FLAVONE C-GLYCOSIDES AND CUCURBITACIN GLYCOSIDES FROM *CITRULLUS COLOCYNTHIS*

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ABSTRACT

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae) is an Iranian medicinal plant that has traditionally been used as an abortifacient and to treat constipation, oedema, bacterial infections, cancer and diabetes. As part of our on-going studies on Iranian medicinal plants, thorough phytochemical investigation was carried out on this plant. The reversed-phase preparative HPLC was employed to isolate compounds from the butanol fraction of the hydro-methanolic (70%) extract of the fruits of the locally grown *C. colocynthis*. Structures of the isolated compounds [**1**-**5**] were elucidated by spectroscopic means. The antioxidant property of the flavonoids **1**-**3** was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Three flavone glucosides, isosaponarin [**1**], isovitexin [**2**] and isoorientin 3'-*O*-methyl ether [**3**] and two cucurbitacin glucosides, 2-*O*-β-D-glucopyranosylcucurbitacin I [**4**) and 2-*O*-β-D-glucopyranosylcucurbitacin L [**5**] were isolated and identified. Flavonoids **1**-**3** showed significant antioxidant properties. Since reactive oxygen species are important contributors to tissue injury, inflammation, cancer and many other ailments, the antioxidant properties of **1**-**3** probably contribute, at least to some extent, to the pharmacological and traditional medicinal uses of the *C. colosynthis*.

Keywords: *Citrullus colocynthis,* Cucurbitaceae, Isosaponarin, Isovitexin , Isoorientin 3'-*O*-methyl ether, Cucurbitacin I, Cucurbitacin L, DPPH.

INTRODUCTION

Citrullus colocynthis (L.) Schrad. (Cucurbitaceae), commonly known as 'bitter apple', 'colosynth', vine-of-Sodom' 'tumba' or 'wild gourd', is a tropical plant that grows abundantly in the south of Iran, and widely in other parts of the world (1). In the traditional medicine of Iran, this plant has been used to treat constipation, oedema, bacterial infections, cancer and diabetes, and as an abortifacient (2). The ethnobotanical uses of this plant include its use as an abortifacient, cathartic, purgative and vermifuse, and for the treatment of fever, cancer, amenorrhea, jaundice, leukemia, rheumatism and tumour (3). In Syria, it has also been used as an insect repellant (3). A number of plant secondary metabolites including cucurbitacins, flavonoids, caffeic acid derivatives and terpenoids have previously been reported from this plant (4-10). As part of our on-going studies on Iranian medicinal plants (11-15), the isolation and identification of the chemical constituents, namely, isoscoparin [**1**], isovitexin [**2**], isoorientin 3'-*O*-methyl ether [**3**], 2-*O*- β -D-glucopyranosyl-cucurbitacin I [**4**], 2-*O*- β -D-glucopyranosyl-cucurbitacin L [**5**] of the endemic Iranian species *C. colocynthis*, and the antioxidant properties of the flavonoids [**1**-**3**] are described.

MATERIALS AND METHODS

General procedures

NMR spectra were recorded on a Bruker NMR Spectrometer (200 MHz for ^{1}H and 50 MHz for ^{13}C NMR) using DMSO-d_6 for the compounds **1** – **3**, and those of **4** and **5** were obtained (500 MHz for ^{1}H and 125 MHz for ^{13}C NMR) on Bruker AVANCE 500 spectrometer using CD_3OD as solvent. The residual solvent peaks were used as internal standards. ESIMS analysis was performed on Finnigan MAT95 spectrometer.

Plant material

Fruits of *Citrullus colocynthis* were collected

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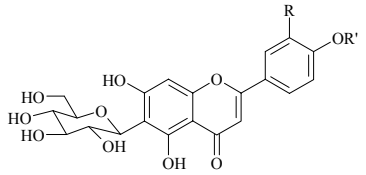
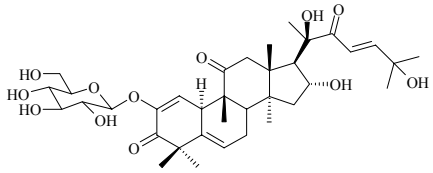


Compound	R	R’
1	H	Glucosyl
2	H	H
3	OMe	H



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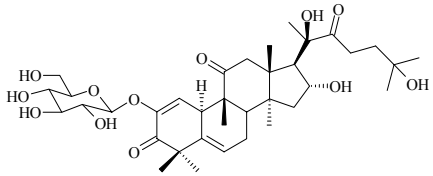

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Figure 1. Structure of compounds [**1-5**] isolated from *C. colocynthis*

during August and September, 2003, from Ahvaz in Khozestan province (Iran) and the identity was confirmed by anatomical examination in comparison with the herbarium specimen retained in the School of Pharmacy, Tabriz University of Medical Sciences, Iran. A voucher specimen (TUM-ADA 87) for this collection has been deposited in the herbarium of the Faculty of Pharmacy, Tabriz University of Medical Science, and also in the herbarium of the Plant and Soil Science Department, University of Aberdeen, Scotland (ABD).

Extraction, isolation and structure elucidation

The pulp was separated from the seeds. The ground pulp (500 g) was macerated in water/methanol (30/70) for 24 hours (three times), and the hydro-methanolic solution was subsequently concentrated by evaporation up to 1/3 of the initial volume. The extract was fractioned by the following solvents with increasing polarity: petroleum ether, chloroform, ethyl acetate, butanol and water fractions. The fractions were concentrated to dryness by rotary evaporator. The butanol fraction (2 g) was subjected to Sep-Pack (ODS) fractionation using a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20 and 100:0). The preparative reversed-phase HPLC analysis (Shim-Pak ODS column 20 µm, 250 mm × 20 mm; mobile phase: 0 to 50 min gradient 25 to 45% MeOH in water; flow-rate: 20 mL/min, detection at 267 nm) of the 40% methanolic Sep-Pack fraction (۲۵۹ mg) resulted in the isolation of compounds **1** (6.4 mg, t_R = 11.3 min), **2** (4.7 mg, t_R = 27.3 min), **3** (7.2 mg, t_R = 29.2 min), **4** (49.8 mg, t_R = 45.8 min) and **5** (35.3 mg, t_R = 51.3 min). The structures of the isolated compounds [**1-5**] were determined by spectroscopic means.

Isosaponarin [**1**]. Gum, UV λ_{max} (MeOH): Table 1; ESIMS *m/z* 595 $[M+H]^+$; ^{1}H NMR (200 MHz, DMSO-d_6): Table 2; ^{13}C NMR (50.0 MHz, DMSO-d_6): Table 2.

Isovitexin [**2**]. Gum, UV λ_{max} (MeOH): Table 1; ESIMS *m/z* 433 $[M+H]^+$; ^{1}H NMR (200 MHz, DMSO-d_6): Table 2; ^{13}C NMR (50.0 MHz, DMSO-d_6): Table 2.

Isoorientin 3’-*O*-methyl ether [**3**]. Gum, UV λ_{max} (MeOH): Table 1; ESIMS *m/z* 463 $[M+H]^+$; ^{1}H NMR (200 MHz, DMSO-d_6): Table 2; ^{13}C NMR (50.0 MHz, DMSO-d_6): Table 2.

2-*O*-β-D-Glucopyranosylcucurbitacin I [**4**]. Gum, ESIMS *m/z* 677 $[M+H]^+$; ^{1}H NMR (500 MHz, CD_3OD): Table 3; ^{13}C NMR (125 MHz, CD_3OD): Table 3.

2-*O*-β-D-Glucopyranosylcucurbitacin L (bryoamaride) [**5**]. Gum, ESIMS *m/z* 679 $[M+H]^+$; ^{1}H NMR (500 MHz, CD_3OD): Table 3; ^{13}C NMR (125 MHz, CD_3OD): Table 3.

Antioxidant assay

2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), molecular formula $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks. The method used by Takao *et al.* (16) was adopted with suitable modifications (17). A solution of DPPH (0.08 mg/mL) in MeOH was used. The compounds **1-3** were dissolved in MeOH to obtain a concentration of 1 x 10^{-1} mg/mL. Dilutions were made to obtained concentrations of $5.00x10^{-2}$, $2.5\ x10^{-2}$, $1.25x10^{-2}$, $6.25x10^{-3}$, $3.13x10^{-3}$, $1.56x10^{-3}$ mg/mL. Diluted solutions (5 mL each) were mixed with DPPH (5 mL) and allowed half hour for any reaction to occur. The UV absorbance was recorded at 517 nm. The experiment was performed in duplicate and average absorption was noted for each concentration. Data were processed using EXCEL and the concentration

that caused a 50% reduction in absorbance (RC_{50}) was calculated. The same procedure was followed for the standard (quercetin).

RESULTS AND DISCUSSION

The reversed-phase preparative HPLC analysis of the butanol fraction of the methanol extract of *C. colocynthis* fruits afforded three flavonoid glycosides, isosaponarin [**1**], isovitexin [**2**] and isoorientin 3'-*O*-methyl ether [**3**] and two cucurbitacin glucosides, 2-*O*-β-D-glucopyranosylcucurbitacin I [**4**] and 2-*O*-β-D-glucopyranosylcucurbitacin L [**5**]. While the chemical structures of **1**-**3** were elucidated by extensive UV analyses (Table 1), ESIMS, and NMR spectroscopic analyses (Table 2), and also by comparing experimental data with respective literature data (4, 18-25), the identity of cucurbitacins **4** and **5** was confirmed unambiguously by a series of 1D and 2D NMR analyses, including ^{13}C DEPT 135, ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY, ^{1}H-^{13}C HMBC and ^{1}H-^{13}C HMQC. The ^{1}H and ^{13}C NMR data of **5** and **6** were in good agreement with the published data (8).

Compounds **1**-**3** displayed characteristic UV absorption maxima for a flavone skeleton (Table 1) (26). The ^{1}H and ^{13}C NMR data (Table 2) for these compounds also confirmed the presence of a flavone nucleus in these molecules (26). The results of the UV analyses (Table 1) on these compounds using various shift reagents were in good agreement with the substitution patterns depicted in the structures of **1**-**3** (26). An ESIMS mass spectrum of **1** revealed $[M+H]^+$ (positive ion mode) ion peak at *m/z* 595, suggesting M_r = 594 and solving for $C_{27}H_{30}O_{15}$. In the ^{1}H and ^{13}C NMR spectra (Table 2), there were signals for a 6-substituted 5,7, 4'-trihydroxy flavone nucleus and also for two glucose moieties. The ^{1}H and ^{13}C chemical shift values were comparable to the published data for the C-glucoside, isosaponarin or isovitexin 4'-*O*-β-D-glucopyranoside [**1**] (18-22). While isovitexin derivatives have previously been isolated from *C. colocynthis*, to our knowledge, this is the first report on the occurrence of isosaponarin [**1**] in this plant. However, compound **1** has previsouly been reported from a number of other genera including *Gentiana, Gypsophila*, *Psoralea* and *Vaccaria* (10).

An ESIMS mass spectrum of **2** displayed the $[M+H]^+$ (positive ion mode) ion peak at *m/z* 433, suggesting M_r = 432 and solving for $C_{21}H_{20}O_{10}$. The ^{1}H and ^{13}C NMR spectra (Table 2) displayed the signals similar to those of **1**, excepting that there were signals for only one glucose unit in **2** (instead of two in **1**). Thus compound **2** was identified as isovitexin, and the ^{1}H and ^{13}C chemical shift values were comparable to the published data for isovitexin [**2**] (23, 24). Isovitexin [**2**] is widely distributed in a number of genera including *Citrullus*, *Geranium*, *Fagopyrum*, *Vitex*, *Polygonum*, *Swertia* and *Passiflora* (10).

An ESIMS mass spectrum of **3** exhibited the $[M+H]^+$ (positive ion mode) ion peak at *m/z* 463, suggesting M_r = 462 and solving for $C_{22}H_{22}O_{11}$. The ^{1}H and ^{13}C NMR spectra (Table 2) displayed, in addition to the signals corresponding to isoorientin (25), a singlet at δ 3.90, integrating for 3H, which could be assigned to a methoxyl group. Comprehensive UV analyses using shift reagents (Table 1) confirmed the attachment of this methoxyl group at C-3'. The spectroscopic data of **3** were in good agreement with those published for isoorientin 3'-*O*-methyl ether (4). While isoorientin is well distributed in the plant kingdom, compound **3** has only been reported from *C. colocynthis* (10).

An ESIMS mass spectrum of **4** exhibited the $[M+H]^+$ (positive ion mode) ion peak at *m/z* 677, suggesting M_r = 676 and solving for $C_{36}H_{52}O_{12}$. The ^{1}H and ^{13}C NMR spectra (Table 3) displayed signals comparable to the published data for 2-*O*-β-D-glucopyranosylcucurbitacin I (8). The extensive 2D NMR analyses including COSY, NOESY, HMQC and HMBC confirmed the identity of this compound. Similarly, an ESIMS mass spectrum of **5** exhibited the $[M+H]^+$ (positive ion mode) ion peak at *m/z* 679, suggesting M_r = 678 and solving for $C_{36}H_{54}O_{12}$. In the ^{1}H NMR spectrum of **5** (Table 2), the two distinct signals for *trans* olefinic protons (as in **4**) were absent. The ^{13}C NMR spectrum (Table 3) also revealed that instead of two olefinic methine signals (as in **4**), signals for two methylene carbons were present. Thus compound **5** was identified as 2-*O*-β-D-glucopyranosylcucurbitacin L, and all data were in good agreement with the published data for this compound (8). Both **4** and **5**, and their aglycones as well as various other cucurbitacins are well distributed in the genus *Citrullus* and the family Cucurbitaceae (10).

The DPPH assay measures the free radical scavenging property of a compound (16). DPPH is a molecule containing a stable free radical. In the presence of an antioxidant which can donate an electron to DPPH, the purple color, which is typical to free DPPH radical, decays, and the change in absorbance at 517 nm is followed spectrophotometrically. In the cases where the structure of the electron donor is not known (e.g., a plant extract), this method can afford data on the reduction potential of the sample, and hence can be helpful in comparing the reduction potential of

Table 1. UV-visible absorption peaks (in nm) of **1-3** in MeOH and their shifts with different reagents

Compounds	MeOH	Shift reagents				
		NaOMe	$AlCl_3$	$AlCl_3$/HCl	NaOAc	NaOAc/ H_3BO_3
1	326.7 sh	364.5	375.5(sh)	375.5(sh)	366.8(sh)	329.0(sh)
	274	279.5	334.5	334.5	279.5	276.4
			302.6(sh)	302.6(sh)		
			281.5	281.5		
2	333.6	398.5	383.0	380.7	392.8	340.94
	272.0	328.0(sh)	349.0	340.9	303.6(sh)	273.6
		278.5	302.8(sh)	301.9(sh)	278.5	
			278.8	279.3		
3	339.5	406.0	389.0	384.3	400.5	392.7(sh)
	272.5	329.6	356.0	348.0	348.9	342.3
		280.0	297.5(sh)	294.0(sh)	278.5	272.5
			276.0	279.0		

Table 2.. ^{1}H (200 MHz , coupling constant *J* in Hz in parentheses) and ^{13}C NMR (50.0 MHz) data of **1-3** in DMSO-d_6

Carbon no.	Chemical shifts δ in ppm					
	δ_H			δ_C		
	1	**2**	**3**	**1**	**2**	**3**
2	-	-	-	164.2	164.2	164.3
3	6.52 s	6.53 s	6.55 s	103.6	103.6	104.0
4	-	-	-	182.8	182.7	182.9
5	-	-	-	161.5	161.5	161.5
6	-	-	-	109.8	109.8	109.7
7	-	-	-	164.4	164.4	164.3
8	6.92 s	6.80 s	6.92 s	94.5	94.5	94.6
9	-	-	-	157.1	157.1	157.1
10	-	-	-	104.2	104.2	104.2
1’	-	-	-	121.9	122.0	122.3
2’	8.06 d (8.6)	7.95 d (8.7)	7.56 d (1.8)	129.3	129.2	109.7
3’	7.20 d (8.6)	6.94 d (8.7)	-	116.8	116.6	148.9
4’	-	-	-	162.1	162.4	151.6
5’	7.20 d (8.6)	6.94 d (8.7)	6.95 d (8.0)	116.8	116.6	116.6
6’	8.06 d (8.6)	7.95 d (8.7)	7.50 dd (1.8, 8.0)	129.3	129.2	121.2
C-glucose						
1’’	4.60 d (9.5)	4.60 d (9.7)	4.56 d (9.7)	73.9	73.9	73.9
2’’	3.20-3.90*	3.20-3.90*	3.20-3.90*	71.5	71.5	71.4
3’’	3.20-3.90*	3.20-3.90*	3.20-3.90*	79.8	79.8	78.8
4’’	3.20-3.90*	3.20-3.90*	3.20-3.90*	71.1	71.1	71.1
5’’	3.20-3.90*	3.20-3.90*	3.20-3.90*	80.0	80.0	80.1
6’’	3.20-3.90*	3.20-3.90*	3.20-3.90*	62.3	62.3	62.4
O-Glucose						
1’’’	5.05 d (7.2)	-	-	102.0	-	-
2’’’	3.20-4.00*	-	-	74.1	-	-
3’’’	3.20-4.00*	-	-	77.1	-	-
4’’’	3.20-4.00*	-	-	70.5	-	-
5’’’	3.20-4.00*	-	-	77.0	-	-
6’’’	3.20-4.00*	-	-	62.1	-	-
OCH_3	-	-	3.90 s	-	-	56.8

* Overlapped peaks.

Table 3. ^{1}H (500 MHz, coupling constant *J* in Hz in parentheses) and ^{13}C NMR (125.0 MHz) data and key ^{1}H-^{13}C long-range correlation observed in the HMBC spectra of **4** and **5**

Carbon no.	Chemical shift δ in ppm				HMBC correlations	
	δ_H		δ_C			
	4	**5**	**4**	**5**	^{2}J	^{3}J
1	6.11 d (2.6)	6.12 d (2.6)	123.8	123.8	C-2	C-3, C-5, C-9
2	-	-	147.4	147.4	-	-
3	-	-	199.9	199.9	-	-
4	-	-	50.7	50.7	-	-
5	-	-	137.6	137.6	-	-
6	5.84 br s	5.84 br s	122.5	122.5	C-5	C-4, C-8
7	2.40 m, 2.04 m	2.40 m, 2.05 m	24.6	24.6		C-9, C-14
8	2.10 m	2.09 m	43.3	43.3	C-9	C-13, C-15
9	-	-	50.5	50.4	-	-
10	3.69 br s	3.69 br s	38.2	38.2		C-6, C-11
11	-		216.6	216.6	-	-
12	3.41 d (14.8)	3.42 d (14.8)	50.1	50.1	C-11	C-14, C-17
	2.65 d (14.8)	2.65 d (14.8)				
13	-	-	50.4	50.4	-	-
14	-	-	50.2	50.2	-	-
15	1.96 dd (12.8, 8.8)	1.95 dd (12.8, 8.8)	46.7	46.7	C-16	C-13, C-17
	1.44 m	1.47 m				
16	4.48 m	4.47 m	71.6	71.7	C-15	C-20, C-13
17	2.59 d (00)	2.60 m	59.8	59.8	C-13	C-21, C-22
18	0.95 s	0.97 s	20.8	20.8	C-13	C-14, C-17
19	1.01 s	1.01 s	20.6	20.6	C-9	C-10, C-8,C-11
20	-	-	80.9	81.0	-	-
21	1.40 s	1.42 s	25.5	25.5	C-20	C-17, C-22
22	-	-	205.2	218.0	-	-
23	6.84 d (15.5)	2.90 ddd (17.4, 9.6)	121.4	33.6	C-22,	C-25
		2.78 ddd (6.2, 9.6)			C-24	
24	6.97 d (15.5)	1.74 m	155.5	38.2	C-25,	C-22
					C-23	
25	-	-	71.8	70.9	-	-
26	1.33 s	1.21 s	29.5	29.3	C-25	C-24, C-27
27	1.31 s	1.21 s	29.5	29.1	C-25	C-24, C-26
28	1.29 s	1.30 s	28.3	28.3	C-4	C-3, C-5, C-29
29	1.27 s	1.28 s	20.8	20.8	C-4	C-3, C-5, C-28
30	1.41 s	1.42 s	18.8	18.8	C-14	C-13, C-15
1’	4.65 d (7.5)	4.66 d (7.5)	101.3	101.3	C-2’	C-2, C-5’
2’	3.42 *	3.42 *	71.9	71.9	C-1’	C-4’
3’	3.44 *	3.44 *	78.3	78.3		C-1’, C-5’
4’	3.53 *	3.53 *	70.9	70.9	C-5’	
5’	3.38 *	3.38 *	80.1	80.1	C-6’	
6’	4.04 dd (12.0, 3.5)	4.06 dd (12.1, 3.5)	62.1	62.1	C-5’	C-4’
	3.86 dd (12.0, 2.5)	3.87 dd (12.1, 2.5)				

*Overlapped peaks; Spectra obtained in CD_3OD

unknown materials. The antioxidant activity of **1-3** was determined by this method and the RC_{50} values were found to be 7.13 x 10^{-2}, 5.62 x 10^{-4} and 3.47 x 10^{-3} mg/mL, respectively. The RC_{50} value of the positive control, quercetin, was 2.78 x 10^{-5} mg/mL.

CONCLUSION

Since reactive oxygen species are important contributors to tissue injury, inflammation, cancer and many other ailments, the antioxidant properties of **1-3** probably contribute, at least to some extent, to the pharmacological and traditional medicinal uses of the *C. colosynthis*.

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