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Qualitative and Quantitative Determination of the Caffeoylquinic Acids on the Korean Mountainous Vegetables Used for Chwinamul and Their Peroxynitrite-Scavenging Effect

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Mountainous vegetables called chwinamul are used in Korea to promote health. Chwinamul was obtained from several plants belonging to the Compositae - e.g., *Kalimeris yomena*, *Aster scaber*, *Solidago virga* var. *gigantea*, *Solidago viragaurea* var. *asiatica*, *Saussurea grandifolia*, *Ainsliaea acerifolia* - were used for our experiments. Analytical methods for simultaneous determination of the caffeoylquinic acids (3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-dicaffeoyl-*epi*-quinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, 3-*O*-caffeoylquinic acid, 3-*O*-*p*-coumaroyl-caffeoylquinic acids) were established for chwinamul. The kinds of constituents were identified from HPLC chromatograms and it was possible to calculate the percentage (w/w) of seven of these compounds in the dried plants and in the extracts. The proportion of caffeoylquinic acids in the extracts ranged from 20.25 to 38.35%. Since it is known that peroxynitrite ($ONOO^-$)-scavenging is beneficial for amelioration of obesity, diabetes mellitus, atherosclerosis and even Alzheimer's disease, assays for peroxynitrite-scavenging activity were performed on the seven chwinamul plants. Of the tested extracts, the MeOH extract of *A. acerifolia* had the most potent effect (IC_{50} 1.49 ± 0.68 μg/mL). These results suggest that chwinamul vegetables can be used for treatment or prevention of peroxynitrite-related diseases.

Key words: Chwinamul, Mountainous vegetable, Compositae, Caffeoylquinic acid, HPLC, Peroxynitrite

INTRODUCTION

Mountainous vegetables called chwinamul are used in Korea to improve health. The area containing mountainous vegetables occupies one eighth of the total cultivated land of in Korea. The following plants are some of those that are commonly used for chwinamul: *Kalimeris yomena* = *Aster yomena* (KS), *Aster scaber* (AS), *Solidago virga* var. *gigantea* (SVG), *Solidago viraaurea* var. *asiatica* (SVA), *Saussurea grandifolia* (SG), *Ainsliaea acerifolia* (AA). All of the above plants belong to the Compositae (Ko et al., 2003). Monoterpenes (Jung et al., 2001), numerous saponins (Nagao et al., 1992; Nagao et al., 1993), and caffeoylquinic acids (Kwon et al., 2000) have been isolated from *A. scaber*, while terpenoids and benzylphenolics (Choi et al., 2005; Sung et al., 1999; Choi et al., 2004) were found in *Solidago virga* var. *gigantea*. In particular, caffeoylquinic acids have been isolated from Compositae plants. Recently, Clifford et al. (2006) reported a method for the identification of caffeoylquinic acids based on HPLC-MS analysis.

Peroxynitrite ($ONOO^-$), which can be formed from the reaction of superoxide anion radical ($\cdot O_2^-$) and nitric oxide (NO) in cells (Radi et al., 1991), causes

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peroxidation of lipids and proteins and are cytotoxic and cause rapid neuronal damage (Haenen et al., 1997). Overproduction of peroxynitrite contributes to several kinds of disease including hypercholesterolemia, atherosclerosis, obesity and diabetes mellitus (Korda et al., 2008; Pacher et al., 2005; Drel et al., 2007). In the present study, we attempted to characterize mountainous vegetables that can scavenge peroxynitrite with the idea that chwinamuls might be capable of treating the aforementioned cardiovascular diseases.

Monocaffeoyl and dicaffeoylquinic acids are usually found as caffeoylquinic acids, though tricaffeoylquinic acids exist unconjugated in nature (Zhao et al., 2006). The most common phenylpropanoid esterifying quinic acid derivative is caffeic acid although *p*-coumaric acid and ferulic acid are also often found. As a parent skeleton, quinic acid is the most common form, and *epi*-quinic acid and *muco*-quinic acid rarely substitute for it. The carboxylic acid at the C-1 position often occurs as a methylated substance. There are reports of a variety of bioactivities of caffeoylquinic acids including antioxidant, anti-inflammatory, anti-hepatotoxic, anti-platelet aggregating, and anti-microbial activities (Zhao et al., 2006).

With this in mind, the present study was performed to investigate possible associations between the content and bioactivity of caffeoylquinic acid derivatives. In particular, it was thought that simultaneous analysis by HPLC would be desirable because several plants are used as chwinamul. Furthermore, because it is not easy to isolate caffeoylquinic acid derivatives and indentify their structures, it would be more efficient to evaluate their quality and quantity using an HPLC method.

MATERIALS AND METHODS

Instruments and reagents

HPLC was performed using a Varian HPLC system that includes a Prostar 210 solvent delivery module, Prostar 325 UV-Vis detector and a 20 μL sample loop (Rheodyne). Separation was achieved on a Shiseido (Chuoku) Capcell Pak C18 column (5 μL, 250 mm × 4.6 mm I.D.). The injection syringe was purchased from Hamilton. All solvents used in the analysis were HPLC grade and obtained from J.T. Baker. Seven standard compounds isolated from *Lactuca indica* L. (Kim et al., 2007; Kim et al., 2008) were assayed. These were obtained from Prof. Kang Ro Lee (College of Pharmacy, SungKyunKwan University, Suwon). The standard compounds used are abbreviated as follows: 3,4-di-*O*-caffeoylquinic acid (3,4-DQ), 3,5-di-*O*-dicaffeoyl-*epi*-quinic acid (3,5-DeQ), 3,5-di-*O*-caffeoylquinic acid (3,5-DQ), 4,5-di-*O*-caffeoylquinic acid (4,5-DQ), 5-*O*-caffeoylquinic acid (5-CQ), 3-*O*-caffeoylquinic acid (3-CQ), 3-*O*-*p*-coumaroyl-caffeoylquinic acids (3-pCQ). The structures of the seven standard compounds are shown in Fig. 1. Dihydrorhodamine 123 (DHR 123) and peroxynitrite were from Molecular Probes (Eugene) and Cayman Chemical Co., respectively.

Plant materials

The following plants were collected in April in Gangwon-do, Korea: *Kalimeris yomena* = *Aster yomena*, *Aster scaber*, *Solidago virga* var. *gigantea*, *Solidago virgaaurea* var. *asiatica* = *Solidago japonica*, *Saussurea grandifolia*, *Ainsliaea acerifolia*. Processed (dried after boiling) *Aster scaber* is commonly used as a Chwinamul. It was made by the Seokwang Food Co., Korea, and purchased from the Wonju market. A commodity derived from a processed *Aster scaber* (pAS) is

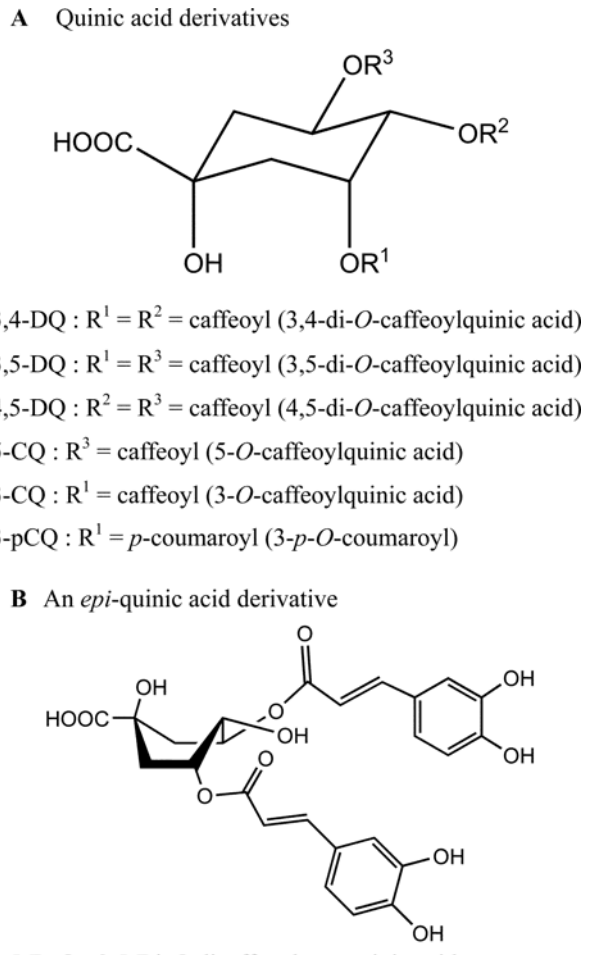

Fig. 1. Structures of seven standard compounds used for HPLC analysis

Chwinamul made by the Seokwang Food Co. in Korea It was processed by boiling followed by drying. We purchased it from a market in Wonju. All plants or plant materials were identified by Dr. Won-Bae Kim (Highland Agriculture Research Institute, Rural Development Administration, Pyeongchang, Gangwon-do).

Extraction

Each test plant material (20 g) together with MeOH (400 mL) were added to a 500 mL Erlenmeyer flask and sonicated at 40°C for 6 h. The extract was filtered using filter paper and concentrated to dryness on a rotary evaporator at 60°C. Afterward, the sticky extract was frozen in the deep freezer (-80°C) and dried on the freeze dryer for 24 h to produce a powdery solid extract. For HPLC analysis, this extract was dissolved in 80% aqueous MeOH and filtered through a 0.50 μm syringe filter. The filtrate (20 μL) was then injected into the analyzer.

HPLC conditions for caffeoylquinic acid analysis

Test samples and standard compounds were dissolved in 80% aqueous MeOH and were filtered through a 0.50 μm syringe filter before injection. The UV detector was fixed at 246 nm. The mobile phase was a mixed solvent of 0.05% phosphoric acid in water (solvent A) and methanol (solvent B). The gradient system was: 0-10 min, 60% A : 40% B; 10-20 min, 50% A : 50% B; 20-30 min, 40% A : 60% B; 30-35 min, 60% A : 40% B. Chromatography was performed at a flow rate of 1.00 mL min^{-1}. Regression equations obtained by calculating peak areas for each concentration (50, 100 and 200 μg/mL) are shown in Fig. 2 including retention times and R^2 values. All R^2 values were > 0.990. Contents of the peak areas (mean ± S.D.) are shown in Table I.

Assay for peroxynitrite-scavenging activity

Peroxynitrite ($ONOO^-$) scavenging activity was assessed by a modification of Kooy's method. This involved monitoring of the highly fluorescent rhodamine 123, which was rapidly produced from non-fluorescent DHR 123 in the presence of $ONOO^-$ (Kooy et al., 1994). In brief, the rhodamine buffer (pH 7.4) consisted of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final DHR 123 concentration was 5 μM. The buffer in this assay was prepared just prior to use and placed on ice. The plant extracts were dissolved in 10% DMSO (f.c. 5 μg/mL). The background and final fluorescent intensities were measured 5 min after treatment with or without the addition of authentic $ONOO^-$ (10 μM) dissolved in 0.3 N sodium hydroxide. The fluorescence intensity of the oxidized DHR 123 was evaluated using a microplate fluorescence reader FL 500 (Bio-Tek Instru-

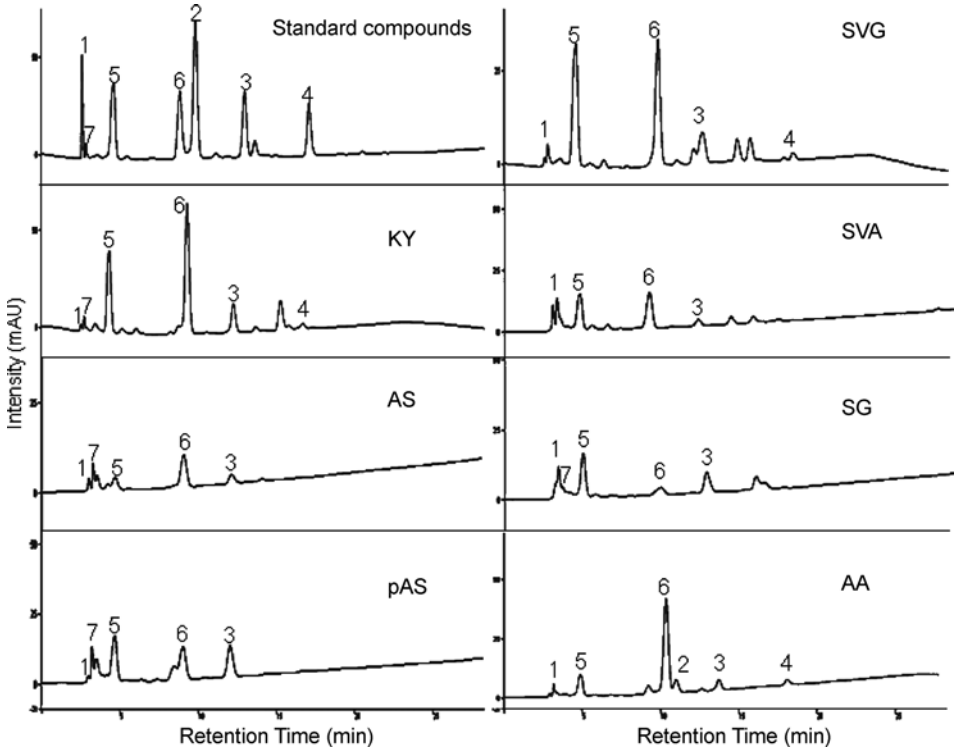

Fig. 2. HPLC profiles of seven chwinamul materials

Table I. Calibration curve equation of the seven caffeoylquinic acids used for HPLC analysis

No	Caffeoylquinic acid	Retention time (min)	Regression Equation	
1	3,4-Di-*O*-dicaffeoylquinic acid	2.7	y = 66.847x - 526.8	R^2 = 0.997
2	3,5-Di-*O*-dicaffeoyl-*epi*-quinic acid	9.6	y = 383.09x - 364.7	R^2 = 0.991
3	3,5-Di-*O*-dicaffeoylquinic acid	12.9	y = 113.68x - 796.4	R^2 = 0.998
4	4,5-Di-*O*-dicaffeoylquinic acid	17.1	y = 141.71x - 162.1	R^2 = 0.996
5	5-*O*-caffeoylquinic acid	4.5	y = 241.02x - 6122	R^2 = 0.992
6	3-*O*-caffeoylquinic acid	8.9	y = 304.05x - 2505.4	R^2 = 0.999
7	3-*O*-*p*-coumaroylquinic acid	2.9	y = 23.022x - 112.3	R^2 = 0.999

y, area, μV; x, concentration, μg mL^{-1}

ments Inc., Winooski, VT) at excitation and emission wavelengths of 480 and 530 nm, respectively. Peroxynitrite-scavenging activity values were calculated as the final fluorescence intensity minus the background fluorescence, *via* the detection of DHR 123 oxidation. L-Penicillamine was employed as a positive control. Data for the peroxynitrite assay are expressed as mean ± S.E.M.

RESULTS

After identifying the peaks of seven standard compounds in the HPLC chromatogram at 50, 100, and 200 μg/mL concentrations using 246 nm, the retention time, regression equation and R^2 values were established as shown in Table I. Retention times are shown in the following order of compounds: 3,4-DQ (2.7 min), 3-pCQ (2.9 min), 5-CQ (4.5 min), 3-CQ (8.9 min), 3,5-DeQ (9.6 min), 3,5-DQ (12.9 min), and 4,5-DQ (17.1 min) (Fig. 2 and Table I). In the chromatogram of *K. yomena*, six peaks were observed except for the peak of 3,5-DeQ. The total weight percentage of a dried weight was 6.17% (155.84 μmol/g dried weight, mean value) while the total percentage of an extract was 38.35% (w/w), which values were the highest of the measured extracts. The compound 5-CQ (53.15 μmol/g) was the most abundant in the chromatogram of *K. yomena*.

In the chromatogram of *A. scaber*, the proportions of dried weight and of the extract were observed as 3.38% (88.36 μmol/g) and 20.61%, respectively. There were no peaks for 3,5-DeQ and 4,5-DQ in the chromatogram and the level of 3-pCQ was 29.40 μmol/g. The total percentage of dried weight of the processed *A. scaber* {2.94% (76.65 μmol/g)} was a little lower than that of *A. scaber* {3.38% (88.36 μmol/g)} while the total percentage of the extract from the processed one was rather higher than in the unprocessed one. The level of 3-pCQ (33.96 μmol/g) in the processed *A. scaber* was also the highest among the peaks (as for *A. scaber*) whereas peaks for 3,5-DeQ and 4,5-DQ were not detected.

In the chromatogram of *S. virga* var. *gigantea*, the total percentage of the dried weight and of the extract were 6.03% (148.11 μmol/g) and 30.55%, respectively; these values were considered relatively high. Chlorogenic acid (5-CQ) had highest levels (63 μmol/g) in the chromatogram of this plant, though peaks for 3,5-DeQ and 3-pCQ were not detected.

The total percentage of the extract from *S. virga-*

Table II. Content of caffeoylquinic acids in the compositae plants used for chwinamul

Compound	KY	AS	pAS	SVG	SVA	SG	AA
3,4-DQ	10.84 ± 1.35[a]	10.86 ± 0.16	7.96 ± 0.22	12.81 ± 1.22	21.27 ± 1.24	20.46 ± 3.91	6.52 ± 0.61
3,5-DeQ	ND[b]	ND	ND	ND	ND	ND	1.62 ± 0.11
3,5-DQ	23.47 ± 1.24	7.57 ± 0.31	9.10 ± 0.23	25.77 ± 0.33	8.06 ± 0.45	14.84 ± 0.39	7.64 ± 0.43
4,5-DQ	7.64 ± 0.41	ND	ND	10.27 ± 0.74	ND	ND	4.23 ± 0.26
5-CQ	53.15 ± 1.78	26.39 ± 0.11	19.72 ± 0.35	63.51 ± 5.14	34.94 ± 0.18	40.80 ± 0.46	17.62 ± 0.70
3-CQ	46.50 ± 2.73	14.14 ± 0.09	5.92 ± 0.02	35.76 ± 2.06	17.56 ± 0.33	15.62 ± 0.26	19.76 ± 1.57
3-pCQ	14.24 ± 3.66	29.40 ± 0.60	33.96 ± 1.27	ND	ND	49.47 ± 3.01	ND
Sum (μmol/g)	155.84 ± 2.14	88.36 ± 0.81	76.65 ± 1.40	148.11 ± 8.51	81.81 ± 1.69	141.19 ± 6.45	57.39 ± 3.50
% of dried weight	6.17 ± 0.12	3.38 ± 0.03	2.94 ± 0.05	6.03 ± 0.33	3.37 ± 0.08	5.49 ± 0.28	2.36 ± 0.14
% of extract	38.35 ± 0.71	20.61 ± 0.20	33.74 ± 0.45	30.55 ± 1.67	20.25 ± 0.48	28.09 ± 1.42	26.32 ± 1.61

[a] Values represent mean ± S.D. based on triplicate experiments, [b]ND : not detected.
Abbreviation: KY(*Kalimeris yomena*), AS (*Aster scaber*), pAS (processed *Aster scaber*), SVG (*Solidago virga* var. *gigantea*), SVA (*Solidago virgaaurea* var. *asiatica*), SG (*Saussurea grandifolia*) and AA (*Ainslaea acerifolia*).

Table III. IC_{50} value for peroxynitrite scavenging activity of chwinamul extracts

Plant name	IC_{50} (μg/mL) ± S.E.M.	Plant name	IC_{50} (μg/mL) ± S.E.M.
KY	1.68 ± 0.37[a]	SVA	3.46 ± 0.13[a]
AS	13.34 ± 0.51[a]	SG	4.46 ± 0.63[a]
pAS	13.81 ± 0.62[b]	AA	1.49 ± 0.68[a]
SVG	2.67 ± 0.34[a]	Penicillamine*	0.89 ± 0.22

$ONOO^-$ scavenging activity was measured by monitoring the oxidation of DHR 123 as described in materials and methods. Data are mean ± S.E.M. of triplicate experiments.
*used as a positive control.
[a]$p < 0.05$ by student's test for values between the sample and the control.
[b]$p < 0.01$ by student's test for values between the sample and the control.

aurea var. *asiatica* was 20.25% and the percentage of dried weight was 3.37%. The most abundant compound was chlorogenic acid (34.94 μmol/g) whereas peaks for 3,5-DeQ, 4,5-DQ, and 3-pCQ were not found.

The total percentage of dried *S. grandifolia* was 5.49% (141.99 μmol/g) while the percentage of the extract was 28.09%. Compounds not observed on the chromatogram were 3,5-DeQ and 4,5-DQ while the most abundant one was 3-pCQ (49.97 μmol/g). The total weight percentage in the dried weight of *Ainsliaea acerifolia* was 2.36% (57.39 μmol/g) but the percentage of the extract was 26.32%. The most abundant compound was 3-CQ (19.76 μmol/g); only 3-pCQ was not detected.

The peroxynitrite-scavenging effects of the seven chwinamuls were determined. Values are shown in Table III. In particular, the IC_{50} values for *K. yomena* and *A. acerifolia* were 1.68 μg/mL and 1.49 μg/mL, respectively, which approached that of the positive control penicillamine (IC_{50} = 0.89 μg/mL). However, the scavenging activities of both unprocessed and processed *A. scaber* were relatively weak (IC_{50} = 13.34 μg/mL and 13.81, respectively).

DISCUSSION

The plant materials employed in this experiment are used for chwinamul in Korea. Under the assumption that these plants may show a peroxynitrite-scavenging effect, this experiment was performed. Furthermore, we hoped to establish HPLC chromatographic profiles of chwinamul extracts so that the plant material of chwinamul could be distinguished by its HPLC pattern. Although Clifford et al. (2006) has established an identification method using an HPLC-MS technique, there have been no quantitative methods for caffeoylquinic acids. The present method could be used as a new method for analyzing chwinamul or other Compositae plants. The absence of certain peaks in the extract of a chwinamul could be an aid in the identification of these plants.

It was reported that dicaffeoylquinic acids are potent inhibitors that prevent nitration of tyrosine residues and NO production due to NF-êB transcriptional inactivation (Olmos et al., 2008). Jeong et al. (2008) reported the peroxinitrite-scavenging effect of thistles and the identification of flavones using HPLC. The neuroprotective effect of caffeoylquinic acids isolated from *A. scaber*, which was used in the present study, was also reported (Hur et al., 2001). Although (-)-3,5-dicaffeoyl-*muco*-quinic acid, (-)-3,5-dicaffeoylquinic acid, (-)-4,5-dicaffeoylquinic acid, (-)-5-caffeoylquinic acid have been isolated from *A. scaber*, and their anti-viral activities have been reported (Kwon et al., 2000), other caffeoylquinic acids were also identified in the present HPLC study.

From *S. virga* var. *gigantea* we isolated sesquiterpene, 3,5-di-*O*-caffeoylquinic acid, methyl 3,5-di-*O*-caffeoylquinate (Choi et al., 2004), triterpene (Moon et al., 2004), benzybenzoate (Choi et al., 2005). However, we found other caffeoylquinic acids than those that have been previously isolated. Our HPLC method made it possible to detect several caffeoylquinic acids simultaneously and even to determine their quantity. Furthermore, caffeoylquinic acids of *K. yomena*, *S. virgaaurea* var. *asiatica*, *A. acerifolia* and *S. grandifolia* were first identified using an HPLC method.

It is assumed that the high levels of caffeoylquinic acids that ranged from 20-38% in chwinamul will be associated with a potent peroxynitrite scavenging effect. The scavenging activities of both *A. scaber* extracts were weak; the IC_{50} values of the unprocessed and the processed samples were 13.34 and 13.81 μg/ml, respectively. Although a relatively lower activity of *A. scaber* may be associated with the saponin content, which has been extensively studied by previous workers (Nagao et al., 1992; Nagao et al., 1993), more studies are required to characterize the detailed biological activities of this plant.

Hyperleptinemia accompanying obesity affects endothelial nitric oxide (NO) and is a serious factor for vascular disorders. Hyperleptinemia triggers an endothelial NO/$ONOO^-$ imbalance characteristic of dysfunctional endothelium observed in other vascular disorders such as atherosclerosis and diabetes (Korda et al., 2008). High blood levels of free fatty acids and glucose, which are often found in diabetes and obesity, can induce the production of superoxide through various mechanisms. Thus, once iNOS is induced in insulin-sensitive tissues, peroxynitrite may be easily

generated from NO in obese and diabetic individuals (Nomiyama et al., 2004). Increased formation of the potent oxidant peroxynitrite has recently been documented in both experimental and clinical diabetic neuropathy (Hoeldtke et al., 2002). Diabetes-induced oxidative stress has been well documented in the circulation and major elements of the peripheral nervous system and has been implicated in nerve conduction deficits, changes in signal transduction and metabolism, impaired neurotrophic support, neurovascular dysfunction and morphological abnormalities characteristics for diabetic neuropathy (Obrosova, 2002; Coppey et al., 2001; Cotter and Cameron, 2003; Cameron et al., 2001; Ziegler et al., 2004). In addition, oxidative and nitrosative stresses induce diabetes mellitus in which peroxynitrite attacks vascular biomolecules and thereby causes cardiovascular disease (Patcher et al., 2005). Decomposition of peroxynitrite in streptozotocin-induced diabetic rats reduces sensory neuropathy (Drel et al., 2007).

Hsu et al. (2006) reported that chlorogenic acid had an IC_{50} value of 72.3 μM for inhibiting the proliferation of 3T3-L1 preadipocytes, implicating an *in vivo* anti-obesity effect. The compound 3,5-DQ was reported to inhibit nitration of tyrosine residues and to reduce NO production due to the inhibition of NF-κB transcriptional activity (Olmos et al., 2008). We have previously reported anti-nociceptive (Park et al., 2007) and anti-hepatotoxic (Choi et al., 2004) effects of 3,4-DQ (IUPAC: 4,5-DQ) isolated from *Ligularia fischeri* var. *spiciformis*. Therefore, it is assumed that the peroxynitrite-scavenging effect and inhibition of NO production by caffeoylquinic acids, including chlorogenic acid, may be beneficial for prevention or treatment of neuropathy, cardiovascular disease, diabetes mellitus and atherosclerosis, which can develop from obesity, or for obesity itself.

Therefore, the mountainous vegetables called chwinamul could be used to treat the above diseases. The activity level of *K. yomena* and *A. acerifolia* in scavenging peroxynitrite was similar to that of the positive control, penicillamine, and higher than that of thistles as reported by Jeong et al. (2008). In addition, it is believed that caffeoylquinic acids are mainly responsible for the scavenging effect. The HPLC method established in the present study could be used for rapid evaluation of the quantity.

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