World Applied Sciences Journal 15 (3): 311-317, 2011
ISSN 1818-4952

Investigation of Phenolic Content and Biological Activities of *Scabiosa atropurpurea* L.

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Abstract: Chemical investigation of phenolic content of the total ethanolic extract (TEE) of aerial parts of *Scabiosa atropurpurea* Linn. (Dipsacaceae) led to the isolation of luteolin 7-O-glucoside (1), luteolin (2), 3-O-caffeoylquinic acid methyl ester (Chlorogenic acid methyl ester) (3) and Luteolin-7-O-β-D-rutinoside (4) for the first time from *S. atropurpurea.* Additionally, the percentage of total phenolics was determined. TEE showed Antihyperglycaemic, Hepatoprotective and Antioxidant activities. The activity was assessed through decreasing glucose levels, decreasing Alanine Transaminase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) levels and increasing blood glutathione. A bioguided fractionation of the total ethanolic extract (TEE) proved high activity in the ethyl acetate fraction (EAF) and TEE.

Key words: *Scabiosa atropurpurea* Linn. · Phenolic compounds · Flavonoids · Anti-diabetic · Hepatoprotective · Antioxidant

INTRODUCTION

Free radicals, from both endogenous and exogenous sources, are implicated in the etiology of several degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer [1]. High consumption of fruits and vegetables is associated with low risk for these diseases, which is attributed to the antioxidant vitamins and other phytochemicals [2]. There is a great deal of interest in plants that contain antioxidants and health - promoting phytochemicals, in view of their health implications. Several studies have shown that the hepatoprotective and antidiabetic effect are associated with antioxidant-rich plant extracts [3]. *Scabiosa atropurpurea* L. traditionally known as Ambarina in Northern Peru [4, 5] and as Escabiosa in north-east Catalonia (Iberian Peninsula) [6] has been used traditionally for its antibacterial activity in bronchitis [4] for menstrual regulation [5] and as veterinary diuretic [6]. *S. atropurpurea* L. possesses different biological activities as analgesic, antipyretic [7], anti-inflammatory and antibacterial [8]. Phytochemical investigations of *S. atropurpurea* L. remained confined to the isolation of irridoid glucosides [9, 10] and the flavone glycoside rhoifoloside [11]. *S. atropurpurea* L. has been introduced in Egypt as an ornamental plant. Due to reported traditional uses in folk medicine as well as lack of literature reports about the phenolic content of *S. atropurpurea* L. the authors planned this work to study those contents and the biological activities of this plant.

MATERIALS AND METHODS

Apparatus: UV spectra were measured using UV-visible, Shimadzu UV 160 A spectrophotometer, Lambomed Inc. Mass spectra were measured using Shimadzu QP -2010 Plus GC/MS. NMR spectra were measured with: Jeol Delta 2 instrument (^{1}H NMR, 500 MHz, ^{13}C, 125 MHz, Japan). The NMR spectra were recorded in DMSO-d_6 and chemical shifts were given in ppm relative to TMS as internal standard.

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Plant Material: The aerial parts (viz: leaves, stems) of *Scabiosa atropurpurea* L. were collected from Botanical Garden of Faculty of Science, University of Alexandria, Egypt in August 2007 and were kindly authenticated by Prof. Dr. S.Z. Heneidy, Prof. of Applied Ecology & Flora, Botany and Microbiology Department, Faculty of Science, Alexandria University. Dry Voucher specimen (no.101) is deposited in the Herbarium of the Botany Department, Faculty of Science, Alexandria University.

Extraction: The air dried and powdered aerial parts of *Scabiosa atropurpurea* L. (1.50 kg) were extracted with 70% EtOH (4 x 2.5 L) by percolation. The ethanolic extract was evaporated under reduced pressure to give (214g) of dark green residue (TEE). The residue was suspended in water (300 ml) and partitioned successively with hexane (8x300 ml), chloroform (8 x 300 ml), ethyl acetate (8x300 ml) and *n*-butanol (8x300 ml), to yield dry residues of 21.5 g (hexane fraction, HF), 9 g (chloroform fraction, CF), 51 g (ethyl acetate fraction, EAF) and 15 g (*n*-butanol fraction, BF), after evaporation.

Isolation: EAF (14.5g) was chromatographed over polyamide column (5 cm x 50 cm, 250 g) with H_2O, H_2O-MeOH mixtures, up to pure methanol. Fractions (100 ml each) were collected and monitored by TLC under UV-light (254 nm and 365 nm) and Aluminum chloride. Similar fractions were pooled to give 7 collective fractions (A-G). Fraction E (0.85g) was subjected to CC on Sephadex LH-20, with MeOH as an eluent. Subfractions (13-16; 5 ml each) containing similar spots including one major spot were pooled. Purified Fraction E (40 mg) was rechromatographed by CC on Sephadex LH-20, with MeOH-H_2O (7:3) as an eluent to give compound 1 (20 mg). Fraction G (1gm) was rechromatographed by CC on Sephadex LH-20, with MeOH as an eluent to yield compound 2 (300mg). BF (15g) was chromatographed over polyamide column (5cm x 50 cm, 250 g) with H_2O, H_2O-MeOH mixtures, up to pure methanol. Fractions (100 ml each) were collected and monitored by TLC under UV-light (254 nm and 365 nm) and *p*-anisaldehyde was used for detection. Similar fractions were pooled to give 7 collective fractions (I-VII).

Fraction III was rechromatographed by CC on Sephadex LH-20, with MeOH as an eluent to give 7 subfractions. Subfractions 2-4 (40 mg) were purified by CC on Sephadex LH-20, with MeOH-H_2O (6:4) as an eluent to give compound 3 (15mg). Fraction IV was rechromatographed on Sephadex LH-20, using MeOH as an eluent to give 19 subfractions. Subfractions 9-11(40 mg) was purified by CC on Sephadex LH-20, with methanol-H_2O (5:5) to give compound 4 (20 mg).

Luteolin 7- O-β-D- Glucoside (1): Yellow amorphous solid. MP: 238-240°C. MS m/z: 448, UV/Vis: (MeOH) nm: 255,270$_{sh}$, 350 NaOMe 265, 410, $AlCl_3$ 275,395$_{sh}$, 325$_{sh}$, 435, $AlCl_3$/HCl 275, 295$_{sh}$, 355$_{sh}$, 385, NaOAc. 260, 375, NaOAc. /H_3BO_3 260, 370 ^{1}HNMR δ ppm (DMSO, 300 MHz) Aglycone: 7.44-(1H, br.s, H-2'), 7.41-(1H, br.s, H-6') 6.8-(1H, d, J=8.1Hz, H-5'), 6.77-(1H, d, J= 1.5Hz, H-8), 6.72- (1H, s,H-3), 6.43(1H, d, J=1.8 Hz,H-6) Sugar: 5.05-(1H, d, J = 6.9Hz, H-1''). ^{13} CNMR (DMSO, 75 MHz) δ ppm 181.764 (C-4), 164.536 (C-7), 162.953(C-2), 161.130 (C-5), 156.922 (C-9), 150.181 (C-4'), 145.832 (C-3'), 121.227 (C-1'), 119.133 (C-6'), 116.005 (C-5'), 113.499 (C-2'), 105.385 (C-10), 103. 092 (C-3), 100.135 (C-1''), 99.613 (C-6), 94.810 (C-8), 77.201 (C-5``), 76.476(C-3''), 73.118 (C-2''), 69.751 (C-4''), 60.779 (C-6'').

Luteolin (2): Yellow amorphous solid MP: 328-330°C. MS m/z 286, UV/Vis:(MeOH) nm:255,270$_{sh}$,290$_{sh}$, 345, 350, NaOMe 265, 400, $AlCl_3$ 265,422, $AlCl_3$/HCl 280, 350, NaOAc. 270, 410, NaOAc./H_3BO_3 275, 380 ^{1}HNMR (DMSO, 500 MHz) δ ppm 7.39-(2H, d, J= 8.1 Hz, H-2', 6'), 6.87-(1H, d, J=7.8Hz, H-5'), 6.65(1H, s, H-3) 6.43-(1H, d, J=2.1Hz, H-8), 6.1-(1H, d, J=2.1Hz, H-6).

3-O-Caffeoylquinic Acid Methyl Ester (3): Brown crystalline solid MP: 220-225°C.ESI/ MS m/z 368, (MeOH) nm: 220,295$_{sh}$, 330, NaOMe 215,265,310$_{sh}$, 375. ^{1}HNMR (DMSO, 500 MHz) δ ppm 7.35- (1H, d, J = 19.85 Hz, β-H), 7.01-(s, H-2'), 6.92-(1H,s,H-6'), 6.71- (1H, d, 7.65Hz, H-5'), 6.17-(1H, d, Jα*β = 15.95Hz, α-H), 5.24-(1H,bs , H-3), 5.1-(1H,d, J = 7.65 H-5), 3.9- (1H,bs H-4), 3.48 (3H, s,OCH_3), 1.82-(4H,m, H-2,6). ^{13} CNMR (DMSO, 125 MHz) δ ppm 166.81 (C-7), 148.91 (C-3'),146.14(C-4'), 145.26 (C-7'), 126.07 (C-1'), 121.84 (C-6'), 116.17 (C-5), 115.185 (C-8), 73.14(C-1), 71.80 (C-5), 71.3087(C-4), 70.8052 (C-3), 49.13 (OCH_3), 39.91 (C-2), 39.74 (C-6).

Luteolin-7- Rutinoside (4): Yellow amorphous solid MP200-202°C. MS m/z 596 (MeOH) nm: 215, 250$_{sh}$, 265$_{sh}$, 349, NaOMe 275, 345, $AlCl_3$ 275, 290$_{sh}$, 310, 420, $AlCl_3$/HCl 260, 290, 350, 385, NaOAc. 260, 267$_{sh}$, 370, 403, NaOAc. /H_3BO_3 260, 365 ^{1}HNMR (DMSO, 500 MHz) δ ppm *Aglycone: 7.63-(1H, d, J= 16Hz, H-2'), 7.41-(1H, d, J= 16Hz, H-6'), 6.75-(1H, d, J=15.3Hz, H-5'), 6.7-(1H, bs, H-6,8), 6.41- (1H, s,H-3) *Sugar: 5.04-(1H, d, J =6.8Hz, H-1''),

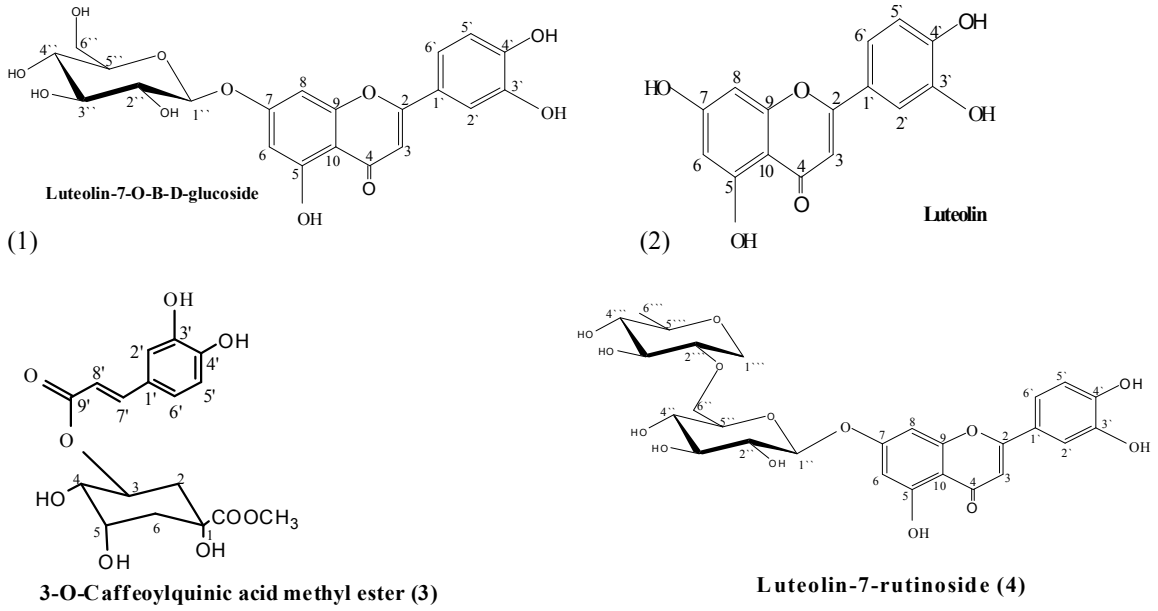

Fig. 1: Structures of compounds 1-4

4.14-(1H, bs, H-1```), 0.88-(3H, d, J= 6.9,H6``) $^{13}$CNMR (DMSO, 125 MHz ) δ ppm 182.4 (C-4), 165.1 (C-7),164.11(C-2), 163.4 (C-5), 157.5(C-9), 150.5 (C-3'), 146 .3(C-4`) 121.8 (C-6'), 119.8 (C-1'), 116.58 (C-5'), 114.1 (C-2'), 105.95 (C-10), 103.6 (C -3), 101.1 (C-1``),100.2 (C-1```), 99.8 (C-6), 95.26 (C-8), 76.6(C-3''), 76.08 (C-5``), 73.58 (C-2''), 72.9 (C-4```), 71.01 (C-3```),69.9 (C-2```) 69.11(C-4``), 67.67 (C-5```), 65.1 (C-6''), 19.4 (C-6```).

Determination of Total Phenolic Content: Total phenolic contents of TEE calculated as gallic acid was spectrophotometrically determined using Folin-Ciocalteu reagent according to the procedure adopted by Singleton *et al.* [12]. The total polyphenol content was calculated using the pre-established standard calibration curve using different concentrations of gallic acid (from 80-250 µg/ml).

Biological Study: Adult male albino rats (130-150 g) were obtained from the animal-breeding unit of National Research Center, Dokki, Giza, Egypt. Rats were fed on standard laboratory diet and water *ad libidum*. Doses of the drugs were calculated according to the method of Paget and Barne's [13] and were administrated orally by gastric tube. The chemicals used in this study were, Alloxan (Sigma Co., USA); Metformin (Cidophage)®, CID Co., Egypt. Vitamin E (Pharco Pharmaceutical Co., Egypt); Silymarin (Sedico, Pharmaceutical Co., Cairo) and Carbon tetrachloride (Analar). The residue of the tested fractions was dissolved in sterile water before administration to rats. Biodiagnostic kits used for the assessment of blood glucose, glutathione level and serum AST (SGOT), ALT (SGPT) and ALP. LD_{50} of ethanol extract previously prepared from *Scabiosa atropurpurea* L. was determined following Karber procedures [14].

For antihyperglycaemic effect, forty eight adult male albino rats were used in the experiment and divided into 8 groups. One group was kept as normal group. All the remaining groups were injected intraperitoneally with a single dose of alloxan (150 mg/kg b. wt.) to induce hyperglycaemia [15]. The second group was kept as diabetic control. Groups three to seven received 200 mg/kg orally of the TEE, HF, CF, EAF and BF of *Scabiosa atropurpurea* L. respectively, on a daily basis. The eighth group was given Metformin orally in a dose of 150 mg/kg daily. Blood glucose level [16] was measured after 4 and 6hrs and results are reported in Table 1.

Hepatoprotective effect of the tested extract as well as its fractions were evaluated and compared with that of Silymarin drug as standard. Liver damage was induced in rats according to the method of Klassen and Plaa [17] by intraperitoneal injection of 5 ml/kg of 25% carbon tetrachloride (CCl_4) in liquid paraffin. The experimental animals were divided into seven groups (six rats per group). All groups received daily oral dose of tested plant fraction for one week prior to liver damage then continued adminstration of drug for one week after liver damage. One group was kept as negative control (received 1 ml saline). Groups 2-6 received 100 mg/kg of TEE, HF, CF,

Table 1: Effect of total ethanolic extract and different fractions of *Scabiosa atropurpurea aerial parts* and Metformin drug on glucose level in diabetic male albino rats (n=6)

	Zero	4 hr	6 hr	
Group	M ±S.E	M ± S.E	M ± S.E	% of change
Normal	92.4±3.20	94.1±2.9	91.7±2.8	-
Diab. Control	248.6±9.40	246.3±8.7	251.2±9.6	-
Diab. treated with TEE	251.7±8.60	214.2±7.1*	149.3±6.4*	40.68
Diab. treated with HF	263.1±11.3	237.6±8.9	214.8±7.4*	18.35
Diab. treated with CF	261.9±8.70	203.2±9.1*	186.8±7.5*	28.67
Diab. treated with EAF	258.7±10.4	191.3±8.5*	136.4±5.8*	47.27
Diab. treated with BF	245.6±8.20	228.9±7.3	203.4±6.2*	17.18
Diab. treated with Metformin	259.4±11.3	186.9±6.8*	93.4±3.7*	63.99

* Statistically significant different from control group at $p < 0.01$

Table 2: Effect of total ethanolic extract and different fractions of *Scabiosa atropurpurea aerial parts* and silymarin drug on serum enzymes level (AST, ALT and ALP) in liver damaged rats

	AST (U/L)				ALT (U/L)				ALP (KAU)			
	Before liver damage		After liver damage		Before liver damage		After liver damage		Before liver damage		After liver damage	
Group	Zero	7d	72h	7d	Zero	7d	72h	7d	Zero	7d	72h	7d
Control	33.4±1.2	31.8±1.2	134.2±6.4	156.3±6.2*•	29.1±0.6	28.8±0.7	141.3±6.8	156.4±7.3*•	7.1±0. 1	7.2±0.1	64.8±2.1	75.3±2.4*•
TEE (100mg/kg)	32.6±0.9	31.4±1.2	71.2±2.3	42.4±2.9*•	28.2±0.6	27.9±0.4	65.2±2.8	49.6±2.4*•	7.4±0.1	7.1±0.1	24.3±0.6	17.8±0.4*•
HF (100mg/kg)	29.8±0.7	30.1±1.1	93.6±4.1	76.3±3.2*•	27.8±0.3	28.2±0.5	71.8±3.3	63.7±3.1*•	7.2±0.1	7.3±0.1	39.8±1.4	33.6±1.3*•
CF (100mg/kg)	28.3±0.7	28.5±0.4	82.8±3.6	68.7±2.4*•	32.4±1.3	32.1±1.1	87.3±3.8	62.5±2.9*•	7.5±0.1	7.4±0.1	36.3±1.1	29.5±0.6*•
EAF (100mg/kg)	27.4±0.8	27.1±0.6	64.3±2.8	51.3±2.8*•	30.2±0.4	29.7±0.4	58.9±2.4	45.5±2.1*•	7.3±0.1	7.2±0.1	28.9±0.7	21.9±0.8*•
BF (100mg/kg)	29.8±0.5	30.2±0.9	104.3±4.7	89.3±3.8*•	29.8±0.7	29.9±0.8	95.6±4.7	86.3±3.8*•	7.3±0.1	7.2±0.7	48.4±1.9	37.8±1.4*•
Silymarin (25mg/kg)	31.9±1.1	30.8±0.9	46.2±1.7	33.4±1.4*	28.7±0.4	28.1±0.5	43.7±1.5	31.5±1.1*	7.3±0.1	6.9±0.1	18.2±0.3	7.4±0.1*

* Statistically significant from zero time at $p < 0.01$

• Statistically significant from 72 h after CCl_4 at $p < 0.01$

Table 3: Effect of total ethanolic extract and different fractions of *Scabiosa atropurpurea* aerial parts and vitamin E drug on GSH in diabetic male albino rats (n=6)

	GSH	
Group	M±S.E	% of change
Normal	36.2±1.3	-
Diab. Control	21.3±0.4*	41.20
Diab. Treated with TEE	35.4±1.3	2.20
Diab. Treated with HF	29.8±0.4*	17.67
Diab. Treated with CF	30.2±1.1*	16.50
Diab. treated with EAF	35.1±1.2	3.03
Diab. treated with BF	26.4±0.4*	27.00
Diabetic + vitamin E	35.8±1.1	1.10

* Statistically significant from zero time at $p < 0.01$

EAF and BF fractions respectively. The seventh group administrated 25 mg/kg silymarin. Blood samples were obtained from the retro orbital venous plexus through the eye canthus of anaesthetized rats. Samples were first collected at zero time and after 7 days of drug injection. Liver was damaged by carbon tetrachloride injection and blood samples were collected again after 72 hrs and after 7 days liver damage. Serum was isolated by centrifugation and divided for analysis of Alanine Transaminase (ALT), Aspartate Amino Transferase (AST) and Alkaline Phosphatase (ALP) [18, 19]. Results are reported in Table 2.

For antioxidant effect, the same experimental design of antihyperglycemic effect was performed except that animals received a dose 100 mg/kg orally of different tested fractions and eighth group administrated 7.5 mg/kg vitamin E. Fresh heparinised blood after 7 days was used for the estimation of blood glutathione level [20] using Biodiagnostic Kit. Results are reported in Table 3.

Statistical Analysis: All data were expressed as the mean ± S.E.M .Means were compared by one-way analysis of variance (ANOVA) [21].

RESULTS AND DISCUSSION

TEE was subjected to fractionation and isolation of the major bioactive phenolic compounds using Polyamide followed by series of Sephadex LH-20 columns. Structures of the isolated compounds were established by comparison of their spectral data (UV, EI/MS, ^{1}H and ^{13}C

NMR) with those from the literature [22] and their identities were further confirmed through co-chromatography with authentic samples. Luteolin (2) and its 7- O-β-D- glucoside (1) was isolated from EAF while chlorogenic acid Methyl ester (3) and luteolin- 7-O β rutinoside (4) were isolated from BF. The percentage of total phenolic was 0.424% of dry aerial parts, calculated as gallic acid. From the biological study, it was clear the responsibility of phenolic compounds for different observed activities. This biological study was undertaken to assess antiperoxidation properties of *S. atropurpurea* L. in alloxan-induced diabetic rats using metformin as reference drug. The present investigation also demonstrates the hepatoprotective potential of TEE and its fractions against CCl_4-induced hepatic damage in rats using Silymarin as reference drug. The results of this study were presented in tables 1 - 3. The biological study revealed that LD_{50} of TEE was 5 g/kg b.wt.

Alloxan produces reactive oxygen species causing rapid destruction of β-cells [23]. In the present investigation, Table 1 showed that significant antihyperglycaemic effect was evident after 6 hrs. The maximum reduction in glucose levels was seen in animal receiving EAF in dose 100 mg/kg followed by TEE with percentage of change 47.27% and 40.68%, respectively.

In the present study (Table 2), TEE was evaluated for the hepatoprotective activity using CCl_4 induced hepatotoxicity in rat. The study revealed a significant increase in the activities of ALT, AST, ALP levels on exposure to CCl_4, indicating considerable hepatocellular injury. Administration of TEE as well as EAF attenuated the increased levels of the serum enzymes, produced by CCl_4 and caused a subsequent recovery towards normalization comparable to the control groups. The effect of TEE wasn't significantly different from Silymarin, ($P < 0.05$) reference group. TEE showed the highest percent protection (71.6% and 92.7% AST, 77.9% and 85.5% ALT and 86.9% and 84.7% ALP after 72 hours and 7 days respectively) followed by the EAF (79.4% and 85.4% AST, 84.4% and 88.8% ALT and 77% and 78.6% ALP after 72 hrs and 7 days, respectively) which could possibly be attributed to their phenolic contents such as luteolin and its derived glycosides [24]. However the data obtained showed a lower percent protection for the chloroform and the hexane fractions. The *n*-butanol fraction showed the least protection as shown in Table 2.

It has been hypothesized that one of the principal causes of CCl_4 induced liver injury is formation of lipid peroxides by free radical derivatives of CCl_4 ($CCl_3^{\bullet}$). Glutathione GSH, the first line of defense against a prooxidant status [25] was decreased significantly in plasma of diabetic rats. Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 induced hepatopathy and anti-diabetic affect. Both TEE and EAF showed significant antioxidant activities (Table 3). The TEE proved the best activity (2.2% of change from control) followed by the EAF (3.03% of change from control) while BF showed the least activity (27% of change from control). The relative potencies of the TEE and EAF were 98.8%, 98.04%, respectively as compared with vitamin E. The above results support hepatoprotective, anti-diabetic and antioxidant activity of TEE. This may be attributed mainly to synergism of different phenolic constituents (1-4) isolated from *S. atropurpurea*.

Luteolin (the major component in Ethyl acetate fraction) and Luteolin-7-glucoside showed anti-diabetic activity through inhibition of α-glucosidase and α-amylase. Luteolin showed stronger antidiabetic effect than acarbose, the most widely prescribed drug, in inhibitory potency, suggesting that it has the possibility to effectively suppress postprandial hyperglycemia in patients with non-insulin dependent diabetes mellitus [26]. On the other hand, Luteolin as well as its 7-glucoside (LUTG) proved to possess antioxidant and hepatoprotective activities [27, 28, 29]. All isolated compound as well as 3-O-caffeoylquinic acid methyl ester, as a phenolic compound with ortho-diphenol structure, showed high Oxygen Radical Absorbance Capacity (ORAC) 3.03 unit/μmol indicating high antioxidant activity [30].

In conclusion, the possible mechanism of hepatoprotective activity of *S. atropurpurea* L. may be due to its free radical scavenging and antioxidant activity, which may be due to the presence of flavonoids (luteolin and its glycosides) and phenolic compound (chlorogenic acid methyl ester) in the extracts. Also the antidiabetic activity showed by the TEE and EAF are related to the antidiabetic activities of the isolated compounds.

ACKNOWLEDGEMENT

The authors would like to thank Dr. S. Z. Heneidy, Prof. of Applied Ecology & Flora, Botany and Microbiology Department, Faculty of Science, Alexandria University for identifying and providing the plant.

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