IJPSR (2015), Vol. 6, Issue 1 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 June 2014; received in revised form, 12 August, 2014; accepted, 28 December, 2014; published 01 January, 2015

NOVEL PROPENYL FLAVONOIDS GLYCOSIDE AND ANTIOXIDANT ACTIVITY OF EGYPTIAN *BAUHINIA RETUSA*

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Keywords:

Bauhinia retusa; Leguminosae; flavonoid glycosides; antioxidant activity

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ABSTRACT: Column chromatography of ethyl acetate of *Bauhinia retusa* afforded a novel flavonoid identified as [rhamnetin-3-O-α- L-rhamnopyranosyl-3'-O-(prop-1-enyl)], in addition to kaempferol, isorhamnetin-3-α-rhamnoside, qurcitrin, quercetin 3-O-β-D-glucopyranosyl-β-D-glucopyranoside, and quercetin 3,7-di-o- β-D-glucoside have been isolated for the first time. Their structures were established from extensive spectroscopic techniques (UV, MS, ^{1}H and ^{13}C-NMR), chemical studies in addition to comparison with literature data and /or authentic samples. More over,qurcitrin, quercetin 3-O-β-D-glucopyranosyl-β-D-glucopyranoside and ethyl acetate fraction exerted pronounced antioxidant activities (SC_{50} µg/ml ,13.5 , 14.7 and 18.7) respectively, compared with vitamin C ($SC_{50,}$,13.9 µg/ml) by DPPH radical scavenging assay.

INTRODUCTION: The genus Bauhinia (Fabaceae, Leguminosae) consists of approximately 300 species, which are commonly known as 'cow's paw' or 'cow's hoof', because of the shape of their leaves. They are widely distributed in most tropical countries, including Africa, Asia and South America [1]. Many plants of the genus have been used frequently in folk medicine as a remedy for different kinds of pathologies, diabetes, pain, cytotoxic, asthma, diuretic, antimalarial as well as antioxidant, hepatoprotective and anti-inflammatory activities [2-7]. Chemically various species of *Bauhinia* have shown the presence of steroids [8], phenan-thraquinone [9] and flavonoids [10, 11] In addition to other minor constituents [12, 13]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(1).478-84

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(1).478-84

The plant of study, *Bauhinia retusa* Roxb. is a deciduous tree distributed in warmer parts of the world found at an altitude of 800 m in the Garhwal Himalayan region, India [14]. The Indian species, have shown the study of the bark and seed to indicate the isolation of some constituents [7, 8, 15-18]. Also the Asian use the plant for some traditional and economical purposes [19-21]. Recently, *B. retusa* was acclimatized and cultivated in Egypt. Therefore, we undertake study of the Egyptian plant to investigate its constituents and uncover their biological potential.

Experimental:

Evaporation of solvents was done at 45^{o}C under reduced pressure, using a Buchi rotary evaporator; UV spectra were measured on Schimadzu UV-260 Spectrophotometer (Japan); EIMS were carried out on Jeol JMS-AX 500, 70 ev and Shimadzu GC/MS-QP5050A, 70 ev;^{1}H and ^{13}C-NMR spectra were run in DMSO-d6 and CD_3OD; at 300 and 75 MHz, respectively using Varian Mercury-VX-300 NMR Spectrometer; Chemical shifts are given in

ppm with TMS as internal standard; silica gel (60 to 120 mesh, Merck) and sephadex LH20 were used for column chromatography, silica gel coated aluminum plates (Merck kieselgel 60 F254, Germany) for TLC and PC (Whatman No. 1);Visualization of the plates was performed using visible light, UV fluorescence ; spraying with anisaldehyde/sulphuric acid reagent or aniline phthalate reagent followed by heating at 100°C for 10 min.; For TLC analysis, the following chromatographic solvent systems were used; $CHCl_3$: MeOH (7:3system I); benzene - ethyl acetate - formic acid - water (3 : 5 : 1.6 : 0.4, system II), Butanol-acetic acid - water (4: 1:5 system111) ; The antioxidant activity of extract and isolated compounds were determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al- Azhar University, Egypt,.

Plant material:

The leaves of *B. retusa* Roxb.were collected in the flowering stage on November 2010 from private garden at the 10th of Ramadan City, Alsharqia governorate, Egypt. The plant was kindly identified by Dr. Abd-ElhalimAbd-Elmagly Mohammed, Agriculture researches center, Ministry of Agriculture and Land Reclamation, Egypt. Voucher specimen is deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

Extraction and Isolation:

The air-dried leaves (1.5Kg) were extracted with 90% ethanol (4Lx3) by cold maceration at room temperature. The alcoholic extract was concentrated under reduced pressure to a syrupy residue (400 ml), this residue was suspended in water (300 ml) and successively extracted with (2L each) of n-hexane, $CHCl_3$, EtOAc and n-BuOH. The ethyl acetate extract (10.0g) was subjected to silica gel column chromatography using benzene as a eluent, followed by ethyl acetate and the polarity was gradually increased until methanol.

Four major fractions were collected. These fractions were further subjected to successive vacuum column chromatography over silica gel and sephadex LH-20columns chromatography to give compounds (1-6).

Compound (1): obtained as yellow powder, R_f 0.87 (system II). UV λ_{max} (MeOH) 267, 368;(+ NaOMe) 278, 317 sh, 415;(+$AlCl_3$) 269, 423;(+$AlCl_3$/HCl)268, 422 ;(+NaOAc) 274, 307, 377;(+NaOAc /H_3BO_3) 268, 308, 365. EIMS m/z(rel%): 286(M+, 100%), 167(17), 153(5), 149(92), 136(8), 121(28). The ^{1}HNMR (300 MHz, DMSO-d6): δ6.18(1H, d, J=1.2 Hz, H-6), 6.43 (1H, d, J=1.2 Hz, H-8), 8.02 (2H, d, J=8.4 Hz, H-6', H-2'), 6.93 (2H, d, J=8.4 Hz, H-5',3') and 12.46 (1H, s, C5-OH)

Compound (2):was obtained as yellow granules, mp181-183^{o}c, R_f 0.68(system II); UV λ_{max} (MeOH) 257, 355; (+ NaOMe) 271, 393 ; (+$AlCl_3$) 273, 317sh, 415; (+$AlCl_3$/HCl) 272, 355, 397; (+NaOAc) 262, 358; (+NaOAc/H_3BO_3) 261, 364; EIMS m/z(rel%):357(M++ H-sugar), 316 (M +-Sugar), 302(100%), 286(5), 177(8), 166(3), 165(2), 152(25),151(3),146(3), 137(19), 136(11), 134(5), 121(26), 108(28); ^{1}HNMR (300 MHz, DMSO-d6): δH, 6.19(1H, d, J=2.1 Hz, H-6), 6.38 (1H, d, J=2.1 Hz ,H-8), 6.85 (1H, d, J=8.4Hz, H-5'), 7.23(1H, d, J=2.4 Hz, H-2'), 7.29 (1H, dd, J=2.4Hz, 8.4 Hz, H-6'), 7.73 (1H, d, J=9Hz, H-1'''), 6.89(1 H, d, J=9,H-2'''), 5.29(1H,s,H-1''), 3.97(3H,s,OCH3),3.53-3.17(m, sugar protons) , 2.48(3H,d,J=1.8,CH3-3''') ,0.80 (3H, d, J= 6.0Hz, CH3 rhamnose protons) and12.64(1H,s,OH-C5);[13]

CNMR(75 MHz, DMSO-d6):δ 177.69 (C-4), 164.18(C-7), 161.24(C-5),159.94(C-1'''), 157.23 (C-2), 156.40(C-9), 148.38(C-4'), 145.15(C-3'), 134.17(C-3), 130.35(C-2'''), 121.06(C-1'), 120.70 (C-6'), 115.61(C-5'), 115.41(C-2'), 104.02(C-10),(C-1''), 98.65(C-6), 93.58(C-8),71.15(C-2''), 70.52(C-3''), 70.32 (C-4''), 70.01(C-5''), 56.42 (OCH3),18.20(C-3''')and 17.45(C-6'')

Compound (3): as pale yellow powder, R_f 0.62 (system II); UV λ_{max} (MeOH) 257, 355;(+NaOMe) 270, 328sh, 393;. (+AlCl3) 273, 328 sh, 428(+AlCl3/HCL) 270, 3 55, 395 ;(+NaOAc)270, 326sh, 369 ; (+NaOAc /H3BO3) 260, 364. EIMS m/z (rel.%): 462(M+,C22H22O11,2),316 (M+ -rhamnose), 302(100%), 273(15), 257(5), 152(12),147(2),146(2), 137(25), 121((6);^{1}HNMR (300 MHz, DMSO-d6): δH, 6.89(1H,d,J=8.1Hz,H-5'),7.24(1H,d,J=2.1Hz,H-2'),7.30(1H,dd, J=2.1Hz, 8.1Hz, H-6'), 5.25(1H,s,H-1''), 3.88(3H,s,OCH3),

3.53-3.25(m, sugar protons),0.83 (3H, d, J= 6.0Hz, rhamnose CH3)and 12.65(1H,s,OH-C5) .

Compound (4): isolated as yellow powder, R_f 0.56(system II). UV λmax(MeOH) 256, 355;(+ $AlCl_3$) 274, 428;(+ $AlCl_3$/HCl) 270, 355, 397;(+NaOAc) 270, 360;(+NaOAc/H_3BO_3) 262, 370. EIMS m/z (% relative abundance) :448(M+,$C_{21}H_{20}O_{11}$, 1), 302(M+-rhamnose,100), 153(10),152(2),136(24).The ^{1}HNMR (300 MHz, DMSO-d6): δH, 6.20(1H,d,J=2.4 Hz, H-6), 6.38 (1H,d,J=2.4 Hz ,H-8), 6.86 (1H,d,J=8.4 Hz,H-5') , 7.24(1H,d,J=2.1 Hz,H-2'), 7.29 (1H, dd, J=2.1Hz ,8.4 Hz,H-6'),5.25(1H,d,J=1.5 Hz,H-1"), 3.53-3.25(m, sugar protons), 0.83 (3H,d,J= 6.0 Hz,CH3 rhamnose protons) and 12.64 (1H, s, OH-C5).^{13}CNMR(75 MHz, DMSO-d6):δ134.17(C-3), 101.79(C-1") and 17.43(C-6").

Compound (5): obtained as canary yellow plate, R_f 0.43 (system II), mp 246-247^{o}C .UV λ_{max}(MeOH) 257,358;(+ NaOMe)272,328sh ,407(+ $AlCl_3$) 274,429 ;(+ $AlCl_3$/HCl) 269,358,400 ;(+ NaOAc) 269,368 ;(+NaOAc/ H_3BO_3) 261 ,376. EIMS m/z (rel. %): 302(M+ -sugars, 100), 152(19). The ^{1}HNMR (300 MHz, DMSO-d6): δH, 6.19(1H, d,J=2.1 Hz, H-6), 6.39 (1H,d,J=2.1 Hz ,H-8), 6.82 (1H,d,J=8.4 Hz,H-5') , 7.50(1H,d,J=1.8 Hz,H-2'), 7.66 (1H, dd, J=1.8 Hz ,8.4 Hz, H-6'), 5.28(1H, d, J=5.1Hz, H-1"), 4.52(1H, d, J=4.5,1"'), 3.78-3.51(m, sugars protons) and12.63(1H,s,OH-C5). ^{13}CNMR(75 MHz, DMSO-d6):δc 177.47(C-4), 164.14(C-7), 161.17(C-5), 161.04(C-2), 158.23 (C-9), 148.54(C-4'), 144.93(C-3'), 133.71(C-3), 122.00(C-1'), 120.86(C-6'), 115.72(C-5'), 115.31 (C-2'),101.36(C-1"), 101.36(C-1'"), and 71.58-64.17(sugars-C) .

Compound (6): as dull yellow powder,R_f0.30 (system II),mp>300 ^{o}C ,UV λ_{max}(MeOH) 257,355 ; (+NaOMe)271,404 ;(+ $AlCl_3$) 273, 319sh,426; (+ $AlCl_3$ /HCl)268,355, 397; (+ NaOAc) 262,326,358 ; (+NaOAc/ H_3BO_3)261,373; EIMS m/z (rel.%): 302(M+ -sugars,2), 284(3), 270(4), 171(19), 162(6), 153(8), 152(2), 149 (17), 136(18), 120(100),118(2). The ^{1}HNMR (300 MHz, DMSO-d6): δH, (1H,d,J=8.4 Hz,H-5') , 7.53(1H, d, J=2.4 Hz, H-2'), 7.64 (1H, dd, J=2.4 Hz, 8.4 Hz,H-6'), 5.44(1H, d, J=7.5 Hz, H-1"), 5.34(1H,d,J=7.5,1"'),4.14-3.34 (m, sugars protons) and 12 .60 (1H,s,OH-C5).

Acid Hydrolysis:

Compounds (**4**), (**5**) and (**6**), (5-7mg each) were separately refluxed, with 7% aqueous sulphuric acid (10 ml) for 2hrs. Then water was added and the mixture was extracted with EtOAc. All aglycones were identical with quercetin on TLC against standard quercetin (system I). The aqueous layer in each case was neutralized with $BaCO_3$, concentrated and subjected to PC (solvent system111), and investigation against authentic sugars, visualized by aniline phthalate spray reagent. Compound (**4**) gave sopt similar to rhamnose (R_f 0.36) while compounds (**5**) & (**6**) gave reddish brown spot for glucose (R_f0.21) (co-Pc and co-TLC).

Antioxidant Activity [(22)]:

This activity was determined by the DPPH assay in triplicate and average values were considered. Freshly prepared (0.004%w/v) methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was prepared and stored at 10C in the dark. A methanol solution of the EtOAc extract and compounds (1-6) were prepared. A 40 uL aliquot of the methanol solution was added to 3ml of DPPH solution. Absorbance measurements were recorded immediately with a UV-visible spectrophotometer (Milton Roy, Spectronic 1201). The decrease in absorbance at 515 nm was determined continuously, with data being recorded at 1 min intervals until the absorbance stabilized (16 min). The absorbance of the DPPH radical without antioxidant (control) and the reference compound ascorbic acid were also measured. All the determinations were performed in three replicates and averaged. The percentage inhibition (PI) of the DPPH radical was calculated according to the formula:

$$PI = [\{(AC- AT)/ AC\} \times 100] \quad (1)$$

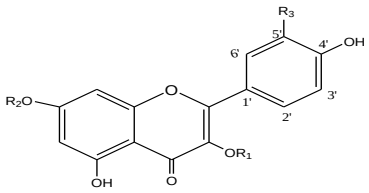
Where AC = Absorbance of the control at t = 0 min and AT = absorbance of the sample+DPPH at t = 16 min (Yen and Duh, 1994). Results were recorded in (**Table 1**).

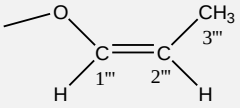
DISCUSSION: Column chromatography of the ethyl acetate extract of *B. retusa* leaf and repeated

chromatographic methods afforded novel compound and five known compounds.

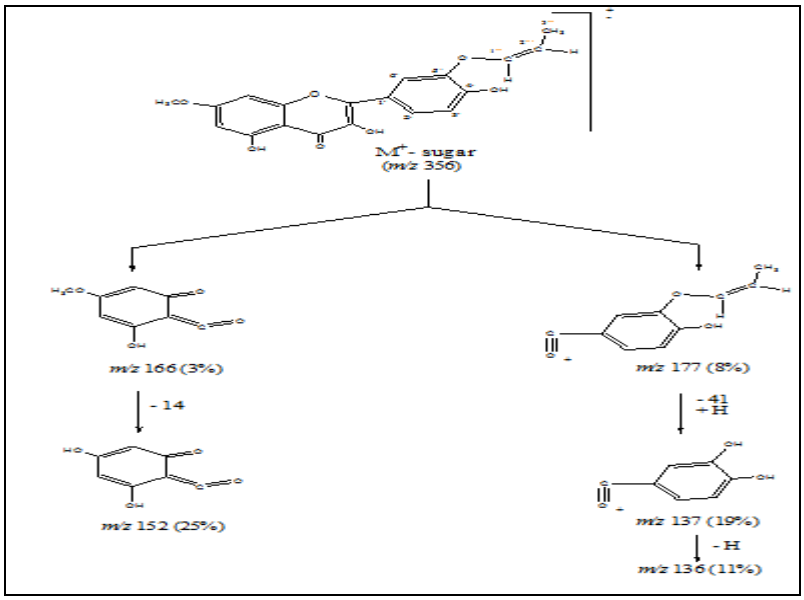
Compound (1): exhibited UV at 267 and 368 and its changes with shift reagents to suggest the presence of 3, 4', 5 and 7 tetrahydroxyflavonol [23-26]. This was confirmed by MS parent ion peak at m/z 286(100%) for $C_{15}H_{10}O_6$. The ^{1}HNMR spectrum displayed signals comparable to the published data for kaempferol [27, 28]. Comparing UV, MS and ^{1}H NMR of compound (1) with available published data were identical with kaempferol [23-29]. This is the first isolation of kaempferol from *B.retusa*.

Compound (2): yellow powder; UV in MeOH (257, 355) and common shift reagents [23-26]. Suggested a flavone with free hydroxyls at C-5 and C-4'. The MS with parent ion at m/z 357(M^++H-sugar) ,fragment at m/z 316 for loss of 41 mass unit indicating the presence of C_3H_5 , Fragment at m/z 177 suggested that one of **B**-ring hydroxyls is substituted by a propenyl group [23, 29] which was confirmed by NMR signals at [δ_H7.73 ppm(1H, d, J=9 Hz), δ_C159.94,C-1'''] and [δ_H6.89 ppm (1H, d, J=9 Hz) ,δ_C 130.35, C-2'''], assigned for two cis protons at H-1''' and H-2''' ,in addition to signal at [δ_H2.48 ppm (3H, d, J= 1.8 Hz),δ_C18.20,C-3''']for3H at 3''' position [24]. The ABX system at 7.23 (1H,d, J=2.4Hz,H-2'), 7.29 (1H,dd, J= 2.4 and 8.4 Hz, H-6'), and 6.85 (1H,d,J=8.4Hz, H-5') confirmed the disubstituted 3 ', 4 'of ring B. Also, MS showed peak at m/z 166 corresponding to ring **A** with one hydroxy and one methoxyl and the fragragments at m/z 152(25) was also significant [23], mass fragmentation **scheme 1.**



Compound	R_1	R_2	R_3
1	H	H	H
2	Rha.	CH_3	
3	Rha.	H	O-CH_3
4	Rha.	H	OH
5	β-D-glu.- β-D-glu.	H	OH
6	β-D-glu.	β-D-glu.	OH

ISOLATED COMPOUNDS FROM THE LEAF OF *BAUHINIA RETUSA*



SCHEME (1): SUGGESTED MASS FRAGMENTATION PATTERN OF COMPOUND 2

TABLE 1: EVALUATION OF ANTIOXIDANT ACTIVITY OF EtOAc EXTRACT AND ISOLATED COMPOUNDS FROM *B. RETUSA* USING DPPH SCAVENGING WITH ASCORBIC ACID AS POSITIVE STANDARD.

SC_{50} µg/ml							
Extract	**Compound Number**						**standard antioxidant**
EtOAc extract	1	2	3	4	5	6	Ascorbic acid
18.7	45.3	37.3	50.8	13.5	14.7	>3000	13.9

The ^{1}HNMR signals at δ_H12.64 for c-5hydroxyl, while position 7 remains for the methoxy group (δ_H 3.97 & δc56.42) in NMR spectrum [23-26]. The glycosidation of compound (**2**) was confirmed by ananomeric proton resonanated as singlet at δ_H5.29ppm indicating that is α-configuration. The up field shift of C-3 (δc134.17, Δ-2 ppm) in ^{13}CNMR confirmed the placement of sugar at position C-3 [31, 32]. Also, ^{13}CNMR clearly showed six carbons, one for methyl groupt at δc17.45, and four carbons at δc (71.15, 70.52, 70.32, 70.01) in addition to anomeric carbon at δc101.78 confirming the presence of rhamnose as sugar moiety . Searching the available literature [23-26, 29-32], this compound was unumbiguiously identified as rhamnetin-3-O-α- rhamnopyranosyl-3'-O-(prop-1-enyl).

To our knowledge, this is the first report of this compound from this plant, genus *Bauhinia* and from nature.

Compound (3):was isolated as yellow granules with uv (257, 355) and shift reagents [23-26] indicating a flavonol with free 7,5 and 4' hydroxy groups and absence of a free ortho-dihydroxy pattern at **B**- ring. MS with molecular ion peak at m/z 462 [$C_{22}H_{22}O_{11}$] followed by fragments at m/z 316(M^+- 146, loss of rhamnose) and 302(M^+-146, -14,1oo% for loss anomeric rhamnose and methyl) indicating its glycosidic nature [29, 33].

^{1} HNMR singlet proton at δ_H 5.25 and δ_H0.83 (d,J= 6.0Hz,Me- rhamnose) indicate the presence of one rhamnose unite, singlet at δ_H3.88 for methoxy group. The placement of rhamnose at 3-OH was confirmed by ^{1}HNMR signal at δ 12.65 (OH-5) and uv bathochromic shift on addition of NaOAc(+13) indicating the free hydroxl at 7-positions [23-26]. From the comparison of the literature data.[23-26, 29, 33, 34] Compound (3) was identified as isorhamnetin-3-α-rhamnoside (quercitin 3'-methoxy 3-o-rhamnoside). This is the first report of this compound from the plant**.**

Compound (4): isolated as yellow granules with MS parent ion at m/z 448, analzing for $C_{21}H_{20}O_{11}$, fragment at m/z 302(M^+-146, loss of rhamnose) [23, 29]. Acid hydrolysis afforded quercetin and rhamnose (PC, TLC). Attachement of sugar at C-3 was confirmed by upfield shift (δ_c < 2) ofδ_c134.17 in C-3 position[32, 35, 36]. The UV, MS, ^{1}HNMRand acid hydrolysis were in agreement with the quercetin-3-α-L-rhamnoside (quercitrin) [23-26, 29, 32, 35, 36]. This is the first report on isolaton of qurcitrin from *B.retusa*.

Compound (5) : was isolated as canary yellow plates , UV with shift reagents ,base peak at m/z 302 and acid hydrolysis indicated quercetin derivatives [23-26, 29]. Its ^{1}HNMR showed a 2H AX and a 3H ABX system characteristic of quercetin [24, 25] , two doublets at δ_H 5.28 (1H, d, J=5.1 Hz, H'') and δ_H4.52(1 H, d, J=4.5,H '') , suggesting the presence of two anomeric protons of a sugar moiety with the β- configuration. [35,37].The appearance of one anomeric signal above δ_H5 pointed to the presence of one aglycone-sugar linkages, the other anomeric signal being located at δ_H4.52, more typical for sugar- sugar linkage [35,37].

Shielding of C-3 (δ_c 133.71) and deshielding for C-2 (δ_c 161.04) indicating glycosylation sites was at C-3 [32]. UV, MS**,** ^{1}H and^{13} C NMR showed identical data with previously published data [23-26, 28, 29, 35-38] confirmed that compound(5) is quercetin 3 – O - β – D – glucopyranosyl – β - D-glucopyranoside. To our knowledge, this is the first report on the presence of this compound in *B.retusa* and genus *Bauhinia.*

Compound (6): is greenish yellow with UV and shift reagents indicated 3,7- disubstituted flavonoid glycoside with free hydroxyl at 5, 3' and 4'[23-26]. Acid hydrolysis afforded glucose and quercetin (TLC and PC) with authentic samples.

MS with peaks at m/z302 (M^+- sugars) and m/z 162 suggesting the presence of qurcetein and hexose sugar [23, 24, 29, 35]. Two anomeric protons were observed at δ_H5.44(d, J=7.5 Hz,H-1") and δ_H 5.34(d, J=7.5,1"') assigned to β-glucose, The chemical shifts at δ_H (5.44 and 5.34) indicated that both glucose moieties are directly attached to the aglcone [37], this was confirmed by UV analysis [23-26]. Based on the above evidence, as well as comparison with published spectral data [23-26, 28, 29, 35-38], the structure of compound (**6**)was assigned as quercetin 3,7-di-o-β-D-glucoside. This is the first report of isolation of this compound from *B.retusa*, from genus *Bauhinia* and second from nature [39].

Antioxidant Activity: Compounds(**4**),(**5**) and ethyl acetate fraction exerted pronounced antioxidant activities (SC_{50} µg/ml,13.5, 14.7 and 18.7) respectively, compared with vitamin C (SC_{50},13.9 µg/ml), while compounds (**2**),(**1**) and (**3**) showed noticeable effect and compound (**6**) is completely inactive (**Table 1**).

This results confirmed by previously reported data [40, 41]. This information may help understand the health benefits of *B.retusa* and may contribute to develop this plant as effective in preventing diseases arising from oxidative damage.

ACKNOWLEDGEMENT: The authors are indebted to Prof. Dr. Mahmoud Khalil, Plant Production Department, Efficiency Productive Institute, Zagazig University, Egypt, for providing us the plant material.

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How to cite this article:
El Sayed ZIA Hassan WHB and Abdel-Monem A: Novel Propenyl Flavonoids Glycoside and Antioxidant Activity of Egyptian *Bauhinia Retusa*. Int J Pharm Sci Res 2015; 6(1): 478-84.doi: 10.13040/IJPSR.0975-8232.6 (1).478-84.