Natural Product Sciences
16(2) : 80-87 (2010)

The Inhibitory Effect of the Caffeoylquinic acid-Rich Extract of *Ligularia stenocephala* Leaves on Obesity in the High Fat Diet-Induced Rat

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Abstract – High performance liquid chromatography (HPLC) analysis on the MeOH extract of *Ligularia stenocephala* leaves identified six caffeoylquinic acids, *viz.* 3,4-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoyl-*muco*-quinic acid, 3,5-di-*O*-caffeoylquinic acid, 4,5-di-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid, and 3-*O*-caffeoylquinic acid using standard compounds, and determined the quantity of each extract. Percentage of total caffeoylquinic acids of the MeOH extract and its BuOH fraction were calculated as 67.83 ± 3.79% and 94.52 ± 1.84%, respectively. Since the caffeoylquinic acid-rich MeOH extract exhibited a potent peroxynitrite-scavenging effect *in vitro* (IC_{50} = 0.87 ± 0.33 μg/ml (mean ± SEM)), the experiment was designed to identify whether or not that extract has an anti-obesity effect on rat obesity induced by high fat diet. Oral administration of the MeOH extract and its BuOH fraction abundant in caffeoylquinic acid decreased the rat body weight to the level of untreated group and decreased abdominal fat pad weight. The atherogenic index and thiobarbituric acid-reactive substance (TBARS) values were restored by treatment, indicating that the caffeoylquinic acid-rich extract probably inhibited hyperlipidemia and oxidative stress caused by high fat diet. These results suggest that *L. stenocephala* in vegetable form or its caffeoylquinic acid-rich fraction (BuOH fraction) as an agent can be used for treatment or prevention of obesity.
Keywords – *Ligularia stenocephala*, caffeoylquinic acid, peroxynitrite, obesity, hyperlipidemia

Introduction

Obesity is increasing in the world due to high-calorie intake and mental stress and it can result in diabetes mellitus and atherosclerosis. Mountainous vegetable plants are employed for treatment of obesity as well as for pharmaceutical, dietary and medical therapy (Jeong*et al.*, 2006), including diabetes mellitus and atherosclerosis. These plants as foods are considered useful for treatment of obesity or obesity-related disease because they have vitamins, fibers and antioxidants. We have reported the anti-obesity effect of *Allium victorialis* (Choi *et al.*, 2005) and *Pleurospermum kamtschaticum* (Jung *et al.*, 2007) and the roots of *Rosa rugosa* (Park *et al.*, 2005).

Peroxynitrite ($ONOO^-$), which can be produced from the reaction of superoxide anion radical ($\bullet O_2^-$) with nitric oxide (NO) in the cell, is a risk factor for cellular damage (Radi *et al.*, 1991). It induces lipid peroxidation, cytotoxicity and rapid neuronal damage (Haenen *et al.*, 1997). In addition, it has been reported that peroxynitrite can cause atherosclerosis, diabetes mellitus, obesity and hypercholesterolemia (Korda *et al.*, 2008; Patcher *et al.*, 2005; Drel *et al.*, 2007). Therefore, the present research was undertaken to examine a mountainous vegetable capable of treating or preventing obesity or obesity-related disease through the mechanism of peroxynitrite-scavenging.

The MeOH extract of *Ligularia stenocephala* was found to have a very strong effect in the course of screening of peroxynitrite-scavenging effects on thistles. Therefore, it was fractionated into Et_2O- and BuOH fractions to reveal bioactive constituents. The leaves of *L. stenocephala* belonging to the family Compositae have been used as an edible wild herb and even cultivated on farms in Korea. In particular, the leaves of this plant are favored by

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Korean people as an edible vegetable with meat dishes. Although anti-thrombotic and anti-diabetic effects have been reported (Yoon *et al*., 2008), we were seeking an inhibitory effect on obesity resulting from a high fat diet. The sample of the MeOH extract of *L. stenocephala* leaves and both Et_2O- and BuOH fractions were given orally to obese rats for two weeks. To examine the effect on obesity, rat body weight and abdominal fat pad (retroperitoneal and epididymal) weights were measured, together with atherogenic index (A.I.) and antioxidant activity. The individual caffeoylquinic acids in the extracts were also analyzed using HPLC.

The two caffeoylquinic acids, 3,4- and 3,5-di-*O*-caffeoylquinic acids, have been isolated from *L. stenocephala* (Yoon *et al*., 2008). Chlorogenic acid and these two caffeoylquinic acids were observed on a TLC chromatogram of this plant during the preliminary study. Other research has reported monocaffeoylquinic acids and dicaffeoylquinic acids in many Compositae plants, along with *muco*-quinic acid or *epi*-quinic acid derivatives instead of quinic acid derivative (Zhao *et al*., 2006). Percentages of total caffeoylquinic acid of the extract and plant material were calculated from the peak area of HPLC chromatograms using the seven standard compounds that have been already isolated from *Lactuca indica* (Kim *et al*., 2007; Kim *et al*., 2008). There have been several known biological activities of caffeoylquinic acids, including antioxidative, anti-inflammatory, vascular relaxant, antimicrobial, anti-hepatotoxic and platelet anti-aggregating. (Zhao *et al*., 2006)

Experimental

Plant Material – The leaves of *L. stenocephala* (Compositae) were collected on April 2009 in Gangwon-do, Korea. The collected leaves were air-dried at the room temperature and pulverized for experiment. The plant was identified by Dr. Won-Bae Kim (Highland Agriculture Research Institute, Rural Development Administration, Pyeongchang, Gangwon-do, Korea). The voucher specimen (# natchem-31) was deposited at the Laboratory of Natural Product Chemistry of the Department of Pharmaceutical Engineering, Sangji University, Wonju, Korea.

Extraction and fractionation – Air-dried leaves of *L. stenocephala* (2 kg) were extracted three times with MeOH (10 L) under reflux at 80 °C for 7 hours each. The extract was concentrated using a rotary evaporator under reduced pressure to give a viscous mass (342 g). This extract (330 g) was suspended in 800 mL H_2O and fractionated with 800 mL Et_2O three times. The residual aqueous layer was then fractionated with 800 mL BuOH three times. Afterward, the Et_2O and BuOH soluble portions were concentrated and dried on a freeze-dryer to produce the Et_2O and BuOH powdery solid fractions (108 g and 119 g, respectively).

Instruments, reagents, and standard compounds for HPLC analysis – Analysis of caffeoylquinic acids contained in the MeOH extract of *L. stenocephala* and its fractions Et_2O and BuOH was conducted on a Varian HPLC system (Walnut Creek, CA, USA) that includes Prostar 210 solvent delivery module, Prostar 325 UV-Vis detector and 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA). Compound separation of the analyzed sample was performed on Shiseido (Chuoku, Tokyo, Japan) Capcell Pak C18 column (5 μL, 250 mm × 4.6 mm I.D.). All solvents used for analysis were the HPLC grade obtained from J.T. Baker (Phillipsburg, NJ, USA). Seven standard caffeoylquinic acids isolated from *Lactuca indica* L. (Kim *et al*., 2007, 2008) were used, supplied by Prof. Kang Ro Lee (College of Pharmacy, SungKyun Kwan University, Suwon, Korea). The seven caffeoylquinic acids are abbreviated as follows: 3,4-di-*O*-caffeoylquinic acid (3,4-DQ), 3,5-Di-*O*-caffeoyl-*muco*-quinic acid (3,5-DmQ), 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), and 3-*O*-*p*-coumaroyl-caffeoylquinic acid (3-pCQ). Structures of the seven standard compounds are shown in Fig. 1.

HPLC condition for caffeoylquinic acids content analysis – For HPLC analysis, test samples (MeOH extract, Et_2O and BuOH fractions) and seven standard compounds were dissolved in 80% aqueous MeOH and were filtered through a 0.50 mm syringe filter and the filtrate (20 mL) injected into the analyzer. 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 the flow rate of 1.00 mL min^{-1}. The UV detector was fixed at 246 nm. The extracts were analyzed by HPLC after R^2 values of regression equations were identified to be more than 0.990.

Assay for peroxynitrite-scavenging activity – Peroxynitrite ($ONOO^-$) scavenging activity was assessed by the modified Kooy's method, which involved the monitoring of highly fluorescent rhodamine 123, which is rapidly produced from non-fluorescent DHR 123 in the presence of $ONOO^-$ (Kooy *et al*., 1994). In brief, the

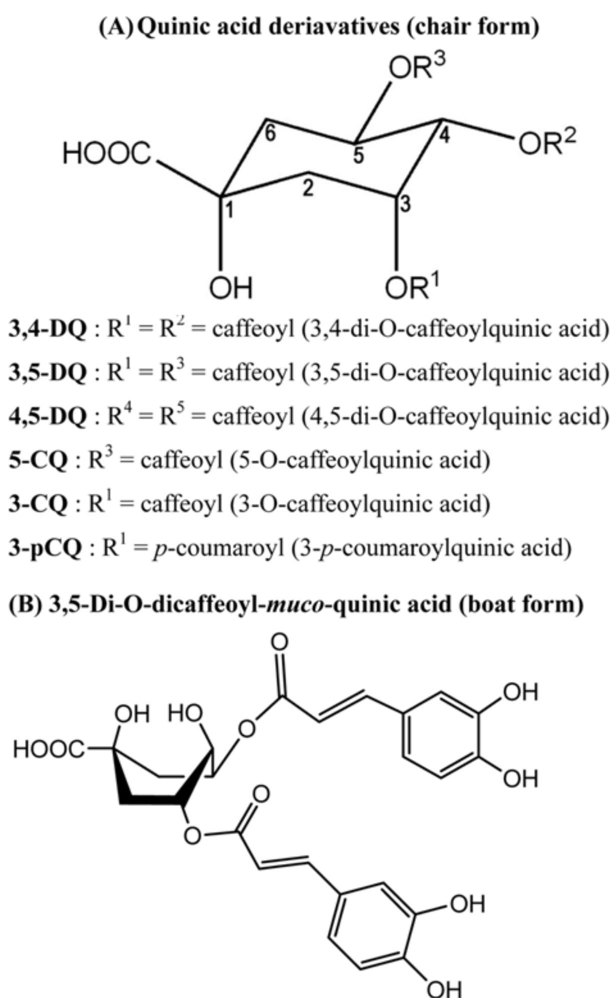

Fig. 1. Structures of compounds **1 - 7** used for quantitative analysis.

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 ìM. The buffer in this assay was prepared 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 minutes after treatment with and 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 Instruments Inc., Winooski, VT, USA) 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 of peroxynitrite assay was expressed as mean ± SD.

Experimental animals and treatment – Four week-old male Sprague-Dawley rats were purchased from Hyochang Science Co. (Daegu, Korea) and housed with free access to water with measured feed in a well-ventilated animal unit (temperature 22 ± 3 °C, humidity 50 ± 10%, light/dark cycle: 12 hours) for a week in the Animal Laboratory of College of Pharmacy (Kyungsung University, Busan, Korea). Rats weighing 140 ± 10 g were used for the animal experiments. During the 24 h before experiment, only water was offered to the animals. The samples, MeOH extract of *L. stenocephala* leaves and its Et_2O and BuOH fractions, were orally administered

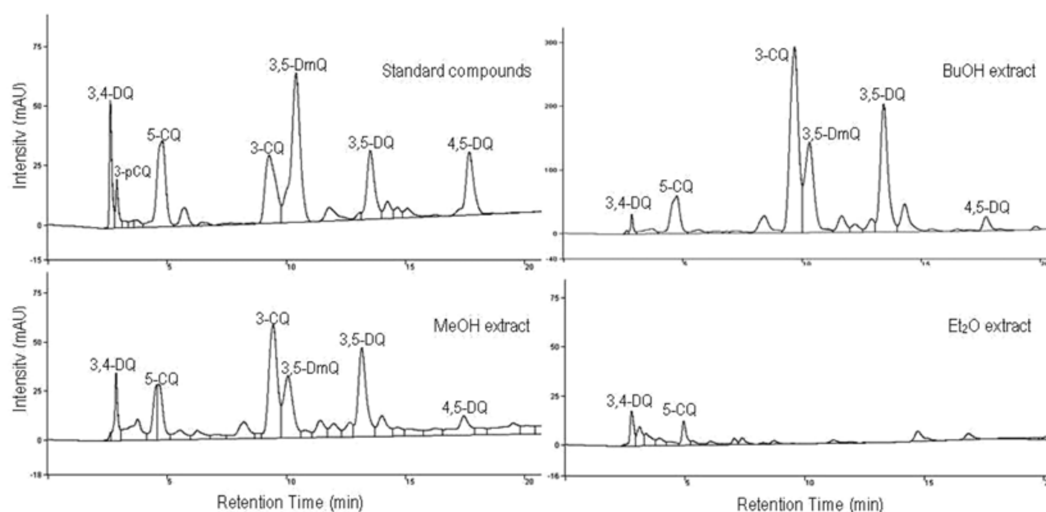

Fig. 2. HPLC chromatograms of seven standard compounds and MeOH extract of *L. stenocephala* and its fractions (Et_2O and BuOH fractions).

using jonde for 2 weeks. In consideration of daily variations in enzyme activity, the animals were sacrificed at a fixed time (10:00 A.M.-12:00 P.M.). These experiments were approved by the University of Kyungsung Animal Care and Use Committee. All procedures were carried out in accordance with the "Guide for Care and Use of Laboratory Animals" published by the National Institutes of Health.

Induction of obesity – The composition of high fat diet is noted in Table 1. To induce obesity, rats were fed with high fat diet for six weeks. After oral administration of the samples in the last two weeks, the anti-obesity effect was examined.

Serum preparation – Animals were anesthetized by CO_2 and blood collected from abdominal aortas. The blood stood for 30 min and was centrifuged for 10 min at 3,000 rpm to obtain sera that were then measured for lipoprotein, hydroxyl radical and superoxide dismutase activity levels.

Measurement of body weight and abdominal fat pad weights – Body weights were checked every week throughout the period of high fat diet. At the final day of sample administration, abdominal fat pads (retroperitoneal and epididymal) were collected and weighed.

Determination of total cholesterol levels – The total serum cholesterol level was measured by using the manufacturer's protocol with an AM 202-K assay kit (Asan Pharm. Co., Korea) prepared per Richmond (1976). Three milliliters of solution (cholesterol esterase 20.5 U/L, cholesterol oxidase 10.7 U/L, sodium hydroxide 1.81 g/L, potassium phosphate monobasic 13.6 g/L, phenol 1.88 g/L) were mixed with 20 µL of serum and incubated at 37 °C for 5 min. For determination of total cholesterol levels, the absorbance of the reaction mixture was measured at 550 nm and the levels were calculated using a standard calibration curve.

Table 1. Composition of basal and hyperlipidemic diet

Ingredient	Basal Diet (%)	Hyperlipidemic Diet (%)
Casein	20.0	20.0
DL-Methionine	0.3	0.3
Corn Starch	15.0	15.0
Sucrose	50.0	34.5
Fiber[1)]	5.0	5.0
Corn oil	5.0	-
AIN-mineral Mixture[2)]	3.5	3.5
AIN-vitamin Mixture[3)]	1.0	1.0
Choline Bitartate	0.2	0.2
Beef Tallow	-	20.5

[1)] Cellulose : Sigma Co. LTD., USA

[2)] Mineral mixture based on the pattern of Rogers and Harper (1965) contain the following (g/kg diet): calcium phosphate dibasic 500.0, sodium chloride 74.0, potassium citrate monohydrate 220.0, potassium sulfate 52.0, magnesium oxide 24.0, magnesium carbonate 3.5, ferric citrate 6.0, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, chromium potassium sulfate 0.55, sucrose, finely powered, to make 1,000

[3)] Vitamin mixture(g/kg diet): thiamine HCl 0.6, biotin 0.02, riboflavin 0.6, cyanocobalamine 0.001, pyridoxine HCl 0.7, retinyl acetate 0.8, nicotinic acid 3.0, DL-tocopherol 3.8, Ca-pantothenate 1.6, 7-dehydrocholesterol 0.0025, folic acid 0.2, methionine 0.005, sucrose, finely powered, to make 1,000

Determination of High density lipoprotein-cholesterol (HDL-C) content – HDL-cholesterol was determined using kit reagent (AM 203-K, Asan) prepared by Noma's enzymatic method. (Noma *et al.*, 1978) The precipitate reagent (0.2 ml) containing 0.1% dextran and 0.1 M magnesium chloride was added in the serum (20 µl), mixed and let stand at room temperature. After centrifugation for 10 min at 3,000 rpm, the supernatant (0.1 ml) was withdrawn and well mixed with 3.0 ml enzymatic solution and incubated for 5 min at 37 °C. Absorbance was measured at 500 nm using the blank solution and their levels were calculated as mg/dl using a standard calibration curve.

Determination of low density lipoprotein (LDL)-cholesterol – LDL-cholesterol level was calculated using Friedewald's equation (Friedewald *et al.*, 1972): LDL-cholesterol = total cholesterol – (HDL-cholesterol + triglyceride/5).

Determination of serum TBARS concentrations – Serum lipid peroxide contents were evaluated by measuring serum TBARS values using the method described by Yagi *et al.* (1987). Serum (20 µl) was mixed with 4.0 ml of 1/12N H_2SO_4, 0.5 ml of phosphotungstic acid was then added, and mixtures were allowed to stand for 5 min to obtain the serum by precipitation. Serum proteins were separated by centrifuge, added to 2 ml 1/12N H_2SO_4 and 0.3 ml 10% phosphotungstic acid, and recentrifuged. Four ml of distilled water and 1.0 ml of thiobarbituric acid-acetic acid (1 : 1) solution were then added to proteins and heated at 95°C for 60 min. It was cooled to room temperature, mixed with 5.0 ml n-BuOH, and centrifuged at 3,000 rpm for 15 min. The absorbance of the formed reddish n-BuOH solution was then determined using a spectrofluorometer (Ex: 515 nm, Em: 553 nm).

Determination of hydroxy radical (•OH) concentrations in serum – Serum hydroxy radical levels were measured using the method of Kobatake *et al.* (1987). Briefly, 333.3 µl of solution composed of 34.8 µl serum, 0.54 M NaCl. 0.1M potassium phosphate buffer (pH 7.4), 10 mM

NaN_3, 7 mM deoxyribose, 5 mM ferrous ammonium sulfate and distilled water was vortexed and allowed to stand at 37°C for 15 min. Of this mixture, 67 ml was added to a solution of 75 µl of 8.1%-sodium dodecyl sulfate, 67 µl of 20% acetic acid and distilled water; 222 µl of 1.2% thiobarbituric acid was then added. This solution was boiled for 30 min and cooled to room temperature before being centrifuged at 700 × g for 5 min. Supernatant absorbance determined at 532 nm using a spectrophotometer and hydroxy radical concentrations were calculated using a standard calibration curve.

Determination of superoxide dismutase (SOD) activity – Blood SOD activities were measured using the method described by Oyanagui *et al.* (1984). A test tube containing 100 µl of serum, 500 µl of distilled water, 200 µl of reagent A (3 mM hydroxylamine/3 mM hypoxanthine), 200 µl of reagent B (7.5 mU/ml xanthine oxidase (XOD), 0.1 mM EDTA-2Na) was gently vortexed and allowed to stand for 30 min. Two ml of reagent C (sulfanilic acid 300 mg/N-1-naphthyl-ethylenediamine 5.0 mg of 16.7% acetic acid) was then added to the reaction mixture, vortexed, and allowed to stand at room temperature for 20 min. For determining serum SOD activity levels, the UV-VIS absorbances of the reaction mixtures were measured at 550 nm and levels were calculated using a standard calibration curve.

Results and discussion

Peroxynitrite-scavenging effect and analysis of caffeoylquinic acids – As shown in Fig. 3, the MeOH extract scavenged the peroxynitrite formation in a dose-dependent fashion. For the MeOH extract, IC_{50} = 0.87 ± 0.33 µg/ml, similar to that of the positive control L-Penicillamine (IC_{50} = 0.89 ± 0.22 µg/ml). These results suggest that the MeOH extract of *L. stenocephala* is a potent peroxynitrite scavenger.

On HPLC chromatogram six kinds of caffeoylquinic acids were detected at the retention times of 2.7 min (3,4-DQ), 9.6 min (3,5-DmQ), 12.9 min (3,5-DQ), 17.1 min (4,5-DQ), 4.5 min (5-CQ), 8.9 min (3-CQ), and 2.9 min (3-pCQ). The four compounds other than 3,4-DQ and 3,5-DQ that have been isolated by Yoon *et al*., (2008), and 3-pCQ were first indentified by HPLC analysis. Percentage of total caffeoylquinic acids of the MeOH extract and the BuOH fraction were 67.83% and 94.52%, respectively, while the Et_2O fraction was very low (6.06%). In contrast, percentage of total caffeoylquinic acid of the plant material was 11.60%. The most abundant compound was 3,5-DQ with percentages of 25.85% in the MeOH extract and 41.32% in the BuOH fraction. HPLC chromatograms of the MeOH extract and both fractions are shown in Fig. 2 and the percentages of the samples in Table 2.

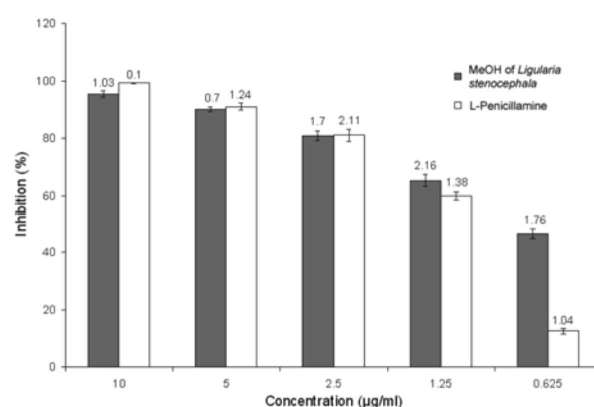

Fig. 3. Peroxynitrite scavenging effect of MeOH extract of *L. stenocephala compared* with L-Penicillamine. Data are mean ± SD of triplicate experiments.

Table 2. Percentage of caffeoylquinic acids in the MeOH extract of *L. stenocephala* and its Et_2O and BuOH fractions

Compound	Caffeoylquinic acids (% of extract)		
	MeOH ext	Et_2O fraction	BuOH fraction
3,4-DQ	8.84 ± 0.19	2.91 ± 0.23	5.16 ± 0.28
3,5-DeQ	3.65 ± 0.28	ND	9.73 ± 0.38
3,5-DQ	25.80 ± 1.51	ND	41.32 ± 1.17
4,5-DQ	1.95 ± 0.10	ND	3.11 ± 0.16
5-CQ	12.31 ± 0.83	3.15 ± 0.03	9.73 ± 0.47
3-CQ	15.27 ± 1.17	ND	25.46 ± 0.42
3-pCQ	ND[b]	ND	ND
Total % of extract	67.83 ± 3.79	6.06 ± 0.26	94.52 ± 1.84
Total % of dried weight	11.60 ± 0.65	0.35 ± 0.01	5.98 ± 0.12

[a] Values represent mean ± S.D. based on triplicate experiments, [b]ND : not detected

Change of body weight and abdominal fat pad weight – To examine *in vivo* anti-obesity effects, high fat diet (Table 1)-induced rats were used. High fat diet was administered for six weeks and samples were treated for the last two weeks of the six-week period. After anti-obesity treatment, the weight changes of body and retroperitoneal and epididymal fat pads were investigated, together with atherogenic index and oxidative stress.

As shown in Fig. 4, the BuOH fraction exhibited the most significant effect on the obesity induced by high fat diet, though the MeOH extract and the Et_2O fraction were also active. The BuOH fraction most significantly reduced the body weight and the abdominal fat pad weights, almost to the level of the untreated group. These results

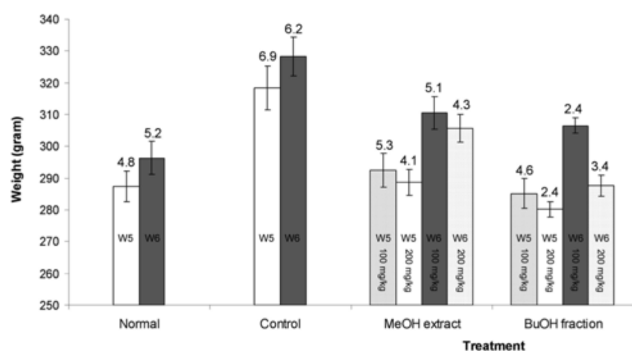

Fig. 4. Effect of the MeOH extract of *L. stenocephala* and its BuOH fraction on body weight in rats fed a normal and high fat diet at week 5 (W5) and 6 (W6). Data are mean ± SD of six experiments.

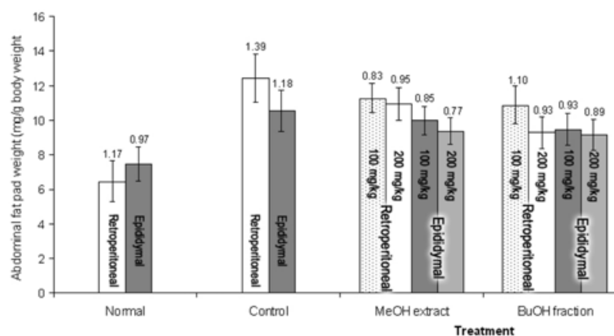

Fig. 5. Effect of the MeOH extract of *L. stenocephala* and its BuOH fraction on the abdominal fat pad (retroperitoneal and epididymal) weight in rats fed a normal and high fat diet. Data are mean ± SD of six experiments.

suggest that the MeOH extract could be considered for a therapeutic against obesity. Since the BuOH fraction contained the highest quantity of caffeoylquinic acids (94.52%), we surmise that those constituents contribute most to the anti-obesity effect. Although Toyoda *et al.* (2005) reported the isolation of benzofuran derivatives from the roots of *L. stenocephala*, these compounds have not been reported from the leaves.

The results on the weight of abdominal (retroperitoneal and epidydymal) fat pads are shown in Fig. 5. The MeOH extract and both fractions significantly reduced those weights increased by high fat diet. In particular, treatment with the caffeoylquinic acid-rich BuOH fraction at 200 mg/kg reduced the retroperitoneal and the epididymal fat pad weights by 53.4% and 45.8%, respectively.

Effect on the atherogenic index – The results on the levels of total cholesterol, HDL-cholesterol and LDL-cholesterol are shown in Table 3. The MeOH extract and both fractions reduced total cholesterol- and LDL-cholesterol levels, though those treatments showed no significant change in HDL-cholesterol. Atherogenic index of the control group was 1.81 ± 0.13 while that of the untreated group was 0.42 ± 0.09. The most significant effect was observed in the treatment group of the BuOH fraction (200 mg/kg) (A.I. = 1.22 ± 0.11).

Effect on TBARS – The levels of TBARS, hydroxyl radical and SOD activity were increased in the control group compared to the untreated group, which suggest the increase of reactive oxygen species (ROS), lipid peroxidation and oxidative stress. The MeOH extract and both fractions prohibited the increase of TBARS and hydroxyl radical levels and increased SOD activity level. In particular, the BuOH fraction (200 mg/kg) was the most effective (Table 4).

Peroxynitrite is produced from the reaction between superoxide anion radical ($\bullet O_2^-$) and nitric oxide (NO) in the cell (Radi *et al.*, 1991). Overproduction of peroxynitrite can result in the occurrence of metabolic or cardiovascular disease such as hypercholerolemia, atherosclerosis, obesity, and diabetes mellitus (Korda *et al.*, 2008; Patcher *et al.*, 2005; Drel *et al.*, 2007). The screening of peroxynitrite-scavenging effects was performed

Table 3. Effect of the MeOH extract of *L. stenocephala* and its Et_2O and BuOH fractions on serum cholesterol and AI in rats fed a hyperlipidemic diet

Treatment	Dose (mg/kg)	cholesterol			AI
		total	HDL	LDL	
Normal	-	59.3 ± 8.27^{c}	41.8 ± 2.16^{b}	2.57 ± 0.97^{c}	0.42 ± 0.09^{e}
Control	-	97.9 ± 9.56^{a}	34.9 ± 1.43^{b}	6.17 ± 0.83^{a}	1.81 ± 0.13^{a}
MeOH ext.	100	89.3 ± 7.25^{ab}	35.0 ± 1.39^{b}	5.87 ± 0.91^{ab}	1.55 ± 0.15^{b}
	200	85.7 ± 6.31^{b}	35.4 ± 2.11^{b}	5.62 ± 0.74^{ab}	1.42 ± 0.10^{bc}
Et_2O Fr.	100	86.3 ± 10.4^{b}	35.1 ± 1.51^{b}	5.65 ± 0.87^{ab}	1.47 ± 0.07^{bc}
	200	84.2 ± 7.83^{b}	35.9 ± 1.60^{b}	5.34 ± 0.91^{ab}	1.36 ± 0.12^{cd}
BuOH fr.	100	84.7 ± 7.11^{b}	35.7 ± 1.30^{b}	5.60 ± 0.85^{ab}	1.37 ± 0.08^{c}
	200	81.6 ± 6.54^{b}	36.7 ± 1.25^{b}	4.72 ± 0.63^{b}	1.22 ± 0.11^{d}

The assay procedure was described in the experimental methods. Values are mean±S.D. for six experiments. Values followed by the same letter are not significantly different ($p < 0.05$).
AI (Atheroscrelosis Index) = (total cholesterol - HDL cholesterol)/ HDL cholesterol

Table 4. Effect of the MeOH extract of *L. stenocephala* and its Et_2O and BuOH fractions on the blood lipid peroxide, the serum hydroxyl radical and superoxide dismutase activities in rats fed a hyperlipidemic diet

Treatment	Dose (mg/kg)	MDA	Hydroxy radical	SOD activity
		nmol/ml of serum	nmol/mg protein	U/g protein
Normal		25.9 ± 4.17^{d}	2.78 ± 0.30^{c}	3.29 ± 0.37^{a}
Control		48.6 ± 3.36^{a}	5.57 ± 0.29^{a}	1.98 ± 0.19^{d}
MeOH ext.	100	45.3 ± 2.98^{ab}	5.26 ± 0.31^{ab}	2.16 ± 0.18^{cd}
	200	41.7 ± 2.37^{bc}	5.15 ± 0.25^{b}	2.37 ± 0.20^{bc}
Et_2O	100	44.6 ± 2.53^{b}	5.21 ± 0.31^{ab}	2.19 ± 0.21^{cd}
	200	41.3 ± 2.46^{bc}	5.05 ± 0.36^{b}	2.41 ± 0.23^{bc}
BuOH	100	41.5 ± 3.11^{bc}	5.13 ± 0.23^{b}	2.25 ± 0.15^{cd}
	200	38.7 ± 2.16^{c}	4.83 ± 0.33^{b}	2.55 ± 0.16^{b}

The assay procedure was described in the experimental methods. Values are mean±S.D. for six experiments. Values followed by the same letter are not significantly different ($p < 0.05$).
SOD 1 Unit: Defined as the amount of enzyme that inhibits the rate of adrenochrome formation to 50%

on mountainous vegetable plants belonging to the family Compositae in our effort to select certain plants with potent anti-obesity effects. The caffeoylquinic acid contents of those plants were also investigated using HPLC. We found that leaves of *L. stenocephala* exhibited low IC_{50} value (0.87 μg/ml) and a high level of caffeoylquinic acids: percentages of total caffeoylquinic acid of the plant material and of the extract were 11.60% and 67.8%, respectively. Furthermore, the percentage of the BuOH extract reached 94.52% and therefore could be called a caffeoylquinic acid-rich fraction. We also found the IC_{50} value of the MeOH extract was similar to that of the positive control L-Penicillamine. At the low concentration (0.625 μg/g), the MeOH extract was much more effective in scavenging peroxynitrite than L-Penicillamine. A number of Korean mountainous vegetable plants belong to the family Compositae are known to contain many caffeoylquinic acids e.g. *Aster scaber* (Kwon *et al*., 2004), *Solidago virga* var. *gigantea* (Choi *et al*., 2004), *Lactuca indica* (Kim *et al*., 2007; Kim *et al*., 2008).

We assumed that the leaves of *L. stenocephala* would be beneficial for lipid metabolism because Koreans use it as an edible vegetable with meat dishes. Since the MeOH extract of *L. stenocephala* was found to be a potent peroxynitrite scavenger, HPLC analysis was performed to find the composition of caffeoylquinic acids and led to the identification of 3,4-DQ, 3,5-DQ, 3,5-DmQ, 4,5-DQ,, 5-CQ, and 3-CQ using standard compounds. Total quantity was very high in the MeOH extract (67.8% of the extract) and even higher in the BuOH fraction (94.52% of the extract). However, one of the standard compounds, 3-pCQ, was not detected on the chromatogram.

Chlorogenic acid derivatives or caffeoylquinic acids can be classified into monocaffeoylquinic acids, dicaffeoylquinic acids and tricaffeoylquinic acids according the number of substituents, i.e. caffeic acid, ferulic acid, and p-coumaric acid; tricaffeoylquinic acids are uncommon. The most common parent skeleton is quinic acid, though *epi*-quinic acid and *muco*-quinic acid occur (Zhao *et al*., 2006). The most well-known caffeoylquinic acid, chlorogenic acid, has high concentration in coffee (Marinova *et al*., 2009) and green tea (Costa *et al*., 2009). Dicaffeoylquinic acids are known as potent inhibitors of nitration reaction of peroxynitrite against tyrosine residue and of NO formation (Olmos *et al*., 2008).

Hyperleptinemia accompanying obesity affects endothelial nitric oxide (NO) and is a serious factor for vascular disorders. Imbalance of NO/peroxynitrite can cause atherosclerosis and diabetes mellitus (Korda *et al*., 2008). Hsu *et al*. (2006) reported that chlorogenic acid potentially inhibited the proliferation of 3T3-L1 preadipocyte (IC_{50} = 72.3 μg/ml) suggesting a possible *in vivo* anti-obesity effect. In addition, Olmos *et al*. (2008) reported the inhibitory effect of 3,5-DQ toward nitration and toward NO production based on the NF-κB transcriptional activity.

There are prospective views on the *in vitro* effect of caffeoylquinic acid (Hsu *et al*., 2006). However, there has been no report on the anti-obesity effect of *L. stenocephala*. In the present study, when the caffeoylquinic acid-rich extract or fraction of *L. stenocephala* with potent peroxynitrite-scavenging effect was investigated for anti-obesity effect *in vivo*, its extracts were found effective on the high fat diet-induced obesity. The extracts significantly reduced the body weight and the abdominal fat pad weight and ameliorated the increase of atherogenic index and decrease of oxidative stress.

From the roots of *L. stenocephala*, the constituents of

ligulariacephalins A, B, C, 5,6-dimethoxy-2-isopropylbenzofuran, euparin, (R)-(-)-hydroxytrementone (Toyoda *et al*., 2005) have been isolated and from the leaves, 3,4-DQ and 3,5-DQ with anti-thrombotic activity (Yoon *et al*., 2008). The present study showed that the BuOH fraction, a caffeoylquinic acid-rich extract containing 94.52% caffeoylquinic acids, had the greatest anti-obesity effect. From it, we conclude that caffeoylquinic acids are mainly responsible for the *in vivo* anti-obesity effect and that *L. stenocephala* or the caffeoylquinic acid-rich fraction of the extract could be used as a therapeutic against obesity through the mechanism of peroxynitrite-scavenging effect.

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Received May 1, 2010
Revised June 10, 2010
Accepted June 15, 2010