Hepatoprotective Triterpenes from Hairy Root Cultures of *Ocimum basilicum* L.

Amani M. Marzouk

Department of Pharmacognosy, Faculty of Pharmacy, University of Mansoura, Mansoura 35516, Egypt. Fax:+2–0 50–2 24 74 96. E-mail: amarzouk2003@yahoo.co.uk

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Six triterpene acids identified as betulinic, oleanolic, ursolic, 3-epimaslinic, alphitolic and euscaphic acids have been isolated from a dichloromethane extract of hairy root cultures of *Ocimum basilicum* L. (Lamiaceae). These cultures were obtained by genetic transformation using *Agrobacterium rhizogenes*. The extract as well as the isolated compounds were evaluated for their hepatoprotective activity by measuring their effect on the oxidative stress status of liver, induced by carbon tetrachloride, in albino rats and in liver homogenate *in vitro*. All tested compounds displayed hepatoprotective activity comparable to oleanolic and ursolic acids.

Key words: Ocimum basilicum, Hairy Root Cultures, Hepatoprotective Triterpenes

Introduction

Ocimum basilicum L. (sweet basil) is a popular lamiaceous herb used as culinary herb and ornamental greenery. It is considered as a major volatile oil crop (Grayer *et al.*, 1996), and most of the biological activities reported for sweet basil like antibacterial, antifungal and antioxidant activities are associated with its volatile oil content (Dube *et al.*, 1989; Wan *et al.*, 1998; Baratta *et al.*, 1998). Limited antioxidant activity of the polar extract of *O. basilicum* L. was attributed to its content of rosmarinic acid (Maarinova and Yanishieva, 1997). The aqueous extract of *O. basilicum* L. has been shown to possess a hepatoprotective effect in traditional medicine (Lin *et al.*, 1995; Kusamaran *et al.*, 1998). Although triterpenes such as oleanolic and ursolic acids, which are known as hepatoprotective agents (Liu, 1995; Jeong, 1999), have been reported from other species of basil like *O. sanctum* (Balanehru and Nagarajan, 1991) and other triterpene acids such as betulinic, alphitolic, pomolic and tormentic acids have been identified from the stems and roots of *O. basilicum* L. (Churng-Werng *et al.*, 1999), the plant has never been evaluated for the hepatoprotective activity of its triterpenoid constituent. In the present study, we report the isolation and identification of six triterpene acids, **1**–**6**, from hairy root cultures of *Ocimum basilicum*. These cultures were obtained by genetic transformation with *Agrobacterium rhizogenes*. The transformed root cultures offer plant material growing *in vitro* under controlled conditions without climatic or geographical limitations. They are also characterized by stability over long periods and high yield of secondary metabolites, like triterpenes, that is comparable to or far exceeding the source plant (Hamill and Lidgett, 1997). The effect of these triterpenes on the oxidative stress status of liver in albino rats, induced by carbon tetrachloride, was screened both *in vivo* and *in vitro*. Oxidative stress is implicated in chronic liver diseases in humans, regardless of the etiology (viral infection, alcohol consumption or metal overload), and serves as a link between liver injury and fibrosis. Membrane damage of hepatic cells could result in the release of oxygen-derived free radicals and other reactive oxygen species (ROS), derived from lipid peroxidation processes, that represent a general feature of sustained inflammatory response and liver injury leading to necrosis and fibrosis (Shimizu, 2005).

Results and Discussion

Transformation and identification of the compounds

TLC screening of different extracts from aerial parts and roots of *Ocimum basilicum* revealed a high content of triterpenes in the dichloromethane extract. Screening of these extracts for their hepatoprotective activity in female albino rats showed that the roots had the highest activity, but the per-

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centage yield of the extract from the roots was low (0.35% w/w, data not shown). Hence a transformed root culture for *O. basilicum* was established as an alternative to the normal root system. Such a culture is known to express the same metabolic profile as the normal roots of the explants and tends to accumulate higher concentrations of secondary metabolites. Hairy roots were induced on leaf and stem segments of *O. basilicum* using a supervirulent strain of *Agrobacterium rhizogenes* (LBA 9402) harbouring a kanamycin-resistant gene as selectable marker. The roots were grown in Murashige and Skoog (1962) liquid media in dim light. Transformants were selected on media containing kanamycin (50 mg l^{-1}). Transformation resulted in a three-fold increase in the percentage of extractives extracted by dichloromethane (DCM) (0.91% w/w) from hairy roots compared to normal roots. TLC screening of this extract revealed more or less the same pattern as that of the normal roots. Chromatographic fractionation led to the isolation of four major triterpenes which were identified by comparing their physical and spectral data (^{1}H NMR, ^{13}C NMR, HMBC, HMQC and ^{1}H-^{1}H COSY) with published ones. These were identified as betulinic acid (**1**) (Chatterjee *et al*., 2000), 3-epimaslinic acid (**3**) (Kojima and Ogura, 1989), 2α-hydroxybetulinic acid (alphitolic acid) (**5**) (Yagi *et al*., 1978; Suksamrarn *et al*., 2006) and euscaphic acid (**6**) (Kuang *et al*., 1989). Oleanolic acid (**2**) and ursolic acid (**4**) were present in the extract as minor components and were identified by comparing their R_f values with those of reference samples and their ^{13}C NMR data with published ones (Mahato and Kundu, 1994). It is worth noting that compounds **2**, **3** and **6** were identified here from *Ocimum basilicum* for the first time.

Compound **3** gave a positive Libermann-Burchard test for triterpenes. The ^{13}C NMR spectrum displayed 30 carbon resonances including a free carboxylic acid carbonyl at δ 181.7 suggesting that it is a triterpene acid. The ^{1}H and ^{13}C NMR (Tables I and II) spectra displayed some of the landmarks of the olean-12-en-28-oic structure: seven methyl singlets in the ^{1}H NMR spectrum resonating at δ 0.68–1.17, a broad singlet at δ 5.19 assigned to H12 and a multiplet at δ 2.75 assigned by HMQC and HMBC to H18. The above information was confirmed by carbon resonances in the ^{13}C NMR spectrum at δ 122.2 and 143.8 assigned to C12 and C13 of oleanane-type triterpenes. A monohydroxylated derivative of oleanolic acid was suggested based on a 16 mass unit increase over oleanolic acid of the molecular ion peak fragment of compound **3** at *m/z* 472 $[M]^+$. Hydroxylation at C2 was confirmed by the presence of two carbinol methine protons in the ^{1}H NMR spectrum at δ 3.89 (dd, J = 4, 10 Hz) and 3.29 (d, J = 4 Hz). These protons were correlated to each other in the COSY spectrum and were assigned by HMBC and HMQC to C2 and C3, respectively. The corresponding carbon atoms were resonating at δ 66.3 and 77.5 in the ^{13}C NMR spectrum, respectively. The orientation of the OH groups was deduced to be α at both positions based on a difference in the δ values between protons at C2 and C3 of 0.60 ppm and a small coupling constant (H2–H3) of 4 Hz (Kojima and Ogura, 1989). These data confirmed compound **3** to be 3-epimaslinic acid (Fig. 1), isolated here from *O. basilicum* for the first time.

Compound **5** gave a positive Libermann-Burchard test indicating a triterpene. The ^{13}C NMR spectrum (Table II) displayed 30 carbon resonances which were discriminated by an APT experiment into six methyl, ten methylene including a vinylic one at δ 109.4, seven methine groups and seven quaternary carbon atoms including a free carboxylic acid carbonyl at δ 179.3. Two doublets in the ^{1}H NMR spectrum (Table I) at δ 4.54 and 4.42 (J = 1.5 Hz) and a broad singlet at δ 1.51 due to a vinylic methyl group suggested that compound **5** has a lup-20-en-28-oic system. This was confirmed by the presence of carbon resonances in the ^{13}C NMR spectrum at δ 150.7 and 109.4 assigned by HMBC and HMQC to C20 and C29, respectively. Two carbinol methine protons observed at δ 3.46 (ddd, J = 4, 4, 9.5 Hz) and 2.74 (d, J = 9.5 Hz) indicated that compound **5** is a monohydroxylated betulinic acid derivative what was confirmed by a molecular ion peak at *m/z* 472 $[M]^+$ in the EI-mass spectrum. These protons were correlated by HMBC and HMQC to C2 and C3, respectively (δ 68.6 and 83.2). The orientation of the OH groups at C2 and C3 were assigned to be α and β, respectively, again based on a difference in the δ values between H3 and H2 of 0.72 ppm and a large coupling constant of 9.5 Hz (Kojima and Ogura, 1989). The collective data of **5** were comparable to those published for 2α,3β-dihydroxy-lup-20-en-28oic acid (alphitolic acid, Fig. 1) (Yagi *et al*., 1978; Suksamrarn *et al*., 2006).

Compound **6** was obtained as white powder with a molecular formula of $C_{30}H_{48}O_5$ as deduced from a quasimolecular ion peak in its ESI-mass spectrum at *m/z* 511 $[M+Na]^+$. It gave a positive Libermann-Burchard test, indicating its triterpenoidal nature. The ^{13}C NMR spectrum (Table II)

Table I. ^1H NMR data of compounds **3**, **5**, **6** (400 MHz in $CDCl_3/CD_3OD$, *J* values in Hz in parentheses).

H	**3**	**5**	**6**
2	3.89, dd (4, 10)	3.46, ddd (4, 4, 9.5)	3.88, dd (5, 10)
3	3.29, d (4)	2.74, d (9.5)	3.28, d (5)
12	5.19, brs	1.52, m 1.64, m	5.25, dd (4.4, 12)
18	2.75, dd (4, 14)	2.86, m	2.44, s
23	1.06, s	0.81, s	0.91, s
24	0.82, s	0.60, s	0.76, s
25	0.77, s	0.71, s	0.65, s
26	0.68, s	0.80, s	1.16, s
27	1.17, s	0.75, s	1.17, s
29	0.92, s	4.42, d (1.5) 4.54, d (1.5)	1.11, s
30	0.86, s	1.51, s	0.85, d (4.8)

Table II. ^{13}C NMR data of compounds **1**–**6** (100 MHz in $CDCl_3/CD_3OD$).

C	**1**	**2**	**3**	**4**	**5**	**6**
1	38.6	38.5	41.3	38.8	46.5	40.0
2	26.9	27.4	66.3	27.3	68.6	66.0
3	77.5	78.7	77.5	78.8	83.2	78.8
4	38.7	38.7	38.3	38.8	40.6	38.2
5	55.3	55.2	48.8	55.4	55.2	46.8
6	18.2	18.3	18.0	18.4	18.2	18.1
7	34.3	32.6	29.7	33.0	34.1	32.6
8	40.6	39.3	39.4	39.6	39.2	41.2
9	50.4	47.6	48.1	47.5	50.3	48.0
10	37.1	37.0	38.2	3.0	38.2	37.6
11	20.	23.1	22.9	23.3	20.9	23.6
12	25.5	122.1	122.2	125.5	25.3	128.9
13	38.2	143.4	143.8	138.0	38.1	138.2
14	42.4	41.6	41.8	42.0	40.6	41.3
15	30.5	27.7	27.7	28.2	30.4	29.7
16	32.2	23.4	23.2	24.3	32.1	26.0
17	56.3	46.6	47.3	48.1	56.1	47.5
18	46.9	41.3	41.1	52.8	48.1	53.2
19	48.9	45.8	46.4	39.1	48.9	73.1
20	150.7	30.6	30.7	38.8	150.7	41.2
21	29.6	33.8	33.9	30.7	29.5	25.4
22	37.1	32.3	32.5	36.7	37.0	32.6
23	27.8	28.1	28.5	28.2	28.2	28.4
24	16.0	15.6	21.9	15.5	16.4	21.8
25	15.8	15.3	16.3	15.7	17.1	16.1
26	15.3	16.8	16.9	16.9	15.7	16.5
27	14.6	26.0	26.0	23.6	14.5	24.5
28	179.5	181.0	181.7	179.5	179.3	181.0
29	109.4	33.1	33.1	16.9	109.4	27.1
30	19.2	23.6	23.6	21.2	19.1	16.2

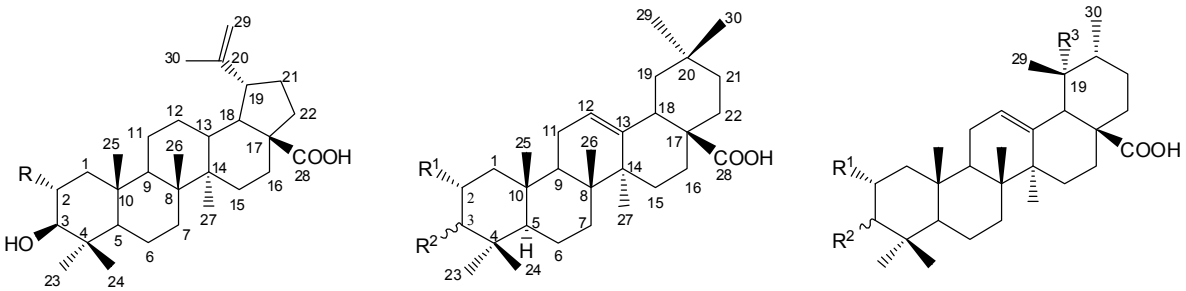

Fig. 1. Chemical structures of compounds **1**–**6** isolated from hairy roots of *Ocimum basilicum* L.

revealed the presence of 30 carbon resonances which were discriminated by the APT experiment supported by HMQC into seven methyl, eight methylene, seven methine groups including a trisubstituted C=C at δ 128.9 and possibly two oxygenated ones at δ 66.0 and 78.8 in addition to 8 quaternary carbon atoms including a free carboxylic acid resonance at δ 181.0, C=C at δ 138.2 and possibly an oxygenated carbon atom at δ 73.1. The ^{1}H NMR data (Table I) were in accordance with information drawn from the ^{13}C NMR spectrum and displayed some of the landmarks of urs-12-ene-type triterpenes: 7 methyl groups resonating between δ 0.65–1.17. These included six singlets and only one doublet at δ 0.85 (J = 4.8 Hz) assigned to the methyl group at position 30 and an olefinic proton signal at δ 5.25 (dd, J = 4.4, 12 Hz). The appearance of H18 (signal at δ 2.44) and methyl protons at position 29 (signal at δ 1.11) as singlets, as assigned by the HMQC experiment, suggested substitution at C19. Hydroxylation at this carbon atom was confirmed by a quaternary carbon resonance at δ 73.1 which showed cross-peaks in the HMBC spectrum with H18 (δ 2.44, s), $CH_3$29 (δ 1.11, s) and $CH_3$30 (δ 0.85, d, J = 4.8 Hz) as well as a fragment ion in the EI-mass spectrum at *m/z* 146 which is characteristic to a tertiary OH function at C19 in the urs-12-ene skeleton (Delgado *et al*., 1989). In the ^{1}H NMR spectrum, two carbinol protons at δ 3.88 (dd, J = 5, 10 Hz) and 3.28 (d, J = 5 Hz) indicated the presence of oxygen. These protons showed cross-peaks to each other in the COSY spectrum and were incorporated by HMQC to carbon resonances at δ 66.0 and 78.8, respectively. These carbon atoms were assigned to positions 2 and 3, respectively. The orientation of the OH groups at C2 and C3 was deduced to be α, based on a difference in chemical shift of 0.60 ppm between H2 and H3 (Kojima and Ogura, 1989) and a small coupling constant between H2 and H3 of 5 Hz. These data along with comparison with those published for tormentic and euscaphic acids (Kuang *et al*., 1989; Da Costa and Dos Santos, 2008) confirmed **6** to be 2α,3α,19α-trihydroxy-urs-12-en-28-oic acid (euscaphic acid, Fig. 1) which was isolated before from *Rosa davurica* and *Euscaphis japonica* (Kuang *et al*., 1989; Takahashi *et al*., 1974), but identified here from *O. basilicum* for the first time.

In vivo hepatoprotective activity

A triterpene-rich CH_2Cl_2 extract from hairy root cultures of *O. basilicum* was screened for its hepatoprotective activity against extracts from the aerial parts and normal roots of the explant. Recently, triterpenes such as oleanolic, ursolic and glycyrrhetinic acids as well as derivatives of them were shown to be effective in inhibiting CCl_4-induced hepatotoxicity in experimental animals (Jeong, 1999; Jeong *et al*., 2002; Wu *et al*., 2008). CCl_4, a well-known model compound for the induction of chemical hepatic injury, requires biotransformation by hepatic microsomal enzymes to produce the hepatotoxic trichloromethyl radicals ($CCl_3^{\bullet}$) and/or $CClOO^-$. The covalent binding of these free radicals to cell proteins is considered as initial step in a chain of events eventually leading to membrane lipid peroxidation and finally to cell necrosis. Injection of CCl_4 to rats significantly increased the levels of liver transaminases (ALT and AST) over the normal group (Table III). Although oral administration of a hairy root extract

Table III. Effects of pretreatment with different basil extracts on CCl_4-induced hepatotoxicity in rats.

Marker	Normal	CCl_4	Aerial parts	Normal roots	Hairy roots	Silymarin[a]
ALT [U/l]	29 ± 4.6	279 ± 4.2	195 ± 38.7	113 ± 4.7	259 ± 1.3	248 ± 3.8
AST [U/l]	167 ± 6.1	365 ± 14.3	290 ± 17.5	276 ± 20.1	329 ± 6.2	349 ± 4.4
MDA [nmol/g liver]	136 ± 7.5	272.7 ± 32.5	149 ± 2.4[b]	140 ± 4.6[b]	144 ± 5.6[b]	108 ± 10.3[b]

Data are expressed as means ± SE.
[a] Silymarin is used as positive control.
[b] Significant from CCl_4 at $p < 0.01$.

Table IV. Inhibitory effect of a hairy roots extract and silymarin on iron ascorbate-induced lipid peroxidation.

Treatment [mg ml^{-1}]	Hairy roots	Silymarin
10.0	34.3 ± 4.6	16.1 ± 1.1
5.0	29.9 ± 8.8	9.9 ± 1.5
1.0	7.9 ± 2.5	4.3 ± 2.1

Data are expressed as percentage inhibition compared to a fully peroxidized control ± SEM.

Table V. Inhibitory effect of compounds isolated from hairy roots on iron ascorbate-induced lipid peroxidation.

Compound	Inhibitory effect at			
	5.0 mg ml^{-1}	1.0 mg ml^{-1}	0.5 mg ml^{-1}	0.1 mg ml^{-1}
1	52.7 ± 15.7	32.5 ± 6.8	19.8 ± 4.7	16.8 ± 2.4
3	38.8 ± 2.3	29.1 ± 7.4	17.6 ± 3.4	13.7 ± 4.8
5	40.9 ± 2.3	22.3 ± 2.8	12.9 ± 3.5	9.6 ± 8.3
6	56.8 ± 1.6	14.22 ± 3.5	11.65 ± 2.1	11.5 ± 4.8
2	50.5 ± 2.2	26.0 ± 3.1	12.9 ± 1.8	11.0 ± 1.9
4	45.4 ± 0.5	27.5 ± 7.3	9.4 ± 4.7	6.6 ± 2.7

Data are expressed as percentage inhibition compared to a fully peroxidized control ± SEM.

at doses of 200 mg kg^{-1} had a low capacity to lower the level of these enzymes; it was effective in lowering the liver oxidative stress which was measured by monitoring the level of malondialdehyde (MDA). MDA is a major product of lipid peroxidation which is used as a marker of oxidative liver damage. The protective effect of the hairy root extract on liver injury was not statistically different from that of silymarin (milk thistle extract), a widely used hepatoprotective natural product (Table III).

Effect of the hairy root extract and the isolated compounds on iron ascorbate-stimulated lipid peroxidation

The hairy root extract and the isolated compounds **1**–**6** were evaluated separately *in vitro* for inhibition of liver oxidative stress in mice liver homogenate using the iron ascorbate system. Liver peroxidation can proceed *in vitro* in a nonenzymatic way. It is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+} (Fukuzawa *et al*., 1993; Bondet *et al*., 2000). The protective activity of the isolated triterpenes (calculated as percentage inhibition of MDA formation compared to a fully peroxidized control) was compared to that of oleanolic and ursolic acids, which are known to be hepatoprotective triterpenes (Jeong, 1999; Tang *et al*., 2005). The hairy root extract and silymarin, a standardized extract from milk thistle which is widely used as hepatoprotective natural product, were also evaluated in the same system. The hairy root extract displayed a good hepatoprotective activity *in vitro* that was 2- to 3-fold that of silymarin (obtained as legalon capsules, CID for Chemical Industries, Egypt) at concentrations of 10 and 5 mg ml^{-1}, respectively (Table IV). All the tested compounds displayed a con-

centration-dependent inhibitory effect on lipid peroxidation, which was comparable to that of oleanolic and ursolic acids. The oleanane derivative 3-epimaslinic acid (**3**) displayed the lowest activity [(38.8 ± 2.3)%], while betulinic acid (**1**) and the ursane derivative euscaphic acid (**6**) displayed the highest activity [(52.7 ± 15.7) and (56.8 ± 1.6)%] at 5 mg ml^{-1}. At the lower concentration of 0.1 mg ml^{-1} all test compounds displayed more or less the same activity (Table V). The results of this preliminary study will lead to further investigations of the nonpolar fraction from the root extract of *O. basilicum* and also consideration of the transgenic root culture as a source for hepatoprotective compounds. Dose adjustment and extract standardization and possibly further purification of the crude extract may give more promising results, especially because they were not statistically different from those of silymarin *in vivo*, while were far better in the *in vitro* assay. Both *in vitro* and *in vivo* results were consistent, but the effect on the levels of the liver injury markers (ALT and AST) should be carefully evaluated, because it might be due to the toxicity of some of the constituents that could not be isolated and identified; especially some triterpenes such as betulinic acid, which is already isolated in this work, are reported to have cytotoxic activity (Chatterjee *et al.*, 2000). This effect on liver enzymes may also be due to the dose used in the assay, which was chosen on the basis of the dose usually used to screen natural products extracts (150–300 mg kg^{-1}). A toxicity study would clarify this point, even though basil has been always looked at as a safe culinary herb.

Experimental

General experimental procedures

Plant materials were collected from plants grown on the campus of Mansoura University, Mansoura, Egypt. Melting points (uncorrected) were recorded on a Yamagimoto micro-melting point apparatus MP-500D. IR spectra were recorded on an FTIR-8100 spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-LA 400 NMR (at 400 and 100 MHz, respectively) spectrometer with $CDCl_3$ and CD_3OD as solvents and TMS as internal standard. EI-mass spectra (20 eV) were recorded on a JEOL JMS-AX500 mass spectrometer. ESI-mass spectra was recorded on a Micromass LCT spectrometer. Column chromatography was performed on normal phase silica gel BW-200 (150–350 μm, Fuji Silysia Chemical Ltd., Japan). TLC was performed on precoated TLC plates with silica gel 60 F_{254} (0.25 mm, Merck, USA). Solvents were of analytical grade, and media and chemicals for tissue culture and thiobarbituric acid assay were obtained from Sigma (UK). Kits for measuring the liver enzymes were obtained from Biodiagnostics (Egypt).

Establishment of transformed root cultures

Transformed roots were obtained by infecting surface-sterilized stem and leaf segments with *Agrobacterium rhizogenes* strain LBA 9402 (a kind gift from Dr. A. Petit, Laboratoire de Biologie de la Rhizosphere, Versailles Cedex, France, in 1998). This strain harbours the wild-type root-inducing plasmid pRi 1855 engineered to contain, in addition, plasmid Bin 19 which harbours a kanamycin-resistant gene as a selectable marker. An overnight bacterial suspension in YMB (Hooykaas *et al.*, 1977) supplemented with 50 μM acetosyringone was used for inoculation into freshly wounded explants. Infected samples were transferred to 1/10 Murashige and Skoog (MS) solidified agar media (Murashige and Skoog, 1962), kept in the dark for 48 h and incubated in dim light at (20 ± 2) °C. The putative hairy roots, which appeared on the infected samples within two weeks were cut and transferred to MS hormone-free, liquid media supplemented with 30 g l^{-1} sucrose. Ampicillin sodium salt at a concentration of 500 mg l^{-1} was added during subsequent subculturing until the cultures were free from the residual bacteria. Transformants were selected on kanamycin-containing media, 50 mg l^{-1}. The obtained axenic roots were maintained on the same liquid media (50 ml in 250 ml-flasks) on gyratory shakers (90 rpm), at (20 ± 2) °C in dim light. The roots were sub-cultured every two weeks. For isolation of the compounds, the roots were inoculated into 5 l-flasks containing 2 l of the same media, aerated with a bubble-type sparger and incubated for a period of four weeks in dim light.

Extraction and isolation

Fresh, four-week-old hairy roots (273.50 g fresh wt) were extracted with MeOH at room temperature (2 l × 3). Evaporation of the solvent under reduced pressure gave the crude extract (3.7 g,

1.35% w/w). The concentrated MeOH extract was suspended in H_2O and extracted with CH_2Cl_2 (1 l × 3). Removal of the solvent under reduced pressure afforded 2.5 g (0.91% w/w) of the CH_2Cl_2 extract, which was chromatographed on a silica gel column and eluted with mixtures of CH_2Cl_2/MeOH of increasing polarity (0–30% MeOH). The collected fractions were monitored by TLC and visualized by spraying with vanillin/H_2SO_4; similar fractions were pooled to yield five main fractions (A–E). These fractions were purified on silica gel columns eluted with hexane/EtOAc mixtures. Fractions A and B (182 mg) afforded betulinic acid (**1**, 37 mg) and oleanolic acid (**2**, 4 mg; hexane/EtOAc, 85:15; R_f 0.75, 0.66; $CHCl_3$/MeOH, 92.5:7.5). Fraction C (127 mg) afforded 3-epimaslinic acid (**3**, 34 mg) and ursolic acid (**4**, 6 mg; hexane/EtOAc, 80:20; R_f 0.58, 0.54; $CHCl_3$/MeOH, 92.5:7.5). Fraction D (205 mg) afforded alphitolic acid (**5**, 105 mg; hexane/EtOAc, 70:30; R_f 0.50; $CHCl_3$/MeOH, 92.5:7.5; R_f 0.49; hexane/EtOAc, 70:30). Fraction E (80 mg) afforded euscaphic acid (**6**, 54 mg; hexane/EtOAc, 60:40; R_f 0.45, 0.50; $CHCl_3$/MeOH, 92.5:7.5).

Betulinic acid (**1**): White crystals, m.p. 296–298 °C. – IR (KBr): υ = 3442, 3427, 2940, 1689, 1641, 1380, 1035 cm^{-1}. – ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$): see Table II. – EI-MS: *m/z* (rel. int.) = 456 (4.2) $[M]^+$, 438 (0.9) $[M–H_2O]^+$, 248 (9.5), 189 (23.0), 85 (75.1), 83 (100), 57 (76.8).

Oleanolic acid (**2**): White crystals, m.p. 298–300 °C. – IR (KBr): υ = 3400, 3200, 2940, 1650 cm^{-1}. – ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$): see Table II. – EI-MS: *m/z* (rel. int.) = 456 (1.4) $[M]^+$, 249 (17.6), 248 (100), 203 (85.4), 189 (17.1), 85 (59.7), 83 (88.5), 57 (29.5), 55 (41.8).

2α,3α-Dihydroxy-olean-12-en-28-oic acid (3-epimaslinic acid) (**3**): White powder. – IR (KBr): υ = 3443, 3423, 2940, 2871, 1693, 1640, 1384, 1274, 1037 cm^{-1}. – ^1H and ^{13}C NMR (400, 100 MHz, $CDCl_3/CD_3OD$): see Tables I, II. – EI-MS: *m/z* (rel. int.) = 472 (0.4) $[M]^+$, 455 (0.2) $[M–OH]^+$, 249 (24.2), 248 (100), 203 (85.0), 189 (19.9), 57 (42.5), 55 (64.0).

Ursolic acid (**4**): Colourless crystals, m.p. 290–292 °C. – IR (KBr): υ = 3448, 2927, 1679 cm^{-1}. – ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$): see Table II. – EI-MS: *m/z* (rel. int.) = 456 (0.4) $[M]^+$, 248 (55.2), 203 (47.0), 189 (52.3), 175 (32.7), 55 (100).

2α,3β-Dihydroxy-lup-20-en-28-oic acid (alphitolic acid) (**5**): White crystals, m.p. 279–281 °C. – IR (KBr): υ = 3442, 3425, 2933, 1685, 1635, 1381, 1047 cm^{-1}. – ^1H and ^{13}C NMR (400, 100 MHz, $CDCl_3/CD_3OD$): see Tables I, II. – EI-MS: *m/z* (rel. int.) = 473 (4.8) $[M+1]^+$, 472 (8.9) $[M]^+$, 454 (18.1) $[M–H_2O]^+$, 248 (90.1), 205 (82.9), 189 (93.0), 57 (48.0), 55 (100).

2α,3α,19α-Trihydroxy-urs-12-en-28-oic acid (euscaphic acid) (**6**): White powder. – IR (KBr): υ = 3450, 3420, 2927, 1690, 1631, 1040 cm^{-1}. – ^1H and ^{13}C NMR (400, 100 MHz, $CDCl_3/CD_3OD$): Tables I, II. – ESI MS: *m/z* (rel. int.) = 511 (29.9) $[M+Na]^+$, 999 (21.0) $[2M+Na]^+$, 443 (58.3) $[M–COOH]^+$, 426 (96.1) $[M–COO–H_2O]^+$. – EI-MS: *m/z* (rel. int.) = 472 (2.5) $[M–OH]^+$, 453 (3.4) $[M–OH–H_2O]^+$, 426 (12.7) $[M–COO–H_2O]^+$, 408 (9.9), 248 (57.2), 203 (44.0), 189 (55.3), 175 (36.7), 146 (7.4), 55 (100).

In vivo hepatoprotective assay

Female albino rats (120–150 g) were allowed free access to a standard meal and tap water and maintained in a controlled environment at (25 ± 2) °C under a 12 h dark/light cycle; they acclimatized at least for one week before starting the experiment. Animals were divided into groups of six each. The treated groups received the corresponding extract four times weekly for three weeks [200 mg kg^{-1}, orally, suspended in carboxymethyl cellulose (CMC)]. The normal group received CMC, 2% w/v. The control group was treated with CCl_4 twice weekly for three weeks (2 ml kg^{-1}, i.p., dissolved in corn oil). 24 h after the last treatment, animals were anesthetized by diethyl ether, and blood was collected by puncture of the retro-orbital plexus to determine the ALT and AST activities. Animals were then killed by cervical dislocation. Livers were dissected, quickly frozen and kept for liver peroxidation analysis.

Hepatotoxicity evaluation

The serum ALT and AST activities were measured using a spectrophotometric diagnostic kit according to the manufacturer's instructions. Liver peroxidation was measured by the formation of the thiobarbituric acid reactive material malondialdehyde (MDA), following a modified thiobarbituric acid reactive species assay (Baratta *et al.*, 1998).

Iron ascorbate-stimulated lipid peroxidation in liver homogenate

The mechanism of iron ascorbate-accelerated lipid peroxidation was discussed by Fukuzawa *et al*. (1993), Bondet *et al*. (2000), and Zadlo *et al*. (2006). The assay was carried out in liver homogenate according to a method described by Jeong *et al*. (2002) with slight modifications. Briefly, a 10% mice liver homogenate in 1.1% w/v KCl was used. The reaction mixture consisted of 250 μl liver homogenate, 50 μl of 0.1 mM ascorbic acid, 50 μl of 4 mM $FeCl_3$, 750 μl thiobarbituric acid (0.8% w/v) in sodium dodecyl sulfate (1.1% w/v), 750 μl acetic acid (20% v/v) and 50 μl of different concentrations of the test substances suspended in sodium dodecyl sulfate (8.1% w/v). The formed malondialdehyde was quantified following a modified thiobarbituric acid reactive species assay as described by Baratta *et al*. (1998). 1,1,3,3-Tetraethoxypropane was used as a standard for calibration of MDA.

Statistical analysis

Differences among all groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test. $N = 6$.

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