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A new triterpene and protective effect of *Periploca somaliensis* Browicz fruits against CCl_4-induced injury on human hepatoma cell line (Huh7)

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The potential hepatoprotective effect of the methanolic extract of *Periploca somaliensis* Browicz fruits, its different fractions (*n*-hexane, chloroform and *n*-butanol) and the major isolated compound ursolic acid was evaluated using the human hepatoma cell line (Huh7) based on the changes in the activity of aspartate aminotransferase, alanine transaminase, glutathione and superoxide dismutase. Each sample was tested at three different concentrations (1000, 100 and 10 μg/mL). All tested samples exhibited a potent concentration-independent cytoprotective effect relative to silymarin as a reference standard. Chromatographic fractionation of the chloroform-soluble fraction of the methanol extract of *P. somaliensis* Browicz fruits afforded two known triterpenes, namely ursolic acid, and 11α,12α-epoxy-3β-hydroxy-olean-13β,28-olide, and a newly discovered one, namely 3β-hydroxy-urs-11-en-13β,28-olide. The structures of the isolated compounds were elucidated by the analysis of 1D and 2D NMR spectral data.

Keywords: *Periploca somaliensis* Browicz; triterpenes; hepatoprotective; Huh7 cell line

1. Introduction

Plants of the genus *Periploca* have been used in Chinese traditional medicine for the treatment of rheumatoid arthritis. An important recent study attributed this effect to their pregnane glycoside contents, which have a potent immunosuppressive effect (Feng et al. 2008). Also, this class of compounds prevented hepatitis in mice and suggested to be used for the treatment of human auto-immune hepatitis (Wan et al. 2008). Other pharmacological activities such as antimicrobial (Mustaq et al. 2011) and cytotoxic effects (Itokawa et al. 1988) were also reported. A previous phytochemical study on *Periploca* species resulted in the isolation of pregnane glycosides (Itokawa et al. 1988; Feng et al. 2008; Wan et al. 2008), triterpenes (Zhang et al. 2006; Ma et al. 2007), cardiac glycosides (Hu et al. 1990; Zhang et al. 2006) and flavonoids (Ma et al. 2007). The absence of chemical or biological studies on *Periploca somaliensis* Browicz prompted the authors to initiate the current investigation on this species. This study assessed the cytoprotective effect of the methanolic extract of the fruits, its different fractions, as well as its major isolated compound in CCl_4-induced injury in human hepatoma cells (Huh7).

2. Results and discussion

The chloroform-soluble fraction of the methanolic extract of *P. somaliensis* Browicz fruits was subjected to phytochemical investigation which resulted in the isolation of three compounds.

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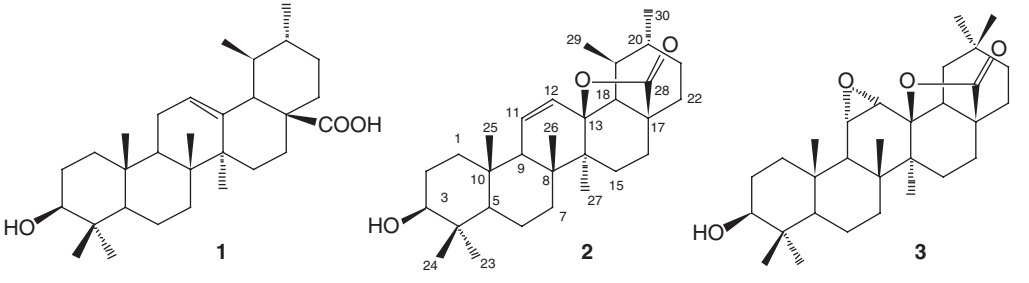
Compound **1** was identified as ursolic acid based on its melting point, mixed melting point, IR spectrum and co-chromatography with an authentic sample.

Compound **2** was isolated as a white amorphous powder with $[a]_D^{25}$ − 0.063° ($CHCl_3$, *c* 0.055). The molecular formula was deduced to be $C_{30}H_{46}O_3$ from the ESI-mass spectrum, and from ^{13}C NMR and DEPT spectra. The IR spectrum of compound **2** showed absorption bands due to a hydroxy group (3432 cm^{-1}) and a carbonyl group (1754 cm^{-1}). The ^1H NMR spectrum of **2** displayed signals of five tertiary methyl groups at δ_H1.15 (H-27), 1.05 (H-26), 0.98 (H-23), 0.90 (H-25) and 0.78 ppm (H-24) and two secondary methyl groups at δ_H 0.99 (H-29) and 0.92 ppm (H-30) indicating its possible ursanetriterpenoidal nature (D'Abrosca et al. 2006; Huang et al. 2012). This was confirmed from ^{13}C NMR and DEPT spectra. Eight degrees of unsaturation were calculated from the molecular formula of compound **2**, due to one olefinic double bond at δ_H 5.51 (dd, J = 10.4, 2.8 Hz, H-11) and 5.95 ppm (d, J = 10.4 Hz, H-12), one carbonyl group at δ_C 179.8 ppm (C-28), and the presence of six ring structures. Five rings were assigned to the ursanetriterpenoidal skeleton, and the additional one is due to 13,28-lactone ring. The long-range correlations (HMBC) between each of the olefinic protons and the strongly downfield carbon atom of C-13 (δ_C 89.6 ppm) indicated a 13,28-lactone ring. The long-range correlations between C-13 and H-18, and H_3-27 provided further evidence for a 13,28-lactone ring. The olefinic bond at C-11/C-12 was evident from the long-range correlations between each of H-11/C-10, C-8, and C-13, and H-12/C-9, C-13, and C-14 in the HMBC spectrum. From the aforementioned discussion and by comparison with the spectral data of the related compounds (Pereda-Miranda & Delgado 1990; Cheng et al. 2011; Huang et al. 2012), compound **2** could be identified as 3β-hydroxy-urs-11-en-13β,28-olide which is a newly isolated compound.

Compound **3** was isolated as a white amorphous powder with $[a]_D^{25}$ + 72.1° ($CHCl_3$; *c* 0.73). The molecular formula of compound **3** was deduced to be $C_{30}H_{46}O_4$ from the HR-ESI mass, ^{13}C NMR and DEPT spectra. The IR spectrum of compound **3** showed absorption bands due to a hydroxy group (3368 cm^{-1}) and a carbonyl group (1768 cm^{-1}). The ^1H NMR spectrum of compound **3** displayed signals due to seven tertiary methyl groups at δ_H1.10 (H-27), 1.06 (H-25), 1.05 (H-26), 0.99 (H-23 and 29), 0.92 (H-30) and 0.80 ppm (H-24). This indicated an oleanane structure of compound **3**, which was confirmed by comparing the spectral data with those of related compounds (Cheng et al. 2011; Lai et al. 2012). ^1H NMR and ^{13}C NMR spectral data of compound **3** showed the absence of the olefinic bond signals and the appearance of a broad singlet at δ_H 3.03 ppm integrated for two protons and their corresponding carbon atoms, according to HSQC, displayed at δ_C 52.7 and 57.3 ppm ascribable for 11α,12α an epoxy group (Xue et al. 2011). The HMBC spectrum showed a long-range correlation between the strongly downfield shifted oxygenated carbon atom (δ_C 87.5 ppm, C-13), the epoxy protons (H-11 and H-12) and 27-CH_3 protons (δ_H 1.10 ppm) which indicated the presence of a 13,28-lactone ring (Pereda-Miranda & Delgado 1990; Ikuta et al. 1995). Therefore, compound **3** was identified as 11α,12α-epoxy-3β-hydroxy-olean-13β,28-olide, previously isolated from the callus cultures of *Paeonia* species (Ikuta et al. 1995), and represents the aglycone moiety of thalicoside F isolated from *Hyptis albida* (Pereda-Miranda & Delgado 1990).



The cytoprotective effect of the methanolic extract of *P. somaliensis* Browicz fruits, its different fractions (*n*-hexane, chloroform and *n*-butanol), in addition to the major isolated compound ursolic acid (**1**) was evaluated. The test was performed for three different concentrations (10, 100 and 1000 μg/mL) using an *in vitro* assay on Huh7 cell line based on the changes in aspartate aminotransferase (AST), alanine transaminase (ALT), superoxide dismutase (SOD) activities and glutathione (GSH) level. The results demonstrated in Table 1 revealed that the exposure of Huh7 cells to CCl_4 significantly increased AST and ALT activities, and decreased the GSH level and SOD activity. Pretreatment with silymarin (10 and 100 μg/mL) significantly reduced CCl_4-induced elevation of AST and ALT activities in the supernatants, at the same time it increased the cellular GSH level and SOD activity. The three different concentrations of the tested samples exhibited a variable promising concentration-independent protective effect against CCl_4-induced damage on Huh7 cells. In general, higher concentration of the tried extracts did offer higher protection. In other instances, higher concentrations exhibited non-statistically significant changes in the protection level offered by lower concentrations. This could be explained by reaching maximum protection by exposure of cells to the low concentrations of the isolated fractions and thus, increasing the concentrations was not met by corresponding protection. All tested samples decreased AST and ALT activities to values close to that of silymarin, which indicates their ability to inhibit the degeneration and necrosis of liver cells from CCl_4-treated cells (Koji et al. 1995). Also, they nearly restored the normal value of the antioxidant GSH enzyme. Moreover, all tested samples except the *n*-butanol fraction significantly reduced the oxidative stress by increasing the SOD activity to a level close to that exhibited by silymarin.

Although, previous studies established more potent hepatoprotective effect for ethyl acetate and *n*-butanol fractions of other related plants, and attributed this effect to the antioxidant

Table 1. Cytoprotective effect of the methanolic extract of *P. somaliensis* Browicz, its different fractions and ursolic acid relative to silymarin using *in vitro* assay on human hepatoma cell line.

		(mean ± SD)			
Test sample	Concentration (μg/mL)	AST (units/mL)	ALT (units/mL)	GSH (mg/10^6 cells)	SOD (units/10^6 cells)
Normal	–	10.35 ± 2.19*	31.29 ± 7.15*	9.19 ± 1.04*	327.37 ± 18.52*
CCl_4-treated	40 mM	37.45 ± 3.17	62.23 ± 20.48	2.37 ± 0.36	181 ± 4.11
n-Hexane fraction	1000	20.51 ± 0.23*	39.46 ± 0.21*	7.86 ± 0.06*	228.12 ± 7.36*
	100	18.18 ± 0.61*	38.02 ± 0.18*	8.62 ± 0.36*	278.95 ± 6.62*
	10	18.80 ± 0.58*	37.58 ± 0.66*	6.99 ± 0.06*	292.20 ± 4.22*
Chloroform fraction	1000	25.39 ± 1.84*	42.45 ± 3.05*	6.19 ± 0.06*	261.70 ± 7.69*
	100	18.41 ± 2.32*	39.02 ± 1.03*	7.24 ± 0.03*	331.04 ± 6.97*
	10	16.32 ± 0.23*	36.19 ± 0.28*	8.64 ± 0.34*	192.33 ± 6.65*
n-Butanol fraction	1000	19.27 ± 3.55*	40.28 ± 0.99*	9.91 ± 0.10*	192.66 ± 7.09*
	100	14.85 ± 0.81*	35.75 ± 0.86*	9.93 ± 0.06*	193 ± 7.54*
	10	17.02 ± 1.23*	36.57 ± 1.15*	8.15 ± 0.07*	192.33 ± 6.65*
Methanolic extract	1000	20.51 ± 0.23*	38.96 ± 1.14*	8.13 ± 0.06*	198.83 ± 11.25*
	100	13.92 ± 0.26*	34.18 ± 0.21*	7.82 ± 0.03*	306 ± 12.16*
	10	20.27 ± 0.23*	39.46 ± 0.28*	7.22 ± 0.03*	275.41 ± 6.76*
Ursolic acid	1000	17.48 ± 0.61*	37.77 ± 0.94*	3.59 ± 0.34*	84.10 ± 2.59*
	100	19.89 ± 0.13*	38.77 ± 0.49*	7.73 ± 0.06*	86.13 ± 3.30*
	10	19.19 ± 0.13*	38.90 ± 0.71*	7.55 ± 0.03*	85.32 ± 2.59*
Silymarin	100	17.57 ± 0.13*	37.14 ± 0.10*	30.93 ± 4.53*	231.33 ± 7.09*
	10	20.66 ± 3.55*	38.52 ± 3.16*	28.13 ± 6.76*	292.75 ± 10.26*

Note: All variables were measured in triplicate, and the experiment was repeated three times.
*Significantly different from CCl_4 at $P < 0.05$.

activity of their phenolic content (Rice-Evans et al. 1996; Cai et al. 2004), in our study the *n*-butanol fraction of *P. somaliensis* Browicz fruits on ALT, AST activities and GSH level was as potent as the other tested samples; moreover, it exhibited a non-significant effect on the SOD activity. This study indicates that the potential cytoprotective effect of the methanolic extract and chloroform fraction is at least partly attributed to their content of ursolic acid as it constitutes the major isolated compound from the chloroform fraction, and/or other triterpene content. This suggestion is supported by previous studies which reported a potent hepatoprotective effect for ursolic acid (Wu et al. 2011).

3. Experimental

3.1. *General*

Electrothermal 9100 apparatus (Electrothermal Engineering Ltd, Southend on Sea, Essex, England) was used for the determination of melting point. Specific rotation was measured at ambient temperature using an Autoplol IV automatic polarimeter (Rudolph Research Analytical, Hackettst, NJ, USA). IR spectra were run in KBr using a Perkin-Elmer (Kyoto, Japan) infrared spectrophotometer FT-IR 1650. HR-ESI-MS (TOF) data were obtained on an Agilent (Palo Alto, CA, USA) Series 1100 SL mass spectrometer. Mass spectra were measured with Thermo scientific, ISQ single quadrapole mass spectrometer (San Jose, CA, USA). All NMR spectra were recorded on Bruker AVANCE AV-600 spectrometer (Bruker, Rheinstetten, Germany) relative to trimethylsilane in dimethylsulfoxide (DMSO).

3.2. *Chemicals for biological study*

Silymarin, carbon tetrachloride (CCl_4), DMSO and thiobarbituric acid reactive substance were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin 0.25%, penicillin G (100 units/mL), streptomycin (100 μg/mL) and phosphate-buffered saline were obtained from Gibco Invitrogen (Carlsbad, CA, USA). Kits for the measurement of AST, ALT, SOD and MDA levels were purchased from Biodiagnostics (Cairo, Egypt).

3.3. *Cell culture*

Human hepatocellular cancer cells (Huh-7) were obtained from VACSERA (Giza, Egypt). Cells were maintained in DMEM supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin and 10% heat-inactivated fetal bovine serum in a humidified, 5% CO_2 atmosphere at 37°C.

3.4. *Assessment of potential hepatoprotective effect*

To assess the potential hepatoprotective effect of the test samples (total methanolic extract, its fractions and ursolic acid), CCl_4-induced (40 mM) hepatic cell injury was induced as previously described (Gonzalez et al. 2011 and modified by Abdallah et al. 2013.

3.5. *Plant material*

Fruits of *P. somaliensis* Browicz were collected from west of Biljurshi, Saudi Arabia, in May 2010. The entire fruits were stored in a dry storage room under controlled temperature (32–35°C) and humidity (30–35%) and were grinded just before extraction. The identity of the plant was authenticated by Dr Farag A. Al-Ghamdi, Department of Biology, Faculty of Science, King

Abdulaziz University, Jeddah, Saudi Arabia. A voucher specimen (No. PS1033) is kept in the herbarium of Faculty of Pharmacy, King Abdulaziz University.

3.6. *Extraction and separation*

The air-dried powdered fruits of *P. somaliensis* Browicz (500 g) were extracted with methanol (4 × 3 L) at room temperature, till exhaustion to obtain a brown residue of 55 g. Part of the residue (50 g) was suspended in distilled water (500 mL) and partitioned successively with *n*-hexane (4 × 500 mL), chloroform (4 × 500 mL) and *n*-butanol (4 × 500 mL) to yield 8.14, 5.95 and 10.24 g, respectively. The chloroform fraction was chromatographed by VLC over silica gel 60 H column (5 cm × 7.5 cm, 30 g) eluted with *n*-hexane, *n*-hexane/chloroform mixtures and chloroform/ethyl acetate mixture with gradual increase in the polarity up to 100% ethyl acetate. Fractions of 200 mL each were collected and monitored by TLC. The spots were visualised after spraying with *p*-anisaldehyde/H_2SO_4 reagent followed by heating. Fractions showing similar TLC profiles were combined to give two main fractions. Fraction 8 (3.5 g) eluted with chloroform/*n*-hexane (3:7) was re-chromatographed over a silica gel column, using *n*-hexane/ethyl acetate (19:1) as an eluent to obtain compound **1** (150 mg). Fractions 11–13 (500 mg) eluted with pure chloroform to ethyl acetate/chloroform (1:4) were re-chromatographed over successive silica gel columns, using chloroform/ethyl acetate mixtures as an eluent to afford compounds **2** (19 mg) and **3** (13 mg).

*Ursolic acid (**1**)*: White powder. M.p. 283–286°C (uncorrected). IR; $\nu_{max} = 3520$ (OH), 1690 (carbonyl), 1630 and 812 cm^{-1} (olefinic).

*3β-Hydroxy-urs-11-en-13β,28-olide (**2**)*: White powder. M.p. 230–236°C (uncorrected). $[a]_D^{25} - 0.063°$ ($CHCl_3$, *c* 0.055). IR: $\nu_{max} = 3432$ (OH), 1754 (γ-lactone) and 1635 cm^{-1} (olefinic). MS: *m/z*: 454.44 $[M]^+$. ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 5.95$ (1H, d, $J = 10.4$ Hz, H-12), 5.51 (1H, dd, $J = 10.4$ and 2.8 Hz, H-11), 3.19 (1H, dd, $J = 10$ and 6 Hz, H-3), 2.13 (1H, dd, $J = 13.5$ and 3 Hz, H-18), 1.15 (3H, s, H-27), 1.05 (3H, s, H-26), 0.99 (d, $J = 6.4$ Hz, H-29), 0.98 (3H, s, H-23), 0.92 (3H, d, $J = 6.4$ Hz, H-30), 0.90 (3H, s, H-25), 0.78 (3H, s, H-24). ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 38.2$ (C-1), 26.97 (C-2), 78.7 (C-3), 38.9 (C-4), 54.7 (C-5), 17.6 (C-6), 31.2 (C-7), 41.9 (C-8), 53.0 (C-9), 36.3 (C-10), 128.7 (C-11), 133.4 (C-12), 89.6 (C-13), 41.6 (C-14), 25.51 (C-15), 22.7 (C-16), 45.0 (C-17), 60.5 (C-18), 38.1 (C-19), 40.2 (C-20), 30.8 (C-21), 31.3 (C-22), 27.7 (C-23), 14.9 (C-24), 17.8 (C-25), 18.8 (C-26), 16.0 (C-27), 179.8 (C-28), 17.8 (C-29), 19.1 (C-30).

*11α,12α-Epoxy-3β-hydroxy-olean-13β,28-olide (**3**)*: White powder. M.P. 258–263°C (uncorrected). $[a]_D^{25} + 72.1°$ ($CHCl_3$; *c* 0.73). IR ν_{max} $cm^{-1} = 3368$ (OH), 1768 (γ-lactone), 868 cm^{-1} (epoxy). HR-ES-MS: *m/z* = 470.3313 $[M]^+$ (calc. 470.33961). ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 3.23$ (1H, dd, $J = 10$ and 6 Hz, H-3), 3.03 (2H, br s, H-11 and 12), 2.30 (1H, dd, $J = 13.5$ and 3 Hz, H-18), 1.10 (3H, s, H-27), 1.06 (3H, s, H-25), 1.05 (3H, s, H-26), 0.99 (6H, s, H-23 and 29), 0.92 (3H, s, H-30), 0.80 (3H, s, H-24). ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 38.1$ (C-1), 26.7 (C-2), 78.7 (C-3), 40.5 (C-4), 54.5 (C-5), 17.5 (C-6), 31.0 (C-7), 40.5 (C-8), 49.5 (C-9), 36.4 (C-10), 52.7 (C-11), 57.3 (C-12), 87.5 (C-13), 41.2 (C-14), 26.6 (C-15), 21.2 (C-16), 43.8 (C-17), 50.6 (C-18), 37.7 (C-19), 31.4 (C-20), 34.2 (C-21), 26.9 (C-22), 27.7 (C-23), 15.0 (C-24), 17.1 (C-25), 20.0 (C-26), 18.8 (C-27), 179.3 (C-28), 33.1 (C-29), 23.5 (C-30).

3.7. *Statistical analysis*

All variables were measured in triplicate, and the experiment was repeated three times. Results were expressed as means ± SD. Data were analysed using one-way analysis of variance (ANOVA), followed by Tukey–Kramer's Post hoc test. Statistical significance was accepted at a level of $P < 0.05$.

4. Conclusion

In conclusion, the results of the present study indicate that the methanolic extract of *P. somaliense* fruits, its different fractions and the major isolated compound ursolic acid exhibited a potent concentration-independent *in vitro* hepatoprotective effect against CCl_4-induced injury on human hepatoma cell line (Huh7). The protective effect was measured based on the changes in the activity of AST, ALT, GSH and SOD comparing to those of the reference standard silymarin. Phytochemical study of the chloroform-soluble fraction of the methanol extract afforded two known triterpenes, namely ursolic acid, and 11α,12α-epoxy-3β-hydroxy-olean-13β,28-olide, and a newly discovered one, namely 3β-hydroxy-urs-11-en-13β,28-olide. The observed hepatoprotective effect of the methanolic extract and chloroform fraction is partly attributed to their content of ursolic acid and/or other triterpene content.

Supplementary material

Experimental details relating to this paper are available online, alongside Figures S1–S9.

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