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POTENT ESTROGENIC FLAVONE GLYCOSIDES FROM *ATRIPLEX SEMIBACCATA*

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تم التعرف على التركيب الكيميائى بطيف الكتلة والرنين النووى المغناطيسى للهيدروجين والكربون وقد بين طيف الكتلة لكل من المركبات الثلاثة. بينما أظهر الرنين النووى المغناطيسى وجود ٣ أو ميثيل كورستين. وبإستخدام الكوزى تم التعرف على الوحدات السكرية وتم تعيين نقط الاتصال بين الوحدات السكرية والشق الاجليكوني بواسطة HMBC. وقد أظهر المركب الأول فعالية ضد ميكروب باسيلس سبتلس والمركب الثانى فعالية ضد باسيلس كوأجيولانس والمركب الثالث فعالية ضد الميكروبين السابقين معا. أوضحت المركبات الثلاثة فعالية قريبة من مركب استراديول وكان المركب الثالث أكثر المركبات فعالية.

*Three known flavone glycosides **1**, **2**, and **3** were isolated for the first time from the whole plant of Atriplex semibaccata. The assignment of the NMR signals was performed by means of ^{1}H-^{1}H COSY, HMQC, and HMBC experiments. The isolated compounds showed potent estrogenic, and promising anti-microbial activities.*

INTRODUCTION

Atriplex semibaccata, family (*Chenopodiaceae*) is very common in salty desert, prostrate or ascending herb with up to 60 cm, leaves thin, up to 4 cm.[1] It is a very important forage at sheep breading in Australia. Most reports say that no members of this genus contains toxins and that all have more or less edible leaves. However, one report say that if very large quantities are eaten they can cause photosensitivity.[2] A few leaves of stronger-flavoured plants of *Atriplex patula* can be added to enhance the tast[3] and its seeds can be used as laxative and the whole plant cures headaches, wandering pains, and the first attacks of rheumatism.[4] In a previous investigation for *Atriplex semibaccata* four saponins were isolated.[5] This paper describes the isolation and structure elucidation of three flavone glycosides **1**, **2**, and **3** in addition to, their potent estrogenic and anti-microbial activities.

EXPERIMENTAL

General

Negative ion MS: MAT 8500 (Finnigan), matrix glycerol. NMR: 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C), reverse probehead, δ at ppm, solvent CD_3OD, CD_3OD signal were used as int. standard (^{1}H: 3.30, ^{13}C: 49.0), temp. 290 K, HMQC: phase-sensetive using TPPI, BIRD sequence, GARP decoupled, HMBC: using TTPI, delay to achieve long range couplings: 71 msec ($J_{C,H}$ = 14 Hz).

CC: silica gel (0.063-0.2 mm); TLC: silica gel (0.25 mm pre-coated plates 60 F254, Merck, The plates were sprayed with 10% H_2SO_4 in MeOH.

Isolation

Atriplex semibaccata was collected in 1999 near Burg El-Arab, Alexandria, Egypt and identified by Dr. M. El-Gebaly from the National Research Centre (NRC) Cairo. A Voucher specimen of the plant is deposited at the Herbarium of the NRC, Department of Chemotaxanomy. Dried powder of the whole plant of *Atriplex semibaccata* (3 kg) was exhaustively extracted with 80% MeOH to give

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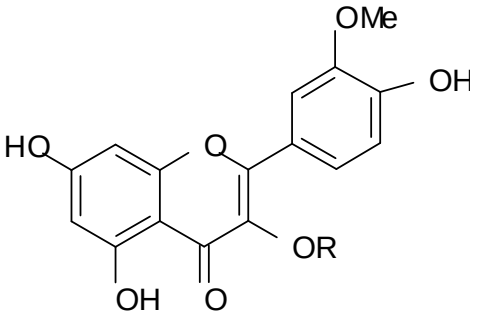
50 g methanolic extract. The methanolic extract was successively partitioned between H_2O and n-hexan, chloroform, and n-butanol. The butanolic fraction was evaporated under red. pressure at 45° to obtain 7 g crude butanol extract. The butanolic extract was chromatographed on silica gel column (1m x 5cm) eluting with $CHCl_3$-MeOH-H_2O in the order of increasing the polarity to afford flavone glycosides fraction F_2 (1 g). F_2 was subjected to MPLC using RP-18 material and MeOH-H_2O (4:6) then sephadex LH-20 using MeOH to give flavone glycosides **1** (5 mg), **2** (10 mg), **3** (4 mg).

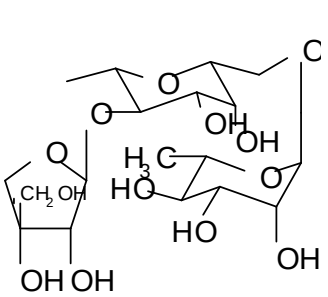
Spectral data

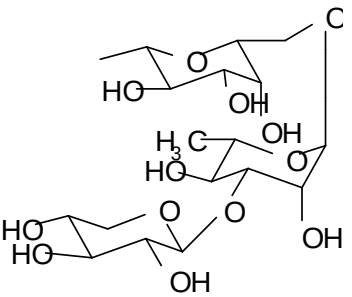
Compound **1** ($C_{28}H_{32}O_{16}$), Mr 624); LSI/MS, negative ion mode m/z (rel. int.): 623 $[M-1]^-$ (83), 477 $[M-1-146]^-$ (33), 315 $[M-1-146-162]^-$ (100). ^{13}C-NMR (Table 1). ^{1}H-NMR (CD_3OD) δ ppm: 6.15 (1H, d, J= 1.8 Hz, H-6), 6.35 (1H, d, J= 1.8 Hz, H-8), 7.98 (1H, d, J= 1.8 Hz, H-2'), 6.9 (1H, d, J= 8.4 Hz, H-5'), 7.6 (1H, dd, J= 8.4, 1.8 Hz, H-6'), 5.17 (1H, d, J= 7.7 Hz, H-1''), 3.76 (1H, H-2''), 3.4 (1H, H-3''), 3.2 (1H, H-4"), 3.5 (1H, H-5"), 3.6/3.7 (2H, H-6"), 4.5 (1H, broad, H-1"'), 3.5 (1H, H-2"'), 3.4 (1H, H-3"'), 3.2 (1H, H-4"'), 3.49 (1H, H-5"'), 1.13 (3H, Me), 3.91 (OMe).

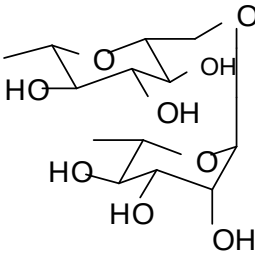
Compound **2** ($C_{33}H_{40}O_{20}$), Mr 756); LSI/MS, negative ion mode m/z (rel. int.): 755 $[M-1]^-$ (100), 623 [M-1-132] (17). ^{13}C-NMR (Table 1). ^{1}H-NMR (CD_3OD) δ ppm: 6.2 (1H, d, J= 1.8 Hz, H-6), 6.4 (1H, d, J= 1.8 Hz, H-8), 8.0 (1H, d, J= 1.8 Hz, H-2'), 6.9 (1H, d, J= 8.4 Hz, H-5'), 7.6 (1H, dd, J= 8.4, 1.8 Hz, H-6'), 5.2 (1H, d, J= 7.8 Hz, H-1''), 3.8 (1H, H-2''), 3.65 (1H, H-3''), 3.73 (1H, H-4"), 3.67 (1H, H-5"), 3.55/3.70 (2H, H-6"), 4.5 (1H, broad, H-1"'), 3.7 (1H, H-2"'), 3.52 (1H, H-3"'), 3.77 (1H, H-4"'), 3.55 (1H, H-5"'), 1.13 (3H, Me), 4.3 (d, J= 7.4 Hz, H-1""), 3.22 (1H, H-2""), 3.3 (1H, H-3""), 3.46 (1H, H-4""), 3.09/3.79 (2H, H-5""), 3.90 (OMe).

Compound **3** ($C_{33}H_{40}O_{20}$), Mr 756); LSI/MS, negative ion mode m/z (rel. int.): 755 $[M-1]^-$ (9), 623 $[M-1-132]^-$ (18). ^{13}C-NMR (Table 1). ^{1}H-NMR (CD_3OD) δ ppm: 6.15 (1H, d, J= 1.8 Hz, H-6), 6.4 (1H, d, J= 1.8 Hz, H-8), 7.9 (1H, d, J= 1.8 Hz, H-2'), 6.9 (1H, d, J= 8.4 Hz, H-5'), 7.6 (1H, dd, J= 8.4, 1.8 Hz, H-6'), 5.5 (1H, d, J= 7.4 Hz, H-1''), 3.6 (1H, H-2''), 3.62 (1H, H-3''), 3.74 (1H, H-4"), 3.18 (1H, H-5"), 3.50/3.68 (2H, H-6"), 4.5 (1H, broad, H-1"'), 3.53 (1H, H-2"'), 3.42 (1H, H-3"'), 3.17 (1H, H-4"'), 3.33/3.77 (2H, H-5"'), 5.4 (1H, H-1""), 3.98 (1H, H-2""), 3.63/3.98 (2H, H-4""), 3.59/3.73 (2H, H-5""), 3.95 (OMe).









Compound **1** Compound **2** Compound **3**

Table 1: ^{13}C-NMR Spectral data for flavone glycosides **1-3**.

C	**1**	**2**	**3**
2	158.7	158.9	158.8
3	135.5	135.5	134.5
4	179.3	179.2	179.2
5	162.8	162.9	163.0
6	99.9	99.9	99.8
7	165.9	166.0	165.8
8	94.9	94.8	94.8
9	158.3	158.4	158.4
10	105.5	105.7	105.9
1’	122.9	122.9	123.4
2’	114.5	114.6	114.6
3’	150.9	150.9	150.6
4’	148.4	148.4	148.3
5’	116.0	115.9	115.9
6’	123.7	123.8	123.5
OCH_3	56.9	56.9	57.0
	glucose	galactose	galactose
1’’	104.9	104.9	100.8
2’’	73.1	73.01	79.0
3’’	78.2	75.2	75.3
4’’	71.6	71.7	70.4
5’’	75.4	75.7	74.8
6’’	67.3	67.9	67.2
	rhamnose	rhamnose	rhamnose
1’’’	101.8	101.9	101.9
2’’’	72.1	71.7	72.3
3’’’	72.2	82.3	72.6
4’’’	73.8	70.1	72.4
5’’’	69.9	69.5	68.4
6’’’	17.9	18,0	17.9
		xylose	apiose
1’’’’		106.5	110.9
2’’’’		75.2	77.9
3’’’’		77.5	80.9
4’’’’		71.04	75.4
5’’’’		66.8	66.5

Procedure of the estrogenic assay

Immature femal sprague-Dawley rats weighing about 55-60 g are ovarectomized, they are kept for about one week on standard laboratory diet and water add libitum. The test compounds **1**, **2**, and **3** are adminestered in 0.5% solution of carboxy methyl cellulose as subcuteneous injection in various doses 0.02, 0.1 and 0.5 µg/kg to groups of 10 rats. Doses of 0.02, 0.1 and 0.5 µg/kg estradiol per animal are used as reference standard. The test compounds are dosed twice daily on two following days at 10.00 a.m and 5.00 p.m At 5.00 p.m of the third day and 10.a.m. of the fourth day vaginal smears are taken using cotton swabs moistered with saline. The smears are transferred to a glass slide and stained for 10 minutes with 5% aqueous methylene blue solution. They are evaluated microscopically according to the following score:

- Diestrus smear, mainly leucocytes, few epithalia cells.
- Mixture of leucocytes and epithalial cells
- Proestrus smear, nucleated or nucleated plus cornified cells.
- Estrus smear, cornfied cell S only. Only animals showing score 2 or 3 are considered to be positive.

RESULT AND DISCUSSION

The n-butanol extract of the whole plant of *Atriplex semibaccata* was obtained as described in experimental part. The n-butanol extract was subjected to column chromatography on silica gel and eluted with chloroform, chloroform-methanol and chloroform-methanol-water to afford main flavonoids fraction. The flavonoid fraction was chromatographed on MPLC using RP-18 and eluted with water-methanol (6:4) to afford three flavonoid glycosides which purified on Sephadex LH-20 using methanol to give compounds **1**, **2**, and **3**.

The LSI mass spectrum for **1**, **2**, **3** exhibited $[M-1]^-$ ion at m/z 623, 755, 755. The ^1H and ^{13}C-NMR spectra for the three compounds showed 3'-O-methyl-quercetine as aglycone.[6] ^1H-NMR for **1**, **2**, **3** showed two singlet at δ 6.2, 6.4 for H-6 and H-8 while the signals at δ 6.9, 7.6, and 7.9 corresponding to H-5', H-6', and H-2'.^{13}C-NMR showed the presence of methoxy group C-3' at δ 56.9. The presence of two sugar moieties in compound **1** was confirmed from two anomeric ^{13}C signals at δ 104.9 and 101.8 while compound **2** showed three anomeric ^{13}C signals at δ 104.9, 101.9, and 106.5 and three anomeric ^{13}C signals of compound **3** at δ 100.8, 101.9, and 110.9 (Table 1). Compounds **1**, **2** and **3** were identified by ^1H-NMR, ^{13}C-NMR data and comparison of the data with reference data.[7-9] Compound **3** was isolated from *Pituranthos tortuosus* (*Apiaceae*) Desf, Benth & Hook[8] while compound **2** isolated from the leaves of *Hammada scoparia* (*Chenopodiaceae*).[9] The presence of galactose moiety in compound **2**, **3** instead of glucose was confirmed by the coupling constant of H-3" of galactose (dd, J= 3.2, 9.4 Hz) and ^{13}C-NMR data. Comparison of ^{13}C- data of the sugars with the published data by Atta ur-Rahman[10] showed a very good agreement (Table 2).

Anti-microbial test

Anti-microbial activities were tested against, *Bacillus subtilis*, *Bacillus coagulans*, *Micrococcus sp*., *Candida albicans*, *Aspergillus niger*, *Macrophomena sp*., *Botrytis sp*. which obtained from the culture collection of the microbiological laboratory, National Research Centre, Cairo, Egypt. The activity was determined by measuring the inhibition zones using agar plate diffusion methods.[11-13] The diameter of inhibition zones was measured after 24 hr for bacteria and yaest and 48 hr for moulds. Purified compounds were tested at 1 mg/1ml. The results are shown in (Table 3). Compound **1** showed high activity against *Bacillus subtilis* and no activity against *Bacillus coagulans*. Compound **2** showed moderate activity against *Bacillus coagulans*, and low activity against *Bacillus subtilis*. Compound **3** showed high activity against *Bacillus coagulans*, and *Bacillus subtilis*. All compounds **1**, **2**, and **3** showed low activity against *Micrococcus sp*., and *Botrytis sp*. and no activity against *Candida albicans*, *Aspergillus niger*, and *Macrophomena sp*.

Estrogenic activity

Flavonoid class have previously been demonstrated to possess estrogenic activity in a number of hormonally responsive systems.[14] Phytochemists determined that apigenin, diosmetin, and kaempferol had the most potent estrogenic activity, which they deemed "nearly equal to those of the isoflavones diadzein...and genistein.[15] This study aims to characterize the estrogenic activity of flavonoid glycosides **1**, **2** and **3**.

Name of the method Vaginal Cornification

Purpose and rational

The Allen –Doisy test for vaginal cornification in rodents[16] is based on the observation of Stockard and Papanicolaou[17] that reported the cyclic vaginal cornification in guinea pigs. The procedure is described in experimental part.

Evaluation The number of positive animals in each dosage group is recorded. Using various doses, ED_{50} values can be calculated. The results are shown in Table (4).

Table 2: ^{13}C- δ values for sugars (bold refer to substituted positions).[10]

C	β-D-Glc	β-D-Gal	β-D-xyl	α-L-Rha	β-D-api
1	105.5	101.7	106.8	101.5	109.1
2	75.5	**77.8**	74.9	71.8	77.7
3	78.3	73.6	78.6	**82.7**	80.4
4	72.6	71.1	71.7	72.9	75.4
5	75.5	76.9	67.5	69.6	65.3
6	**68.8**	61.6		18.5	

C = carbon atoms of the sugar, Glc = glucopyranose, Gal = galactopyranose, Rha = rhamnopyranose, xyl = xylopyranose, api = apiofuranose.

Table 3: Anti-microbial activities of the isolated compounds.

Compound	Inhibition Zone Width						
	M1	M2	M3	M4	M5	M6	M7
1	+ ++	-	+	-	-	-	+
2	+	++	+	-	-	-	+
3	+ ++	+ ++	+	-	-	-	+

\+ the activity < 0.5 ++ the activity from 0.6 to 1.0 +++ the activity more than 1.0

M1: *Bacillus subtilis*, M2: *Bacillus coagulans*, M3: *Micrococcus sp*., M4: *Candida albicans*, M5: *Aspergillus niger*, M6: *Macrophomena sp*., M7: *Botrytis sp*.

Table 4: Estrogenic activities for the isolated compounds **1**, **2**, **3**.

Dose	1	2	3	Estradiol	Control
0.02 µg/animal	1	1	2	3	0
0.1 µg/animal	6	6	7	8	0
0.5 µg/animal	9	9	9	10	0
E D_{50}	1.8 µg/kg	1.8 µg/kg	1.5 µg/kg		

Conclusions

The three tested compounds **1**, **2**, and **3** showed a potent estrogenic activities nearly to that of estradiol. The most potent one is compound **3**.

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