Natural Product Sciences
19(1) : 15-19 (2013)

Analysis of Flavonoid Contents in the Fruits of *Acanthopanax* Species using HPLC

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Abstract – Analysis of flavonoid contents in the fruits of *Acanthopanax* species (*A. chiisanensis*, *A. divaricatus*, *A. koreanum*, *A. senticosus*, and *A. sessiliflorus*) was conducted by high performance liquid chromatography. A Discovery® C18 (4.6 × 250 mm, 5 μm) column was used with a gradient mobile phase of water and acetonitrile (90 : 10 to 60 : 40 for 60 min) and UV detection was conducted at 350 nm. The contents of rutin, hyperin, quercetin, afzelin, and kaempferol were 0.063~0.540, 0.494~7.480, 0.584~0.704, 0.388~0.567, 0.190~0.471 mg/g, respectively, in the fruits of *Acanthopanax* species. Total content of flavonoids in the fruits of *Acanthopanax* species was highest in those of *A. chiisanensis*. Furthermore, hyperin was the most abundant compound in the fruits of *Acanthopanax* species. Consequently, our results demonstrate that the fruits of *Acanthopanax* species containing flavonoids have promising potential as a new income source of agriculture and industry in medicinal natural products, health supplements, and beverages.
Keywords – *Acantopanax*, Flavonoid, HPLC, Hyperin.

Introduction

Acanthopanax species are perennial herbaceous genus of the family Araliaceae that are widely distributed in East-Asia, such as Korea, China, Russia, and Japan (Boon and Smith, 1999). Most of *Acanthopanax* species grows to 2~4 m in height, bears five leaflets, the flowering is from July to September, and the fruit ripening in October (Lee, 2003). The dried roots and stem barks of *Acanthopanax* species have been used for a long time as a sedative and tonic to treat rheumatism and hepatitis, liver disease and diabetes, chronic bronchitis, stress, ischemic heart disease, tumor, hypertension, and gastric ulcers (Fujikawa *et al*., 1996; Kang *et al*., 2005; Ni and Liu, 2006).

Until now, the study of *Acanthopanax* species has been focused on stems, roots, and leaves. There are only a few studies on the fruit of *Acanthopanax* species. Most of the fruit of *Acanthopanax* species is round and be used on health supplements, and beverages in Korea (Lee, 2003; Kim *et al*., 2006). Also, the biological activities of *Acanthopanax* fruits are antitumor, immunostimulating (Lee *et al*., 2003), antioxidant, and antimicrobial (Kim *et al*., 2006). The phytochemicals of fruits from *Acanthopanax* species are composed of sterols (β-sitosterol and stigmasterol), a nitrogen compound (sessiline), lignins (sesamin, savinin, eleutherosides B and E), a coumarin (scoparone), terpenoids (ursolic acid, chiisanoside, and methyl betulin), a phenolic compound (protocatechuic acid), a flavonoid (hyperin) (Kim and Lee, 1990; Yook *et al*., 1992; Lee *et al*., 2002; Lee *et al*., 2002). The quantitative analysis of phytochemicals in fruits from *Acanthopanax* species are eleutherosides B and E in *A. sessiliflorus* (Kim *et al*., 2006), chiisanoside and hyperin in *Acanthopanax* species (Lee *et al*., 2007; Lee *et al*., 2010).

Among various phytochemical constituents in *Acanthopanax* species, flavonoids such as afzelin, antoside, isoquercitrin, hyperin, kaempferol, quercitrin, and rutin have previously been isolated from *Acanthopanax* species (Yasue *et al*., 1968; Kitajima *et al*., 1989; Chung and Hahn, 1991; Shirasuna *et al*., 1997; Lee *et al*., 2002). Flavonoids have been used as natural antioxidants and for their health-promoting properties in humans (Bekker *et al*., 2006). Flavonoids having various biological activities are important compounds in *Acanthopanax* species. Until now, many studies have reported on the analysis of triterpenoids, lignans, and phenylpropanoids constituents of *Acanthopanax* species (Shin and Lee, 2002; An *et al*.,

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2008; Lee *et al*., 2011; Kim *et al*., 2012), but there are few reports on flavonoid analysis in *Acanthopanax* species.

In this study, analysis of flavonoids (rutin, hyperin, quercetin, afzelin, and kaempferol) in the fruits of *Acanthopanax* species was conducted using high performance liquid chromatography (HPLC).

Experimental

Plant materials – The fruits of *Acanthopanax* species (*A. chiisanensis*, *A. divaricatus*, *A. koreanum*, *A. senticosus,* and *A. sessiliflorus*) were cultivated and collected at Gongju, Korea, and botanically identified by Prof. S. H. Cho, Gongju National University of Education, Korea.

Apparatus and chemicals – Mass spectrometry (MS) was performed using a Jeol JMS-600W (Tokyo, Japan) mass spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker Avance 300, 400, and 500 NMR (Rheinstetten, Germany) spectrometer using tetramethylsilane (TMS) as an internal standard. Evaporation was conducted using an Eyela rotary evaporator system (Tokyo, Japan) under reflux *in vacuo*. TLC was performed with precoated silica gel 60 F_{254} plates (Art. 5715, Merck Co., Darmstädt, Germany). The compounds on the TLC plate were visualized by spraying with 10% sulfuric acid in methanol followed by heating at 100°C to detect spot color. HPLC chromatograms were recorded with a Waters Breeze system (Massachusetts, USA) equipped with a Waters 1525 binary HPLC pump and 2489 system UV/VIS detector. Water and acetonitrile used in this research were of HPLC grade, and all other reagents were of analytical grade.

Preparation of flavonoids – Compounds **1 - 5** were isolated from *Fagopyrum tataricum*, *Acanthopanax chiisanensis*, *Vaccinium koreanum*, and *Rhododendron mucronulatum* for. *albiflorum* by repeated column chromatography as reported previously by our team. Compound **1** (rutin) was isolated from the ethyl acetate fraction of *F. tataricum* (Mok *et al*., 2011). Compound **2** (hyperin) was isolated from the ethyl acetate fraction of *A. chiisanensis* (Lee *et al*., 2008). Compound **3** (quercetin) was isolated from the butanol fraction of *V. koreanum* (Lee *et al*., 2008). Compounds **4** and **5** (afzelin and kaempferol, respectively) were isolated from the ethyl acetate fraction of *R. mucronulatum* for. *albiflorum* (Mok and Lee, 2013).

Sample preparation – For analysis of flavonoids (rutin, hyperin, quercetin, afzelin, and kaempferol) in the fruits of *Acanthopanax* species (*A. chiisanensis*, *A. divaricatus*, *A. koreanum*, *A. senticosus,* and *A. sessiliflorus*), each 50 g of fruits from *Acanthopanax* species was extracted with 50% MeOH (3 × 100 mL) by reflux and evaporated *in vacuo*. The residue was dissolved in 1 mL of MeOH and filtered with a 0.45 μm filter. The resulting solution was used for HPLC analysis.

HPLC conditions – HPLC separation of flavonoids for qualitative and quantitative analysis was performed using a reverse phase system. A Discovery® C18 (4.6 × 250 mm, 5 μm) column was used with a mobile phase consisting of water (0.1% acetic acid) and acetonitrile. The elution program was a gradient solvent system of water and acetonitrile (90 : 10 to 60 : 40 for 60 min). UV detection was conducted at 350 nm. The injection volume was 10 μL and the flow rate was 1 mL/min. All injections were performed in triplicate.

Calibration curve – A stock solution (1 mg/mL) of each flavonoid was prepared in MeOH, successively reducing the solution content to 50% to create different concentrations. The contents of the analytes were determined from the corresponding calibration curves. The calibration functions of the flavonoids were calculated using the peak area (Y), concentration (X, μg/10 μL), and mean values (n = 5) ± standard deviation.

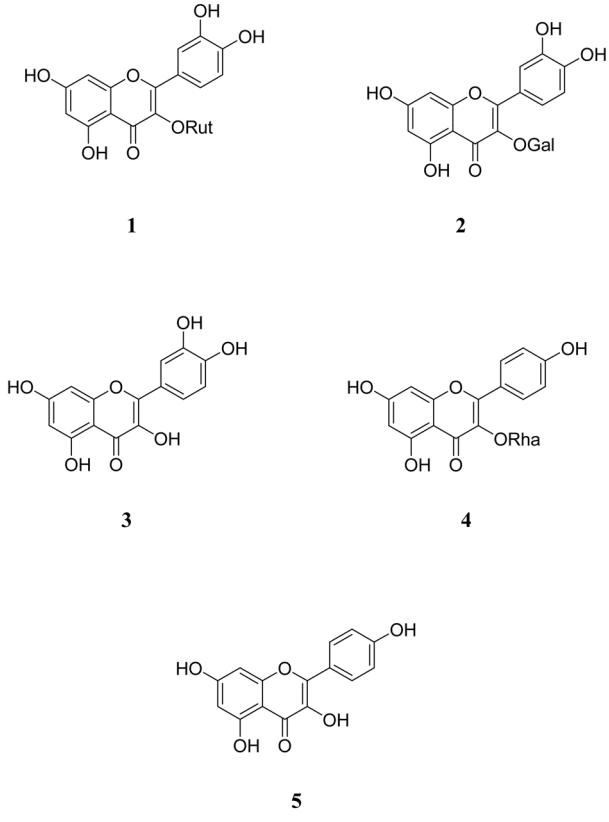

Fig. 1. Chemical structures of compounds **1 - 5**.

Table 1. Calibration curves for compounds **1 - 5**

Compound	t_R	Calibration equation [a]	Correlation factor, r^2 [b]
1	20.3	Y = 398,291.7345X + 1,500.1193	0.9999
2	20.9	Y = 6,586.1167X – 12,154.2627	0.9999
3	30.5	Y = 4,696.8372X – 13,277.3222	0.9999
4	37.2	Y = 18,089.3016X – 33,399.1890	0.9999
5	45.6	Y = 72,658.2176X – 5,993.2176	0.9999

[a] Y = peak area, X = concentration of standard (μg/ml).
[b] r^2 = correlation coefficient for three data points in the calibration curve (n = 5).

Table 2. Contents of compounds **1-5** in the MeOH extracts of the fruits of *Acanthopanax* species

Sample	Content (mg/g)					
	1	**2**	**3**	**4**	**5**	Total
A. chiisanensis	0.224 ± 0.011	7.480 ± 0.277	0.615 ± 0.003	0.567 ± 0.009	0.190 ± 0.002	9.076 ± 0.294
A. divaricatus	0.105 ± 0.007	4.414 ± 0.066	0.704 ± 0.009	0.474 ± 0.002	0.288 ± 0.002	5.986 ± 0.053
A. koreanum	0.540 ± 0.008	0.774 ± 0.020	trace	0.389 ± 0.001	0.471 ± 0.014	2.175 ± 0.015
A. senticosus	0.063 ± 0.002	0.494 ± 0.003	0.587 ± 0.001	trace	0.212 ± 0.002	1.355 ± 0.007
A. sessiliflorus	0.326 ± 0.011	3.209 ± 0.011	0.584 ± 0.001	0.388 ± 0.000	0.338 ± 0.005	4.845 ± 0.025
Total	1.259 ± 0.002	16.370 ± 0.254	2.490 ± 0.006	2.188 ± 0.010	1.499 ± 0.017	

Data are represented as the mean ± S.D. (n = 4) in mg/g of the dried extract samples.

Results and Disscusion

Simultaneous determination of flavonoids in the fruits of *Acanthopanax* species was conducted by HPLC. Compounds **1-5** (Fig. 1) were previously isolated from *A. chiisanensis, A. divaricatus*, *A. koreanum*, and *A. sciadophylloides* (Kitajima *et al*., 1989; Chung and Hahn, 1991; Shirasuna *et al*., 1997; Lee *et al*., 2008). Rutin (**1**) has been reported to have antidiabetic, antioxidative, and antihypertensive activities (Lee *et al*., 2000; Alsaif, 2009; Rie *et al*., 2010). In addition, there are many reports on antiapoptotic effects, anti-inflammatory, and anticancer (Middleton *et al*., 2000; Zhang *et al*., 2010; Kim *et al*., 2011) of hyperin (**2**); antiviral, antihypertensive, and neuroprotective activities (Johari *et al*., 2012; Rogovski *et al*., 2012) of quercetin (**3**); antimicrobial and antioxidant activities (Tatsimo *et al*., 2012) of afzelin (**4**); and antimicrobial, and antidiabetic effetcs (Zang *et al*., 2011; Jindal and Kumar, 2012) of kaempferol (**5**).

The HPLC separation of flavonoids for qualitative and quantitative analysis was conducted using a reverse phase system with a mobile phase consisting of water and acetonitrile (90 : 10 to 60 : 40 for 60 min). Standard calibration curves for compounds **1 - 5** are shown in Table 1. Using an optimized analytical method, flavonoids in the fruits of *Acanthopanax* species were successfully determined simultaneously. The contents of rutin (**1**), hyperin (**2**), quercetin (**3**), afzelin (**4**), and kaempferol (**5**) were detected in the fruits of *A. chiisanensis* (0.224, 7.480, 0.615, 0.567, and 0.190 mg/g, respectively), *A. divaricatus* (0.105, 4.414, 0.704, 0.474, and 0.288 mg/g, respectively), *A. koreanum* (0.540, 0.774, trace, 0.389, and 0.471 mg/g, respectively), *A. senticosus* (0.063, 0.494, 0.587, trace, and 0.212 mg/g, respectively), and *A. sessiliflorus* (0.326, 3.209, 0.584, 0.388, and 0.338 mg/g, respectively). Also, the total content of flavonoids was detected in the fruits of *A. chiisanensis* (9.076 mg/g), *A. divaricatus* (5.986 mg/g), *A. koreanum* (2.175 mg/g), *A. senticosus* (1.355 mg/g), and *A. sessiliflorus* (4.845 mg/g), respectively. In our results, the most abundant flavonoid in the stems was hyperin (16.370 mg/g), and the total content of flavonoids was highest in *A. chiisanensis* (9.076 mg/g) (Table 2). In particular, the total content of flavonoids in *A. chiisanensis* was 7 times than that of *A. senticosus.* The content of hyperin was similar to the previous our study. The content of hyperin from *Acanthopanax* species fruits was detected the highest in the *A. chiisanensis* and the least in the *A. senticosus* (Lee *et al.,* 2010).

Consequently, our results demonstrate that the fruits of *Acanthopanax* species containing flavonoids have promising potential as a new income source of agriculture and

industry in medicinal natural products, health supplements, and beverages in Korea.

Acknowledgement

We thank the National Center for Inter-University Research Facilities (Seoul National University, Republic of Korea) for the NMR and MS measurements.

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Received December 23, 2012
Revised January 15, 2013
Accepted February 22, 2013