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SHORT COMMUNICATION

Online High Performance Liquid Chromatography (HPLC)-ABTS$^+$ Based Assay and HPLC-Electrospray Ionization Mass Spectrometry Analysis of Antioxidant Phenolic Compounds in *Salsola komarovii*

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Abstract Online high performance liquid chromatography (HPLC)-2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS$^+$) based assay system for phenolic antioxidants in *Salsola komarovii* extracts was applied. HPLC elute was mixed with a stabilized solution of ABTS$^+$ reagents, and the negative peaks, indicating the antioxidant activity, were monitored by measuring the decrease in absorbance at 734 nm. HPLC with electrospray ionization mass spectrometry was used to identify the seven flavonoids and two phenolic amides in the *S. komarovii* extracts. The antioxidant compounds were identified as rutin, isoquercitrin, astragalin, and isorhamnetin.

Keywords antioxidant · flavonoids · online high performance liquid chromatography-2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation based assay · phenolic amides · *Salsola komarovii*

Introduction

Plants are a rich source of natural antioxidants including polyphenolic compounds, tocopherol (Vitamin E), ascorbic acid (Vitamin C), and carotenoids. In particular, polyphenols are isolated from higher plants as mostly antioxidants, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Parr and Bolwell, 2000). Antioxidant can prevent the oxidation process, the initial step in the development of degenerative diseases, cancer, cardiovascular diseases, among others (Block et al., 1992; Ames et al., 1993; Vinsion et al., 1995). Thus, antioxidants have recently become a topic of increasing interest.

Among the many antioxidant capacity assays available, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS$^+$) and 2,2-diphenyl-1-picryl-hydrazylhydrate (DPPH) assays are based on electron-transfer reaction. The reagents are made up of oxidants that can abstract an electron from the antioxidant, causing change in color of the reagent. Due to their operational simplicity, these assays have been used in many research laboratories to study antioxidant capacity (Huang et al., 2005). However, the offline ABTS assay involves a process that isolates and determines of antioxidants due to the complex composition of the plants. This step is costly and inefficient. To address this problem, an online high performance liquid chromatography (HPLC)-ABTS$^+$ assay using separation and activity identification has recently been developed. This technique is able to rapidly detect radical scavenging compounds with a minimum sample preparation (Nuengchamnong et al., 2005) and has been applied to the evaluation of antioxidant compositions in fruits and vegetables (Bandoniene and Murkovic, 2002; Stewart, 2005).

The genus *Salsola* is one of the largest genera in the family Chenopodiaceae, which includes about 200 species occurring in arid regions of Africa, Asia, and Europe. Phytochemical studies on plant members of this genus resulted in the isolation of flavonoids, alkaloids, acetophenones, coumarines, and sterols (Monica et al., 2007). *Salsola* species, a well-known folk

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medicine in China, used for treatments of hypertension, cancer, and inflammation (Al-Saleh et al., 1993; Nikiforov et al., 2002). However, only few studies on the biological activity of *Salsola komarovii* have been performed. In the present study, the antioxidant constituents of *S. komarovii* were determined using the online HPLC coupled $ABTS^+$ based assay and HPLC-mass spectroscopy (MS) for rapid detection and identification.

The aerial parts of *S. komarovii* were collected from Gangneung, Korea and identified by one of the authors (C.Y. Kim). The voucher specimens (Salsola) were deposited in the Functional Food Center, KIST Gangneung Institute, Gangneung, Korea. Dried *S. komarovii* (1 g) was extracted with 50 mL of ethyl acetate by ultrasonic extraction at room temperature for 3 h and filtered through filter paper into a 50-mL volumetric flask. HPLC grade acetonitrile, water and ethanol were purchased from Fisher Scientific (Pittsburgh, PA). ABTS and potassium persulfate, and formic acid (FA) were obtained from the Sigma-Aldrich Company (St. Louis, MO). Other solvents were purchased from Daejung Chemicals & Metals Co. Ltd (Siheung, Korea).

The online $ABTS^+$ based HPLC analysis was modified using the $ABTS^+$ assay methods of Stewart et al. (2005). A 2-mM $ABTS^+$ stock solution containing 3.5 mM potassium persulphate was prepared by diluting the stock 8-fold in methanol followed by incubation overnight at room temperature in darkness to allow for stabilization of the radicals. The HPLC system was equipped with an Agilent Series 1200 liquid chromatography, a vacuum degasser, a binary pump, an autosampler, a column oven, and diode array detection (DAD) and MWD detectors (Agilent, Waldbronn, Germany). A Waters XTerra® MS C18 column (2.1 × 150 mm, 3.5 μm, Waters, Milford, MA) was also used. The gradient started with 0.1% FA in acetonitrile/0.1% FA in water (10:90, %, v/v) to 40:60 (%, v/v) for 50 min. Chromatogram was recorded at 280 nm. The sample injection volume was 10 μL, and the flow rate was 0.2 mL/min. After passing through the column, the HPLC elute arrived at a "T" piece, where the $ABTS^+$ was added. The $ABTS^+$ flow rate was set at 0.8 mL/min delivered by an additional Agilent 1200 Pump. After a thorough mixing at 40°C with a 1-mL loop, the absorbance was measured using a UV-detector at 734 nm. The data were then analyzed using the ChemStation (Agilent Technologies).

The polyphenols were identified by Thermo HPLC-MS systems (Thermo Fisher Scientific Inc., SanJose, CA). HPLC with electrospray ionization mass spectrometry system, which consisted of an ACCELA photodiode array detector, an autosampler, a quarternary pump, and LCQ FLEET ion trap mass spectrometer equipped with an electrospray ionization source, was employed. In addition, Thermo Xcalibar software was used for data acquisition and processing. The HPLC conditions were the same as those previously described in on-line $ABTS^+$ based assay HPLC analysis section**.** Mass spectrometer conditions are as follows: positive and negative ion mode; mass range, *m/z* 100–1000; capillary voltage, 49 V; tube lens, 100 V; Sheath gas flow rate (N_2), 35 arb; Aux gas flow rate (N_2), 12 arb; capillary temp, 300°C.

The ethyl acetate extracts of *S. komarovii* were applied into the HPLC system. Following separation, the HPLC eluate was mixed with a stabilized solution of $ABTS^+$ reagents, and the solution was set to a UV-Vis detector monitoring absorbance of 734 nm. The radical solution is deep blue in color, and any quenching of the $ABTS^+$ radical resulted in a loss of color indicated by a negative peak in the absorbance profile monitored at 734 nm (Stewart et al., 2005). Nine major compounds were detected in their HPLC with DAD chromatogram (Fig. 1), among which four negative peaks were detected, indicating that the corresponding four components were antioxidants in *S. komarovii*.

Positive and negative ion mode electrospray ionization mass spectrometry **(**ESI-MS) were used to identify the seven flavonoids and two phenolic amides. HPLC-photodiode array (PDA)-ESI/MS analysis of the *S. komarovii* extract was carried out, and the results are shown in Table 1 and Fig. 2. The presence in all mass spectra of an ion at *m/z* 303 or 287 or 317 indicated that the peaks 1–6 were quercetin, kaempferol or isorhamnetin derivatives. Peaks 1, 3, and 4 showed $[M+H]^+$ ions at *m/z* 611, 595, and 625, respectively. Other characteristic fragment ions $[M+H-162]^+$ and $[M+H-(162+146)]^+$ indicated neutral losses of rhamnose (Rha) and glucose (Glc). The UV spectrums of peaks 1 and 4 showed

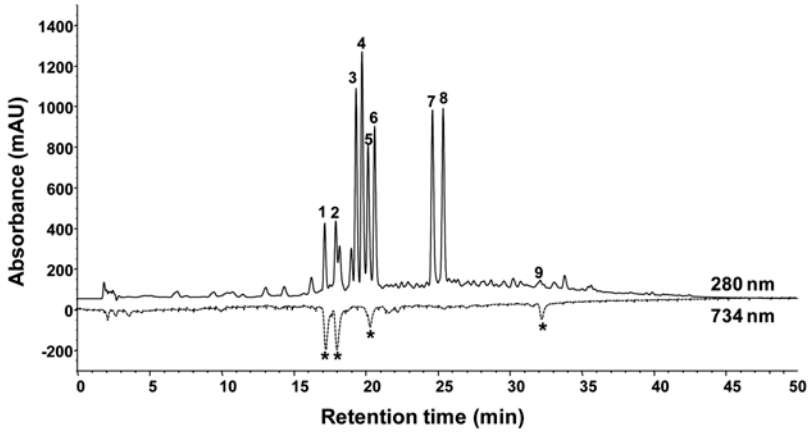


Fig. 1 Online HPLC-ABTS+ analysis of *S. komarovi* EtOAc extract. 1, Rutin; 2, Isoquercitrin; 3, Kaempferol-3-*O*-rutinoside; 4, Isorhamnetin-3-*O*-rutinoside; 5, Astragalin; 6, Isorhamnetin-3-*O*-glucoside; 7, *N-trans*-feruloyl tyramine; 8, *N-trans*-feruloyl-3-*O*-methyldopamine; 9, Isorhamnetin.

Table 1 Peak assignment for the analysis of *S. komarovii*

Peak	RT (min)	$[M+H]^+$ (*m/z*)	fragment ion (*m/z*)	$[M-H]^-$ (*m/z*)	λ_{max} (nm)	Identification
1	17.1	611	465, 303	609	256, 354	Rutin
2	17.9	465	303	463	256, 354	Isoquercitrin
3	19.3	595	449, 287	593	265, 348	Kaempferol-3-*O*-rutinoside
4	19.7	625	479, 317	623	256, 354	Isorahmnetin-3-*O*-rutinoside
5	20.1	449	287	447	265, 354	Astragalin
6	20.6	479	317	477	254, 356	Isorhamnetin-3-*O*-glucoside
7	24.6	314	177	312	290, 317	*N*-*trans*-feruloyl tyramine
8	25.4	344	177	342	289, 317	*N*-*trans*-feruloyl-3-*O*-methyldopamine
9	32.1	317	-	315	265, 368	Isorhamnetin

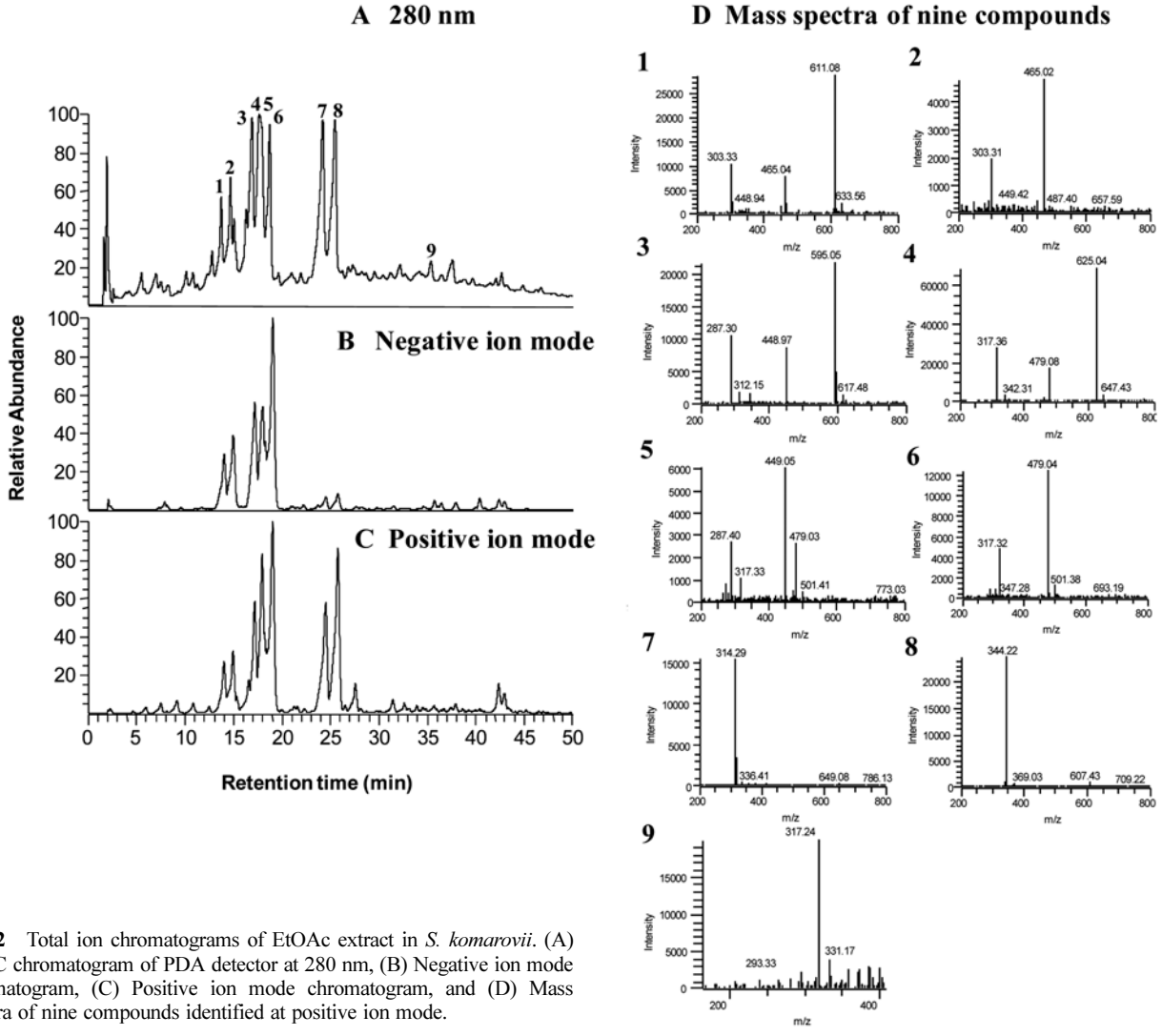
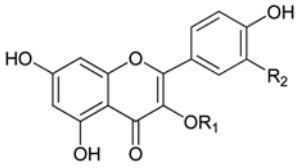
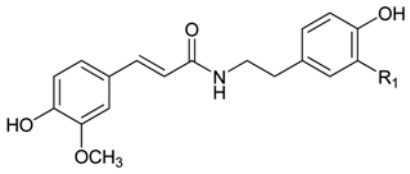


Fig. 2 Total ion chromatograms of EtOAc extract in *S. komarovii*. (A) HPLC chromatogram of PDA detector at 280 nm, (B) Negative ion mode chromatogram, (C) Positive ion mode chromatogram, and (D) Mass spectra of nine compounds identified at positive ion mode.

maximum absorption at 256 and 354 nm, respectively. These compounds were identified as rutin and isorahmnetin-3-*O*-rutinoside based on the UV and MS spectra and previously reported literature (Bilia et al., 2002; Wang et al., 2003). Peak 3 displayed UV absorptions with maximum at 265 and 348 nm and was identified as kaempferol-3-*O*-rutinoside (Papp et al., 2004). Peaks 2, 5, and 6 indicate $[M+H]^+$ ions at m/z 465, 449, and 479, and major fragment ion at m/z 303, 287, and 317 (corresponding to the aglycone) due to the loss of a hexose unit and were identified as isoquercitirin, astragalin, and isorhamnetin-3-*O*-glucoside, respectively. Peaks 2, 5, and 6 displayed UV spectrum with maximum at 256, 354 nm, 265, 354 nm and 254, 356 nm,



Rutin (**1**), R_1=Rutinose, R_2=OH
Isoquercitrin (**2**), R_1=Glc, R_2=OH
Kaempferol-3-*O*-rutinoside (**3**), R_1=Rutinose, R_2=H
Isorhamnetin-3-*O*-rutinoside (**4**), R_1=Rutinose, R_2=OCH_3
Astragalin (**5**), R_1=Glc, R_2=H
Isorhamnetin-3-*O*-glucoside (**6**), R_1=Glc, R_2=OCH_3
Isorhamnetin (**9**), R_1=H, R_2=OCH_3



N-trans-feruloyl tyramine (**7**), R_1=H
N-trans-feruloyl-3-*O*-methyldopamine (**8**), R=OCH_3

Fig. 3 Structures of flavonoids (1-6, 9) and phenolic amides (7, 8) in the *S. komarovi* extract.

respectively (Bilia et al., 2002; Gall et al., 2003). The UV spectrum of peak 9 showed a maximum absorbance at 265 and 368 nm, and the mass analysis of peak 9 showed an $[M+H]^+$ ion at *m/z* 317 and $[M-H]^-$ at *m/z* 315 in ESI-MS with positive and negative ion modes. This compound was identified as isorhamnetin based on UV and MS spectra and previously reported literature (Duan et al., 2011). The MS of peak 7 showed a molecular ion $[M+H]^+$ at *m/z* 314, and a base peak at *m/z* 177 which corresponded to a feruloyl moiety. Peak 8 indicated a $[M+H]^+$ ion at *m/z* 344, and a base peak at *m/z* 177. Peaks 7 and 8 contain ferulic acid as a common structure, and tyramine (peak 7) or dopamine (peak 8). The UV spectrum of peak 7 was very similar to that of peak 2 with a maximum at 290 and 317 nm. Peaks 7 and 8 were identified as *N-trans*-feruloyl tyramine and *N-trans*-feruloyl methyldopamine by comparing their MS and UV data with a reference (Tanaka et al., 1989).

Peaks 1, 2, 5, and 9 indicate the presence of antioxidant activity in the *S. komarovii* extracts (Fig. 1). Antioxidant activity of flavonoids depends on the structure and substitution pattern of the hydroxyl group. The structure of flavonoids for effective radical scavenging has the 3',4'-orthodihydroxy configuration in ring B and 4-carbonyl group in ring C. In addition, the presence of 3-OH group, giving a catechol-like structure in ring C, also improves the antioxidant activity of flavonoids. Quercetin, the aglycone of peaks 1 and 2, has identical numbers of hydroxyl groups, with 3',4'-orthodihydroxy configuration in ring B and 5,7-dihydroxy groups in ring A (Wojdylo et al., 2007). The aglycone of kaempferol (peak 5) and isorhamnetin (peak 9) have a hydroxyl group at position 3 (Kim and Lee, 2004). This result showed that the plants with high levels of quercetin, kaempferol, and isorhamnetin had high antioxidant activity.

Rutin, isoquercitirin, astragalin, and isorhamnetin exhibited the antioxidant activity in online HPLC-$ABTS^+$ assay. These compounds have also been reported to show high scavenging activity against the $ABTS^+$ free radical-generating system (Han et al., 2004; Ascension et al., 2008).

The results of the present study indicate that *S. komarovii* is rich in polyphenolic constituents and demonstrates good antioxidant activity as measured by HPLC coupled with an on-line $ABTS^+$ based assay system. Seven flavonoids and two phenolic amides were identified using HPLC-ESI/MS, and their antioxidant activities were detected simultaneously. The on-line coupled HPLC-$ABTS^+$ based assay and HPLC-ESI/MS analysis allow rapid identification of antioxidants in natural products.

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