Non-Phenolic Antioxidant Compounds from *Buddleja asiatica*

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The methanol extract of the leaves of *Buddleja asiatica* Lour. (Loganiaceae) showed antioxidant activity toward the well known *in vitro* antioxidant tests such as total antioxidant capacity by the phosphomolybdenum method, free radical scavenging activity by the 1,1-diphenyl-2-picrylhydrazyl scavenging assay (DPPH assay) and hydrogen peroxide scavenging methods. Due to the high scavenging activity of the *n*-butanol successive fraction toward DPPH and H_2O_2 (SC_{50} = 11.99 and 18.54 μg/ml, respectively), this extract was subjected to chromatographic separation and isolation. Four non-phenolic compounds were isolated and identified on the basis of spectroscopic and chemical analyses: 1-*O*-β-D-glucopyranosyl-2-methoxy-3-(2-hydroxy-triaconta-3,12-dienoate)-glycerol (**1**), 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-[β-D-glucopyranosyl-(1→2)]-β-D-fucopyranosyl-olean-11,13(18)-diene-3β,23,28-triol (**2**), 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-β-D-fucopyranosyl-olean-11,13(18)-diene-3β,23,28-triol (**3**), and 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucuronopyranosyl-acid-olean-11,13(18)-diene-3β,23,28-triol (**4**). The four compounds were evaluated as antioxidant agents using the three antioxidant bioassay tests.

Key words: Buddleja asiatica, Antioxidant, Triterpenoidal Glycosides

Introduction

The genus *Buddleja* (family Loganiaceae) comprises about 100 species native of the tropical lands of America, Asia and Africa. Several *Buddleja* species have been used in traditional medicine in many parts of the world. Roots, barks and leaves of these plants are used against different kinds of ailments such as dysentery, against eye or skin inflammation, as diuretic and antiseptic agents, antispasmodic, wound healing and anti-inflammatory agents (Cortés *et al.*, 2006; Houghton *et al.*, 2003; Houghton, 1984). Previous phytochemical investigations on *Buddleja* led to the isolation of flavonoids, iridoids, phenylpropanoids, sesquiterpenoids and saponins (Bowers *et al.*, 1991; Arciniegas *et al.*, 1997; Houghton *et al.*, 2003; Guo *et al.*, 2004).

Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective drugs have been recently shown to have an antioxidant or antiradical scavenging mechanism as part of their activity (Exachou *et al.*, 2006). Recently, attention has been focused on a wide array of natural antioxidants replacing synthetic antioxidants, which are restricted due to their side effects such as carcinogenicity. In addition natural antioxidants are able to scavenge or prevent formation of free radicals and to protect cells from oxidative damage (Huang *et al.*, 2005). In the search for new sources of natural antioxidants during the last few years medicinal plants have been extensively studied for their antioxidant and radical scavenging activities (Zaporozhets *et al.*, 2004; Sanchez-Moreno, 2002).

The roots, stems and leaves of *Buddleja asiatica* Lour. (Loganiacea) have been used as a traditional Chinese medicine for the treatment of fever, ache, diarrhea and articular rheumatism (Chen *et al.*, 2005). On continuation of our work for searching and phytochemically investigating medicinal plants, the methanolic extract and the *n*-butanol successive fraction of *B. asiatica* showed high antioxidant activity toward three *in vitro* antioxidant bioassays including determination of the total antioxidant capacity, by the phosphomolybdenum method, and the free radical scavenging ability, using the artificial radical 1,1-diphenyl-2-picrylhydrazyl (DPPH assay), and the hydrogen peroxide scavenging method. The preliminary phytochemi-

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cal tests of this plant revealed the presence of phenolics and saponins as the principle constituents. Due to the increasing interest in finding vegetables or medicinal plants containing saponins or non-phenolic compounds with antioxidant properties we aimed to isolate and identify some of the non-phenolic compounds from this plant. The isolated compounds were evaluated toward the *in vitro* bioassay tests.

Experimental

General

Melting points (uncorrected) were measured using the digital melting point apparatus Electrothermal IA 9200 (Electrothermal Engineering LTD, UK) and UV/Vis spectra using the Ultraviolet-visible spectrophotometer Milton Roy, Spectronic 601. ^{1}H and ^{13}C NMR spectra were recorded in CD_3OD and DMSO-d_6 solution containing TMS as internal standard on Bruker Avance 300 and 400 spectrometers equipped with a 5 mm normal configuration $^{13}C\{^{1}H\}$ probe with standard sequences operating at 300 and 400 MHz for ^{1}H and 75 and 100 MHz for ^{13}C NMR. The multiplicities of carbon atoms or the number of attached protons for a ^{13}C signal were determined using a DEPT-135 experiment. Mass spectra were recorded using a HPLC-MS instrument equipped with an Agilent G 1978A dual ESI and APCI mode ion source. Different sizes of open glass chromatographic columns were used packed with silica gel 60 (70–230 mesh, Merck) and Sephadex LH-20 (Sigma). TLC was performed over precoated silica plates (GF_{254}, Merck) and the spots were visualized by spraying with 40% sulfuric acid/methanol reagent followed by heating the plate at 110 °C for 15 min.

Plant material

The leaves of *B. asiatica* were collected at Agriculture Botanical Garden, Faculty of Agriculture, Cairo University, Egypt, in December 2004 and identified by Mrs. Traes Labib, General Manager and Head of Specialists of Plant Taxonomy in El-Orman Botanical Garden, Giza, Egypt. A voucher specimen was deposited at the Laboratory of Medicinal Chemistry, Theodor Bilharz Research Institute, Warrak El-Hader, Egypt. The plant was dried in shade, finally powdered with an electric mill and kept for biological and chemical investigation.

Extraction and isolation

1 kg of the powdered leaves was extracted three times with methanol (7 l) at room temperature. The solvent was evaporated under reduced pressure using a rotatory evaporator affording 183 g (18.3%) methanolic extract. The methanolic extract was dissolved in a small amount of distilled water and then successively extracted (partition fractionation) with petroleum ether (60–80 °C) (2 × 1 l) followed by *n*-butanol (2 × 1 l). The solvents were evaporated to afford petroleum ether (27 g, 2.7%) and *n*-butanolic (63 g, 6.3%) extracts. About 50 g of the *n*-butanolic extract were subjected to open glass column chromatography using a column (5 × 120 cm) packed with silica gel 60 (70–230 mesh) as adsorbent material. The column was washed with petroleum ether and then eluted with chloroform (100%) followed by a gradient of $CHCl_3$/MeOH till pure methanol. Fractions of 250 ml each were collected, analyzed by TLC (precoated silica gel GF_{254}; solvent systems C_6H_6/MeOH 85:25 v/v and *n*-butanol/Me_2CO/AcOH/H_2O 35:35:20:10 v/v/v/v) and grouped into three major groups, A–C. Group A (120 mg) was collected from a column eluted with CH_3Cl/MeOH 95:5 and then subjected to a Sephadex LH-20 open glass column (2 × 30 cm) affording compound **1** (12 mg, 0.0012%). Groups B (4.73 g) and C (2.3 g) were collected from a column eluted with CH_3Cl/MeOH 85:15 and 80:20, respectively. About 500 mg of each group were subjected to a Sephadex LH-20 open glass column (2 × 30 cm) using a gradient of methanol and water affording compound **2** (44 mg, 0.042%) and compound **3** (39 mg, 0.037%) from group B and compound **4** (27 mg, 0.013%) from group C.

Acid hydrolysis

5 mg of compounds **1**–**4** were hydrolyzed by reflux with 2 M HCl/1,4-dioxane (1:1, 2.5 ml), for 3 h. Dioxane was removed under reduced pressure and the remaining reaction mixture was extracted with dichloromethane (3 × 3 ml). After removing the organic layer, the aqueous layer was neutralized by sodium bicarbonate and evaporated to dryness, then extracted with a very small amount of pyridine. The sugar units were detected by means of TLC with authentic sugar samples (solvent system: EtOAc/*n*-BuOH/H_2O 20:70:10 v/v/v).

Partial acid hydrolysis by TLC

Partial acid hydrolysis was performed according to the method of Uniyal *et al.* (1991) and used to determine the kind of terminal sugar of the sugar moiety of the glycoside. The compound under investigation was applied to TLC (silica gel) and left in a HCl atmosphere at 30 °C for 1 h. After elimination of HCl vapour, the authentic sugars were applied to the chromatoplate. The chromatoplate was developed with the solvent system $CHCl_3$/MeOH/Me_2CO/H_2O 3:3:3:1 v/v/v/v and spots were detected by spraying with 40% methanolic H_2SO_4 followed by heating.

Compound ***1****:* Amorphous powder; m.p. 255–258 °C; R_f = 0.62 (C_6H_6/MeOH 85:25 v/v). – Acid hydrolysis afforded D-glucose. – ESI-MS: *m/z* = 736.6 $[M+Na]^+$, 712.7 $[M-H]^-$, 681 $[(M-H)-(OCH_3)]^-$, 532 $[(M-H)-(Glc-H_2O)]^-$, 475 $[(M-H)-(CH(OCH_3)-CH_2-O-Glc)]^-$, 325 $[(M-(CH=CH-(CH_2)_7-CH=CH-(CH_2)_{16}-CH_3)]^-$ and 265 $[M-(CH_2-(CH_2)_6-CH=CH-CH(OH)-COO-CH_2-CH(CH_3O)(CH_2-O-Glc)]^-$. – ^{1}H NMR (400 MHz, CD_3OD): δ = 5.76 (H-3″), 5.72 (H-4″), 5.51 (H-12″), 5.47 (H-13″), 5.40 (H-2, glycerol), 4.27 (1H, d, *J* = 7.8 Hz, Glc), 1.22–2.07 (long chain methylene of fatty acid), 0.91 (3H, t, *J* = 7 Hz, CH_3-30″). – ^{13}C DEPT NMR (100 MHz, CD_3OD): glycerol unit: δ = 69.88 (CH_2, C-1), 74.01 (CH, C-2), 64.66 (CH_2, C-3), 54.86 (OCH_3); glucose unit: δ = 104.88 (CH, C-1′), 73.32 (CH, C-2′), 78.16 (CH, C-3′), 71.82 (CH, C-4′), 75.18 (CH, C-5′), 62.92 (CH_2, C-6′); 2-hydroxy unsaturated esterified fatty acid unit: δ = 177.32 (COO, C-0″), 73.09 (CH, C-2″), 132.17 (CH, C-3″), 134.52 (CH, C-4″), 131.32 (CH, C-12″), 130.79 (CH, C-13″), 26.03–36.02 (CH_2, 5″–10″, 14″–28″ long chain methylene of fatty acid), 23.82 (CH_2, C-29″), 14.51 (CH_3, C-30″).

Compound ***2****:* Amorphous powder; m.p. 200–205 °C; R_f = 0.72 (*n*-butanol/Me_2CO/AcOH/H_2O 35:35:20:10 v/v/v/v). – Acid hydrolysis afforded D-fucose, D-glucose and L-rhamnose as sugar moieties. – ESI-MS: *m/z* (negative ion mode) = 1071.6 $[M-H]^-$, 909 $[(M-H)-162]^-$, 925 $[(M-H)-146]^-$, 763 $[(M-H)-(146+162)]^-$; *m/z* (positive ion mode) = 1095.5 $[(M+Na)]^+$, 949 $[(M+Na)-146]^+$, 787 $[(M+Na)-146-162]^+$, 643 $[(M+Na+H_2O)-146-162-162]^+$. – ^{1}H NMR (400 MHz, CD_3OD): δ = 6.42 (1H, dd, *J* = 10.6, 2.5 Hz, H-12), 5.60 (1H, d, *J* = 10.6 Hz, H-11), 4.88 (1H, d, *J* = 1.5 Hz, Rha), 4.86 (1H, d, *J* = 7.8 Hz, Glc\), 4.61 (1H, d, *J* = 7.7 Hz, Glc), 4.48 (1H, d, *J* = 7.7 Hz, Fuc), 0.99, 0.96, 0.95, 0.79, 0.74, 0.73 (3H, s, CH_3-27, -24, -30, -25, -29, -26). – ^{13}C and ^{13}C DEPT (100 MHz, CD_3OD) data of aglycone and sugars: see Tables II and III.

Compound ***3***: Amorphous powder; m.p. 247–252 °C; R_f = 0.66 (*n*-butanol/Me_2CO/AcOH/H_2O 35:35:20:10 v/v/v/v). – Acid hydrolysis afforded D-fucose, D-glucose and L-rhamnose as sugar moieties. – ESI-MS: *m/z* (negative ion mode) = 1071.5 $[M-H]^-$, 925 $[(M-H)-146]^-$, 763 $[(M-H)-(146+162)]^-$. – ^{1}H NMR (400 MHz, CD_3OD): δ = 6.42 (1H, dd, *J* = 10.7, 2.7 Hz, H-12), 5.60 (1H, d, *J* = 10.7 Hz, H-11), 4.87 (1H, d, *J* = 1.5 Hz, Rha), 4.85 (1H, d, *J* = 7.8 Hz, Glc), 4.62 (1H, d, *J* = 7.7 Hz, Glc), 4.48 (1H, d, *J* = 7.7 Hz, Fuc), 1.01, 0.96, 0.95, 0.79, 0.74, 0.73 (3H, s, CH_3-27, -24, -30, -25, -29, -26). – ^{13}C and ^{13}C DEPT (100 MHz, CD_3OD) data of aglycone and sugars: see Tables II and III.

Compound ***4****:* Amorphous powder; m.p. 216–218 °C; R_f = 0.61 (*n*-butanol/Me_2CO/AcOH/H_2O 35:35:20:10 v/v/v/v). – Acid hydrolysis afforded D-glucuronic acid, D-glucose, D-xylose and L-rhamnose as sugar moieties. – ESI-MS: *m/z* (positive ion mode) = 1095.4 $[M+Na]^+$, 949 $[(M+Na)-146]^-$, 793 $[(M-H)-146+132]^+$, 631 $[(M-H)-146+132+162]^+$; *m/z* (negative ion mode) = 1071.4 $[M-H]^-$, 925 $[(M-H)-146]^-$, 939 $[(M-H)-132]^-$. – ^{1}H NMR (300 MHz, DMSO-d_6): δ = 6.38 (1H, dd, *J* = 10.5, 2.6 Hz, H-12), 5.53 (1H, d, *J* = 10.6 Hz, H-11), 4.88 (1H, br, Rha), 4.65 (1H, d, *J* = 7.8 Hz, Xyl), 4.51 (1H, d, *J* = 7.8 Hz, Glc), 4.32 (1H, d, *J* = 8 Hz, H-1 GlcA), 0.91, 0.89, 0.84, 0.72, 0.63, 0.59 (3H, s, CH_3-27, -24, -30, -25, -29, -26). – ^{13}C NMR (75 MHz, DMSO-d_6) data of aglycone and sugars: see Tables II and III.

Antioxidant assays

The antioxidant activities of each methanolic and *n*-butanolic fraction as well as of the isolated compounds **1**–**4** were evaluated using the following antioxidant assays.

Determination of total antioxidant capacity

The total antioxidant capacities of the extracts and the isolated compounds were evaluated by the phosphomolybdenum method according to Prieto *et al.* (1999). 0.3 ml of each sample solution and ascorbic acid (100 μg/ml) were combined with 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium

phosphate and 4 mM ammonium molybdate). A typical blank solution contained 3 ml of reagent solution and the appropriate volume of the same solvent used for the sample. All tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had been cooled to room temperature, the absorbance of the solution of each sample was measured at 695 nm against the blank using a UV/Vis spectrophotometer. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Scavenging ability towards DPPH radical (DPPH assay)

The free radical scavenging activity of the tested extracts and compounds based on the scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl). The DPPH assay was performed as described by Shirwaikar *et al.* (2006). 2 ml of various concentrations of extracts/compounds were added to 2 ml solution of 0.1 mM DPPH. An equal amount of methanol and DPPH served as control, and also ascorbic acid was used as positive control. After 20 min of incubation at 37 °C in the dark, the absorbance was recorded at 517 nm. The experiment was performed in triplicate. The DPPH radical scavenging activity was calculated according to the equation: % DPPH radical scavenging activity = $1 - (A_{sample}/A_{control}) \times 100$, where A_{sample} and $A_{control}$ are the absorbance of sample and control. The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values were determined. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity.

Scavenging of hydrogen peroxide

The ability of the extract/compounds to scavenge hydrogen peroxide was determined according to Ruch *et al.* (1989) and Gülçin *et al.* (2004). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). 3.4 ml of samples of different concentrations (5–150 μg/ml) in phosphate buffer (pH 7.4) were added to 0.6 ml hydrogen peroxide solution. A control solution consisting of 3.4 ml phosphate buffer (pH 7.4) and 0.6 ml hydrogen peroxide solution (40 mM) was prepared. Also, for each concentration a separate blank sample was prepared for background subtraction. The absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenged of hydrogen peroxide by samples was calculated using the equation: % scavenged (H_2O_2) = $1 - (A_{sample}/A_{control}) \times 100$, where A_{sample} and $A_{control}$ are the absorbance of sample and control. The SC_{50} (concentration of sample required to scavenge 50% of H_2O_2) values were determined.

The SC_{50} values were calculated, using the SPSS 13.0 program, by probit-graphic interpolation of six concentration levels.

Results and Discussion

*Isolation and identification of compounds **1**–**4***

The aqueous fraction of the methanol extract of the leaves of *B. asiatica* was successively fractionated using petroleum ether and *n*-butanol. The *n*-butanolic fraction showed high scavenging activity toward DPPH and H_2O_2 (Table I). Also, pre-

Table I. Total antioxidant capacity, DPPH scavenging activity and H_2O_2 scavenging activity of the methanol and *n*-butanol extracts and of the isolated compounds **1**–**4**.

Extract/compound	Total antioxidant capacity[a] [mg equivalent to ascorbic acid/g extract]	DPPH free radical scavenging activity[b]	H_2O_2 scavenging activity[b]
		SC_{50} [μg/ml]	SC_{50} [μg/ml]
Methanol extract	383	16.28	23.17
n-Butanol extract	402	11.99	18.54
1	443	43.08	25.21
2	435	72.90	27.21
3	481	76.38	32.68
4	506	60.83	18.39

[a] Antioxidant activity monitored by the phosphomolybdenum method.
[b] SC_{50}, concentration in μg/ml required for scavenging the DPPH radical (100 μg/ml) or H_2O_2 (40 mM) by 50%.

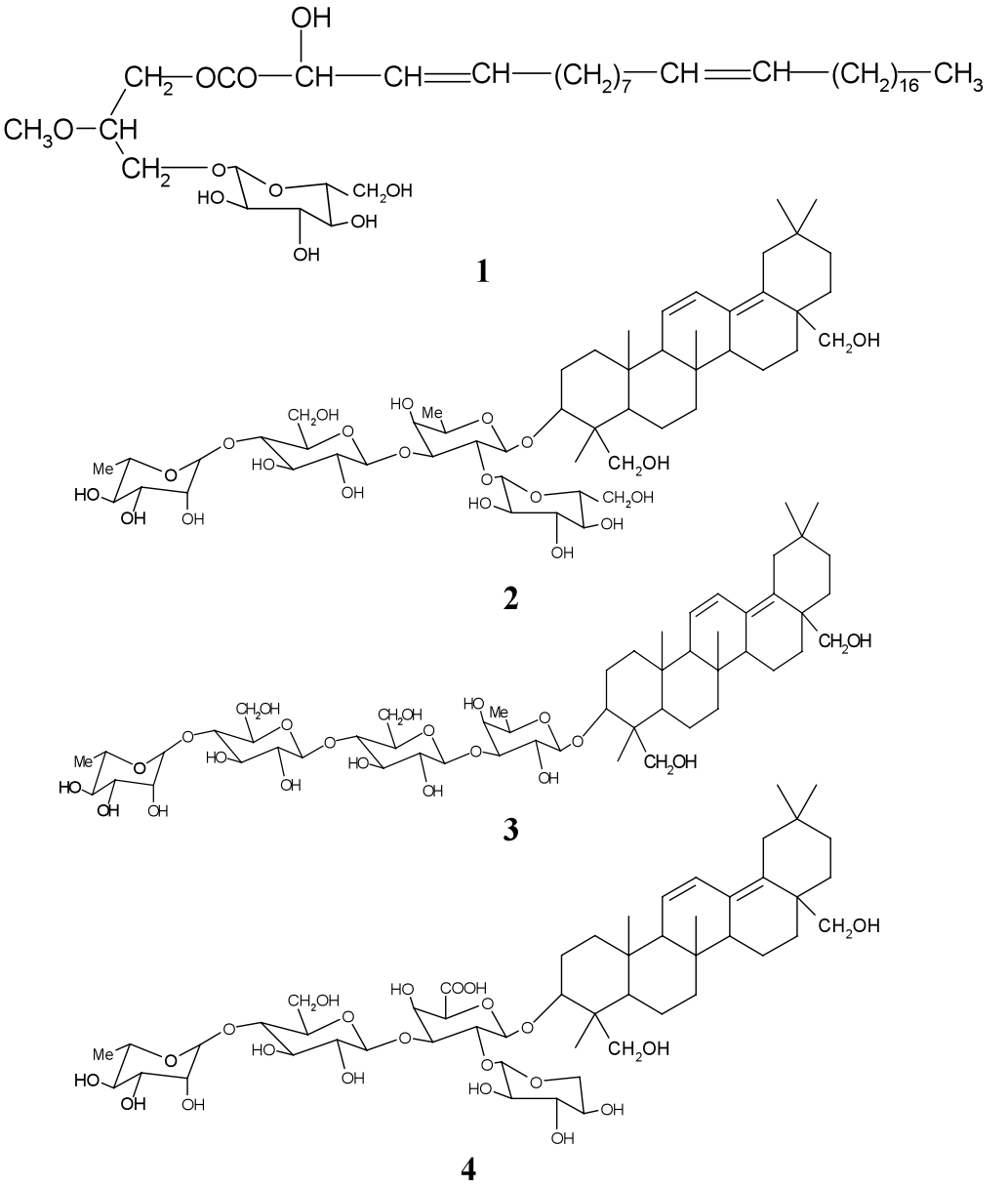

Fig. 1. Chemical structures of compounds **1**–**4**.

liminary phytochemical tests of the butanol extract showed the presence of phenolics and saponins as the principle constituents. The well known properties of phenolic compounds as antioxidant agents and also the increasing interest in finding vegetable saponins or non-phenolic compounds with antioxidant properties as previously studied in some saponin-rich plants, *e.g. Panax quinquefolium* (Li *et al.*, 1999), Vietnamese ginseng (Huong *et al.*, 1998), *Pueraria thunbergiana* (Park *et al.*, 2002), and *Camellia sinensis* (Sur *et al.*, 2001), encouraged us to chromatographically isolate and purify some non-phenolic compounds of the *n*-butanol extract using a combination of silica gel column chromatography and Sephadex LH-20 column chromatography. Four compounds, **1**–**4**, were iso-

lated and their structures were identified by interpretation of obtained data from spectroscopic and chemical analyses.

Compound **1** was obtained as amorphous powder. The ESI-mass spectrum showed an ion peak at *m/z* 712.7 $[M\text{-}H]^-$ in negative ion mode and at *m/z* 736.6 $[M+Na]^+$ in positive ion mode. The ESI-MS spectrum in combination with ^{13}C DEPT NMR analysis indicated that **1** has a molecular mass of 714 with the molecular formula $C_{40}H_{73}O_{10}$. The neutralized aqueous layer obtained from acid hydrolysis of **1** afforded D-glucose as sugar moiety on comparison with authentic sugar moieties by TLC or PC. Comprehensive NMR and mass spectra interpretation of compound **1** guided by review of literature (De Souza *et al.*, 2007; Vlahov, 1996; Scherer *et al.*, 1992; Knothe *et al.*, 1995) indicated that this compound consists of a glycerol unit attached to a long chain unsaturated 2-hydroxy fatty acid forming an ester at C-3 of glycerol, methoxy group with C-2 of glycerol and β-D-glucose with C-1 of glycerol unit. From all experimental data compound **1** could be identified as 1-*O*-β-D-glucopyranosyl-2-methoxy-3-(2-hydroxy-triaconta-3,12-dienoate)-glycerol (Fig. 1).

Compound **2**, obtained as amorphous powder showed a positive reaction in Libermann-Burchard and Molisch tests. The positive ESI-mass spectrum of **2** showed a quasimolecular ion $[M+Na]^+$ at *m/z* 1095.5, while the negative ESI-mass spectrum exhibited a molecular ion $[M\text{-}H]^-$ at *m/z* 1071.6. The ^{13}C DEPT NMR spectrums showed 54 carbon signals attributed to 8 methyl, 13 methylene, 25 methin groups and 8 quaternary carbon atoms. From ESI-MS and ^{13}C DEPT NMR analyses it can be consider that the molecular formula of **2** is $C_{54}H_{88}O_{21}$ with the molecular mass 1073. Acid hydrolysis of **2** afforded D-glucose, L-rhamnose and D-fucose, while partial hydrolysis afforded D-glucose and L-rhamnose identified by co-TLC and paper chromatography with authentic samples. The NMR data showed that signals of the aglycone were in good agreement with the known olean-11,13(18)-diene-3β,23,28-triol (Seifert *et al.*, 1991; Mahato and Kundu, 1994; Avila and De Vivar, 2002). The presence of a 3-*O*-glycosidic linkage was identified by the downfield shift of C-3 of the aglycone at δ_C 85.80 (Seifert *et al.*, 1991; Guo *et al.*, 2004, 2000). The comprehensive interpretation of NMR (Tables II and III) and mass fragmentation data revealed that the structure of **2** could be identified as 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-[β-D-glucopyranosyl-(1→2)]-β-D-fucopyranosyl-olean-11,13(18)-diene-3β,23,28-triol (Fig. 1). This compound was previously isolated from the aerial parts of *Verbascum songaricum* (Seifert *et al.*, 1991) and *Buddleja scordiodes* (Avila and De Vivar, 2002), but this is the first isolation from *B. asiatica*.

Compound **3**, obtained as amorphous powder, showed a positive reaction when tested in Libermann-Burchard and Molisch reactions. The ESI-mass spectrum in negative ion mode showed a strong ion at *m/z* 1071.5 and also a strong peak at *m/z* 1095.4 in positive ion mode indicating a molecular mass of 1073, which with ^{13}C DEPT NMR analysis gave the empirical formula $C_{54}H_{88}O_{21}$.

Table II. ^{13}C NMR and ^{13}C DEPT NMR (100 MHz, CD_3OD) chemical shifts of the aglycone of compounds **2** and **3** and ^{13}C NMR (75 MHz, DMSO-d_6) chemical shifts of compound **4**.

C	**2**		**3**		**4**
	δ	DEPT	δ	DEPT	δ
1	37.45	CH_2	37.44	CH_2	37.38
2	26.48	CH_2	26.49	CH_2	26.18
3	85.80	CH	85.79	CH	83.15
4	41.54	C	41.55	C	42.33
5	48.22*	CH	48.12*	CH	46.50
6	19.02	CH_2	19.01	CH_2	18.14
7	33.09	CH_2	33.09	CH_2	32.18
8	39.18	C	39.17	C	41.71
9	55.75	CH	55.74	CH	53.91
10	40.95	C	40.96	C	41.80
11	127.24	CH	127.23	CH	125.88
12	126.50	CH	126.50	CH	125.09
13	137.70	C	137.69	C	135.30
14	44.44	C	44.44	C	46.28
15	33.17	CH_2	33.21	CH_2	32.54
16	25.33	CH_2	25.33	CH_2	25.13
17	43.51	C	43.51	C	42.70
18	136.04	C	136.04	C	135.19
19	36.12	CH_2	36.11	CH_2	36.82
20	33.78	C	33.78	C	35.45
21	33.18	CH_2	33.17	CH_2	34.70
22	29.85	CH_2	29.84	CH_2	29.01
23	64.92	CH_2	64.93	CH_2	62.83
24	12.77	CH_3	12.77	CH_3	12.00
25	18.97	CH_3	18.96	CH_3	20.40
26	16.91	CH_3	16.91	CH_3	16.73
27	21.04	CH_3	21.03	CH_3	23.89
28	63.95	CH_2	63.96	CH_2	62.02
29	24.92	CH_3	24.92	CH_3	24.23
30	32.82	CH_3	32.91	CH_3	31.74

* Signals superimposed with solvent in the normal ^{13}C NMR experiment but appeared in the DEPT NMR experiment due to solvent suppression.

Table III. ^{13}C NMR and ^{13}C DEPT NMR (100 MHz, CD_3OD) chemical shifts of the sugar parts of compounds **2** and **3** and ^{13}C NMR (75 MHz, DMSO-d_6) chemical shifts of compound **4**.

C	**2**		**3**		**4**
	δ	DEPT	δ	DEPT	δ
	3-*O*-Fuc		3-*O*-Fuc		3-*O*-GlcA
1	104.74	CH	104.73	CH	103.01
2	76.55	CH	72.31	CH	80.86
3	84.59	CH	84.58	CH	83.00
4	72.36	CH	72.36	CH	72.11
5	71.36	CH	71.35	CH	76.80
6	17.40	CH_3	17.39	CH_3	171.94
	Glc (1→2) Fuc		Glc (1→3) Fuc		Xyl (1→2) GlcA
1	103.59	CH	105.44	CH	103.35
2	76.35	CH	75.53	CH	74.14
3	76.87	CH	76.88	CH	76.68
4	72.42	CH	79.55	CH	70.74
5	78.40	CH	77.21	CH	68.78
6	63.60	CH_2	61.89	CH_2	
	Glc (1→3) Fuc		Glc (1→4) Glc		Glc (1→3) GlcA
1	105.15	CH	103.60	CH	102.03
2	75.51	CH	75.21	CH	74.49
3	76.92	CH	76.04	CH	75.58
4	79.57	CH	79.61	CH	77.03
5	78.24	CH	77.94	CH	76.29
6	61.90	CH_2	62.60	CH_2	61.87
	Rha (1→4) Glc		Rha (1→4) Glc		Rha (1→4) Glc
1	102.95	CH	102.95	CH	100.64
2	72.51	CH	72.54	CH	71.06
3	72.76	CH	72.77	CH	71.96
4	73.86	CH	73.84	CH	74.13
5	70.81	CH	70.81	CH	69.51
6	17.91	CH_3	17.93	CH_3	17.84

Acid hydrolysis of **3** gave the same products like acid hydrolysis of compound **2**. The ESI-mass spectra of **3** showed fragments at *m/z* 925 $[(M-H)-146]^-$ and 763 $[(M-H)-146-162]^-$ and the disappearance of *m/z* 909 indicating that the sugar moiety is a straight chain. There is a great similarity between the NMR spectrum of **2** and **3**, especially in the aglycone part, but in the sugar moieties there is a little difference: the downfield shift of C-2 of fucose disappeared and only a downfield shift of C-3 appeared at δ_C 84.58 with two carbon atom signals at δ_C 79.55 and 79.61 for C-4 of the two glucose units. The comprehensive interpretation of NMR (Tables II and III) and mass fragmentation data revealed that compound **3** could be identified as 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-β-D-fucopyranosyl-olean-11,13-(18)-diene-3β,23,28-triol (Fig. 1).

Compound **4** was obtained as amorphous powder. It gave a positive reaction in Libermann-Burchard and Molisch tests. The ESI-mass and NMR spectra indicated that **4** has the molecular formula $C_{53}H_{84}O_{22}$ with the molecular mass 1073. The ^{13}C DEPT NMR spectrum showed 53 carbon signals attributed to 7 methyl, 13 methylene, 24 methine groups and 9 quaternary carbon atoms. Acid hydrolysis of **4** gave the same aglycone like acid hydrolysis of **2** and **3**, but afforded D-xylose, L-rhamnose, D-glucose and D-glucuronic acid as sugar moieties. The downfield shift of C-3 of the aglycone at δ_C 85.73 indicated that the tetrasaccharide chain was connected at this position (Guo *et al.*, 2000; Hu *et al.*, 1995). The comprehensive interpretation of NMR (Tables II and III) and mass fragmentation data revealed that compound **4** could be identified as 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-[β-D-xylopyranosyl-(1→2)]-β-D-glucuronopyranosyl-acid-olean-11,13(18)-diene-3β,23,28-triol (Fig. 1).

Antioxidant assays

The free radicals and reactive oxygen species have been proposed to induce cellular damage and to be involved in several human diseases, such as

cancer, arteriosclerosis, inflammatory disorders as well as in the aging process (Kumaran and Karunakaran, 2007). Recently, there is increasing demand to evaluate the antioxidant properties of plant extracts or isolated products from plant origin rather than looking for synthetic antioxidants. Among different classes of natural products, phenolic compounds are extensively studied for their antioxidant activity (Wang *et al.*, 2005, 1998) and there are few works on other natural product classes as antioxidant agents (Yan *et al.*, 1996; Yoshiki *et al.*, 2001; Jung *et al.*, 2004).

Table I shows the antioxidant properties of the methanolic and *n*-butanolic extracts from *B. asiatica* and of the four isolated non-phenolic compounds **1**–**4** determined by three antioxidant *in vitro* assays including total antioxidant capacity using the phosphomolybdenum method (Prieto *et al.*, 1999), free radical scavenging ability using the DPPH assay (Shirwaikar *et al.*, 2006) and hydrogen peroxide scavenging methods (Gülçin *et al.*, 2006a).

Total antioxidant activity

The results in Table I show that the antioxidant capacity of the methanolic and *n*-butanolic extracts and of compounds **1**–**4** were 383, 402, 443, 435, 481 and 506 expressed by the number of equivalents of ascorbic acid. This method bases on the reduction of Mo(VI) to Mo(V) by the extract/compound and the formation of a green phosphate/Mo(V) complex at lower pH values and was measured as maximal absorption at 695 nm.

Free radical scavenging

Table I shows that the methanolic and *n*-butanolic extracts and compounds **1**–**4** have radical scavenging activities with SC_{50} = 16.28 and 11.99 μg/ml for the methanolic and butanolic extracts and 43.08, 72.90, 76.38 and 60.83 μg/ml for the four isolated compounds, respectively. This method depends on the reduction of DPPH (violet colour) in the presence of hydrogen-donating antioxidants to a yellow stable diamagnetic molecule (diphenylpicryl hydrazine) (Gülçin *et al.*, 2006b). The reduction capability of DPPH radicals is determined by the decrease in absorbance at 517 nm induced by antioxidants.

Scavenging of hydrogen peroxide

It must be noted that H_2O_2 itself is not very reactive, but sometimes it is toxic to cells because it may give rise to hydroxyl radicals in the cells (Gülçin *et al.*, 2006a). Therefore, removal of H_2O_2 is very important for antioxidant defense in cells or food systems. Table I shows that the methanolic and *n*-butanolic extracts and the compounds **1**–**4** have H_2O_2 scavenging activity with SC_{50} = 23.17 and 18.54 μg/ml for the methanolic and butanolic extracts and 25.21, 27.21, 32.68 and 18.39 μg/ml for the four isolated compounds, respectively. The activity depends on the presence of good electron donors, which are accelerating the conversion of H_2O_2 to H_2O. As good electron donors, they show good reduction power.

The antioxidant properties of compounds **2**–**4** are in good agreement with previously reported data on the same class of natural products (Gülçin *et al.,* 2004, 2006b). Finally, these compounds or the extract can be used as accessible sources of natural antioxidants after studying their antioxidant activity *in vivo*.

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