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ISOFLAVONES AND A SAPONIN FROM *CROTALARIA THEBAICA* (DEL.) DC GROWING IN EGYPT

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أسفر فحص خلاصة الأجزاء الهوائية الجافة لنبات الكروتالاريا ثبيكا عن فصل إثنين من الايزوفلافونيدات الحرة وهما بيوكانين-أ-وجينستين ، وأيزوفلافون-أ-جلوكوزيد (بيوكاين-أ-جلوكوزيد)، وإثنين من الايزوفلافون-ك-جلوكوزيد وهما ٨-ك-جلوكوزيد الجينستين ، ٨،٦-داى-ك-جلوكوزيد بيوكاين أ. وذلك بالاضافة إلى صابونين تم تعريفه على أنه روبينيوزيد-ث-ميثيل استر. وقد تم التعرف على جميع المركبات المفصولة باستخدام الطرق الكيميائية والطيفية المختلفة.

Further investigation of the dried aerial parts of Crotalaria thebaica (Del.) DC. led to the isolation of two isoflavone aglycones; Biochanin A (1) and Genistein (2), an isoflavone-O-glucoside; Biochanin A-7-O-β-glucoside (3) two isoflavone C-glycosides identified as 8-C-glucosyl genistein (4) and 6,8 di-C-glucosyl biochanin A (5) and a saponin glycoside identified as robinioside C methylester (6).

The identification of the isolated compounds was based on chemical and spectral studies.

INTRODUCTION

The genus *Crotalaria* (F. *Leguminosae* sub-family *Papilionaceae*) comprises about 550 species distributed mainly in the temperate or tropical regions[1,2] the genus is represented in Egypt by 5 species.[3] The genus *Crotalaria* is characterized by its pharmacologically active pyrrolizidine alkaloids (PAs).[4-8] Beside PAs, several pigments have been isolated from the genus *Crotalaria* including flavonoids, prenylated chalcones, isoflavonoids, prenylated flavonoids and their glycosides[9-25] and saponins.[26,27] In the early studies on this plant, PAs,[27,28] lignans,[27] saponins[27-29] and flavones C-glycosides[30], were isolated. Continuation of the study on this plant thought to be interesting.

EXPERIMENTAL

General experimental procedures

Melting points were uncorrected and determined by electrothermal model 550 spectrophotometer Thamson THN 60 eV. IR spectra were recorded in KBr using Unicam SP1025 spectrometer. UV spectra were measured in MeOH and different ionizing and complexing reagents using Unicam 1750 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded in DMSO-d_6 and pyridine-d_5, unless otherwise mentioned, at 400 MHz and 100 MHz, respectively, using Bruker AM-400 spectrometer, chemical shifts are given in δ value with TMS as internal standard. Mass spectra were carried out using Hitachi M-80 spectrometer (Japan) for EIMS and JOEL MS-SX 102 (JOEL-Japan) for recording FAB-MS.

For CC Amberlite IR A-45 (weak anion exchange resin), silica gel (E. Merck) and irregular reversed phase (RP 18-37) were used. CIG column system (22 mm i.d. X 30 cm, Kusano Scientific Co., Tokyo, Japan) was used for final purification. Silica gel 60 F_{254} (E. Merck) was used for TLC. PC was performed using Whatman paper No. 3 and No. 1. 5% methanolic $AlCl_3$ and 10% H_2SO_4 were used as spraying reagents. The following solvent systems were used:

I) Chloroform-methanol. (a) 90:10 (b) 85:15
II) Chloroform-methanol-water. 75:23:2

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III) 15% acetic acid.
IV) Butanol-acetic acid-water 4:1:5
V) Chloroform-methanol-water 65:35:5

Plant material

The aerial parts of *Crotalaria thebaica* (Del.) DC. were collected from El-Hafafit near Aswan in April 1994 during flowering, the plant was kindly identified by Dr. Nabil El-Hadidy, Professor of Taxonomy, Faculty of Science, Cairo University. The collected aerial parts were air-dried, powdered and kept in well closed containers till used.

Extraction and isolation

The air-dried powdered aerial parts (about 2.5 kg) of *Crotalaria thebaica* were exhaustively extracted with 70% ethanol, the conc. alcoholic extract was diluted with distilled water (500 ml) and extracted with EtOAc. The EtOAc fraction was evaporated under reduced pressure [Fraction A, 22.3 g]. The aqueous fraction left after extraction with EtOAc was concentrated to the least possible volume [Fraction B, 34 g].

Fractionation of fraction A

20 g of the dry extract (Fr. A) were fractionated over silica gel CC (750 g, 7x150 cm). Elution was started with chloroform containing gradiently increasing amounts of methanol. Fractions 250 ml, each, were collected, concentrated and monitored by TLC silica gel (systems I, II) and/or PC (system III). Purification of the obtained compounds was carried out by repeated CC using SiO_2 and RP-18 using MPLC and/or preparative PC using Whatman No. 3, where compounds **(1-5)** were isolated.

Fractionation of fraction B

30 g of the concentrated extract of fraction B were treated with diazomethane and fractionated over an Amberlite column (2L) using water-methanol gradient. The fractions eluted with water-methanol (3:2 and 1:1) containing a mixture of saponins were mixed and refractionated on SiO_2 column (120x5 cm) using chloroform-methanol gradient. The fractions eluted with chloroform-methanol (70:30) upon repeated MPLC using SiO_2 and RP-18 columns afforded compound **(6)** (60 mg).

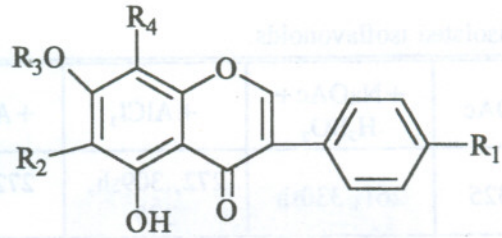
Compound (1): Obtained as fine needle crystals (15 mg), m.p. 216-218° (MeOH), it gave purple colour under UV light. UV data in methanol and with different ionizing and complexing reagents were cited in Table (1). EIMS: m/z (rel. int %) showed M^+ at m/z 284 (base peak), other peaks at m/z 267 (14), 266 (7), 253 (12), 238 (27), 203 (3), 190 (12), 167 (14), 142 (12), 138 (40) and 118 (50). 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR (DMSO-d_6) were cited in Tables (2) and (3) respectively.

Compound (2): Obtained as colourless needles (12 mg), m.p. 297-299° (MeOH), it gave purple colour under UV light. UV data in methanol and with different ionizing and complexing reagents were cited in Table (1). EIMS: m/z (rel. int %) showed M^+ at m/z 270 (base peak), other peaks at 253, 252, 203 and 153. 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR (DMSO-d_6) were cited in Tables (2) and (3) respectively.

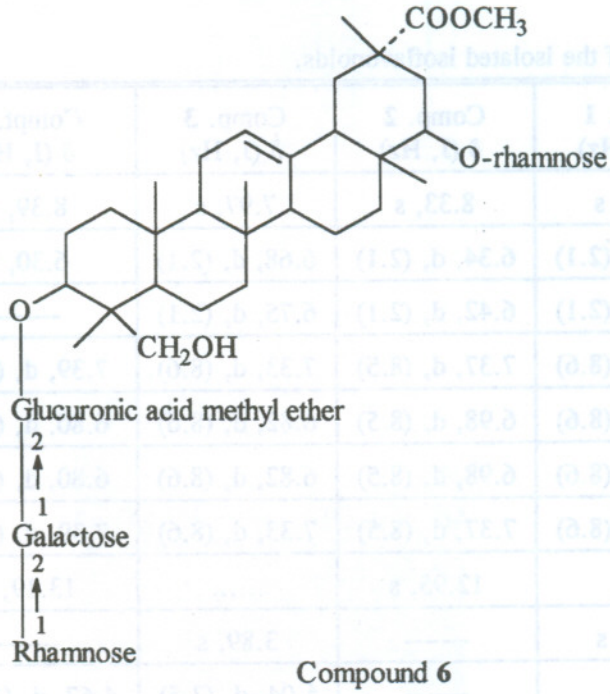
Compound (3): Obtained as amorphous powder (28 mg). It gave a purple colour under UV light and positive Molish's test. The UV data with standard reagents, 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR (CD_3OD) were cited in Tables (1, 2 and 3) respectively.

Compound (4): Obtained as amorphous powder (18 mg). It gave purple colour under UV light and positive Molish's test. The UV data in methanol and with standard reagents were cited in Table (1). The 400 MHz ^{1}H-NMR and 100 MHz ^{13}C-NMR (DMSO-d_6) were cited in Tables (2) and (3) respectively. EIMS showed fragments at m/z 415 (M^++1-18), and other significant peaks at m/z 379 (100), 351 (14), 324 (40), 285 (71), 254 (20).

Compound (5): Amorphous powder (22 mg), it gave purple colour under UV light and positive Molish's test. The UV data with standard reagents, 400 MHz ^{1}H-NMR (DMSO-d_6) and



	R_1	R_2	R_3	R_4
Compound **1**	OCH_3	H	H	H
Compound **2**	OH	H	H	H
Compound **3**	OCH_3	H	glucose	H
Compound **4**	OH	H	H	glucose
Compound **5**	OCH_3	glucose	H	glucose



Compound **6**

Table 1: UV spectral data of the isolated isoflavonoids.

Comp.	λ_{max} MeOH	+NaOAc	+NaOAc+ H_3BO_3	+$AlCl_3$	+$AlCl_3$/HCl	+NaOMe
1	248sh, 260, 327sh	272, 325	261, 330sh	272, 309sh, 375	272, 309sh, 373	248sh, 273 330
2	262, 300sh, 328	272, 326	262, 336sh.	273, 305sh, 370	272, 309sh, 373	279, 328sh.
3	262, 330sh	262, 328sh	262, 325sh	274, 310sh, 375	274, 310sh, 372	249sh, 270, 370
4	264, 330sh	280, 328sh	264, 332sh	276	272	
5	262, 328	271, 321	261, 320	273, 305	273, 303, 380	267, 368

Table 2: ^{1}H NMR Data of the isolated isoflavonoids.

Proton	Comp. **1** δ (J, Hz)	Comp. **2** δ (J, Hz)	Comp. **3** δ (J, Hz)	Compt. **4** δ (J, Hz)	Comp. **5** δ (J. Hz)
H-2	8.21, s	8.33, s	7.97, s	8.39, s	8.11, s
H-6	6.36, d, (2.1)	6.34, d, (2.1)	6.68, d, (2.1)	6.30, s	-------
H-8	6.44, d, (2.1)	6.42, d, (2.1)	6.75, d, (2.1)	-------	-------
H-2`	7.38, d, (8.6)	7.37, d, (8.5)	7.33, d, (8.6)	7.39, d, (8.7)	7.37, d, (8.6)
H-3`	6.84, d, (8.6)	6.98, d, (8.5)	6.82, d, (8.6)	6.80, d, (8.7)	6.84, d, (8.6)
H-5`	6.84, d, (8.6)	6.98, d, (8.5)	6.82, d, (8.6)	6.80, d, (8.7)	6.84, d, (8.6)
H-6`	7.38, d, (8.6)	7.37, d, (8.5)	7.33, d, (8.6)	7.39, d, (8.7)	7.37, d, (8.6)
5-OH	.....	12.95, s	.....	13.19, s	13.19, s
OCH_3	3.90, s	-------	3.89, s	-------	3.84, s
H-l``	------	-------	5.04, d, (7.5)	4.67, d, (10.0)	4.94, d, (9.9)
H-2``-H-6``	------	-------	3.2-3.9	3.2-4.0	3.1-4.1 12H, m

Table 3: ^{13}C-NMR spectral data of the isolated isoflavonoids.

Carbon	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5
2	153.65	153.88	152.86	153.93	154.72
3	122.42	122.74	124.35	122.11	124.57
4	180.60	180.58	177.72	180.63	182.63
5	162.52	157.6	163.64	161.18	158.89
6	98.82	98.98	98.60	98.82	106.58
7	163.88	164.38	162.67	163.37	164.72
8	94.44	92.76	97.50	104.65	100.40
9	157.58	157.60	158.63	157.52	163.49
10	104.55	104.50	111.30	104.36	104.58
1`	125.25	121.31	127.26	121.72	123.28
2`	131.58	130.21	131.61	130.23	131.40
3`	116.22	115.16	116.15	115.20	116.38
4`	160.14	162.1	160.45	157.52	163.72
5`	116.22	115.16	116.15	115.20	116.38
6`	131.58	130.21	131.61	130.23	131.40
OCH_3	55.80		56.78		60.80
1``(1```)			101.92	73.40	73.82, 74.36
2``(2```)			74.84	70.84	72.98, 72.65
3``(3```)			77.99	78.89	80.17
4``(4```)			71.48	70.86	72.00, 71.89
5``(5```)			78.60	81.81	82.66, 84.95
6``(6```)			62.63	61.58	62.95

100 MHz ^{13}C-NMR (CD_3OD) were cited in Tables (1, 2 and 3) respectively. EIMS did not show the molecular ion peak but showed fragments at m/z 325 (10), 285 (100), 238 (13), 190 (5), 167 (8).

Compound (6): Amorphous powder (60 mg), It gave a positive Molish's test, -ve FAB-MS, m/z, 1145 [M-H], 999 [(M-H)-rhamnose]. IR νcm^{-1} (KBr) 3440, 1720 and 1616. The 400 MHz ^{1}H-NMR (δ ppm,C_5D_5N) (0.71, 0.92, 1.02, 1.21, 1.38, 1.42 (each 3H, s, CH_3 x 6) 1.71 (3H, d, J= 5.5 Hz, CH_3 -rhamnose), 1.74 (3H, d, J= 6.2 Hz, CH_3-rhamnose), 3.62 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 4.91 (1H, d, J= 7.4 Hz, glucuronic acid, H-1), 5.25 (1H, br.s, H-12), 5.44 (1H, br.s, rhamnose H-1), 5.81 (1H, d, J= 7.7 Hz, galactose H-1), 6.30 (1H, br.s, rhamnose H-1) other protons appeared between δ 3.2-5.2.

Hydrolysis of compound (6): 30 mg of compound **(6)** were subjected to acid hydrolysis to give aglycone [**(6a)** 8.5 mg] as colourless needles m.p. 253-255°, IR (KBr) ν cm^{-1} 3444, 1716, 1612 cm^{-1}, EIMS, M^+ at m/z 502 other peaks at m/z 443, 442, 278, 224 and 219. 400 MHz ^{1}H-NMR (C_5D_5N) δ 0.97, 1.02, 1.21, 1.24, 1.56, 1.67 (each 3H, s, 6 x CH_3), 3.67 (3H, s, OCH_3), 3.89 (1H, br.t), 3.71 and 4.53 (1H each, ABq, J= 10.6 Hz, 24-H_2) and 5.35 (1H, brt, H-12).

Acid hydrolysis

Compound **(6)** was heated with 10% aqueous HCl (2 ml), in a sealed tube, at 80°, for about 4 hr. The sapogenin was extracted with Et_2O. The aqueous layer was neutralized by Amberlite MB-3 resin and dried. Sugars were identified by comparison with authentic samples.

RESULTS AND DISCUSSION

From the 70% ethanol extract of *Crotalaria thebaica* (Del.) DC, five isoflavonoids have been isolated in addition to one saponin. The isoflavonoid nature of the isolated compounds was deduced from their UV spectra (λ_{max} at *ca* 262 nm and *ca* 290 sh.) and from ^{1}H-NMR (characteristic low-field singlet assigned to an isoflavonoid C_2-H at *ca* δ 8.2 ppm).

Compound (1)

The solubility with the colour under UV and the effect of different standard reagents indicated that it is an isoflavone aglycone with free hydroxyl groups at 7 and 5 positions.[31] ^{1}H-NMR showed isoflavone pattern with H-2 at δ 8.21, methoxy signal at δ 3.90 (3H, s) and p-substituted B-ring Table (2). ^{13}C-NMR Table (3) showed signal identical with those reported for 5,7-dihydroxy-4`-methoxy isoflavone. EIMS confirmed the molecular weight of this compound.

From all the above mentioned data (UV, EIMS, ^{1}H-NMR and ^{13}C-NMR), compound **(1)** was identified as 5,7-dihydroxy-4`-methoxy isoflavone (Biochanin A). This is the first report for isolation of this compound from *C. thebaica* Del (DC).

Compound (2)

The solubility and the colour under UV of compound **(2)** are similar to those of compound **(1)**. UV spectra with standard reagents indicated that compound **(2)** is an isoflavone with free hydroxyl groups at 5, 7 and 4` positions.[31] ^{1}H-NMR (Table 2) and ^{13}C-NMR (Table 3) are similar to those of compound **(1)** except in the disappearance of the methoxy signal and this was confirmed from the molecular weight in the MS. From all the above mentioned data, compound **(2)** was identified as genistein.

Compound (3)

The UV colour reaction in presence of standard reagents indicated that it is an isoflavone glycoside with free hydroxyl at position 5, upon acid hydrolysis it gave glucose (PC, system IV and TLC, system V) hence it is O-glucoside.

The ^{1}H-NMR Table (2) displayed a typical AA`BB`system for p-substitued B-ring at δ 7.33 (2H, d, J= 8.6 Hz) and δ 6.82 (2H, d, J= 8.6 Hz), two doublet signals at δ 6.75 (1H, d, J= 2.1 Hz) and δ 6.68 (1H, d, J= 2.1 Hz) assigned

for H-8 and H-6, respectively, and the singlet signal at δ 7.97 (1H, s) for H-2. The anomeric sugar proton appeared at δ 5.04 (1H, d, J= 7.5 Hz, H-1``) indicated the ß-configuration. The singlet signal at δ 3.89 was assigned for 4`-OCH_3.

^{13}C-NMR (CD_3OD) Table (3) showed 20 signals for 22 carbons. The signals at δ 101.92, 78.60, 77.99, 74.84, 71.48 and 62.63 were assigned for ß-glucopyranoside. Other signals were assigned for isoflavone, Biochanin derivative.

From all of the above mentioned data (UV, ^{1}H-NMR and ^{13}C-NMR) compound **(3)** was identified as Biochanin A -7-O-ß-glucoside.

Compound (4)

The usual colour reaction with the UV spectrum in the presence of standard reagents indicated that it is an isoflavone glycoside with free hydroxyls at positions 5, 7 and 4`.[31] It was stable to acid hydrolysis and hence it is a C-glycoside.[32,33]

The ^{1}H-NMR (Table 2) displays a typical AA`BB` system for 4` hydroxy substitution pattern of ring-B by showing two doublets at δ 6.80 (2H, d, J= 8.7 Hz for H-3`, H-5`) and δ 7.39 (2H, d, J= 8.7 Hz for H-2`, H-6`) of the B-ring. The two singlet signals at δ 6.30 and 8.39 (1H, s, each) assigned for H-6 and H-2 of the isoflavone nucleus and hence the sugar moiety was present at position 8. The doublet signal at δ 4.67 (1H, d, J= 10 Hz) was assigned for the anomeric proton of the sugar moiety present in ß-configuration.

The ^{13}C-NMR data Table (3) showed the presence of 13 signals assigned for 15 carbons of genistein and 6 carbon signals assigned for ß-glucopyranoside[34]. The signal at δ 98.82 assigned to C-6 while the signal at δ 104.65 assigned for C-8 which is downfield shifted due to C-glycosilation[35]. The assignments of other signals were cited in Table (3). The glucose was ß-linked to the aglycone on the bases of ^{1}H-NMR data (H-1, J= 10 Hz) and ^{13}C-NMR data (δ 73.40, ß-C-glucopyranosyl bonded to an aromatic ring). The MS showed peak at m/z 416 for M^+-H_2O.

From all of the above mentioned data it is clear that compound **(4)** is 8-C-glucosyl genistein. This is the first report for isolation of this compound from Genus *Crotalaria*.

Compound (5)

The usual colour with UV spectrum in the presence of standard reagents indicated that it is an isoflavone with free hydroxyls at 5 and 7 positions[31]. It was stable to acid hydrolysis and hence it is a C-glycoside. [32,33]

The 400 MHz ^{1}H-NMR (CD_3OD) Table (2) showed a typical pattern for AA`BB` system by showing two doublets at δ 7.37 (2H, d, J= 8.6 Hz) and δ 6.84 (2H, d, J= 8.6 Hz), one singlet proton at δ 8.11 (1H, s) assigned for H-2 of isoflavone and one anomeric proton at δ 4.94 (1H, d, J= 9.9 Hz), the other anomeric proton disappeared under water signal. The methoxyl group appeared at δ 3.84, other sugar protons appeared between δ 4.1-3.1.

^{13}C-NMR (Table 3) showed only three doublet signals in the aromatic region, two signals for p-substituted aromatic system at δ 131.40 and δ 116.38 assigned for 2`, 6` and 3`, 5` carbons of ring B and one at δ 154.72 assigned for C-2 of the isoflavone. The two singlet signals at δ 100.39 and δ 104.56 for C-6 and C-8 were lowerfield shifted indicating the site of glycosylation. The signal at δ 60.80 was assigned for the C-4` methoxy group. The other signals were assigned as cited in Table (3).

From all the above mentioned data, compound **(5)** was identified as 6,8-di-C-glucosyl biochanin A, this is the first report for isolation of this compound from Genus *Crotalaria*.

Compound (6)

It was obtained as a white amorphous powder, it gave positive tests for sterols and/or triterpenes and glycosides. The IR spectrum showed the presence of hydroxyl (3444 cm^{-1}) and carbonyl (1720 cm^{-1}) groups and olefinic system (1616 cm^{-1}).

The -ve FAB-MS showed M^+-1 at m/z 1145 and a peak at m/z 999 for the loss of methylpentose as terminal sugar. The ^{1}H-NMR (C_5D_5N) showed six singlet methyl signals, the two doublet signals at δ 1.71 (3H, d, J= 5.5 Hz) and 1.74 (3H, d, J= 6.2 Hz) were assigned for the two methyl signals for two rhamnose

Table 4: ^{13}C-NMR data of compound **(6)** (C_5D_5N).

C No.	δ (Mult.)	C No.	δ (Mult.)	C No.	δ (Mult.)
1	38.5 (t)				
2	26.4 (t)	28	21.0 (q)	α-L-Rh.	
3	91.5(d)	29	178.5 (s)	1	98.4 (d)
4	43.8 (s)	30	23.5 (q)	2	72.5 (d)
5	55.8 (d)	OCH_3	52.0 (q)	3	72.9 (d)
6	18.4 (t)	GLC-UA		4	73.6 (d)
7	33.1 (t)	1	105.2 (d)	5	70.2 (d)
8	39.8 (s)	2	78.1 (d)	6	18.4 (q)
9	47.5 (d)	3	76.8 (d)		
10	36.4 (s)	4	73.4 (d)		
11	23.9 (t)	5	77.5 (d)		
12	123.4 (d)	6	170.2 (s)		
13	144.1 (s)	$COO\underline{CH_3}$	52.2 (q)		
14	42.5 (s)	B-D-galactopyranosyl			
15	25.9 (t)	1	101.8 (d)		
16	28.4 (t)	2	76.5 (d)		
17	37.3 (s)	3	76.4 (d)		
18	44.1 (d)	4	70.9 (d)		
19	40.6 (t)	5	76.4 (d)		
20	42.1 (s)	6	61.8 (t)		
21	40.0 (t)	α-L-Rh.			
22	79.2 (d)	1	102.3 (d)		
23	22.8 (q)	2	72.2 (d)		
24	63.5 (t)	3	72.4 (d)		
25	15.6 (q)	4	74.1 (d)		
26	16.8 (q)	5	69.1 (d)		
27	25.3 (q)	6	18.8 (q)		

sugar units. The two doublet signals at δ 4.91 (1H, d, J= 7.4 Hz) and δ 5.81 (1H, d, J= 7.7 Hz) and the two singlet signals at δ 5.44 and 6.30 (1H, br.s. each) were assigned for four sugars anomeric protons ß-glucuronic acid, ß-galactose and two α-L-rhamnose sugars. The two signals at δ 3.62 and 3.76 (3 H, s, each) were assigned for two methoxyl groups while the signals at δ 5.25 was assigned for H-12 olefinic proton. ^{13}C-NMR (C_5D_5N), Table (4), showed signals characteristic for aglycone and 4 sugar moieties assigned for ß-glucuronopyranosyl 6-O-methylester, ß-galactopyranosyl and two α-L-rhamnopyranosyl. The ^{13}C-NMR also suggesting the 1-2 linkage between rhamnopyranosyl and galactopyranosyl and also 1-2 linkage between ß-galactopyranosyl and ß-6-O-methyl glucuronopyanosyl moieties [35]. The ^{13}C-NMR of compound **(6)** is similar to Robinioside-C-methylester [36].

Acid hydrolysis of compound **(6)** gave an aglycone **(6a)**. Its IR indicated the presence of hydroxyl group (s), ketonic group and olefinic bond.

The EIMS of **(6a)** showed M^+ at m/z 502 corresponding to the molecular formula $C_{31}H_{50}O_5$, other peaks at m/z 443 for the loss of $COOCH_3$ group, and retro Diels-Alder fragments at m/z 224 (for A/B rings) and m/z 278 (for D/E rings).

^{1}H-NMR (C_5D_5N) showed the presence of 6 methyl groups, one methoxy group, one hydroxymethyl group and one olefinic proton, so as compound **(6a)** was identified as oxytrogenin methylester.[36]

From all of the above mentioned data, compound **(6)** was identified as 3-O-α-L-rhamnopyranosyl (1→2) ß-galactopyranosyl (1→2) ß-glucuronopyranosyl oxytrogenin methylester 22-O-α-L rhamnopyranoside (Robinioside-C-methylester).

This is the first report for the isolation of this compound from the Genus *Crotalaria*.

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