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ORIGINAL ARTICLE

Pharmacological Mechanisms of the Water Leaves Extract of *Lysiphyllum strychnifolium* for its Anti-Inflammatory and Anti-Hyperuricemic Actions for Gout Treatment

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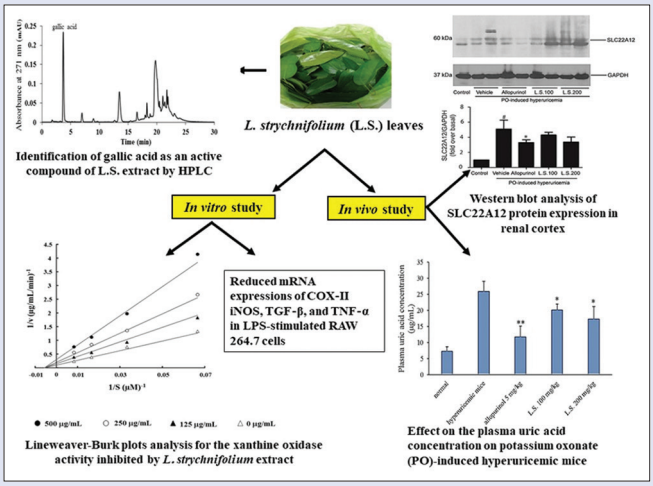
ABSTRACT

Background: There have been anecdotal reports from Thai hyperuricemic patients that the leaves of *Lysiphyllum strychnifolium* could reduce plasma uric acid level and relieve inflammation of gout. However, no research to support these effects has been conducted. **Objectives:** This study was aimed to evaluate the anti-inflammatory and hypouricemic effects of *L. strychnifolium* leaves extract and to investigate the pharmacological mechanisms of these effects. **Materials and Methods:** The anti-inflammatory effect of *L. strychnifolium* was evaluated in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Effects of *L. strychnifolium* on xanthine oxidase (XO) were examined *in vitro* and *in vivo* using potassium oxonate (PO)-induced hyperuricemic mice. In addition, the antioxidant activity of *L. strychnifolium* was determined. **Results:** *L. strychnifolium* significantly reduced the mRNA expression of cyclooxygenase-II, inducible nitric oxide synthase, transforming growth factor-β, and tumor necrosis factor-α in LPS-stimulated RAW 264.7 cells ($P < 0.05$). It exhibited a noncompetitive inhibition of XO activity with IC_{50} and *Ki* of 231 μg/ml and 177 μg/ml, respectively. Oral administration of *L. strychnifolium* (100 and 200 mg/kg) significantly lowered the plasma uric concentration ($P < 0.05$) in PO-induced hyperuricemic mice and inhibited 56.9% and 66.3% of the hepatic XO activity, respectively, compared to control hyperuricemic mice ($P < 0.05$). *L. strychnifolium* did not significantly decrease the protein expression of solute carrier family 22 member 12 in the renal cortex. Total phenolic and flavonoid contents were determined to be 197.8 ± 5.8 mg gallic acid equivalence/g extract and 32.2 ± 1.2 mg quercetin equivalent/g extract, respectively. The IC_{50} of the inhibition of 2,2-diphenyl-1-picrylhydrazyl radical was 44.32 μg/ml. **Conclusion:** The present study first provided scientific evidence for the anti-inflammatory, anti-hyperuricemic and antioxidant effects of *L. strychnifolium* leaves extract *in vitro* and *in vivo*, suggesting the possibility of this plant to treat gout.

Key words: Anti-inflammatory, antioxidant, *Lysiphyllum strychnifolium*, RAW 264.7 cells, xanthine oxidase

SUMMARY

- *Lysiphyllum strychnifolium* leaves contain gallic acid as the active compound
- *Lysiphyllum strychnifolium* significantly reduced the mRNA expression of inflammatory mediators, i.e., cyclooxygenase-II, inducible nitric oxide synthase, transforming growth factor-β, and tumor necrosis factor-α in lipopolysaccharide-stimulated RAW 264.7 cells
- *Lysiphyllum strychnifolium* exhibited an anti-hyperuricemic effect *via* mechanisms of inhibitory effect on hepatic xanthine oxidase in a non-competitive manner.



Abbreviations used: ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); COX-II: Cyclooxygenase-II; DMEM: Dulbecco's modified Eagle medium; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; FBS: Fetal bovine serum; FRAP: Ferric reducing antioxidant power; GAE: Gallic acid equivalence; GLUT9: Glucose transporter 9; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PO: Potassium oxonate; P/S: Penicillin/streptomycin; QE: Quercetin equivalent; SLC22A12: Solute carrier family 22 member 12; TNF-α: Tumor necrosis factor-α; TGF-β: Transforming growth factor-β; URAT: Urate-anion transporter 1; XO: Xanthine oxidase.

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INTRODUCTION

Hyperuricemia is a condition pertaining to supersaturated extracellular urate caused by dysfunction of purine metabolism and/or disorder of uric acid excretion. Consequent deposition of urate crystals in the

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joints and tissues can induce an inflammatory response. The treatment of hyperuricemia, therefore, focuses on decreasing the uric acid level and suppressing inflammation.[1] Current anti-hyperuricemic agents employed in clinical settings are xanthine oxidase (XO) inhibitors, such as allopurinol and febuxostat, as well as uricosuric agents. Recently, it has been found that urate reabsorption at the proximal tubules plays a key role in the renal urate excretion.[2] Multiple lines of evidence indicated that some urate transporters in the proximal tubule, such as solute carrier family 22 member 12 (SLC22A12) or urate-anion transporter 1 [URAT1] and SLC2A9 or glucose transporter 9 [GLUT9], mediate the renal urate reabsorption.[1] Uricosuric agents such as probenecid and benzbromarone act on the urate transports and inhibit the urate reabsorption at the renal proximal tubules. Colchicine has been used to treat acute inflammation in gout. However, these drugs are commonly associated with adverse effects, such as fever, skin rashes, allergic reactions, hepatitis, and nephropathy, which may limit their clinical uses.[1] Recent studies have hypothesized that the production of uric acid catalyzed by XO generates free radicals which might adversely affect mitochondrial function and ATP production, leading to cardiovascular diseases.[2] Therefore, alternative medicines which exhibit urate-lowering, anti-inflammation, and antioxidant effects have been extensively explored in recent studies,[2,3] and most such candidates are natural products which have already been used as a folk remedy.

Lysiphyllum strychnifolium is classified as a family of *Leguminosae* and has been known as "Khayan" or "Ya Nang Dang" in North East of Thailand. Since it was formerly classified in the genus *Bauhinia*, the synonym of *Bauhinia strychnifolium* has been also used.[4,5] In Thai traditional medicine, the stems and roots of *L. strychnifolium* have been used to treat cancer, fever, and allergy.[4] Boiling of its leaves and stems with water has been used to detoxify heavy metals, pesticides, and alcohol. Nowadays, dried stems or leaves of *L. strychnifolium* are distributed as commercial products in the form of tea or dried powder for infusion.

Several bioactive compounds including trilobatin, quercetin, 3,5,6,3',5'-pentahydroxy-flavanonol-3-O-α-L-rhamnopyranoside, 3,5,7-trihydroxy-chromoone-3-O-α-L-rhamnopyranoside, β-sitosterol, stigma sterol and gallic acid have been observed in *L. strychnifolium*.[6] Previous studies revealed that the extract of *L. strychnifolium* stems exhibited anti-cancer activity against cancer cell lines and possessed a greater antioxidant activity than that of green tea.[7,8] Recently, the hypouricemic and anti-inflammatory properties have been claimed by Thai hyperuricemic patients who often drink fresh *L. strychnifolium* leaves boiled in water. However, experimental evidence for the anti-hyperuricemic and anti-inflammatory activities of *L. strychnifolium* have been lacking so far.

Therefore, the present study was conducted to evaluate the hypouricemic effect of *L. strychnifolium* leaves extract by determining the inhibitory activity on XO *in vitro* and by measuring the uric acid-lowering activity using potassium oxonate (PO)-induced hyperuricemic mice *in vivo*. Moreover, the effect of *L. strychnifolium* leaves extract on the protein expression of SLC22A12 in the kidney of hyperuricemic mice was evaluated to explore the anti-hyperuricemic effect on renal transporters. The antioxidant effect of *L. strychnifolium* leaves extract on free radicals was also assessed. Furthermore, the mRNA expression of inflammatory markers, i.e., cyclooxygenase-II (COX-II), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) was quantitated in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Chemicals

Allopurinol, acetonitrile, gallic acid, Folin-Ciocalteu reagent, carboxymethylcellulose-sodium (CMC-Na), quercetin, Trolox, sodium acetate trihydrate, sodium octane sulfonate, uric acid, XO enzyme from bovine milk, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), PO were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Aluminum chloride, ascorbic acid, potassium persulfate, and sodium bicarbonate were obtained from Ajax Finechem, Australia. All other chemical reagents were of analytical grade and used without further purification.

Plant material

L. strychnifolium leaves were harvested from Mahasarakham Province, Thailand, in February - April 2016. They were identified by Assist. Prof. Wanida Caichompoo, Faculty of Pharmacy, Mahasarakham University, Thailand, with the voucher number monosodium urate (MSU). PH-LEG-BS01 and its reference specimen have been deposited at the herbarium of the institute.

L. strychnifolium leaves were collected, cleaned, dried at 50°C for 48 h, and cut into small pieces. Water extraction was carried out by an infusion method. Dried leaves (100 g) were extracted in boiling water (1 L) for 15 min. The pooled extract was filtered through a Whatman filter paper (No. 1), and then concentrated using a freeze dryer (Labconco, Kansas, MO, USA). The residue was lyophilized into powder and stored in a sealed container protected from light at −20°C until used for assays.

Determination of gallic acid content in *Lysiphyllum strychnifolium* extract by a high-performance liquid chromatography

Gallic acid was used as a chemical marker of *L. strychnifolium* leaves extract. The content of gallic acid in the extract was determined using a previously reported high-performance liquid chromatography (HPLC) method with some modifications.[9] Methanol solution containing known concentrations of gallic acid (1.56–50 μg/ml) were prepared and used for calibration. *L. strychnifolium* (100 mg) leaves were mixed with 20 ml of 12% hydrochloric acid for 30 min under reflux on a water bath. The mixture was extracted by 25 ml of diethyl ether 3 times, evaporated to dryness, reconstituted in 5 ml of methanol, and filtered through a 0.45 μm nylon membrane. The obtained filtrate was injected into the HPLC in triplicate.

The HPLC system consisted of a system controller SCL-10AVP, a detector ultraviolet (UV)-visible SPD-10A, a dual piston solvent delivery pump LC-10AD and an auto-injector SIL-10A (Shimadzu, Kyoto, Japan). The analytical column used was a BDS Hypersil C18 column (150 mm × 4.6 mm, i.d. 5 μm) (Thermo Fisher Scientific, Waltham, MA, USA), connected with a BDS Hypersil C18 guard column (10 mm × 4 mm, i.d. 5 μm) (Thermo Fisher Scientific). The mobile phase was a mixture of 0.05% phosphoric acid (solvent A) and methanol (solvent B). The gradient elution, with the total run-time of 30 min, was set as follows: 10% B for 10 min, 10%–30% B (3 min), 30%–60% B (5 min), 60%–20% B (3 min), 20%–10% B (3 min) and 10% B (6 min). The flow rate was 1.0 ml/min, UV detection at 271 nm, and the volume of injection 20 μl.

Anti-inflammatory effect of *Lysiphyllum strychnifolium* extract in lipopolysaccharide-stimulated macrophages

Cell culture

RAW 264.7 macrophage cells were provided by Dr. Primchanien Moongkarndi, Faculty of Pharmacy, Mahidol University in Thailand, and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1.0% penicillin/

streptomycin (P/S), and incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C. After RAW 264.7 cells were grown to 80% confluent condition, the cells were passaged by the treatment with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution to maintain cells exponential growth stage.

Cell viability

Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described.[10] RAW 264.7 cells were cultured in 96-well plate at a starting density of 1×10^4 cells/well and incubated overnight. The RAW 264.7 cells were divided into two groups, as follows: (1) Control group, which was exposed to vehicle only (distilled water containing 0.2% dimethyl sulfoxide [DMSO]); (2) treated group, which was treated for 24 h with various concentrations of *L. strychnifolium* (0.01–500 μg/ml in distilled water containing 0.2% DMSO). After the incubation, the culture medium was replaced with 100 μl of MTT solution (1 mg/ml dissolved in DMEM) and the RAW 264.7 cells were incubated for an additional 4 h. The medium was removed by aspiration, and 100 μl DMSO was added to dissolve the insoluble formazan crystals. Finally, the sample was thoroughly mixed, and the absorbance was measured at 570 nm using a microplate reader (Infinite M200, Tecan, Switzerland). All experiments were performed in triplicate, and the results were expressed as percentage of viable cells compared with control cells.

mRNA expression of inflammatory genes

RAW 264.7 cells were seeded at a density 1×10^6 cells/well in DMEM supplemented with 10% FBS and 1% P/S, and maintained in a humidified 37°C, 5% CO_2 incubator for 24 h, as previously described.[11] Then, the culture medium was replaced with DMEM supplemented with 1% FBS and 1% P/S. The RAW 264.7 cells were divided into four groups, as follows: (1) Normal cells not stimulated with LPS; (2) Cells stimulated with 1 μg/ml LPS for 12 h; (3) Cells treated with *L. strychnifolium* extract (1 μg/ml) for 4 h, followed by stimulation with 1 μg/ml LPS for 12 h; (4) Cells treated with 0.1 μM dexamethasone (as positive control) for 4 h, followed by stimulation with 1 μg/ml LPS for 12 h.

The total RNA from RAW 264.7 cells was extracted using the Gene JET RNA Purification Kit (Thermo Fisher Scientific). The expression levels of COX-II, iNOS, TGF-β, and TNF-α were determined by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) using one-step RT-qPCR kits (KAPA Biosystems, Wilmington, MA, USA) according to the manufacturer's protocol. The gene-specific primers for RT-qPCR (mouse) were designed as follows [Table 1]. RT-qPCR was performed under the following conditions: Reverse transcription at 42°C for 5 min; RT inactivation and DNA polymerase activation at 95°C for 2-5 min; combined annealing, extension and data acquisition at 95°C for 3 s and at 55°C for 30 s (40 cycles); last extension step at 72°C for 1 min followed by at 25°C for 2 min. The expression of targeted genes was normalized to GAPDH and expressed as fold over the nontreated group.

Effect of *Lysiphyllum strychnifolium* extract on xanthine oxidase activity *in vitro*

Effect of *L. strychnifolium* extract on XO activity was assayed *in vitro* according to a previous study.[16,17] *L. strychnifolium* extract was dissolved in DMSO (0.2% in the reaction mixture) and diluted with distilled water to obtain the final concentrations of 25–500 μg/ml. Then, 20 μL of freshly-prepared XO enzyme (0.1 U) dissolved in 0.1 M pyrophosphate (pH 7.4) was gently mixed with of 1 ml of *L. strychnifolium* extract, buffer (as a negative control) or allopurinol (0.0625–5 μg/ml, as a positive control). Subsequently, the reaction mixture was incubated for 10 min at 25°C. Then, 2 ml of 120 μM xanthine (as a substrate) dissolved in 0.1 M pyrophosphate buffer (pH 7.4) was added to initiate the enzymatic reaction and incubated at 25°C for 10 min. The reaction was terminated by adding 1 ml of 1 N HCl. The concentration of uric acid generated in the reaction mixture was determined by HPLC, as described later.

Table 1: The gene specific primers for quantitative reverse transcriptase polymerase chain reaction (mouse)

Gene specific primer	Sequences	Reference
GAPDH		
Sense	5′-GCCTGCTTCACCACCTTC-3′	[12]
Antisense	5′-GGCTCTCCAGAACATCATCC-3′	
COX-2		
Sense	5′-TGCATGTGGCTGTGGATGTCATCAA-3′	[13]
Antisense	5′-CACTAAGACAGACCCGTCATCTCCA-3′	
iNOS		
Sense	5′-GTGTTCCACCAGGAGATGTTG-3′	[13]
Antisense	5′-CTCCTGCCCACTGAGTTCGTC-3′	
TGF-β1		
Sense	5′-TGGAGCAACATGTGGAACTC-3′	[14]
Antisense	5′-TGCCGTACAACTCCAGTGAC-3′	
TNF-α		
Sense	5′-TACTGAACTTCGGGGTGATTGGTCC-3′	[15]
Antisense	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	

COX-2: Cyclooxygenase-2; iNOS: Inducible nitric oxide synthase, TGF-β: Transforming growth factor-β; TNF-α: Tumor necrosis factor-α

Percent inhibition of the XO-mediated uric acid formation by *L. strychnifolium* extract or allopurinol was calculated using the following equation:

$$\text{Inhibitory activity of XOD}(\%) = \left(1 - \frac{UA_{sample}}{UA_{control}}\right) \times 100$$

Where "$UA_{control}$" is the concentration of uric acid in the negative control, and "UA_{sample}" is the concentration of uric acid in the sample or in the positive control. The IC_{50}, defined as the concentration required inhibiting XO activity by 50%, was calculated by a linear regression analysis of the percentage of the inhibitory activity-log concentration curve.

The Lineweaver-Burk plot analysis was performed to assess the type of XO inhibition by *L. strychnifolium*. XO activity was measured with increasing concentrations of xanthine (15, 30, 60, and 120 μM) in the absence or presence of *L. strychnifolium* leaves extract (125, 250, and 500 μg/ml), as previously described.[16] The inhibitory constant (*K*i) for the XO inhibition by *L. strychnifolium*, as well as Michaelis constant (*K*m) for the substrate xanthine, were determined by a nonlinear least-squares regression of an enzyme-kinetic equation to the observed data using Solver Add-in equipped with Microsoft Excel 2010. For the nonlinear optimization, the generalized reduced gradient algorithm was employed.

Effects of *Lysiphyllum strychnifolium* extract on plasma uric acid level in potassium oxonate-induced hyperuricemic mice

Forty male ICR-Swiss mice (30–40 g, 6-week-old) were supplied by National Laboratory Animal Center (Mahidol University, Thailand) and housed at the animal center of Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, at a constant temperature (23°C ± 2°C) with a 12 h light-dark cycle, and had free access to standard diet and water *ad libitum*. Mice were acclimatized for 7 days before the experiment. The animal care and experimental protocol were approved by the Committee on the Ethics of Animal Experiments of Faculty of Pharmacy, Mahidol University (Permission Number: PYT 008/2559).

Mice were randomly divided into five groups. Group 1 was not induced to be hyperuricemic and administered with water only (control mice). Group 2, 3, 4, and 5 were induced to be hyperuricemia with intraperitoneal (*i.p.*) injection of 300 mg/kg PO dissolved in normal saline. These groups were administered with water, allopurinol (5 mg/kg) and *L. strychnifolium* leaves extract dissolved in water (100 and 200 mg/kg), respectively.[17] The treatment was consecutively given to each mouse for 7 days. On the last day, after *i.p.* injection with PO, xanthine dissolved in 0.5% CMC-Na was orally administered to increase uric acid levels in the blood. After 2 h, each mouse was sacrificed with CO_2-inhalation, and whole blood was collected into a heparinized tube by heart puncture. After blood was centrifuged at 3000 g for 10 min, plasma was obtained and stored at −20°C for further determination of uric acid concentrations by HPLC.

After whole blood was collected, mice livers were immediately excised and washed three times in ice-cold normal saline solution. Then, they were homogenized in 3 ml of ice-cold 80 mM pyrophosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min at 4°C. After the upper layer was removed, the remaining part was further centrifuged at 10,000 g for 60 min at 4°C. The obtained supernatant was kept in −20°C until the determination of XO activity *ex vivo*, as described below. Simultaneously, the kidney cortex tissues were rapidly and carefully separated on an ice-plate and stored in liquid nitrogen for the further Western blot analysis.

Effect of *Lysiphyllum strychnifolium* extract on residual activity of hepatic xanthine oxidase activity *ex vivo*

XO activity in the excised liver was determined by measuring the production rate of uric acid from xanthine.[16,17] A portion (100 µl) of the liver homogenate and 900 µl of 80 mM sodium pyrophosphate buffer pH 7.4 were mixed and incubated at 25°C for 10 min. Then, 500 µl of 120 µM xanthine solution was added, mixed and incubated for 10 min, respectively. The reaction was terminated after 0 and 10 min by adding 100 µl of 1 N HCl. Total protein concentration in the liver homogenate was determined according to Bradford test kit (Thermo Scientific Fisher). The liver XO enzyme activity was expressed as µmol/min/mg protein.

Protein expression of Solute carrier family 22 member 12 in the kidney cortex

The protein expression of SLC22A12 was evaluated by Western blotting as previously described.[17,18] Renal cortex was isolated and homogenized in NP-40 lysis buffer containing 20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 20% glycerol, 100 µM phenylmethylsulfonyl fluoride, 5 µg/ml aprotinin and 5 µg/ml leupeptin. After centrifugation, protein concentration of renal cortex homogenates was assayed using a Bio-Rad protein assay kit with bovine serum albumin as a standard. Samples were mixed with sodium dodecyl sulfate (SDS) loading buffer and warmed at 90°C for 5 min before resolution by SDS-polyacrylamide gel electrophoresis (PAGE) gels. Separated proteins were transferred from SDS-PAGE gels to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). Before incubating with the primary antibody, the membrane was incubated with 5% skim milk for 1 h to block nonspecific binding sites. Then, the membrane was incubated with the antibodies for SLC22A12 (1:1000 dilution) or GAPDH (1:2000 dilution) overnight. The expression of GAPDH was served as a loading control. Proteins were visualized with horseradish peroxidase-conjugated secondary antibodies and a chemiluminescence detection system (GE Healthcare, Piscataway, NJ).

Quantification of uric acid concentrations by high-performance liquid chromatography

To determine the uric acid content in plasma collected from mice *in vivo*, a plasma sample (100 µl) was mixed with acetonitrile (100 µl), centrifuged at 3000 g for 5 min at 4°C, and the supernatant was then filtered through a membrane filter (0.45 µm), and the resultant filtrate (20 µl) was injected into the HPLC system as described below, to determine the uric acid concentration.[16] A calibration curve of uric acid in plasma was obtained using blank plasma spiked with known concentrations of uric acid ranging 5–50 µg/ml, after subtracting the intrinsic uric acid content.

To determine the uric acid content in the reaction mixtures *in vitro* and those generated from liver homogenates *ex vivo*, the reaction mixtures were centrifuged 3000 g for 10 min and filtered through a membrane (0.22 µm). The uric acid concentrations were measured by HPLC.

Uric acid concentrations in the above samples were determined by a reversed-phase HPLC with TSkgel ODS-80Ts column (150 mm × 4.6 mm, 5 µm; Tosoh Bioscience, Tokyo, Japan) on an HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a quaternary pump LC (LC-20AT) with degasser (DGU-20A3), UV-visible detector (SPD-20A), and communication module (CBM-20A). The analytical column was maintained at 25°C.[17] The HPLC isocratic mobile phase consisted of acetonitrile and 1 mM octane sulfonic acid in 7 mM K_2HPO_4 at pH 3.0 (2.5: 97.5) with the flow rate of 1.0 ml/min. The sample (20 ml) was injected into the HPLC system, and the absorbance was recorded at 292 nm.

Determination of total phenolic and flavonoid contents and antioxidant activity in *Lysiphyllum strychnifolium* extract

Total phenolic content assay

Total phenolic contents in the *L. strychnifolium* leaves extract were performed using a rapid microplate Folin-Ciocalteu method.[19] Gallic acid was dissolved in deionized water in the range of 50–790 ng/ml. Folin-Ciocalteu working solution was prepared by 10-fold dilution with deionized water. A portion (20 µl) of the sample solution (1 mg/ml) or Gallic acid solution (1 mg/ml) was mixed with 50 µl of Folin-Ciocalteu working solution, then Na_2CO_3 (80 µl) was added and mixed thoroughly. The reaction mixture was allowed to stand for 2 h at ambient temperature, and the absorbance at 765 nm was determined by UV spectrophotometry by a microplate reader (Infinite M200, Tecan, Switzerland). The phenolic content in the extract was expressed regarding mg gallic acid equivalent (GAE) per gram of extract (mg of GAE/g of extract).

Total flavonoid content assay

A portion (10 µL) of the extract dissolved in methanol (1 mg/ml) or quercetin (1 mg/ml), as a standard, was mixed with 2% (w/v) $AlCl_3$ solution (100 µl) and incubated in 96-well plate for 10 min at room temperature.[19] The absorbance at 415 nm was analyzed using the microplate reader. The flavonoid content in the extract was expressed as mg quercetin equivalent (QE) per gram of extract (mg of QE/g of extract).

2,2-Diphenyl-1-picrylhydrazyl hydrate radical scavenging activity assay

DPPH scavenging activity was analyzed as described in the previous study.[20] In a 96-well plate, 100 µl of extract solution (1 mg/ml) or ascorbic acid (as a positive control) was added with 100 µl of DPPH solution. The mixture was incubated at room temperature in the dark for 30 min. The absorbance was recorded at 517 nm using the microplate reader. The percentage of scavenging activity was calculated as follows:

$$\%\,\text{DPPH radical scavenging activity} = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100$$

Where OD_{blank} and OD_{sample} represent the absorbance of DPPH solution without and with the herbal extract, respectively. The IC_{50} was determined by the correlation between % DPPH radical scavenging activity and log-concentration.

2,2-Azinobis 3-ethyl-benzothiazoline-6-sulfonic acid assay

ABTS assay was performed as described in the previous study with some modification.[20] *L. strychnifolium* extract in methanol (1 mg/ml) was mixed with 200 µl of the ABTS solution in 96 well-plate in the dark. Then, the absorbance was measured at 734 nm using the microplate reader. Trolox was used for the standard curve. Results are expressed in mg Trolox equivalents antioxidant capacity/g extract.

Ferric reducing antioxidant power assay

Extract solution (500 µl) was mixed with 500 µl of potassium phosphate buffer (0.2 M, pH 6.6) and 500 µl of 1% (w/v) potassium ferricyanide solution.[20] The mixture was incubated at 50°C for 20 min, and the reaction was terminated by adding 2 ml of trichloroacetic acid. Then, 100 µl of supernatant was mixed with 100 µl of deionized water before the addition of 20 µl of 0.1% (w/v) ferric chloride solution. The reaction was incubated for 30 min in the dark before measuring the absorbance at 700 nm using the microplate reader. Ferrous sulfate was used as a standard. The absorbance was expressed in mmol ferrous sulfate equivalents/g extract.

Statistical analysis

Data were expressed as the mean ± standard error of the mean. Data were analyzed by one-way analysis of variance, followed by Dennett's *post-hoc* analysis using the statistical program SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Differences in the mean values were considered to be statistically significant at $P < 0.05$.

RESULTS

Determination of gallic acid content in *L. strychnifolium* extract by high-performance liquid chromatography

The obtained extract of *L. strychnifolium* leaves was dried powder with brownish, and the percent yield was 23.81%. The HPLC chromatogram of *L. strychnifolium* extract showed a prominent peak at the retention time of 3.83 ± 0.03 min, which corresponds to Gallic acid reference standard [Figure 1]. A highly significant correlation between added gallic acid (x) and chromatographic peak area (y) was represented by the equation: y = 48461x + 16967 (r^2 = 0.999). The content of Gallic acid in *L. strychnifolium* leaves extract was 2.56 ± 0.56%w/w.

Anti-inflammatory effect of *Lysiphyllum strychnifolium* extract in lipopolysaccharide-stimulated macrophages

Cell viability

After the treatment of RAW 264.7 macrophage cells with *L. strychnifolium* extract at the concentrations ranged over 0.01–500 µg/ml for 24 h, the percentage of viable cells were calculated and compared to the number of surviving cells in the control group (no treatment with vehicle only). As shown in Figure 2, *L. strychnifolium* did not affect the cell viability at the concentrations of 0.01 to 10 µg/ml. Based on this result, the concentration of 10 µg/ml of *L. strychnifolium* was selected for the subsequent experiments.

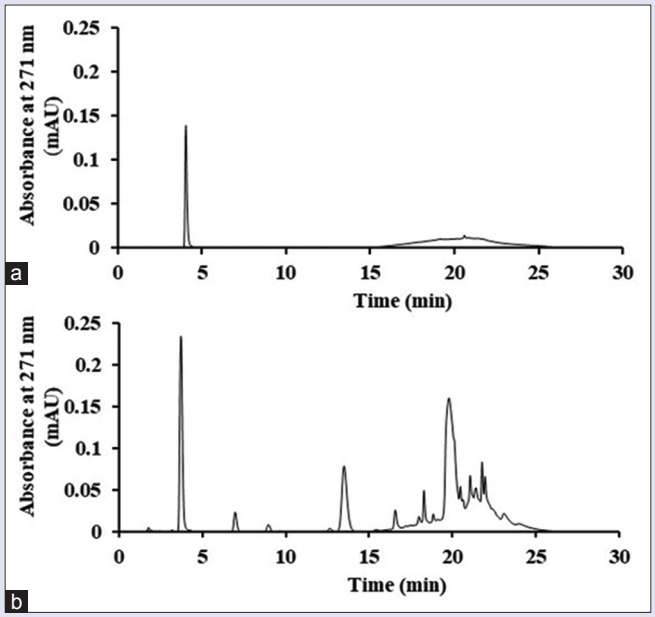

Figure 1: High-performance liquid chromatography chromatograms of *Lysiphyllum strychnifolium* extract (a) and standard gallic acid (b)

mRNA expression of inflammatory genes

As presented in Figure 3, the treatment of RAW 264.7 cells with LPS (1 µg/ml) significantly increased the COX-II, iNOS, TGF-β and TNF-α mRNA levels compared to the control group. Pretreatment with *L. strychnifolium* extract (1 µg/ml) significantly inhibited the LPS-induced mRNA expression of COX-II, iNOS, TGF-β, and TNF-α, which had similar effects as those of dexamethasone (0.1 µM).

Effect of *Lysiphyllum strychnifolium* extract on xanthine oxidase activity *in vitro*

L. strychnifolium inhibited the XO activity with IC_{50} of 231 µg/ml, while that of allopurinol (positive control) was 2.51 µg/ml. Lineweaver-Burk plot analysis presented in Figure 4 indicated that *L. strychnifolium* inhibited XO in a noncompetitive fashion *in vitro*. The observed data were thus fitted to the noncompetitive type equation for enzyme inhibition as follows:

$$v = \frac{V_{max} S}{(Km+S)\left(1+\frac{I}{Ki}\right)}$$

Where v and V_{max} represent the initial and maximum velocities (µmol/min) of uric acid formation, respectively, Km is the Michaelis constant (µg/ml) for xanthine substrate, Ki is the inhibition constant (µM) for *L. strychnifolium* extract, and S and I are the substrate concentration (µM) and inhibitor concentration (µg/ml), respectively. The Ki and Km values were calculated to be 246.71 µg/ml and 177.34 µg/ml, respectively.

Effect of *Lysiphyllum strychnifolium* extract on plasma uric acid level in potassium oxonate-induced hyperuricemic mice

As shown in Figure 5a, PO administration induced a significant elevation of plasma uric acid level as compared to control mice ($P < 0.01$). Allopurinol significantly attenuated the plasma uric acid concentration ($P < 0.01$). *L. strychnifolium* extract (100 and 200 mg/kg) significantly lowered

the plasma uric concentration ($P < 0.05$). Any toxicity on body weight, behaviors or visual inspection of organs was not noticeable.

Effect of *Lysiphyllum strychnifolium* extract on residual activity of hepatic xanthine oxidase activity *ex vivo*

A highly significant correlation between uric acid concentration in the liver homogenate (x) and chromatographic peak area (y) was represented by the equation: y = 65685x + 89477 ($r^2 = 0.998$). As shown in Figure 5b, an *i.p.* administration of PO significantly elevated the hepatic XO activity and accelerated the production rate of uric acid as compared to normal control mice ($P < 0.01$). An oral administration of allopurinol (5 mg/kg) significantly reduced the hepatic XO activity in hyperuricemic mice, resulting in the suppressed production of uric acid by approximately 88%. The treatment with *L. strychnifolium* extract at oral doses of 100 and 200 mg/kg significantly lowered the hepatic XO activity by approximately 56.9 and 66.3%, respectively, as compared to control hyperuricemic mice ($P < 0.05$).

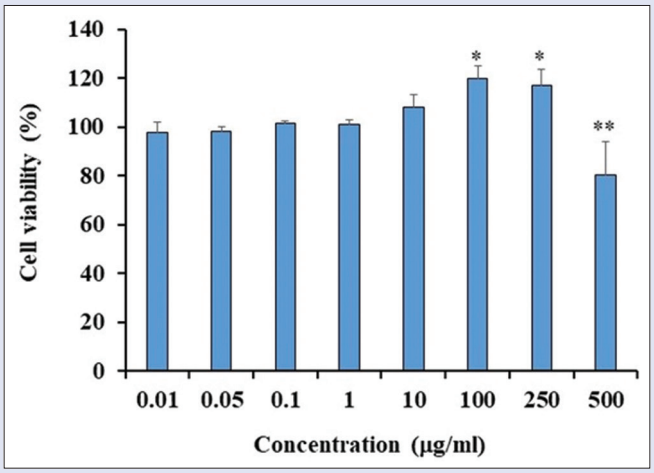

Figure 2: Effect of *Lysiphyllum strychnifolium* extract on the viability of RAW 264.7 cells. RAW 264.7 cells were treated with *Lysiphyllum strychnifolium*; L.S. (0.01 – 500 µg/ml) for 24 h. Cell viability was quantified, expressed as a percentage of cell viability, and shown as the mean ± standard error of the mean of four independent experiments * and ** represent $P < 0.05$ and $P < 0.01$ respectively, compared to control

Protein expression of solute carrier family 22 member 12 in the kidney cortex

The effect of *L. strychnifolium* on SLC22A12 protein expression in the kidney cortex was examined in PO-induced hyperuricemic mice. As shown in Figure 6, repeated *i.p.* administrations of PO (300 mg/kg) for 7 days caused a significant increase in the SLC22A12 protein expression compared to the control group, suggesting that PO induced SLC22A12 protein synthesis. Subsequent oral administration of allopurinol (5 mg/kg/day) resulted in a significant decrease in the SLC22A12 protein expression in the kidney cortex. However, repeated oral administrations of *L. strychnifolium* at doses of 100 and 200 mg/kg/day for 7 days did not decrease the SLC22A12 protein at a statistically significant level.

Determination of total phenolic and flavonoid contents and antioxidant activity in *Lysiphyllum strychnifolium* extract

Table 2 summarizes the total phenolic, flavonoid contents and antioxidant activity of *L. strychnifolium*, determined by previously described assays. The efficacy for the inhibition of DPPH radical determined by IC_{50}

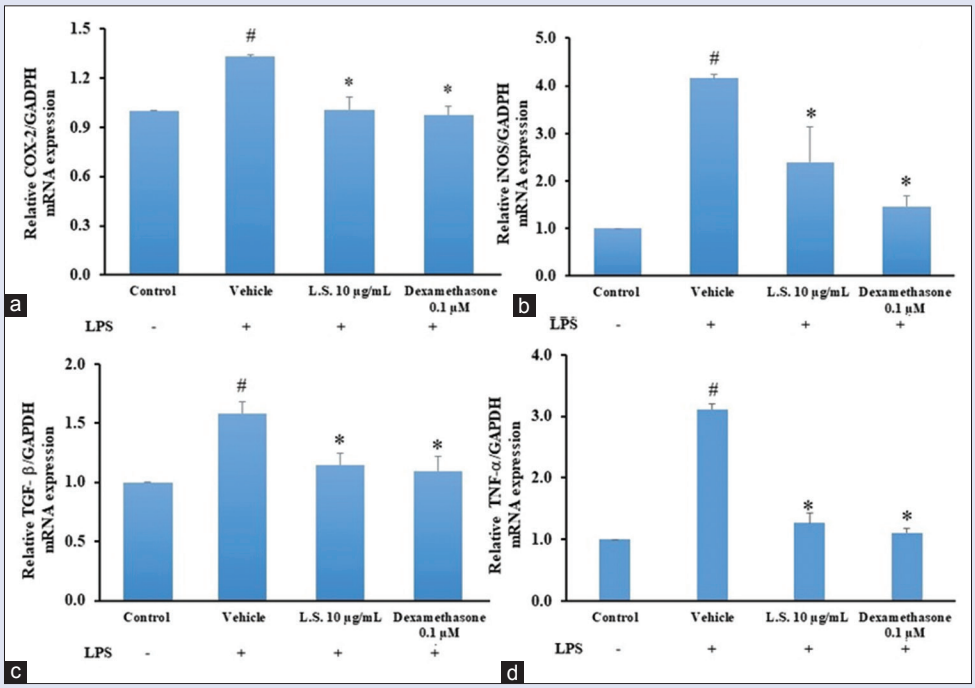

Figure 3: Effect of *Lysiphyllum strychnifolium* extract on the mRNA expression of lipopolysaccharide-induced inflammatory mediators in RAW 264.7 cells. RAW 264.7 cells were pre-treated with *Lysiphyllum strychnifolium* for 3–4 h before stimulated by 1 µg/ml lipopolysaccharide for 12 h. The total RNA was extracted from the cells and the mRNA expression of inflammatory mediators was analyzed using specific primers. GAPDH was served as a loading control. The relative cyclooxygenase-II (a), inducible nitric oxide synthase (b), transforming growth factor-β (c), and tumor necrosis factor-α (d) mRNA levels were quantified and expressed as fold increase over control group. Data are shown as the mean ± standard error of the mean of three independent experiments. #$P < 0.05$ compared to control; *$P < 0.05$ compared to lipopolysaccharide; L.S. represents *Lysiphyllum strychnifolium*

Table 2: Total phenolic and flavonoid contents and antioxidant activity of *L. strychnifolium*

Assays	Values
Content	
Total phenols	197.82±5.78 mg GAE/g extract[a]
Total flavonoids	32.22±1.23 mg QE/g extract[b]
Antioxidant activity	
DPPH (IC_{50})	44.32 µg/mL
ABTS	559.11±23.79 mg TEAC/g extract
FRAP	1.16±0.07 mmol $FeSO_4$/g extract

[a] and [b] represent represent the linear regression equations of y=0.0053x + 0.0146 (r^2=0.9984) and y=0.0101x + 0.029 (r^2=0.9935), respectively. GAE: Gallic acid equivalence; TEAC: Trolox equivalent antioxidant capacity; QE: Quercetin equivalent, IC_{50}: Inhibitory concentration at 50%; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; FRAP: Ferric reducing antioxidant power

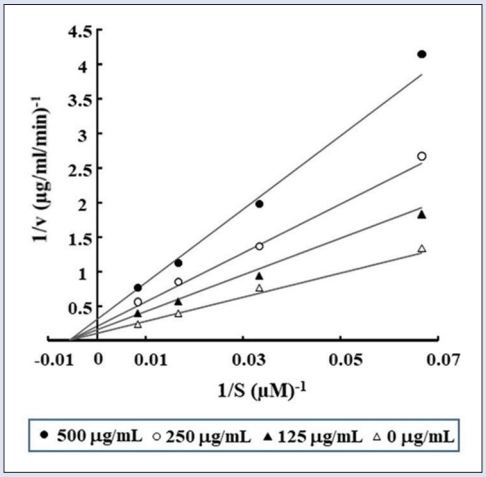

Figure 4: Lineweaver-Burk plots analysis for the xanthine oxidase activity inhibited by *Lysiphyllum strychnifolium* extract. The symbols △, ▲, ○, and ● represent *Lysiphyllum strychnifolium* concentrations of 0, 125, 250, and 500 µg/ml, respectively

values of *L. strychnifolium* was approximately 13% as compared to that of ascorbic acid (3.25 µg/ml).

DISCUSSION

To induce hyperuricemia in mice in this study, PO (300 mg/kg, *i.p.*) was administered for 7 days, followed by a treatment with xanthine solution (300 mg/kg, *p.o.*) on the last day (day 7). As a result, the plasma uric acid level was markedly increased by approximately five times as compared to the control group. The rate of uric acid production in the homogenated livers extracted from untreated PO-induced hyperuricemic mice was also significantly higher than that of the normal control mice, indicating that hyperuricemia was induced successfully. It is interesting to note that the expression of SLC22A12 protein in the kidney cortex was increased in this hyperuricemic animal model. Subsequently, oral administrations of *L. strychnifolium* (100 and 200 mg/kg) in the PO-induced hyperuricemic mice significantly lowered plasma uric concentration. The hypouricemic effect in the standard control, allopurinol, was also clearly observed. No toxicity was evident in mice treated with *L. strychnifolium* extract, which was consistent with a previous report that toxicity was not observed after repeated oral administrations of *L. strychnifolium* for 7 days in mice at the daily dose of 3000 mg/kg.[21]

To explore the underlying mechanisms of hypouricemic effect of *L. strychnifolium* extract, we evaluated the XO activity *in vitro* and *in vivo*. Our *in vitro* finding indicated that *L. strychnifolium* extract inhibited the enzyme activity of XO in a dose-dependent manner. A previous study[22] indicated that phenolic content of the plant was significantly correlated with the XO inhibitory activity. We found that the phenolic content of *L. strychnifolium* extract was 197.82 ± 5.78 mg GAE/g extract. Kinetic analysis with Lineweaver–Burk plots [Figure 4] revealed that the inhibition of XO activity by *L. strychnifolium* extract was a non-competitive type, which is often observed in natural products possessing XO inhibitory activity.[22,23] Due to high amounts of phenolic and flavonoid compounds (e.g., gallic acid) in the *L. strychnifolium* extract, its antioxidant activity against DPPH formation and free radical scavenging activity were demonstrated by ABTS and ferric reducing antioxidant power assays, respectively, in accordance with a

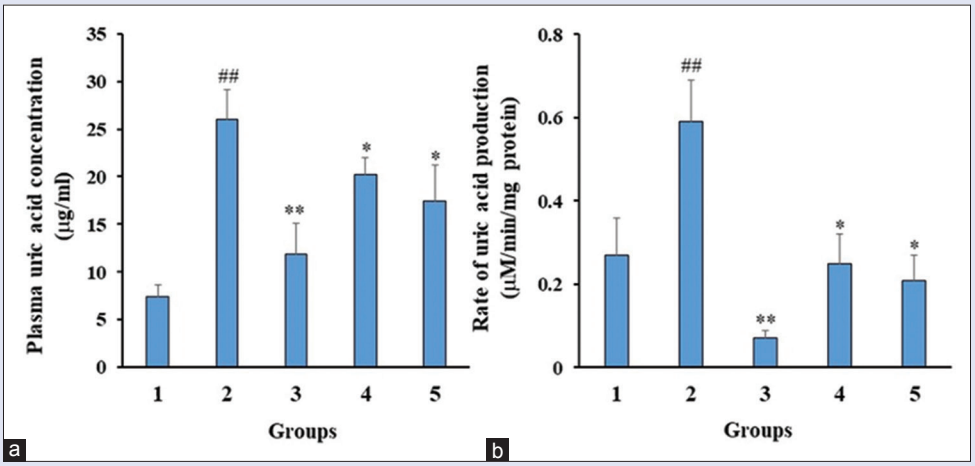

Figure 5: Effects of *Lysiphyllum strychnifolium* on the plasma uric acid concentration on potassium oxonate-induced hyperuricemic mice (a) and rate of uric acid production in homogenated mice livers (b). Group 1 represents normal control group; Group 2 means untreated-potassium oxonate induced hyperuricemic mice; Group 3 represents potassium oxonate-induced hyperuricemic mice treated with allopurinol (5 mg/kg); groups 4 and 5 represent potassium oxonate-induced hyperuricemic mice treated with 100 and 200 mg/kg of *Lysiphyllum strychnifolium*, respectively. Data are expressed as mean ± standard error of the mean (n = 6–8). ##represents $P < 0.01$, compared to normal control mice, respectively, compared to normal control mice. * and ** represent $P < 0.05$ and $P < 0.01$ respectively, compared to untreated potassium oxonate-induced hyperuricemic mice

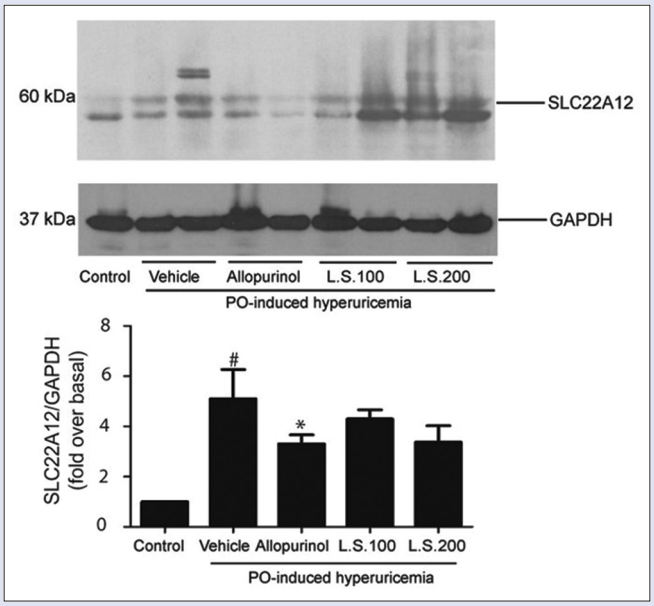

Figure 6: Western blot analysis of SLC22A12 protein expression in the renal cortex of potassium oxonate-induced hyperuricemic mice treated with vehicle, allopurinol (5 mg/kg), and *Lysiphyllum strychnifolium* (100 and 200 mg/kg) for 7 days. The protein expression was expressed as the fold increase over control group, and shown as the mean ± standard error of the mean (n = 4). $^{\#}P < 0.05$ compared to normal control mice and $^{*}P < 0.05$ compared with untreated potassium oxonate-induced hyperuricemic mice. L.S.100 and L.S.200 represent the treatments by 100 mg/kg and 200 mg/kg of *Lysiphyllum strychnifolium*, respectively

previous study.[8] Moreover, gallic acid has been reported to exhibit the XO inhibitory activity and anti-inflammatory effect in RAW 264.7 cells stimulated by LPS.[24,25] Previous studies suggested that flavonoids with the structure of planar flavones and flavonoids with a 7-hydroxyl group, i.e., quercetin and gallic acid, were able to inhibit the XO activity.[26,27] These results are consistent with our finding that the abundance of gallic acid, flavonoids and phenolic components in the water extract of *L. strychnifolium* may be responsible for the inhibitory effect of the XO activity.

LPS stimulates macrophages to secrete excess pro-inflammatory cytokines by binding to the toll-like receptor 4 and CD14 receptors complex on the cell surface.[28] Pro-inflammatory cytokines have a crucial role in the inflammatory reaction to MSU crystals in the hyperuricemia of gout.[29] Therefore, suppression of LPS-induced pro-inflammatory cytokines is a potential target to prevent inflammatory diseases including hyperuricemia-induced chronic inflammation in gout.

Among the pro-inflammatory cytokines, TNF-α and TGF-β are regarded as important mediators which can elicit an acute inflammatory process and cause tissue destruction.[28] COX-II causes an acute inflammation and pain in gout, and iNOS is a mediator for oxidation to be up-regulated in the inflammation process. We, therefore, selected TNF-α, TGF-β, COX-II, and iNOS as inflammatory markers to investigate the anti-inflammatory effect of *L. strychnifolium* extract. In the present study, we found that *L. strychnifolium* extract did not show cytotoxicity in RAW 264.7 macrophage cells up to 10 μg/ml. It markedly inhibited the mRNA expression of COX-II, TNF-α, iNOS, and TGF-β in LPS-induced RAW 264.7 macrophage cells and its inhibitory effect was comparable to that of dexamethasone. This is consistent with the present finding that *L. strychnifolium* contains gallic acid as a major bioactive constituent, which is known to exhibit anti-inflammatory effects.[25,27] Possible effects of *L. strychnifolium* on interleukin (IL)-1β, IL-1α and NOD-like receptors protein 3 (NLRP3) inflammasome complex should be further investigated, sinceecent studies have indicated that IL-1β, IL-1α, as well as NLRP3 inflammasome complex, play key roles in the regulation of pro-inflammatory cytokine in MSU crystal deposition in gout.[29] Therefore, possible effects of *L. strychnifolium* on IL-1β, IL-1α, and NLRP3 should be further investigated. Moreover, the inhibitory effect of *L. strychnifolium* on the production of various pro-inflammatory cytokines should be confirmed under *in vivo* conditions.

Several renal transporters have been identified to regulate the excretion and reabsorption of urate in renal tubules, and the inhibition of transporter-mediated urate reabsorption, (especially *via* SLC22A12 and SLC2A9) has been the novel target of anti-hyperuricemic treatment.[1,2] In the present study, allopurinol significantly suppressed the protein expression of SLC22A12 in the kidney cortex, which is consistent with a previous study.[30] *L. strychnifolium* extract also decreased the protein level of SLC22A12, but did not significantly suppress the protein expression of SLC22A12 [Figure 6], suggesting that the inhibition of renal reabsorption *via* this urate transporter may not be directly responsible for the hypouricemic effect of *L. strychnifolium*. From the *in vitro* and *in vivo* findings obtained in this study, it was considered that *L. strychnifolium* extract caused the reduction of plasma uric acid mainly by regulating the hepatic XO activity. However, the effect on other renal transporters (i.e., GLUT9 and OAT1/3) by *L. strychnifolium* should be further examined.

This is the first study that examined the anti-inflammation and anti-hyperuricemic activities of *L. strychnifolium* extract. The results obtained in this study may serve as scientific evidence to rationalize the clinical use of the plant extract as an alternative medicine for treating inflammatory reactions of gout, which is a newly affirmed therapeutic target of this plant. Since no apparent toxicity of *L.* strychnifolium extract was observed, it may be warranted to clinical use. Indeed, a further study to examine the clinical efficacy and safety of *L. strychnifolium* extract in hyperuricemic patients is needed. Moreover, since some patients may use *L. strychnifolium* extract during treatment with an anti-gout drug (typically allopurinol), the efficacy and safety of such co-administration cases should be investigated.

CONCLUSION

The present study demonstrated that the water leaves extract of *L. strychnifolium* inhibited the mRNA productions of COX-II, iNOS, TGF-β, and TNF-α in the LPS-induced RAW 267.4 macrophage cells. The plant extract exhibited a potent inhibitory effect on the XO activity in a non-competitive manner and demonstrated a significant anti-hyperuricemic effect in the experimental hyperuricemic model. Therefore, *L. strychnifolium* extract could potentially be used as an alternative medicine for the anti-inflammatory treatment of gout.

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Conflicts of interest

There are no conflicts of interest.

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