the monodesmosidic triterpenes **2** and **6** was in agreement with that has previously been reported [13]. The results indicated that the presence of the sugar moiety is essential for antifungal activity and that the presence of the acetoxyl group(s) on the sugar moiety resulted in loss of antifungal activity.

Acknowledgements

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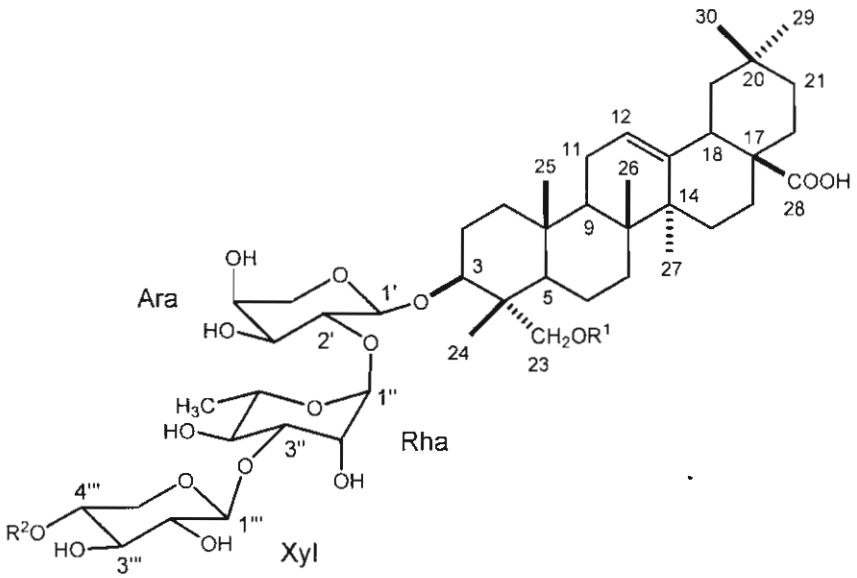
Table 1

Antifungal activity of compounds **2** and **6**

Fungus	MIC (μg/ml)	
	Compound **2**	Compound **6**
Microsporum spp.	54.0	NA
Candida albicans ATCC 10231	6.8	NA
C. krucei	6.8	85.0
C. tropicalis	27.0	NA
Trichophyton mentagrophytes	13.5	42.5
T. rubrum	6.8	10.6
Acremonium spp.	6.8	21.3
Aspergillus fumigatus	NA	NA
A. flavus	NA	NA
Penicillium spp.	NA	NA

NA, no activity at 100 μg/ml.



1 R^1 = H, R^2 = Ac (Mukurozi-saponin E_1)
2 R^1 = R^2 = H (Sapindoside B)
3 R^1 = H, no glycosidic unit (Hederagenin)
4 R^1 = R^2 = Ac (23-*O*-Acetylhederagenin 3-*O*-(4-*O*-acetyl-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside))
5 R^1 = Ac, R^2 = H (Rarakoside)
6 R^1 = H, no xylose unit (α-Hederin)

Anticmycobacterial and cytotoxic activities of anthraquinones from *Prismatomeris filamentosa*

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Abstract

Ten anthraquinones were isolated and identified from the roots of *Prismatomeris filamentosa* (Rubiaceae) and these included 1-hydroxy-2-methylanthraquinone (**1**), damnacanthal (**2**), 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**3**), nordamnacanthal (**4**), rubiadin (**5**), 2-formyl-1-hydroxyanthraquinone (**6**), lucidin-ω-methyl ether (**7**), rubiadin-1-methyl ether (**8**), lucidine-3-*O*-β-primeveroside (**9**) and rubiadin-3-*O*-β-primeveroside (**10**). The isolated compounds were evaluated for antimycobacterial and cytotoxic activities. All tested compounds excepted **8** exhibited antimycobacterial activity against *Mycobacterium tuberculosis* with **6** being the most active quinone (MIC 0.78 μg/ml). Compound **6** exhibited weak activity against the KB cells, whereas **2**, **6** and **7** showed strong toxicity against the BC cells. Compounds **2**, **4**, **6** and **7** were highly active against the NCI-H187 cytotoxicity assay, with **2** being the most active compound (IC_{50} 1.20 μg/ml). The results indicated that the aldehydic functionality at the 2-position of anthraquinone contributed to antimycobacterial activity and cytotoxicity.

Keywords: *Prismatomeris filamentosa*, Anthraquinone; Antimycobacterial activity; Cytotoxic activity.

1. Introduction

The genus *Prismatomeris* (Rubiaceae) includes about 29 species growing in tropical areas [1] and only six species have been found in Thailand [2]. *P. filamentosa* is a tree, 10–12 m in height, distributed in the northeastern part of Thailand. It is known as Pra-San-Tia in Ubon Ratchathani province. Four species of *Prismatomeris* genus have been investigated worldwide including *P. malayana* [3], *P. tetrandra* [4,5], *P. sessiliflora* [6] and *P. fragrans* [7]. Neither phytochemical nor biological investigation of this plant species has been reported. As part of our ongoing project on bioactive compounds from Thai medicinal plants for the treatment of tropical diseases, we have investigated this plant species and it was found that the hexane and chloroform extracts of the roots exhibited antimycobacterial activity against *Mycobacterium tuberculosis* $H_{37}Ra$ (MIC range 25 50 μg/ml). The chloroform extract showed cytotoxic activity against human small cell lung cancer (NCI-H187) cells with IC_{50} value of 11.2 μg/ml. The present report deals with the isolation and biological activity evaluations of the anthraquinones **1–10** from this plant species.

2. Experimental

2.1. General

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer, operating at 400 MHz (^{1}H) and 100 MHz (^{13}C). For spectra taken in $CDCl_3$, the residual nondeuterated solvent signals at 7.24, and the solvent signals at 77.00 were used as references for ^{1}H and ^{13}C NMR spectra, respectively. ESMS spectra was measured

with a Finnigan LC-Q mass spectrometer. Unless indicated otherwise, column chromatography and TLC were carried out using Merck silica gel 60 (finer than 0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

2.2. Plant material

The twigs of *Prismatomeris filamentosa* were collected from Sri Muang Mai district, Ubon Ratchathani province, Thailand. A voucher specimen (Aroon Jankam, No. 001) is deposited at the Faculty of Science, Chiang Mai University, Thailand.

2.3. Extraction and isolation

The pulverized, dry roots (850 g) were extracted successively with *n*-hexane, $CHCl_3$ and MeOH in a Soxhlet extraction apparatus to give the hexane (1.7 g, 0.20 %), $CHCl_3$ (8.0 g, 0.94 %) and MeOH (104.6 g, 12.30 %) extracts. The hexane and $CHCl_3$ extracts gave positive results in the antimycobacterial and cytotoxicity assays and were selected for the isolation of active constituents. The crude MeOH extract which was inactive from preliminary screening was also subjected to further study.

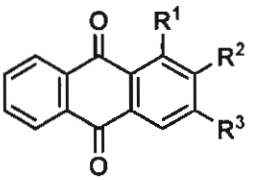
The hexane extract (1.7 g) was subjected to column chromatography eluted with a gradient system of hexane and hexane–CH_2Cl_2 and CH_2Cl_2-MeOH. Eluates with similar TLC patterns were combined to give seven fractions (H1-H7). Fraction H4 was repeatedly column chromatographed eluting with a gradient system of hexane-CH_2Cl_2 (50:50 20:80) to afford 1-hydroxy-2-methylanthraquinone (**1**) (1 mg) as pale yellow amorphous.

Fraction H5 was subjected to three repeated column chromatography with similar eluting solvent systems to give pale yellow solid of damnacanthal (**2**) (31 mg).

The $CHCl_3$ extract (8.0 g) was fractionated by column chromatography over silica gel, eluting with *n*-hexane–CH_2Cl_2, CH_2Cl_2 and CH_2Cl_2–MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 10 combined fractions (C1–C10) were obtained. Fraction C4 was identified as pale yellow amorphous of 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**3**) (1 mg). Fraction C3 was subjected to repeated column chromatography eluting with hexane-CH_2Cl_2 (60:40) to afford dark yellow solid of nordamnacanthal (**4**) (82 mg). Fraction C5 was rechromatographed twice using hexane–CH_2Cl_2 and CH_2Cl_2–MeOH as eluents, with increasing amount of the more polar solvent to afford two subgroups. Subgroup 2 was rechromatographed under isocratic conditions (0.05% MeOH in CH_2Cl_2), to give rubiadin (**5**) (57 mg) as dark yellow solid. Fraction C9 was subjected to repeated column chromatography using CH_2Cl_2 as eluent to give 5 subfractions. Subfraction 1 was rechromatographed under isocratic conditions (50% CH_2Cl_2 in hexane), to give pale yellow amorphous of 2-formyl-1-hydroxyanthraquinone (**6**) (3 mg). Subfraction 5 was rechromatographed twice using hexane–CH_2Cl_2 and CH_2Cl_2–MeOH as eluents, with increasing amount of the more polar solvent to afford pale yellow amorphous of lucidin-ω-methyl ether (7) (20 mg).

The MeOH extract (104.6 g) was column chromatographed eluting with CH_2Cl_2, CH_2Cl_2–MeOH with increasing amount of the more polar solvent. The eluates were examined by TLC and 15 groups of eluting fractions (fractions M1–M15) were obtained. Fraction M2 was rechromatographed twice eluting with CH_2Cl_2, CH_2Cl_2–MeOH with increasing amount of the more polar solvent to yield pale yellow solid of rubiadin-1-methyl ether (**8**) (6 mg). Fraction M7 was a dark yellow solid which was identified to be

lucidine-3-*O*-β-primeveroside (**9**) (34 mg). Fraction M15 was subjected to repeat column chromatography eluting with CH_2Cl_2, CH_2Cl_2–MeOH with increasing amount of the more polar solvent to afford rubiadin-3-*O*-β-primeveroside (**10**) (35 mg) as brownish orange amorphous.



1 R^1 = OH, R^2 = CH_3, R^3 = H
2 R^1 = OCH_3, R^2 = CHO, R^3 = OH
3 R^1 = OH, R^2 = CH_2OH, R^3 = OCH_3
4 R^1 = OH, R^2 = CHO, R^3 = OH
5 R^1 = OH, R^2 = CH_3, R^3 = OH
6 R^1 = OH, R^2 = CHO, R^3 = H
7 R^1 = OH, R^2 = CH_2OCH_3, R^3 = OH
8 R^1 = OCH_3, R^2 = CH_3, R^3 = OH
9 R^1 = OH, R^2 = CH_2OH, R^3 = O-prim
10 R^1 = OH, R^2 = CH_3, R^3 = O-prim

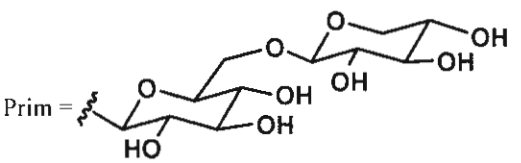


Fig. 1. Anthraquinones isolated from the roots of *Prismatomeris filamentosa*.

2.4. Bioassay

2.4.1. Antimycobacterial assay

Antimycobacterial activity was assessed against *Mycobacterium tuberculosis* $H_{37}Ra$ using the Microplate Alamar Blue Assay (MABA) [8]. The lowest drug concentration effecting an inhibition of ≥ 90% was considered the MIC. The standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.004, 0.06 and 2.5 μg/ml, respectively.

2.4.2. Cytotoxicity assay

The cytotoxicity assays against human epidermoid carcinoma of the mouth (KB), human breast cancer (BC) and human small cell lung cancer (NCI-H187) cells were performed employing colorimetric method [9]. The standard drug ellipticine exhibited IC_{50} values against these cell lines at 1.33, 1.46 and 0.39 μg/ml, respectively.

Table 1
Biological activities of isolated anthraquinones.

Compound	Antimycobacterial activity (MIC, μg/mL)	Cytotoxic activity (IC_{50}, μg/mL)		
		KB	BC	NCI-H187
2	12.5	Inactive[b]	4.9	1.2
4	6.25	Inactive[b]	Inactive[b]	2.1
5	200	Inactive[b]	Inactive[b]	Inactive[b]
6	0.78	10.6	3.1	4.1
7	50	Inactive[b]	4.0	2.3
8	Inactive[a]	Inactive[b]	Inactive[b]	Inactive[b]
9	200	Inactive[b]	Inactive[b]	Inactive[b]
10	100	Inactive[b]	Inactive[b]	Inactive[b]

[a] Inactive at > 200 μg/ml; [b] Inactive at ≥ 20 μg/ml.

3. Results and discussion

The crude extracts were separated and purified to afford ten anthraquinones. These compounds were identified by spectroscopic methods and by comparison with those of literature data: 1-hydroxy-2-methylanthraquinone (**1**) [10], damnacanthal (**2**) [11,12], 1-hydroxy-2-hydroxymethyl-3-methoxyanthraquinone (**3**) [13], nordamnacanthal (**4**) [14,15], rubiadin (**5**) [6,14], 2-formyl-1-hydroxyanthraquinone (**6**) [16], lucidin-ω-methyl

ether (7) [17], rubiadin-1-methyl ether (**8**) [6,12], lucidine-3-*O*-β-primeveroside (**9**) [18] and rubiadin-3-*O*-β-primeveroside (**10**) [18].

The results of bioactivity tests of the isolated compounds are shown in Table 1. Compounds **1** and **3** were not sufficient for biological testing. Compounds **2**, **4** and **6** exhibited potent antimycobacterial activity against *M. tuberculosis*, with **6** being the most active compound (MIC 0.78 μg/ml) whereas **5**, **7**, **9** and **10** exhibited weak antimycobacterial activity. For cytotoxic activity, compound **6** exhibited weak activity against KB cells, whereas **2**, **6** and 7showed strong cytotoxic activity against BC cells with IC_{50} values of 4.9, 3.1 and 4.0 μg/ml, respectively. Compounds **2**, **4**, **6** and **7** were strongly active against the NCI-H187 cytotoxicity assay, with damnacanthal (**2**) being the most active compound (IC_{50} 1.2 μg/ml). It is worth to note that anthraquinones with 2-formyl moiety exhibit antimycobacterial and cytotoxic activity in this study.

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