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Metabolism of parthenin by *Beauveria bassiana* ATCC 7159

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Two new metabolites were isolated from the biotransformation reaction of parthenin by *Beauveria bassiana* ATCC 7159 along with the known naturally occurring sesquiterpene lactones hymenolin and dihydrocoronopilin. The new metabolites: are 3β,4β,6β-trihydroxy-10αH,11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone and 3α,6β-dihydroxy-4β-hydroperoxy-10αH, 11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone. The structure elucidation of these compounds was achieved by different spectroscopic methods.

1. Introduction

Parthenin (**1**) is the major sesquiterpene lactone in many plants belonging to the Asteraceae like *Parthenium hysterophorous* (Rodriguez et al. 1971) and *Artemisia maritima* (Cantrell et al. 2001). Plants containing parthenin have been reported to be responsible for allergic contact dermatitis in humans as well as being toxic to domestic animals. This was attributed to the presence of the α-methylene-γ-lactone moiety in parthenin which is a characteristic feature of this type of sesquiterpene lactones (Chhabra et al. 1999). Parthenin possesses anticancer, antibacterial, antiamoebic and antimalarial, allelopathic and antifungal activities (Das et al. 1999). Biotransformation using microorganisms provides many advantages over chemical derivatization. These include high catalytic activity and high regio- and stereo specificity (Kieslich 1976). Moreover, some microorganisms, especially fungi, have been successfully used as *in vitro* models for mammalian drug metabolism, among which is *Beauveria bassiana* (Clark et al. 1985). This article describes the metabolism of parthenin isolated in high yield from *Dichrocephala integrifolia* (Asteraceae) (Abdel Halim et al. 2004) by *Beauveria bassiana* ATCC 7159. The fermentation method, isolation and structural elucidation of the microbial metabolites are discussed.

2. Investigations, results and discussion

Parthenin (**1**) was subjected to screening studies using a large number of fungi, bacteria and yeasts. These microorganisms are *Absidia pseudocylinderospora* ATCC 24169, *Amycolata autotrophica* ATCC 35203, *Aspergillus alliaceus* UI 315, *Aspergillus niger* ATCC 9142, *Aspergillus ochraceous* ATCC 1008, *Bacillus cereus* NRRL 14591b, *Bacillus megaterium* ATCC 14581, *Beauveria bassiana* ATCC 7159, *Botrytis alli* NRRL 2502, *Candida tropicalis* UI 2312, *Comamonas testosteroni* ATCC 11996, *Cunninghamella echinulata* ATCC 8688a, *Cunninghamella elegans* ATCC 9245, *Curvularia lunata* NRRL 2178, *Gliocladium viride* ATCC 10097, *Mortierella isabellina* ATCC 38063, *Mucor mucedo* UI 5513, *Nocardia species* NRRL 5646, *Rhizopus stolonifer* NRRL 1478, *Rhodtorula rubra* ATCC 20129, *Sepedonium chrysanthosporum* ATCC 13378, *Streptomyces griseus* ATCC 13273 and *Thamnidium elegans* ATCC 18191. *Beauveria bassiana* ATCC 7159 was found to be the only species of the tested organisms to transform **1** to several metabolites. All other organisms were able to produce only metabolites **2** and **3** (TLC screening). Scaling up of the biotransformation reaction with *Beauveria bassiana* ATCC 7159 led to the isolation and identification of four metabolites.

The ^{13}C-spectrum of metabolite **2** exhibited fifteen carbon signals. DEPT experiment discriminated these signals into three methyls, two methylenes, six methines and four quaternaries. The EIMS spectrum of **2** showed a molecular ion peak at m/z 264, analyzed for $C_{15}H_{20}O_4$, which has two mass units more than the substrate. This indicated the likely reduction of either the endocyclic or the exocyclic double bonds, the carbonyl group or opening the lactone ring. In the ^{1}H NMR spectrum, the disappearance of the two doublets at δ 5.59 and 6.29 (J = 2.6 Hz), which are characteristic for the exocyclic methylene at position 13, and the appearance of a secondary methyl doublet at δ1.31 (J = 7.6 Hz), assigned for protons at C-13 and a proton multiplet at δ 2.44 assigned for H-11 suggested the reduction of the exomethylene group. Furthermore, the disappearance of the carbon resonances at δ 121.5 and 140.1 assigned for C-13 and C-11 respectively, and the appearance of the new carbon resonances at δ 16.2 and 40.6, assigned for 13- and 11-positions respectively, supported this hypothesis. The configuration of the newly formed methyl group was deduced to be α-oriented from NOE experiment, where the NOE spectrum showed correlations between H-6, H-7 and H-13. This conclusion is consistent with results obtained by Shimoma et al. (1998) who reported that H-6 is always α-oriented in the ambrosanolide-type pseudoguianolides, where the lactone ring is β-oriented *cis*-fused at C-6, 7 positions. The ^{1}H NMR data of metabolite **2** were almost identical to those reported for hymenolin (Balza and Towers 1988; Shimoma et al. 1998). Thus the structure of **2** was confirmed to be the naturally occurring pseudogianolide hymenolin. The same

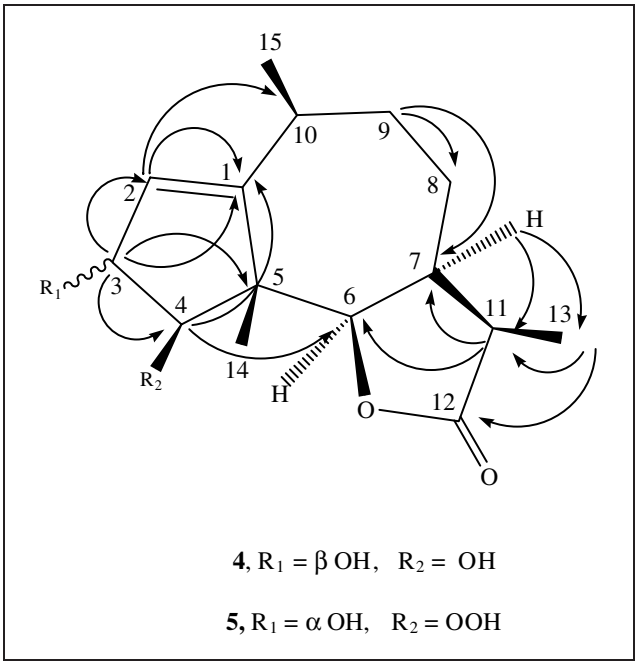


Fig. 1: Selected HMBC correlations of **4** and **5**

metabolite was probably isolated by Bhutani and Thakur (1991) from a biotransformation reaction of parthenin by the same organism (*Beauveria bassiana*) but the methyl group at position 11 was wrongly assigned a β-configuration, based only on the coupling constant of H-11 and H-13 (8 Hz).

The ^{1}H NMR and ^{13}C- data of metabolite **3** were almost similar to those of **2**, except for the disappearance of the two olefinic protons at δ 7.51 and 6.18 and their corresponding carbons at δ 162.8 and 131.4, respectively. Instead, new upfield multiplets at δ 1.65, 2.62 and 2.38 could be assigned to protons at positions 2 and 3, respectively. These signals were correlated to the carbon resonances at δ 33.0 and 32.0 by HMQC. These data indicated the reduction of both the endocyclic and exocyclic double bonds of parthenin to give the naturally occurring dihydrocoronopilin (Geissman et al. 1967).

The EIMS of metabolite **4** showed a molecular ion peak at m/z 266 indicating a molecular formula $C_{15}H_{22}O_4$ as concluded from HREIMS measurement. The ^{1}H NMR spectrum (Table 1) showed the absence of the two olefinic doublets at δ 7.5 and 6.2 (J = 6.0 Hz), which were characteristic for positions 2 and 3 of parthenin, respectively. A single olefinic proton signal appeared as a doublet at δ 5.65 (J = 2.1 Hz). This signal was correlated to a carbon at δ 129.8 and was assigned to C-2 by HMQC. The appearance of an olefinic quaternary carbon (^{13}C and DEPT spectra) at δ 153.5 and the deshielding effect exhibited on H-10 at δ 2.85 (δ + 0.50, as compared to δ 2.34 and 2.33 in **1** and **2**, respectively) as well as the absence of the deshielding effect of the OH group at C-1 on both C-5 and C-10 (Table 1) confirmed double bond migration to position 1. The ^{1}H NMR spectrum exhibited also two carbinolic proton resonances at δ 4.64 (dd, J = 5.5, 2.5 Hz) and 4.12 (d, J = 5.2 Hz). These resonances were correlated to the carbon signals at δ 73.2 and 78.6 (HMQC) which could be assigned to positions 3 and 4, respectively. Hydroxylation at these sites was confirmed through HMBC (Fig. 1), where cross peaks were observed between H-3, C-1, C-2, C-4 and C-5; between H-2, C-1, C-10 and C-4; between H-4, C-1 and C-6. The NOE spectrum of **4** (Fig. 2) showed correlations between H-3, H-4 and H-6 and between H-6, H-7 and H-13, which concluded the configuration of the hydroxyl groups at C-3 and C-4 to be β-oriented, while the methyl group at C-11 is α-oriented. These data proved that the structure of metabolite **4** has to be 3β,4β,6β-trihydroxy-10αH-11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone which is a new natural product.

Metabolite **5** is the most polar compound of parthenin metabolites. The ^{13}C NMR spectrum of **5** (Table) displayed 15 carbon resonances. All the signals were comparable to those of **4** except for the deshielded carbon signals at δ 88.5, 88.0 and 78.4 which were assigned by HMBC and HMQC to C-4, 6 and 3, respectively. The deshielding of C-4 in metabolite **5** by 10 ppm (from δ 78.6 to 88.5 as compared to **4**) refers to its possible peroxidation (Jung et al. 2001; El-Gamal et al. 2005) and causes the deshielding of C-3 and C-6 by 5 and 3 ppm, respectively as compared to **4**. It gave a positive test on TLC for OOH^- radical (see experimental, part 3.7). The HREIMS spectrum of **5** did not show a molecular ion peak. However, peaks due to loss of oxygen (fragment at m/z 266), in addition to fragments at m/z 264 [M-H_2O], m/z 249 [M-OOH] and m/z 248 [M-H_2O_2] which are characteristic to peroxide derivatives of sesquiterpene lactones (Zedro et al. 1987; Vajs et al. 2000) were detected. The orientation of the substituting groups at C-3 and C-4 as well as at C-11 were concluded based on observed enhancements in the NOE spectrum as shown in Fig. 2. It showed correlations between H-4 and H-6, between H-6 and H-7 and between H-7 and H-13, so the orientation of the hydroxyl group at position 4 was decided to be β, while the methyl group at position 13 was assigned α-configuration. No correlation was observed between H-3 and H-4, hence the OH group at position 3 was α-configured. Thus the structure of **5** could be formulated as 3α,6β-dihydroxy-4β-hydroperoxy-10αH-11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone, which is a new microbial metabolite of parthenin.

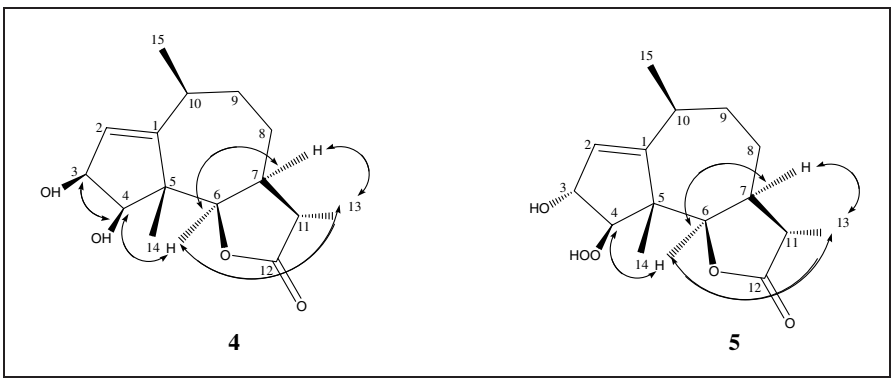


Fig. 2
Selected NOE correlations of **4** and **5**

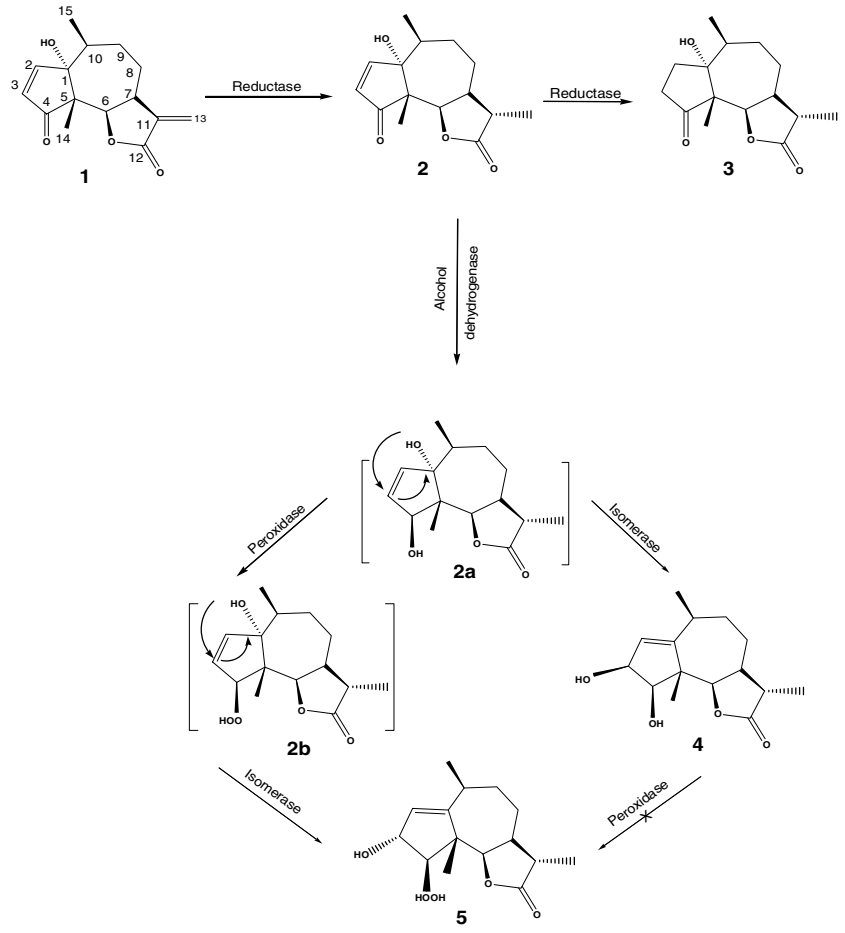
Table: ^{1}H and ^{13}C NMR data of parthenin and its metabolites*

	1		2		3		4		5	
	H	C	H	C	H	C	H	C	H	C
1		84.4		84.4		84.8		153.5		151.8
2	7.50, d (6.0)	162.3	7.50, d (5.9)	162.8	1.65, m, 2.62, m	33.0	5.65, d (2.1)	129.8	5.54, dd (1.0, 2.0)	129.1
3	6.20, d (6.0)	131.9	6.18, d (5.9)	131.4	2.38, m	32.0	4.64, dd (5.5, 1.5)	73.2	4.57, d (5.6)	78.4
4	–	209.8	–	210.8	–	217.9	4.12, d (5.2)	78.6	4.04, d (5.6)	88.5
5		59.0		58.9		58.8		56.8		55.8
6	5.01, d (8.0)	78.4	5.01, d (8.0)	79.1	4.94, d (8.0)	79.9	4.16, d (8.5)	85.0	4.48, d (8.2)	88.0
7	3.5, m	44.0	2.64, m	47.6	2.37, m	48.2	2.41, m	46.9	2.44, m	47.2
8	1.80, 2.23, m	28.3	1.89, m	29.6	1.80, m	25.6	1.83, 1.85, m	22.9	1.80, 1.92, m	22.4
9	1.73, m	29.8	1.66, 2.04, m	25.9	1.62, m	30.1	1.62, 1.69, m	29.2	1.65, 1.71, m	29.5
10	2.34, m	40.7	2.33, m	41.6	2.33, m	42.99	2.85, m	36.6	2.92, m	35.9
11	–	140.1	2.44, m	40.6	2.46, m	42.7	2.50, m	39.6	2.48, m	40.4
12		170.3		180.4		180.3		179.7		179.3
13	5.59, d (2.6) 6.29, d (2.6)	121.5	1.31, d (7.6)	16.2	1.31, d (7.6)	14.8	1.22, d (6.6)	14.5	1.23, d (6.6)	14.5
14	1.29, s	18.3	1.31, s	18.4	1.19, s	17.6	1.26, s	16.9	1.16, s	15
15	1.05, d (7.5)	17.4	1.13, d (7.6)	17.7	1.29,d (7.6)	16.1	1.23, d (7.6)	22.8	1.20, d (7.5)	23.6

* At 500 and 125 MHz in $CDCl_3$ as solvent and TMS as internal standard. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in Hz.
d = doublet, dd = double doublet, m = multiplet, s = singlet.

Thus, four metabolites were isolated from the biotransformation reaction of parthenin with *Beauveria bassiana* ATCC 7159 which is one of the most commonly used microorganisms known to mimic mammalian drug metabolism (Azerad 1999). Several enzyme-mediated steps seem to be involved in the chemical modification of parthenin, where reduction, isomerization of double bond and peroxidation obviously have taken place. Reductase

Scheme Possible metabolic pathway of parthenin by *Beauveria bassiana ATCC 7159*



likely converts **1** to **2** and **3**, then alcohol dehydrogenase, which is responsible for the reversible conversion of ketones to alcohols (Faber 1997; Azerad 2000), converts **2** into an intermediate metabolite **2a** which produces **4** by isomerase, or produces another intermediate, **2b**, by peroxidase. Metabolite **5** could be produced by isomerase from **2b** rather than peroxidation of **4** (Scheme). It is worth to note that the isolated metabolites might prove less toxic than the substrate, since all of them have been reduced at C-11. The toxicity of most of these pseudoguianolides is attributed to the presence of an intact α-methylene -γ-lactone moiety (Chhabra et al. 1999) and has to be verified.

3. Experimental

3.1. Instrumentation

Melting points: Yamagimoto micro-melting point apparatus MP-500D (values are uncorrected). Specific rotation: Horiba SEPA-300 digital polarimeter (I = 5 cm), UV spectra: Shimadzu UV-1600 spectrometer. IR spectra: FTIR-8100 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-LA 500 NMR (at 500 and 125 MHz, respectively) spectrometer, $CDCl_3$ as a solvent and TMS as internal standard. EIMS (20 ev) and HREIMS were recorded on a JEOL JMS-GCMATE mass spectrometer. Normal phase column chromatography: normal phase silica gel BW-200 (Fuji Silysia Chemical Ltd., 150–350 μm). Reversed phase column chromatography: chromatorex ODS DM102OT (Fuji Silysia Chemical Ltd., 100–200 μm). TLC: precoated TLC plates with silica gel 60 F_{254} (Merck, 0.25 mm) for normal phase and silica gel RP-18 F_{254S} (Merck, 0.25 mm) for reversed phase. HPLC was performed on a Shimadzu LC-6AD apparatus equipped with a Shimadzu RID-6A refractive index detector. TMC-Pack ODS-A (250 × 4.6 mm) and (250 × 20 mm) columns were used for analytical and preparative purposes, respectively.

3.2. Substrate

Parthenin (**1**) used in this study was isolated from the ethyl acetate extract of *Dichrocephala integrifolia* (L. F.) O. Kuntze (Asteraceae), growing wild in Yemen (Abdel Halim et al. 2004). The isolated material was identified by comparison of its different spectroscopic data to those published for parthenin (Rodriguez et al. 1971).

3.3. Fermentation method

Fermentation in liquid cultures was carried out according to the standard two-stage fermentation protocol (Betts et al. 1972). For screening experiments, solid cultures kept on either potato dextrose agar (PDA) or maltose sabaraud agar (MSA) of the following organisms: *Absidia pseudocylinderospora* ATCC 24169, *Amycolata autotrophica* ATCC 35203, *Aspergillus alliaceus* UI 315, *Aspergillus niger* ATCC 9142, *Aspergillus ochraceous* ATCC 1008, *Bacillus cereus* NRRL 14591b, *Bacillus megaterium* ATCC 14581, *Beauveria bassiana* ATCC 7159, *Botrytis alli* NRRL 2502, *Candida tropicalis* UI 2312, *Comamonas testosteroni* ATCC 11996, *Cunninghamella echinulata* ATCC 8688a, *Cunninghamella elegans* ATCC 9245, *Curvularia lunata* NRRL 2178, *Gliocladium viride* ATCC 10097, *Mortierella isabellina* ATCC 38063, *Mucor mucedo* UI 5513, *Nocardia species* NRRL 5646, *Rhizopus stolonifer* NRRL 1478, *Rhodtorula rubra* ATCC 20129, *Sepedonium chrysanthosporum* ATCC 13378, *Streptomyces griseus* ATCC 13273 and *Thamnidium elegans* ATCC 18191 were used to inoculate 100 ml flasks containing one fifth of their volume of the following medium: 2% glucose, 0.5% soybean meal, 0.5% yeast extract, 0.5% NaCl, 0.5% K_2HPO_4. The pH was adjusted to 7.0 before autoclaving for 20 min at 121 °C and 15 psi. The inoculated flasks were incubated at 27 °C and 250 rpm for 72 h (stage I cultures) before being used to inoculate stage II culture flasks, in which 10% inoculum volumes of stage I were used to inoculate another sterile medium and were incubated under the same conditions for 24 h. Parthenin (30 mg) dissolved in 1.5 ml of a CH_2Cl_2-DMSO (1 : 1) was added to the 24 h-old stage II cultures in 50 μl aliquots to give a final concentration of 1 mg substrate per flask. The flasks were incubated again and sampled periodically for analysis.

3.4. Sampling

Samples of 1 ml were taken after 12, 24, 36 and 48 h and then every other day for 2 weeks following substrate addition. Each sample was extracted by shaking with 0.5 ml of EtOAc and spun at 3000 × g for 1 min in a desktop centrifuge. The extracts were concentrated and spotted on Si gel GF_{254} TLC plates and developed with CH_2Cl_2-MeOH (95 : 5) and visualized after spraying with vanillin/H_2SO_4, followed by heating with a heating gun until maximum development of the spots colour.

3.5. Preparative scale conversion

Parthenin (400 mg) was added to stage II cultures (*Beauveria bassiana* ATCC 7159, which gave the best results in screening) prepared as above in 1 l flasks at concentration of 1 mg of substrate per ml of culture medium. After incubation for 2 weeks under the same conditions, the broth and the fungal mycelia were combined and exhaustively extracted with EtOAc 3 × 1 l. The extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to yield a crystalline brownish residue of 641.0 mg.

3.6. Isolation and purification of the metabolites

The extract (641.0 mg) was column chromatographed (45 g Si gel, 1.5 × 60 cm). Elution was achieved with MeOH/CH_2Cl_2 mixtures, starting with 100% CH_2Cl_2 and collecting 20 ml fractions to afford five fractions. Fraction B eluted with CH_2Cl_2-MeOH (98 : 2) afforded a mixture of the substrate, metabolites **2** and **3** (225 mg, one yellow spot, $R_f = 0.61$ in 5% MeOH/CH_2Cl_2). Elution with CH_2Cl_2-MeOH (97 : 3) gave fraction C which contained mainly metabolite **4** (97.0 mg, semisolid, $R_f = 0.30$, in 5% MeOH/CH_2Cl_2, as violet spot). Elution with CH_2Cl_2-MeOH (96 : 4) afforded a mixture of metabolites **4** and **5** (fraction D, 87.0 mg, semisolid, $R_f = 0.30$ and 0.23 in 5% MeOH/CH_2Cl_2, as violet spots). Further purification of the metabolites as well as the fractions containing the substrate was carried out as follows: fraction B was separated on reverse phase silica gel column chromatography (10 g, MeOH/H_2O, 20, 50 and 70%, 50 ml each) to give **1** (50 mg) and a crude fraction of **2** and **3** (150 mg). Metabolites **2** and **3** were further purified by HPLC (50% MeOH/H_2O, flow rate 8.0 ml/min) to give **2** (110 mg, R_t 39 min) and **3** (18 mg, R_t 41 min). Fraction C (97.0 mg) was subjected to reverse phase silica gel column chromatography (5.0 g, 40% MeOH/H_2O) to give metabolite **4** (85 mg). It was purified by HPLC (45% MeOH/H_2O, flow rate 8.0 ml/min) to give pure **4** (85 mg, R_t 41 min). Fraction D (87.0 mg) was purified by reverse phase silica gel column chromatography (5.0 g, 45% MeOH/H_2O) to give metabolite **5** (47 mg) which was similarly purified by HPLC (45% MeOH/H_2O) to afford metabolite **5** (29 mg, R_t 29 min).

3.7. Test for OOH^- radical

A TLC method for detection of the hydroperoxy radical was adopted from a qualitative test for peroxides (Vogel 1996) as follows: the developed chromatogram was air dried, sprayed with a freshly prepared spray reagent prepared as follows: 50 ml of 1% alcoholic solution of ferrous ammonium sulphate $(NH_4)_2Fe(SO_4)_2$ were mixed with 5 ml of 1 M H_2SO_4 and added to 5 ml of 0.1 M alcoholic solution of NH_4SCN. A dark red coloured spot was considered a positive result. Ascaridol and hydrogen peroxide were used as positive controls.

3.8 Hymenolin (**2**)

Colourless fine plates (from MeOH); mp 187–188 °C; $[\alpha]_D^{25} + 21.0°$ (MeOH; c = 0.60); UV λ_{max} 291 nm (log ε 4.5); IR ν_{max} (cm^{-1}): 3600 (OH), 1770 (lactone C=O), 1700 (α,β-unsaturated C=O). ^{1}H and ^{13}C NMR (500 and 125 MHz, respectively, $CDCl_3$) data, see Table. EIMS, 20 ev, m/z (relative intensity): 264 $[M]^+$ (10), 246 $[M\text{-}H_2O]^+$ (13), 218 (17), 193 (24), 111 (100).

3.9. 3β,4β,6β-Trihydroxy-10αH-11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone, (**4**)

Colourless fine crystals (from MeOH); m.p. 174–176 °C; $[\alpha]_D^{25} + 41.0°$ (MeOH; c = 0.4); UV λ_{max} 215 nm (log ε 3.60); IR ν_{max} (cm^{-1}): 3600 (OH), 1755 (lactone C=O). ^{1}H and ^{13}C NMR (500 and 125 MHz, respectively, $CDCl_3$) data, see Table. HREIMS: m/z 266.1517 (calc. for $C_{15}H_{22}O_4$: 266.1512); EIMS, 20 ev, m/z (relative intensity): 266 $[M]^+$ (25), 248 $[M\text{-}H_2O]^+$ (95), 175 (80), 135 (100).

3.10. 3β,6β-Dihydroxy-4β-perhydroxy-10αH-11α-methyl-ambrosa-1-en-12-oic acid-γ-lactone, (**5**)

Colourless fine crystals (from MeOH); m.p. 160–162 °C; $[\alpha]_D^{25} + 18.0$ (MeOH; c = 0.3); UV λ_{max} 213 nm (log ε 3.10); IR ν_{max} (cm^{-1}): 3580 (OH), 1750 (lactone C=O). ^{1}H and ^{13}C NMR (500 and 125 MHz, respectively, $CDCl_3$) data, see Table. EIMS, 20 ev, m/z (relative intensity): 266 $[M\text{-}O]^+$ (3), 249 $[M\text{-}OOH]^+$ (19), 248 $[M\text{-}H_2O_2]^+$ (100), 233 $[266\text{-}OOH]^+$ (21), 204 (38), 175 (64), 135 (43).

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