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Validation of High-Performance Liquid Chromatography Analysis on Phenolic Substances of *Cirsium setidens* and Sedative Effect of Pectolinarin as the Active Principle

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Abstract – This study was performed to determine the composition of phenolic substances contained in the leaves of *Cirsium setidens* (Compositae), validate the high-performance liquid chromatography (HPLC) method, and determine the *in vivo* sedative effect of the main component pectolinarin. Six phenolic compounds isolated from *C. setidens* were spectroscopically identified as chlorogenic acid (**1**), hyperoside (**2**), 3,4-di-*O*-caffeoylquinic acid (**3**), caffeic acid methyl ester (**4**), linarin (**5**), and pectolinarin (**6**) and then used as standard compounds for HPLC analysis. HPLC proved to be precise, accurate, and sensitive for the simultaneous analysis of the phenolic substances. In particular, six compounds showed good regression ($R^2 > 0.999$) within test ranges and recovery was in the range of 95.4 - 104.8%. The content of pectolinarin was considerably higher (156.48 mg/g) than those of other phenolic substances including the other flavone glycoside, linarin (18.99 mg/g). The contents of other phenolic substances, in order, were chlorogenic acid (8.41 mg/g), 3,4-di-*O*-caffeoylquinic acid (5.74 mg/g), hyperoside (4.33 mg/g), and caffeic acid methyl ester (0.51 mg/g). Oral administration with compound **6** (10 and 20 mg/kg) enhanced the sleeping time induced by pentobarbital in mice, indicating that it has a sedative effect.
Keywords – *Cirsium setidens*, Compositae, phenolic, pectolinarin, HPLC, validation, sedative

Introduction

We have studied mountainous vegetables mainly belonging to the family Compositae in terms of phytochemistry and pharmacology, since those foods are highly consumed as healthy foods in Korea. These are generally abundant in polyphenolic substances. We have reported the pharmacological activities (Nugroho and Bachri *et al.*, 2010; Nugroho and Lee *et al.*, 2010) and the content of such compounds in mountainous vegetables, including caffeoylquinic acids and flavonoids (Nugroho *et al.*, 2009; Nugroho *et al.*, 2010).

We have also reported a significant *in vivo* hepatoprotective effect of pectolinarin isolated from *Cirsium setidens* Nakai used for treatment of hemostasis, hematemesis, heamaturia, and hypertension as a Korean famous mountainous vegetable during our studies on the mountainous vegetables (Yoo *et al.*, 2008). Presently, we attempted to qualitatively and quantitatively identify the composition polyphenolic substances in *C. setidens*, since the high concentration of pectolinarin was predicted during the studies on this plant.

Numerous studies have reported on the phytochemical and pharmacological characteristics of flavonoids, in particular on the pharmacology of flavones or flavone glycosides, including eupatilin (5,7-dihydroxy-6,3',4'-trimethoxyflavone) with gastroprotective action from Artemisia species (Song *et al.*, 2008), pectolinarin (5,7-dihydroxy-6,4'-dimethoxyflavone 7-O-rutinoside) with hepatoprotective (Yoo *et al.*, 2008) and anti-inflammatory (Lim *et al*., 2008) actions, and diosmin with vascular-protective action (Bouskeia *et al.*, 1995). In addition, hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) binds with γ-aminobutyric acid (GABA) as an inhibitory neurotransmitter receptor and opens chloride channel of postsynaptic neurons, exerting central nervous system (CNS) inhibitory actions (Kavvadias *et al.*, 2004). In addition, gastroprotective actions of flavones or their

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glycosides have been reported (Nilsson *et al.*, 2008; Mota *et al.*, 2009). The gastroprotective flavones reduce gastric acid secretion by inhibiting H^+-K^+-ATPase on the pariental cell of stomach and by stimulating prostaglandin E_2 biosynthesis (Beil *et al.*, 1995; Alcaraz *et al.*, 1985).

In this study, we report the isolation, identification, and quantitative analysis of phenolic substances in *C. setidens* together with the *in vivo* sedative effect of pectolinarin. Validation using high-performance liquid chromatography (HPLC) method was performed to ensure that the methodology was sufficiently selective, accurate, and reproducible for quality control. Since the fresh and processed leaves of *C. setidens* are available in the market in Korea, the two plant materials were analyzed under the name of unprocessed- and processed ones throughout this article.

Materials and Methods

Instruments and reagents – HPLC was performed using a Varian HPLC system (Varian, Walnut Creek, CA, USA) consisted of a Prostar 210 solvent delivery module and Prostar 325 UV-Vis detector. Separation was achieved on a Shiseido (Chuoku, Tokyo, Japan) Capcell Pack C18 column (5 μm, 250 mm × 4.6 mm I.D.). All solvents used in the analysis were HPLC grade obtained from J.T. Baker (Phillipsburg, NJ, USA).

Plant material – The leaves of *Cirsium setidens* (Compositae) were collected from a farm in Youngweol-gun, Gangwon-do, Korea. This plant was identified by Professor Sang-Cheol Lim (Department of Horticulture and Landscape Architecture, Sangji University, Korea). A voucher specimen was deposited in the laboratory of Natural Product Chemistry, Department of Pharmaceutical Engineering, Sangji University, Korea). This was dried and powdered with a blender to use it as a plant material, and is presently designated as the unprocessed one. On the other hand, the fresh leaves of *C. setidens* were dried after boiling in water for 10 min to obtain the processed one that was the same as the product in the market, and was designated as the processed one.

Extraction, fractionation, and isolation – Dried leaves of *C. setidens* (350 g) were extracted with methanol (MeOH) under reflux for 5 hours, for three times. The extracted solution was filtered and evaporated under reduced pressure on a rotatory evaporator to give a viscous extract. It was further subjected to freeze-drying to yield 32.6 g. The extract was fractionated into seven fractions using a Diaion HP-20 column (6.5 cm × 35 cm). In this column, 2000 ml water was poured into the column to remove sugars, ions, or other very polar substances. Then, 1500 ml 50% MeOH and 2500 ml MeOH were supplied in this order and collected (500 ml each). After concentration, seven fractions were obtained: Fr. A (1.40 g), Fr. B (3.95 g), Fr. C (8.36 g), Fr. D. (4.12 g), Fr. E (4.56 g), Fr. F (3.62 g), and Fr. G (1.34 g). Using some of these fractions, six compounds (**1** - **6**) were isolated. In brief, Fr. C was chromatographed on silica gel column (5.5 cm × 35 cm) using $CHCl_3$-MeOH-H_2O (6 : 4 : 1, lower phase) to yield chlorogenic acid (**1**, CGA, 38 mg). Fr. D was subjected to an ODS column (4.5 cm × 30 cm) using 55% aqueous MeOH to yield caffeic acid methyl ester (**4**, CAME, 28 mg). Using the same column with higher methanolic solvent (60% MeOH), Fr. E (4.25 g) was chromatographed to produce 3,4-di-*O*-caffeoyl-quinic acid (**3**, 3,4-DQ, 46 mg), linarin (**5**, 86 mg), and pectolinarin (**6**, 1.62 g). Separation of Fr. F using an ODS column (3.5 cm × 35 cm) and the solvent (60% MeOH) produced hyperoside (**2**, 76 mg).

Chlorogenic acid: Amorphous brown solid, ^{1}H- NMR (Cheminat *et al*., 1988), and ^{13}C-NMR (Lin *et al.*, 1999).

Hyperoside: Yellowish powder, mp 253 - 254 ºC, FAB-MS; ^{1}H- and ^{13}C-NMR (Lee *et al.*, 2003).

3,4-di-*O*-caffeoylquinic acid: Amorphous brown solid, ^{1}H- and ^{13}C-NMR (Timmerman *et al.*, 1983).

Caffeic acid methyl ester: White solid, mp 150 - 152 ºC, ^{1}H- and ^{13}C-NMR (Cuong *et al.*, 2009).

Linarin: mp 273 - 275 ºC, ^{1}H- and ^{13}C-NMR (Roh *et al.*, 2000).

Pectolinarin: White amorphous powder from MeOH, mp 250 - 253 ºC; ^{1}H- and ^{13}C-NMR (Yoo *et al.*, 2008).

Sample preparation – The two processed and the two unprocessed plant materials (10 g each) weighed exactly were put in the four different bottles. The solvent MeOH (200 ml each) were added to the two bottles containing a processed- and an unprocessed one, respectively; the solvent 30% MeOH in the other two bottles. They were extracted with an ultrasonicator at 50 ºC for 6 h, filtered, and subjected to drying using a rotatory evaporator and further freeze-drying to produce powdery extracts. Two MeOH extracts of the processed- (extraction yield, 1.32 g) and unprocessed (1.61 g) fractions together with the two 30% MeOH extracts of processed- (1.62 g) and unprocessed ones (2.49 g) were used for quantitative analysis by HPLC.

HPLC conditions – Test samples and standard compounds were dissolved in MeOH using an ultrasonicator and a vortex mixer, and then filtered through a 0.50 μm syringe filter before injection. The mobile phase was a mixed solvent of 0.05% trifluoroacetic acid (TFA)

in water (solvent A) and 60 : 40 methanol-acetonitrile + 0.05% TFA (solvent B). The gradient elution system was as follows: (A)/(B) = 58/42 (0 min; hold for 8 min) → 40/60 (24 min) → 0/100 (26 min; hold for 4 min) → 58/42 (32 min; hold for 8 min to equilibrate the column condition). Column temperature was maintained at 40 ºC using a temperature controller. Analysis was performed at a flow rate of 1.00 ml/min with detection wavelength fixed at 254 nm.

Method validation of quantitative analysis – The analytical method was validated according to the International Conference on Harmonization guidelines by determination of the linearity, limit of detection (LOD) and limit of quantification (LOQ), precision, repeatability, and accuracy of each analyte.

Standard solution and the linearity – Linearity was examined with standard solutions. Individual solutions of six standard compounds were prepared by dissolving in methanol. Working standard solutions were prepared by serial dilution with the same solvent to get six different concentrations in the range of 1.56 - 40.00 μg/ml. The calibration curve and linearity was determined by plotting the mean of peak area (y axis) versus concentration (x axis) of each analyte in that range which expressed by a calibration equation.

LOD and LOQ – By injecting an aliquot (20 μl) of the serial dilution of six individual standard solutions, LODs and LOQs under the present HPLC method were determined at signal to noise (S/N) of 3 and 10, respectively.

Precision, repeatability, and accuracy studies – Precision of the chromatographic method was assessed by measuring the intra- and inter-day variability of the MeOH extract of unprocessed sample. The intra-day variability was determined by analyzing that sample within 24 hours. The solution were injected five times, and the relative standard deviation (RSD) value was calculated for concentration of each analyte contained in that extract, and considered as a measure of precision. The inter-day variability was performed on three consecutive days with each sample injected five times a day. To confirm the repeatability, five different working solutions prepared from the same sample (MeOH extract of the unprocessed one) were analyzed. Variations were expressed as relative standard deviation (RSD). To evaluate the accuracy, a recovery test was carried out in MeOH extract spiked with standard compounds. The accuracy was evaluated by calculating the mean recovery (%) of the standard compounds from the spiked extract solution versus the non-spiked extract sample.

Animals – ICR mice weighing 25 ± 3 g were purchased from Daehan Biolink (Eumseong, Republic of Korea), housed in a controlled environment at 22 ± 3 ºC and 40 - 60% humidity under 12 hour light/dark cycle, and cared for according to the Guide for the Care and Use of Laboratory Animals issued by the American Institute of Laboratory Animal Resources.

Pentobarbital-induced sleeping time – Mice were fasted for 24 hours prior to the experiment. Sample solutions were prepared by dissolving samples in 4% Tween 80 for treatment. The MeOH extract (100 and 200 mg/kg) and pectolinarin (10 and 20 mg/kg) were orally administered with jonde to mice daily for two weeks. Thirty minutes after the final treatment of samples, animals were intraperitoneally injected with pentobarbital (42 mg/kg). Latency for the loss of righting reflex and its total duration were recorded. However, animals in whom the loss of righting reflex was not caused within 15 min were excluded in this experiment (Wolfman *et al.*, 1996; Darias *et al.*, 1998).

Results and Discussion

Isolation and identification of phenolic substances – The MeOH extract of the leaves of *C. setidens* was fractionated into seven fractions using Diaion HP-20 column chromatography and then separated using silica gel-, Sephadex LH-20, and/or ODS column chromatography to yield six compounds. Based on the spectroscopic data, compounds **1 - 6** were identified as chlorogenic acid (CGA) (**1**), hyperoside (**2**), 3,4-di-*O*-caffeoylquinic acid (3,4-DQ) (**3**), caffeic acid methyl ester (CAME) (**4**), linarin (**5**), and pectolinarin (**6**). Except for pectolinarin, the compounds have not been previously reported from *C. setidens*. The structures of **1 - 6** are shown in Fig. 1.

Optimization of extraction method – The two solvents, MeOH and 30% MeOH, were used to extract the processed- and unprocessed plant materials. MeOH was better to extract phenolic substances because most peak areas were larger than 30% MeOH.

Optimization of HPLC condition – Column, mobile phases, and detection wavelengths were considered for HPLC chromatographic resolution. A Capcell Pack C18 column (5 μm, 250 mm × 4.6 mm) was chosen, because it produced better peak separations and sharper peaks than other columns. As solvents (A and B) of a mobile phase, 0.05% TFA in water as a solvent A and 0.05% TFA in MeOH-acetonitrile (60 : 40) as a solvent B, were chosen and run according to the programmed gradient elution, since the selected mobile phase system produced better peak shapes and resolution than MeOH-H_2O system and

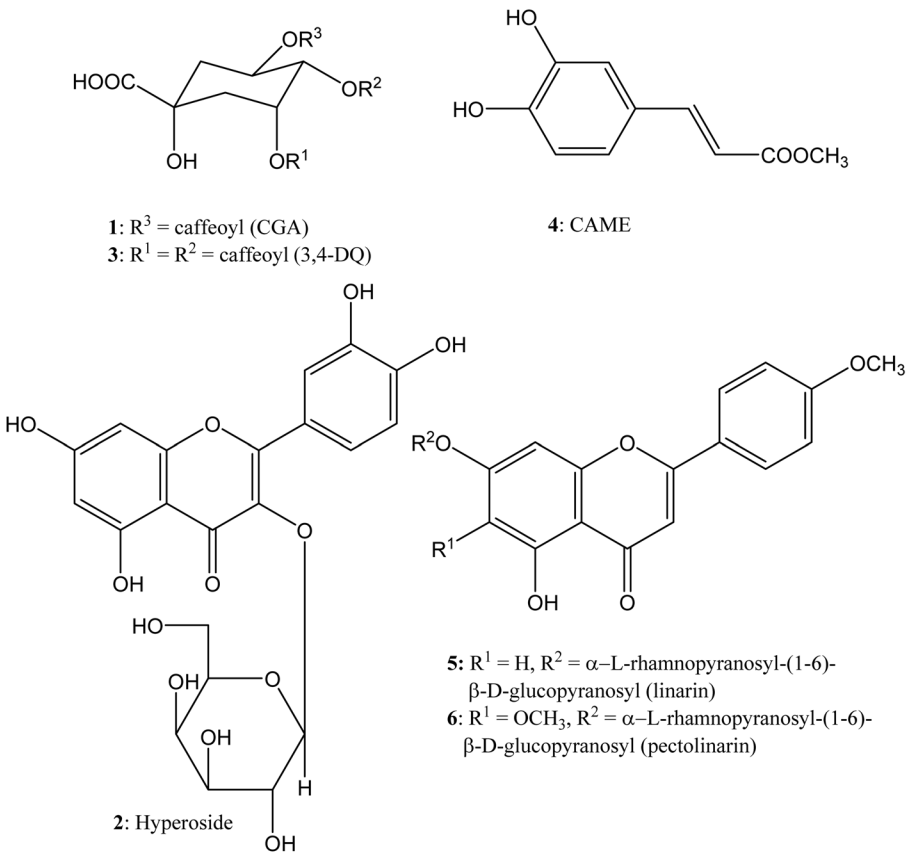

Fig. 1. Chemical structures of (**1**) chlorogenic acid; (**2**) hyperoside; (**3**) 3,4-di-*O*-caffeoylquinic acid; (**4**) caffeic acid methyl ester; (**5**) linarin; (**6**) pectolinarin.

Table 1. Calibration curves, detection limits and quantification limits of the analytes

Analyte	Calibration equation (linear model)[a]	Linear range (μg/ml)	R^{2} [b]	LOD[c] (μg/ml)	LOQ[d] (μg/ml)
CGA	y = 85.929x + 11.292	2.50-40.00	0.9998	0.74	2.47
Hyperoside	y = 217.040x + 46.329	2.34-37.50	0.9999	0.14	0.46
3,4-DQ	y = 133.617x + 45.500	1.88-30.00	0.9999	0.23	0.76
CAME	y = 172.389x + 44.580	1.72-27.50	0.9999	0.12	0.40
Linarin	y = 315.523x – 81.790	1.56-31.25	0.9998	0.50	1.67
Pectolinarin	y = 105.305x – 18.403	1.88-37.50	0.9997	0.89	2.97

[a]y, peak area at 254nm; x, concentration of the standard (μg/ml); [b]R^2, correlation coefficient for 5 data points in the calibration curves (n = 3); [c] LOD, limit of detection (S/N = 3); [d] LOQ, limit of quantification (S/N = 10).

MeOH-CH_3CN systems. Use of acid (TFA) made peak shapes better due to the ionization inhibition of phenolic substances. Column temperature, flow rate, and detection wavelength were maintained at 40 °C, 1.00 ml/min, and 254 nm to obtain chromatograms at constant condition.

Method validation of quantitative analysis – Linearity, precision, repeatability, and accuracy were evaluated according to the International Conference on Harmonization guideline to validate the present HPLC method. To validate the linearity, serial dilutions of six individual standard solutions in the range of 1.56 - 40.00 μg/ml were injected to HPLC and calibration curve equations were calculated. As shown in Table 1, linearity was established based on the R^2 values > 0.999 and linear ranges were also determined. LODs and LOQs of six compounds were < 0.89 μg/ml and < 2.97 μg/ml. Experiments on intra- and inter-day variations were conducted to validate the precision of the method. In addition, sample solutions were injected five times to find the repeatability by calculating RSD. As shown in Table 2, intra-day

Table 2. Analytical results of intra-day and inter-days variability and repeatability for the analytes in MeOH extract of unprocessed sample

Analyte	Intra-day variability		Inter-days variability		Repeatability (n = 5)		
	Content (mg/g)	RSD (%)	Content (mg/g)	RSD (%)	Content (mg/g)	RC[a]	RSD (%)
CGA	8.41	1.38	8.48	1.52	8.32	0.053	2.85
Hyperoside	4.33	2.06	4.42	2.31	4.57	0.029	3.21
3,4-DQ	5.74	1.92	5.79	2.21	5.95	0.038	2.95
CAME	0.53	2.39	0.52	3.35	0.53	0.003	3.94
Linarin	18.99	0.69	18.89	0.92	19.21	0.123	1.50
Pectolinarin	156.48	0.29	156.18	0.49	156.91	1.000	0.83

[a]RC, relative concentration.

Table 3. Recovery of each analyte determined by standard addition method (n = 3)

Analyte	Initial concentration (μg/ml)	Amount added (μg)	Concentration after addition (μg/ml)		Recovery (%)	RSD (%)
			Expected	Measured		
CGA	15.80	15.00	30.80	29.55	95.94	2.23
Hyperoside	8.56	18.70	27.26	26.55	97.39	2.12
3,4-DQ	10.89	15.00	25.89	24.75	95.60	1.95
CAME	1.26	6.87	8.13	8.52	104.79	2.51
Linarin	35.50	120	155.5	147.30	94.73	0.70
Pectolinarin	305.78	300	605.78	584.516	96.49	0.15

The data was present as average of three determinations. The sample type used to carry out the recovery study was extract of unprocessed plant material extracted by 100% of methanol.

variations of compounds **1 - 6** were < 2.39% and inter-day variations were < 3.35%. Repeatability was < 3.94%. A recovery test was used to determine accuracy. The test was carried out in MeOH extract spiked with standard compounds**.** The accuracy was evaluated by calculating the mean recovery (%) of the standard compounds from the spiked extract solution versus the non-spiked extract sample. As shown in Table 3, average recoveries of six compounds were present in the range of 94.73% and 104.79%, and the RSDs were < 2.51%. Therefore, the present HPLC method enabled the precise, accurate, and sensitive simultaneous determination of the phenolic saubstances of *C. setidens*

Sample analysis – Phenolic substances of the leaves of *C. setidens* were analyzed by HPLC using standard compounds isolated from the same plant material. As shown in Table 4, relative retention times (RRT) and the RSD values considering pectolinarin as a reference compound were determined. In two different type of plant material (processed and unprocessed) with different solvent of extraction (30%- and 100% MeOH), content of pectolinarin was much higher than other phenolic substances contained in the extract, including the other flavone glycoside, linarin, as shown in Table 5. In the case of MeOH extract of unprocessed plant material, content of pectolinarin was 156.48 mg/g, equivalent to 25.19 mg/g of the dried weight of plant material. It was much higher than linarin, 18.99 mg/g of extract (equivalent to 3.06 mg/g of the dried weight of plant material). The contents of other phenolic substances were detected, in order of quantity, as chlorogenic acid (8.41 mg/g), 3,4-DQ (5.74 mg/g), hyperoside (4.33 mg/g), and caffeic acid methyl ester (0.51 mg/g). The content of pectolinarin in the MeOH extract was higher than the 30% MeOH extract, indicating that the former solvent was more efficient for extraction than the latter. In addition, the concentration of pectolinarin in the unprocessed one was higher than in the processed one, indicating that processing for the preservation accompanies a small loss of phenolic substances including pectolinarin.

Table 4. Relative Retention Time.

Analyte	RRT	RSD (%)
CGA	0.22	0.21
Hyperoside	0.36	0.16
3,4-DQ	0.41	0.17
CAME	0.56	0.12
Linarin	0.94	0.12
Pectolinarin	1.00	0.10

Based on our evaluation of phenolic substances in *C.*

Table 5. Content of analytes of unprocessed (U) and processed (P) plant materials with two different solvents of extraction

Analyte	Content of analyte											
	U (100% MeOH)[a]			U (30% MeOH)			P (100% MeOH)			P (30% MeOH)		
	Extc.[b]	DW[c]	RSD[d]	Extc.	DW	RSD	Extc.	DW	RSD	Extc.	DW	RSD
CGA	8.41	1.35	1.38	7.28	1.81	2.21	5.96	0.39	2.34	5.26	0.42	1.95
Hyperoside	4.33	0.69	2.06	2.01	0.50	2.81	1.95	0.13	2.78	1.83	0.15	2.45
3,4-DQ	5.74	0.92	1.92	4.26	1.06	1.79	2.65	0.18	1.64	1.87	0.15	2.03
CAME	0.51	0.08	3.33	0.63	0.16	3.21	0.48	0.03	2.85	0.56	0.04	2.79
Linarin	18.99	3.06	0.69	8.32	2.07	0.90	9.28	0.61	1.22	6.90	0.56	1.18
Pectolinarin	156.48	25.19	0.29	73.26	18.24	0.51	115.62	7.63	0.42	89.14	7.22	0.37

[a]Value in the parentheses are solvent for extraction; [b]Extc., content of analyte of extract (mg/g); [c]DW, content of analyte of the dried weight of plant material (mg/g); [d]RSD, (%) for n = 5.

Table 6. Effect of pretreatment of the MeOH extract of unprocessed sample and its main component pectolinarin on the latency time for the loss of righting reflex and its total duration in pentobarbital-induced mice

Treatment	Dose (mg/kg, *p.o.*)	Latency time (min)	Total duration (min)
Control	–	3.64 ± 0.46[a]	65.4 ± 6.1[d]
MeOH extract	100	3.87 ± 0.33[a]	73.1 ± 8.7[d]
	200	3.68 ± 0.40[a]	98.7 ± 7.8[c]
Pectolinarin	10	3.63 ± 0.25[a]	95.2 ± 3.9[c]
	20	3.52 ± 0.37[a]	113.2 ± 10.3[b]
Chlorpromazine	50	2.36 ± 0.43[b]	211.8 ± 24.6[a]

Mice were fasted for 24 hr prior to the experiment. Samples were orally administered daily for two weeks. Thirty min after the final treatment of samples, animals were intraperitoneally injected with pentobarbital (42 mg/kg). Data are mean ± SD (n=6). Values sharing the same superscript letter are not significantly different each other ($p<0.05$) by Duncan's multiple range test. CPZ: Chlorpromazine

setidens and our HPLC method validation, pectolinarin could be a major and marker compound for the quality control of *C. setidens*. Polyphenolic substances including caffeoylquinic acids and flavone glycosides, which are commonly rich in Compositae, are pharmacologically active against aging-related diseases such as hepatitis, diabetes mellitus, obesity, and hyperlipidemia. Flavones or flavone glycosides that are bioactive or developed as medicinal drugs have been reported. A flavone, hispidulin, of *Salvia officinalis* L. (Labiatae; also called sage) has agonistic action against the GABA receptor to bestow anxiolytic, sedative, and hypnotic actions (Kavvadias *et al.*, 2004). Well-known pharmacologically active flavones or their glycosides include eupatilin with gastroprotective action (Song *et al.*, 2008), diosmin with vascular-protecting action (Bouskeia et al., 1995), and pectolinarin with hepatoprotective (Yoo *et al.*, 2008) and anti-inflammatory (Lim *et al.*, 2008) actions. Therefore, the leaves of *C. setidens* with high level of pectolinarin could be also beneficial for several diseases such as hepatitis, inflammation, and others.

Sedative activity – Loss of righting reflex in pentobarbital-induced mice and its total duration were used to test sedative effect of the MeOH extract and pectolinarin. As shown in Table 6, 100 and 200 mg/kg of MeOH extract and 10 and 20 mg/kg pectolinarin exhibited no reduction of latency time for the loss of righting reflex compared to the control, but showed marked increases of total duration. Chlorpromazine, which was used as a positive control, decreased the latency time and increased the total duration. The increase of pentobarbital-induced loss of righting reflex is reported to be related to the interaction with different neurotransmitter systems, GABA (Lolli *et al.*, 2007; Cryan *et al.*, 2004) and 5-HT (serotonin) (Zhao *et al.*, 2004), although the sedative mechanism of MeOH extract and pectolinarin is unclear. However, the present results demonstrate that pectolinarin could be a major sedative compound of *C. setidens*. The sedative effect of pectolinarin has not been reported before.

In conclusion, *C. setidens*, one of Korean famous mountainous vegetables, could be used as a functional food or developed as a medicinal drug based on the high concentration of pectolinarin**.** In addition, the present verified method could be used to simultaneously analyze phenolic substances in *C. setidens* for quality control.

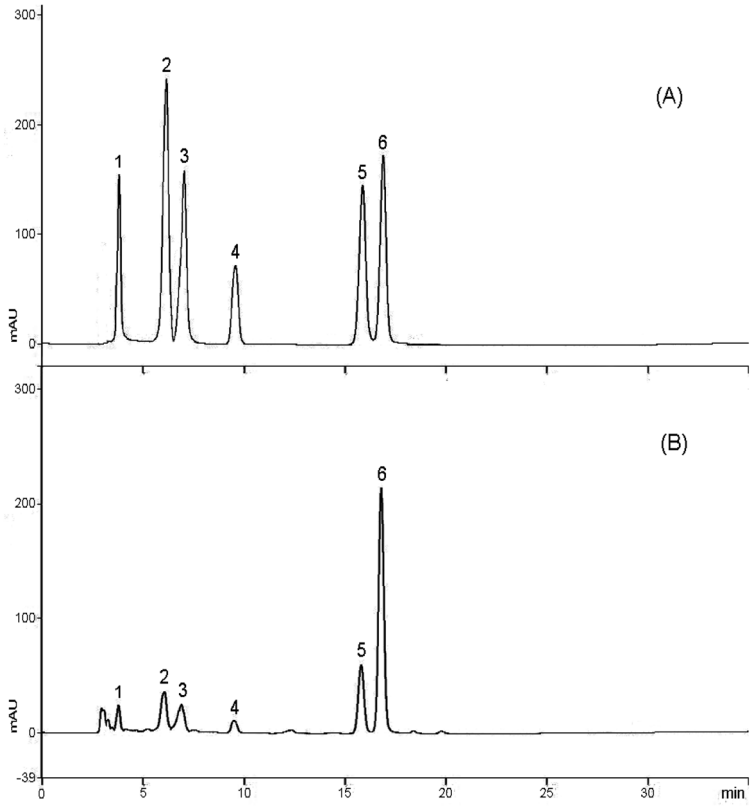

Fig. 2. (A) Chromatogram of mixture standard compounds: (**1**) chlorogenic acid; (**2**) hyperoside; (**3**) 3,4-di-O-caffeoylquinic acid; (**4**) caffeic acid methyl ester; (**5**) linarin; (**6**) pectolinarin. (B) Chromatogram of MeOH extract of unprocessed sample.

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