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Quantitative Determination of Five Phenolic Peroxynitrite-scavengers in Nine Korean Native Compositae herbs

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Abstract – Peroxynitrite ($ONOO^-$)-scavenging activities of nine Compositae herbs consisting of three *Ixeris*, two *Youngia*, two *Cirsium* and one of each *Lactuca* and *Taraxacum* species were evaluated. The contents of their $ONOO^-$ scavengers in the extracts were also determined on a HPLC using seven standard compounds, chlorogenic acid (CGA), chicoric acid (CA), luteolin 7-glucoside (luteolin-7-glc), luteolin 7-glucuronide (luteolin-7-glcU), luteolin, linarin and pectolinarin. Five of those compounds exhibited potent $ONOO^-$-scavenging activities: IC_{50}, CA (0.76 μM), CGA (1.34 μM), luteolin (0.81 μM), luteolin-7-glc (0.86 μM) and luteolin-7-glcU (3.13 μM). Both CA and luteolin-7-glc were highly contained in *I. dentata* (19.71 mg/g and 13.58 mg/g, respectively), *I. dentata* var. *albiflora* (17.58 mg/g and 23.83 mg/g, respectively) and *I. sonchifolia* (65.71 mg/g and 6.99 mg/g, respectively). Among the nine herbs, those three *Ixeris* species had very low IC_{50} values over the range of 0.48 - 1.74 μg/mL, suggesting that they could be potential therapeutic vegetables, particularly for preventing diabetic complications or obesity, which can be caused by an excess production of $ONOO^-$.
Keywords – *Ixeris* species, wild vegetable, chicoric acid, luteolin, HPLC

Introduction

Peroxynitrite ($ONOO^-$), which is formed through the reaction of nitric oxide with a superoxide anion radical, causes severe cytotoxicity, although nitric oxide itself is not cytotoxic. Excess production of $ONOO^-$ induces cytotoxicity and neurotoxicity.[1] Initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane Na^+/K^+ ATPase activity, inactivation of membrane sodium channels, and other oxidative protein modifications contribute to the cytotoxic effect of $ONOO^-$.[2] Furthermore, those reactions trigger cellular responses ranging from subtle modulations of cell signaling to overwhelming oxidative injury, committing cells to necrosis or apoptosis. $ONOO^-$ generation represents a crucial pathogenic mechanism in conditions such as stroke, myocardial infarction, chronic heart failure, diabetes, circulatory shock, chronic inflammatory diseases, cancer, and neurodegenerative disorders.[3]

Endothelial dysfunction induced by $ONOO^-$ in obesity and diabetic diseases can result in cardiovascular diseases. Therefore, such a nitrosative stress due to a very reactive $ONOO^-$ can cause diabetic complications related to cardiovascular dysfunction.[4] Pires *et al*.[5] reported the reduction of body weight by treatment of a peroxynitrite scavenger, Mn (III) tetrakis (4-benzoic acid) porphyrin chloride, in obese mice. In addition, it was found that the reduction of nitrosative stress contributes to the amelioration of neurotoxicity, cytotoxicity, and vascular endothelial toxicity.[6]

In general, caffeoylquinic acids which are a group of polyphenols exhibit strong $ONOO^-$-scavenging activities.[7] We have also studied the natural products with $ONOO^-$ scavengers by analyzing caffeoylquinic acids from several plant resources.[8,9] Further, it was demonstrated that the antiulcer effect of ellagic acid, a potent $ONOO^-$-scavenger, frequently occurs in nuts and fruits, such as strawberry, raspberry, grape, and blackberry.[10] Therefore, development of $ONOO^-$ scavengers from plant resources will be beneficial for cardiovascular dysfunctions, such as athero-

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sclerosis, cardiac failure, and hypertension, as well as for the risk of stroke accompanied by obesity or diabetic complications or for gastrointestinal diseases. $ONOO^-$-scavengers are particularly important because humans lack the enzyme capable of scavenging it.

Koreans have used *Ixeris dentata* Nakai, *Youngia sonchifolia* Hance, *Lactuca indica* L. or others primarily as wild vegetables of Compositae.[11] Although these plants are taxonomically or morphologically close to one another, their comparative studies have not been performed on $ONOO^-$ scavenging activities. Well-known $ONOO^-$ scavengers, chlorogenic acid (CGA)[7], luteolin 7-glucoside (luteolin-7-glc), and luteolin[12] together with chicoric acid (CA) and luteolin-7-glcU were used for both analysis and assay. We aimed to lead the most potent extract from those nine plant materials which were widely distributed in Korea. For the comparative study, we included both pectolinarin and linarin as the test compounds in this research. It would be meaningful to analyze $ONOO^-$ scavengers by HPLC, because plant constituents individually contribute to the genuine $ONOO^-$-scavenging activity. In addition, the analytical data could be conveniently used for biomedical purposes, because pharmacological activities of numerous plant constituents are commonly known. In the present study, we studied the extracts of nine mountainous vegetables (*Ixeris dentata* Nakai, *I. dentata* var. *albiflora* Nakai, *I. sonchifolia* Hance, *Youngia denticulata* Kitamura, *Y. japonica* (L.) DC, *Lactuca indica* L., *T. officinale* Weber, *C. setidens* Nakai, and *C. pendulum* Fisch. ex DC) widely distributed in Korea from Compoistae in $ONOO^-$-scavenging assay as well as in HPLC analysis.

Experimental

Plant materials – Leaves of *Ixeris dentate* (voucher number, natchem-#42), *Ixeris dentata* var. *albiflora* (natchem-#43), *Ixeris sonchifolia* (syn. *Youngia sonchifolia*, natchem-#45), *Youngia japonica* (natchem-#55), *Lactuca indica* (natchem-#61), *Taraxacum officinale* (natchem-#62), *Cirsium pendulum* (natchem-#74), and *Cirsium setidens* (natchem-#75) were collected in May 2012. Leaves of *Youngia denticulata* were collected on September 2013, considering its flowering period. All plant materials were collected from a mountain area near Wonju City. The plants were identified by Prof. Dr. Sang-Cheol Lim (Department of Horticulture and Landscape Architecture, Sangji University). Voucher specimens were deposited at the Laboratory of Natural Products Chemistry, Sangji University. The collected plants were separately dried in dark area and crushed for extraction.

Instruments and reagents – An HPLC system consisting of two Prostar 210 pumps, a Protar 325 UV-Vis detector and a Shiseido Capcell PAK C18 column (5 μm, 4.6 mm × 250 mm, Japan) was used for analysis. The HPLC solvents of H_2O, MeOH, and CH_3CN were purchased from J.T.Baker Co. (Phillisburg, NJ, USA). The reagents used for $ONOO^-$-scavenging assay were diethylenetriaminepentaacetic acid (DTPA) from Sigma Chemical Co. (St. Louis, MO, USA), dihydrorhodamine 123 (DHR 123) from Molecular probes (Eugene, OR, USA) and $ONOO^-$ from Cayman Chemicals Co. (Ann Arbor, MI, USA).

Extraction and identification of the compounds – Dried, crushed, and lyophilized plant material (leaf part) (5 g) were soaked in 250 mL of 70% MeOH and extracted under reflux on a heating mantle for 5 h. After cooling, each extract solution was filtered through a filter paper. The extracted solution (2 mL) was filtered again through a disposable syringe filter (0.50 μm, Dismic-25JP Advantec, Japan) prior to the injection into the HPLC system. The extract was evaporated *in vacuo* using a rotary evaporator and dried on a freeze-dryer in order to obtain the extraction yield. The lyophilized extracts were also used for the $ONOO^-$-scavenging test.

Based on literatures and our experiences on the identification of phenolic compounds in several Compositae plants, seven standard compounds were used to detect the constituents of nine extracts. Three compounds (luteolin-7-glc, luteolin-7-glcU, and pectolinarin) were obtained by isolation (purity ≥ 98% by HPLC) and CGA (Lot No. 104K0722), CA (060M1184V), and luteolin (11K4085) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and linarin from Extrasynthese (France, Lot No. 11011412). The detection of the standards in the extracts was performed by HPLC and TLC. Their chemical structures are presented in Fig. 1.

$ONOO^-$-scavenging assay – The measurement of peroxynitrite-scavenging activity was performed by modifying the method described by Kooy *et al*.[13] This method is based on the measurement of highly fluorescent rhodamine 123 converted from non-fluorescent DHR-123 under the presence of $ONOO^-$. In brief, this assay was based on the method using a rhodamine buffer (pH 7.4, 50 mM sodium phosphate diabasic, 50 mM sodium phosphate monobasic, 90 mM chlorides, 5 mM potassium chloride, 100 μM DTPA). The final concentration of DHR 123 was 5 μM. In this assay, the buffer was preserved in ice bath prior to use, and the samples were dissolved in 10% dimethylsulfoxide. The intensity of the background fluorescence was measured in 0.3N NaOH under the

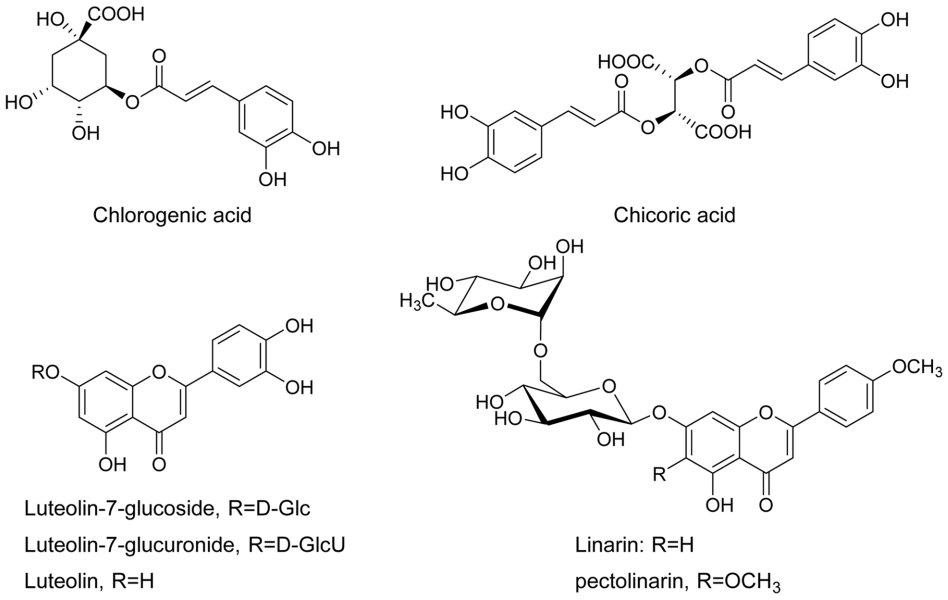

Fig. 1. Structures of standard compounds used for HPLC analysis.

presence or absence of 10 μM $ONOO^-$. Then, the fluorescent intensity was measured from the excitation and emission at 480 nm and 530 nm using a microplate fluorescencereader FL 500 (Bio-Tek Instruments Inc., Winooski, VT, USA). $ONOO^-$-scavenging activities were calculated from the final fluorescent intensity minus the intensity of the background. The positive control was L-penicillamine.

HPLC analytical method – The mobile phase consisted of 0.05% (v/v) trifluoroacetic acid (TFA) in H_2O (eluent A) and 0.05% (v/v) TFA in MeOH and CH_3CN (60:40, v/v; eluent B). The gradient program was as follows: 15% B to 65% B (35 min), 65% B isocratic (5 min), 65% B to 100% B (2 min), 100% B isocratic (4 min), 100% B to 15% B (3 min), and 15% B isocratic (6 min). The total run time was 55 min at a flow rate of 1 mL/min and at 40 °C of column temperature. Chromatograms were recorded during a period of 0 - 40 min. The injection volume for each sample was 20 μL and simultaneous monitoring was performed at 254 nm. UV wavelength 254 nm was more sensitive for the simultaneous detection of caffeoylquinic acid, caffeoyl tartaric acid, and luteolin analogues.

To assess the linearity of the regression equations, standard stock solutions (1000 μg/mL) were prepared by dissolving each standard compound in MeOH. Stock solutions were serially diluted in order to prepare working solutions of 1.56 - 50.0 μg/mL (CGA, luteolin-7-glc, luteolin, linarin, and pectolinarin) and 3.13 - 100.0 μg/mL (CA and luteolin-7-glcU). Calibration curve equations were determined by plotting the peak area (*y* axis) versus the concentration (*x* axis), and the liniarities of equations were evaluated by R^2 values. LOD (limit-of-detection) and LOQ (limit-of-quantification) were determined by signal-to-noise (S/N) ratios of 3 and 10, respectively.

Results and Discussion

Five compounds, CGA, CA, luteolin-7-glc, luteolin-7-glcU, and luteolin, displayed potent activities over the IC_{50} ranges of 0.23 - 1.45 μg/mL, although two substances, linarin and pectolinarin, had relatively higher IC_{50} values (Table 1). In particular, the IC_{50} of CA was the lowest, considering the value of the μM unit. Therefore, those five compounds can be considered as the active components of the tested plant materials.

After testing the $ONOO^-$-scavenging effects of nine extracts at concentrations of 0.4, 2, 10 and 50 μg/mL, the scavenging percentage and IC_{50} values were displayed in Table 2. The activities of *Ixeris dentata*, *I. dentata* var. *albiflora*, and *I. sonchifolia* with a range of IC_{50} 0.48 - 1.74 μg/mL were very potent, whereas those of other plants were relatively weak. Of these, the activity of *I. dentata* var. *albilfora* (IC_{50} 0.48 μg/mL) was the strongest. However, the activity of dandelion (*T. officinale*) was not so strong, although it is a very well-known crude drug in Western countries.[14]

The following four parameters of mobile phase composition, gradient elution, UV wavelength, and column temperature were considered for the optimization of the

Table 1. Peroxynitrite-scavenging activities of compounds

Compound	Peroxynitrite-scavenging (%)					IC_{50} (μg/mL)
	0.08 μg/mL	0.4 μg/mL	2 μg/mL	10 μg/mL	50 μg/mL	
CGA	12.67 ± 8.55[a]	56.56 ± 4.38	78.70 ± 1.85	–	–	0.47 (1.34)[b]
CA	21.75 ± 1.37	53.62 ± 3.47	85.34 ± 0.69	–	–	0.36 (0.76)
Lut-7-glc	17.01 ± 3.50	54.34 ± 3.41	88.80 ± 1.27	–	–	0.39 (0.86)
Lut-7-glcU	–	22.53 ± 5.13	64.29 ± 2.08	86.87 ± 2.05	–	1.45 (3.13)
Luteolin	10.80 ± 9.65	86.85 ± 3.70	96.15 ± 0.27	–	–	0.23 (0.81)
Linarin	–	–	32.56 ± 1.21	66.89 ± 1.22	97.45 ± 0.24	4.60 (7.77)
Pectolinarin	–	–	35.86 ± 1.36	69.94 ± 0.68	90.65 ± 0.59	4.03 (6.47)
L-Penicillamine[c]	–	34.18 ± 4.98	62.74 ± 0.62	86.73 ± 0.35	–	1.28 (8.62)

[a]Value represents mean ± S.D. (n = 2); [b]Unit of the value in the parenthesis is μM; [c]Positive control.

Table 2. Peroxynitrite-scavenging activities of extracts

Extract	Peroxynitrite-scavenging (%)				IC_{50} (μg/mL)
	0.4 μg/mL	2 μg/mL	10 μg/mL	50 μg/mL	
Ixeris dentata	16.99 ± 1.37*	56.22 ± 0.77	86.57 ± 0.33	–	1.74
Ixeris dentata var. albiflora	48.19 ± 4.34	81.88 ± 1.59	95.54 ± 0.38	–	0.48
Ixeris sonchifolia	47.37 ± 0.11	81.78 ± 0.60	93.17 ± 0.13	–	0.52
Youngia denticulata	–	21.03 ± 3.46	48.71 ± 0.96	89.44 ± 0.66	10.37
Youngia japonica	–	15.53 ± 0.47	64.11 ± 1.90	87.38 ± 1.51	7.67
Lactuca indica	–	25.67 ± 3.23	79.77 ± 0.59	96.89 ± 0.10	5.59
Taraxacum officinale	–	19.92 ± 2.03	68.10 ± 0.23	91.20 ± 0.14	6.99
Cirsium setidens	18.89 ± 3.29	34.99 ± 2.25	71.75 ± 5.26	–	5.27
Cirsium pendulum	16.89 ± 1.46	37.67 ± 2.82	73.97 ± 0.84	–	4.71

*Value represents mean ± SD (n = 2).

HPLC method. The eluents, 0.05% TFA in H_2O and 0.05% TFA in MeOH-CH_3CN (60:40) as solvents A and B, respectively, showed better resolution, and were environment-friendly and economic compared to other solvents. The addition of TFA with a 0.05% concentration in A- and B solvents contributed to sharper peaks on the chromatograms, probably due to the deionization of phenolic substances. Gradient elution included the peaks due to a wide range of polarity within the shortest retention time. The use of a fixed column temperature and flow rate at 40 °C and 1.00 mL/min led to good chromatograms. The HPLC chromatograms of the mixed standard sample solution and the seven 70% MeOH extracts were shown in Fig. 2. The 70% MeOH extract was prepared since 70% MeOH solvent produces more powdery extract than 100% MeOH and that the extraction rate of flavonoids is similar between the two solvents.

In order to determine the contents of the five $ONOO^-$ scavengers, experiments on linearity and sensitivity were performed. Regression equations of the compounds were determined as shown in Table 3. Further, linearities were proven from the R^2 value of more than 0.9996. Sensitivity was also high from the LOD and LOQ values over the range of 0.07 - 0.89 μg/mL and of 0.22 - 1.44 μg/mL, respectively.

The contents of CGA, CA, luteolin-7-glc, luteolin-7-glcU, luteolin, linarin, and pectolinarin in nine extracts are shown in Table 4. The sum of these substances was especially high in *I. sonchifolia* (182.03 mg/g). In the extracts of *C. setidens* and *C. pendulum*, CGA, CA, luteolin-7-glc, luteolin-7-glcU, and luteolin were observed, but the remaining two compounds (linarin and pectolinarin) were detected. Although, the other plants did not contain linarin and pectolinarin; however, they had CGA, CA, luteolin-7-glc, luteolin-7-glcU, and luteolin. Of the CGA and CA, the former compound was quantitatively lower and the latter one was higher. The levels of CA were particularly high in *I. sonchifolia* (65.71 mg/g) and *T. officinale* (36.02 mg/g). Out of these compounds, the contents of luteolin-7-glc and luteolin-7-glcU were high, although the level of luteolin was generally very low (0.24 - 4.80 mg/g). These results suggest that luteolin was present mainly in the

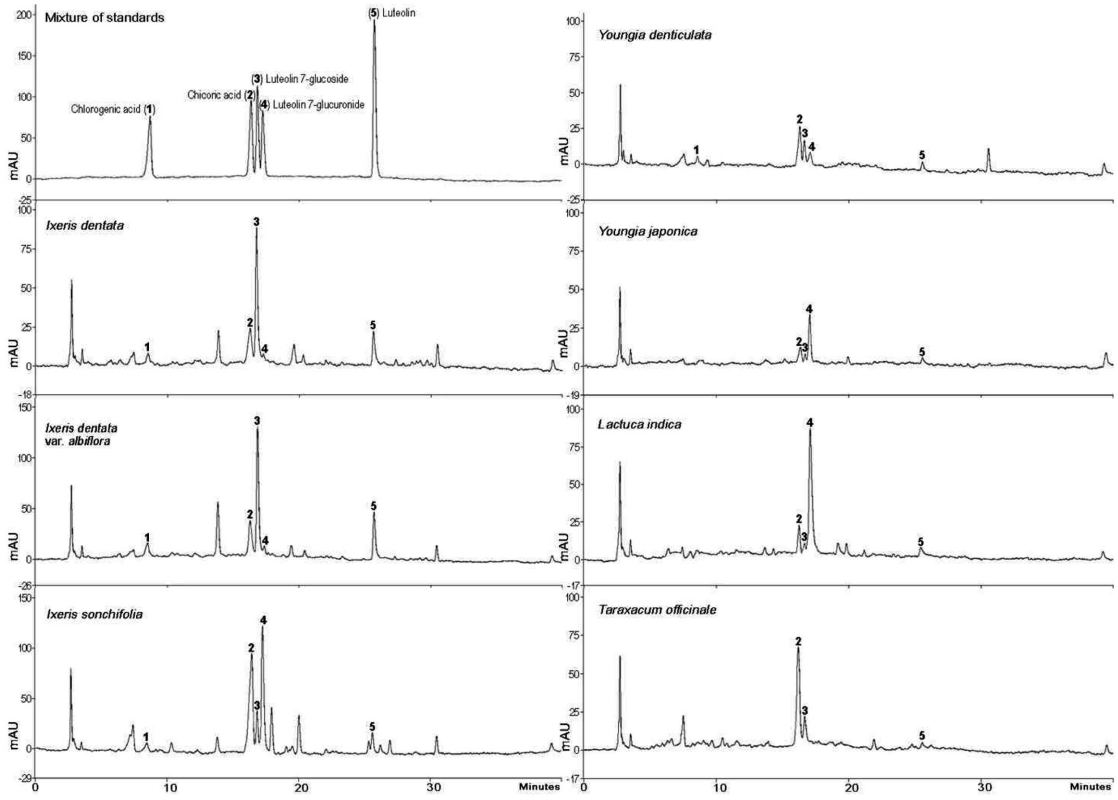

Fig. 2. HPLC chromatograms of mixed standard compounds and 70% MeOH extracts of the seven Compositae herbs.

Table 3. Linearities and limits of detection and quantification (LOD - LOQ) of analytes

Analyte	t_R (min)	Equation of the linear regression	Linear range (μg/mL)	R^2	LOD (μg/mL)	LOQ (μg/mL)
CGA	8.65	$y = 166.80x + 58.46$	1.56 - 50.0	0.9998	0.19	0.63
CA	16.40	$y = 84.43x + 35.13$	3.13 - 100.0	0.9997	0.65	2.17
Luteolin 7-glc	16.73	$y = 200.82x + 40.73$	1.56 - 50.0	0.9998	0.25	0.82
Luteolin 7-glcU	17.05	$y = 48.71x + 46.73$	3.13 - 100.0	0.9999	0.89	2.96
Luteolin	25.46	$y = 399.67x + 64.06$	1.56 - 50.0	0.9996	0.07	0.22
Linarin	24.85	$y = 309.18x + 30.86$	1.56 - 50.0	0.9998	0.23	0.76
Pectolinarin	25.68	$y = 103.76x + 60.10$	1.56 - 50.0	0.9997	0.43	1.44

Table 4. Content of analytes in the extracts

Extract	Content of analyte in extracts (mg/g)							
	CGA	CA	Luteolin 7-glc	Luteolin 7-glcU	Luteolin	Linarin	Pecto-linarin	Total
Ixeris dentata	2.33	19.91	13.58	4.15	2.71	–	–	42.68
Ixeris dentata var. albiflora	4.33	17.58	23.83	6.41	4.80	–	–	56.96
Ixeris sonchifolia	3.04	65.71	6.99	104.68	1.61	–	–	182.03
Youngia denticulata	0.98	14.12	3.59	10.46	0.54	–	–	29.69
Youngia japonica	–	8.39	0.99	20.93	0.44	–	–	30.61
Lactuca indica	–	10.00	0.66	79.20	1.12	–	–	90.89
Taraxacum officinale	–	36.02	1.60	–	0.24	–	–	37.58
Cirsium setidens	–	–	–	–	–	8.32	73.26	81.58
Cirsium pendulum	–	–	–	–	–	20.95	76.64	97.59

glycosidic form.

Dandelion is a local name of a famous herb medicine, *Taraxacum officinale*, in Western countries. The herb of *T. officinale* has been used to mainly treat hepatic disease in Chinese medicine. This crude drug has been known to have a variety of pharmacological activities, including diuretic, choleretic, anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-allergic, anti-hyperglycemic, and anti-thrombotic.[14] We have also studied several mountainous vegetables mainly belonging to the Korean native *Aster* species, called chwinamul, on $ONOO^-$-scavenging activities[7,8] as well as the contents of phenolic substances.[15]

Of the nine herbs tested, the three of the plants studied, *I. dentata*, *I. dentata* var. *albiflora*, and *I. sonchifolia*, exhibited potent $ONOO^-$-scavenging activities in the present study. The activities of these three plants were stronger than those of *T. officinale* (dandelion). In addition, five compounds, CGA, CA, luteolin-7-glc, luteolin-7-glcU, and luteolin, were shown to be potent $ONOO^-$-scavengers. However, linarin and pectolinarin, exhibited considerably weaker activities.

CA and luteolon-7-glc, were quantitatively abundant in *I. dentata* and *I. dentata* var. *albiflora*. In particular, the content of CA was very high in *I. sonchifolia* (65.71 mg/g), which can also be considered to be the most significantly active substance. Although the content of phenolic substances in *I. sonchifolia* was reported, CA was not quantitatively determined.[16] In *T. officinale*, the content of CA was 36.02 mg/g; but the levels of the other compounds were very low. This indicates that CA might be the main substance in *T. officinale*. Schütz *et al*.[17] has also reported that CA was a main compound in the same plant. However, in *C. setidens* and *C. pendulum*, the two substances, linarin and pectolinarin, existed in high proportions, whereas the other five phenolic substances were not detected.

Certain pharmacological activities of CA have been known: apoptosis-inducing activity against preadipocyte cells,[18] anti-stress activity,[19] and anti-viral activity,[20] although its $ONOO^-$-scavenging activity has not been known. Park *et al*.[21] reported potent anti-inflammatory activity due to the synergistic action between CA and luteolin. Luteolin-7-glcU has antigastritis,[22] antidepressant,[23] and anti-mutagenic activities.[24] Further, it possesses inhibitory activities on the protein expression of inducible nitric oxide synthase and cyclooxygenase in lipopolysaccharide-induced macrophage RAW 264.7 cells.[25]

Pacher *et al*.[4] suggested that cardiovascular dysfunctions, such as neuropathy, nephropathy, and hypertension, or the risk of stroke of patients with obesity or diabetic complications, are caused from the excess production of $ONOO^-$. Therefore, *I. dentata*, *I dentata* var. *albiflora*, and *I. sonchifolia*, which were confirmed to be potent $ONOO^-$ scavengers through this study, will be beneficial for patients suffering from diabetic cardiovascular dysfunction.

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