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Full Length Research Paper

New acylated flavonol glycoside from *Ceratonia siliqua L.* seeds

A. Gohar*, S. R. Gedara and H. N. Baraka

Pharmacognosy Department, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.

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New flavonol glycoside, 4`-*p*-hydroxybenzoylisorhamnetin-3-O-α-L-rhamnopyranoside named ceratoside (5), together with the known kaempferol-3-O-α-L-rhamnopyranoside (afzelin) (3), quercetin-3-O-α-L-arabinofuranoside (auriculain) (4) quercetin-3-O-α-L-rhamnopyranoside (6), β-sitosterol (1) and β-sitosterol-3-O-β-D-glucoside (2) were isolated for the first time from *Ceratonia siliqua* L. seeds. The identity of these compounds was established through UV, IR, MS and NMR spectral data. The antioxidant activity of the organic solvent fractions together with some isolated compounds were carried out and showed significant results.

Key words: *Ceratonia siliqua*, carob, flavonol glycosides, antioxidants

INTRODUCTION

Ceratonia siliqua L. Fabaceae, the carob tree, is a Mediteranean plant. Components of the carob pod have been used as a sweetener, as a food ingredient in the production of confectionery, beverages, bread or pasta (Marakis, 1996; Avallone et al., 1997). Many recent activities are reported for carob pods such as antihyperglycemic (Jung-In et al., 2005), antioxidant (Cantalejo, 2001; Fouly et al., 2004; Makris and Kefalas, 2004), immunomodulating (Cantalejo, 2001) and antiproliferative on mouse hepatocellular carcinoma cell line (Corsi, et al., 2002).

The nutritive value of the carob pods was attributed to its high levels of carbohydrates (45%), appreciable amounts of protein (3%), and low levels of fat (0.6%) (Avallone et al., 1997; Ayaz et al., 2007). The green carob pods contain a major leucoanthocyanins in highly polymerised leucodelphinidins and proanthocyanidin together with hydrolysable tannins, crystalline galloyl glucose compounds. In the ripe pods as well as the leaf and the wood extracts, gallic acid, epigallocatechin-3-gallate and epicatechin-3-gallate were detected. A total of 24 polyphenol compounds were identified with a yield of 3.94 g/kg (dry weight). Gallotannins 1,6-di-, 1, 2,6-tri- and 1,2,3,6-tetra-O-galloyl-β-D-glucose were isolated from the Carob fibre. The profile was dominated by gallic acid in various forms: free gallic acid (42% of polyphenols by weight), gallotannins (29%), methyl gallate (1%) and catechin while simple phenols, mainly cinnamic acid, made up about 2% of the total (Avallone et al., 1997; Corsi et al., 2002; Balban, 2004; Joslyn et al., 2006; Ayaz et al., 2007). The butanol soluble fraction of the methanol extract of the carob cotyledons contain five C-glycosylflavones including schaftoside, isoschaftoside, neoschaftoside, isoschaftoside-4'-O-glucoside and schaftoside-4`-O-glucoside (Batista and Gomes, 1993). Flavonoids represented 26% of the polyphenols and the major components were identified as the glycosides myricetin and quercetin-3-O-α-L-rhamnoside (Joslyn et al., 2006; Vaya and Mahmood, 2006). The antioxidant activity of the carob pod is attributed to the presence of such phenolic compounds (Owen et al., 2003; Ayaz et al., 2007).

To date it is still unknown whether other compounds may be responsible for this activity. The aim of this study was therefore to investigate in addition to the antioxidant activity of the seeds, its chemical constituents.

MATERIALS AND METHODS

General

Melting points were measured by Hot-Stage melting point microscope (Sybron, USA). UV spectra were measured in spectroscopic methanol using Beckmann DU-7 Spectrophotometer. IR (KBr-discs) spectra were measured by Nicolet MX-1 FT-IR spectrometer. ^{1}H- and ^{13}C-NMR experiments were run in DMSO-d_6 at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR on JOEL TNM- IR (KBr-discs) spectra were measured by Nicolet MX-1 FT-IR spectrometer. ^{1}H- and ^{13}C-NMR experiments were run in DMSO-d_6 at 400

*Corresponding author. E-mail: gohar1952@yahoo.com.

MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR on JOEL TNM-LA, FT-NMR system, Japan, using TMS as internal standard. Chromatographic separation was performed using silica gel 60 for column (E-Merck, Germany). TLC was performed on silica gel GF_{254} (E-Merk, Germany, 0.2 mm thickness).

Chemicals

Authentic sample, β-sitosterol, β-sitosterol-3-O-glucoside, kaempferol and quercetin were obtained from isolated and identified materials in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University. Authentic sugars, glucose, galactose, rhamnose, xylose and arabinose were obtained from El-Nasr Pharmaceutical and Chemical Company, Egypt. Shift reagents viz., sodium methoxide, aluminum chloride and HCl were prepared according to the standard methods (Mabry et al., 1970). Chromatograms were visualized under UV light (Ultra-Violet Lamp 254 and 366 nm, Desaga, Germany) and by spraying developed plates with 1% vanillin-H_2SO_4 for β-sitosterol, β-sitosterol-3-O-glucoside, 5% aluminum chloride and alcoholic potassium hydroxide for flavonoids and aniline hydrogen phthalate for sugars. For TLC analysis, the following solvent systems were used: pet. ether- ether 6:4 (system I), chloroform - methanol 9.5:0.5 (system II), chloroform - methanol 9:1 (system III) and chloroform - methanol 8:2 (system IV).

Plant material, extraction and chromatographic isolation

The *Ceratonia siliqua* L. pods were purchased from a local market in El Mansoura, Egypt in 2006. The seeds separated from the pods and grounded in a suitable mill.

Extraction and isolation

Dried powdered seeds (75 g) were extracted with cold methanol (3 L) and the extract was evaporated to dryness in a rotary evaporator at 40°C. The residue (22 g) was suspended in distilled water and extracted with chloroform followed by ethyl acetate.

The chloroform extract (9.5 g) was chromatographed on silica gel column. Elution was started with hexane, hexane- ethyl acetate gradient. The eluted fractions (250 ml each) were collected, concentrated and screened by TLC. Similar fractions pooled together. Fractions 14 - 16 eluted with hexane - ethyl acetate (95:5) were crystallized from methanol to afford compound **1** (30 mg). Fractions 32 - 34 eluted with hexane- ethyl acetate (50:50) gave compound **2** (20 mg).

The ethyl acetate soluble fraction (5 g) was chromatographed on silica gel column, using ethyl acetate followed by ethyl acetate- methanol gradient. The eluted fractions (250 ml each) were collected, concentrated and screened by TLC. Similar fractions pooled together. Fractions 11 – 14, eluted with 10% ethyl acetate methanol mixture, contained two flavonoid spots and so, fractions 15 – 18 contained another two-flavonoid spots. Rechromatography of the first fraction on silica gel column and elution with CH_2Cl_2 – CH_3OH mixture (gradient), 100 ml fractions were collected, afforded compound **3** (fractions 5 – 6, eluted with 10% mixture, 15 mg) and compound **4** (fractions 8 – 13, eluted with 10% mixture, 16 mg). Rechromatography of the second fraction on silica gel column and elution with CH_2Cl_2 – CH_3OH mixture (gradient), 100 ml fractions were collected, afforded compound **5** (fractions 6 – 9, eluted with 10% mixture, 28 mg) and compound **6** (fractions 12- 14, eluted with 20% mixture, 54 mg).

Acid hydrolysis

About 2 mg of the tested substance dissolved in 5% w/v hydrochloric acid, mixed with an equal volume of ethanol and refluxed on a steam bath for two hours. The solvent distilled off and the hydrolysate shacked with ether. The ether extract was dried over anhydrous sodium sulphate and the solvent was distilled off. The residue was dissolved in methanol and kept for identification of the aglycone moiety. The acidic mother liquor containing the sugar was neutralized with silver oxide and filtered. The precipitate was washed several times with few ml of water. The filtrate and the washing were combined and evaporated to dryness. The residue dissolved in one ml of pyridine and used for identification of sugar moiety (Kikuchi and Matsuda, 1996). Sugars were identified though TLC and GLC

GLC Analysis of the trimethylsilyl ether derivatives of the sugars

It was performed on Pachard Gas Chromatograph Model 427 equipped with dual flame ionization detector. The stationary phase was SE-30 (10%) on Chromosorb W, 80 – 100 mesh, packed in a coiled glass column 150 cm long and 4 mm internal diameter. The flow rates of H_2, air and N_2 were 40, 300 and 35 ml/min respectively. The temperature of the column, injector port and the detector were 205, 250 and 260°C respectively in isothermal technique. The pyridine solution of the sugar was mixed with 0.2 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane, shaken for 30 s. and allowed to stand for 5 min.

Measuring the antioxidant activity

ABTS [2, 2-azinobis(3-ethylbenzothiazoline 6-sulphonate] radical cation ($ABTS^{+}$) scavenging activity was measured according to the method described by Re et al., 1999. ABTS was dissolved in water to a 7 mM concentration and the ABTS radical cation was produced by adding potassium persulfate to a final concentration of 2.45 mM. The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.50 ± 0.02. Different plant extracts, total extract and compound C-6 and C-5 were prepared by dissolving 1 mg each in 0.5 ml methanol and 0.5 ml phosphate buffer. To determine the scavenging activity, 1 ml of diluted $ABTS^{+}$ solution was added to 50 µl of plant extract or tested isolated compounds (or water for the control), and the absorbance at 734 nm was measured 6 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

Inhibition percentage (%IP) = [*A*c – *A*s /*A*c] × 100

Where *A*c and *A*s are the absorbance of the control and of the tested samples respectively.

Identification of the isolated compounds

β-Sitosterol (1): White needles (CH_3OH). - M.p. 135-137°C. - R_f = 0.59 (system I). - IR: ν_{max} = 3350, 2940, 1530, 1460, 1380, 1360 cm^{-1}.

3. 7. 2. β-Sitosterol-3-O-glucoside (2): White needles (CH_3OH / $CHCl_3$ mixture). - M.p. 294-296°C. - R_f = 0.35 (system II). - IR: ν_{max} = 3420, 2940, 1460, 1380, 1080, 1030 cm^{-1}.

Kaempferol-3-O-α-L-rhamnopyranoside (3): Yellow needles (CH_3OH). – M. p. 227 - 229°C. - R_f = 0.27 (system III). – UV λ_{max} CH_3OH 343, 282^{sh}, 265; + CH_3ONa 390, 320^{sh}, 272; + $AlCl_3$ 392, 346, 304^{sh}, 273; $AlCl_3$ + HCl 393, 344, 302^{sh}, 274; NaOAc 370, 304^{sh}, 272; NaOAc + H_3BO_3 365, 284^{sh}, 345 nm. - ^{1}H- and ^{13}C-NMR (Table 1).

Table 1. NMR Data of Compounds **3** - **6** (DMSO-d_6).

Position #	3		4		5		6	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	-	157.8	-	156.8	-	157.9	-	157.9
3	-	134.0	-	134.0	-	134.0	-	134.9
4	-	177.6	-	177.6	-	177.5	-	178.3
5	-	161.9	-	161.9	-	162.0	-	161.8
6	6.19 s	98.5	6.19 s	98.5	6.21 s	98.5	6.21 br. s	98.4
7	-	164.1	-	164.2	-	164.5	-	164.5
8	6.39 s	93.4	6.39 s	93.4	6.43 s	93.4	6.33 br. s	93.4
9	-	156.8	-	156.8	-	157.3	-	157.1
10	-	104.1	-	104.3	-	104.5	-	104.6
1`	-	121.7	-	121.8	-	122.1	-	121.6
2`	7.76 (d, *J* = 8.4)	130.2	7.48 m	115.1	7.44 m	112.0	7.32 m	115.6
3`	6.91 (d, *J* = 8.4)	115.4	-	145.1	-	150.2	-	145.0
4`	-	161.5	-	145.1	-	148.3	-	148.4
5`	6.91 (d, *J* = 8.4)	115.4	6.88 (d,*J* = 8.0)	115.2	6.94 (d,*J* = 6.2)	115.1	6.91 (d, *J* = 8.4)	115.0
6`	7.76 (d, *J* = 8.4)	130.2	7.48 m	121.6	7.39 m	123.5	7.32 m	121.5
1``	5.36 br. s.	102.0	5.46 br.s	108.6	5.37 br.s.	102.0	5.30 br. s	102.2
2``	4.22 dd (*J* =1.83, 3.34)	70.5	4.32 d (*J*= 2.1)	81.9	4.21 dd (*J* =1.8, 3.4)	70.5	4.22 m	70.8
3``	3.33- 3.70 m	70.7	3.49-3.86 m	76.4	3.33- 3.70 m	70.5	3.20 - 3.76 m	70.7
4``	3.33- 3.70 m	70.8	3.49-3.86 m	86.0	3.33- 3.70 m	70.7	3.20 - 3.76 m	70.7
5``	3.33- 3.70 m	69.9	3.49-3.86 m	60.6	3.33- 3.70 m	70.8	3.20 - 3.76 m	70.6
6``	0.91 d (*J* = 5.4)	16.4	-	-	0.91 d (*J* = 5.4)	16.3	0.93 d (*J* =5.4)	16.4
OCH_3	-	-	-	-	3.94 s	55.2	-	-
1```	-	-	-	-	-	122.2	-	-
2```	-	-	-	-	7.76 (d,*J* = 8.5)	130.6	-	-
3```	-	-	-	-	6.93 (d,*J* = 8.5)	115.2	-	-
4```	-	-	-	-	-	160.0	-	-
5```	-	-	-	-	6.93 (d,*J* = 8.5)	115.2	-	-
6```	-	-	-	-	7.76 (d,*J* = 8.5)	130.6	-	-
C=O	-	-	-	-	-	165.0	-	-

Quercetin-3-O–α–L-arabinofuranoside (4): Yellow needles (CH_3OH). – M. p. 252 – 253°C. - R_f = 0.24 (system III). - UV λ_{max}: CH_3OH 360, 299^{sh}, 262; + CH_3ONa 399, 330, 272; + $AlCl_3$ 422, 332^{sh}, 305^{sh}, 275; $AlCl_3$ + HCl 395, 358^{sh}, 304^{sh}, 271; NaOAc 385, 324, 274; NaOAc + H_3BO_3 374, 261 nm. ^{1}H- and ^{13}C-NMR (Table 1).

4`-p-hydroxylbenzoylisorhamnetin-3-O-α-L-rhamnpyranoside (5): Yellow amorphous powder (CH_3OH). – R_f = 0.85 (system IV). – Positive FAB-MS: *m/z* = 583 $[M+1]^+$, 414 [M - OCH_3 - O – $C_7H_5O_2$ (*p*-hydroxybenzoyl)], 404 [(M – Rh – OCH_3], 299 [(M – Rh – *p*-hydroxybenzoic acid]$^+$ and 268 [(M – Rh – *p*-hydroxybenzoic acid- OCH_3]$^+$, 152, 154, 147, 137. - UV λ_{max}: CH_3OH

Table 2. The antioxidant activity of the tested materials.

Material	Absorption	% IP
Control	0.50	---
Vitamin C	0.082	83.6
Methanolic	0.099	80.2
Chloroform	0.176	64.8
Ethyl acetate	0.16	68.0
Butanol	0.145	71
Compound-6	0.089	82.2
Compound-5	0.085	83

348, 265; + CH_3ONa 396, 327^{sh}, 272; + $AlCl_3$ 400, 334^{sh,} 300$^{sh'}$ 274; $AlCl_3$ + HCl 393, 351^{sh}, 300 ^{sh}, 273; NaOAc 371, 364^{sh}, 308^{sh}, 272; NaOAc + H_3BO_3 350, 324, 275 nm. - ^{1}H- and ^{13}C-NMR (Table 1).

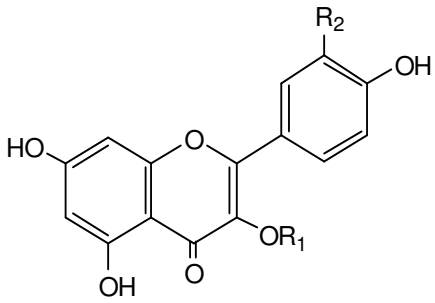
Quercetin-3-O-α-L-rhamnopyranoside (6): Yellow needles (CH_3OH). – M. p. 191 - 193°C. - R_f = 0.73 (system IV). - UV λ_{max}: CH_3OH 354, 300^{sh}, 263; + CH_3ONa 395, 325^{sh}, 272; + $AlCl_3$ 433, 329^{sh}, 301^{sh}, 275; $AlCl_3$ + HCl 403, 352^{sh}, 301^{sh}, 272; NaOAc 372, 320^{sh}, 272; NaOAc + H_3BO_3 367, 260 nm. - ^{1}H- and ^{13}C-NMR (Table 1).

RESULTS AND DISCUSSIONS

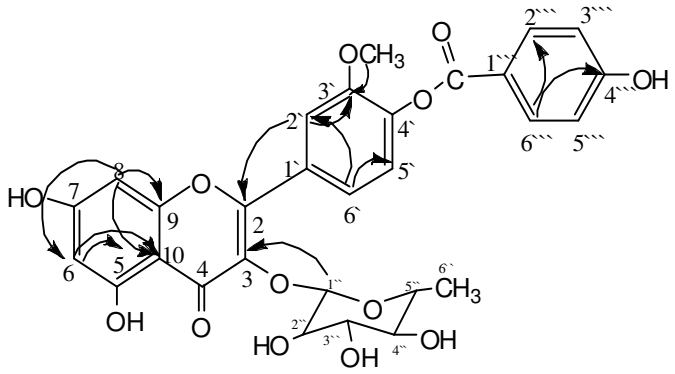
Column chromatography of the chloroform fraction afforded β-sitosterol (**1**) and β-sitosterol-3-O-glucopyranoside (**2**), both compounds were identified by comparison of their mp. and IR as well as co-chromatography with authentic samples.

Column chromatography of the ethyl acetate fraction afforded Compounds **3** – **6**. They gave yellow colour with $AlCl_3$ spray and yellowish green fluorescence in UV light 366 nm. In addition, they gave positive Molisch's test. This proved that these compounds could be flavone or flavonol glycosides. The compounds **3** and **6** was proved to be kaempferol-3-O-α-L-rhamnopyranoside and quercetin-3-O-α-L-rhamnopyranoside respectively by comparing their UV and ^{1}H and ^{13}C-NMR data (Table 1) with those reported for similar compounds (Mabry et al., 1970; Harborne and Mabry 1982; Agrawal and Bansal, 1989), both compounds were not previously reported in the seeds of the titled plant.

Analysis of ^{1}H and ^{13}C-NMR data of **4** (Table 1) revealed that the aromatic signals are close to those reported for quercetin moiety (Harborne and Mabry, 1982; Agrawal and Bansal 1989). The sugar signals in ^{13}C-NMR, δ 108.6, 81.9, 76.4, 86.0 and 60.6 are comparable with those reported for α-L-arabinofuranose (Agrawal and Bansal, 1989). ^{1}H-NMR showed the presence of anomeric proton signal at δ 5.46 (1H, br. *s*) indicated the presence of α- linked sugar. The sugar moiety was proved to be acylated at C-3 of the aglycone as deduced from the correlation between the anomeric proton at δ 5.46 and the C-3 at δ 134.0 in HMBC. This was further confirmed from ^{13}C-NMR by the downfield shift of C-2 by ca +9.2



	R_1	R_2
Compound 3	Rhamnose	H
Compound 4	Arabinose	OH
Compound 6	Rhamnose	OH



Compound 5

Figure 1. Isolated flavonoids and HMBC correlation of compound 5.

comparing with that of quercetin (Harborne and Mabry, 1982). Compound **4** gave UV absorption data identical with those reported for quercetin-3-O-α-L-arabinofuranoside (Mabry et al., 1970; Harborne and Mabry, 1982). Therefore, compound **4** could be quercetin-3-O-α-L-arabinofuranoside (auriculain), which is not previously reported in the titled plant.

The ^{1}H- and ^{13}C-NMR of Compound **5** (Figure 1) revealed beside the signals assigned to isorhamnetin (Mabry et al., 1970; Harborne and Mabry, 1982), two doublets at δ 6.93 (2H, d, *J* = 8.5 Hz) and δ 7.76 (2H,d, *J* = 8.5 Hz) correlated in HMQC to the carbon signals at δ 115.2 and δ 130.6 respectively. These signals together with the CO signal at δ 165.0 and the signal at δ 160 (C4''') were consistent with those of *p*- hydroxyl benzoic acid. The proton signal at δ 3.94 (3H, *s,* O-CH_3) correlated in HMQC to the carbon signal at δ 55.2 assigned for an aromatic OCH_3 at C-3' as indicated by HMBC correlation of the OCH_3 protons and the C-3' resonated at δ 150.2. The position of the *p*-hydroxy benzoyl moiety was proved to

be acylated at C-4' as suggested by the presence of free 5- and 7-OH groups as deduced from UV spectrum. Moreover, 3-OH was glycosidated as deduced from HMBC correlation between the anomeric proton at δ 5.37 (br. *s*., H-1``) which showed a cross peak with C-3 at δ 134.0. In addition, the upfield shift of H-2` proton by ca – 0.53 ppm relative to the unacylated isorhamnetin-3-O-β-D-xylosyl (1→ 6) β-D- glucoside due to the effect of *p*- hydroxy benzoic acid (Tomas-Lorente et al., 1992).

The singlets at δ 5.37 (1H) and the doublet at δ 0.91 (3H, d, *J* = 5.4 Hz) were assigned to the anomeric (H-1``) proton and H-6`` of α- rhamnose moiety respectively. The presence of O-CH_3, *p*- hydroxybenzoic acid and the rhamnose moieties were confirmed from FAB-MS as it showed the presence of characteristic fragments at 414 [M - O-CH_3 – O-*p*-hydroxybenzoyl], 404 [(M+1) – Rh – O-CH_3] and 268 (M – Rh – *p*-hydroxybenzoic acid - O-CH_3).

These data indicated that **5** could be 4`-*p*-hydroxy benzoylisorhamnetin-3-O-α-L-rhamnopyranoside named ceratoside. This is the first report for the isolation of this compound from any natural source.

Testing the antioxidant activity of the total methanol extract, chloroform, ethyl acetate and the butanol fractions together with compound **5** and **6** showed significant results as shown in Table 2.

The antioxidant activity of the total extract**,** compound **5** and **6** (Table 2) was comparable to that of most common antioxidant ascorbic acid. The higher value of the total methanol extract could be attributed to the fact that the tannoid materials, soluble in methanol, are considered potent natural antioxidant (Makris and Kefalas, 2004). Due to health benefits, natural antioxidants have been extensively employed in recent years (Yen et al., 2003). Therefore, methanol extract of the carob seeds could be a potential source for natural antioxidants

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