Cytotoxic activity of *Buddleja asiatica*

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Abstract: Phytochemical investigations of *Buddleja asiatica* leaves have led to the isolation of a new compound: 3,4 dihydroxy phenylethyl alcohol 8-*O*[(4'-*O*-feruoyl)-α-L-rhamnopyranosyl-(1"→3') –β-D-glucopyranosyl-(1"'→6')]- β-D-glucopyranoside **1**, along with 5 known metabolites: *E*-acteoside, *E*-iso acteoside, rutin, ajugol and luteolin . All metabolites were isolated for the first time from the genus *Buddleja.* The structures were determined by spectroscopic methods (UV, ESI-MS, ^{1}H, ^{13}CNMR, ^{1}H-^{1}H COSY, HSQC, and HMBC). The investigated methanol extract and compounds (**1-4)** showed significant cytotoxic activity against a HepG2 cell line.
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1. Introduction

The genus *Buddleja* belonging to the family Scrophulariaceae, comprises approximately 100 species. In Pakistan, it is represented by four species (Abdullah, 1974). *Buddleja asiatica* is a tender *deciduous shrub endemic* to a vast area of the *East Indies*. The shrub can be found in *India*, *Nepal*, *Bangladesh*, *China*, *Taiwan*, *Burma*, *Thailand*, *Laos*, *Cambodia*, *Vietnam*, *Malasia*, New *Guinea*, and the *Philippines*. *B. asiatica* has been used medicinally in different regions in the past and present. It has been used for skin complaints (Pande *et al*., 2007) and as an abortifacient (Nepal, 1970). A paste of its roots is used as a tonic when mixed with rice water (Gage, 1904). This plant is also used as a medicine for skin disease, abortion and as a cure for loss of weight (Guerrero, 1921). Roots and leaves of this plant are employed to treat head tumor (Hartwell, 1970). A fusion of roots is used in the treatment of malaria (Reis and Von,1973). The leaves of *B. asiatica* have a hypertensive effect on cats and dogs, probably due to α-adrenoceptor blocking activity. It is also reported that essential oil of the leaves has *in-vitro* antifungal activities (Garg and Dengre, 1992) and the flowers are used in the treatment of cystitis and cold (Lee *et al*., 2008) and to treat edema (Zheng and Xing, 2009). The extracts of *B. asiatica* also showed cyclo-oxygenase inhibitory activities in elicited rat peritoneal leukocytes (Liao *et al*., 1999). The genus *Buddleja* has been reported to possess anti-inflammatory, antibacterial and cytotoxic properties (Yoshida *et al*., 1978; Houtghon and Hikino 1989; Kosaku *et al.,* 1993). It has been reported to contain terpenoids, flavonoids, phenylethanoids, lignans, neolignans and saponins (Houghton 1985; Houghton *et al.,* 2003). Due to the interesting phytochemical and ethnopharmacological observations, the genus *Buddleja* has been investigated extensively; a valuable review on chemical composition and biological activity has been published by Houghton 1984 and Houghton *et al*., 2003.

In the course of our interest on phytochemical and biological activities of medicinal plants, we report herein the isolation and structural elucidation of some chemical constituents from the leaves of *B. asiatica. Moreover* the investigated methanol extract and major isolates (1-4) were tested against the HepG2 tumor cell line.

2. Experimental

2.1-Equipment

The NMR spectra were recorded at 300, 500 (^{1}H) and 75, 125 (^{13}C) MHz, respectively, on a Varian Mercury 300, and JEOL GX- NMR spectrometer, and δ values are reported in ppm relative to TMS in the convenient solvent. ESI-MS analyses were measured on a Finnigan LCQ deca LC/ MS and double focusing sector field MAT 90 MS *spectrometer (Finnigan, Bremen, Germany).* UV spectra of pure samples were recorded, separately, in MeOH using different diagnostic UV shift reagents using a Shimadzu UV 240 spectrophotometer. For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany) and polyamide 6S (Riedel de Haën AG, Seelze, Germany). For paper chromatography Whatman No. 1 sheets (England) were used.

2.2-Plant material: *Buddleja asiatica* leaves collected from plants growing in El-Orman Botanical Garden, Giza, Egypt. Plant Leaves were authenticated by Dr. Wafaa M. Amer, Department of Botany, Faculty of Science, Cairo University, Giza, Egypt. Voucher specimens (Reg. No.: B–1) are kept in the herbarium, Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt.

2.3-Measurement of potential cytotoxicity by SRB assay.

Potential cytotoxicity of the methanol extract of *B. asiatica* leaves and the isolated compounds **1-4** were tested at the National Cancer Institute, Egypt using the method of (Skehan *et al*., 1990) Cells were plated in a 96-well plate (104 cells/well) for 24 hrs before treatment to allow the attachment of cells to the wall of the plate. Different concentrations of the fractions under investigation (0, 1, 2.5, 5 and 10 µg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose and they were incubated for 48 hrs at 37 ºC in 5% CO_2. After 48 hrs cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer and the color intensity was measured in an ELISA reader. The survival curve of the tumor cell line was plotted for each tested methanol extract and major isolate.

2.4-Extraction and isolation

The air-dried powdered leaves of *(*500 g) were exhaustively extracted with 85% MeOH (5 x 1.5 l and 2 x 2.5 l), under reflux (70 °C). The aqueous methanol extract was concentrated under reduced pressure and defatted with light petroleum ether (60 – 80 °C, 5×1 l) to give a viscous extract which was dissolved in water, and the water-insoluble residue was removed by filtration. The water-soluble portion was desalted by precipitation with excess MeOH to give a dry brown residue (50 g) that was suspended in H_2O and fractionated on a polyamide column (110 × 5 cm, 250 g) using a stepwise gradient from H_2O, H_2O/MeOH mixtures up to pure MeOH for elution. Based on comparative paper chromatography (Co-PC) with the use of UV light, 1% $FeCI_3$, or Naturstoff spray reagent for detection, the individual 55 fractions were pooled into five collective fractions (A – E). Fraction A (H_2O, 10 g) was found to be a dark brown material with no phenolic character. Fraction B (10 – 30% MeOH, 12 g) was fractionated on cellulose with MeOH as an eluent, followed by a Sephadex LH- 20 column using BIW (*n*-BuOH/2-propanoI/H_2O, 4:1:5 v/v/v, organic layer) to afford pure **1** (57mg). Two major spots were detected in fraction B (30-50 % MeOH, 9g), which was subjected to repeated CC on Sephadex LH- 20 with 60-80% aqueous EtOH as an eluent. This fractionation resulted in pure **2** (40 mg) and **3** (35 mg). Crude **4** was crystallized from Fr.C (50-70 % MeOH, 8 g) and purified by repeated crystallization from MeOH to yield a pure sample of **4** (35 mg). Fraction D (50 – 70% MeOH, 8 g) was subjected to repeated CC on cellulose and Sephadex LH-20 with 20 – 60% aqueous MeOH as an eluent, resulting in pure samples of **5** (17 mg). Fr. E (70 –100% MeOH, 7 g) was rechromatographed on Sephadex LH-20 with MeOH to afford a pure sample of **6** (19 mg). All separation processes were followed by 2D-PC and CoC using Whatman No. 1 paper with *n*-BuOH/ AcOH/H2O (4:1:5, top layer) (S1) and 15% aqueous AcOH (S2) as solvent systems.

2.5- 3, 4 dihydroxy phenylethyl alcohol 8-*O* [(4'-*O*-feruoyl)-α-L-rhamnopyranosyl-(1"→3') –β-D-glucopyranosyl-(1'"→6')]-β-D-glucopyranoside:

Yellow amorphous powder, Rf = 0.73 (S1), 0.65 (S2– Negative ESI-MS: m/z = 799.18 $[M-H]^-$, 623.77 [M-H-deoxy feruloyl]$^-$, 461.08 [M-H-deoxy feruloyl glucoside]-, 299.51 [M-H-deoxy feruloyl glucoside glucoside]$^-$, 153.46 [M-H-deoxy feruloyl glucoside glucoside rhamnoside]$^-$,193.01 [ferulate]$^-$ and 178.94 [caffeate]$^-$

^{1}H NMR and ^{13}C NMR: Table I.

3-Results and Discussion

Chromatographic separation of *B.asiatica* methanol extract resulted in the isolation of a new compound 3,4 dihydroxy phenylethyl alcohol 8-*O*[(4'-*O*-feruoyl)-α-L-rhamnopyranosyl-(1"→3') –β-D-glucopyranosyl-(1'"→6')]- β-D-glucopyranoside **1** along with five known metabolites: *E*-acteoside **2**, *E*-iso acteoside **3** (Han *et al*., 2012), rutin **4** (agrawal, 1989), ajugol (Abd ElMoaty, 2010) **5,** luteolin **6** (Agrawal, 1989).

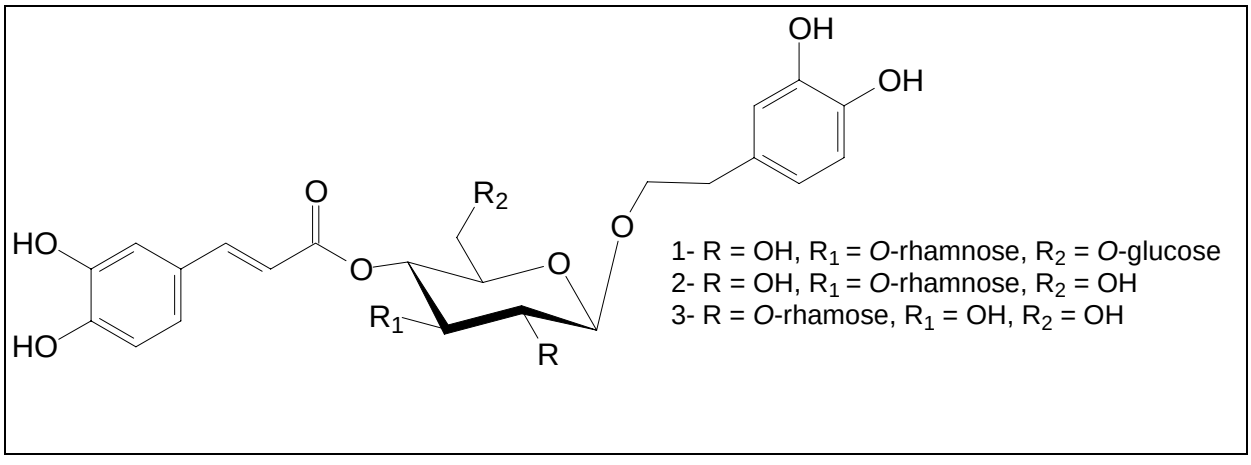
Compounds **1** was expected to be 3,4 dihydroxyphenylethyl alcohol feruloylrhamnosyl diglycosides on the basis of its chromatographic properties, UV spectra and acid hydrolysis. The negative ESI-MS of **1** exhibited a molecular ion peak at *m/z* 799.18 $[M-H]^-$, together with six diagnostic fragments at m/z 623.77 [M-H- deoxy feruloyl]$^-$, 461.08 [M-H-deoxy feruloyl glucoside]$^-$, 299.51 [M-H-deoxy feruloyl glucoside glucoside]$^-$, 153.46 [M-H-deoxy feruloyl glucoside glucoside rhamnoside]$^-$ „193.01 [ferulate]$^-$ and 178.94 [caffeate]$^-$. In the aromatic region of its ^{1}HNMR spectrum an A^2X^2-spin coupling system of H-7"" and H-8"" at δ 7.50 and 6.18 ppm, respectively (each d, J = 15.9) together with an ABM one of H-2"", H-6"" and H-5"" at δ 6.96 (d, J = 2.0), 6.86 (dd, J = 2.0, 8.1) and 6.68 (d, J = 8.1) ppm, respectively and a methoxy at 3.76 (s) in the aliphatic region were indicative an *E*-feruoloyl moiety. The characteristic signals of the aglycone moiety (3,4 dihydroxyphenyl alcohol) were assigned in the form of an ABM spin coupling system at 6.61 (d, J = 2.0),, 6.58 (d, J = 8.1), and 6.47 (dd, J = 2.0, 8.1), ppm for H-2, H-5 and H-6, respectively in the aromatic region and its characteristic AX-system of two triplets for H-7 and H-8 at 2.69 and 3.94, respectively in the aliphatic region. The ^{1}HNMR showed three anomeric proton signals at 4.29 ppm (d, J = 8.1, H1'), at 5.08 (brs, H-1'') and at 4.20 ppm (d, J = 7.8, H1'") together with the signal at 1.00 (3H, d, J = 6.1, CH3-6"). The sugar moieties were deduced to adopt α-$^1C_4$ and β-$^4C_1$-

pyranose stereostructure in case of rhamnosyl and glucosyl moieties, respectively on the basis of *J*-values of the anomeric protons and *δ*-values of their ^{1}H and ^{13}C-resonances (Table 1). The deshielded location of C-6' at 69.5 suggesting the (1'''→6') interglycosidic link. This evidence was further conformed by observation of the three bond correlation peak between the CH_2OH proton signal at δ 3.83 and the C-1''' signal at δ 104.6 in the HMBC spectrum. The glucose rhamnose linkage was assigned as (1''→3') on the basis of the three bond correlation peak between H1'', (δ 5.08.) and C-3' (*δ* 81.7) in the HMBC spectrum. In addition, the attachment of the *E*- feruloyl group to C4' was deduced from the characteristic downfield shift of H-4' triplet signal at 4.91 relative to unsubstituted glucose (Nakanishi 1990; Hirobe *et al*., 1997; Kamel *et al*., 2003; Wang *et al*., 2004; Kanchanapoom *et al*., 2005), together with the long range correlation between H-4' at 4.91 and the carbonyl carbon of the feruloyl moiety at 168.5. Additional evidence for the feruloyl moiety was obtained from the three bond correlation peak between the methoxy proton signal at δ 3.67 and the C-3'''' signal at δ 148.2 in the HMBC spectrum. The assignment of all ^{1}H and ^{13}C-resonances was proved by the aid of HSQC and HMBC correlation peaks and comparison with the corresponding published data for structural related compounds (Gao *al*., 2000; Li *et.al*., 2008; Li *et al*., 2011). Therefore **1** was identified as 3,4 dihydroxy phenylethyl alcohol 8-*O*[(4'-*O*-feruoyl)-α-L-rhamnopyranosyl-(1''→3') –β-D-glucopyranosyl-(1'''→6')]- β-D-glucopyranoside.

Table I. ^{1}H, ^{13}C NMR and HMBC spectral data of compound **1** (500/125 MHz, DMSO-d_6)

	δH	δC	HMBC
1		131.5	
2	6.61d (2)	117.1	C-4,6,7
3		146.1	
4		144.6	
5	6.58d (8.1)	116.4	C-3,1
6	6.47dd (2.0, 8.1)	121.3	C-4, 2,7
7	2.69 t (7.0)	35.5	
8	3.64,3.94 m	72.4	
1'	4.29 d (8.1)	104.2	C-3'
2'	3.31m	76.1	C-4'
3'	3.72 m	81.7	C-5',1',1''
4'	4.91t (9.6)	70.4	C-6',2', 9''''
5'	3.65*	74.6	C-3', 1
6'	3.85 m	69.4	C-4', 1'''
1''	5.08 brs	103.1	C-3'', 3'
2''	3.83 m	72.3	C-4''
3''	3.48*	72.0	C-5'', 1''
4''	3.20*	73.8	C-6'',2''
5''	3.45m	70.6	C-3'''
6''	1.00 d (6.1)	18.4	C-4'''
1'''	4.20 d (7.8)	104.6	C-3''', 6'
2'''	3.11*	75.1	C-4'''
3'''	3.13*	77.8	C-5''', 1'''
4'''	3.18m	71.4	C-6''',2'''
5'''	3.25m	77.9	C-3'''
6'''	3.46*	62.6	C-4'''
1''''		127.6	
2''''	6.96 d (2.0)	115.3	C-6'''',4'''', 7''''
3''''		148.2	
4''''		149.8	
5''''	6.86 dd (8.1, 2.0)	116.5	C-1'''', 3''''
6''''	6.68 d (8.1)	123.3	C-2'''', 7'''',4''''
7''''	7.50 d (15.9)	148.3	C-4'''', 9'''',2''''
8''''	6.18 d (15.9)	114.7	C-1''''
9''''		168.5	
OCH_3	3.67 s	56.1	C-3''''

*Unresolved proton resonances, *δ* in ppm and *J* values (Hz), are given in parentheses. All carbon and proton resonances were assigned on the basis of 2D (^{1}H-^{1}H COSY, HSQC and HMBC).



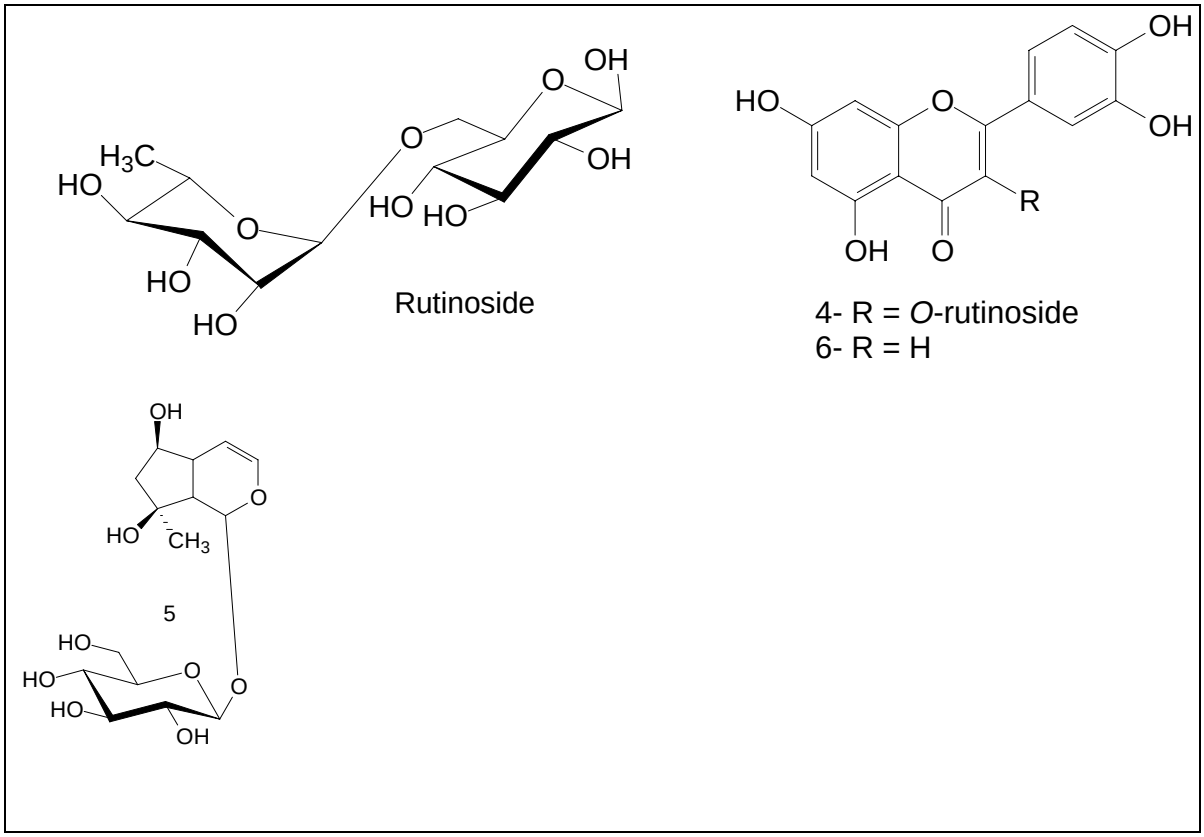


Fig 1. Structures of isolated compounds

The methanol extract and compounds **1-4** were cytotoxic for HepG2 cells and **2** was the most cytotoxic agent (IC_{50} = 1.07, 0.93, 0.8, 0.84 and 1.65, respectively, Figure 2. Although different extracts of *B. asiatica* have been investigated as antimicrobial (Ali *et al.*, 2011) to our knowledge, this work is the first trial to investigate the cytotoxicity of the methanol extract of *B. asiatica* leaves against HepG2 solid tumor cell lines. The strong anti-cancer activity of *B. asiatica* leaves methanol extract may be attributed to the corresponding activities of the extract constituents. Phenylethanoid glycoside (**1-3**) is reported to be protector from induced lipid peroxidation (Chiou *et al.*, 2004), and hepatotoxic (Lee *et al.*, 2004) that was attributed to the large number of phenolic hydroxy groups. Recently, phenylethanoid was found to possess anti-proliferative activity against B16F10. It is suggested that the 3,4-dihydroxy-phenethyl alcohol group might be more responsible for their activities than the feruoyl group (Nagao *et al.*, 2001; He *et al.*, 2001) suggested that hydroxy groups of the aromatic rings appear to play a role in the anticancer effect of phenylethanoid. The weak cytotoxicity of the isolated flavonoid **4** may be attributed to the presence of sugar moieties.

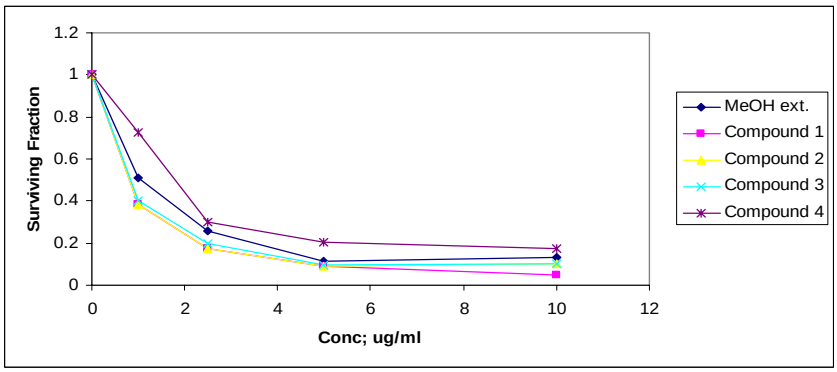


Fig 2. The cytotoxic activity of *P. asiatica* MeOH extract and compounds **1- 4** against HepG2 cell line.

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