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Phytochemical Study of *Lotus ornithopodioloides* L.

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Abstract – Phytochemical investigation of the aerial parts of *Lotus ornithopodioloides* L. resulted in the isolation of six known compounds. The structures were determined utilizing physical, chemical, spectral methods as well as direct comparison with reference materials whenever possible. The compounds were identified as: *β*-sitosterol; the two triterpenes oleanolic and betulinic acids; the two cyanogenic glycosides lotaustralin and linamarin in addition to the flavonol diglycoside kaempferitin.
Keywords – *Lotus ornithopodioloides* (Fabaceae), *β*-sitosterol, triterpene acids, cyanogenic glycosides, kaemferitin

Introduction

About 100 members of the genus *Lotus* are present in the north temperate regions especially the Mediterranean and West Asia. About 18 members of the genus *Lotus* are present in the Egyptian flora (Boulos, 1999). In folk medicine, plants of the genus *Lotus* are used as contraceptives, prophylactics and treatment of sexually transmitted disorders and peptic ulcers (El-Mousallami, *et al*., 2002). *Lotus halophilus* has good antimicrobial activity against Gram-positive, Gram-negative bacteria and fungi (Mahasneh, 2002). Previous phytochemical investigations of the genus *Lotus* revealed the presence of cyanogenic glycosides and flavonoids as the major secondary metabolites. The aerial parts are rich in flavone and flavonol derivatives (Abdel-Ghani, *et al*., 2001; El-Mousallami, *et al*., 2002; Reyanaud and Maurice, 1982; Strittmatter, *et al*., 1992; Yang, *et al*., 1989; Ali, *et al*., 2000). The roots are usually rich in isoflavone derivatives (Yang, *et al*., 1989; Mahmoud, *et al*., 1990; Abdel-Kader, *et al*., 2006; Abdel-Kader, 2001).

Experimental

General – Melting points were determined on a mettler FP 80 Central Processor supplied with a Mettler FP 81 MBC Cell Apparatus, and were uncorrected. Ultraviolet absorption spectra were obtained using a Hewlett-Packard HP-845 UV-Vis spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance DRX-500 instrument at 500 MHz for protons and 125 MHz for carbons using the residual solvent signal as internal standard. Coupling constants (*J*) are in Hz. Standard Bruker pulse programs were used for DEPT, 2D NMR COSY, HMBC and HSQC spectra. EI-MS were obtained using Finnegan MAT 300 mass spectrometer. ESI-MS were obtained using Liquid Chromatography/MS Spectrometer (Quattro micro API) equipped with direct prob and a Z- spray electrospray ion source (Micromass®, Quattro micro ™, Waters). Silica gel (70 - 230 mesh , ASTM, Merck) and RP-18 Silica gel F_{254} (Whatmann) were used for column chromatography, Pre-coated silica gel 60 F_{254} plates, 0.25 mm thick, Merck, were used for TLC and PTLC.

Plant material – The plants of *Lotus ornithopodioloides* L. were collected in April, 2005 from the borders of cultivation near Rosetta. The Plant was identified by Dr. S. Kamal, Prof. of plant Taxonomy, Faculty of Science, University of Alexandria. A voucher specimen is deposited in the Pharmacognosy Department, Faculty of Pharmacy, University of Alexandria, Egypt.

Extraction and isolation – The air-dried powdered aerial parts of *Lotus ornithopodioloides* L. (950 g) were exhaustedly extracted with 90% EtOH at room temperature. The alcoholic extract was evaporated under vacuum then water was added to produce a 30% alcoholic extract (300 ml) which was successively extracted with petroleum ether (3 × 300 mL), $CHCl_3$ (3 × 400 mL) and EtOAc (3 × 200 mL).

Part of the petroleum ether soluble fraction (2 g) was

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fractionated over silica gel column (200 g, 2.5 cm i.d.) eluting with C_6H_{14} and C_6H_{14}/EtOAc mixtures in a gradient elution system. Fraction A (450 mg) eluted with C_6H_{14}/EtOAc (20 : 5) was rechromatographed over silica gel column (30 g, 1 cm i.d.) eluting with $CHCl_3$ then $CHCl_3$/MeOH mixtures. 40 fractions of 50 ml each were collected, screened by TLC and similar fractions were pooled. Fractions 20 - 28 (200 mg) eluted with $CHCl_3$ afforded 34 mg of **1** upon crystallization from $CHCl_3$/MeOH mixture. Fractions 38 - 40 (180 mg) eluted with 2% MeOH in $CHCl_3$ afforded 66 mg of **2** upon crystallization from $CHCl_3$/MeOH mixture.

Fraction B (280 g) eluted with C_6H_{14}/EtOAc (20 : 10) was repurified over flash silica gel column (100 g, 2.5 cm i.d.) eluting with $CHCl_3$ then $CHCl_3$/MeOH mixtures. Fractions 15 - 19 eluted with 2% MeOH in $CHCl_3$ afforded 14 mg of **3** upon crystallization from $CHCl_3$/MeOH mixture.

The EtOH extract (4.1 g) was subjected to a silica gel column chromatography using $CHCl_3$ then different ratios of $CHCl_3$-MeOH mixtures till 100% MeOH. Fraction C (800 mg) eluted with $CHCl_3$-MeOH (90 : 10) afforded 388 mg of compund **4** as a major constituent. Fraction D (300 mg) eluted with $CHCl_3$-MeOH (85 : 15) was rechromatographed on medium pressure RP18 silica gel column elution started with 30% MeOH in H_2O increasing the polarity with MeOH. Fractions 12 - 15 (160 mg) eluted with 40% MeOH in H_2O afforded 36 mg of **4**. Fractions 20 - 23 (50 mg) were subjected to PTLC on silica gel plates using EtOAc/MeOH/H_2O (30 : 5 : 4) as solvent system to afford 10 mg of **5** and 9 mg of **6**.

β-sitosterol (1) – $C_{29}H_{50}O$, white powder, m.p. 138 - 140 °C ($CHCl_3$). Selected ^{1}H- NMR ($CDCl_3$): δ 3.45 (1H, *m*, H-3), 2.19 (1H, *bt*, *J* = 17 Hz, H-4), 2.24 (1H, *dd*, *J* = 4, 17 Hz, H-4), 5.28 (1H, *bd*, *J* = 4 Hz, H-5), 0.66 (3H, *s*, H-18), 0.99 (3H, *s,* H-19), 0.91 (3H, *d*, *J* = 6.5 Hz, H-21), 0.80 (3H, *d*, *J* = 7.8 Hz, H-26), 0.78 (3H, *d*, *J* = 8 Hz, H-27), 0.81 (3H, *t*, *J* = 7.7 Hz, H-29). ^{13}C-NMR: Table 1. EI-MS (rel. abund. %): 414 (84), 396 (46), 329 (59), 303 (36), 273 (35), 255 (61), 213 (57) 159 (100).

Oleanolic acid (2) – $C_{30}H_{48}O_3$, white crystals, m.p. 194 - 195 °C (MeOH). Selected ^{1}H- NMR ($CDCl_3$): δ 3.19 (1H, *m*, H-3), 5.24 (1H, *s*, H-12), 2.78 (1H, *dd*, *J* = 3, 12 Hz, H-18), 0.95 (3H, *s*, H-23), 0.65 (3H, *s*, H-24), 0.87 (3H, *s,* H-25), 0.71 (3H, *s*, H-26), 1.09 (3H, *s*, H-27), 0.86 (3H, *s*, H-29), 0.89 (3H, *s*, H-30). ^{13}C-NMR: Table 1. ESI-MS (rel. abund. %): 479 (M^++Na, 100).

Betulinic acid (3) – $C_{30}H_{48}O_3$, white powder, m.p. 283 - 285 °C (MeOH). Selected ^{1}H- NMR (DMSO d_6): δ 2.96 (2H, *m*, H-3 and H-19), 2.23 (1H, *m*, H-13), 0.87 (6H, *s,* H-23 and H-25), 0.80 (3H, *s*, H-24), 0.65 (3H, *s*, H-26), 0.94 (3H, *s*, H-27), 4.56 (1H, *s*, H-29), 4.69 (1H, *s*, H-29), 1.64 (3H, *s*, H-30). ^{13}C-NMR: Table 1. ESI-MS (rel. abund. %): 479 (M^++Na, 100).

Table 1. ^{13}C-NMR data of compounds 1– 3 in $CDCl_3$*

Position	1	2	3
1	37.3	38.8	38.8
2	31.7	27.7	27.4
3	71.8	79.0	79.0
4	42.3	38.4	38.9
5	140.9	55.3	55.3
6	121.7	18.3	18.3
7	31.9	32.5	34.4
8	31.9	41.0	40.7
9	50.2	47.7	50.7
10	36.5	37.1	37.2
11	21.1	22.9	20.9
12	39.8	122.7	25.5
13	40.4	143.0	38.4
14	56.8	45.9	42.5
15	24.3	27.2	30.6
16	28.2	22.9	32.2
17	56.1	46.6	56.4
18	12.0	41.6	49.3
19	19.4	45.9	46.9
20	36.2	30.6	150.2
21	18.8	33.8	29.7
22	34.0	32.7	37.0
23	26.1	28.1	28.0
24	45.9	15.5	15.3
25	29.2	15.3	16.0
26	19.8	17.2	16.1
27	19.0	26.0	14.7
28	23.1	183.0	180.7
29	11.8	33.1	109.7
30	–	23.6	19.4

* Assignments based on DEPT, HSQC, and HMBC experiments and comparison with literature.

Kaempferitin (4) – $C_{27}H_{30}O_{14}$, pale yellow shiny needle crystals, m.p. 201 - 202 °C (MeOH). $UV_{\lambda max}^{MeOH}$: 265, 328, 343; NaOMe: 265, 211, 392; $AlCl_3$: 275, 300, 346, 396; $AlCl_3$/HCl: 275, 298, 342, 396; NaOAc: 264, 349. NMR data (Table 2). FAB-MS (rel. abund. %): 579 ([M^++1], 16).

Lotaustralin [2-(β-D-glucopyranosyloxy)-2-methylbutyronitrile] (5) – $C_{11}H_{19}O_6N$, white needles, m.p. 138 - 139 °C (MeOH). ^{1}H- NMR (Acetone-d_6): δ 1.69 (3H, *s*, H-3), 1.90 (1H, *d*, *J* = 7.0 Hz, H-4), 1.98 (1H, *d*, *J* = 7.0

Table 2. ^{1}H- and ^{13}C-NMR data (δ ppm, *J* in parenthesis in Hz) of **4** in CD_3OD*

Pos.	^{1}H	^{13}C
2	–	159.81
3	–	136.53
4	–	179.81
5	–	163.01
6	6.35 (*d*, *J* = 1.80)	100.60
7	–	163.58
8	6.62 (*d*, *J* = 1.80)	95.67
9	–	158.10
10	–	107.61
1'	–	122.47
2'	7.69 (*d*, *J* = 9.0)	131.99
3'	6.83 (*d*, *J* = 9.0)	116.61
4'	–	161.74
5'	6.83 (*d*, *J* = 9.0)	116.61
6'	7.69 (*d*, *J* = 9.0)	131.99
1''	5.29 (*s*)	103.56
2''	4.13 (br *d*, *J* = 1.5)	71.73
3''	3.60 (*dd*, *J* = 8.5, 3.0)	72.09
4''	3.24 (*m*)	73.66
5''	3.24 (*m*)	71.94
6''	1.16 (*d*, *J* = 6.0)	18.07
1'''	5.46 (br *s*)	99.94
2'''	3.92 (br *d*, *J* = 1.5)	71.94
3'''	3.73 (*dd*, *J* = 3.0,8.5)	72.15
4'''	3.38 (*m*)	73.26
5'''	3.50 (*m*)	71.13
6'''	0.84 (*d*, *J* = 6.0)	17.67

* Assignments based on DEPT, HSQC, and HMBC experiments and comparison with literature.

Hz, H-4), 1.13 (3H, *t*, *J* = 7.0 Hz, H-5), 4.76 (1-H, *d*, *J* = 8.0 Hz, H-1'), 3.29 (1-H, *m*, H-2'), 3.53 (1-H, *m*, H-3'), 3.42 (1-H, *m*, H-4'), 3.46 (1-H, *m*, H-5'), 3.70 (1H, *dd*, *J* = 2.0, 6.0 Hz, H-6') 3.88 (1H, *dd*, *J* = 2.0, 6.0 Hz, H-6'). ^{13}C-NMR (Acetone-d_6): 120.0 (C-1), 74.6 (C-2), 25.5 (C-3), 34.5 (C-4), 9.0 (C-5), 100.3 (C-1'), 74.2 (C-2'), 78.0 (C-3'), 71.7(C-4'), 77.7 (C-5'), 62.4 (C-6'). FAB-MS *m/z* (rel. int. %): 261 (M^+, 23).

Linamarin [2-(β-D-glucopyranosyloxy)-2-methypropionitrile] (6) – $C_{10}H_{17}O_6N$, white shiny needles, m.p. 144 - 145 °C (MeOH). ^{1}H-NMR (Acetone-d_6): δ 1.70 (3H, *s*, H-3), 1.68 (3H, *s*, H-4), 4.64 (1-H, *d*, *J* = 7.5 Hz, H-1'), 3.22 (1-H, *dd*, *J* = 7.5, 8.0 Hz, H-2'), 3.35 (1-H, *m*, H-3'), 3.33 (1-H, *m*, H-4'), 3.42 (1-H, *m*, H-5'), 3.70 (1H, *dd*, *J* = 3.0, 11.5 Hz, H-6') 3.88 (1H, *bd*, *J* = 11.5 Hz, H-6'). ^{13}C-NMR (Acetone-d_6): 122.2 (C-1), 72.7 (C-2), 28.6 (C-3), 27.8 (C-4), 101.2 (C-1'), 72.9 (C-2'), 78.0 (C-3'), 71.4(C-4'), 77.9 (C-5'), 62.7 (C-6'). FAB-MS *m/z* (rel. int. %): 248 ([M^++1], 21).

Results and Discussion

^{1}H- and ^{13}C-NMR (experimental and Table 1) of **1** showed six methyl signals. Two appears as singlets at δ_H 0.66, 0.99 and δ_C 12.0, 19.4; three as doublets at δ_H 0.78, 0.80, 0.91 and δ_C 19.0, 19.8, 18.8; one as triplet at δ_H 0.81 and δ_C 11.8 respectively.

These methyl arrangements as well as the presence of only 29 carbon signals were typical for a steroidal skeleton. The proton at δ_H 5.28 correlated to carbon at δ_C 121.7 in an HSQC experiment as well as the quaternary carbon resonance at δ_C 140.9 were assigned for exocyclic double bond at C-6. The data reported for *β*-sitosterol were in full agreement with those of **1** (Good and Akihisa, 1997).

The ^{1}H- and ^{13}C-NMR of **2** (experimental and Table 2) showed 30 carbon signals with 7 methyl singlets diagnostic for a typical triterpenoidal skeleton with one methyl group replace by carboxylic function (δ_C 183.0). The proton and carbon signals at δ_H 3.19 (1H, *m*) and δ_C 79.0 were assigned for hydroxylated C-3. The quaternary carbon signal at δ_C 143.0 and the methine at δ_C 122.7 correlated in an HSQC experiment with proton triplet at δ_H 5.24 were assigned to an exocyclic double bond at C-12. The data of **2** were typical with those reported for oleanolic acid (Ahmed and Rahman, 1994).

The ^{1}H- and ^{13}C-NMR of **3** (experimental and Table 1) showed 30 carbon signals with 6 methyl singlets, one carboxylic function (δ_C 180.7) and one terminal methylene (δ_H 4.56, 4.69 and δ_C 109.7). These data indicated the presence of a triterpene acid with a lupane skeleton. The data of **3** were consistent with those reported for betulinic acid (Ahmed and Rahman, 1994).

UV data with different shift reagents as well as ^{1}H- and ^{13}C-NMR (experimental) indicated that **4** is a 3,7-diglycosylated kaempferol The presence of two methyl doublet (*J* = 6.0 Hz) at δ_H 1.16, 0.84 and δ_C 18.07, 17.67, respectively in the ^{1}H- and ^{13}C-NMR were diagnostic for two rhamnosyl moieties. HMBC experiments confirmed the attachment of rhamnose at C-3 where correlation were observed between H-1'' at δ_H 5.29 with C-3 at δ_C 136.53 and at C-7 since correlations exist between H-1''' at δ_H 5.46 with C-7 at δ_C 163.58. Acid hydrolysis of **4** afforded the aglycone kaempferol and the sugar rhamnose. The data of **4** were identical with those reported for kaempferol 3,7-di-*O*-*α*-L-rhamnopyranoside (kaempferitin) (Seif El-

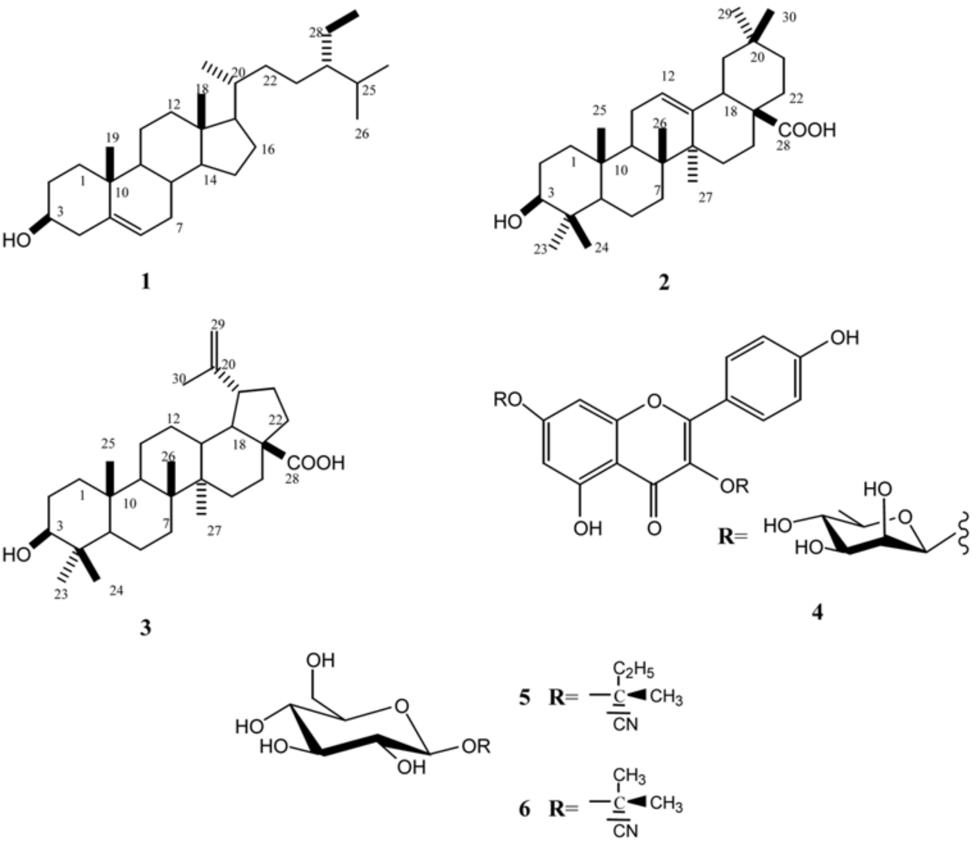

Fig. 1. Structures of compounds **1** - **6**.

Den, *et al.*, 1987). Kaempferitin was reported to exhibit antibacterial effects against *E. coli*. (Abdel-Ghani, *et al.*, 2001), strong antioxidant potential (De Sousa, *et al.*, 2004) and antidiabetic activity (Jorge, *et al.*, 2004). In the genus *Lotus*, It was previously reported from *lotus corniculatus* (Abdel-Ghani, *et al.*, 2001; Reyanaud and Maurice, 1982).

Compounds **5** and **6** have molecular formulae $C_{11}H_{19}O_6N$ and $C_{10}H_{17}O_6N$ as deduced from FAB-MS *m/z* at 261 [M^+] and 248 [M^+ +1] as well as eleven and ten carbon signals in ^{13}C-NMR respectively (experimental). ^{1}H- and ^{13}C-NMR spectra of compound **5** revealed, in addition to sugar signals, the presence of methyl singlet at δ_H 1.69 and δ_C 25.5, CH_2-CH_3 signals at δ_H 1.13 (3H, *t*, $J = 7.0$ Hz), 1.90, 1.98 (1H each, *d*, $J = 7.0$ Hz) and δ_C 9.0, 34.5 respectively. The two quaternary carbon signals at δ_C 74.6 and 120.0 were assigned for oxygenated and cyanohydrin's carbons. All the assignments were based on DEPT, COSY, HSQC and HMBC experiments.

Comparison with the published data indicated that **5** is lotaustralin (Pitsch, *et al.*, 1984; Valen, 1979). In **6** the CH_2-CH_3 signals were replaced by another methyl singlet at δ_H 1.70, δ_C28.61. The data of **6** based on 2D-NMR experiments were consistent with those reported for linamarin (Valen, 1979). These two cyanogenic glycosides are of common occurrence in *Lotus* species (Abdel-Ghani, *et al.*, 2001; Reyanaud and Maurice, 1982; Seigler, 1975a; Seigler, *et al.*, 1975b; Yang, *et al.*, 1989).

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