Identification of the biologically active constituents of *Camellia japonica* leaf and anti-hyperuricemic effect *in vitro* and *in vivo*

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Abstract. *Camellia japonica* L. is a plant of which the seeds are used as a folk medicine, and it is native to South Korea, Japan and China. In previous study, triterpenes, flavonoids, tannins and fatty acids which have antiviral, antioxidant and anti inflammatory activity were reported from *C. japonica* leaf and flower. In Korea, the seed from this plant is used as a traditional medicine and in folk remedies for the treatment of bleeding and inflammation. However, the major issue associated with the use of the seed as a medicinal and/or functional food ingredient is its application to the pharmaceutical and food industry. First, the productivity of seed extract is very much less than that of the leaf. Second, the beneficial usage of the seed extract as an alternative medicine and functional source is not yet clear. Thus, in this study, we focused on another part of the plant, the leaf, and found that the extract of *Camellia japonica* leaf has a high concentration of vitamin E, rutin and other biologically active compounds related to hyperuricemia. We aimed to investigate the biological activities, namely the antioxidant activities, xanthine oxidase (XO) inhibitory activity and anti-hyperuricemic effects of extract from *C. japonica* leaf and the phytochemicals contained therein. Ethanol extracts of *C. japonica* leaf (ECJL) were prepared, and the extract was used with respect to antioxidant activities, total phenolic contents and XO inhibitory activity. The *in vivo* XO inhibitory activity and anti-hyperuricemic effects of the extract were evaluated in mice with potassium oxonate-induced hyperuricemia. To clarify the marker compounds that are responsible for the anti-hyperuricemic effects, several key constituents were identified using gas chromatography-mass spectrometry (GC-MS) and and liquid chromatography-mass spectrometry (LC-MS). ECJL was found to have strong antioxidant activities, and *in vitro* XO inhibitory activity. The results of the *in vivo* experiments using mice demonstrated that ECJL at the doses of 100 and 300 mg/kg inhibited hepatic XO activity and significantly attenuated hyperuricemia. To the best of our knowledge, the present study is the first report on the XO inhibitory and anti-hyperuricemic effects of ECJL, which can be therapeutically applied in the treatment of hyperuricemia and gout.

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Introduction

Camellia japonica L. (*C. japonica*) is a tree belonging to the Theaceae family and is cultivated and has been used in traditional medicine in Japan, China, and Korea for the treatment of stomachic illness, bleeding and inflammation (1,2). The usage of the seed and flower from *C. japonica* was first reported in 'Dongui Bogam' which was thought to have been written by the doctor of oriental medicine, Heo Jun in 1613 (3).

C. japonica is a perennial herb that grows in forests in the southern region of Korea. Recently, the northern limits of *C. japonica* have widely increased due to the greenhouse effect on Earth's atmosphere in South Korea (4). Additionally, the provincial governments of South Korea have made many efforts to industrialize *C. japonica* in the field of food and medicinal

sources. From 2004, green tea from *C. japonica* has been developed as a food source in Korea so called 'Dong-Baek Cha' which was commercialized in 2007 (5). However, the specific and beneficial usage of the leaf as a functional food source and/or plant medicine has not yet been determined.

Chemically, this species has shown to be constituted of triterpenes, saponins, glycosylated flavonoids and tannins (1,6-8). Several of these derivatives have been shown to exhibit biological activity, such as antioxidant (7), antifungal (9), antiviral (10) and cytotoxic potential (8).

In our preliminary experiments, we screened approximately 500 plant extracts and selected the extract of *C. japonica* leaf as a xanthine oxidase (XO) inhibitor to find an alternative medicine for hyperuricemia. We investigated the active constituents and the biological activities of extracts from *C. japonica* leaf for the development of possible alternatives to allopurinol.

Hyperuricemia is a condition in which the levels of uric acid in the blood exceed the normal range, which is due to the regular intake of food with a high purine content and is invariably accompanied by gout, chronic kidney disease and other metabolic syndromes (11). Uric acid is the final metabolite of the purine compound, which is formed by the oxidation of hypoxanthine to xanthine and xanthine to uric acid by XO (12). The overproduction of uric acid by XO leads to hyperuricemia, which is the main cause of gout. Gout is a metabolic disorder that is closely associated with high levels of uric acid in the body, which can cause inflammation, gouty arthritis and uric acid nephrolithiasis (13). Gout has been reported to afflict >2 million men and women in the US alone (14). The prevalence of gout is rapidly increasing in other regions, including Northeast Asia (15), likely due to changes in dietary habits.

The aim of the present study was to investigate the potential of extract of *C. japonica* leaf (ECJL) to inhibit XO and to act as an antioxidant *in vitro* and *in vivo*. Moreover, the extract was investigated for its effects on the elimination of uric acid in serum and the inhibition of XO in mice with potassium oxonate-induced hyperuricemia. Screening of the extract for its XO inhibitory activity followed by their potential to reduce serum uric acid levels and XO activity may play an important role in identifying a potent chemical entity for the treatment of gout and related inflammatory disorders. To clarify the biological marker in ECJL, we describe the identification of bioactive constituents that include rutin, vitamin E, all-trans-squalene, neophytadiene, linolenic acid, n-hexadecanoic acid, n-octacosane, n-eicosane, and 6,9-pentadecadien-1-ol using gas chromatography-mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analysis.

Materials and methods

Plant materials. The leaves of *C. japonica* were provided from Jeollanamdo Wando Arboretum, in Jeonnam, Korea. *C. japonica* leaves were collected on Joyag island, Korea (126˚56'50.07"E longitude and 34˚22'31.27"N latitude). A voucher specimen (MNUCSS-CJ-01) was deposited at Mokpo National University (Muan, Korea). The leaves were separated for use in the present study. The air-dried and powdered *C. japonica* leaf (10 g) was extracted twice with ethanol (100 ml) at room temperature for 3 days. The resultant ethanol solution was evaporated, dried and stored at -50˚C. The sample was used for *in vitro* and *in vivo* experiments, and for the analysis of the active constituents.

Animals. Male ICR mice (4 weeks old) were purchased from Orient Bio Co. (Sungnam, Korea). The mice were retained in a clean room at a temperature of 20-23˚C with 12-h light (07:00-19:00) and dark (19:00-07:00) cycles, and a relative humidity of 50±5%. The mice were housed in ventilated mice cages (Tecniplast, West Chester, PA, USA) under filtered and pathogen-free air, with food (Agribrands Purina Korea, Inc., Seoul, Korea) and water available *ad libitum*. All animal experiments were carried out according to the guidelines of the Animal Investigation Committee of Jeonnam Bioindustry Foundation (Naju, Korea) (approval no. JINR1517).

Profiling of constituents by GC-MS analysis. The analytical methods for the analysis based on GC-MS have been previously reported (16). The analysis of scanned organic compounds was performed using an Agilent 7890 Gas Chromatograph System, coupled to a quadrupole Agilent 5975C electron ionization (EI) (70 eV) mass spectrometric detector (Agilent Technologies, Inc., Palo Alto, CA, USA). An Agilent HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) was utilized for the identification of organic compounds. Briefly GC-MS was tuned using perfluorotribuylamine (PFTBA) by mass fragments of 69.0, 219.0, 502.0 m/z under EI conditions. The GC oven was heated using the following program: isothermal at 65˚C for 10 min and 10 min^{-1} to 300˚C with He as the carrier gas. The transfer line was heated at 300˚C, and the mass spectrometer was then operated in scan mode (50-550 amu). All mass spectra were compared with the data system library (NIST 2008). The summarized operation parameters for the GC are shown in Table I.

Profiling of constituents by HPLC analysis. Constituent profiling of ECJL was performed with HPLC. All HPLC analyses were performed using the Alliance 2695 HPLC System (Waters Corp., Millford, MA, USA) equipped with a photodiode array detector. The Agilent ZORBAX Extend-C18 (5 μm, 150 mm l. x 5 mm i.d.) analytical column was used with a mobile phase consisting of solvent A (acetonitrile) and solvent B (water containing 0.2% phosphoric acid). Gradient elution (from 10/90 to 100/0, v/v) was performed at a flow rate of 1.0 ml/min (Table II). Column temperature was maintained at 25˚C. The detection wavelength was set at 270 nm for rutin. The solvent was filtered through a 0.22-μm filter and degassed. The sample injection volume was 10 μl.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical assay. The determination of the antioxidant activity of ECJL was performed by the DPPH radical scavenging method. DPPH radicals have an absorption maximum of 517 nm, which disappears with reduction by an antioxidant compound. ECJL solution (1 ml) containing 1-20 mg of ECJL was added to a 0.4 mM DPPH ECJL solution (1 ml). The solution was mixed and allowed to react at room temperature in the dark for 10 min. The absorbance at 517 nm was measured using a microplate reader (PerkinElmer, Inc., Waltham, MA, USA). The radical scavenging activity was calculated as a percentage using the following equation: DPPH radical scavenging

Table I. Analysis condition of GC-MS.

Parameter	Condition		
Column	Agilent HP-5MS fused silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness)		
Carrier	Helium		
Split	1:5		
Injection volume	1 μl		
MS source	230˚C		
MS quad	150˚C		
Thermal aux	300˚C		
Electron ionization	70 eV		
Mass range	50-550 amu		
Scan method	Full scan		
	Rate (˚C/min)	Value (˚C)	Hold time (min)
Analytical temperature			
Initial		65	10
Ramp	10	300	22
Total	55.5		

GC-MS, gas chromatography-mass spectrometry.

Table II. Analytical conditions of HPLC for analysis of ECJL.

Parameters	Conditions		
Column	Zorbax extended-C18 (C18, 4.6 mm x 150 mm, 5 μm)		
Flow rate	1 ml/min		
Injection volumn	10 μl		
UV detection	270 nm		
Run time	30 min		
	Time (min)	% A[a]	% B[b]
Gradient	0	90	10
	7	90	10
	15	70	30
	22	70	30
	25	0	100
	30	90	10
	35	90	10

[a]0.2% Phosphoric acid; [b]acetonitrile. HPLC, high-performance liquid chromatography; ECJL, extracts of *C. japonica* leaf.

activity (%) = [1 - (A_{sample}/A_{blank})] x100. The DPPH free radical scavenging activities of the samples were compared in terms of their IC_{50} (μg/ml) values, as previously described (17).

Determination of reducing power. The reducing power of ECJL was determined according to a modified reducing power assay method. The sample (0.1 ml) was added to 0.2 M sodium phosphate buffer (0.5 ml) and 1% potassium ferricyanide (0.5 ml), and this mixture was incubated at 50˚C for 20 min. Following incubation, 10% trichloroacetic acid solution (0.5 ml) was added to the reaction mixture, and it was centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with distilled water (0.5 ml) and a 0.1% iron (III) chloride solution (0.1 ml), and the absorbance at 700 nm of the resulting solution was measured. The reducing powers of the samples were expressed as vitamin C equivalents, as previously described (17).

Determination of total phenolic content. The total phenolic content was determined by Folin-Ciocalteu assay, as previously described (18). Water solution (1 ml) containing 5 mg of ECJL or standard was mixed with 1 ml of 2% sodium carbonate solution and 1 ml of 10% Folin-Ciocalteu's phenol reagent. After 10 min, the absorbance was measured at 750 nm using a microplate reader (PerkinElmer, Inc.). The measurement was compared to calibration curve of gallic acid. The results were expressed as milligrams of gallic acid equivalents per gram of sample, as previously described (17).

Determination of XO inhibitory activity in vitro. The XO inhibitory activity was measured by monitoring uric acid formation in a XO system, as previously described (19). The assay system consisted of 0.6 ml phosphate buffer (100 mM; pH 7.4), 0.1 ml sample, 0.1 ml XO (0.2 U/ml) and 0.2 ml xanthine (1 mM; dissolved in 0.1 N NaOH). The reaction was initiated by adding the enzyme with or without inhibitors, and the change in absorbance of the mixture at 290 nm for 15 min was measured against a reagent blank. A 0.2 ml aliquot of 1 N HCl was used to terminate the enzymatic reaction. Allopurinol was used as a positive control.

Establishment of the mouse model of hyperuricemia and drug administration. Six groups of mice (n=5 for each group) were treated once daily for 7 days as follows: the mice in the 2 negative control groups (NOR and HU groups) received 0.3% CMC-Na aqueous solution; the mice in the positive control group (ALLO group) received allopurinol solution at a dose of 10 mg/kg; the mice in the ECJL 30, ECJL 100, and ECJL 300 groups received the ECJL solution at doses of 30, 100 and 300 mg/kg, respectively. Hyperuricemia was induced in the mice by potassium oxonate, a uricase inhibitor (20). Briefly, ECJL (30, 100 and 300 mg/kg) or allopurinol (10 mg/kg) were dissolved in 0.3% CMC-Na aqueous solution. The resultant drug solutions were orally administered once per day for 7 days. Food, except water, was withdrawn from the mice at 1.5 h prior to drug administration, and the mice were intraperitoneally injected with potassium oxonate (300 mg/kg) 1 h before the final drug administration on the 7th day in order to induce hyperuricemia. Blood samples were collected via the tail vein of the mice at 1 h after the final drug administration on the 7th day. The blood samples were allowed to clot for approximately 1 h at room temperature and then centrifuged at 10,000 x g for 15 min to obtain serum. The serum samples were stored at -80˚C until use. The serum uric acid concentration was measured using standard diagnostic kits (Abcam, Cambridge, UK). Each assay was performed in triplicate.

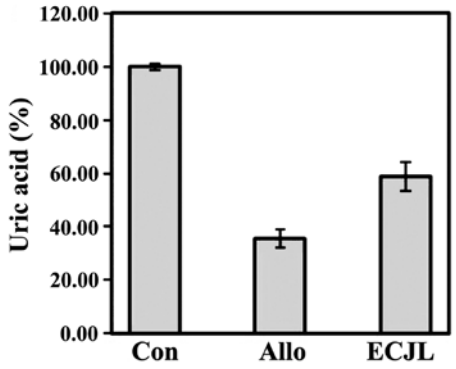

Figure 1. XO inhibitory activity of ECJL *in vitro*. Inhibitory effects by 30 μg/ml allopurinol, 2 mg/ml ECJL. Each value is represented as the mean ± SD of 3 separate experiments in triplicate measurements. XO, xanthine oxidase; ECJL, extracts of *C. japonica* leaf.

Table III. Antioxidant activities of ECJL.

	DPPH IC_{50} (μg/ml)	Total phenol (mg/g eq. gallic acid)	Reducing power (ascorbic acid eq. μg/50 μg ECJL)
ECJL	38.53±0.72	46.6±1.6	13.34±1.7
Ascorbic acid	6.95±0.3		

ECJL, extracts of *C. japonica* leaf.

Determination of XO inhibitory activity in vivo. The residual activity of XO in the mouse liver and plasma were spectrophotometrically determined by monitoring uric acid formation from xanthine (12). The mouse livers (0.5 g) were homogenized in an 1 ml aliquot of 50 mM sodium phosphate buffer (pH 7.4). The homogenates were centrifuged at 3,000 x g for 10 min at 4˚C. After removing the lipid layer, the supernatant was centrifuged at 10,000 x g for 60 min at 4˚C. The resultant supernatant was then used for determining XO residual activity and the total protein concentration. A 0.12 ml aliquot of xanthine solution (250 mM) was added to a test tube containing 10 μl liver homogenate and 0.54 ml potassium oxonate solution (1 mM) in 50-mM sodium phosphate buffer (pH 7.4) that were previously incubated at 35˚C for 15 min. The reaction was termined after 0 and 30 min by the addition of a 0.1 ml aliquot of 0.6 M HCl. Thereafter, the test tube was centrifuged at 3,000 x g for 5 min, and the absorbance of the supernatant was measured at 295 nm using a UV/VIS spectrophotometer (Biochrom Libra S12; serial no. 116997; Biochrom Ltd., Cambridge, UK). The total protein concentration was determined spectrophotometrically by the Bradford method (21). XO activity was expressed as micromoles of uric acid formed per minute (U) per milligram protein.

Statistical analysis. A value of P<0.05 was considered to indicate a statistically significant difference and was determined using a t-test between the two means for the unpaired data or an ANOVA (post hoc test: Tukey's multiple range test) among the 3 or more means for the unpaired data. All data were expressed as the means ± standard deviation and rounded to 2 decimal places.

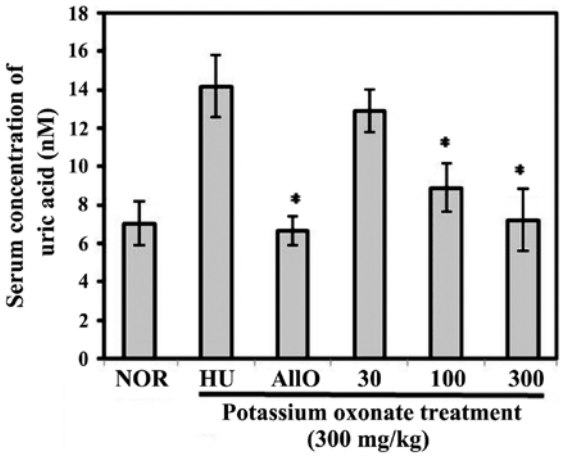

Figure 2. Serum uric acid levels following the oral administration of saline in normal mice (NOR) or following the oral administration of saline (HU), allopurinol at a dose of 10 mg/kg (ALLO) or ECJL at a dose of 30 mg/kg (30), 100 mg/kg (100) or 300 mg/kg (300) in mice with potassium oxonate-induced hyperuricemia over a period of 7 days. The rectangular bars and their error bars represent the means ± SD, respectively (n=5). *Significantly different from other groups (p<0.05, ANOVA *a posteriori* Tukey's multiple range test). ECJL, extracts of *C. japonica* leaf.

Results

Antioxidant activity and total phenolic contents of ECJL. The antioxidant potential of ECJL was determined by DPPH and reducing power assays. Plants have been reported to be useful sources of phytochemicals, such as phenolics, and various health benefits, such as antioxidant activity have been suggested (22). In addition, the various antioxidant activities of natural resources significantly correlate with their major compound contents, such as polyphenols. Therefore, in this study, we determined the total phenolic contents of the extract obtained from ECJL.

The DPPH scavenging effect is a widely used method to evaluate the free radical scavenging ability of plant extracts. Table III shows the measured DPPH radical scavenging activity. A low IC_{50} value indicates a strong antioxidant activity in ECJL. The IC_{50} value of the extract was 38.53±0.72 μg/ml.

Fe^{3+} was transformed into Fe^{2+} in the presence of extracts to measure the reductive capability. At 50 μg/ml of the extract, the value, which is expressed in vitamin C equivalents, of the reducing power of vitamin C was 13.34±1.7 μg/50 μg ECJL.

Total phenol compounds, as determined by the Folin-Ciocalteu method, as previously described (17), are reported as gallic acid equivalents by reference to a standard curve (r^2=0.999). Table III shows the total phenolic content of the extract. The amount of the phenolic compounds in the extract was 46.6±1.6 mg/g eq. gallic acid.

XO inhibitory activity of ECJL in vitro. Fig. 1 shows the XO inhibitory activity of ECJL. XO inhibitory activity was expressed as the suppression rate of uric acid production. Allopurinol (as a positive control) at a concentration of 30 μg/ml significantly inhibited XO activity by 64.6±3.4%. Notably, the dose-dependent XO inhibitory activity of ECJL significantly increased. ECJL at a concentration of 2 mg/ml inhibited XO activity by 41.3±5.5%.

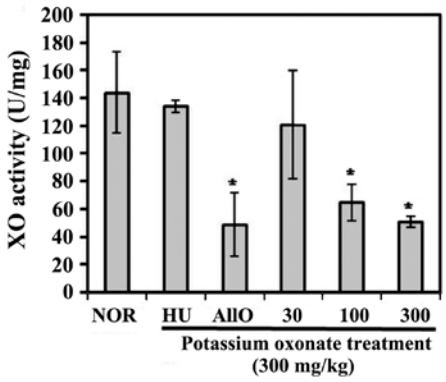

Figure 3. Hepatic XO activity following the oral administration of saline in normal mice (NOR) or following the oral administration of saline (HU), allopurinol at a dose of 10 mg/kg (ALLO) or ECJL at a dose of 30 mg/kg (30), 100 mg/kg (100) or 300 mg/kg (300) in mice with potassium oxonate-induced hyperuricemia over a peiod of 7 days. The rectangular bars and their error bars represent the means ± SD, respectively (n=5). *Significantly different from the HU group ($p<0.05$, ANOVA *a posteriori* Tukey's multiple range test). XO, xanthine oxidase; ECJL, extracts of *C. japonica* leaf.

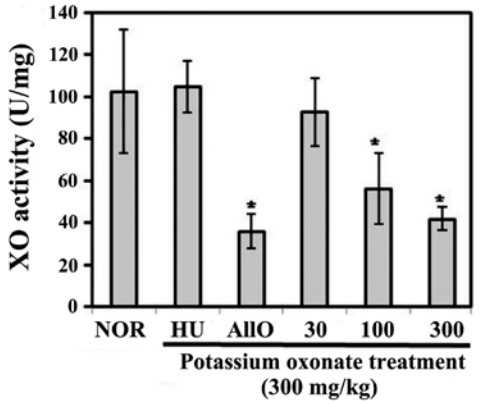

Figure 4. Serum XO activity following the oral administration of saline in normal mice (NOR) and after oral administration of saline (HU), allopurinol at a dose of 10 mg/kg (ALLO) or ECJL at a dose of 30 mg/kg (30), 100 mg/kg (100) or 300 mg/kg (300) in mice with potassium oxonate-induced hyperuricemia over a period of 7 days. The rectangular bars and their error bars represent the means ± SD, respectively (n=5). *Significantly different from the HU group ($p<0.05$, ANOVA *a posteriori* Tukey's multiple range test). XO, xanthine oxidase; ECJL, extracts of *C. japonica* leaf.

Anti-hyperuricemic effects of the extract in vivo: serum uric acid levels. Fig. 2 shows the effects of ECJL on the serum uric acid levels in mice with potassium oxonate-induced hyperuricemia. The concentrations were significantly higher in the mice with hyperuricemia compared to the normal mice, indicating that the mouse model of hyperuricemia was successfully established at 1 h after the intraperitoneal injection of potassium oxonate, a urate oxidase inhibitor, which is consistent with the findings of a previous study (20). The serum uric acid concentrations in the normal mice were comparable with those in the hyperuricemic mice that were administered allopurinol or the 3 different doses of ECJL (30, 100 and 300 mg/kg over a period of 7 days.

In our *in vivo* model, the level of serum uric acid was effectively increased by potassium oxonate at a dose of 300 mg/kg via intraperitoneal injection (14.19±1.62 μM). The serum levels of uric acid in the allopurinol-treated group and ECJL treatment groups (30, 100, 300 mg/kg) were 6.65±0.74, 12.89±1.13, 8.89±1.17 and 7.23±1.63 μM, respectively. Allopurinol at a dose of 10 mg/kg suppressed the serum uric acid levels by 53% and ECJL at a dose of 300 mg significantly suppressed the uric acid levels by 49.1%, similar to allopurinol.

Anti-hyperuricemic effects in vivo: hepatic and serum XO activity. Figs. 3 and 4 show the effects of ECJL on the hepatic and serum XO activity in the mice with potassium oxonate-induced hyperuricemia. Pre-treatment with allopurinol for 1 week (oral administration) and ECJL at the doses of 100 and 300 mg/kg significantly reduced hepatic XO activity by 66.1, 55.2 and 64.7%, respectively (Fig. 3). Similarly, the serum XO activity in the mice pre-treated orally with allopurinol and ECJL at the doses of 100 and 300 mg/kg was reduced by 65, 45.2 and 59.3%, respectively.

However, there were no significant differences in hepatic and plasma XO activity between the normal and hyperuricemic control groups. Taken together, our findings demonstrated that ECJL at the dose of 300 mg/kg exerted XO inhibitory activity similar to that of allopurinol at a dose of 10 mg/kg.

XO inhibitory activity of the main components identified from ECJL. In the present study, we identified active compounds related to XO inhibitory activity from *C. japonica* leaf using GC-MS and liquid chromatography analysis. We identified one XO inhibitor, namely rutin (5.87%) using liquid chromatography. We identified 8 active compounds, which were vitamin E (25.35%) n-eicosane (10.2%), neophytadiene (0.91%), all trans-squalene (3.32%), n-octacosane (2.65%), 6,9-pentadecadien-1-ol (1.51%), α-linolenic acid (1.41%), and n-hexadecanoic acid (0.61%) using GC-MS.

Figs. 5 and 6 show typical GC-MS and HPLC chromatograms, respectively, which show the phytochemical constituents. After clarifying the active substances, we expected that the potent XO inhibitory activity of ECJL was due to the synergism of antioxidant and XO inhibitory substances.

Discussion

In our preliminary experiments, we screened 500 plant extracts, and selected ECJL as a candidate for an XO inhibitor. We fractionated the extract using solvent extraction and found that the ethanol fraction (ECJL) exhibited the most prominent XO inhibitory activity in water/ethanol extraction. The ethanol fraction exhibited strong DPPH radical scavenging activity and reducing power (unpublished data). Based on these results, the ethanol fraction was selected for further *in vitro* and *in vivo* experiments.

Our results revealed that total phenolics were enriched in ECJL. Phenolics have been shown to possess antioxidant and anti-inflammatory activities (23).

A number of phenolics have been shown to possess antioxidant and XO inhibitory activity, and to have the ability to decrease uric acid levels in serum. Thus, the solvent extraction condition was optimized with respect to the antioxidant and XO inhibitory activity (unpublished data). Table III shows that ECJL contained phenolic compounds (46.6±1.6 mg/g eq. gallic acid).

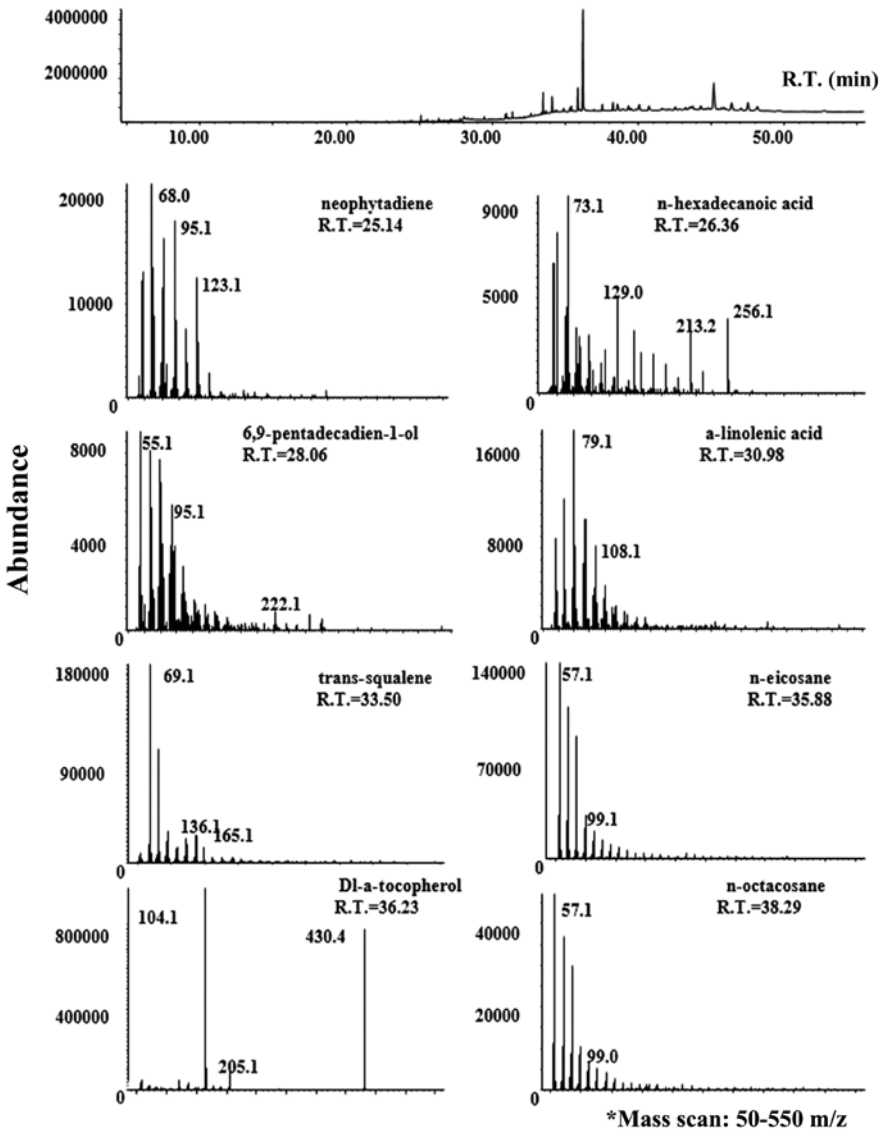

Figure 5. Representative GC-MS chromatogram showing the bioactive constituent profiles of ECJL. GC-MS, gas chromatography-mass spectrometry; ECJL, extracts of *C. japonica* leaf.

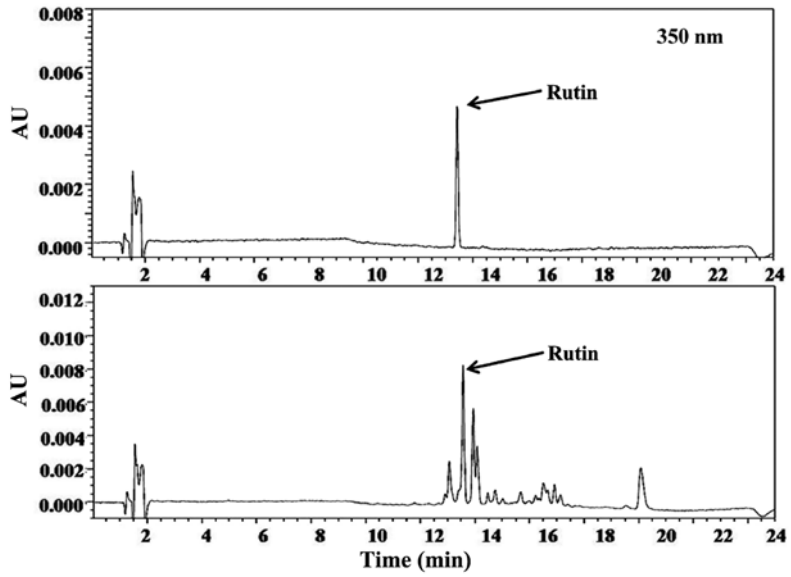

Figure 6. Representative HPLC chromatogram showing the bioactive constituent profiles of ECJL (RUT: rutin). HPLC high-performance liquid chromatography; ECJL, extracts of *C. japonica* leaf.

Hyperuricemia is an abnormal condition which involves high levels of uric acid, and the major factor is known to be the overproduction of uric acid by XO (24). In general, the extract that has antioxidant activity is closely related to XO inhibitory activity and the recovery of oxidative damage. The breakdown of purine metabolism is known to be responsible for hyperuricemia. Hyperuricemia is mainly caused by decreased renal uric acid excretion or excessive hepatic uric acid generation (25). XO can catalyze the oxidation of hypoxanthine to uric acid, primarily in the liver (26). Gout is a representative disease that is closely related to hyperuricemia. Excessive levels of serum uric acid are the main risk factors for uric acid crystal deposition in joints and kidneys, resulting in uric acid nephrolithiasis and gouty arthritis (27). Thus, the serum uric acid level and hepatic XO activity were evaluated to examiney the anti-hyperuricemic effects of ECJL that was prepared in this study.

Figs. 2-4 show that ECJL at doses of 100 and 300 mg/kg significantly reduced the serum uric acid levels and inhibited the hepatic and serum XO activity. These results clearly indicate that the oral administration of ECJL markedly attenuates the hyperuricemic state in mice. In particular, there were no significant differences in hepatic and serum XO activity between the normal and hyperuricemic control groups. This result suggests that intraperitoneal pre-treatment with potassium oxonate, a known uricase inhibitor, may not affect XO activity in mice. Thus, ECJL controls hyperuricemia via the inhibition of XO in the liver and serum in potassium oxonatetreated mice.

The mechanisms of action of ECJL could be explained through analytical study of the active constituents in ECJL. Fig. 5 shows that we identified the important bioactive markers related to antioxidant activity and hyperuricemi, such as vitamin E (25.35%), neophytadiene (0.91%), all trans-squalene (3.32%), α-linolenic acid (1.41%), n-eicosane (10.2%), n-octacosane (2.65%), 6,9-pentadecadien-1-ol (1.51%), and n-hexadecanoic acid (0.61%) using GC-MS.

Vitamin E was the major compound in ECJL. Catignani *et al* found that the increase in XO activity in the liver was due to the level of vitamin E and involved the accumulation of the enzyme protein in vitamin E-deficient rabbits (28). Mohd Fahami *et al* reported that palm vitamin E also reduced XO acitivity in gastric regions (29). Thus, vitamin E may be the first key compound as a strong antioxidant agent and XO regulator in ECJL.

Squalene has been reported to possess antioxidant properties. Huang *et al* reported that squalene is a highly effective oxygen-scavenging agent (30).

Among the identified fatty acids, linolenic acid was analyzed. Park *et al* reported that linolenic acid downregulated inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression and thereby reduced nitric oxide (NO) and prostaglandin E2 production in lipopolysaccharide (LPS)-activated RAW264.7 cells. These findings indicate the potential therapeutic use of linolenic acid as an anti-inflammatory agent (31). Linolenic acid is not closely related to hyperuricemia. Besides, linolenic acid affects secondary inflammatory damage, such as uric acid nephrolithiasis and gouty arthritis.

Fig. 6 shows that we identified rutin as an XO inhibitor using bioassay-guided liquid chromatography. The content of rutin in ECJL was determined to be 5.87% (w/w). A previous study reported that rutin at the dose of 50-100 mg/kg significantly decreased the biomarkers of serum urate, creatinine and blood urea nitrogen and serum, and kidney uromodulin levels in hyperuricemic mice (32). Azuma *et al* reported that 5 flavonoids were purified from the buthanol extract of *C. japonica* leaf and identified them as quercetin, kaempferol, apigenin, rutin and quercetrin (33). Previous studies have reported that quercetin inhibits XO activity in a competitive manner, while apigenin inhibits it in a mixed manner (20,34). As shown in a previous sudy, rutin, when administered orally to mice with oxonate-induced hyperuricemia, was able to draw out dose-dependent hypouricemic effects by exerting significant inhibitory effects on xanthine dehydrogenase/xanthine oxidase (XDH/XO) activities (35). In the present study, we identified rutin by liquid chromatography. Considering previous reports of active markers and purification techniques, further studies are warranted for the isolation and identification of active compounds from ECJL.

Allopurinol is commonly used to treat and control hyperuricemia. However, its use can cause side-effects to patients, and these are reported as the main cause for the termination of anti-hyperuricemic therapy. This necessitates an alternative herbal medicine that is therapeutically effective for hyperuricemia and gout (12). In the present study, we aimed to evaluate the anti-hyperuricemic activities of ECJL, and to determine the optimal dosage in experimental models *in vivo* to predict oral administration in humans. ECJL was effective in reducing uric acid levels and inhibiting liver/serum XO activity, which indicates that ECJL may exert its anti-hyperuricemic effects mainly through this mechanism. ECJL reduced uric acid levels at two evaluated doses, at 100-300 mg/kg, significantly inhibiting liver and serum XO activity.

In general, we considered the oral intake of dried *Camellia japonica* leaf of 10 g daily for the treatment of gout. Due to the yield of extract being 14.17%, the oral dose for a human weighing 60 kg is 1,417 mg/day (23.61 mg/kg/day). The conversion factor between humans and mice is 12.33 (36). Therefore, if the effective dose for mice is 300 mg/kg/day, the human equivalent dose is 1,459.85 mg/60 kg/day as ECJL, or 10.3 g/60 kg/day as dried *Camellia japonica* leaf. In the present study, mice with hyporuricemia were treated with 30, 100 and 300 mg/kg ECJL and the effective oral dose was drawn in the range of 100-300 mg/kg. We concluded that the oral intake of 3-10 g of *C. japonica* leaf is beneficial to prevent and/or decrease the possibility of the occurrence of hyperuricemia related disease.

Taken together, we found beneficial effects of ECJL from the results of biological evaluation through antioxidant assay and XO assay *in vitro* and *in vivo*. We also identified the active compounds that contribute to the antioxidant, anti-inflammatory, and XO inhibitory properties of ECJL from the results of GC-MS and HPLC analysis. In the future, we aim to determine whether ECJL has beneficial effect on gouty arthritis.

In conclusion, in the present study, *C. japonica* leaf from Korea was selected based on the possibility of its development for plant medicine and/or functional food materials as an anti-hyperuricemic agent. Our study revealed that the ethanol extract of *C. japonica* leaf was a positive sample which exerts a potent XO inhibitory effect similar to that of allopurinol. This study is a significant contribution to the knowledge of bioactive markers from the *C. japonica* leaf as potential sources for the medical industry, and presents data to explain the effects of ECJL in the treatment of hyperuricemia.

References

1. Yoshikawa M, Murakami T, Yoshizumi S, Murakami N, Yamahara J and Matsuda H: Bioactive saponins and glycosides. V. Acylated polyhydroxyolean-12-ene triterpene oligoglycosides, camelliasaponins A1, A2, B1, B2, C1, and C2, from the seeds of *Camellia japonica* L.: Structures and inhibitory activity on alcohol absorption. Chem Pharm Bull (Tokyo) 44: 1899-1907, 1996.
2. Yoshikawa M, Morikawa T, Asao Y, Fujiwara E, Nakamura S and Matsuda H: Medicinal flowers. XV. The structures of noroleanane- and oleanane-type triterpene oligoglycosides with gastroprotective and platelet aggregation activities from flower buds of *Camellia japonica*. Chem Pharm Bull (Tokyo) 55: 606-612, 2007.
3. Heo J: Dongui Bogam. Naeuiwon edition, Bubinmunhwasa publisher, Seoul, Korea, 1613.
4. Cha SS, Lee KE, Lee SH, Choi MJ and Shim JK: Decomposition and nutrient dynamics of leaf litter of *Camellia japonica* L. in Korea. Korean J Environ Ecol 30: 110-117, 2016 (In Korean).
5. Hwang EJ, Cha YJ, Park MH, Lee JW and Lee SY: Cytotoxicity and chemosensitizing effect of Camellia (*Camellia japonica*) tea extracts. J Korean Soc Food Sci Nutr 33: 487-493, 2004 (In Korean).
6. Yang JL, Ha TK, Dhodary B, Pyo E, Nguyen NH, Cho H, Kim E and Oh WK: Oleanane triterpenes from the flowers of *Camellia japonica* inhibit porcine epidemic diarrhea virus (PEDV) replication. J Med Chem 58: 1268-1280, 2015.
7. Onodera K, Hanashiro K and Yasumoto T: Camellianoside, a novel antioxidant glycoside from the leaves of *Camellia japonica*. Biosci Biotechnol Biochem 70: 1995-1998, 2006.
8. Thao NT, Hung TM, Lee MK, Kim JC, Min BS and Bae K: Triterpenoids from *Camellia japonica* and their cytotoxic activity. Chem Pharm Bull (Tokyo) 58: 121-124, 2010.
9. Nagata T, Tsushida T, Hamaya E, Enoki N, Manabe S and Nishino C: Camellidins, antifungal saponins isolated from *Camellia japonica*. Agric Biol Chem 49: 1181-1186, 1985.
10. Park JC, Hur JM, Park JG, Hatano T, Yoshida T, Miyashiro H, Min BS and Hattori M: Inhibitory effects of Korean medicinal plants and camelliatannin H from *Camellia japonica* on human immunodeficiency virus type 1 protease. Phytother Res 16: 422-426, 2002.
11. Zhao M, Zhu D, Sun-Waterhouse D, Su G, Lin L, Wang X and Dong Y: In vitro and in vivo studies on adlay-derived seed extracts: Phenolic profiles, antioxidant activities, serum uric acid suppression, and xanthine oxidase inhibitory effects. J Agric Food Chem 62: 7771-7778, 2014.
12. Lemos Lima Rde C, Ferrari FC, de Souza MR, de Sá Pereira BM, de Paula CA and Saúde-Guimarães DA: Effects of extracts of leaves from *Sparattosperma leucanthum* on hyperuricemia and gouty arthritis. J Ethnopharmacol 161: 194-199, 2015.
13. Chu YH, Chen CJ, Wu SH and Hsieh JF: Inhibition of xanthine oxidase by *Rhodiola crenulata* extracts and their phytochemicals. J Agric Food Chem 62: 3742-3749, 2014.
14. Kramer HM and Curhan G: The association between gout and nephrolithiasis: The National Health and Nutrition Examination Survey III, 1988-1994. Am J Kidney Dis 40: 37-42, 2002.
15. Choi HJ, Lee CH, Lee JH, Yoon BY, Kim HA, Suh CH, Choi ST, Song JS, Joo HY, Choi SJ, *et al*: Current gout treatment and flare in South Korea: Prophylactic duration associated with fewer gout flares. Int J Rheum Dis: Aug 27, 2014 (Epub ahead of print).
16. Bae MS, Shin JS, Lee KY, Lee KH and Kim YJ: Long-range transport of biomass burning emissions based on organic molecular markers and carbonaceous thermal distribution. Sci Total Environ 466-467: 56-66, 2014.
17. Kim YH, Cho ML, Kim DB, Shin GH, Lee JH, Lee JS, Park SO, Lee SJ, Shin HM and Lee OH: The antioxidant activity and their major antioxidant compounds from *Acanthopanax senticosus* and *A. koreanum*. Molecules 20: 13281-13295, 2015.
18. Seo JH, Kim JE, Shim JH, Yoon G, Bang MA, Bae CS, Lee KJ, Park DH and Cho SS: HPLC analysis, optimization of extraction conditions and biological evaluation of *Corylopsis coreana* Uyeki flos. Molecules 21: 94, 2016.
19. Arimboor R, Rangan M, Aravind SG and Arumughan C: Tetrahydroamentoflavone (THA) from *Semecarpus anacardium* as a potent inhibitor of xanthine oxidase. J Ethnopharmacol 133: 1117-1120, 2011.
20. Huo LN, Wang W, Zhang CY, Shi HB, Liu Y, Liu XH, Guo BH, Zhao DM and Gao H: Bioassay-guided isolation and identification of xanthine oxidase inhibitory constituents from the leaves of *Perilla frutescens*. Molecules 20: 17848-17859, 2015.
21. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254, 1976.
22. Perron NR and Brumaghim JL: A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem Biophys 53: 75-100, 2009.
23. Liu LM, Cheng SF, Shieh PC, Lee JC, Chen JJ, Ho CT, Kuo SC, Kuo DH, Huang LJ and Way TD: The methanol extract of *Euonymus laxiflorus*, *Rubia lanceolata* and *Gardenia jasminoides* inhibits xanthine oxidase and reduce serum uric acid level in rats. Food Chem Toxicol 70: 179-184, 2014.
24. Wu XH, Yu CH, Zhang CF, Anderson S and Zhang YW: *Smilax riparia* reduces hyperuricemia in mice as a potential treatment of gout. Am J Chin Med 42: 257-259, 2014.
25. Gurwitz JH, Kalish SC, Bohn RL, Glynn RJ, Monane M, Mogun H and Avorn J: Thiazide diuretics and the initiation of anti-gout therapy. J Clin Epidemiol 50: 953-959, 1997.
26. Ardan T, Kovaceva J and Cejková J: Comparative histochemical and immunohistochemical study on xanthine oxidoreductase/xanthine oxidase in mammalian corneal epithelium. Acta Histochem 106: 69-75, 2004.
27. Grassi D, Ferri L, Desideri G, Di Giosia P, Cheli P, Del Pinto R, Properzi G and Ferri C: Chronic hyperuricemia, uric acid deposit and cardiovascular risk. Curr Pharm Des 19: 2432-2438, 2013.
28. Catignani GL, Chytil F and Darby WJ: Vitamin E deficiency: Immunochemical evidence for increased accumulation of liver xanthine oxidase. Proc Natl Acad Sci USA 71: 1966-1968, 1974.
29. Mohd Fahami NA, Ibrahim IA, Kamisah Y and Mohd Ismail N: Palm vitamin E reduces catecholamines, xanthine oxidase activity and gastric lesions in rats exposed to water-immersion restraint stress. BMC Gastroenterol 12: 54, 2012.
30. Huang ZR, Lin YK and Fang JY: Biological and pharmacological activities of squalene and related compounds: Potential uses in cosmetic dermatology. Molecules 14: 540-554, 2009.
31. Park SY, Seetharaman R, Ko MJ, Kim DY, Kim TH, Yoon MK, Kwak JH, Lee SJ, Bae YS and Choi YW: Ethyl linoleate from garlic attenuates lipopolysaccharide-induced pro-inflammatory cytokine production by inducing heme oxygenase-1 in RAW264.7 cells. Int Immunopharmacol 19: 253-261, 2014.
32. Chen YS, Hu QH, Zhang X, Zhu Q and Kong LD: Beneficial effect of rutin on oxonate-induced hyperuricemia and renal dysfunction in mice. Pharmacology 92: 75-83, 2013.
33. Azuma CM, dos Santos FCS and Lago JHG: Flavonoids and fatty acids of *Camellia japonica* leaves extract. Rev Bras Farmacogn 21: 1159-1162, 2011.
34. Pauff JM and Hille R: Inhibition studies of bovine xanthine oxidase by luteolin, silibinin, quercetin, and curcumin. J Nat Prod 72: 725-731, 2009.
35. Zhu JX, Wang Y, Kong LD, Yang C and Zhang X: Effects of Biota orientalis extract and its flavonoid constituents, quercetin and rutin on serum uric acid levels in oxonate-induced mice and xanthine dehydrogenase and xanthine oxidase activities in mouse liver. J Ethnopharmacol 93: 133-140, 2004.
36. Shin JW, Seol IC and Son CG: Interpretation of animal dose and human equivalent dose for drug development. J Korean Orient Med 31: 1-7, 2010.