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Capillary electrophoretic method for the determination of diterpenoid isomers in *Acanthopanax* species

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Abstract

Acanthoic, continentalic and kaurenoic acids are bioactive diterpenoids that are structural isomers isolated from *Acanthopanax* species. Due to the interest in their potent biological activity, an analytical method of diterpenoids was developed for the quality control and the classification of *Acanthopanax* species. Capillary electrophoresis was used to separate and quantify the isomers. The three compounds were successfully separated from each other and from the matrices in the extracts of leaves, stems and roots of *Acanthopanax* species. The contents of acanthoic, continentalic and kaurenoic acids showed taxonomical differences in *Acanthopanax* species. Relatively higher concentrations of diterpenoids were found from *A. koreanum* and *A. trifoliatus*, while only trace amounts were found from the four other species tested: *A. senticosus*, *A. senticosus* f. *inermis*, *A. chiisanensis*, and *A. divaricatus* var. *albeofructus*. The contents of diterpenoids in association with lignans and triterpenoids in the *Acanthopanax* species could provide a chemotaxonomical index able to be used in the classification and discrimination of the species.

Keywords: *Acanthopanax* species; Acanthoic acid; Continentalic acid; Kaurenoic acid; Capillary electrophoresis

1. 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 [1]. Isolation and structural studies on diterpenoids [2–4], triterpenoid saponins [5], and phenolic components [6] from *Acanthopanax* species have been reported previously. Of these components, diterpenoids such as acanthoic, kaurenoic and continentalic acids were reported as important ingredients in *Acanthopanax* species for their potent biological activities [7–10].

Acanthoic acid ((−)-pimara-9(11),15-diene-19-oic acid), which belongs to pimaradiene diterpenoid, was first isolated from the root of *A. koreanum* [2] and showed a potent anti-inflammatory and anti-fibrosis effect [7], an inhibitory effect of IL-8 production [8], and a protective effect against liver injury [9]. Due to the potent biological activity, acanthoic acid was considered as a good lead compound to develop herbal drugs or nutraceutics from natural products. Kaurenoic acid ((−)-kaur-16-en-19-oic acid) belongs to kaurane type diterpenoid. It was isolated from the leaves of *A. tricodon* [11] and from the root bark of *A. koreanum* [2] with acanthoic acid. It showed anti-inflammatory effect [10], smooth muscle relaxant effect [12], and anti-fungal activity [13]. Another structural isomer of acanthoic acid, continentalic acid ((−)-pimara-8(14),15-diene-19-oic acid), was reported early from *Aralia cordata* [14], *Gnaphalium gaudichaudianum* [15] and *A. trifoliatus* [4]. The only difference between acanthoic and

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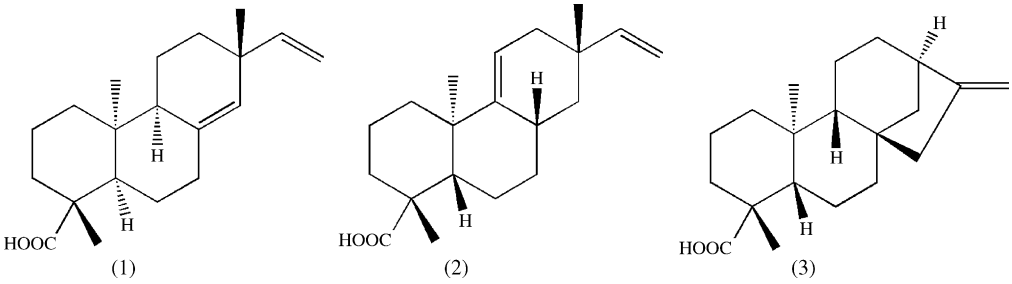

Fig. 1. Chemical structures of continentalic (1), acanthoic (2), and kaurenoic (3) acids.

continentalic acids is the position of the double bond in the skeleton.

The genus *Acanthopanax* includes 10 species, 3 forma and 2 varieties in Korea [16]. Extracts of *Acanthopanax* species are used as ingredients in health foods without any classification of the taxonomical differences of the species. However, we have previously reported differences in the content of chemical components between *Acanthopanax* species [17,18]. For effective utilization and quality control of the natural resources, analysis of the active compounds in *Acanthopanax* species could be very important. Although several reports have dealt with the determination of kaurenoic acid from *Annona glabra* by HPLC [19], from *Xylopia* species by HPLC [20], from *Mikania glomerata* by GC [21] or from *Montanoa* species by HPLC [22], no report about the simultaneous determination of diterpenoid isomers has been published. In our preliminary trials, acanthoic, kaurenoic and continentalic acids could not be separated simultaneously by HPLC because of their close molecular structure and physical properties (Fig. 1). Capillary electrophoresis (CE) could be an alternative separation method to HPLC that is able to be used for the determination of numerous compounds in plant extracts [23–26]. In this experiment, acanthoic, continentalic and kaurenoic acids were separated by CE and the taxonomical differences of *Acanthopanax* species were evaluated on the basis of the concentration of diterpenoids.

2. Experimental

2.1. Instruments and chemicals

CE analysis was performed on a ^{3D}CE (Hewlett Packard, Germany) equipped with a DAD detector. Data were collected and analyzed on an HP Vectra computer equipped with an HP Chemstation system. ATI Model 370 (Orion, MA, USA) was used for measuring the pH. Methanol (Tedia, OH, USA), borax (Sigma, MO, USA) and hydrochloric acid (Merck, Germany) used in this work were of HPLC grade. β-Cyclodextrin sulfobutyl ether (DS 6.5) was purchased from Cydex Co. (KS, USA). Milli-Q (Millipore, MA, USA) treated water with resistivity more than 18 MΩ cm was used throughout the experiment. Acanthoic and continentalic acids were isolated from the root of *A. koreanum* [2] and kaurenoic acid from *A. trifoliatus*, as reported previously [4]. Their structures were identified by ^{1}H NMR, ^{13}C NMR and MS. The purity of the isolated compounds confirmed by HPLC was more than 99%.

2.2. Plant materials

The plant materials were collected from various regions in Korea and Vietnam. *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 from a farm operated by Susin Co. in Cheonan, Chungnam Province. *A. chiisanensis* (CNU-A05) was from Chiri mountain in Jeonbuk Province and *A. trifoliatus* (CNU-A06) was collected in Langson Province, Vietnam. They were identified by Prof. Young Ho Kim in the College of Pharmacy, Chungnam National University (CNU). The collected samples were separated in leaves, stems, roots and fruits and stored at a cool and dark place. The voucher specimens were deposited at the herbarium in the College of Pharmacy, CNU.

2.3. Electrophoresis

To separate the compounds by CE, a fused silica, uncoated capillary 50 μm inner diameter, 38.5 cm in length (30 cm effective length) was used with borate buffer (0.05 M, pH 8.5) containing β-cyclodextrin sulfobutyl ether at a concentration of 30 mg/ml. The system was programmed to rinse capillary at the beginning and between runs by flush with sodium hydroxide (0.1 M), distilled water and running buffer for 2 min each. Sample injection was performed by pressure at 50 mbar for 5 s. The electrophoretic procedure was developed at voltage of 18 kV with positive polarity. Detection was performed at the wavelength of 195 nm. Cartridge temperature was set at 20 °C.

2.4. Sample preparation

A powdered sample (0.5 g) was extracted with 20 ml of dichloromethane in ultrasonic bath three times for 40 min. The extract was then washed two times with 20 ml of water. The organic layer was collected and dichloromethane was evaporated under nitrogen stream. The residue was reconsti-

tuted in 1 ml of 50% methanol and centrifuged (Microspin, Hanil, Korea) at 15,000 × *g* for 10 min. The supernatant was suitably diluted with 50% methanol and injected directly to the capillary. Stock standard solutions of acanthoic, continentalic and kaurenoic acids were prepared by dissolving each compound in 50% methanol (1.0 mg/ml) and suitably diluted.

2.5. Optimization of CE conditions for separation

To separate the three compounds it was necessary to add appropriate selector to the buffer, because it was difficult to separate them without any selector. To optimize the type of selector, cyclodextrin (CD) derivatives, which are the most common selectors in the field of chiral separation by CE, such as β-CD, methyl β-CD, dimethyl β-CD, carboxymethyl β-CD, acetyl β-CD, hydroxypropyl β-CD, hydroxytrimethylammoniumpropyl β-CD, succinyl β-CD, sulfobutyl ether β-CD, α-CD, hydroxypropyl-α-CD, γ-CD, and hydroxypropyl-γ-CD were tested in various concentrations. Furthermore the effects of the pH and concentration of the buffer and applied voltage on the separation were also tested.

2.6. Precision and recovery test

For recovery testing, known amounts of continentalic, acanthoic and kaurenoic acid standards were added to powdered sample of *A. senticosus*, which contained no detectable amount of the interested compounds, and then quantified by CE. Intraday and interday precisions were calculated from the analysis of the standard in five consecutive runs and in five consecutive days, respectively.

3. Results and discussion

3.1. Optimization of CE conditions for separation

The three compounds, acanthoic, kaurenoic and continentalic acids, could not be separated by CE in the absence of any selector (Fig. 2a) or neutral selector (Fig. 2b and c). The neutral selectors could shorten the migration time, because they move together with electroosmotic flow, while the negatively charged sample molecules move towards the anode. This is an indication that these three acids could be well incorporated in the CD cavity. The negatively charged CDs could increase the migration time of the compounds and provide the possibility of separation, because these CDs move in the opposite direction of the electroosmotic flow. Carboxymethyl β-CD could not separate continentalic acid from kaurenoic acid (Fig. 2d), whereas sulfobutyl ether β-CD could separate the three compounds with reasonable resolution (Fig. 2e). None of the other tested CDs could separate the three compounds. When the concentration of sulfobutyl ether β-CD was increased from 10 mg/ml to 50 mg/ml, the migration time increased; however the resolution was best for 30 mg/ml of

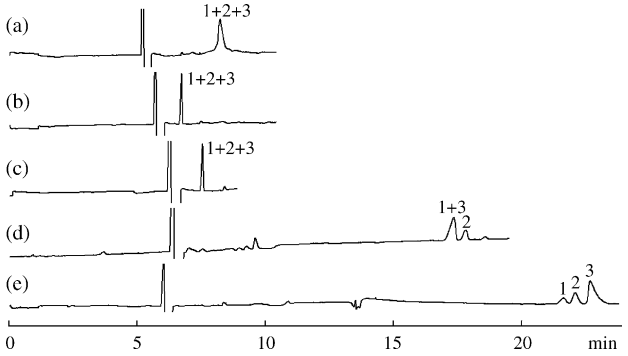

Fig. 2. Representative electropherograms for the separation of continentalic (1), acanthoic (2), and kaurenoic (3) acids. The electrolyte used was borate buffer (0.05 M, pH 8.5) without (a) or with selectors such as 20 mg/ml dimethyl β-CD (b), 30 mg/ml hydroxypropyl β-CD (c), 20 mg/ml carboxymethyl β-CD (d), and 30 mg/ml sulfobutyl ether β-CD (e). CE was run with a fused silica, uncoated capillary (50 μm × 38.5 cm, 30 cm effective), an applied voltage of 8 kV, and detection at 195 nm.

CD (Fig. 3a). To increase the resolution and reduce the analysis time, the CE method was further optimized with the pH and buffer concentration. As shown in Fig. 3b and c, the optimum pH was 8.5 and the optimum buffer concentration was about 70 mM. However, the experiments were carried out with 50 mM buffer because an elevated current value and increased noise level were observed with 70 mM buffer. Increased resolution and decreased migration time were observed when the applied voltage was increased up to 18 kV; however, peak broadening and decreased resolution, caused by Joule heating, were observed with voltage elevated above 18 kV.

3.2. Linearity, recovery and precision

The calibration functions of continentalic, acanthoic and kaurenoic acid standards calculated with peak area (y, mAU) and concentration (x, μg/ml) were $y = 0.76x - 1.20$ ($r^2 = 0.9948$), $y = 0.74x - 0.82$ ($r^2 = 0.9934$) and $y = 0.81x - 0.16$ ($r^2 = 0.9966$), respectively, over the concentration range of 15–150 μg/ml for continentalic and acanthoic acids, and of 30–300 μg/ml for kaurenoic acid. The intraday and interday precisions of the analysis, calculated by five determinations are presented in Table 1. The intraday and interday precisions of the diterpenoids ranged from 2.5% to 11.7% and from 4.8% to 15.0%, respectively. These values were rather larger than those from other analytical data in CE [25,26]. The main reason could be the difficult control for the homogeneous sampling and for the exact extraction from the plant materials. The recovery efficiency in the CE analysis was in the range of 88–99% as shown in Table 2. The observed linearity and the results of recovery testing indicated that this CE method is suitable and applicable for qualitative and quantitative evaluation of the *Acanthopanax* species. The detection limits of continentalic, acanthoic and kaurenoic acids were 100, 50 and 50 ng/ml, respectively, at a signal to noise ratio of 3.

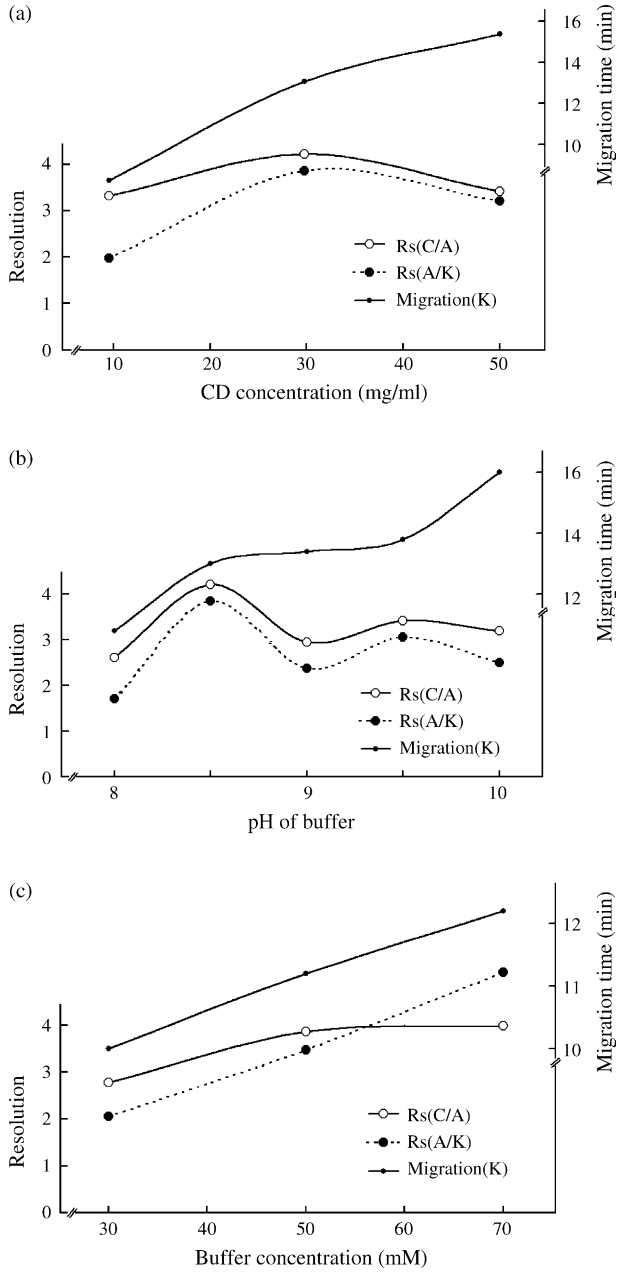

Fig. 3. Effects on migration time and resolution of (a) concentration of sulfobutyl ether β-CD, (b) pH, and (c) buffer concentration. Solid line with dots; migration time of kaurenoic acid, solid line with open circles; resolution between continentalic and acanthoic acids [Rs(C/A)], dotted line with closed circles; resolution between acanthoic and kaurenoic acids [Rs(A/K)]. Only one parameter was varied among the parameters, concentration (50 mM) and pH (8.5) of buffer, concentration of sulfobutyl ether β-CD (30 mg/ml) and applied voltage (18 kV).

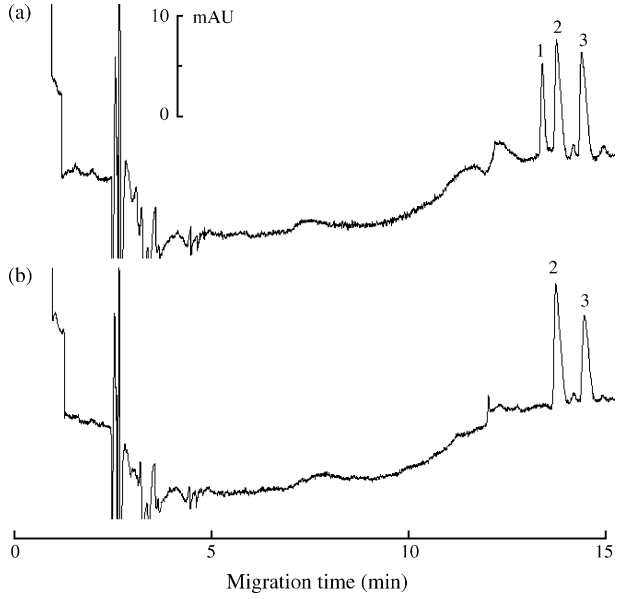

Fig. 4. Representative electropherogram of (a) a standard mixture of continentalic (1), acanthoic (2) and kaurenoic acids (3) in 0.1 mg/ml, and (b) a root extract of *A. koreanum*.

3.3. Analysis of diterpenoids in the Acanthopanax species

The diterpenoids in the roots, stems and leaves in six *Acanthopanax* species were analyzed with borate buffer (0.05 M, pH 8.5) containing 30 mg/ml sulfobutyl ether β-CD and an applied voltage of 18 kV, and were detected at 195 nm. The three compounds, acanthoic, continentalic and kaurenoic acids, were separated at the base line with reasonable resolution (Fig. 4). The contents of the diterpenoids are presented in Table 3. The *Acanthopanax* species used in this experiment could be categorized into two groups in relation with the amount of diterpenoids. One group consisted of *A. koreanum* and *A. trifoliatus* with a higher diterpenoid content (>0.1%), while the other group, *A. senticosus*, *A. senticosus* f. *inermis*, *A. divaricatus* var. *albeofructus* and *A. chiisanensis* had a lower diterpenoid content (<0.1%). However, *A. koreanum* and *A. trifoliatus* could be discriminated by their contents of acanthoic and continentalic acids. Interestingly, acanthoic acid was found only in the root of *A. koreanum*, while its positional isomer, continentalic acid only in *A. trifoliatus*. Due to the potent hepatoprotective [9] and anti-inflammatory [8] effects of acanthoic acid, *A. koreanum* was considered as an important resource for the development of

Table 1
The intraday and interday precisions of the diterpenoid analysis

Standard (mg/ml)	Intraday, CV (%)			Interday, CV (%)		
	Continentalic acid	Acanthoic acid	Kaurenoic acid	Continentalic acid	Acanthoic acid	Kaurenoic acid
0.05	8.17	7.11	2.48	11.61	9.43	11.95
0.20	5.42	5.25	5.83	4.81	9.36	7.28
0.40	11.66	10.24	9.70	15.02	9.32	8.16

Table 2
The recovery of diterpenoids from the leaves of *A. senticosus*

Added (mg/g)	Found (mg/g)			Recovery (%)		
	Continentalic acid	Acanthoic acid	Kaurenoic acid	Continentalic acid	Acanthoic acid	Kaurenoic acid
0.5	0.46	0.47	0.44	91.2	94.7	88.8
2.0	1.75	1.84	1.98	87.6	92.0	98.8
4.0	3.68	3.88	3.88	92.1	96.9	95.2

Table 3
Concentration of continentalic acid, acanthoic acid and kaurenoic acid in the *Acanthopanax* species

Species	Parts	Continentalic acid	Acanthoic acid	Kaurenoic acid
A. koreanum	Roots	0	0.92 ± 0.05	0.79 ± 0.04
	Stems	0	0	0.12 ± 0.02
	Leaves	0	0	0.08 ± 0.01
A. trifoliatus	Roots	0.23 ± 0.03	0	0.09 ± 0.01
	Stems	0.05 ± 0.01	0	0.17 ± 0.02
	Leaves	0	0	<0.01
A. senticosus	Roots	<0.01	0	0
	Stems	<0.01	<0.01	0
	Leaves	0	0	0
A. senticosus f. *inermis*	Roots	<0.01	<0.01	0
	Stems	0	0	0
	Leaves	0	<0.01	<0.01
A. divaricatus var. *albeofructus*	Roots	0	0	0
	Stems	0	0	0
	Leaves	0	0	0
A. chiisanensis	Roots	0	0	0
	Stems	0	<0.01	0
	Leaves	0	<0.01	0

Data are given as mean ± S.D. ($n = 3$) in % dried sample.

health foods and herbal drugs in Korean biodiversity. *A. senticosus* was categorized in a group having a lower content of diterpenoid, although this species had a close genotype with *A. koreanum* by using random Amplified Polymorphic DNA (RAPD) analysis [27]. Classification of *Acanthopanax* species according to the contents of diterpenoids, eleutherosides B and E, and chiisanoside as we reported before [17,18], was in good accord with the taxonomy. The chemotaxonomical index based on the contents of diterpenoids in association with those of eleutherosides B and E, and chiisanoside could be used in the classification and discrimination of *Acanthopanax* species.

4. Conclusion

A capillary electrophoretic procedure has been developed for the determination of diterpenoids in *Acanthopanax* species. Acanthoic, continentalic and kaurenoic acids were successfully separated on a fused silica, uncoated capillary, 50 μm inner diameter, 38.5 cm in length (30 cm effective length) with borate buffer (0.05 M, pH 8.5) containing 30 mg/ml sulfobutyl ether β-CD. *A. koreanum* and *A. trifoliatus* showed relatively high diterpenoid content (>0.1%) in comparison with the other tested species, indicated that these two species carried the chemotaxonomical similarity. However, acanthoic acid was found from *A. koreanum*, while its positional isomer, continentalic acid, from *A. trifoliatus*.

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