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Quantitative Determination of Eleutheroside B and E from *Acanthopanax* Species by High Performance Liquid Chromatography

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Reversed-phase high performance liquid chromatographic method was applied for the determination of eleutheroside B and E in the various *Acanthopanax* species collected in Korea. The stationary phase used was Zorbax 300 SB C_{18} and a mobile phase program was used, which started at 6% acetonitrile for 2 min, and then a linear gradient was operated for the next 18 min to 17% acetonitrile at a flow rate of 1.0 ml/min. The column effluent was monitored at UV 210 nm. Identification was carried out by comparing the retention time and the LC/MS spectrum of each peak corresponding to eleutheroside B and E from sample with those of standards. In general, the contents of eleutheroside B and E in stems were higher than those in roots. *Acanthopanax* species could be classified into two groups based upon the contents of eleutheroside B and E: one group contains no or very little eleutheroside B and another contains both eleutheroside B and E.

Key words: *Acanthopanax* species, Eleutheroside B, Eleutheroside E, Gradient elution, HPLC

INTRODUCTION

Acanthopanax species (Araliaceae) are widely distributed in Korea, Japan, China and the far-eastern region of Russia. The root and stem barks of these plants have been used as a tonic, and sedative as well as in the treatment of rheumatism, and diabetes (Perry and Metzger, 1980). Extensive biological studies were carried out and reported several pharmacological activities such as the induction of apoptosis (Hibasami *et al.*, 2000), gastric ulcer protection (Fujikawa *et al.*, 1996), antitumor (Wang *et al.*, 1992), immunomodulation (Wang *et al.*, 1991; Shen *et al.*, 1991; Shan *et al.*, 1999), and radioprotective effects (Miyanomae and Frindel, 1988).

Isolation and structural studies on several diterpenoids (Kim *et al.*, 1988), triterpenoid saponins (Park *et al.*, 2000), and phenolic components (Nishibe *et al.*, 1990) from *Acanthopanax* species have been reported previously. Among these, the lignan compounds, eleutheroside B (syringin) and eleutheroside E ((-)syringaresinol-di-O-β-D-glucoside), are known to be main active principles of *Acanthopanax* species (Takasugi *et al.*, 1985). The genus *Acanthopanax* includes ten species, three forma, and two varieties in Korea (Yook *et al.*, 1976). *Acanthopanax senticosus* (=*Eleutherococcus senticosus*, Siberian ginseng) is a woody shrub found only in northeast Asia. Extracts of the root barks are commonly used as ingredients in health foods and for medicinal purposes, but the plant is endangered by over-harvesting, and this is exacerbated by its slow growth. However, other species such as *A. koreanum*, *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 similarity in their constituents and biological activities. From this chemotaxonomical standpoint, we analyzed contents of eleutheroside B and E components in the roots and stems of various *Acanthopanax* plants growing in Korea by reversed-phase HPLC and compared their contents.

MATERIALS AND METHODS

Materials and instruments

Dried stems and roots of *A. senticosus* were collected from Cheongok Mountain in Kangwon province. *A. kore-*

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anum, *A. senticosus* forma *inermis*, *A. divaricatus* var. *albeofructus* were collected from a farm operated by Susin Co. in Cheonan, Kyeongki Province and *A. chiisanensis* was collected from Chiri Mountain in Jeonbuk Province. The voucher specimens are deposited at the herbarium in College of Pharmacy, Chungnam National University. Eleutheroside B and E were isolated from *A. koreanum* and used as standards. 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 experiments. 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 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.

Preparation of eleutheroside B and E standards

Eleutheroside B and E were isolated from the root of *A. koreanum* as reported previously (Kim, 1986) and identified by ^{1}H-NMR, ^{13}C-NMR and MS. The purity confirmed by HPLC was more than 99.5%

Chromatography and identification

The HPLC separation of eleutherosides for qualitative and quantitative analysis was performed using a reversed-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 at 30°C. The two solvent gradient elution with acetonitrile and water at a flow rate 1.0 ml/min was employed to separate the components. Detection was carried out at UV 210 nm. The identification of eleutheroside B and E in the sample was carried out by analyzing the mass spectrum of peak corresponding to eleutheroside B and E in the HPLC effluent.

Effect of solvent and time on extraction

The dried stems of *A. senticosus* were coarsely powdered and 3 g of samples were extracted with chloroform, ethylacetate, acetonitrile, acetone, ethanol, methanol, and water under reflux for 3 h, respectively. Separately, the extraction was also carried out with 50 and 75% ethanol and methanol in water for various time periods. The extract was then placed in a volumetric flask, diluted to 50 ml with methanol and centrifuged at 13,000 rpm for 10 min. The 10 μl supernatants were injected into the HPLC. The extraction efficiencies calculated on the basis of the amounts of eleutherosides were compared.

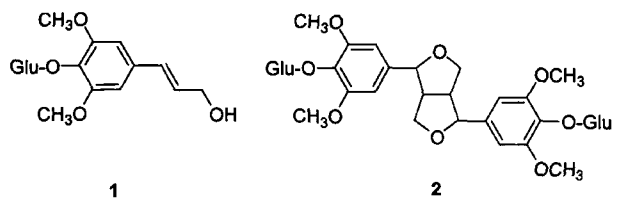

Fig. 1. Chemical structures of eleutheroside B (**1**) and E (**2**).

Analysis of eleutherosides

The dried sample of *Acanthopanax* species was separated in roots and stems, and 3 g of coarsely powdered each samples were extracted with 50% ethanol under reflux for 1 h. The extracts were then diluted and centrifuged as above and analyzed by HPLC.

RESULTS AND DISCUSSION

Resolution and identification of eleutheroside B and E

To select an optimal mobile phase composition for the analysis of eleutheroside B and E, several isocratic and gradient runs with various concentrations of acetonitrile in water were performed. Based upon the resolution obtained and retention time of eleutheroside B and E under several different HPLC analyses, it was found that an acetonitrile gradient with increasing concentration was the best to achieve the proper separation. Accordingly, the selected HPLC conditions were arrived as follows. With a starting mobile phase of 6% acetonitrile, run for 2 min, linear gradient then increased over 18 min to 17% acetonitrile. A chromatogram of *A. senticosus* extract obtained under this condition is shown in Fig. 2, which

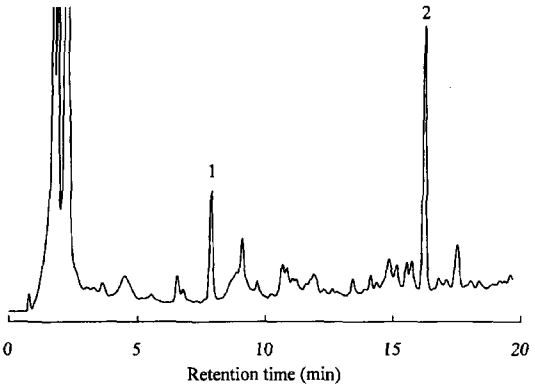

Fig. 2. Chromatogram of *A. senticosus* extract in HPLC. Zorbax 300SB C_{18} column was used with gradient elution as a starting mobile phase of 6% acetonitrile, run for 2 min, linear gradient then increased over 18 min to 17% acetonitrile. Peaks 1 and 2 represent eleutheroside B and E, respectively.

indicates the base line separation of eleutheroside B and E within 17 min. The two peaks designated **1** and **2** in the chromatogram of *A. senticosus* extract were confirmed as eleutheroside B and E, respectively, by comparing retention times and mass spectra with those of authentic samples (Fig. 3). The base peak of eleutheroside B was found at m/z 193 with loss of O-glucose from the molecular ion while that of eleutheroside E was found at m/z 401 corresponding to the loss of O-glucose and glucose.

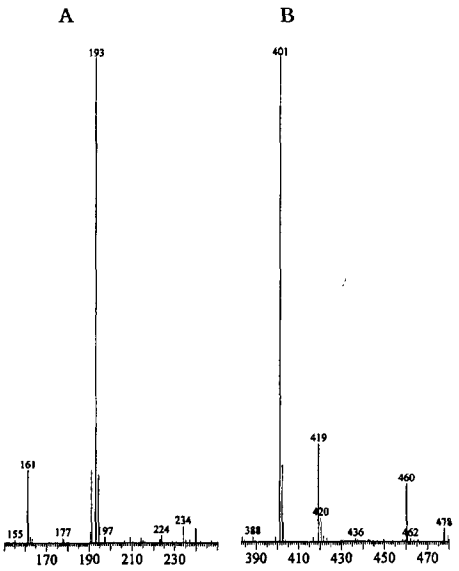

Fig. 3. Mass spectra of the components of *A. senticosus* by LC/MS. The peaks eluted at 7.9 min (A) and 16.2 min (B) were corresponded to eutheroside B with m/z 193 $[M\text{-}O\text{-}Glc]^+$ and eleutheroside E with m/z 401 $[M+H\text{-}O\text{-}Glc\text{-}Glc]^+$, respectively. MS conditions: Ionization; APCI with 4.0 kV (400 °C), nebulizer gas flow rate; 2.5 l/min, CDL voltage; -25 V, deflector voltage; 50 V.

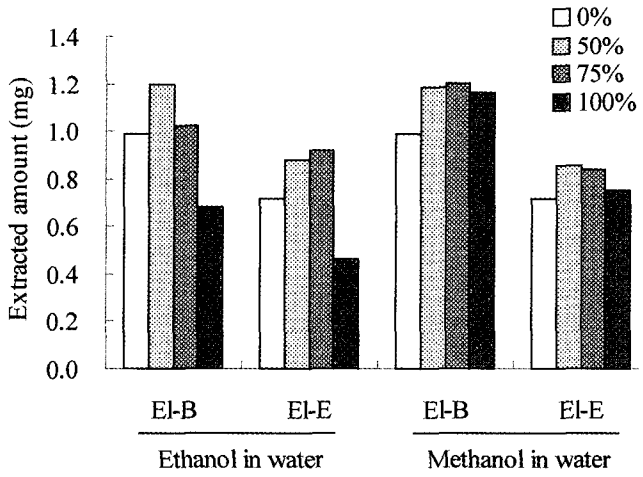

Fig. 4. Extraction efficiency of eleutheroside B (El-B) and E (El-E) from the stem of *A. senticosus*. Each sample was extracted with 100%, 75%, 50% and 0% concentration of ethanol or methanol in water, separately.

Extraction efficiency of eleutherosides

The coarsely powdered stems of *A. senticosus* were extracted with several solvent, namely, chloroform, ethylacetate, acetonitrile, acetone, ethanol, methanol, and water. Among these the polar solvents showed the best extraction efficiencies. The amounts of eleutheroside B and E extracted were affected by the concentration of ethanol or methanol in water (Fig. 4). The concentration effect of ethanol in extraction was greater than that of methanol. The highest extraction efficiency of eleutheroside B and E was achieved with 50% of ethanol. As shown in Fig. 5 the amounts of eleutheroside B and E extracted with 50% ethanol under various extraction time indicated that 1 h is sufficient enough to extract. From these experiments it could be concluded that the best solvent for extracting eleutheroside B and E was 50% ethanol with an extraction time of 1 h.

Linearity, recovery and limit of detection

The calibration functions of eleutheroside B and E standard calculated with peak height (y, mAU) and concentration

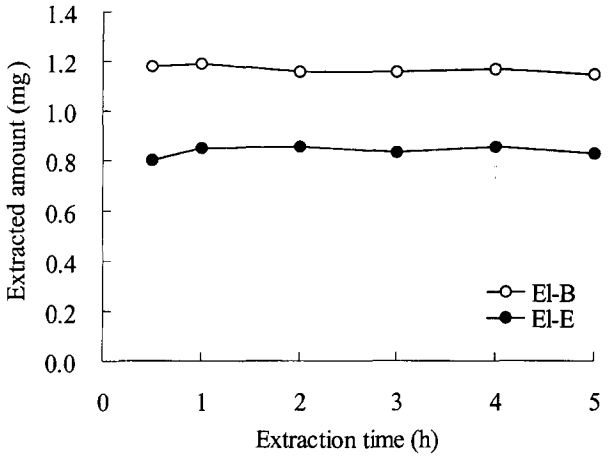

Fig. 5. Extraction efficiency of eleutheroside B (–○–) and E (–●–) by the extraction time on the stem of *A. senticosus*. 50% ethanol was used as extraction solvent.

Table I. The recovery of eleutheroside B from the roots of *A. senticosus* forma *inermis* by the reversed-phase HPLC method

Eleutheroside B			Eleutheroside E		
Added (mg/g)	Found (mg/g)	Recovery (%)	Added (mg/g)	Found (mg/g)	Recovery (%)
0.177	0.167	94.4	0.168	0.164	97.7
0.443	0.445	100.5	0.420	0.417	99.2
0.885	0.895	101.2	0.840	0.857	102.1

Data are given as single determination.

Table II. Concentration of eleutheroside B and E in *Acanthopanax* species

Acanthopanax species	Eleutheroside B		Eleutheroside E	
	Stem	Root	Stem	Root
A. koreanum	0.621 ± 0.014	0.478 ± 0.009	0.885 ± 0.010	0.538 ± 0.014
A. senticosus	1.203 ± 0.024	0.445 ± 0.011	0.854 ± 0.020	0.561 ± 0.011
A. divaricatus var. albeofructus	0	0	1.804 ± 0.040	1.016 ± 0.034
A. senticosus forma *inermis*	0	0	1.789 ± 0.069	0.496 ± 0.008
A. chiisanensis	0	0	0.690 ± 0.014	0.974 ± 0.030

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

(x, μg/ml) were $y=0.86x + 1.48$ ($r=0.9996$) and $y=1.74x + 1.98$ ($r=0.9996$), respectively, over the concentration range 5 to 200 μg/ml. For recovery testing, known amount of eleutheroside B and E standards were added to the root of *A. senticosus* forma *inermis*, which contained no eleutheroside B, and then quantified eleutheroside B and E by HPLC. The efficiency of recovery in the HPLC analysis was more than 94% as shown in Table I. The observed linearity and the results of recovery testing indicate that this HPLC method is suitable and applicable for qualitative and quantitative evaluation of the *Acanthopanax* species. The detection limits of eleutheroside B and E were 100 and 50 ng/ml, respectively, at a signal to noise ratio of 3.

Analysis of eleutherosides in the *Acanthopanax* species

The content of eleutheroside B and E in the stems and roots of various *Acanthopanax* species was analyzed using the reversed-phase HPLC method. The variation in the contents of eleutheroside B and E in *Acanthopanax* species is presented in Table II. Generally, the content of eleu-theroside B and E was higher in the stem than that in the root for all *Acanthopanax* species except *A. chiisanensis*. *A. koreanum* and *A. senticosus* contained eleutheroside B and E in both the stems and roots, while other species contained only eleutheroside E. The contents of eleutheroside B and E in the stems and roots of *A. koreanum* were similar with those in *A. senticosus*, even their taxonomical classifications and growing conditions are very different. Therefore, it may be possible to develope *A. koreanum* as a market substitutive of *A. senticosus*. In particular, *A. koreanum* can be cultivated very quickly and easily in Korea than *A. senticosus*, thereby the cost of extracting the essential components from *A. koreanum* would be less than that of *A. senticosus*.

A. divaricatus var. *albeofructus*, *A. senticosus* forma *inermis* and *A. chiisanensis* did not show the peak corresponding to eleutheroside B, but they showed much amounts of eleutheroside E in both stems and roots, compared with the other species examined. From the biosynthetic point of view, eleutheroside B and E could be synthesized from phenylpropanoid via the shikimic acid pathway. It is quite unusual to produce only eleutherosdie E without the production of eleutheroside B in those three species. The reason why those species showed only eleutheroside E in our HPLC analysis could be due to the species specific extensive metabolic flux of eleutheroside B to eleutherside E. Therefore the content of eleutheroside B would remain under the detection limit at early winter. Eleutheroside E is reported to have a counteracting effect on stressed animals, androgenic effects in immature male mice, and of increasing the RNA content of seminal vesicles and the prostate (Farnsworth *et al.*, 1985). The stem of *A. divaricatus* var. *albeofructus* and *A. senticosus* forma *inermis* have a large amount of eleutheroside E, and they could be resources for the development of anti-fatigue drugs and androgenic effects of these plant species.

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