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Anti-Inflammatory and Cytotoxic Constituents of *Bauhinia retusa*

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ABSTRACT
Genus *Bauhinia* (Caesalpiniaceae) is used traditionally for the treatment of wounds, ulcers, diabetes, general pain, inflammation and infections. The aim of the study was to investigate the anti-inflammatory and cytotoxic activities of the bioactive fractions of the leaves of *Bauhinia retusa* and to identify the chemical constituents in these active fractions by different spectroscopic techniques. The anti-inflammatory activity of chloroform and ethyl acetate soluble fractions (Br-1 and Br-2) of *B. retusa* and isolated compounds was determined in terms of their ability to inhibit NF-kB and iNOS activity and to decrease oxidative stress in cellular systems. Their cytotoxicity was also determined against a set of 6 mammalian cell lines. Seven compounds were isolated from Br-1 and Br-2, namely methyl gallate, (1) ethyl gallate, (2) kaempferol, (3) quercetin, (4) kaempferol 3-O-α-L-rhamnoside, (5) quercetin 3-O-α-L-rhamnoside (6) and β- sitosterol (7), all reported for the first time from this species. Br-1 and Br-2 were found to decrease oxidative stress and inhibit NF-kB and iNOS activities. Among the isolated compounds, 1-4 were more effective in decreasing oxidative stress and inhibiting iNOS activity than compounds 5-7. None of the compounds inhibited NF-kB. The Br-1 and Br-2 showed cytotoxicity towards KB, SK-OV-3 and LLC-PK1 cell lines. Compounds 1, 2 and 4 were toxic to LLC-PK1 cells. Phytochemical investigation of the leaves of *Bauhinia retusa* has led to the isolation of seven compounds. Some of which showed promising anti-inflammatory, antioxidant and cytotoxic effects. The reported anti-inflammatory properties of this plant could be attributed to the presence of these constituents.

Key words: Anti-inflammatory, *Bauhinia retusa*, Caesalpiniaceae

INTRODUCTION

Bauhinia is a genus of deciduous trees or shrubs of Caesalpiniaceae family, having about 300 species distributed in the warmer parts of world, including Africa, Asia and South America (Filho, 2009). According to a literature survey, several uses in traditional medicine have been reported for different *Bauhinia* species, such as, treating ailments, wounds, ulcers and diabetes and as a diuretic agent (Kirtikar and Basu, 2003; Castro-Castillo *et al*., 1982; Melendez, 1978). Plants belonging to genus *Bauhinia* are also frequently used in folk medicine to treat infectious diseases and several experimental studies have confirmed their antimicrobial potential, especially against pathogenic fungi and bacteria.

Bauhinia retusa is a medium sized tree which is cultivated in Egypt for its showy flowers and ornamental foliage. A literature survey revealed that flavonoid glycosides (Yadava and Jain, 2003; Yadav and Verma, 2010), amino acids (Prakash and Misra, 1983), lignin rhamnoside, (Semwal and Sharma, 2011a), eudesmane sesquiterpene glucoside, β-sitosterol and stigmasterol have been isolated from the different parts of the Indian *B. retusa* (Semwal and

Sharma, 2011b). The flavonoid glycoside isolated from *B. retusa* showed anti-inflammatory and antioxidant activities (Yadava and Jain, 2003; Yadav and Verma, 2010), while the sesquiterpene lactone glucoside gave moderate antibacterial activity (Semwal and Sharma, 2011a). The important traditional uses prompted us to carry out pharmacological and phytochemical studies on Egyptian *B. retusa*. As a result of these studies, the isolation and structure elucidation of seven compounds from the ethyl acetate and chloroform soluble fractions, known as methyl gallate (1), (Rashed and Butnariu, 2014), ethyl gallate (2), (Zhang *et al*., 2014), kaempferol (3), (Rashed and Butnariu, 2014), quercetin (4), (Rashed and Butnariu, 2014), kaempferol 3-O-α-L-rhamnoside (5) (Zhang *et al*., 2014), quercetin 3-O-α-L-rhamnoside (6) (Zhang *et al*., 2014) and β-sitosterol (7) (Prakash and Prakash, 2012) have been reported. This is the first report to isolate these compounds from *B. retusa*.

MATERIALS AND METHODS

Plant material: The leaves of *B. retusa* Roxb. were collected in the flowering stage in 2011 from a private garden at the 10th of Ramadan City, Alsharqia governorate, Egypt. The plant was kindly identified by Dr. Abd-Elhalim Abd-Elmagly Mohammed, Agriculture researches center, Ministry of Agriculture and Land Reclamation, Egypt. Voucher specimen (No. 25) is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

General experimental procedure: The ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on a BrukerAMX-500 spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts are in ppm (δ), relative to TMS as an internal standard and scalar coupling constants (J) reported in Hertz. ESI-MS analyses were measured on an Agilent Triple Quadrupole 6410 QQQ LC/MS mass spectrometer with ESI ion source (gas temperature is 350°C, nebulizer pressure is 60 psi and gas flow rate is 12 L min^{-1}), operating in the negative and positive scan modes of ionization through direct infusion method using $CH_3OH\backslash H_2O$ (1:1 v/v) at a flow rate of 0.4 mL min^{-1}. Column chromatography was carried out on sephadex LH-20 and silica gel (E. Merck, Darmstadt, Germany). Thin Layer Chromatography (TLC) was performed on precoated TLC plates (Aluminium sheets, silica and RP-18 F254, Merck, Germany); the detection was done at 254 nm and by spraying with ceric sulphate reagent.

Extraction and isolation: The air-dried leaves (1.5 kg) were extracted with 90% ethanol (4L×3) by cold maceration at room temperature. The alcoholic extract was concentrated under reduced pressure to a syrupy residue (350 mL). This residue was suspended in water (250 mL) and successively extracted with petroleum ether (27 g), chloroform (6 g), ethyl acetate (8 g) and n-butanol (5 g). A part of chloroform soluble fraction (4 g) was loaded on a silica gel column and the elution was successively carried out with chloroform and mixture of chloroform-methanol in increasing order of polarity leading to two major sub-fractions CI-CII. Fraction C-I which was eluted with chloroform showed one major spots on TLC. It was further subjected to column chromatography using chloroform-methanol (9.8:0.2) as eluent to afford compound 7 (15 mg). Fraction C-II obtained from chloroform-methanol (9.5:0.5) was further purified by column chromatography eluting with chloroform-methanol (9.1:1.0) to afford compounds 1 (40 mg) and 2 (10 mg). The ethyl acetate soluble fraction (7.0 g) was chromatographed on Sephadex LH-20 and the elution was successively carried out with water and mixtures of water and methanol in decreasing order of polarity till 100% methanol, to afford two sub-fractions. Fraction E-I was rechromatographed on a silica gel column, eluted with chloroform-methanol (9:1) to afford compounds 3 (15 mg) and 4 (12 mg). The sub-fraction E-II obtained from water-methanol (8:2) was a binary mixture which was separated through Sephadex LH-20 column by using water-methanol (7:3) to afford compounds 5 (10 mg) and 6 (15 mg).

Assay for the inhibition of cellular oxidative stress: Human hepatoma cell line (HepG2) was cultured in DMEM supplemented with 10% FBS and antibiotics (50 units mL^{-1} penicillin and 50 μg mL^{-1} streptomycin). The cellular antioxidant activity was measured in HepG2 cells according to the method described by Wolfe and Liu (2007). The assay measures the ability of test samples to prevent intracellular generation of peroxyl radicals in response to 2, 2 -azobis (2-amidinopropane) dihydrochloride (ABAP, Sigma-Aldrich, St Louis, MO, USA), a generator of peroxyl radicals. The assay is more relevant biologically than a chemical assay because it represents the complexity of biological system and accounts for cellular uptake, bioavailability and metabolism of the antioxidant agent under test. For the assay, HepG2 cells were seeded in the wells of a 96-well plate (60,000 cells/well) and incubated for 24 h for confluency. The test samples were diluted in serum free medium containing 25 μM 2',7'-dichlorofluorescin diacetate (DCFH-DA, Invitrogen, Carlsbad, CA, USA). The cells were washed with PBS and treated with the test samples for 1 h. The medium containing samples was removed and ABAP (600 μM) was added and plate was immediately placed on a SpectraMax plate reader for kinetic reading every 5 min for 1 h (37°C, emission at 538 nm and excitation at 485 nm). Quercetin (Sigma-Aldrich, St Louis, MO, USA) was included as the positive control. The Area Under the Curve (AUC) of fluorescence versus time was used to calculate percent decrease in oxidative stress.

$$\text{Decrease in oxidative stress (\%)} = 100 - \left[\frac{\text{AUC sample}}{\text{AUC control}} \times 100\right]$$

Assay for inhibition of iNOS activity: Mouse macrophage cell line (RAW264.7) was cultured in phenol red free RPMI medium with 10% bovine calf serum and 100 U mL^{-1} penicillin G sodium and 100 μg mL^{-1} streptomycin. For the assay, cells were seeded in the wells of 96-well plates

(50,000 cells/well) and incubated for 24 h for a confluency of 75% or more. Test samples diluted in serum free medium were added and after 30 min of incubation, lipopolysaccharides (LPS, 5 μg mL^{-1}) was added and cells were further incubated for 24 h. The concentration of Nitric Oxide (NO) in the cell supernatant was determined by measuring the level of nitrite using Griess reagent. The inhibition of nitrite production by the extract and the pure compounds was calculated in comparison to vehicle control. IC_{50} values were obtained from dose curves. Parthenolide (Sigma-Aldrich, St Louis, MO, USA) was used as positive control (Zhao *et al*., 2014).

Reporter gene assay for inhibition of NF-kB activity: Human chondrosarcoma cell line (SW1353) was cultured in 1:1 mixture of DMEM/F12 supplemented with 10% FBS, 100 U mL^{-1} penicillin G sodium and 100 μg mL^{-1} streptomycin. For the assay, cells were washed once in an antibiotic and FBS-free DMEM/F12 medium and then resuspended in antibiotic-free DMEM/F12 medium containing 2.5% FBS. NF-kB luciferase plasmid construct was added to the cell suspension at a concentration of 50 μg mL^{-1} and incubated for 5 min at room temperature. The cells were transfected by electroporation at 160 V and one 70-msec pulse using BTX disposable cuvettes model 640 (4-mm gap) in a BTX Electro Square Porator T 820 (BTX I, San Diego, CA). The transfected cells were plated in 96-well plates at a density of 1.25×10^5 cells per well in 200 μL of DMEM/F12 containing 10% FBS and antibiotics. After 24 h incubation, cells were treated with various concentrations of test samples for 30 min and then induced with PMA (70 ng mL^{-1}) for 8 h. Luciferase activity was measured using a Luciferase Assay Kit (Promega, Madison, WI, USA). Light output was detected on a SpectraMax plate reader. The inhibition of NF-kB activity by the extract and pure compounds was calculated compared to vehicle control. IC_{50} values were obtained from the dose curves. Parthenolide was included as positive control (Zhao *et al*., 2014).

Assay for cytotoxicity: The *in vitro* cytotoxic activity was determined against a panel of four human cancer cell lines (SK-MEL, KB, BT-549, SK-OV-3) and two noncancerous kidney cell lines (LLC-PK_1 and VERO). All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were seeded at a density of 25,000 cells/well and incubated for 24 h. Test samples were added at different concentrations and cells were again incubated for 48 h. At the end of incubation, the cell viability was determined using Neutral Red dye according to a modification of the procedure of Borenfreund *et al.* (1990). Doxorubicin was used as a positive control while DMSO was used as the negative (vehicle) control.

RESULTS AND DISCUSSION

Separation and identification of compounds from the ethyl acetate and chloroform soluble fractions of Bauhinia retusa: Compound 1 was identified as methyl gallate and compound 2 was identified as ethyl gallate. Compounds 3, 4, 5 and 6 were identified as flavonols and their rhamnosides, i.e., kaempferol (3), quercetin (4), kaempferol 3-O-α-L-rhamnoside (5), quercetin 3-O-α-L-rhamnoside (6). Compound 7 was identified as β-sitosterol. All of these compounds were isolated for the first time from *B. retusa* and their structures were shown in Fig. 1.

Methyl gallate (1): white amorphous powder: ^{1}H-NMR (CD_3OD, 500 MHz) δ: 6.94 (2H, s, H-2,6), 3.73 (3H, s, OCH_3).

Ethylgallate (2): $C_9H_{10}O_5$, white amorphous powder, ^{1}H-NMR (CD_3OD, 500 MHz) δ: 6.99 (2H, s,H-2, 6), 4.21 (2H, q, J = 7.2 Hz, -OCH_2CH_3), 1.28 (3H, t, J = 7.2 Hz,-OCH_2CH_3).

Kaempferol (3): Yellow powder. ^{1}H-NMR (CD_3OD, 500 MHz):δ 8.11 (2H, d, J = 8 Hz, H-2',6'), 6.96 (2H, d, J = 8 Hz, H-3',5'), 6.47 (1H, d, J = 2 Hz, H-8), 6.19 (1H, d, J = 2 Hz, H-6). EI-MS: m/z 286.

Quercetin (4): Yellow powder. ^{1}H-NMR (CD_3OD, 500 MHz), δ 7.74 (1H, d, J = 8, 2 Hz, H–2'), 7.55 (1H, d, J = 2 Hz, H-6'), 6.92 (1H, d, J = 8 Hz, H-5'), 6.42 (1H, d, J = 1.2 Hz, H-8), 6.15 (1H, d, J = 1.2 Hz, H-6). EI-MS: m/z 302.

Kaempferol 3-O-α-L-rhamnoside (5): Yellow gummy solid, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 8.0 (2H, d, H-2'/6', J = 8.5), δ 6.9 (2H, d, H-3'/5', J = 8.5), δ 6.5 (1H, d, J = 2 Hz, H-8), 6.2 δ (1H,d, J =2.5 Hz, H-6), 5.4 (1H, d, J = 7.5, H-1''), 3.80-3.10 (5H, m, remaining sugar protons).

Quercetin 3-O-α-L-rhamnoside (6): Yellow gummy solid, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ 7.26 (2H, m, H-2'/6'), 6.83 (1H, d, J = 9 Hz, H-5'), 6.49 (1H,d, J = 2.5 Hz, H-8), 6.14 (1H, d, J = 2.5Hz, H-6), 5.25 (1H, br s, H-1'') 0.78 (3H, d, J = 6Hz).

β-sitosterol (7): Crystallized colorless needles, ^{1}H-NMR ($CDCl_3$, 500 MHz) d: 5.32 (1H, m, H-6), 3.36 (1H, m, H-3a), 0.92 (3H, s, CH_3-19), 0.88 (3H, d, $J_{21,20}$ = 6.5 Hz, CH_3-21), 0.83 (3H, d, $J_{26,25}$ = 6.5 Hz, CH_3-26), 0.81 (3H, d, $J_{27,25}$ = 6.5 Hz, CH_3-27), 0.77 (3H, t, $J_{29,28}$ = 7.0 Hz, CH_3-29), 0.63 (3H, s, CH_3-18). EI-MS: m/z 414.

Anti-inflammatory and cytotoxic activities: The chloroform and ethyl acetate fractions (Br-1 and Br-2) of *B. retusa* leaves extract showed a decrease in cellular oxidative stress (45% decrease at 500 μg mL^{-1}) and inhibition of NF-kB and iNOS activities with IC_{50} values in the range of 16-28 and 38-40 μg mL^{-1}, respectively (Table 1). Among the isolated compounds, methyl gallate (1) and ethyl gallate (2), were the most effective in decreasing oxidative stress with 78-80% decrease at 250 μg mL^{-1}. Out of the four flavonols, kaempferol (3) and quercetin (4), were more effective than their corresponding glycosides 5-6 as shown in Table 1.

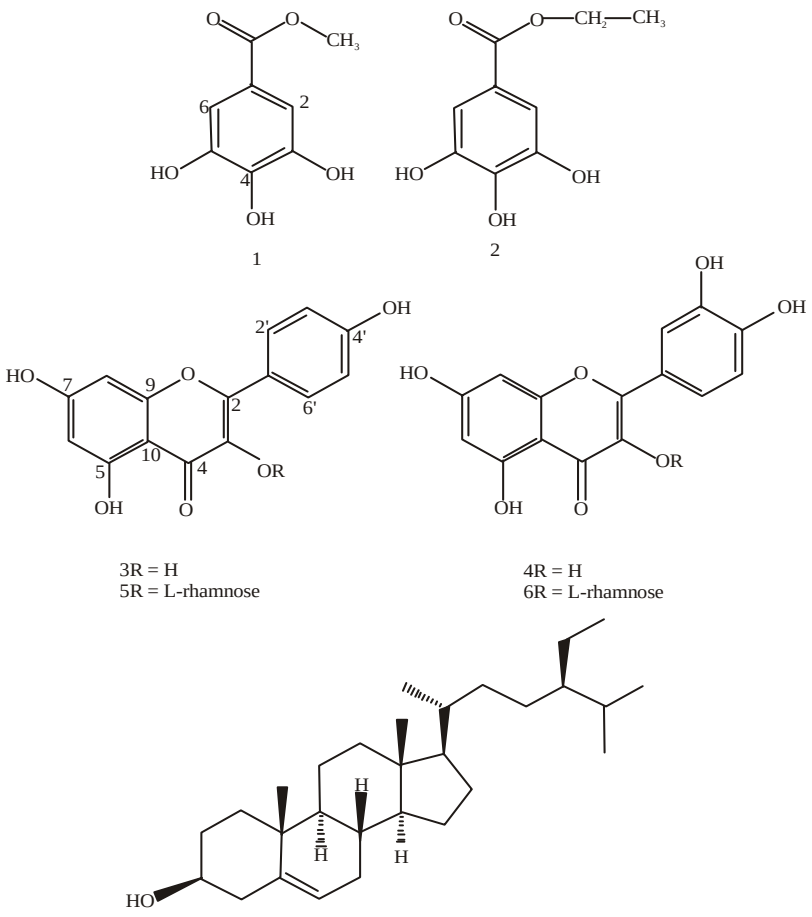

Fig. 1: Structures of compound 1-7

Table 1: Anti-inflammatory activity of *B. retusa* fractions and isolated compounds

Sample name	*Decrease in oxidative stress (%)	Inhibition of NF-kB activity IC_{50} in (μg mL^{-1})	Inhibitionof iNOS activity IC_{50} in (μg mL^{-1})
Br-1	45	28	38
Br-2	45	16	40
1	80	NA	29
2	78	NA	50
3	74	NA	32
4	71	NA	19
5	47	NA	NA
6	30	NA	NA
7	NA	NA	NA
Parthenolide***		0.5	0.3
Quercetin***	74		

*: At 500 μg mL^{-1} for extract and 250 μg mL^{-1} for pure compounds, **: Positive control, NA: No activity

Methyl gallate and ethyl gallate inhibited iNOS activity with IC_{50} values of 29 and 50 μg mL^{-1}, respectively while quercetin and kaempferol inhibited iNOS with IC_{50} values of 19 and 32 μg mL^{-1}, respectively. However the corresponding glycosides were not active. Despite the NF-kB inhibitory activity of the fractions, no inhibition of NF-kB was seen with any of the compounds.

Ethyl acetate and chloroform fractions were toxic to epidermal carcinoma (KB) and ovarian cancer (SK-OV-3) cell lines with IC_{50} values in the range of 57-90 μg mL^{-1}. None of the isolated compounds decreased the viability of cancer cell line to 50% at the highest concentration of 25 μg mL^{-1} (IC_{50} >25 μg mL^{-1}). Methyl gallate, ethylgallate and quercetin were toxic to kidney cells (LLC-PK_1) with IC_{50} values of 14, 4.5 and 17 μg mL^{-1} as shown in Table 2.

Results of evaluation of biological activity of *B. retusa* leaves and its constituents indicate the anti-inflammatory potential of this plant and support its traditional use in various conditions of inflammation such as pain, wound, ulcers and diabetes. These results are in agreement with previously reported anti-inflammatory activity of methyl and ethyl gallates (Chae *et al*., 2010; Mehla *et al*., 2011) and the flavonol aglycones; kaempferol and quercetin (Garcia-Mediavilla *et al*., 2007; Wang *et al*., 2006).

Table 2: Cytotoxic activity of *B. retusa* fractions and isolated compounds

	Cell lines					
Sample name	SK-MEL	KB	BT-549	SK-OV-3	VERO	LLC-PK1
IC_{50} in µg mL^{-1}						
Br-1	NA	57	NA	90	NA	62
Br-2	NA	70	NA	84	NA	68
1	>25	>25	NA	>25	NA	14
2	17.5	>25	>25	>25	NA	4.5
3	NA	NA	NA	>25	NA	>25
4	>25	NA	NA	>25	NA	17
5	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA
7	>25	NA	NA	NA	NA	NA
Doxorubicin***	1.5	1.8	2.2	1.0	>10	0.85

***: Positive control

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