INTERNATIONAL RESEARCH JOURNAL OF PHARMACY ISSN 2230 – 8407
Available online http://www.irjponline.com
Research Article

FLAVONOIDAL CONTENT OF *VANGUERIA INFAUSTA* EXTRACT GROWN IN EGYPT: INVETIGATION OF ITS ANTIOXIDANT ACTIVITY

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Article Received on: 01/02/2011 Revised on: 20/02/2011 Approved for publication: 11/03/2011

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ABSTRACT
Chromatographic purification of methanolic extract of *Vangueria infausta* leaves grown in Egypt led to isolation of five flavonoids namely luteolin-7-*O*- rutinoside, apigenin- 7-*O*- rutinoside, luteolin-4` -*O*- glucoside, quercetin-3-*O*-glucoside and quercetin. Also, the extract revealed both free radical (DPPH), H_2O_2 scavenging activities and reducing power potential as compared to rutin. The extract showed promising activity that may limit free radical and oxidative damage.
KEYWORDS: *Vangueria infausta*, flavonoid, DPPH

INTRODUCTION
Vangueria infausta Burch (family Rubiaceae) is a tree, grows well on semiarid soils and in rain forests, but has a worldwide distribution. This plant is found mostly in southern Africa but is also native to East Africa and Madagascar[1,2]. The fruits of *V. infausta* are eaten by both people and wild animals while different parts of this plant have been used traditionally for treatment of malaria, wounds, menstrual, uterine problems, and genital swellings among others[3,4]. Recent pharmacological reports have shown that extracts from leaves and roots of this plant exhibited significant antiplasmodial activity[5]. It was reported previously the isolation of (-)-epicatechin, epiafzelechin, dihydrokaempferol, quercetin, luteolin, dihydroquercetin-3'-Oglucoside, daidzein and genistein together with 5,7,3',5",7",4"'-hexahydroxy (4'-*O*-3"')-biflavone and methylcylohex-1-ene from the plant[6]. The present study aimed to investigate the flavonoidal content of *Vangueria infausta* grown in Egypt and evaluation of its antioxidant activity.

MATERIALS AND METHODS
Plant material
The leaves of *Vangueria infausta* Burch. family Rubiaceae was collected from El-Zohria Research Garden, Cairo, Egypt. A voucher specimen was deposited at Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University, Nasr city, Cairo, Egypt.

Apparatus and chemicals used for phytochemical study
Polyamide of the caprolactam type (MN Polyamide SC6, Mcharay Nagel) for column chromatography (CC) that was used for fractionation and purification. Sephadex LH-20, 25–100 Am (Pharmacia, Biotech). TLC was performed on silica gel 60 F_{254} sheets of 0.25 mm thickness, (E-Merck Company, Germany). PC (descending): was performed on Whatman no. 1 paper, using solvent systems: (1) H_2O, (2) 15% HOAc, (3) BAW (n-BuOH-HOAc-H_2O, 4:1:5, upper layer); (4) C_6H_6-n-BuOH-H_2O- pyridine (1:5:3:3, upper layer) (5) 40% HOAc. Solvents 3 and 4 were used for sugar analysis and solvent 5 for preparative isolation on Whatman no. 3mm paper. UV absorptions were measured on a Shimadzu UV-visible recorder spectrophotometer model–UV 240. ^1H NMR spectra were acquired on a Jeol EX-500 spectrometer (1107 T) operating at 500 MHz for ^1H resonances relative to TMS.

Extraction and isolation
The ground leaves of *Vangueria infausta* Burch.(500 g) were extracted exhaustively at room temperature using petroleum ether, chloroform and methanol (3 extractions for each with 3L). Concentrated MeOH extract (30 g) was applied to polyamide column and eluted with H_2O followed by H_2O/ MeOH mixtures of decreasing polarities and finally with MeOH to yield 15 fractions. Fraction 10 (from 40% column fraction) was subjected to Sephadex LH-20 fractionation and PPC chromatography using 40% HOAc yielding compound 1. Fraction 14 (from 50% column fraction) was subjected to silica

column using $CHCL_3$-MeOH-H_2O (80-18-2) as eluent followed by repeated Sephadex LH-20 fractionation and PPC chromatography using 40% HOAc, yielding compounds 2, 3, 4 and 5.

Luteolin-7-O- rutinoside 1: (25mg) yellow amorphous powder, mp 320-326 ^oC. UV λmax (MeOH) nm: 225, 258, 347.5 (NaOMe) 262.5, 298, 398, ($AlCl_3$) 272.5, 297, 331, 421, ($AlCl_3$/HCl) 269,293, 361,387 (NaOAc) 261, 268,379,423, (NaOAc/H_3BO_3)261.5, 370.5. ^1H NMR (500 MHz, DMSO-d_6) δ7.34 (1H,*dd*, *J*= 8, 2.5 Hz, H-6'), 7.29 (1H,*d*, *J*= 2.5 Hz, H-2') , 6.73 (1H,*d*, *J*= 8 Hz, H-5') , 6.72 (1H,*d*, *J*= 2 Hz, H-8) , 6.61 (1H,*d*, *J*= 2Hz, H-6) , 6.38 (1H,*s* ,H-3), 5.01 (1H,*d*, *J*= 3Hz , H1'') and, 4.51 (1H,*d*, *J*= 1.1 Hz , H-1'''), 1.03 (3H,*d*, *J*=6.1 Hz, H-6'''), 3.2- 3.8 (ramaining sugar protons) ^{13}C NMR (500 MHz, DMSO-d6): -4), 164.7 (C-7),163.0 (C-2), 157.1 (C-9), 160.0 (C-5), 150.1 (C-4'), 145.4 (C-3'), 120.7 (C-6'), 121.0 (C-1'), 116.1 (C-5'), 113.6 (C-2'), 105.2 (C-10), 99.2 (C-6), 95.2 (C-8), 101.1(C-1''), 76.6 (C-3''), 75.8 (C-5''), 73.2 (C-2''), 69.8 (C-4''), 66.2 (C-6''), 100.6 (C-1'''), 72.2 (C-4'''), 70.9 (C-3'''), 70.5 (C-2'''), 68.5 (C-5'''), 18 (C-6''').

Apigenin- 7-O- rutinoside 2*:* (22mg) yellow amorphous powder, mp 310-312 ^oC, UV λmax (MeOH) nm: 273, 330 (NaOMe) 278, 313, 400, ($AlCl_3$) 275, 300, 352, 384, ($AlCl_3$/HCl) 275,299, 346,387 (NaOAc) 276, 351, (NaOAc/H_3BO_3) 276, 352. ^1H NMR (500 MHz, DMSO-d_6) δ7.33(2H,*dd*, *J*= 8.5 Hz, H-2', 6'), 7.31(2H,*dd*, *J*= 8.5 Hz, H-3', 5') , 7.1 (1H,*d*, *J*= 2 Hz, H-8) , 6.63 (1H,*d*, *J*= 2Hz, H-6) , 6.43 (1H,*s,* H-3) , 5.2 (1H,*d*, *J*=7.7 Hz, H-1''), 5.01 (1H,*d*, *J*= 1.1 Hz, H-1'''), 1.02 (3H,*d*, *J*=6 Hz, H-6'''). ^{13}C-NMR (100 MHz, DMSO- d_6) δ ppm 182.8 (C-4), 164.1 (C-2), 164.9 (C-7) , 161.5 (C-9), 162.6 (C-4'), 157.3 (C-5), 128.4 (C-6', C-2'), 123.1 (C-1'), 115.1 (C-3', C-5'), 105.7 (C-10) , 100.2 (C-6), 95.1 (C-8), 101.1(C-1''), 74.0 (C-3''), 76.1 (C-5''), 74.2 (C-2''), 69.9 (C-4''), 66.5 (C-6''), 101.6 (C-1'''), 72.9 (C-4'''), 68.9 (C-3'''), 74.5 (C-2'''), 69.2 (C-5'''), 17.9 (C-6''').

Luteolin-4`-O- glucoside 3: (14mg) yellow amorphous powder, mp 308-312 ^oC. UV λmax(MeOH) nm: 259, 352 (NaOMe) 262.5, 313.5, 396, ($AlCl_3$) 272, 298, 397, ($AlCl_3$/HCl) 251,395, 346 (NaOAc) 259, 385, (NaOAc/H_3BO_3) 263, 365. ^1H NMR (500 MHz, DMSO-d_6): δ 7.32 (1H,*dd*, *J*= 8, 2 Hz, H-6'), 7.15 (1H,*d*, *J*= 2 Hz, H-2'), 6.68 (1H,*d*, *J*= 2 Hz, H-8) , 6.43 (1H,*s* ,H-3), 6.4 (1H,*d*, *J*= 8 Hz, H-5'), 6.31(1H,*d*, *J*= 2Hz, H-6) and 5.01 (1H,*d*, *J*= 1.5 Hz,H-1''). ^{13}C NMR (500 MHz, DMSO-d_6): δ ppm 181.9 (C-4), 163.4 (C-7), 164.4 (C-2), 157.0 (C-9), 161.6 (C-5), 148.7 (C-4'), 147.1 (C-3'), 124.9 (C-6'), 124.8 (C-1'), 118.6 (C-5'), 116.2 (C-2'), 103.9 (C-10), 99.0 (C-6), 94.1 (C-8), 101.4 (C-1''), 75.9 (C-3''), 74.8 (C-5''), 73.3 (C-2''), 69.9 (C-4''), 60.8 (C-6'').

Quercetin -3-glucoside 4*:* (18 mg) yellow crystals, mp 318-320 ^oC., UV λmax (MeOH) nm: 258, 357 (NaOMe) 267, 313.5, 406, ($AlCl_3$) 274, 298, 433, ($AlCl_3$/HCl) 267,300, 346 (NaOAc) 269, 380, (NaOAc/H_3BO_3) 261, 379 . ^1H NMR (500 MHz, DMSO-d6) δ 7.62 (1H,d, J= 2.5 Hz, H-2'), 7.51 (1H,dd, J= 8, 2.5 Hz, H-6') , 6.77 (1H,d, J= 8 Hz , H-5') , 6.24 (1H,d, J= 2 Hz ,H-8) , 6.04 (1H,d, J= 2Hz , H-6), and 5.37 (1H,d, J= 1.5 Hz , H-1'''). ^{13}C NMR (500 MHz, DMSO-d_6): δ ppm 176.8 (C-4) , 164.6 (C-7), 161.3 (C-5) , 156.4 (C-2), 152.2 (C-9), 148.2 (C-4') , 144.5 (C-3'), 133.6 (C-3) , 121.4 (C-6') , 121.1 (C-1') , 115.1(C-2'),116.5 (C-5'), 104.1 (C- 10), 98.8 (C-6) 93.6 (C-8), 101.38 (C-1''), 62.3 (C-6''),77.4 (C-5'') ,76.9(C-3''), 74.2 (C-2''), 70.3 (C-4'').

Quercetin 5*:* (10 mg) yellow crystals, m.p 322-324 ^oC, UV λmax (MeOH) nm: 255, 301 and 371, (NaOMe) 245, 330, ($AlCl_3$) 272, 301, 454., ($AlCl_3$/ HCl) 270,357, 426, (NaOAc) 275, 324,387, (NaOAc/ H_3BO_3) 262,385. ^1H NMR (500 MHz, DMSO-d_6) δ 7.63 (1H , d ,J=8.5 H-2'), 7.50 (1H , d, J=8.5 , H-6'), 6.85 (1H , d, J=8.5 ,H-5')., 6.37 (1H , d, J=2.5, H-8) and 6.15 (1H ,d ,J=2.5, H-6). ^{13}C NMR (500 MHz, DMSO-d_6): δ ppm 176.3 (C-4) , 164.5 (C-7), 161.2 (C-5) , 156.6 (C-2), 148.2 (C-9), 147.2 (C-4') , 145.5 (C-3'), 136.2 (C-3) , 122.4 (C- 6') , 120.4 (C-1') , 116.1(C-2'), 115.5 (C-5') , 103.4 (C-10), 98.8 (C-6), 93.7 (C-8).

Investigation of antioxidant activity in vitro

DPPH$^•$ radical scavenging assay

Radical scavenging activity of plant extracts against stable DPPH$^•$ (2,2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich Chemie, Steinheim, Germany) was determined spectrophotometrically. When DPPH$^•$ reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep—violet to light—yellow) were measured at 520 nm on a UV/visible light spectrophotometer. Radical scavenging activity of extracts was measured by slightly modified method of Brand-Williams et al. (1995)[7], as described below. Extract solutions were prepared by dissolving 0.025 g of dry extract in 10 ml of methanol. The solution of DPPH$^•$ in methanol ($6x10^{-5}$ M) was prepared daily, before UV measurements. Three ml of this solution were mixed with 200,400, 800 and 1600 µg extract solution in 1 cm path length disposable microcuvettes similar concentrations of rutin were used as reference standard. The samples were kept in the dark for 15 min at room temperature and then the decrease in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH$^•$ solution was prepared and measured daily. The experiment was carried out in triplicate. Radical

scavenging activity was calculated by the following formula:

% inhibition = $[(A_B - A_A)/A_B] \times 100$

Where: A_B —absorption of blank sample (t=0 min);
A_A —absorption of tested extract solution (t=15 min).

Assay of reducing power

The reductive capability of the extract was quantified by the method of Oyaizu et al. (1986)[8]. One ml of (Extract) 100, 200, 300 and 400 µg/ml in distilled water was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide $[K_3 Fe (CN)_6]$. Similar concentrations of standard rutin were used as standard. The mixture was incubated at 50°C for 20 min. Then, the reaction was terminated by adding 2.5 ml of 10% trichloroacetic acid. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.5 ml of 0.1%$FeCl_3$. Blank reagent is prepared as above without adding extract. The absorbance was measured at 700 nm in a spectrophotometer against a blank sample. Increased absorbance of the reaction mixture indicated greater reducing power.

Hydrogen peroxide scavenging effects

The H_2O_2 scavenging effect of the extract was determined according to Ruch et al.(1989)[9]. A solution of H_2O_2 (4 mM) was prepared in phosphate buffer (pH 7.4). H_2O_2 concentration was determined spectrophotometrically from absorption at 230 nm in a spectrophotometer. The extracts (50,100,200 and 300 µg/ml) in distilled water were added to a H_2O_2 solution (0.6 ml, 4 mM). Absorbance of H_2O_2 at 230 nm was determined after ten min against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 of *Vangueria infausta* extracts and standard compounds:

H_2O_2 scavenging effects (%) = $[(A_B - A_A)/A_B] \times 100$

Where: A_B —absorption of blank sample (t=0 min);
A_A —absorption of tested extract solution (t=10min).

RESULTS AND DISCUSSION

Identification of compounds

Compounds 1-5 were identified as luteolin-7-O-rutinoside (1), apigenin- 7-O- rutinoside (2), luteolin-4`-O- glucoside (3), quercetin -3-glucoside (4) and quercetin (5) by comparing their , ^{1}H NMR, ^{13}C NMR, UV spectrum in methanol and different shift reagents with published data[10-15].

Acid hydrolysis of compound **1** yielded glucose and rhamnose (comparative paper chromatography Co-PC) and luteolin (Co-PC, UV spectra and ^{1}H NMR), UV spectral analysis in methanol and on addition of shift reagents confirmed the aglycone to be luteolin substituted at position 7 similarly compound **3** yielded glucose and luteolin aglycone on acid hydrolysis (Co-PC, UV spectra and ^{1}H NMR).

Compound **2** yielded glucose and rhamnose (Co-PC) and apigenin (Co-PC and ^{1}H NMR) UV spectral analysis in methanol and on addition of shift reagents confirmed the aglycone to be substituted at position 7. While compounds **4** and **5** yielded quercetin aglycone with additional glucose from compound **4** at position 3.

DPPH• radical scavenging activity

DPPH• is usually used as a substrate to evaluate antioxidative activity of antioxidants (Gulcin 2006)[16]. Fig. (2) represents the percent of free radical scavenging activity of *Vangueria infausta* extract in to relation to its concentration. There was an increase in the free radical scavenging activity with the increase in extract concentration. These results indicated *Vangueria infausta* extract have a noticeable effect on scavenging free radical. Based on the data obtained from this study the extract under study is free radical inhibitors or scavengers, as well as primary antioxidants that react with free radicals, which may limit free radical damage occurring in the human body.

Reducing power

Reducing capacity of the extract components may serve as a significant indicator of its potential antioxidant activity[17]. Fig. (3) shows the reductive capabilities of the *Vangueria infausta* extract compared to rutin. For the measurements of the reductive ability, we investigated the Fe^{3+}_Fe^{2+} transformation in the presence of *Vangueria infausta* samples using the method of Oyaizu (1986)[8]. The plant extract could reduce the most Fe^{3+} ions. The reductive potential of both extract and standard increased with increasing concentration. The antioxidant activity of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[18].

Hydrogen peroxide scavenging effects

The scavenging ability of *Vangueria infausta* extract on H_2O_2 is shown in Fig. (4) and compared with rutin as standard, 50, 100, 200 and 300 µg/ml of the extract and rutin exhibited 49.4, 66.8, 81.9, 89.4 and 65.6, 80.6, 85, 95% scavenging activity on H_2O_2 respectively.These results showed that *Vangueria infausta* extract had scavenging activity on H_2O_2.

In conclusion, the results of the present work revealed that the methanolic extract of *Vangueria infausta* leaves may be helpful in the elimination of free radicals and oxidative stress which could be attributed to its flavonoidal content.

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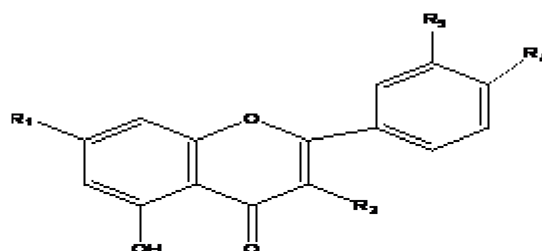


Compounds	R_1	R_2	R_3	R_4
1	O-gluc-Rham.	H	OH	OH
2	O-gluc-Rham	H	H	OH
3	OH	H	OH	O-gluc.
4	OH	O-gluc.	OH	OH
5	OH	OH	OH	OH

Fig 1: Structures of flavonoids isolated from *Vangueria infausta* Burch.

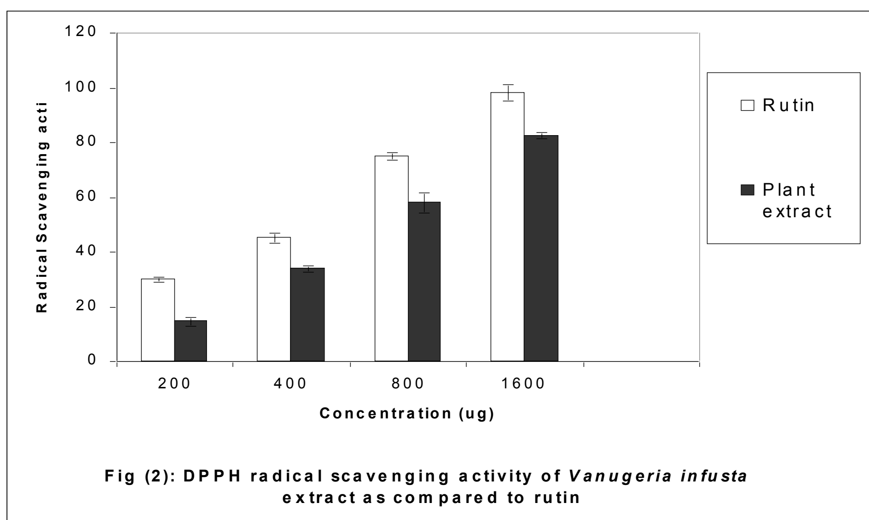
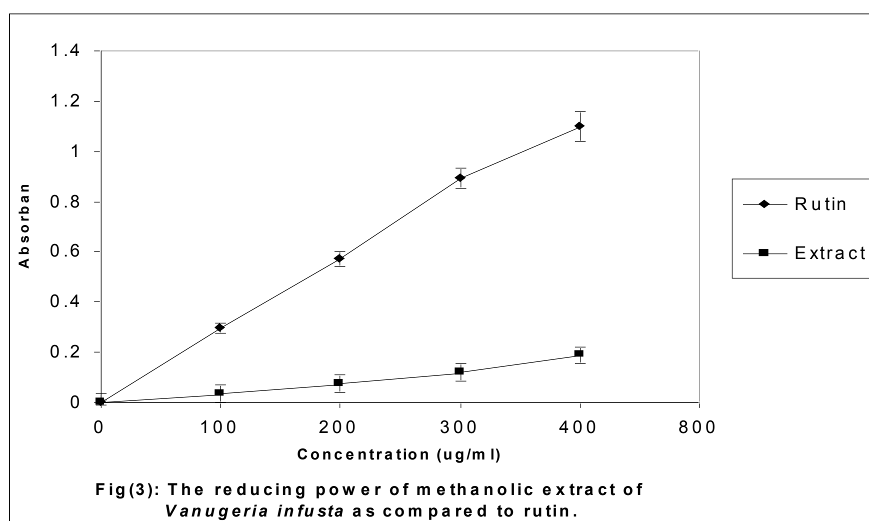


Fig (2): DPPH radical scavenging activity of *Vanugeria infusta* extract as compared to rutin



Fig(3): The reducing power of methanolic extract of *Vanugeria infusta* as compared to rutin.

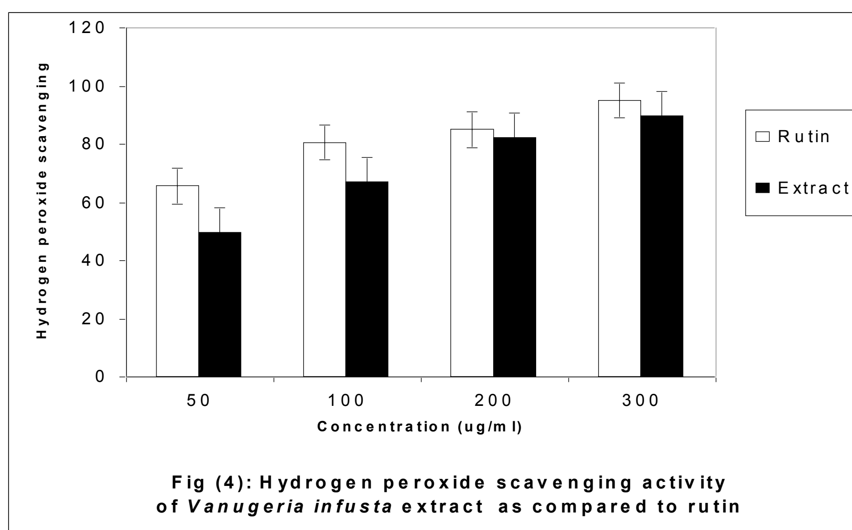


Fig (4): Hydrogen peroxide scavenging activity of *Vanugeria infusta* extract as compared to rutin

Source of support: Nil, Conflict of interest: None Declared