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The Pharmaco-chemical Study on the Plant of *Ixeris* spp.
2. Flavonoids and Free Amino Acid Composition of *Ixeris sonchifolia*

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Abstract

From the leaves of *Ixeris sonchifolia* (Compositae), luteolin and its glucoside and apigenin glucuronide were isolated and their structures were characterized on the basis of spectral data. Besides these flavonoids, the composition and relative content of free amino acids and related compounds, compared to standards determined under identical conditions was also investigated using automatic amino acid analyzer. Major free amino acids were glutamic acid, aspartic acid, serine, proline, valine and arginine.

Key words : *Ixeris sonchifolia* (Compositae), luteolin, cynaroside, apigenin glucuronide, free amino acid, ^{13}C-NMR

INTRODUCTION

Ixeris sonchifolia (Compositae) is a perennial herb which is cultivated widely and the whole plants are used as a traditional Kimchi in Korea. According to the dictionary of Chinese drugs[1], they have been used for strengthening of stomach, as sedatives and diuretic agents. The phytochemical study was previously reported that the whole plants contain sugars, amino acids, fatty acid and polyphenols[2].

In the course of biological screening of Chinese drugs in our laboratory it was found that the methanol extract from the leaves of *I. sonchifolia* on serum constituents in hypercholesterolemic rats caused a significant lowering in serum cholesterol[3]. Monitoring fractionation of the methanol extract with animal tests showed that the ethylacetate soluble fraction and interphase material exhibited strong hypocholesterolemic activities[4].

The interphase material was subjected to column chromatography to yield two flavone glycosides (**2** and **3**) together with a luteolin (**1**).

We now report the isolation of these flavonoids from the interphase material of the methanol extract and the composition and relative content of free amino acids and related compounds from this plant part.

MATERIALS AND METHODS

Instruments

All melting points (mp) were measured on a Thomas Hoover 6406-H apparatus and are uncorrected. The infrared (IR) spectra were determined in KBr tablets on a Bomem MB-10 FT-IR spectrophotometer, and the ultraviolet (UV) spectra were run with CE 599 Universal automatic scanning spectrophotomenter. The proton nuclear magnetic resonance (^{1}H-NMR) and carbon nuclear magnetic resonance (^{13}C-NMR) spectra were obtained on either a Brucker-AM 300 or a Varian FT-80A spectrometer, using TMS as an internal standard and chemical shifts are given as δ (ppm). The fast atom bombardment (FAB) mass spectrum was taken with Kratos

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MS 25 RFA spectrometer. For thin layer chromatography (TLC), Kieselgel 60 F_{254} sheets (Merck) were used.

Analysis of free amino acids

The amino acid samples were analyzed quantitatively in an automatic amino acid analyzer (LKB 4150 ALPHA) using lithium buffer solutions. The buffer formulations and operational conditions are the same as in *Allium tuberosum*[5].

Preparation of free amino acid samples[6]

The leaves of *I. sonchifolia* obtained commercially were homogenized in cold 5% trichloroacetic acid (TCA) and the precipitated proteins removed by centrifugation. TCA was then removed by shaking with Et_2O. The residual solution was then evaporated under reduced pressure and the amino acid compounds then dissolved in lithium buffer solution (pH 2.20).

Extraction, fractionation and isolation

The dried leaves (1.0kg) of commercially available *I. sonchifolia* were extracted with MeOH under reflux. The MeOH extract was fractionated to yield several fractions as shown in Scheme 1. The interphase material (8g) was chromatographed on Silica gel column using $CHCl_3$-MeOH mixture to yield compounds **1** (50mg), **2** (250mg), and **3** (500 mg) in the order of elution.

Compound 1 (luteolin)

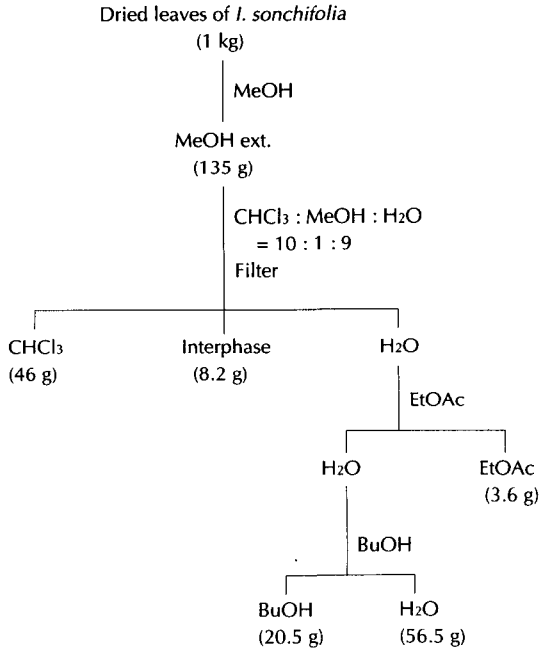
mp 330° C, Mg/HCl, Zn/ HCl ; positive, IR (KBr, cm^{-1}) ; 3400~3200 (OH), 1660 (α ,β-unsaturated C =O), 1605, 1498 (aromatic C=C), UV λ_{max}^{MeOH}nm (log ε) ; 253 (4.29), 267 (4.01), 349 (4.38), $\lambda_{max}^{MeOH+NaOMe}$ nm ; 266 (4.59), 401 (4.63), $\lambda_{max}^{MeOH+AlCl_3}$nm ; 274 (4.53), 426 (4.59), $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm ; 275 (4.23), 355 (4.24), 385 (4.25), $\lambda_{max}^{MeOH+NaOAc}$ nm ; 269 (4.39), 384 (4.32), $\lambda_{max}^{MeOH+NaOAc+H_3BO_3}$nm ; 259 (4.40), 370 (4.53), ^{1}H-NMR (DM SO-d_6, 80MHz) δ ; see text

Compound 2 (luteolin 7-0-glucoside, cynaroside)

mp 250~2° C, Mg/HCl, Zn/HCl, Molisch test ; positive, IR (KBr, cm^{-1}) ;3400~3200 (OH), 1650 (C =O), 1595, 1490 (C=C), 1100~1000 (glycoside), UV λ_{max}^{MeOH}nm (log ε) ; 256 (4.32), 267 (4.28), 358 (4.41), $\lambda_{max}^{MeOH+NaOMe}$ nm ; 264 (4.34), 397 (4.45), $\lambda_{max}^{MeOH+AlCl_3}$ nm ; 275 (4.39), 297 (4.04), 428 (4.53), $\lambda_{max}^{MeOH+AlCl_3+HCl}$ nm ; 275(4.30), 297 (4.08), 360 (4.30), 368 (4.32), $\lambda_{max}^{MeOH+NaOAc}$nm ; 261 (4.37), 403 (4.37), $\lambda_{max}^{MeOH+NaOAc+H_3BO_3}$ nm ; 260 (4.48), 374 (4.47), ^{1}H-NMR(DMSO-d_6, 300 MHz) δ ;13.1 (1H, brs, 5-OH), 7.54 (1H, d, J= 7.7 Hz, H-6′), 7.52 (1H. s, H-2′), 6.99 (1H,d, J=7.7 Hz, H-5′), 6.89 (1H,d, J=1.8 Hz, H-8), 6.84 (1H, s, H-3), 6.54 (1H, d, J=1.8Hz, H-6), 5.16 (1H, d, J=6.8 Hz, anomeric), ^{13}C-NMR ; see text

Compound 3 (apigenin 7-0-glucuronide)

mp 321~22° C, Mg/HCl, Zn/HCl, Molisch test ; positive, FABMS (m/z) ; 469[M+Na]$^+$, 447[M+H]$^+$, IR (KBr, cm^{-1}) ; 3400~3200 (OH), 1720 (acid), 1655 (C=O), 1616, 1499 (C=C), 1100~1000 (glycoside), ^{1}H-NMR (DMSO-d_6+D_2O, 300MHz) δ ; 7.84 (2H, d, J=8.2Hz, H-2′, 6′), 6.91 (2H, d, J=8.2Hz, H-3′,



Scheme 1. Extraction and fractionation of *I. sonchifolia*.

5′), 6.78 (1H, s, H-3), 6.66 (1H,brs, H-8), 6.41 (1H, brs, H-6), 5.03 (1H, d, J=6.3Hz, anomeric). 3.74~3.30 (4H, m, H-2″-5″), ^{13}C-NMR ; see Table 1

Acid hydrolysis of compounds 2 and 3

Each flavonoid (20mg) was refluxed with 5% H_2SO_4 for 4hr. Workup in the usual way, followed by crystallization from MeOH afforded luteolin from **2** and apigenin from **3** which were confirmed by direct comparison with authentic samples (TLC, mmp and UV). The aqueous mother liquor was neutralized with $BaCO_3$, filtered and concentrated *in vacuo*. D-glucose from **2** and D-glucuronic acid from **3** were detected by TLC.

RESULTS AND DISCUSSION

Flavonoids

Column chromatography of the interphase material of the methanol extract yielded compounds **1**, **2**, and **3** in the order of elution. The compound 1, mp 330° C, showed characteristic bands at 3400~3200 (OH), 1660 (α,β-unsaturated C=O), 1605 and 1498 (aromatic) cm^{-1} in its IR spectrum.

The UV (in MeOH) spectrum showed strong absorption peaks characteristic of a flavone at 253 and 349nm[7], which were shifted by addition of NaOMe (266, 401nm), NaOAc (269, 384nm), NaOAc+ H_3BO_3 (259, 370nm), $AlCl_3$ (274, 426nm) and $AlCl_3$ +HCl (275, 355 and 385nm). The ^{1}H-NMR spectrum of **1** exhibited signals at δ 6.45 (1H, d, J=2.0 Hz) and 6.12 (1H, d, J=2.0Hz) ascribable to H-8 and H-6 on the A-ring of a flavone skeleton and the signals at 7.72 (1H, dd, J=7.0 and 2.0Hz), 7.26 (1H, d, J=2.0Hz) and 6.83 (1H, d, J=7.0Hz) assignable to the protons of a 1, 3, 4-trisubstituted benzene ring. The signal at δ 6.72 (1H, s) suggested the presence of a flavone moiety. These spectral data were in agreement with those for the structure of 3′, 4′, 5, 7-tetrahydroxy flavone (=luteolin). This was further confirmed by direct comparison with an authentic sample (mmp, co-TLC and ^{1}H-NMR).

Compound 2, mp 250~52° C gave a positive reaction in Molisch test besides flavone color reactions. Acid hydrolysis of **2** yielded luteolin as the aglycone and D-glucose as the sugar. The ^{1}H-NMR spectrum showed only one anomeric proton signal, indicating the presence of one mole of sugar in **2**. The band II shift of compound 2 by the effect of NaOAc was not observed in the UV spectrum. It was, thus, suggested that the sugar might be attached to 7-hydroxyl group[7]. This was further confirmed by the inspection of ^{13}C-NMR spectrum (Table 1). The configuration and conformation of sugar moiety was determined by the J value of the anomeric proton signal (See Materials and Methods). Compound 2 was, therefore, identified as luteolin 7-0-β-D-glucoside (cynaroside). Compound 3, mp 321~22° C gave a positive reaction in Molisch test besides flavone color reactions and showed glycoside bands (1000 ~1100cm^{-1}) in its IR spectrum. Acid hydrolysis yielded apigenin, mp 296~8°, which was identified by direct comparison with an authentic sample as the genin and D-glucuronic acid as the sugar. The positive FAB mass spectrum showed signals at m/z

Table 1. ^{13}C-NMR spectral data of 2, 3 and related compounds

Compound / Carbon No.	2	3	Apigenin[9]	Apigenin 7-glucuronide[10]
2	164.48	164.14	165.5	164.72
3	103.16	102.86	104.3	102.68
4	181.85	181.74	183.2	182.47
5	161.12	161.65	162.0	160.36
6	99.56	99.56	100.3	100.08
7	162.94	162.88	164.9	162.83
8	94.76	-	95.6	95.98
9	156.93	156.91	158.7	156.91
10	105.35	106.10	105.1	105.99
1′	121.38	120.59	122.7	121.15
2′	113.56	128.32	129.8	128.43
3′	145.77	115.98	117.3	116.14
4′	149.94	161.65	161.8	160.36
5′	116.00	115.98	117.3	116.14
6′	119.15	128.32	129.8	128.43
1″	99.96	99.56		100.08
2″	73.13	72.90		72.5C
3″	76.41	74.33		76.01
4″	69.61	71.85		69.19
5″	77.16	76.27		76.99
6″	60.67	172.98		175.77

469[M+Na]$^+$ and m/z 447[M+H]$^+$ consistent with a molecular formula $C_{21}H_{18}O_{11}$ for the glucuronide. In the ^{1}H-and ^{13}C-NMR (Table 1) spectra of **3**, only one anomeric proton signal at δ 5.03 (d, J=6.3Hz)and a set of carbon signals between 71.85ppm and 172.98ppm, including an anomeric carbon signal 99.56ppm, indicated the presence of one mole of β-D-glucuronopyranosyl unit.

The 300 MHz ^{1}H-NMR spectrum of **3** also showed expected signals in the aromatic region : two singlets at δ 6.41 and 6.66 for H-6 and H-8 respectively ; one singlet at δ 6.78 for H-3 ; and two doublets integrating for two protons each at δ 6.91 ; and 7.84 (J=8.2Hz) for H-3′, H-5′ and H-2′, H-6′ respectively. The marked downfield shift in the signals for H-6 and H-8 suggested localization of the glucuronide moiety at C-7[8]. This suggestion was supported by the inspection of ^{13}C-NMR data (Table 1), from which the ^{13}C-NMR shift of **3** corresponded well to the shifts for a 7-conjugated apigenin as suggested by shielding of C-7 (-2.02ppm) and deshieling of C-10 (+1.0ppm). Thus, compound 3 is apigenin 7-0-β-D-glucuronopyranoside.

This is the first report of their occurrence from this plant part.

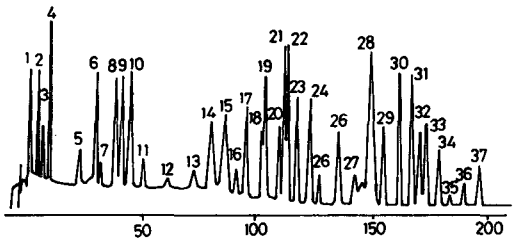


Fig. 1. Separation of standard amino acids and related compounds by automatic amino acid analyzer.

Peaks ;

1. phosphoserine	2. taurine
3. phosphoethanolamine	4. urea
5. unknown	6. aspartic acid
7. hydroxyproline	8. threonine
9. serine	10. glutamic acid
11. glutamine	12. α-aminoadipic acid
13. proline	14. glycine
15. alanine	16. α-aminobutyric acid
17. valine	18. cystine
19. methionine	20. cystathionine
21. isoleucine	22. leucine
23. tyrosine	24. phenylalanine
25. β-aminoisobutyric acid	26. γ-aminobutric acid
27. ethanolamine	28. ammonia
29. DL+allohydroxylysine	30. ornithine
31. lysine	32. 1-methylhistidine
33. histidine	34. 3-methylhistidine
35. anserine	36. carnosine
37. arginine	

Free amino acids and related compounds

Besides flavonoids, we now also report amino acid composition of this plant extract using TCA precipitation method[6] by means of automatic amino acid analyzer. The composition of free amino acids and related compounds of *Ixeris sonchifolia*, compared to standard amino acids determined under identical conditions are presented in Figs. 1 and 2 and summarized in Table 2.

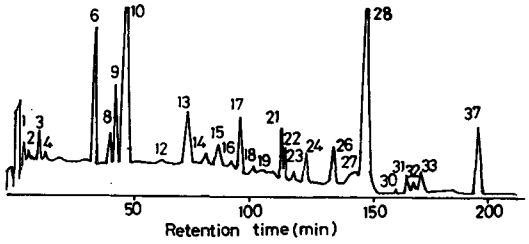


Fig. 2. Free amino acids and related compounds from *Ixeris sonchifolia*.

Numerical name of peaks is the same as in Fig. 1.

Table 2. Composition and relative content of free amino acids and related compounds from *Ixeris sonchifolia*

Composition	Rt*	Composition	Rt*
Phosphoserine	1.53	Cystine	1.09
Taurine	0.88	Methionine	0.88
Phosphoethanolamine	2.19	Isoleucine	1.53
Urea	1.75	Leucine	1.53
Aspartic acid	5.91	Tyrosine	1.31
Threonine	1.53	Phenylalanine	2.84
Serine	4.38	γ-Aminobutyric acid	4.16
Glutamic acid	17.07	Ethanolamine	1.09
α-Aminoadipic acid	1.09	Ammonia	23.85
Proline	5.47	Ornithine	0.65
Glycine	1.53	Lysine	1.09
Alanine	3.06	1-methylhistidine	1.09
α-Aminobutyric acid	1.09	Histidine	1.09
Valine	4.60	Arginine	6.34

*Rt ; relative content (%)

The plant extract contained a number of proteineous, non-proteineous (taurine, phosphoserine and phosphoethanolamine) free amino acids and other related compounds. Among the proteineous free amino acids, glutamic acid (17.07%), proline (5.47 %), serine (4.38%) and alanine (3.06%), representative of delicious and sweet taste and arginine (6.34%), aspartic acid (5.91%) and valine (4.60%) exhibiting bitter taste were predominantly present in this extract. The whole plants of *Ixeris sonchifolia* are used as a traditional Kimchi in Korea because of its bitterness. But, the bitter principles of this plant are not identified. Whether the composition and relative content of these amino acids are attributed to the bitterness of this plant or not, it is not possible to estimate the bitter principles from the present results, even though free amino acids can taste responsible. Further comprehensive chemical study will be needed.

In the case of non-proteineous amino acids, the relative contents of phosphoserine, phosphoethanolamine and taurine were 1.53%, 2.19% and 0.88%, respectively. It is of significance that the concentrations of phosphoethanolamine and phosphoserine, the potential celluar phosphate donors in phospholipid biosynthesis, are much higher than those of taurine.

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Ixeris 속 식물의 약화학적 연구
2. 고들빼기의 플라보노이드 성분과 유리 아미노산 조성

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요 약

고들빼기 잎으로부터 플라보노이드인 luteolin, luteolin 7-0-glucoside 및 apigenin 7-0-glucuronide를 분리하고 그들의 구조를 기기적인 분석방법에 의하여 동정하였으며 또한, 유리 아미노산과 관련 화합물들의 조성과 상대함량을 표준품과 동일조건하에서 아미노산 자동분석기로 비교 검토하였다. 가장 함량이 많은 유리 아미노산들은 glutamic acid, aspartic acid, serine, proline, valine 그리고 arginine이었다