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Studies on Index Compounds for HPLC Analysis of *Glycyrrhiza macedonica*

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As index compounds for HPLC analysis of *Glycyrrhiza macedonica* BOISS. et ORPH., from the aerial parts, 7 compounds were isolated and from the subterranean parts, 5 compounds including 4 new compounds were isolated. Of the 12 compounds, 8 compounds were identified as schaftoside, rutin, bioqurcetin, isoquercitrin, kaempferol-3-*O*-rhamnosylgalactoside, nicotiflorin, astragalin and yunganoside L2 and the 4 new compounds (macedonosides A - D) were formulated as 3-*O*-β-D-glucuronopyranosyl-(1→2)-β-D-glucuronopyranosyl-3β, 21α-dihydroxy-11-oxo-12-oleanen-29-oic acid, -isoechinatic acid, -macedonic acid, and -3β-hydroxy-22-oxo-11, 13(18)-oleanadien-29-oic acid, respectively.

Keywords : *Glycyrrhiza macedonica* ; Leguminosae ; aerial part ; subterranean part ; MeOH extract ; HPLC analysis

In a previous report, we demonstrated that MeOH extracts of the aerial parts of 163 samples of *Glycyrrhiza (G.) uralensis, G. glabra, G. pallidiflora, G. echinata,* and *G. macedonica*, grown in 43 medicinal plant research stations and gardens in Japan, can be grouped into 7 types according to the HPLC profiles with the peaks mostly of 17 flavonoids and isoflavonoids.[1] Furthermore, we reported the index compounds for HPLC analysis of EtOAc extracts of the subterranean parts of *G. uralensis* and *G. glabra*.[2,3] A preliminary HPLC analysis of MeOH extracts of aerial and subterranean samples of *G. macedonica* BOISS. et ORPH. (= *G. foetida* JACQ. = *G. echinata* var. *frearitis* BOISS. = *G. frearitis* ORPH. ex BOISS.)[4] provided reproducible peak patterns. The present report deals with characterization of the index compounds for HPLC analysis of the aerial and subterranean parts of *G. macedonica*.

EXPERIMENTAL

General The equipments used in this investigation were as follows : a Yanagimoto micromelting apparatus (for melting points, uncorrected) ; a Shimadzu UV-2100 (for UV spectra, measured at 25℃); a JASCO digital polarimeter (for specific rotation, measured at 25℃); a Perkin-Elmer 1720X-FT IR spectrometer (for IR spectra); a Hitachi M-80 spectrometer (for MS spectra) ; a Varian Mercury 300, unity Inova -500 (for NMR spectra, measured in pyridine-d_5, on the δ scale using tetramethylsi-

Table 1. Morphological Features of *G. macedonica* Cultivated in the Garden of Osaka University of Pharmaceutical Sciences

Stems	1.5 - 2.0 m, branching glabrous but dotted with small glands in upper parts.
Leaves	8 - 14 cm long, short-petioled with 3 - 6 pairs of leaflets, malodorous in summer.
Leaflets	obovate, 2 - 4 cm long, 0.7 - 2 cm wide, cuneate at base, short-mucronate at apex dotted on both sides with small glands.
Peduncles	glandular, 1 - 3 cm long.
Inflorescence	denseracemes, compact, oblong, 1.5 - 3 cm long.
Flowers	6 - 7.5 mm long, pale violet, subsessile, calyx ca 2.5 - 3 mm long, teeth slightly shorter than tube, two upper teeth much shorter than the others, dotted with yellowish glands, standard> wing ≫ keel, pale violet limb of standard, oblong - ovate, tapering into short claw, pale violet limb of wing, lanceolate, tapering into thin claw, deep violet limb of keel, lanceolate, tapering into thin claw.
Pods	tightly crowded in compact subspherical heads ca 2 - 4 cm long, valves, oblong -obate ca 11 - 15 mm long, 5 - 6 mm wide, sparsely covered with thin and rather long reddish prickles since young.

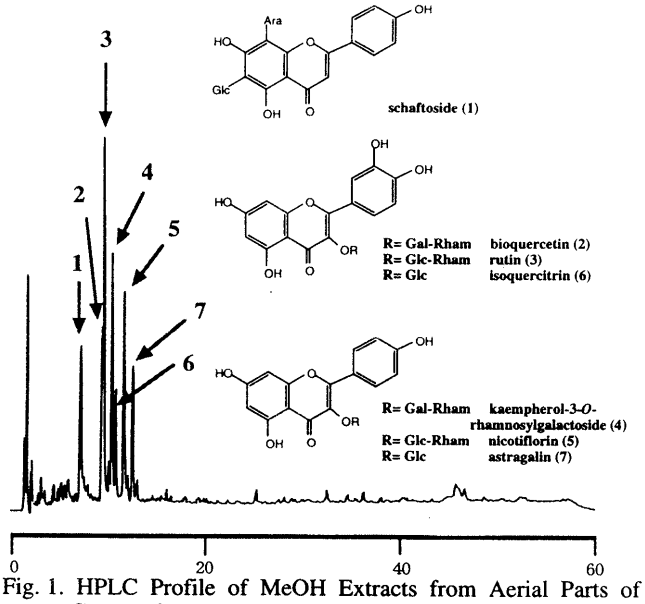


Fig. 1. HPLC Profile of MeOH Extracts from Aerial Parts of *G. macedonica* and Structures of the Index Compounds

Photo 1

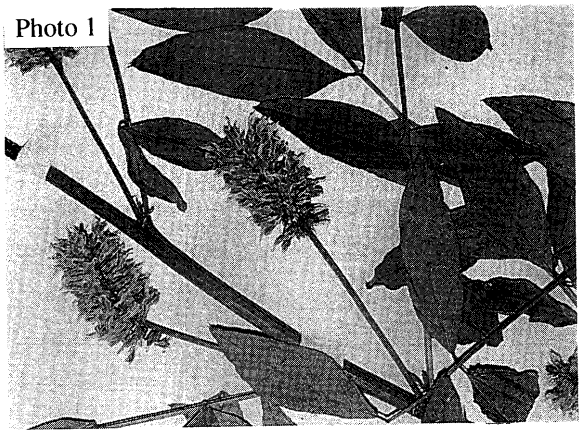
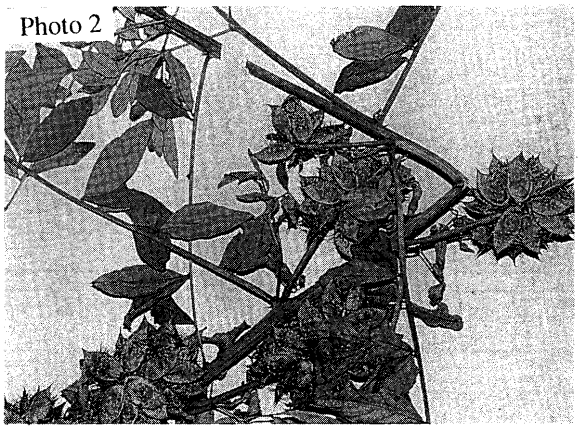


Photo 2



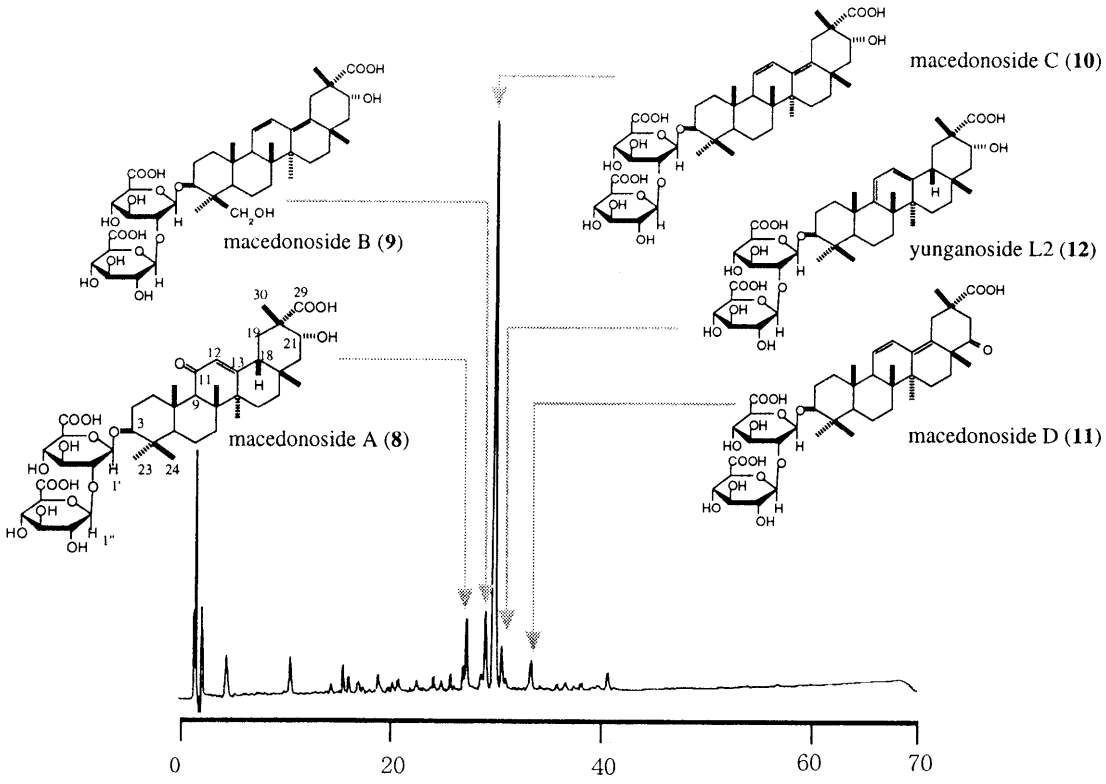


Fig. 2. HPLC Profile of MeOH Extracts from Subterranean Parts of *G. macedonica* and Structures of the Index Compounds

lane as an internal standard). Chromatography was carried out on a Diaion HP-20, a silica gel (Wakogel C-200) and an ODS-A YMC column. HPLC was performed on a JASCO PU-980 pump equipped with a JASCO 875-UV detector, 254 nm, and a JASCO Model 807-IT Integrator. TLC was carried out on precoated Kieselgel $60F_{254}$ (Merck) or RP-$8F_{254}$ (Merck) reversed-phase plates with *n*-BuOH-H_2O-AcOH (7:2:1) and MeOH−H_2O (1:1) as developing solvents, and the spots were detected either by spraying of 40% H_2SO_4 followed by heating, or by exposure under a UV lamp.

Materials for HPLC analysis

Rhizomic parts of *G. macedonica* obtained from Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, were transplanted to the Medicinal Plant Research Station of Osaka University of Pharmaceutical Sciences and cultivated for 4 years. Aerial samples of this plant were collected in June, July and August, in 1996, 1997 and 1998 , and their MeOH extracts were submitted to HPLC analysis(Fig.1).[1] Subterranean parts were collected in October, 1997 and September, 1998, and the MeOH extracts were submitted to HPLC analysis (Fig.2) and the isolated index compounds were characterized. The HPLC peak pattern analysis conditions were as follows : column ; CrestPak C-18s (*i.d.* 4.6×150 mm, JASCO Co. Ltd.) ; column temperature ; 40 ℃ ; flow rate ; 1 ml/min ; detection ; UV 254 nm. The gradient mobile phase consisted of 1% acetic acid in water (A) and 1% acetic acid in acetonitrile (B)(from A／B=90／10 to A／B=30／70 in 65 min).

Botanical identification of *G. macedonica*

The external morphological features of buds from the rhizomes in the middle of April (the garden station of Osaka University of Pharmaceutical Sciences, Takatsuki, Osaka, Japan), were as shown in Table 1. By the characters of the leaf samples with flowers and pods (photo 1, 2) the plant was identified as *G. macedonica*. Vouchers specimens are deposited in the Herbarium of the faculty of Sciences, Tohoku University and in the Osaka University of Pharmaceutical Sciences.

Chemical identification of schaftoside (1), bioqurcetin (2), rutin (3), kaempferol 3-*O*-rhamnosylgalactoside (4), nicotiflorin (5), isoquercitrin (6) and astragalin (7) from the aerial parts

Dried aerial parts (500 g) of *G. macedonica* were extracted with hot MeOH (1000 ml) three times. After evaporation of the solvent *in vacuo*, the extract was analyzed by HPLC to provide 7 major peaks. Then, it was subjected to preparative HPLC to give compounds **1** - **7**, which were identified as schaftoside (**1**), bioqurcetin (**2**), rutin (**3**), kaempferol-3-*O*-rhamnosylgalactoside (**4**), nicotiflorin (**5**), isoquercitrin (**6**), and astragalin (**7**), respectively by the direct comparison of HPLC or by the comparison of their spectral data with those reported.[1]

Isolation of macedonosides A (8), B (9), C (10), D (11), and yunganoside L2 (12)[5] from the subterranean parts

Dried subterranean parts (500 g) of *G. macedonica* were extracted with hot 50 % EtOH three times. HPLC of the extract provided 5 peaks. Chromatography on silica gel and subsequent preparative HPLC gave compounds **8** - **12**. HPLC conditions were as follows : column ; CrestPak C-18 (*i.d.* 10.0×250 mm, JASCO Co. Ltd.) ; column temperature ; 40 ℃ ; flow rate ; 2 ml/min ; detection ; UV254nm. The mobile phases were flowed gradiently with 1% acetic acid in water (A) and 1% acetic acid in acetonitrile (B), from A／B=90／10 to A／B=30／70 in 90 min.

macedonoside A (8) colorless amorphous powder ; mp 213 - 215℃ ; $[\alpha]_D$ +14.0° (*c*=0.12, MeOH) ;UV(MeOH) λ max nm (log ε) : 251(4.04) ; $C_{42}H_{62}O_{17}$. pos. HR-SIMS *m/z*: 861.3898 ($[M+Na]^+$): error, 1.7 mmu.; IR ν (KBr) cm^{-1}: 3402, 1657.; ^1H- and ^{13}C-NMR(pyridine-d_5): Table 2, 3.

macedonoside B (9) pale yellow amorphous powder ; mp 208 - 210℃ ; $[\alpha]_D$ -53.8° (*c*=1.07, MeOH) ;UV(MeOH) λ max nm (log ε) : 241 (4.07), 249 (4.12), 257 (3.96); $C_{42}H_{62}O_{17}$. pos. HR-SIMS *m/z*: 839.4052 ($[M+H]^+$): error, -1.0 mmu.; IR ν (KBr) cm^{-1}: 3432, 1734, 1419.; ^1H- and ^{13}C-NMR(pyridine-d_5): Table 2, 3.

macedonoside C (10) pale yellow amorphous powder ; mp 218 - 220℃ ; $[\alpha]_D$ -33.1° (*c*=1.10, MeOH) ;UV(MeOH) λ max nm (log ε) : 241 (4.15), 249 (4.20), 257 (4.06); $C_{42}H_{62}O_{16}$. pos. HR-SIMS *m/z*: 845.3962 ($[M+H]^+$): error, -0.2 mmu.; IR ν (KBr) cm^{-1}: 3401, 1735, 1419.; ^1H- and ^{13}C-NMR(pyridine-d_5): Table 2, 3.

macedonoside D(11) pale yellow amorphous powder ; mp 219 - 220℃ ; $[\alpha]_D$ -21.6° (*c*=0.55, MeOH) ;UV(MeOH) λ max nm (log ε) : 250 (4.96) ; $C_{42}H_{62}O_{16}$. pos. HR-SIMS *m/z*: 843.3798 ($[M+Na]^+$): error, 2.3 mmu.; IR ν (KBr) cm^{-1}: 3420, 1706, 1349.; ^1H- and ^{13}C-NMR(pyridine-d_5): Table 2, 3.

yunganoside L2 (12) colorless amorphous powder ; mp 210 - 212℃ ; $[\alpha]_D$ +53.0° (*c*=0.31, MeOH) ;UV(MeOH) λ max nm (log ε) : 279(3.63) ; $C_{42}H_{62}O_{16}$. pos. HR-SIMS *m/z*: 845.3928 ($[M+Na]^+$): error, -0.4 mmu.; IR ν (KBr) cm^{-1}: 3410, 1723.

RESULTS AND DISCUSSION

The plants examined in this study were obtained from Tsukuba Medicinal Plant Station (TS-2729-82-177, as *G. echinata* from Italy). Kyoto Herbal Garden, Takeda Chemical Industry has the same plant as *G. foetida* DESF.[9] from Italy. Both plants were identified as *G. macedonica* BOISS. et ORPH. by observation of the external morphological features, and by examination of leaf samples with flowers and pods. The HPLC profiles of MeOH extracts of the aerial parts and of the subterranean parts were similar. The morphological features are different from the descriptions of *G. foetida* DESF., especially, in the height of stems and color of the standard petal.

The aerial parts and the subterranean parts of *G. macedonica* were submitted to HPLC analysis. The MeOH extract of the aerial parts provided the reproducible HPLC profile partially similar to those of *G. pallidiflora* and *G. echinata*. By the direct comparison of the HPLC and spectral data, the peaks were identified as shaftoside (**1**) and isoquercitrin (**6**), which had been isolated from *G.*

Table 2 ^{1}H-NMR Data of **8** - **11** in Pyridine-d_5 (ppm)

	macedonoside A (**8**)	macedonoside B (**9**)	macedonoside C (**10**)	macedonoside D (**11**)
1	3.11 br.d (13.5), 1.05*	0.76*, 1.62*	0.78*, 1.62*	0.72*, 1.61*
2	2.34*, 2.10 m	2.02 m, 2,27 m	1.94*, 2.27 m	1.90 m, 2.23 m
3	3.37 dd (4.5, 11.5)	3.46*	3.30 dd (4.5, 11.5)	3.27 dd (4.5, 11.5)
5	0.70 br.d (11.5)	0.90*	0.70*	0.68 br.d (12.0)
6	1.30*	1.58*, 1.74*	1.27*, 1.50*	1.24*, 1.50*
7	1.18*, 1.46*	1.18*, 1.26*	1.21*	1.20*, 1.27*
9	2.45 br.s	1.85 br.s	1.85 br.s	1.77 br.s
11	-	5.58 br.d (10.5)	5.60 br.d (10.5)	5.57 br.d (10.0)
12	5.90 s	6.90 dd (3.0, 10.5)	6.88 dd (2.5, 10.5)	6.70 dd (2.5, 10.0)
15	1.16*, 1.69*	0.94 br.d (9.5), 1.65*	0.95*, 1.62*	1.05 m, 1.66*
16	1.29*	1.44*, 1.66*	1.42 m, 1.62*	2.02 m, 2.10 m
18	2.37* m	-	-	-
19	α : 3.19 t (14.0), β : 1.73*	α : 3.47 d (14.0), β : 1.93 d (14.0)	α : 3.46 d (14.5), β : 1.91 d (14.5)	α : 3.44 br.d (14.0), β : 2.37 d (14.0)
21	4.52 m	4.04 dd (4.5, 11.5)	4.00 dd (4.5, 12.0)	α : 3.09 dd (2.5, 15.5), β : 2.63 d (15.5)
22	1.87 dd (3.0, 12.0), 1.79* dd (3.0, 12.0)	2.10 m	2.01 t (12.0), 2.09 dd (4.5, 12.0)	-
23	1.41 s	1.47 s	1.38 s	1.33 s
24	1.27 s	4.40*, 3.70 d (11.5)	1.18 s	1.14 s
25	1.25 s	0.83 s	0.82 s	0.80 s
26	1.08 s	0.74 s	0.73 s	0.74 s
27	1.49 s	0.98 s	0.95 s	0.85 s
28	0.92 s	1.20 s	1.19 s	1.38 s
30	1.47 s	1.69 s	1.66 s	1.47 s
1'	5.09 d (7.5)	5.09 d (7.5)	5.06 d (7.5)	5.01 d (7.5)
2'	4.32 dd (7.5, 8.5)	4.38*	4.27*	4.23 dd (7.5, 8.5)
3'	4.45 dd (8.5, 8.5)	4.45*	4.39 dd (8.5, 8.5)	4.40 dd (8.5, 8.5)
4'	4.61 dd (8.5, 8.5)	4.61*	4.57 dd (8.5, 8.5)	4.52*
5'	4.67*	4.67 d (9.0)	4.56*	4.58 *
1"	5.48 d (7.5)	5.75 d (7.5)	5.39 d (7.5)	5.34 d (7.5)
2"	4.30 d (7.5, 8.5)	4.33*	4.24*	4.19 dd (7.5, 8.5)
3"	4.37 dd (8.5, 8.5)	4.62*	4.30*	4.28 dd (8.5, 8.5)
4"	4.69 dd (8.5, 8.5)	4.62*	4.58*	4.54*
5"	4.65*	4.62*	4.56*	4.55*

*: overlapped signals
Figures in parentheses are coupling constants in Hz.

pallidiflora and *G. echinata*, rutin (**3**) and kaempferol 3-*O*-rhamnosylgalactoside (**4**), which had been isolated from *G. echinata*, astragalin (**7**), which had been isolated from *G. pallidiflora*, bioquercetin (**2**) and nicotiflorin (**5**). **2** and **5** were detected only in *G. macedonica*, and thus, these are the index compounds for distinguishing this species from *G. pallidiflora* and *G. echinata*.

Table 3 ^{13}C-NMR Data of **8**, **9**, **10**, **10a**, and **11** in Pyridine-d_5 (ppm)

	8	**9**	**10**	**10a**	**11**
1	39.68	38.29	38.21	38.46	38.08
2	26.94	26.71	26.64	27.82	26.57
3	89.29	89.99	89.45	77.99	89.32
4	40.13	44.28	39.74	39.46	39.68
5	55.55	55.74	55.38	55.26	55.25
6	30.30	19.43	18.49	18.77	18.37
7	33.02	32.98	32.63	32.60	32.46
8	45.57	40.76	40.73	40.71	40.33
9	62.21	54.60	54.56	54.65	54.34
10	37.42	36.45	36.56	36.79	36.44
11	199.76	126.45	126.51	126.54	127.50
12	128.50	126.84	126.68	126.67	125.98
13	170.10	134.49	135.61	134.49	135.13
14	44.06	42.65	42.61	42.57	42.19
15	27.02	24.69	24.63	24.56	24.09
16	30.50	36.32	36.27	36.14	28.17
17	33.23	36.98	36.90	36.95	49.37
18	47.29	135.70	135.29	135.18	133.34
19	35.13	35.28	35.22	35.03	35.74
20	48.38	49.94	49.82	49.76	45.65
21	72.19	73.28	73.09	73.05	47.74
22	43.27	49.83	49.54	49.21	212.63
23	28.25	22.85	27.88	28.46	27.81
24	17.08	63.22	16.34	16.00	16.30
25	17.01	18.05	18.31	18.31	18.27
26	19.01	16.72	16.85	16.86	16.80
27	23.21	20.41	20.35	20.34	20.04
28	29.32	26.47	26.42	26.31	26.96
29	179.98	179.59	179.59	179.58	178.24
30	21.13	25.27	25.18	25.09	25.58
1'	105.26	104.97	105.26		105.23
2'	84.70	81.63	84.73		84.68
3'	77.63	77.83	77.71		77.75
4'	73.51	73.41	73.37		73.09
5'	77.94	78.05	78.53		78.54
6'	172.62	172.38	172.13		172.10
1"	107.14	105.51	107.08		107.04
2"	77.04	75.98	76.88		76.88
3"	78.67	77.89	77.61		77.64
4"	73.23	73.34	73.31		73.31
5"	77.81	77.96	77.51		77.55
6"	172.33	172.56	172.51		172.47

The 50 % EtOH extract of the subterranean parts of *G. macedonica* provided 5 peaks on the HPLC profile of compounds (**8** -**12**), which were isolated and the structures were formulated.

The ^{1}H and ^{13}C NMR spectra of **8** were similar to those of glycyrrhizin, except that **8** had a 21 α-hydroxy group and a carboxyl group at C-29 instead of C-30. NOEs between H-21 β and H-30, H-18 and H-30, and H-18 and H-28 suggested the structure of E ring. The presence of 3 β-*O*-[β-D-glucuronopyranosyl-(1→2)- β-D-glucuronopyranosyl] group was confirmed by HMBC spectrum (HMBC correlation between H-3 α and C-1', H-1' and C-3, H-2' and C-1", and H-1" and C-2'), that was recognized similarly in the sugar moieties of **9** - **12**. Thus, the structure of **8** named macedonoside A was established as 3-*O*-[β-D-glucuronopyranosyl-(1→2)- β-D-glucuronopyranosyl]-3 β,21 α-dihydroxy-11-oxo-12-oleanen-29-oic acid.

The ^{1}H and ^{13}C NMR spectra of **9** were similar to those of macedonoside C (**10**), except that **9** had 6 tertiary methyl groups and a hydroxymethyl group, instead of 7 tertiary methyl groups in **10**. NOEs between H-3 α and H-23, H-5 and H-23, and H-24 and H-25 showed that the aglycone was isoechinatic acid [7]. Thus, the structure of **9** named macedonoside B was established as 3-*O*-[β-D-glucuronopyranosyl-(1→2)- β-D-glucuronopyranosyl]-isoechinatic acid. Deviation in the chemical shifts of C-1', C-2', C-1", and C-2" of **9** from the corresponding values of **8**, **10**, and **11** was due to the presence of 24-hydroxymethyl group.[5]

By acidic hydrolysis compound **10** gave D-glucuronic acid and an aglycone (**10a**), which was identified as macedonic acid [7] by the comparison of the physical constants and the spectral data with those reported. Thus, the structure of **10** named macedonoside C was established to be β-D-glucuronopyranosyl-(1→2)- β-D-glucuronopyranosyl macedonic acid.

The spectral data (SI-MS, IR, UV, ^{1}H and ^{13}C

NMR) of **11** were similar to those of **10**, except that **11** had 22-carbonyl group instead of 21 α-hydroxy group. From the fact that W form long range coupling between H-19 α and H-21 α, and HMBC correlations between H-21 and C-29, H-21 and C-22, and H-28 and C-22 were observed, the structure of **11** named macedonoside D was established to be 3-*O*-[β-D-glucuronopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β-hydroxy-22-oxo-11,13(18)-oleanadien-29-oic acid.

Compound **12** was identified as yunganoside L2 by the comparison of the physical constants and the spectral data with those reported.

Compound **10** was isolated also from the subterranean parts of *G. pallidiflora* and *G. echinata*, but it was not detected in *G. uralensis* and *G. glabra*. The amounts of **10** in several samples from different culture stations are shown in Table 4. High macedonoside C content was characteristic of *G. macedonica*, which seems useful for distinguishing this species from others.

Table 4 Macedonoside C (**10**) Contents in *G. echinata*, *G. pallidiflora*, and *G. macedonica* by HPLC

Origins (Medicinal Plants Gardens)	Plant Species	Content(%)
Nayoro Medicinal Plant Research Station, National Institute of Health Sciences	*G.echinata*	1.4
	G.echinata	0.9
	G.pallidiflora	2.1
Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences	*G.pallidiflora*	2.0
	G.pallidiflora	1.9
	G.macedonica	5.8
Kyoto Herbal Garden,Takeda Chemical Industries	*G.echinata*	0.5
	G.macedonica	5.0
Osaka University of Pharmaceutical Sciences	*G.echinata*	0.7
	G.pallidiflora	1.3

HPLC conditions : column ; CrestPak C-18s (*i.d.* 4.6×150mm, JASCO Co. Ltd.) ; column temperature ; 40℃ ; flow rate ; 0.8ml/min ; detection ; UV254nm. The mobile phases ; acetic acid (1→15) : acetonitrile (3 : 2)

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