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Determination of Chiisanoside in *Acanthopanax* Species by High Performance Liquid Chromatography

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Abstract – The content of chiisanoside in the *Acanthopanax* species was determined by reversed-phase high performance liquid chromatographic method. Chiisanoside was separated from the other components in the plant extracts using Zorbax 300 SB C_{18} column with gradient elution of acetonitrile. Identification of chiisanoside was carried out by comparison in the LC/MS spectrum of separated peak from extract with that of standard. By HPLC analysis in this experiment, *Acanthopanax* species could be classified into two groups based upon the content of chiisanoside-one with low concentration of chiisanoside, such as *A. senticosus* and *A. koreanum*, and another with high concentration of chiisanoside, such as *A. senticosus* f. *inermis*, *A. divaricatus* var. *albeofructus*, and *A. chiisanensis*.
Keywords – *Acanthopanax* species, chiisanoside, HPLC, LC-MS

Introduction

Acanthopanax species (*Araliaceae*) are widely distributed in Korea, Japan, China and the far-eastern region of Russia. The barks and small branches of this species are used in medicine for people in their dotage, for paralysis, arthritis, rheumatism, lameness, high blood pressure, and as a tonic (Perry *et al.*, 1980). The whole plant including leaves, stems and roots has been also widely taken as health supplements in Korea. There are eighteen kinds of plants belonging to the genus *Acanthopanax* in Korea including ten species, five forma, and three varieties (Yook, 2001). Although *Acanthopanax senticosus* (=*Eleutherococcus senticosus*, Siberian ginseng) has been well-known as an adaptogenic medicine, this plant is endangered by over-harvesting and exacerbated by its slow growth. Other species such as *A. koreanum*, *A. chiisanensis*, *A. divaricatus* var. *albeofructus* and *A. senticosus* forma *inermis* are well cultivated in southern Korea and it may well be possible to substitute the extract of *A. senticosus* with that of other better-growing *Acanthopanax* species, on the basis of superiority in their constituents and biological activities. Isolation and characterization of several diterpenoids (Kim *et al.*, 1988), triterpenoid saponins (Park *et al.*, 2000 and Oh *et al.*, 2002), and phenolic components (Nishibe *et al.*, 1990) from *Acanthopanax* species have been reported previously.

Chiisanoside, the main component of some *Acanthopanax* species, has been first isolated (Hahn *et al.*, 1984) from *A. chiisanensis*. It was reported to posses anticancer (Yook *et al.*, 1996), anti-hepatotoxic and antidiabetic activity (Kim *et al.*, 1980), an effect of mitogen-induced proliferation of lympocytes (Kim *et al.*, 1999). Chiisanogenin, the metabolites of chiisanoside by human intestinal bacteria, showed also some biological activities such as protection of ulcer of stomach and liver damage (Bae *et al.*, 2001).

Hence the importance of chiisanoside has been increased for the development of new lead compound in some biological activities. For the purpose of the effective utilization of natural resources we tried to determine the chiisanoside contents in various *Acanthopanax* plants. Cho *et al.* (1999) reported about the isolation and quantitative analysis of chlorogenic acid and chiisanoside from *Acanthopanax* species, but there were some discrepancies in the contents of chiisanoside compared with our results. In this report a modified HPLC method for the dertermination of chiisanoside was described and applied to some *Acanthopanax* species.

Materials and Methods

Instruments and chemicals – The chromatographic system for quantitative analysis consisted of a SCL-10A system controller, an LC-10AD pump, a SPD-10MVP diode array detector (Shimadzu, Japan), column temperature

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controller (Waters, CA, USA) and a Rheodyne 7725 injector with a 20 μl sample loop. For qualitative analysis a QP8000 LC/MS (Shimadzu, Japan) with an atmospheric pressure chemical ionization (APCI) interface, LC-10AD pump and SPD-10A UV/VIS detector were used. Methanol (Tedia, OH, USA) acetonitrile (J. T. Baker, NJ, USA.) used in this work were of HPLC grade and other reagents were of analytical grade. Milli-Q (Millipore, MA, USA) treated water (with resistivity more than 18 MΩ cm) was used throughout the experiment.

Plant materials – The plant materials were collected from various regions in Korea and identified by Prof. Young Ho Kim at the College of Pharmacy, Chungnam National University. *A. senticosus* (CNU-A01) was collected from Cheongok mountain in Kangwon province. *A. koreanum* (CNU-A02), *A. senticosus* forma *inermis* (CNU-A03), *A. divaricatus* var. *albeofructus* (CNU-A04) were gratefully provided by Susin Ogapi Co. in Cheonan, Kyeongki Province and *A. chiisanensis* (CNU-A05) was collected from Chiri mountain in Jeonbuk Province. The collected samples were separated in leaves, stems and roots, and stored at a cool and dark place. The voucher specimens were deposited at the herbarium in the College of Pharmacy, Chungnam National Umniversity.

Preparation of chiisanoside standard – The methanolic extract of the leaves of *A. divaricatus* var. *albeofructus* passed through a Diaion-HP 20 column by elution with H_2O, 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, and MeOH successively. 80% MeOH eluate was concentrated under reduced pressure to give a residue, which was subjected to silica gel column ($CHCl_3$-MeOH-H_2O, 10:3:0.1) and YMC reversed-phase C18 column (MeOH-H_2O, 10:7), successively. Chiisanoside was obtained as white powder by recrystallization in MeOH. Chemical structure was identified by ^{1}H-NMR, ^{13}C-NMR and MS. The purity confirmed by HPLC was more than 99.5%

Sample preparation – The air dried and coarsely powdered sample (3 g) was extracted with 30 ml of 75% ethanol under reflux for 1 h. The extract was placed in a volumetric flask. The residue was then washed two times with 75% ethanol and the liquid was added to the volumetric flask adjusted 50 ml. The solution was filtered through 0.2 μm membrane filter prior to HPLC injection.

Chromatography and identification – The HPLC separation of chiisanoside for qualitative and quantitative analysis was performed using a reverse phase system. A Zorbax 300SB C_{18} (4.6×150 mm, Hewlett-Packard Co., CA, USA) chromatographic column was used and column temperature was maintained to be 40°C. The two solvent gradient elution of acetonitrile and water at a flow rate 1.0 ml/min was employed to separate the component. Detection was carried out at UV 205 nm. The identification of chiisanoside was carried out by analyzing the mass spectrum of the peak corresponding to chiisanoside in the HPLC effluent.

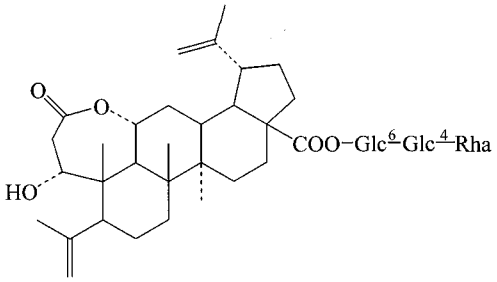

Fig. 1. Chemical structure of chiisanoside.

Results and Discussion

Separation and identification of chiisanoside – To select an optimal mobile phase for the analysis of chiisanoside, several isocratic and gradient runs with various concentrations of acetonitrile in water were performed. Based upon the resolution obtained and retention time of chiisanoside gradient elution with a starting mobile phase of 25% acetonitrile, run for 1 min isocratic, linear gradient and increment over 7 min to 32% acetonitrile, and hold time of 6 min with 32% acetonitrile was used. A chromatogram of *A. divaricatus* var. *albeofructus* extract obtained under this condition is shown in Fig. 2b, which indicates the base line separation of chiisanoside. The peak, appeared in about 9.3 min, on the chromatogram of *A. divaricatus* var. *albeofructus* extract (Fig. 2b) could be confirmed as chiisanoside by comparing retention times and mass spectra with those of authentic sample (Fig. 2a). The base peak of chiisanoside was found at m/z 483 with loss of Glc-Glc-Rha from the molecular ion and another peak at m/z 953 corresponding to the loss of H^+.

Method validation – The calibration functions of chiisanoside standard was y (peak height) = 96.56x (mg/ml) + 0.86 (r = 0.9995) over the concentration range 5 μg/ml to 3.5 mg/ml (Fig. 3). Table 1 showed the precision of this method. The intra-day precision of the analysis was obtained from the separated injections of three different concentrations of standard (0.1, 1.0 and 2.0 mg/ml). Interday precision was also calculated from the analysis results of the standards in 5 consecutive days. The intra-day precisions were all less than 5%. The interday precision of 0.1 mg/ml standard (10.66%) were larger than expected. For recovery testing, known amount of chiisanoside standards was spiked to the

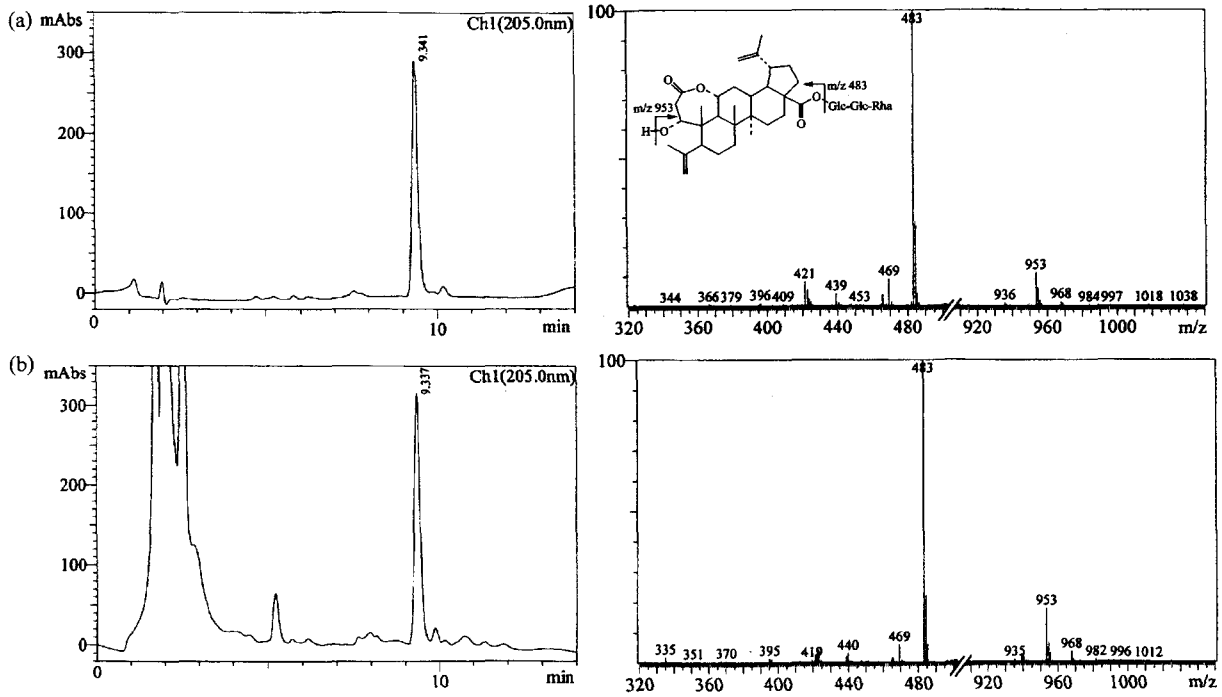

Fig. 2. Chromatogram and mass spectra of separated peak (R_t~9.3 min) of authentic chiisanoside (a) and an extract of *A. divaricatus* var. *albeofructus* separated on Zorbax 300SB C_{18} column with gradient elution (b). MS conditions: Ionization; APCI-Negative, probe temperature; 400°C, probe voltage; −4.0 KV, CDL voltage; 70 V, deflector voltage; −60 V, nebulizer gas flow; 2.5 L/min.

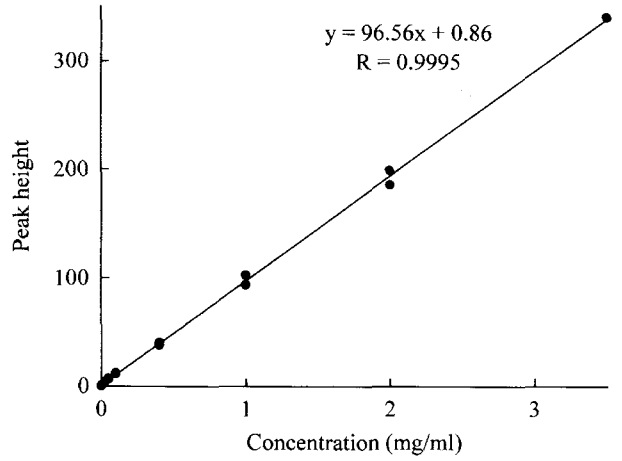

Fig. 3. Calibration curve of chiisanoside in the range of 5 μg/ml to 3.5 mg/ml.

leaves of *A. koreanum*, which was not contained chiisanoside, and then determined by HPLC. Recovery efficiency in the HPLC analysis was more than 96% as shown in Table 2. The limit of detection of chiisanoside was 0.2 μg/ml with a signal-to-noise ratio of three to one. Both accuracy and precision criteria indicated that this method was suitable and applicable for the determination of chiisanoside in *Acanthopanax* species by HPLC.

Analysis of chiisanoside in the *Acanthopanax* species – The content of chiisanoside in the stems, roots and leaves of various *Acanthopanax* species was analyzed using the reversed-phase HPLC method. No detectable amounts of chiisanoside were found from stems and roots

Table 1. The intra-day and interday precisions of the standard analysis

standard (mg/ml)	Intra-day		Interday	
	Mean±SD	CV(%)	Mean±SD	CV(%)
0.1	0.12±0.01	4.45	0.12±0.01	10.66
1.0	0.99±0.04	4.08	1.05±0.07	6.72
2.0	1.95±0.02	1.18	1.94±0.05	2.39

Table 2. The recovery of chiisanoside from the leaves of *A. koreanum*

Added (mg/g)	Found (mg/g)	Recovery (%)
5.0	4.6	92.0
25.0	24.3	97.2
50.0	48.5	97.0

Table 3. Concentration of chiisanoside in the leaves of *Acanthopanax* species

Acanthopanax species	Chiisanoside
A. senticosus f. *inermis*	64.1±0.23
A. divaricatus var. *albeofructus*	62.7±0.16
A. chiisanensis	59.5±0.82
A. senticosus	0.23±0.01
A. koreanum	0

Data are given as mean±S.D. (n=3-4) in mg/g dried sample.

of any tested *Acanthopanax* species. The variation in the contents of chiisanoside in the leaves of *Acanthopanax* species is presented in Table 3. Relatively high concentration of chiisanoside (60-64 mg/g) was observed from the leaves of *A. divaricatus* var. *albeofructus, A. senticosus* f. *inermis* and *A. chiisanensis*, while very low concentration (0-0.2 mg/g) was detected from *A. koreanum* and *A. senticosus*. The *Acanthopanax* species tested could be divided in two groups by the chiisanoside content. We could categorized the tested plants in the same way by the elutheroside B and E contents in the previous study (Kang *et al.*, 2001). These results were in good agreements with the chemotaxonomical data by RAPD analysis (Park, 2002). *A. koreanum* and *A. senticosus* are classified into a minor cluster group in genetic dendrogram, while *A. chiisanensis, A. divaricatus* var. *albeofructus, and A. senticosus* f. *inermis* are belong to major cluster group showing high genetic similarity which yield high contents of 3,4-seco-lupane triterpene compounds. From these results, it can be concluded that *A. chiisanensis, A. divaricatus* var. *albeofructus, and A. senticosus* f. *inermis* are good resources to produce chiisanoside.

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