Flavone Composition and Antihypercholesterolemic and Antihyperglycemic Activities of *Chrysanthemum coronarium* L.

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Five flavones were isolated from *Chrysanthemum coronarium* L., four of which were isolated for the first time from the genus *Chrysanthemum*. Two were the flavonoid aglycones 5,7-dihydroxy-3,6,4′-trimethoxyflavone (**1**) and scutellarin-6,7-dimethyl ether (**2**). A new flavonoid glycoside, apigenin-7-*O*-[2″(6‴-*O*-*β*-D-acetylglucopyranosyl)]-6″-*O*-acetylglucopyranoside (**3**), along with two known ones, *i. e.* apigenin-7-*O*-(2″-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside (**4**) and 6-methoxy quercetin-7-*O*-*β*-D-glucopyranoside (**5**), were identified. Structures were elucidated by NMR and MS. The therapeutic value of petroleum ether, ethyl acetate, and methanol extracts, respectively, in rats suffering from hypercholesterolemia − as a consequence of high-fat diet − and hyperglycemia − as a consequence of hypercholesterolemia and low doses of streptozotocin − was investigated through determination of biochemical markers and histopathology. The ethyl acetate and methanol extracts showed remarkable results, followed by the petroleum ether extract.

Key words: Chrysanthemum coronarium, Flavones, Hypercholesterolemia

Introduction

Chrysanthemum coronarium L. (Asteraceae) is widely distributed in the Mediterranean region, Western Africa, and Asia. The plant is regarded in East Asia as a health vegetable, because the edible portion contains abundant nutrients, has a fresh flavour, and has been used as herbal medicine (El-Masry *et al.*, 1984). Sesquiterpene lactones, campesterol, and heterocyclic compounds were the main compounds isolated from the flowers and aerial parts of the plant (Lee *et al.*, 2002; Choi *et al.*, 2007; Song *et al.*, 2008). Harborne *et al.* (1970) reported the presence of the 7-glucoside of quercetagetin 3′-methyl ether in flowers of *C. coronarium*, and caffeoylquinic acids were identified by liquid chromatography-diode array detection-atmospheric pressure chemical ionization-mass spectrometry (LC/DAD-APCI/MS) (Lai *et al.*, 2007). Additionally, apigenin, luteolin, luteolin-3′-methyl ether, and quercetin-3-methyl ether were reported in exudates of species in the *Chrysanthemum* complex (Valant-Vetschera *et al.*, 2003). Here we report the isolation of one new and two known flavonoid glycosides along with two aglycones. All five compounds were isolated for the first time from the genus *Chrysanthemum*.

Chronic diseases represent 73 % of mortality and 60 % of global morbidity burden. There is emerging evidence that diabetes mellitus, obesity, hypertension, and hyperlipidemia also contribute to national morbidity and mortality in Egypt and Saudi Arabia (Strong *et al.*, 2005). Natural products have been long considered promising antidiabetic agents because of their potent effects and low toxicities. With the distinctive traditional medical opinions and natural medicines mainly based on herbs, a combination of traditional

and herbal treatment performed well in clinical trials and is showing a bright future in the treatment of diabetes mellitus (Li *et al.*, 2004; Afifi-Yazar *et al.*, 2011). In the current study, an evaluation of the efficacy of successive extracts of *C. coronarium* on hypercholesterolemic and hyperglycemic rats through the assessment of biochemical markers and a histopathological investigation was carried out.

Materials and Methods

General

UV spectra were measured using a Shimadzu UV 240 spectrophotometer (Tokyo, Japan). ^{1}H NMR, ^{13}C NMR, ^{1}H,^{1}H-COSY, HMBC, and HSQC spectra were measured on a Bruker Avance 500 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a cryogenic TCI probe (5 mm) (Bruker), or on a Bruker DRX 500 spectrometer using an inverse-detection probe (5 mm). Operating frequencies were 500.13 MHz for acquiring ^{1}H NMR and 125.75 MHz for ^{13}C NMR spectra. The NMR spectra were recorded in CD_3OD or DMSO-d_6, and chemical shifts are given in δ (ppm) relative to tetramethylsilane (TMS) as internal standard. Electrospray ionization mass spectrometry (ESIMS) and high-resolution ESIMS (HRESIMS) were done with an Orbital XL instrument (Thermo-Fisher, San Jose, CA, USA), and electron ionization mass spectrometry (EIMS) was carried out with a Finnigan MAT 8500 instrument (Thermo-Fisher). Silica gel 60 (70 – 230 mesh ASTM; Fluka, Steinheim, Germany) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} precoated plates (Fluka). The chromatograms were visualized under UV light at 254 and 366 nm before and after exposure to ammonia vapour, as well as spraying with $AlCl_3$ or anisaldehyde/sulfuric acid reagent. The solvent systems (v/v) were as follows: S_1, acetic acid/H_2O (3:7); S_2, *n*-butanol/acetic acid/H_2O (4:1:5); S_3, distilled H_2O; S_4, *n*-butanol saturated with H_2O.

Plant material

Flowering aerial parts of *Chrysanthemum coronarium* L. were collected in a mountain region of Alkurr Wadi, Tabuk area, Saudi Arabia, in spring 2009. The plant was identified by Dr. Amal M. Fakhri Abdelsalam, Plant Culture Biology Department, Tabuk University, Tabuk, Saudi Arabia. A voucher specimen has been deposited at the herbarium of the National Research Centre, Cairo, Egypt.

Extraction and isolation

The dried plant material (2.0 kg) was successively extracted with petroleum ether and ethyl acetate followed by methanol using a Soxhlet extractor. The petroleum ether extract (47.2 g) was obtained as an oily residue. The ethyl acetate extract (21.0 g) was subjected to silica gel CC, eluted with benzene and a benzene/ethyl acetate gradient with increasing polarity. The fractions collected with benzene/ethyl acetate (9:2) afforded a yellow powder (57.0 mg). This powder gave two compounds on TLC which separated by Sephadex LH-20 CC, using methanol as eluent, to yield compounds **1** (21.0 mg) and **2** (15.0 mg). The methanol extract (52.11 g) was subjected to silica gel CC using chloroform and a chloroform/methanol gradient with increasing polarity. Fractions 1 – 20, collected with chloroform/methanol (9:1), contained a crude compound which was purified by preparative paper chromatography using S_1 as eluent to afford the pure compound **3** (16.0 mg). Fractions 26 – 118, collected with chloroform/methanol (9:2), gave an impure yellow compound on TLC, which was purified by passage through two successive Sephadex LH-20 columns followed by crystallization in methanol to give the pure compound **4** (22.0 mg). Fractions 151 – 162 gave a yellow precipitate which was further purified by silica gel CC, using chloroform/methanol as eluent, to afford compound **5** (32.0 mg).

Apigenin-7-O-[2″(6‴-O-β-D-acetylglucopyranosyl)]-6″-O-acetylglucopyranoside (**3**): HRESIMS: m/z = 679.22713 $[M+H]^+$, 271.06309 $[M+H-2$ (acetylglucose)$]^+$. – UV(MeOH): λ_{max} = 268.5, 330.5; (NaOMe) 273, 303, 380; ($AlCl_3$) 272, 299, 351, 383; ($AlCl_3$ + HCl) 276, 299, 340, 383; (NaOAc) 267, 330, 400; (NaOAc + H_3BO_3) 267, 330 nm. – ^{1}H and ^{13}C NMR: see Table I.

*Acid hydrolysis of compound **3***

A solution of 5 mg of compound **3** in 10 mL MeOH/1 M HCl (1:1) was boiled under reflux for 4 h, concentrated under reduced pressure, and diluted with H_2O (10 mL). This solution was extracted with ethyl acetate, and the residue recovered from the organic phase yielded the aglycone. The remaining aqueous

layer was neutralized with 5 % aqueous $NaHCO_3$ solution, concentrated *in vacuo*, and sugars were then identified by comparative TLC with authentic standards on silica gel F_{254} and with chloroform/methanol/acetic acid/H_2O (8:3:5:2) as developing solvent system. The nature of the sugar was confirmed by TLC using cellulose F_{254}, *n*-butanol/pyridine/acetic acid/ethyl acetate/H_2O (50:20:10:25:20), and aniline hydrogen phthalate spray reagent (Shalaby *et al.*, 2011).

Animals

One hundred and fifty male Wistar rats, aged 8 – 12 weeks and weighing 110 – 120 g, provided by the Animal House of the National Research Centre, Cairo, Egypt, were used. Rats were housed in a temperature-controlled environment (26 – 29 °C), with a fixed light/dark cycle for two weeks to acclimatize. They were allowed free access to food and water. This study had been approved by the Ethical Committee of the National Research Centre. The rats were randomly divided into ten groups of fifteen animals each.

Bioassays

Dose regimens and route of administration

The successive extracts of *C. coronarium* were administered orally daily for 45 d at the dose of 500 mg/kg body weight (BW) (Erdemoglu *et al.*, 2003). The standard antihyperlipidemic reference drug, flufastatin, was given orally at a dose of 20 mg/(kg BW d) (Lei *et al.*, 2011), and the antihyperglycemic reference drug, glibenclamide, at a dose of 3 mg/kg BW (Andrade-Cetto and Wiedenfeld, 2004).

Experimental design

Group 1 served as normal control and received normal diet and water. Groups 2, 3, and 4, separately, received orally normal diet and the petroleum ether, ethyl acetate, and methanol extract, respectively. Group 5 [hyperglycemia associated with hyperlipidemia (H-H) group] received orally a high-fat diet and cholesterol at a dose of 30 mg/0.3 mL, five times a week for 4 weeks (Adaramoye *et al.*, 2008), and the animals were injected streptozotocin (STZ) at 35 mg/kg BW (Srinivasan *et al.*, 2005). Groups 6 – 8 (H-H groups) were treated orally with the mentioned successive extracts of *C. coronarium*. Group 9 (H-H group) received orally the standard antihyperlipidemic reference drug flufastatin at a dose of 20 mg/(kg BW d). Group 10 received the antihyperglycemic reference drug glibenclamide at a dose of 3 mg/(kg BW d). After 4 weeks of the respective diet, the rats were fasted overnight and then intraperitoneally injected a freshly prepared solution of STZ (35 mg/kg BW). Serum glucose levels were determined every 3 d. At the completion of the study, rats were fasted overnight, each rat was anaesthetized with diethyl ether, and blood samples were collected. The serum was prepared by centrifugation and used for the determination of the lipid profile. The rats were then sacrificed by cervical dislocation, the livers rapidly removed, washed in saline, dried on filter paper, weighed, and homogenized in phosphate buffer for further biochemical analyses. Livers were also separated for histopathological examination.

Chemicals

The chemicals were high analytical grade products from Sigma-Aldrich (St. Louis, MO, USA), Merck (Darmstadt, Germany), BDH (Poole, UK), Riedel de Haën (Seelze, Germany), and Fluka (Buchs, Switzerland). The kits used were purchased from Biosystems (Barcelona, Spain) and Biodiagnostic Chemical Company (Giza, Egypt).

Biochemical studies

Profiles of the following serum lipids were established: total cholesterol (TC) (Meiattini, 1978), triglycerides (TG) (Fossati and Principe, 1982), low-density lipoprotein-cholesterol (LDL-c) (Schriewer *et al.*, 1984), high-density lipoprotein-cholesterol (HDL-c) (Burstein *et al.*, 1970), very low-density lipoprotein-cholesterol (VLDL-c), as well as total lipids (Zollner and Kirsch, 1962). Profiles of fasting blood glucose levels were established using commercially available glucose kits based on the glucose oxidase method (Trinder, 1969). The antioxidant status of serum was assessed by determination of glutathione (GSH), lipid peroxide [as malondialdehyde (MDA)] (Wood, 1970), and nitric oxide (NO) (Allen *et al.*, 2005). The inflammatory mediator factors, interleukin-10 (IL-10) (Dubiński and Zdrojewicz, 2007), tumour necrosis factor-α (TNF-α), and C-reactive protein (CRP), were estimated using diagnostic ELISA kits. Liver function tests of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were conducted (Belfield and Goldberg, 1971) using colori-

metric diagnostic kits. The albumin level was determined using a diagnostic kit, and the total serum protein content was assayed according to Bradford (1976).

Histopathological analysis

Liver slices were fixed in 10% formaldehyde and embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin and eosin (H & E), then examined under a light microscope for the determination of pathological changes.

Statistical analysis

All data are expressed as mean $\pm$ SD of ten rats in each group. Statistical analyses were carried out by one-way analysis of variance (ANOVA) and Costat computer program coupled with post-hoc [least significance difference (LSD)] analysis. Unshared letters indicate significant correlation at $P < 0.05$.

Results

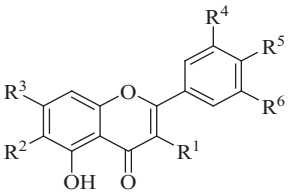
Five flavonoid compounds were identified in the *C. coronarium* extracts (Fig. 1). Four known flavonoids, previously not isolated from this plant, were the two flavonoid aglycones 5,7-dihydroxy-3,6,4′-trimethoxyflavone (**1**) (Agrawal and Bansal, 1989) and scutellarin-6,7-dimethyl ether (**2**) (Lai *et al.*, 2007), and the two flavonoid glycosides apigenin-7-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-glucopyranoside (**4**) (Agrawal and Bansal, 1989; Agrawal, 1992) and 6-methoxy quercetin-7-*O*-β-D-glucopyranoside (**5**) (Agrawal and Bansal, 1989; Agrawal, 1992). The isolated compounds were identified by UV, MS, ^{1}H and ^{13}C NMR analyses. The analytical data were in agreement with those reported in the literature.

The UV spectrum of compound **3** in MeOH and shift reagents (NaOMe, $AlCl_3$/HCl, NaOAc/H_3BO_3) revealed the possibility of an apigenin glycosidically linked at position 7 (Mabry *et al.*, 1970). The molecular formula was deduced from the HRESI mass spectrum as $C_{31}H_{34}O_{17}$. ^{1}H NMR data showed the occurrence of an AB spin system of ring A at δ_H 6.47 and 6.78 ppm for H-6 and H-8, respectively. Ring B exhibited an AA'XX' spin system of a *p*-substituted phenyl ring at δ_H 6.94 (d, $J = 8.7$ Hz, H-3′/5′) and 7.93 ppm (d, $J = 8.4$ Hz, H-2′/6′) (Table I). The signal of two anomeric protons at δ_H 5.26 (d, $J = 7.0$ Hz, H-1″) and 4.52 ppm (d, $J = 7.5$ Hz, H-1‴) indicated the presence of two sugar moieties. The values of the coupling constants $^3J_{\text{H-1}''-\text{H-2}''}$ and $^3J_{\text{H-1}'''-\text{H-2}'''}$ ($J = 7.0$ and 7.5 Hz) reflected the β-configuration of the sugar moieties. By ^{1}H-^{1}H-COSY, HSQC, and HMBC experiments, the sugars were identified as glucose moieties. The cross-signals of the ^3J long-range couplings between H-1″ (glucose) → C-7 (δ_C 164.2 ppm) and H-1‴ (terminal glucose) → C-2″ (δ_C 82.8 ppm) confirmed the connectivity of the inner glucose to the apigenin at C-7 and of the terminal glucose at C-2″ of the inner glucose. The downfield shift of C-6″ and C-6‴ of both glucose moieties at δ_C 69.3 and 69.1 ppm, respectively, revealed the presence of two substituted groups. The substituents were identified as acetyl groups by the carbonyl ester signals at δ_C 170.2/170.1 ppm and HMBC cross-peaks of the hydroxymethylene groups of glucose to the carbonyl ester groups. The acid hydrolysis yielded glucose in the aqueous phase and apigenin in the organic phase. The above data,

Table I. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compound **3**.

C/H	δ_H	δ_C^a
2		162.5
3	6.9, s	104.6
4		181.9
5		161.3
6	6.47, d, $J = 2.1$ Hz	99.5
7		164.2
8	6.78, d, $J = 2.1$ Hz	94.8
9		156.8
10		105.3
1′		120.9
2′	7.93, d, $J = 8.4$ Hz	128.5
3′	6.94, d, $J = 8.7$ Hz	115.9
4′		161.1
5′	6.94, d, $J = 8.7$ Hz	115.9
6′	7.93, d, $J = 8.4$ Hz	128.5
1″	5.26, d, $J = 7.0$ Hz	97.9
2″	3.3, m	82.8
3″	3.53, dd, $J = 10$, 11 Hz	75.7
4″	3.73, m	69.7
5″	3.43, m	73.7
6″	4.36, brd, $J = 11.4$ Hz; 3.9, m	69.3
1‴	4.52, d, $J = 7.5$ Hz	103.1
2‴	3.27, m	73.1
3‴	3.54, m	75.9
4‴	3.76, t, $J = 9.5$ Hz	69.8
5‴	3.43, m	74.5
6‴	4.09, dd, $J = 4.8$, 12 Hz; 4.36, brd, $J = 11.4$ Hz	69.1
CO		170.1/170.2
CH_3	1.95, 2.0, s	20.5/20.4

[a] Assignments are based on HSQC and HMBC experiments.



1 $R^1 = R^4 = R^6 = H$; $R^2 = R^3 = OCH_3$; $R^5 = OH$
2 $R^1 = R^2 = R^5 = OCH_3$; $R^3 = OH$; $R^4 = R^6 = H$
4 $R^1 = R^2 = R^4 = R^6 = H$; $R^3 = Glc\text{-}Glc$; $R^5 = OH$
5 $R^1 = R^4 = H$; $R^2 = OCH_3$; $R^3 = R^5 = R^6 = OH$

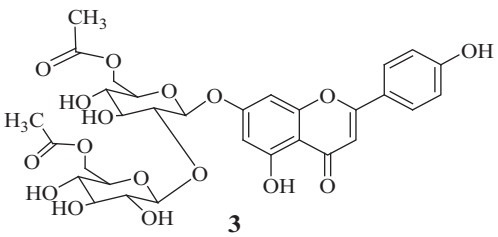


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Fig. 1. Chemical structures of the flavones isolated from *Chrysanthemum coronarium*: 5,7-dihydroxy-3,6,4′-trimethoxy-flavone (**1**), scutellarin-6,7-dimethyl ether (**2**), apigenin-7-*O*-[2″(6‴-*O*-*β*-D-acetylglucopyranosyl)]-6″-*O*-acetylglucopyranoside (**3**), apigenin-7-*O*-(2″-*O*-*β*-D-glucopyranosyl)-*β*-D-glucopyranoside (**4**), and 6-methoxy quercetin-7-*O*-*β*-D-glucopyranoside (**5**).

Table II. Lipid profiles in normal rats (negative control), hypercholesterolemic-hyperglycemic (H-H) rats (positive control), and in normal and H-H rats, respectively, treated with either of the three extracts from *C. coronarium* (500 mg/kg BW).

Group	TC [mg/dL]	TG [mg/dL]	HDL-c [mg/dL]	LDL-c [mg/dL]	Total lipids [mg/dL]
Negative control	89.45 ± 8.66^{a}	56.78 ± 4.34^{c}	31.21 ± 1.48^{c}	12.49 ± 9.24^{d}	1000.00 ± 52.60^{e}
PE extract	84.78 ± 6.15^{a}	64.00 ± 6.00^{c}	27.25 ± 4.77^{c}	22.07 ± 2.69^{d}	1105.26 ± 52.65^{e}
EA extract	88.23 ± 7.22^{a}	65.34 ± 10.08^{c}	27.74 ± 3.09^{c}	25.10 ± 5.10^{d}	1105.26 ± 105.25^{e}
M extract	90.34 ± 12.33^{a}	57.32 ± 5.88^{c}	32.01 ± 2.18^{c}	12.19 ± 7.34^{d}	1000.00 ± 50.12^{e}
Positive control (H-H rats)	225.00 ± 13.20^{f}	156.90 ± 10.76^{m}	4.95 ± 2.27^{h}	152.14 ± 11.56^{i}	1964.90 ± 109.55^{j}
H-H + PE extract	130.70 ± 7.98^{h}	69.75 ± 4.78^{c}	27.25 ± 2.27^{c}	31.96 ± 12.05^{e}	1070.20 ± 60.79^{e}
H-H + EA extract	133.67 ± 8.30^{h}	94.06 ± 5.67^{k}	30.21 ± 2.27^{c}	24.18 ± 7.99^{e}	1087.73 ± 80.40^{e}
H-H + M extract	130.00 ± 7.67^{h}	60.30 ± 5.12^{c}	34.19 ± 1.50^{c}	15.80 ± 3.30^{d}	1140.33 ± 80.40^{e}
H-H + Flufastatin	100.65 ± 9.24^{a}	58.21 ± 3.85^{c}	18.82 ± 5.62^{g}	48.20 ± 4.15^{m}	1410.06 ± 42.35^{h}
H-H + Glibenclamide	123.80 ± 7.22^{h}	100.21 ± 11.34^{k}	18.82 ± 5.62^{g}	58.23 ± 4.65^{n}	1421.00 ± 52.65^{h}

PE, petroleum ether; EA, ethyl acetate; M, methanol; H-H, hypercholesterolemia-hyperglycemia.
Data are means ± SD of ten rats in each group. Statistical analysis was carried out using the Costat computer program coupled with post-hoc LSD analysis. Unshared letters indicate significant differences at $P < 0.05$.

assign the structure of compound **3** as apigenin-7-*O*-[2″(6‴-*O*-*β*-D-acetylglucopyranosyl)]-6″-*O*-acetylglucopyranoside, which is a new compound.

The bioactivity assays data did not reveal significant changes in total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, and total lipids levels in normal rats fed with either of the three extracts of *C. coronarium*. As expected, in hypercholesterolemic-hyperglycemic rats, levels of total cholesterol, triglycerides, LDL-cholesterol, and total lipids were increased, while that of HDL-cholesterol was decreased as compared to the normal control group (Table II).

Administration of the three extracts to the rats counteracted development of the symptoms of hypercholesterolemia-hyperglycemia. The standard antihyperlipidemic drug flufastatin had a significant lowering effect on the lipid profile of the hypercholesterolemic-hyperglycemic rats as compared to the hypercholesterolemic-hyperglycemic rats, and caused a significant increase as compared to the normal control rats. The antihyperglycemic reference drug glibenclamide gave the same results (Table II). Furthermore, serum levels of the liver marker enzymes AST, ALT and ALP, total protein content, albumin and blood glucose levels did not change significantly in rats treated with the petroleum ether and methanol extracts (Table III). The ethyl acetate extract caused a significant increase in liver function enzymes as compared to the normal control, and a significant decrease as compared to the hypercholesterolemic-hyperglycemic rats. A significant increase in all liver marker enzymes and blood glucose levels was ob-

Table III. Enzymes of liver function, total protein content, albumin and glucose levels in the sera of normal (negative control), hypercholesterolemic-hyperglycemic (H-H), and extract-treated rats.

Group	AST [U/L]	ALT [U/L]	ALP [U/L]	Total protein [mg/dL]	Albumin [g/L]	Glucose [mg/dL]
Negative control	43.31 ± 1.02^{a}	64.86 ± 3.63^{b}	46.00 ± 1.80^{c}	2.99 ± 0.89^{f}	36.33 ± 3.33^{k}	106.23 ± 4.08^{f}
PE extract	43.98 ± 0.38^{a}	69.74 ± 3.94^{b}	45.75 ± 8.42^{c}	3.12 ± 0.19^{f}	36.90 ± 4.42^{k}	99.43 ± 2.99^{f}
EA extract	44.43 ± 0.58^{a}	67.08 ± 1.17^{b}	42.50 ± 2.64^{c}	2.65 ± 0.44^{f}	39.89 ± 4.26^{k}	$110.76 \pm 2.9\ 9^{f}$
M extract	42.31 ± 2.00^{a}	62.86 ± 1.63^{b}	44.00 ± 1.40^{c}	2.94 ± 0.56^{f}	40.23 ± 6.02^{k}	106.00 ± 4.30^{f}
Positive control (H-H rats)	59.97 ± 1.76^{h}	86.63 ± 1.53^{g}	94.00 ± 4.27^{i}	1.55 ± 0.05^{h}	23.06 ± 3.44^{l}	297.83 ± 2.99^{i}
H-H + PE extract	45.98 ± 1.01^{a}	66.64 ± 1.54^{b}	49.00 ± 2.17^{c}	2.23 ± 0.22^{f}	31.23 ± 2.64^{k}	96.03 ± 2.99^{f}
H-H + EA extract	52.42 ± 1.34^{h}	73.74 ± 1.17^{g}	59.50 ± 2.78^{i}	2.25 ± 0.15^{f}	33.54 ± 3.54^{k}	118.63 ± 1.93^{f}
H-H + M extract	46.64 ± 1.38^{a}	66.19 ± 0.44^{b}	46.33 ± 1.42^{c}	2.60 ± 0.89^{f}	34.39 ± 4.09^{k}	108.50 ± 5.19^{f}
H-H + Flufastatin	53.10 ± 2.04^{h}	72.18 ± 2.55^{g}	50.50 ± 2.19^{c}	2.45 ± 0.58^{f}	30.61 ± 3.82^{k}	189.63 ± 2.89^{j}
H-H + Glibenclamide	41.13 ± 1.34^{a}	63.17 ± 1.00^{b}	43.33 ± 1.12^{c}	3.00 ± 0.67^{f}	32.67 ± 4.88^{k}	$111.25 \pm 6.10^{f*}$

PE, petroleum ether; EA, ethyl acetate; M, methanol; H-H, hypercholesterolemia-hyperglycemia.
Data are means ± SD of ten rats in each group. Statistical analysis was carried out using the Costat computer program coupled with post-hoc LSD analysis. Unshared letters indicate significant differences at $P < 0.05$.

Table IV. Glutathione content, lipid peroxide and nitric oxide levels in rat livers of normal (negative control), hypercholesterolemic-hyperglycemic (H-H), and extract-treated rats.

Group	Glutathione [nmol/mg protein]	Malondialdehyde [nmol/mg protein]	Nitric oxide [μmol/mg protein]
Negative control	22.67 ± 5.70^{h}	19.30 ± 0.06^{c}	1.62 ± 0.04^{d}
PE extract	24.06 ± 6.12^{h}	19.10 ± 0.04^{c}	1.66 ± 0.07^{d}
EA extract	24.21 ± 2.26^{h}	19.03 ± 0.03^{c}	1.56 ± 0.06^{d}
M extract	23.34 ± 2.34^{h}	19.10 ± 0.01^{c}	1.44 ± 0.01^{d}
Positive control (H-H rats)	13.23 ± 1.46^{g}	33.98 ± 0.35^{f}	5.00 ± 0.55^{a}
H-H + PE extract	19.67 ± 1.12^{h}	21.23 ± 0.06^{c}	1.45 ± 0.03^{d}
H-H + EA extract	20.23 ± 2.43^{h}	21.08 ± 0.033^{c}	1.60 ± 0.04^{d}
H-H + M extract	22.09 ± 2.00^{h}	21.06 ± 0.02^{c}	1.44 ± 0.06^{d}
H-H + Flufastatin	19.20 ± 0.11^{h}	19.00 ± 0.01^{c}	1.34 ± 0.05^{d}
H-H + Glibenclamide	20.90 ± 0.15^{h}	19.92 ± 0.04^{c}	1.66 ± 0.04^{d}

PE, petroleum ether; EA, ethyl acetate; M, methanol; H-H, hypercholesterolemia-hyperglycemia.
Data are means ± SD of ten rats in each group. Statistical analysis was carried out using the Costat computer program coupled with post-hoc LSD analysis. Unshared letters indicate significant differences at $P < 0.05$.

Table V. Levels of proinflammatory mediators and C-reactive protein in the sera of normal (negative control), hypercholesterolemic-hyperglycemic (H-H), and extract-treated rats.

Group	IL-10 [pg/mL]	TNF-α [pg/mL]	CRP [μg/mL]
Negative control	63.59 ± 4.90^{a}	119.82 ± 4.10^{b}	6.05 ± 0.60^{c}
PE extract	66.52 ± 4.65^{a}	113.52 ± 7.90^{b}	5.744 ± 0.80^{c}
EA extract	64.03 ± 6.00^{a}	109.11 ± 7.90^{b}	5.67 ± 0.96^{c}
M extract	60.50 ± 7.00^{a}	110.02 ± 5.13^{b}	6.15 ± 0.50^{c}
Positive control (H-H rats)	94.58 ± 4.90^{d}	179.05 ± 4.90^{e}	11.5142 ± 4.90^{f}
H-H + PE extract	54.03 ± 5.91^{a}	149.59 ± 6.90^{b}	7.158 ± 6.60^{c}
H-H + EA extract	51.11 ± 5.00^{a}	149.38 ± 3.78^{b}	6.5788 ± 5.00^{c}
H-H + M extract	55.13 ± 4.88^{a}	143.59 ± 4.90^{b}	7.05 ± 2.80^{c}
H-H + Flufastatin	49.58 ± 5.97^{a}	173.00 ± 6.34^{b}	7.15 ± 3.19^{c}
H-H + Glibenclamide	49.50 ± 3.39^{a}	170.44 ± 5.60^{b}	7.16 ± 2.09^{c}

PE, petroleum ether; EA, ethyl acetate; M, methanol; H-H, hypercholesterolemia-hyperglycemia.
Data are means ± SD of ten rats in each group. Statistical analysis was carried out using the Costat computer program coupled with post-hoc LSD analysis. Unshared letters indicate significant differences at $P < 0.05$.

served in hypercholesterolemic-hyperglycemic rats, concomitant with a significant decrease in total protein content and albumin level. It should be pointed out that the *C. coronarium* extracts caused insignificant alterations in the levels of all parameters studied in the hypercholesterolemic-hyperglycemic rats, except for the ethyl acetate fraction which produced significant increases in the serum levels of the liver enzymes AST, ALT, and ALP as compared to the normal control group (Table III). Flufastatin did not normalize the liver enzyme activities AST and ALT and the blood sugar level, but rather normalized ALP, total protein content, and the albumin level. In contrast, glibenclamide normalized the levels of AST, ALT, ALP, total protein, albumin, and blood glucose.

With respect to oxidative stress, levels of the stress markers MDA, GSH, and NO were hardly affected by the three *C. coronarium* extracts. In hypercholesterolemic-hyperglycemic rats, GSH was

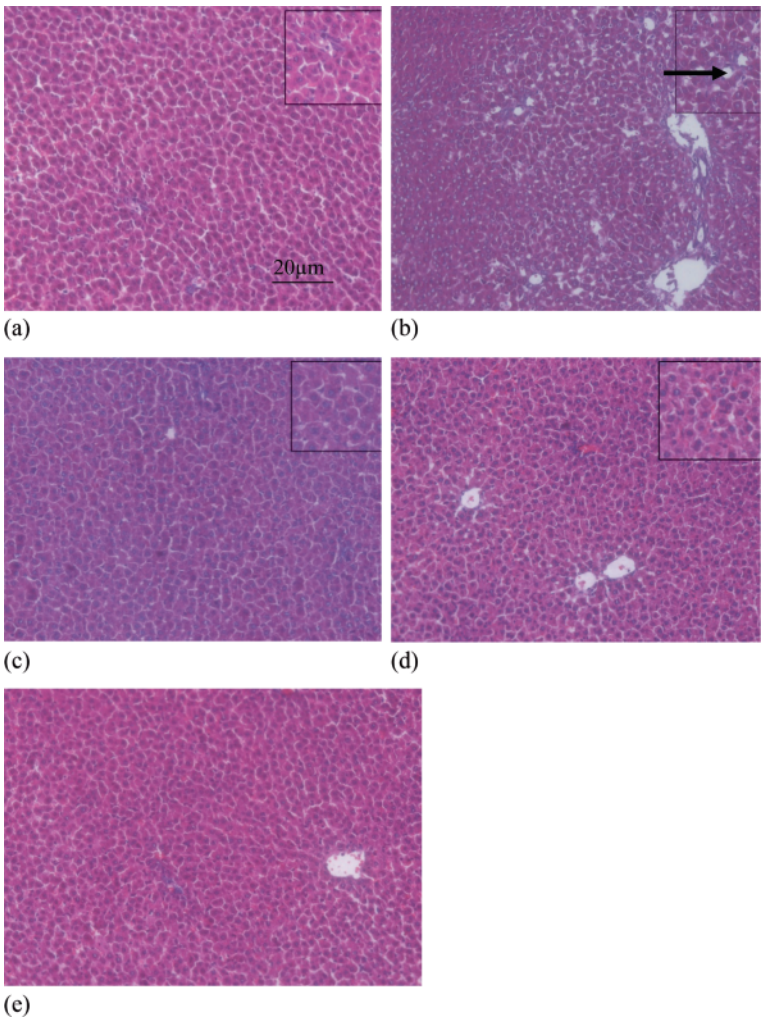

Fig. 2. Hematoxylin/eosin-stained liver sections. (a) Negative control rat, showing normal hepatic lobular architecture. (b) Hypercholesterolemic-hyperglycemic rat, showing vacuolar degeneration of hepatocytes (steatosis) (black arrow). (c) Hypercholesterolemic-hyperglycemic rat treated with petroleum ether extract, showing almost normal hepatic lobular architecture. (d) Hypercholesterolemic-diabetic rat treated with ethyl acetate extract, showing mild steatotic changes (vacuolar degeneration of hepatocytes). (e) Hypercholesterolemic-diabetic rat treated with methanol extract, showing almost normal hepatocytes. The inset is a 2-fold magnification.

almost depleted, while significant increases in liver MDA and NO levels were recorded (Table IV), and the three extracts were more effective in improving the oxidative status than the reference drugs flufastatin and glibenclamide.

Levels of the proinflammatory mediators TNF-α and CRP and the anti-inflammatory mediator IL-10 were not significantly affected by the three extracts compared to the normal control group (Table V). Significant increases in the levels of the proinflammatory mediators, and a decrease in that of the anti-inflammatory mediator, were observed in hypercholesterolemic-hyperglycemic rats, and mediator levels were largely normalized in these rats upon treatment with the three extracts, as well as with flufastatin and glibenclamide.

Histological examination revealed a normal hepatic lobular architecture in control rats, with hepatocytes being arranged in thin plates. In hypercholesterolemic-hyperglycemic animals, vacuolar degeneration of hepatocytes (steatosis) was observed, which was restored to almost normal architecture in rats treated with the three extracts (Figs. 2a–2e).

Discussion

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids. The phytochemical investigation of *C. coronarium* afforded two flavonoid aglycones from the ethyl acetate extract and three flavonoid glycosides from the methanol extract. The ethyl acetate and methanol extracts exhibited remarkable antihypercholesterolemic and antihyperglycemic activities.

Many studies have reported that flavonoids act as antihyperglycemic and antihypercholesterolemic compounds (Bhatia *et al*., 2011; Dharmarajan and Arumugam, 2012; Merina *et al*., 2011). They have been suggested to lower blood cholesterol levels by different mechanisms. Kim *et al*. (2008) suggested that flavonoids may indirectly enhance the phosphorylation of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase and thus diminish the endogenous cholesterol production. HMG-CoA reductase is the rate-limiting enzyme of the cholesterol biosynthesis (Boll *et al*., 1999). Inhibitory effects of flavonoids on the synthesis of cholesterol differ depending on the type and size of substituents and the position of their side chains in the flavonoid backbone structure, and they also depend on the type of cells that are treated.

The flavone apigenin has antihypercholesterolemic properties and has been reported to inhibit the production of cholesterol in hepatocyte and breast cancer cell cultures, respectively (Kim *et al.*, 2008). A quercetin glycoside was found to be a potent inhibitor of alkaline phosphatase activity (Salib *et al*., 2011). Park *et al.* (2002) demonstrated in rats that rutin, another glycosylated quercetin, promoted the excretion of fecal sterols, thereby decreasing the absorption of dietary cholesterol as well as lowering plasma and hepatic cholesterol concentrations. Also, it caused a reduction in plasma triglyceride levels of diet-induced hypercholesterolemic hamsters. Quercetin was found to inhibit hepatic cholesterol biosynthesis *in vitro* and to have a hypocholesterolemic effect *in vivo* (Auger *et al*., 2005). In the present study, two glycosylated apigenin compounds, **3** and **4**, and the glycosylated quercetin derivative **5** were obtained from the methanol extract.

The flavone glucoside isoorientin was reported to exhibit significant hypoglycemic and antihyperlipidemic activities at 15 mg/kg BW (Sezik *et al*., 2005). A number of flavones, such as 7,8-dihydroxy-flavone, luteolin, and hypolaetin-8-glucoside, significantly inhibited ALT release from the liver (Añón *et al*., 1992). A flavone derivative, which effectively elevated HDL levels and lowered triglyceride levels in hyperlipidemic rats, was likely to do so by affecting a series of genes, receptors, and proteins related to HDL (Guo *et al*., 2006).

Methoxylated flavones are known to have potent hypocholesterolemic properties (Kurowska and Manthey, 2004; Green *et al*., 2011). They were reported to reduce lipid levels *in vivo* and reduce the number of absorptive cells, villus length, and hepatic steatosis in hypercholesterolemic rats (Green *et al*., 2011). In the current study, three methoxylated flavones, *i. e.* **1**, **2**, and **5**, were isolated.

Excessive storage of fat, in the liver and the diabetic state, affects liver functions and increases the susceptibility to free radical attack. Treatment with the ethyl acetate and methanol extract, containing the flavone aglycones **1** and **2** and the glycosides **3**, **4**, and **5**, respectively, may prevent oxidative damage by detoxifying reactive oxygen species, thus reducing hyperlipidemia (Bhatia *et al.*, 2011), with a concomitant decrease in ALT, AST, and ALP enzyme activities. The decreased ALT and AST enzyme activities in serum, as a result of the treatment with the ethyl acetate and methanol extracts, might be ascribed to the ability of phenolic compounds to maintain membrane integrity

(Añón *et al.*, 1992) and thereby restrict the leakage of these enzymes (Li *et al.*, 2004).

The petroleum ether and methanol extracts may have reduced lipid peroxidation by acting as antioxidants and hence aiding the endogenous antioxidant enzymes involved in the scavenging/inactivation of the reactive oxygen species or redox metal ions before lipid peroxidation takes place.

A structure-activity relationship study has revealed that the presence of the 3′- and 4′-OH groups in the B-ring and a double bond between C-2 and C-3 in flavones are important factors for their recognition and binding by glycogen phosphorylase (GP). Flavones inhibited dephosphorylated GP in a noncompetitive manner and may bind to the enzyme's allosteric site. They could have the potential to contribute to the protection or improvement of control of diabetes type 2 (Afifi-Yazar *et al.*, 2011).

The hypolipidemic and antidiabetic activities of these flavones are likely due to their radical-scavenging, antioxidant, and antihepatotoxic properties (Li *et al.*, 2004).

The results of this study demonstrate that such plant extracts could have a potential as drugs or in functional food in the treatment of hypercholesterolemia and hyperglycemia.

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