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Studies on the Constituents from the Herbs of *Ajuga multiflora* (II)

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Abstract – Continuing to previous report, seven compounds were isolated from the aerial parts of *Ajuga multiflora*. The structures of them were established as Di-2-ethylhexyl phthalate (1), ursolic acid (2), sterol glucoside (3), 20-hydroxyecdysone (4), makisterone A (5), cyasterone (6) and apigenin 7-glucuronide (7), respectively.

Key words – *Ajuga multiflora*; Labiatae; cyasterone; 20-hydroxyecdysone; makisterone A; apigenin 7-glucuronide.

In previous paper[1)], we reported the isolation of apigenin and two iridoid glucosides, 8-*O*-acetylharpagide and harpagide from *A. multiflora*. In further phytochemical work on this plant, we isolated seven compounds.

This paper describes the structure elucidation of these compounds.

Experimental

General experimental procedures – The mps were taken on a Yanaco micro-melting point apparatus and are uncorrected. The IR spectra were determined in KBr tablets on a Mattson Polaris TM (FT-IR) spectrophotometer and the UV spectra were run with a Varian DMS 200 UV-Vis spectrophotometer. The EI-MS and FAB-MS spectra were recorded on a JMS SX-102A and JMS HX-110/110A (JEOL) spectrometer. The ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker DRX-500 or Brucker AMX-300 spectrometer with TMS as an intenal standard and chemical shifts are given a ppm. TLC chromatography was performed on precoated Kieselgel 60 F_{254} plates (Merck, 5715).

Plate material – See previous report[1)].

Extraction and isolation – The chopped herbs of *A. multiflora* (2.5 kg) were extracted with MeOH under reflux (three times, 12 h each). The combined MeOH extracts were evaporated under reduced pressure, to give a brown residue (302 g), which was partitioned with *n*-hexane, EtOAc, *n*-BuOH and water, successively. EtOAc fraction (11 g) was chromatographed on silica gel with increasing concentration of MeOH in $CHCl_3$ as eluents to give six compounds (1~6). *n*-BuOH fraction (50 g) was chromatographed on silica gel with $CHCl_3$-MeOH-H_2O (52:28:8, lower layer) as eluent to obtain compound 7.

Compound 1 – Yellowish oil. UV λ_{max} (MeOH) (logε) 224 (4.0), 274 (3.2). EI-MS *m/z* 390 $[M]^+$, 149 [phthalic anhydride+H]$^+$ (base peak), 104 $[2\times C_4H_9]^+$, 57 $[C_4H_9]^+$. ^1H-NMR (300 MHz, $CDCl_3$) δ 0.87 (6H, t, J=7.3 Hz, $2\times CH_3$), 0.91 (6H, t, J=7.4 Hz, $2\times CH_3$), 1.24~1.46 (16H, m, $8\times CH_2$) 1.66 (2H, m, 3'and 3"-CH), 4.21

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(4H, dd, J=6.0, 3,6 Hz, 2′ and 2″-CH_2), 7.50 (2H, dd, J=6.0, 3.3 Hz, 3 and 4-CH), 7.69 (2H, dd, J=6.0, 3.3 Hz, 2 and 5-CH). ^{13}C-NMR (75.5 MHz, $CDCl_3$) δ 11.6 (2×CH_3), 14.6 (2×CH_3), 23.6 (CH_2), 24.4 (CH_2), 29.5 (CH_2), 31.0 (CH_2), 39.3 (C-3′ and 3″), 68.7 (C-2′ and 2″), 129.4 (C-3 and 4), 131.5 (C-2 and 5), 133.1 (C-1 and 6), 168.3 (C-1′ and 1″).

Compound 2-A white amorphous powder form MeOH, mp 290~292℃, LB test: positive. IR ν_{max} (KBr) 3445 (OH), 1692 (carboxylic C=O), 1628 (C=C) cm^{-1}. EI-MS *m/z* 456 $[M]^+$, 438 $[M-H_2O)^+$, 248 (D/E ring, base peak), 203 $[248-COOH]^+$. ^{1}H-NMR (300 MHz, $CDCl_3$+DMSO-d_6) δ 0.77, 0.82, 0.92, 0.98, 1.08 (each 3H, s, CH_3), 0.85 (3H, d, J=6.4 Hz, CH_3), 0.93 (3H, d, J=8.6 Hz, CH_3), 2.19 (1H, d, J=11.2 Hz, H-18), 3.18 (1H, dd, J=5.5, 7.7 Hz, H-3), 5.23 (1H, brs, H-12). ^{13}C-NMR (75.5 MHz, $CDCl_3$ +DMSO-d_6) see Table II.

Compound 3-A white amorphous powder from MeOH, mp 298~299℃, LB test: positive, Molisch test: positive. ^{1}H-NMR (300 MHz, pyridine-d_5) δ 0.66 (3H, s, 18-CH_3), 0.84, 0.87, 0.89 (each 3H, s, 29, 27, 26-CH_3), 0.93 (3H, s, 19-CH_3), 0.98 (3H, d, J=6.4 Hz, 21-CH_3), 5.04 (1H, d, J=7.7 Hz, anomeric H), 5.35 (1H, brd, J=4.5 Hz, H-6).

Compound 4-A white needles from aquous MeOH, mp 237~239℃, LB test: positive. IR ν_{max} (KBr) 3429 (OH), 1651 (α,β-unsaturated C=O) cm^{-1}. FAB-MS *m/z* (rel. int.) 503 $[M+Na]^+$ (23.59), 481 $[M+H]^+$ (57.44), 463 $[M+H-H_2O]^+$ (91.62), 445 $(M+H-2H_2O)^+$ (75.97), 427 $[M+H-3H_2O]^+$ (32.55), 409 $[M+H-4H_2O]^+$ (5.75), 391 $[M+H-5H_2O]^+$ (5.50), 363 (C-20/C-22 fission) (22.51). ^{1}H-NMR (500 MHz, CD_3OD) see Table I. ^{13}C-NMR (125 MHz, CD_3OD) see Table II.

Compound 5-A white amorphous powder from MeOH, mp 260~262℃, LB test: positive. IR ν_{max} (KBr) 3431 (OH), 1658 (α,β-unsaturated C=O) cm^{-1}. FAB-MS *m/z* (red. int.) 517 $[M+Na^+]$ (10.36), 495 $[M+H]^+$ (59.11), 477 $[M+H-H_2O]^+$ (15.58), 459 $[M+H-2H_2O]^+$ (25.55), 441 $[M+H-3H_2O]^+$ (10.04), 363 (C-20/C-22 fission) (20.21). ^{1}H-NMR (500 MHz, CD_3OD) see Table I. ^{13}C-NMR (125 MHz, CD_3OD) see Table II.

Compound 6-A white needles from MeOH, mp 164~165℃, LB test: positive. IR ν_{max} (KBr) 3430 (OH), 1750 (γ-lactone), 1649 (α,β-unsaturated C=O) cm^{-1}. FAB-MS *m/z* (rel. int.) 521 $(M+H)^+$ (25.24), 503 $[M+H-H_2O]^+$

Table I. ^{1}H-NMR spectral data of compounds 4~6 (500 MHz)[a]

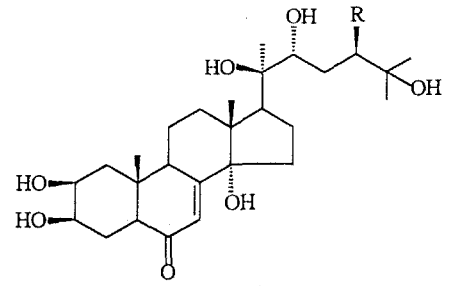
Position	4[b]	5[b]	6[c]
H-2	3.85 (1H, m)	3.85 (1H, m)	4.16 (1H, m)
H-3	3.97 (1H, brd, 2.2)	3.97 (1H, brd, 2.1)	4.21 (1H, brd, 2.2)
H-5	2.40 (1H, m)	2.39 (1H, m)	3.00 (1H, dd, 13.1, 3.5)
H-7	5.83 (1H, d, 2.4)	5.83 (1H, d, 2.2)	6.28 (1H, d, 2.2)
H-9	3.17 (1H, m)	3.17 (1H, t, 8.1)	3.60 (1H, dd, 9.8, 8.4)
H-17	2.40 (1H, m)	2.37 (1H, m)	2.87 (1H. t. 9.2)
H-18	0.91 (3H, s)	0.92 (3H, s)	1.24 (3H, s)
H-19	0.99 (3H, s)	0.99 (3H, s)	1.08 (3H, s)
H-21	1.22 (3H, s)	1.21 (3H, s)	1.57 (3H, s)
H-22	3.34 (1H, d, 10.7)		3.94 (1H, brd, 9.3)
H-25		3.48 (1H, d, 10.6)	2.38 (1H, m)
H-26	1.21 (3H, s)[d]	1.18 (3H, s)[d]	
H-27	1.22 (3H, s)[d]	1.17 (3H, s)[d]	1.36 (3H, d, 7.0)
H-28		0.96 (3H, d, 6.8)	4.03 (1H, qd, 6.1, 3.2)
H-29			1.32 (3H, d, 6.1)

[a]Chemical shifts (δ) are expressed in ppm from internal standard (TMS) and coupling constant (J) are in Hz. [b]measured in CD_3OD. [c]measured in pyridine-d_5. [d]Assignment may be interchangeable.

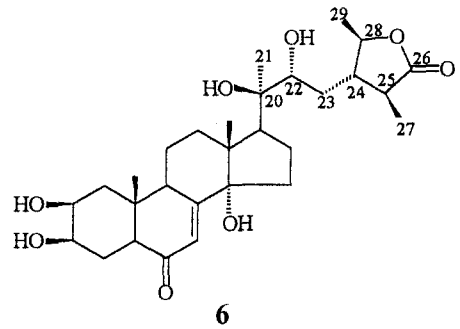
Table II. ^{13}C-NMR spectral data of compounds 2, 4, 5 and 6 (125 MHz)

position	2[a]	4[b]	5[b]	6[c]
C-1	38.9	37.9	37.9	38.0
C-2	27.5	69.2	69.2	68.1
C-3	78.8	69.0	69.0	68.1
C-4	39.5	33.0	33.0	32.5
C-5	55.8	52.3	52.3	51.4
C-6	18.6	206.9	206.9	203.4
C-7	33.3	122.6	122.7	121.9
C-8	39.4	168.4	168.4	165.8
C-9	47.7	35.6	35.6	34.5
C-10	37.2	39.8	39.8	38.7
C-11	23.5	22.0	22.0[e]	21.1
C-12	125.6	33.3	33.4	32.1
C-13	138.6	49.1[d]	49.0[d]	48.2
C-14	42.3	85.7	85.7	84.2
C-15	28.3	32.3	32.3	31.9
C-16	34.4	22.0	21.9[e]	21.4
C-17	47.8	51.0	51.0	50.0
C-18	52.9	18.5	18.6	17.9
C-19	39.1	24.9	24.9	24.5
C-20	39.0	78.4	78.5	76.8
C-21	30.9	21.6	21.5	21.0
C-22	37.0	78.9	75.9	74.0
C-23	28.5	27.8	35.0	34.5
C-24	16.1	42.9	42.2	48.7
C-25	15.8	71.8	74.3	42.5
C-26	17.4	29.5[e]	26.6	179.2
C-27	23.8	30.2[e]	28.0	15.9
C-28	180.2		15.4	79.8
C-29	17.4			19.4
C-30	21.5			

[a]measured in $CDCl_3$+DMSO-d_6. [b]measured in CD_3OD. [c]measured in pyridine-d_5. [d]overlapped with intensive solvent multiplet. [e]assignment may be interchangeable.



4 R = H
5 R = CH_3



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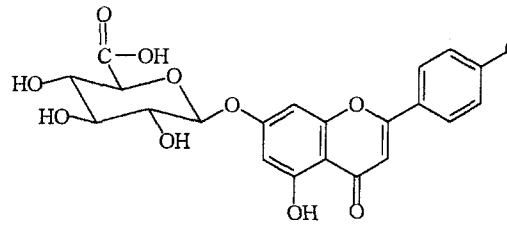

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(21.53), 485 $[M+H-2H_2O]^+$ (6.0), 363 (C-20/C-22 fission) (22.05). ^{1}H-NMR (500 MHz, pyridine-d_5) see Table I. ^{13}C-NMR (125 MHz, pyridine-d_5) see Table II.

Compound 7 – A yellow amorphous powder from MeOH, mp>300℃, $FeCl_3$, Mg/HCl tests: positive, Molish test: positive. IR ν_{max} (KBr) 3423 (OH), 1655 (α,β-unsaturated C=O), 1607, 1499 (C=C), 1071 (glycosidic CO) cm^{-1}, UV λ_{Max} (50% MeOH) (logε) 268 (4.28), 338 (4.34). FAB-MS *m/z* 447 $[M+H]^+$, 271 [genin+H]$^+$. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 5.10 (1H, d, *J*=7.5 Hz, anomeric proton), 6.41 (1H, brs, H-6), 679 (1H, brs, H-8), 6.90 (2H, d, *J*=10.0 Hz, H-3′ and 5′), 7.88 (2H, d, *J*=10.0 Hz, H-2′ and 6′). ^{13}C-NMR (125 MHz, DMSO-d_6) δ 164.2 (C-2), 102.9 (C-3), 181.9 (C-4), 160.4 (C-5), 99.4 (C-6), 162.9 (C-7), 94.6 (C-8), 156.9 (C-9), 106.0 (C-10), 120.6 (C-1′), 128.4 (C-2′ and C-6′), 116.0 (C-3′ and C-5′), 161.7 (C-4′), 99.5 (C-1″), 72.9 (C-2″), 76.3 (C-3″), 71.9 (C-4″), 74.3 (C-5″), 173.0 (C-6″).

Results and Discussion

Column chromatography of EtOAc and *n*-BuOH fractions of MeOH extract afforded seven compounds, three of which were identified as Di-2-ethylhexyl phthalate 1, ursolic

acid 2 and sterol glucoside 3 by comparison of spectral data with those of the reported in literature[2-4] as well as direct comparison with authentic samples. Since 1 and related phthalates are widely used in the plastics industry and are indicators of environmental pollution, 1 may not be a consituent of this plant.

Compound 4 was positive in the Liebermann-Burchard reaction and showed a strong hydroxyl group absorption band and α,β-unsatruated ketone absorption band in its ir spectrum. In the FAB-MS spectrum, 4 exhibited the pseudomolecular ion peak at *m/z* 503 $[M+Na]^+$ and 481 $[M+H]^+$ corresponding to the molecular formular $C_{27}H_{44}O_7$. The ^{1}H-nmr spectrum displayed signals due to five tertiary methyl groups, three signals due to protons attached to a carbon bearing hydroxyl groups and one olefinic proton at δ 5.83 (d, *J*=2.4 Hz). The ^{13}C-nmr spectrum showed signals for 27 carbon atoms. The multiplicity assignments were made by DEPT experiments. The fragment ion peak at *m/z* 363 arising from the C-20/C-22 cleavage in the ms spectrum and the charcateristic ^{13}C-nmr signals at δ 206.9 (s), 168.4 (s), 122.6 (d), 69.2 (d) and 69.0 (d) in accord with the occurrence of a 2β,3β-dihydroxy-7-en-6-one system strongly suggested that this compound is a phytoecdysteroid. Detailed analysis of ^{1}H-^{1}H COSY and HMQC data as well as the comparison with literature data[5] enabled to confirm 4 is 20-hydroxyecdysone (ecdysterone).

Compound 5 showed an ir spectrum similar to that of 4, suggesting it to be an ecdysteroid. Its molecular weight was deduced to be 494 by FAB-MS spectrum and the C-20/C-22 fission ion peak was also shown at *m/z* 363. On the comparison of nmr spectrum with that of 4, one additional methyl group was observed in 5. The ^{13}C-nmr signals of 5 for C-1→C-21 are superimposable to those of 4. And two terminal methyl singlets (26-CH_3 and 27-CH_3) were observed in the ^{1}H-nmr spectrum. Thus, one additional methyl group must be located on C-23 or C-24. The signal for C-24 at δ 42.9 (t) in 4 is displaced by δ 42.2 (d) in 5 but the multiplicity for C-23 (t) is not changed. thus, C-28 methyl group is located on C-24. The comparison of the ^{13}C-nmr data of makisterone A and 24-epimakisterone A reported by Miller *et. al*[6] with those of 5 resulted that the data of 5 resembles those of makisterone A in all respects.

Compound 6 gave positive Liebermann-Burchard test and showed γ-lactone absorption band at 1750 cm^{-1} in the ir spectrum. In the FAB-MS spectrum, 6 exhibited the pseudomolecular ion peak at *m/z* 521 $[M+H]^+$ and the ion peak at *m/z* 363 arising from the C-20/C-22 cleavage. In the ^{1}H-nmr spectrum, two tertiary methyl groups at δ 1.21 (s) and δ 1.22 (s) shown in 4 were disappeared. Instead, Two secondary methyl groups at δ 1.32 (d, *J*=6.1 Hz) and 1.36 (d, *J*=7.0 Hz) were observed. In the ^{13}C-nmr spectrum, The signals at δ 179.2 (C-26) and 79.8 (C-28) strongly indicated the presence of lactone moiety. In the light of above findings, 6 is identified as cyasterone and the literature data supported the result.[7]

Compound 7 was positive in the $FeCl_3$, Mg/HCl and Molisch tests, suggesting that it is flavonoid glycoside. On acid hydrolysis 7 liberated D-glucoronic acid and an aglycone, apigenin. In the FAB-MS spectrum, the pseudomolecular ion at *m/z* 447 $[M+H]^+$ was oberved. Thus, 7 is an apigenin monoglucuronide. On the comparison of the ^{13}C-nmr chemical shifts of 7 with those of apigenin, the signals correspoonding to C-6, C-7 and C-8 of 7 revealed glycosidation shifts at C-6

(+0.8 ppm), C-7 (-1.2 ppm) and C-8 (+0.7 ppm), suggesting that glucuronic acid unit was attached at C-7 of apigenin. The configuration of sugar moiety was determined by J value of the anomeric proton signal. Accordingly, the structure of 7 was elucidated as apigenin 7-*O*-β-D-glucuronoside.

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