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In vitro and *in vivo* evidence of hypouricemic and anti-inflammatory activities of *Maclura cochinchinensis* (Lour.) Corner heartwood extract

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Maclura cochinchinensis (Lour.) Corner (MC) heartwood extracts have been used for the treatment of gout, hyperuricemia, and inflammation in Thai traditional medicine. Despite their traditional use, their mechanisms of action remain unknown. The aim of this study was to determine the mechanisms of MC heartwood extract activity using both *in vitro* and *in vivo* models**.** The extraction methods were optimized to yield the highest contents of biochemical compounds and antioxidant activities. The effects of MC heartwood extract on xanthine oxidase and its enzyme kinetics were determined *in vitro* and the antihyperuricemic effect was evaluated in potassium oxonate (PO)-induced hyperuricemic mice. The anti-inflammatory effect of MC heartwood extract was also tested against lipopolysaccharide-induced proinflammatory mRNA upregulation in RAW 264.7 mouse macrophage cells. Soxhlet extraction of MC heartwood with 70% ethanol produced stronger antioxidant activity, and higher total phenolic and flavonoid contents than conventional methods did (maceration or decoction). By using HPLC, we found that MC contains morin as a major constituent, which may account for its pharmacological activities. Moreover, administration of MC heartwood extract (500 mg/kg) markedly decreased uric acid levels in PO-induced hyperuricemic mice ($p < 0.05$). MC heartwood extract inhibited the hepatic activity of xanthine oxidase *ex vivo* by approximately 53%. In addition, MC heartwood extract markedly downregulated mRNA expression of inflammatory mediators (TNF-α, TGF-β, iNOS, and COX-2) and this inhibition was comparable with that of dexamethasone. Therefore, MC heartwood extract is a promising candidate as a natural treatment for inflammation and the hyperuricemia that causes gout.
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1. Introduction

Maclura cochinchinensis (Lour.) Corner, a scrambling branched or woody climber, belongs to the Moraceae family. It is also named *Cudrania cochinchinensis* and *Cudrania javanensis*, and is commonly called cockspur thorn or "Kae Lae" in Thai. It is widely distributed in many countries in Asia, such as Southern China, Japan, Korea, Taiwan, India, and Thailand.[1] The heartwood of *M. cochinchinensis* (MC) has been used in Thai traditional medicine as a tonic agent and to treat chronic fever, skin infections, diarrhea, and lymph node abnormalities.[2] MC exhibits various biological effects, such as antioxidant and antibacterial activities. The chloroform extract of MC heartwood was shown to exhibit antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and dermatophytes with minimum inhibitory concentration (MIC) values ranging from 125 to 250 μg/mL.[2] Furthermore, methanolic extracts of MC heartwood showed anti-herpes simplex virus (HSV) activity with a half-maximal effective concentration (EC_{50}) value of 50.8 μg/mL.[3,4] Moreover, MC heartwood is a component of the Thai herbal remedy given postnatally for seven days to strengthen the womb, which has been established in the National list of essential drugs of Thailand.[5] Several phytochemical constituents of MC heartwood have been reported, including morin, oxresveratrol, and hydroxyresveratrol.[6,7] Among them, morin is a major compound

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that possesses several biological properties such as antioxidant, anti-inflammatory, and antidiabetic activities.[7–9] In Thailand, some patients use MC heartwood extract as a traditional remedy for the treatment of diabetes, inflammation, and gout. However, mechanistic pharmacological evidence supporting its medical relevance has been limited to date.

Regarding the pathophysiology of uric acid, its maximum solubility in the blood is 6.4-6.8 mg/dL and hyperuricemia causes the deposition of urate crystals. Urate is reabsorbed and excreted in the renal proximal tubules, where several types of urate transporters such as urate/anion exchanger 1 (URAT1) and glucose transporter type 9 (GLUT9) are located and may be responsible for the renal handling of urate.[10] Hyperuricemia may occur because of decreased excretion or increased production of uric acid and recognized as the main risk factor for gout. The current treatment for hyperuricemia is based on the inhibition of xanthine oxidase (XOD), which is the target of the well-known drug for gout, allopurinol. However, severe adverse events, such as hypersensitivity reactions, have been a concern.[11] Furthermore, a recent study found that the deposition of monosodium urate (MSU) crystals in chronic hyperuricemia can induce an inflammatory reaction (*i.e.*, production of inflammatory related mediators such as interleukin-1 (IL-1)).[12] Lowering uric acid levels via XOD inhibition has been reported to correlate with attenuation of oxidative stress. Oxidative stress leads to endothelial dysfunction and contributes to the pathophysiology of diabetes, hypertension, arteriosclerosis, and chronic heart failure.[12,13] Thus, studies on natural products that exhibit hypouricemic, anti-inflammatory, and antioxidant effects are needed to provide essential data to develop alternative medicines for the treatment of gout.

Therefore, the objectives of this study were to determine the molecular mechanisms of MC heartwood extract with respect to its antioxidative, hypouricemic, and anti-inflammatory activities using *in vitro* and *in vivo* models. Moreover, enzyme kinetics of XOD were measured using Lineweaver-Burk plots to determine the type of enzyme inhibition by MC heartwood extract.

2. Materials and methods

2.1. Chemicals

Allopurinol, Acetonitrile, gallic acid, morin, Folin-Ciocalteu reagent, carboxymethylcellulose (CMC-Na), quercetin, trolox, sodium acetate trihydrate, sodium octane sulfonate, uric acid, xanthine oxidase enzyme from Bovine milk, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-S-triazine and potassium oxonate were purchased from Sigma Aldrich Chemical. (St. Louis, MO, USA). Aluminium chloride, ascorbic acid, potassium persulfate and sodium bicarbonate were obtained from Ajax Finechem, Australia. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were obtained from Gibco, USA. All other chemical reagents were of analytical grade and used without further purification.

2.2. Plant material

The heartwood of *M. cochinchinensis* (MC) were obtained from Amphoe Mueang, Nakhon Pathom Province by Assist. Prof. Dr. Boonyadist Vongsak, Faculty of Pharmaceutical Sciences, Burapha University in August 2016. These materials were identified by comparing with an authentic sample by Mr. Sarawit Phisutthinusart. The voucher specimens (SCFC-0816-01) have been deposited in Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Thailand. The dried heartwoods of *M. cochinchinensis* were cut into small pieces and ground into moderate to coarse powder, and then kept in dry place until extraction.

2.3. Extraction methods

2.3.1. Soxhlet extraction

The MC heartwood powder (20 g) was refluxed using a Soxhlet apparatus in three different solvents, namely 70% ethanol, ethyl acetate, and water at a ratio of 1:20 (MC:solvent) for 4 h/extraction. The solutions obtained were filtered through Whatman No. 1 filter paper, and then concentrated and dried at 45 °C using a rotary evaporator. The percentage yield was calculated. The MC heartwood extracts obtained were stored at −20 °C until further experimentation.

2.3.2. Maceration

The MC heartwood powder (20 g) was soaked in 70% ethanol or ethyl acetate at a ratio of 1:20 (MC: solvent) for 24 h. The marc was re-extracted three times with the same method. The solutions obtained were filtered through Whatman No. 1 filter paper, and then concentrated and dried at 45 °C using a rotary evaporator. The percentage yield was calculated. The MC heartwood extracts obtained were stored at −20 °C until further experimentation.

2.3.3. Decoction

The MC heartwood powder (20 g) was extracted by boiling thrice in water at a ratio of 1:20 (MC: solvent) for 15 min/extraction. The pooled extract was filtered through Whatman No. 1 filter paper, and then concentrated and dried at 45 °C using a rotary evaporator. The percentage yield was calculated. The MC heartwood extracts obtained were stored at −20 °C until further experimentation.

2.4. Phytochemical analysis

2.4.1. Quantitative analysis of the bioactive marker, morin, by HPLC

Morin was considered the major bioactive compound and its content was determined by HPLC. This HPLC analysis was performed according to a previous study with some modifications.[14] The HPLC system (Shimadzu, Japan) consisted of a quaternary HPLC pump (LC-10AD) with degasser (DGU-14A3), UV−Visible detector (SPD-10AV), autosampler (SIL-10AD), and system controller (SCL-10A). The separation was performed with a Thermo Fisher Scientific Hypersil™ BDS C_{18} column (4.6 × 100 mm i.d., 5 μm) with a C_{18} guard column. The elution used an isocratic solvent system of 0.5% acetic acid in water: acetonitrile (80:20). The flow rate was 1.0 mL/min with a controlled temperature of 25 °C. The UV−Vis detector was set at 355 nm and the injection volume was 5 μL for every sample and standard.

The morin standard solutions and MC heartwood extracts were prepared by dilution with methanol to a concentration of 1 mg/mL. The working standard solutions were obtained by appropriate dilution of the stock solution with methanol to concentrations of 7−900 μg/mL; these were filtered through 0.45 μm nylon syringe filters before injection and analysis. All samples were measured in triplicate.

2.5. Determination of antioxidant activity

2.5.1. DPPH free radical scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was freshly prepared in methanol at a final concentration of 152 μM. Each plant extract was diluted in methanol at varying concentrations. In a 96-well plate, 100 μL of each extract was added to each well followed by 100 μL of methanolic DPPH solution. Ascorbic acid was used as a standard and was treated under the same condition as the samples. The mixtures were allowed to stand at room temperature in the

dark for 30 min. The absorbance was recorded at 517 nm using a microplate reader (Tecan, Switzerland). Each extract was assayed in triplicate. The percentage of scavenging activity was calculated as follows[15]:

$$\% \text{ inhibition} = [(A_c - A_s)/A_c] \times 100 \quad (1)$$

Where A_c is the absorbance of the control solution at 517 nm and A_s is the absorbance of the sample solution at 517 nm.

2.5.2. Ferric reducing antioxidant power (FRAP) assay

Each sample (500 μL) was mixed with 500 μL of 0.2 M potassium phosphate buffer (pH 6.6) and 500 μL of 1% (w/v) potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. Then, 2 mL of trichloroacetic acid was added to stop the reaction. In a 96-well plate, 100 μL of the supernatant of the above mixture was added to 100 μL deionized water and 20 μL 0.1% (w/v) ferric chloride solution. The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at 700 nm using a microplate reader (Tecan). Ferrous sulfate was used for the standard curve. The activity was expressed as mmol ferrous sulfate equivalents/100 g extract.[16]

2.5.3. ABTS radical scavenging assay

The stock solutions included 7 mM ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and 2.45 mM potassium persulfate. The working solution was then prepared by mixing the two stock solutions in equal volumes and allowing them to react for 12–16 h in the dark at room temperature. The solution was then diluted by mixing 1 mL ABTS solution with 24 mL methanol to obtain an absorbance of 1.100 ± 0.020 units at 734 nm using a microplate reader (Tecan). Fresh $ABTS^{\bullet+}$ solution was prepared for each assay. Ten microliters of each sample was mixed with 200 μL $ABTS^{\bullet+}$ radical cation solution in 96-well plates. The absorbance was measured at 734 nm after 6 min in the microplate reader. All determinations were carried out at least three times and in triplicate. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used for the standard curve. The results are expressed in mg Trolox equivalent antioxidant capacity (TEAC)/g extract.[16]

2.5.4. Total phenolic content

Each sample (20 μL) was mixed with 50 μL of Folin-Ciocalteu reagent (diluted 1:10 in water) in a 96-well plate for 3 min. Eighty microliters of sodium carbonate (75% w/v of Na_2CO_3) was added, and then incubated for 2 h in the dark at 25 °C.[17] The absorbance was measured using a microplate reader (Tecan) at λmax = 765 nm. Each extract was assayed in triplicate. Gallic acid was used for the standard curve (the same procedure was repeated for the standard solutions). Total phenolic content in the extracts is expressed in terms of gallic acid equivalents (mg of GAE/g extract).

2.5.5. Total flavonoid content

One hundred microliters of sample solution (1 mg/mL in dimethyl sulfoxide (DMSO)) was mixed with 100 μL of 2% (w/v) aluminum chloride solution. The mixture was incubated for 10 min at room temperature. The absorbance was analyzed at 415 nm using a microplate reader (Tecan).[17] Each sample was measured in triplicate. Quercetin was used as the standard, following the same procedure as used with the samples. The content of flavonoid in the extract is expressed as mg quercetin equivalents (QE)/g extract.

2.6. Hypouricemic effect of MC heartwood extract

2.6.1. Effect of MC heartwood extract on XOD activity in vitro

The inhibition of XOD activity was analyzed based on the decrease in uric acid formation at 37 °C using an HPLC system at 295 nm. The test samples were dissolved in DMSO (final concentration of 0.1% (v/v)) to final concentrations that were appropriate for the tested dose. The reaction mixture was comprised of 0.1 M pyrophosphate (pH 7.4), 0.08 units of XOD enzyme, and test sample (10–100 μg/mL MC heartwood extract, allopurinol as a positive control, or buffer as a negative control). The reaction was started by adding 120 μM xanthine solution and incubating at 25 °C for 10 min. The reaction was then terminated by addition of 1 mL of 1 N HCl, and the concentrations of uric acid were determined by HPLC.[18] The inhibitory activity of MC heartwood extract on XOD was calculated as an IC_{50} value, determined by a linear regression analysis of the concentration-response curve (percent inhibition versus log-concentration). Each IC_{50} value is the mean of three experiments.

2.6.2. In vitro enzyme kinetic analysis of XOD inhibition by MC heartwood extract

The type of XOD inhibition by MC heartwood extract was determined by Lineweaver-Burk plot analysis. Three concentrations of MC heartwood extract (15, 30, and 45 μg/mL) were assessed with different concentrations of xanthine solution (15, 30, 60, and 120 μM). The XOD enzyme reaction was performed as described in section 2.6.1 above. The inhibitory constant (*Ki*) for XOD inhibition by MC heartwood extract was determined by a non-least squares regression of the observed data using Microsoft Excel 2010 equipped with Solver Add-in, using the following equation.[19]

$$v = \frac{V\max \cdot S}{Km + S\left(1 + \frac{I}{Ki}\right)} \quad (2)$$

where v and v_{max} represent the initial and maximum velocities of the uric acid formation, respectively (mmol/min), K_m represents the Michaelis constant, and *S* and *I* represent the substrate (μM) and inhibitor concentrations (μg/mL), respectively. For the non-linear optimization, the generalized reduced gradient (GRG) algorithm was employed.

2.6.3. In vivo effects of MC heartwood extract on plasma uric acid levels in potassium oxonate (PO)-induced hyperuricemic mice

Male ICR mice (30–40 g, 6 weeks old) were housed at the animal centre of the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, at a controlled temperature (23 ± 2 °C) with a 12 h light-dark cycle and access to standard diet and water *ad libitum*. Mice were acclimatized for 7 days prior to starting the experiment. The animals received care in accordance with the Guide for the Care and Use of Laboratory Animals, National Laboratory Animal Centre, Mahidol University. The animal care and experimental protocol were approved by the animal ethics committee of the Faculty of Pharmacy, Mahidol University (permission number PYT 008/2560).

The mice were randomly divided into 5 groups (6 mice per group). Group 1 was the normal control mice that had no treatment and were orally administered water. Groups 2 to 5 were made hyperuricemic by intraperitoneal (*i.p.*) injection with 300 mg/kg potassium oxonate (uricase inhibitor) freshly dissolved in normal saline. Fifteen minutes before injection of potassium oxonate, each group was orally administered the following: Group 2: water; Group 3: allopurinol (5 mg/kg); Groups 4 and 5: MC heartwood extract at doses of 200 and 500 mg/kg, respectively. Each treatment was given consecutively for 7 days. On the last day, 60 min after

injection with potassium oxonate, the mice were orally administered 300 mg/kg xanthine (as a XOD substrate) dispersed in 0.5% sodium carboxymethyl cellulose (CMC-Na). After 2h, the mice were euthanized by CO_2-inhalation and whole blood was collected via heart puncture. The blood sample was centrifuged at 3000 *g* for 10 min at 4 °C. The plasma was stored at −20 °C until analysis. Subsequently, the livers were rapidly excised and washed in cold 0.9% normal saline for further extraction.

2.6.4. Effects of MC heartwood extract on residual activity of XOD in liver homogenates

The XOD activity was measured in the excised liver of each treated mouse. The livers were homogenized in 80 mM sodium pyrophosphate buffer, pH 7.4, and centrifuged at 3000 *g* for 10 min at 4 °C. The upper lipid layer was discarded and the lower solution was centrifuged at 10,000 *g* for 60 min at 4 °C. The obtained supernatants of the liver homogenates were stored at −80 °C until further analysis.

The total protein concentration in the liver homogenates was determined using the Bradford Protein Assay (Sigma-Aldrich, St. Louis, MO, USA). The procedure for assaying XOD activity in the liver homogenates was followed according to previous studies.[18,19] Briefly, the liver homogenate (150 μL) was mixed with 80 mM sodium pyrophosphate buffer, pH 7.4 and incubated at 25 °C for 10 min. Then, 1000 μL of 120 μM xanthine solution was added, mixed, and incubated for 10 min. After adding 100 μL of 1 N HCl to terminate the reaction, the sample was centrifuged at 3000 *g* for 10 min. The supernatant was filtered with a membrane filter and the uric acid concentration measured by HPLC, as indicated in section 2.6.5 below.

2.6.5. Determination of uric acid concentration by HPLC

Uric acid levels were measured by reversed-phase HPLC with a BDS Hypersil C_{18} column (150 × 4.6 mm, i.d. 5 μm) (Thermo Fisher Scientific, Waltham, MA, USA), connected to a BDS Hypersil C_{18} guard column (10 × 4 mm, i.d. 5 μm) (Thermo Fisher Scientific). The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a quaternary pump LC (LC- 20AT) with a degasser (DGU-20A3), UV−Visible detector (SPD-20A), and communication module (CBM-20A); the analytical column was constantly maintained at 25 °C. The analytical method was modified from a previous study.[19] The determination of uric acid in the *in vitro* XOD activity study was performed using standard uric acid solutions ranging from 1.5 to 50 μg/mL, with the equation: $y = 67188x + 3095.7$ ($r^2 = 0.99$). The amount of uric acid in plasma samples and liver microsomes was quantified using a calibration curve of spiked uric acid in blank plasma and liver microsomes from 3.12 to 50 μg/mL, with the calculated calibration curve of $y = 26581x + 5819.3$ ($r^2 = 0.99$) and $y = 28315x + 2355.2$ ($r^2 = 0.99$), respectively.

2.7. Anti-inflammatory effect of MC heartwood extract in lipopolysaccharide (LPS) - induced proinflammatory responses of RAW 264.7 mouse macrophage cells

2.7.1. Cell culture

RAW 264.7 mouse macrophage cell lines were cultured and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C in a humidified atmosphere of 5% CO_2.[20] After the RAW 264.7 cells reached 80% confluence, the cells were passaged by treatment with 0.25% trypsin-EDTA to maintain exponential cell growth. The medium was changed to serum-free DMEM and the cells were incubated for at least 30 min prior to initiating experiments.

2.7.2. Cell viability test

Cell viability or cytotoxicity was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.[21] Briefly, RAW 264.7 cells were seeded in 96-well plates at a density of 1×10^4 cells/well in 200 μL DMEM supplemented with 1% FBS and 1% P/S, and were incubated overnight. The cells were divided into control and treatment groups. The control group was exposed to varied concentrations of vehicle (70% ethanol), while the treatment groups were treated with various concentrations of MC heartwood extract (0.01−500 μg/mL diluted in 70% ethanol) for 24 h. After incubation, the culture medium was replaced with 100 μL MTT solution (1 mg/mL dissolved in DMEM) and the RAW 264.7 cells were incubated for an additional 4 h at 37 °C. Then, the MTT solution was removed by aspiration and 100 μL DMSO was added to dissolve the insoluble formazan product. The samples were mixed thoroughly and the absorbance of each well was measured at 570 nm with a microplate reader (Tecan). The number of viable cells was calculated as a percentage of the number of control cells. All assays were performed in triplicate and were repeated four times.

2.7.3. Induction of inflammatory processes in RAW 264.7 cells

The inflammatory cascades in RAW 264.7 cells were initiated by addition of LPS from *Salmonella enterica* serotype Typhimurium. Briefly, RAW 264.7 cells were seeded in 6-well plates at a density 1×10^6 cells/well in DMEM supplemented with 10% FBS and 1% P/S and were maintained in a humidified 37 °C, 5% CO_2 incubator for 24 h· Then, the culture medium was replaced with DMEM supplemented with 1% FBS and 1% P/S. The cells were divided into four groups, as follows: 1) no LPS stimulation; 2) stimulated with 1 μg/mL LPS for 12 h; 3) pretreated with diluted MC heartwood extract (10 μg/mL) for 3−4 h before stimulation with 1 μg/mL LPS for 12 h; and 4) pretreated with 0.1 μM dexamethasone (as a positive control) for 3−4 h before stimulation with 1 μg/mL LPS for 12 h. The culture medium was then removed and the cells were washed with ice-cold phosphate-buffered saline (PBS).

2.7.4. Measurement of mRNA expression of inflammation-related genes by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA from RAW 264.7 cells was extracted using the GeneJET RNA Purification Kit (Thermo Fisher Scientific) following the instructions of the manufacturer. The expression levels of inflammation-related genes, including tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were determined by RT-qPCR using KAPA SYBR® FAST One-step RT-qPCR kits (KAPA Biosystems) according to the manufacturer's protocol. The gene-specific primers for RT-qPCR (mouse) are shown in Table 1. RT-qPCR was performed under the following conditions: reverse transcription at 42 °C for 5 min; reverse transcriptase 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 55 °C for 30 s (40 cycles); last extension step at 72 °C for 1 min followed by 25 °C for 2 min. Relative mRNA expression levels were evaluated by the comparative cycle threshold (CT) method and normalized to an endogenous reference, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.8. Statistical data analysis

Data are expressed as means ± standard errors of the mean (SEMs). Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests for post-hoc analysis. Values of $p < 0.05$ were considered to be statistically significant.

Table 1
The gene specific primers for RT-qPCR (mouse)

Gene specific primers	Sequences	References
Mouse GAPDH	sense, 5′-GCCTGCTTCACCACCTTC-3′ antisense, 5′-GGCTCTCCAGAACATCATCC-3′	[22]
Mouse COX-2	sense, 5′-TGCATGTGGCTGTGGATGTCATCAA-3′ antisense, 5′-CACTAAGACAGACCCGTCATCTCCA-3′	[23]
Mouse iNOS	sense, 5′-GTGTTCCACCAGGAGATGTTG-3′ antisense, 5′-CTCCTGCCCACTGAGTTCGTC-3′	[23]
Mouse TNF-α	sense, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ antisense, 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	[24]
Mouse TGF-β1	sense, 5′-TGGAGCAACATGTGGAACTC-3′ antisense, 5′-TGCCGTACAACTCCAGTGAC-3′	[25]

3. Results

3.1. Extraction yield and antioxidant content with different extraction methods

The extraction yields obtained by three different extraction methods are presented in Table 2. It was observed that maceration with 70% ethanol presented the highest extraction yield (30.2%). The total phenolic content and antioxidant activities of the different extraction methods from MC heartwood extract are shown in Table 3. Among all extraction methods, the soxhlet extraction with 70% ethanol significantly showed the highest total phenolic content and ABTS scavenging activity ($p < 0.05$).

3.2. Phytochemical analysis

The HPLC chromatogram of MC heartwood extract shows a prominent peak at a retention time of 6.88 min, which corresponds to the morin reference standard (Fig. 1). The calibration curve of the morin standard generated the regression equation, $y = 11668x - 8204.7$, with a good coefficient of determination (r^2 of 0.9982). The content of morin in each MC extract is shown in Table 4. Although Soxhlet extraction with ethyl acetate gave the highest morin content (1.10 ± 0.08% w/w), its antioxidant activities were less than those of Soxhlet extraction with 70% ethanol. Therefore, Soxhlet extraction with 70% ethanol was the most suitable extraction method, and gave higher yield, antioxidant activities, and morin content than did maceration and decoction. MC heartwood extract produced by Soxhlet extraction with 70% ethanol was chosen for further experiments.

3.3. Hypouricemic effect of MC heartwood extract

3.3.1. Effect on in vitro XOD activity

MC heartwood extract inhibited XOD activity *in vitro* in a concentration-dependent manner; the IC_{50} was determined to be 32.24 μg/mL (Fig. 2), while that of allopurinol was 2.58 μg/mL.

3.3.2. Enzyme kinetics of XOD inhibition

Fig. 3 shows the Lineweaver-Burk analysis of MC heartwood extract inhibition of XOD activity. The results indicate that the V_{max} increased according to the concentration of MC heartwood extract, but the K_m did not change. This indicates that MC inhibits XOD in a non-competitive manner. The K_i value was calculated to be 29.52 μg/mL.

Table 2
Extraction yield of different extraction methods of *M. cochinchinensis* heartwood extract.

Extraction methods	Solvent	Yield extraction (%)
Soxhlet extraction	70% ethanol ethyl acetate	25.2
	water	11.3
		15.6
Maceration	70% ethanol ethyl acetate	30.2
		12.6
Decoction	water	20.2

3.3.3. In vivo hypouricemic effect on plasma uric acid levels in PO-induced hyperuricemic mice

Fig. 4a shows the hypouricemic effect of MC heartwood extract in PO-induced hyperuricemic mice. The uric acid level in the plasma of the control hyperuricemic group was significantly increased after an *i.p.* injection of PO compared with that of the normal control group ($p < 0.05$), indicating successful hyperuricemic induction. Allopurinol treatment (group 3) reduced the concentration of plasma uric acid by approximately 45%, compared with that of the non-treated hyperuricemic group ($p < 0.05$). Administration of MC heartwood extract (200 and 500 mg/kg) decreased uric acid concentrations by approximately 32% and 43%, respectively, compared with that of the untreated hyperuricemic control group, suggesting that the hypouricemic effect of MC was dose-dependent. Moreover, the hypouricemic effect of MC heartwood extract at 500 mg/kg was not significantly different from that of allopurinol.

3.3.4. Ex vivo effects of MC heartwood extract on residual activity of XOD in liver homogenates

Injection of PO significantly stimulated the rate of uric acid production from liver homogenate (Fig. 4b). By inhibiting XOD activity, treatment with allopurinol extensively inhibited the formation of uric acid by 84% compared with that of the untreated hyperuricemic group ($p < 0.05$). Treatment with MC heartwood extract (200 and 500 mg/kg) decreased the formation of uric acid (by inhibiting XOD activity in liver homogenates) by 15% and 53%, respectively, but these values were not significantly different from that of the untreated hyperuricemic group.

3.4. Anti-inflammatory effect of MC heartwood extract in RAW 264.7 cells

Using MTT colorimetric assays, we found that MC heartwood extract was not toxic to RAW 264.7 cells at the concentration range of 0.01–10 μg/mL (Fig. 5). Therefore, 10 μg/mL MC heartwood extract was selected for subsequent experiments. The ability of MC heartwood extract to inhibit mRNA expression of LPS-induced inflammatory genes in RAW 264.7 macrophage cells was investigated using RT-qPCR. The mRNA expression of inflammatory-related genes, including TNF-α, TGF-β1, iNOS, and COX-2, were increased after stimulation with LPS for 12 h (Fig. 6a–d). Interestingly, treatment with MC heartwood extract (10 μg/mL) significantly inhibited LPS-stimulated TNF-α, TGF-β1, iNOS, and COX-2 mRNA expression compared with control mRNA levels ($p < 0.05$). This

Table 3
Total phenolic, total flavonoid contents, DPPH radical scavenging activity, FRAP, and ABTS assay of *M. cochinchinensis* heartwood extracts.

M. cochinchinensis heartwood extract	Total phenolic content mg GAE/g extract	Total flavonoid content mg QE/g extract	DPPH IC_{50}(μg/mL)	FRAP (mmol/100g extract)	ABTS (g TEAC/g extract)
Soxhlet extraction					
−70% ethanol	38.54 ± 0.08^{c}	173.90 ± 6.12^{b}	6.98 ± 0.93^{b}	7.91 ± 0.38^{b}	1.82 ± 0.00^{c}
-ethyl acetate	$34.93 \pm 0.84^{a,b}$	$213.06 \pm 12.42^{b,c}$	$5.10 \pm 0.95^{a,b}$	7.23 ± 0.08^{b}	$1.74 \pm 0.14^{b,c}$
-water	$35.28 \pm 0.83^{a,b}$	94.18 ± 14.11^{a}	3.96 ± 0.10^{a}	7.47 ± 0.30^{b}	1.58 ± 0.04^{b}
Maceration					
−70% ethanol	34.10 ± 0.34^{a}	$150.33 \pm 23.14^{a,b}$	7.55 ± 1.08^{b}	5.83 ± 0.14^{a}	1.36 ± 0.06^{a}
-ethyl acetate	$36.69 \pm 1.06^{b,c}$	258.95 ± 29.10^{c}	7.77 ± 0.80^{b}	9.32 ± 0.32^{c}	$1.71 \pm 0.05^{b,c}$
Decoction	36.28 ± 0.27^{b}	98.77 ± 24.88^{a}	7.40 ± 0.87^{b}	7.16 ± 0.16^{b}	$1.76 \pm 0.06^{b,c}$

Data are expressed as mean ± SD (n = 3).
Values in the same column followed by a different letter (a-c) are significantly different ($p < 0.05$).
GAE = gallic acid equivalent, TEAC = Trolox Equivalent Antioxidant Capacity.

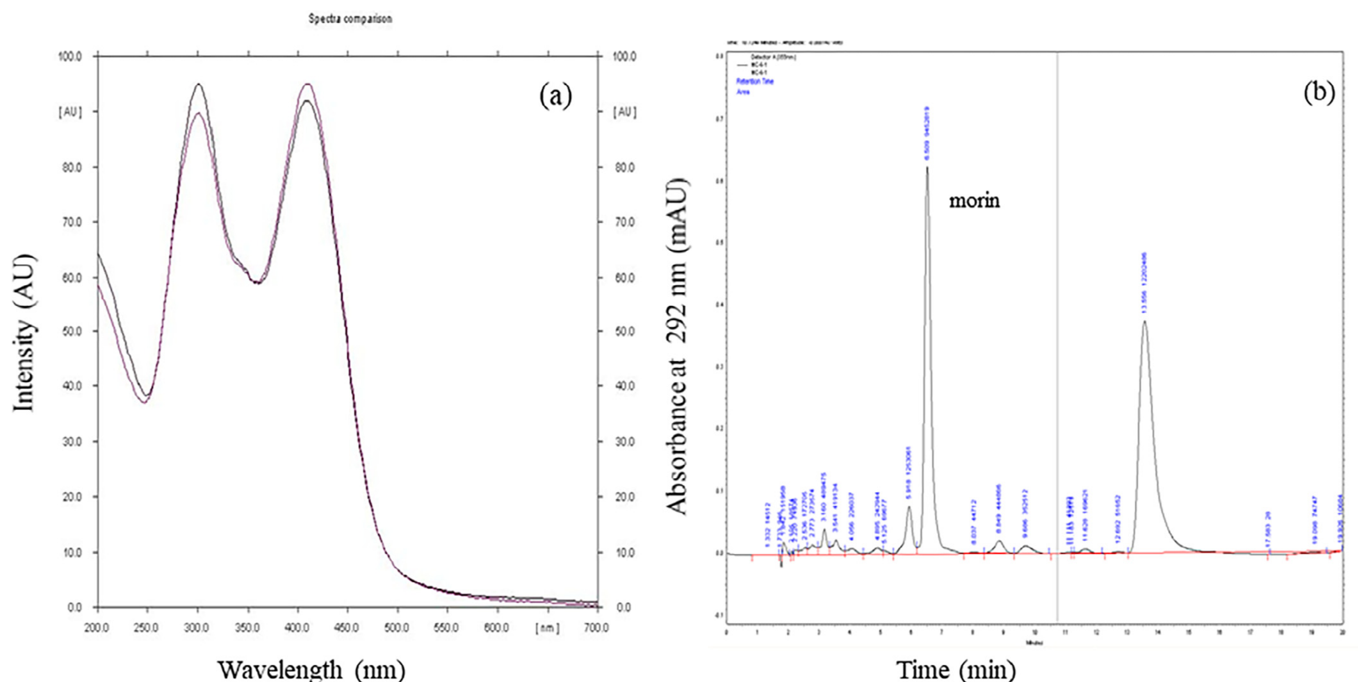

Fig. 1. HPLC chromatograms of 70% ethanolic *Maclura cochinchinensis* heartwood extract by the Soxhlet extraction method.

Table 4
The content of morin from different extraction method of *M. cochinchinensis* heartwood extract determined by HPLC analysis.

Method of extraction	Morin content (% w/w)
Soxhlet extraction	
- 70% ethanol	1.02 ± 0.04
- ethyl acetate	1.10 ± 0.08
- water	0.49 ± 0.04
Maceration	
- 70% ethanol	0.84 ± 0.05
- ethyl acetate	0.96 ± 0.04
Decoction	0.47 ± 0.01

Data are expressed as mean ± SD (n = 3).

anti-inflammatory effect was comparable to that of dexamethasone.

4. Discussion

In recent years, several lines of evidence have suggested that hyperuricemia and gout are independent risk factors and may play a role in the development and pathogenesis of several metabolic diseases, including hypertension, stroke, and atherosclerosis. Whether these risk factors are causally related to these important chronic comorbidities remains uncertain.[12,13] The combination of relevant therapies to lower uric acid levels, inhibit inflammatory responses, and prevent oxidative stress, for example, was suggested as an effective treatment for gout.

The Soxhlet extraction with 70% ethanol, which was the most suitable extraction method, giving high extraction yields, antioxidant activities, and morin content, was selected as the extraction method for MC. A previous study revealed that high polarity solvents such as ethanol and distilled water produced extracts with high total phenolic content and antioxidant activity.[26,27] Seventy percent ethanol extract contained higher morin levels than did aqueous extract, using the same extraction method, likely due to similar polarity between these compounds and the extraction solvent.

We demonstrated in this study that MC heartwood extract was able to inhibit XOD activity in a non-competitive manner and markedly reduced plasma uric acid levels in PO-induced hyperuricemic mice through inhibition of hepatic XOD activity. MC

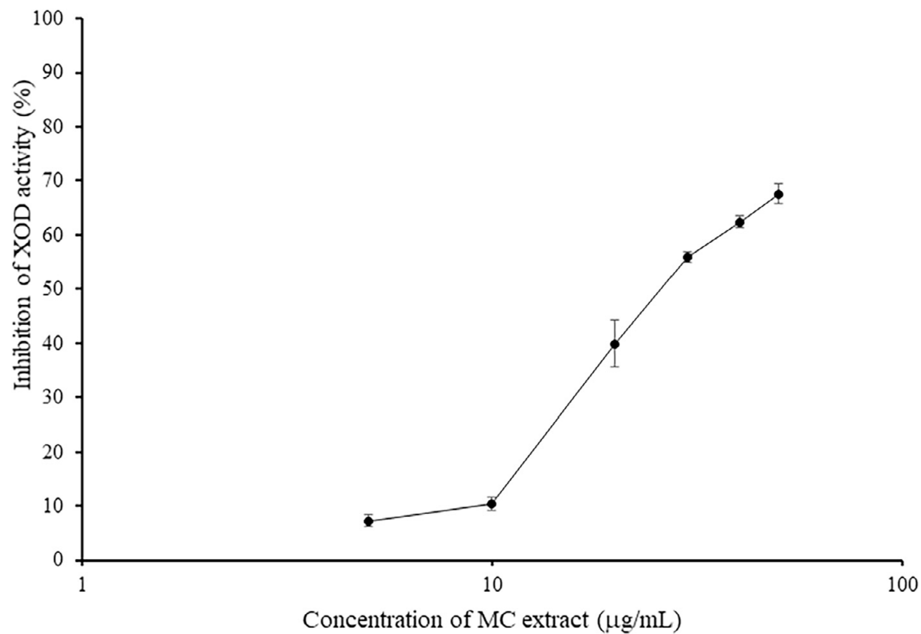

Fig. 2. The percentage inhibition of xanthine oxidase (XOD) activity and concentrations of *Maclura cochinchinensis*(MC) heartwood extract.

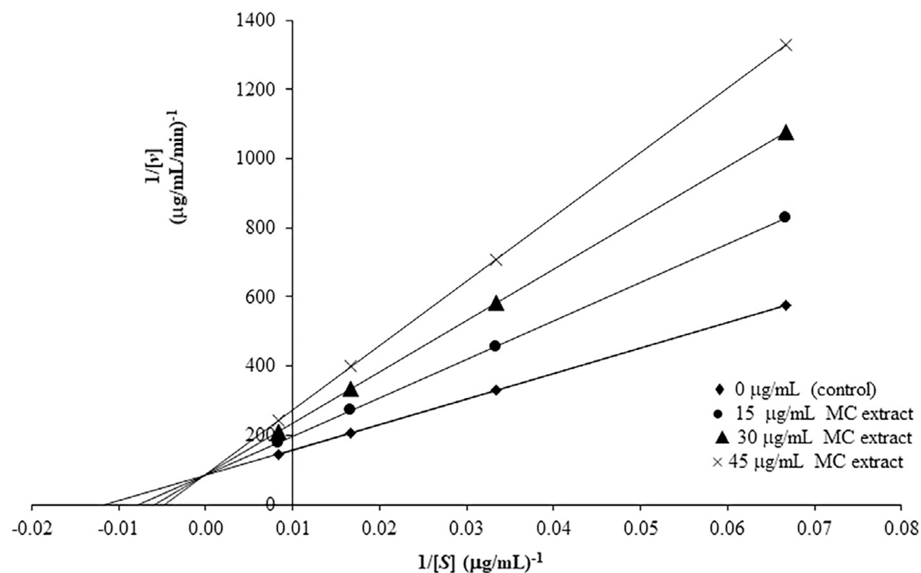

Fig. 3. Lineweaver-Burk plots for the kinetic analysis of xanthine oxidase activity inhibited by *Maclura cochinchinensis (*MC*)* heartwood extract. The symbols ◆, ●, ▲ and × represent MC heartwood extract at concentrations of 0 (control), 15, 30, and 45 µg/mL, respectively.

heartwood extract at a dose of 500 mg/kg exhibited potent hypouricemic effects in plasma that were similar to those of the standard drug, allopurinol; however, the inhibition of residual hepatic XOD activity was less effective with MC heartwood extract (Fig. 4). These observations suggest that the hypouricemic effect of MC heartwood extract is caused, at least in part, by the inhibition of XOD, which is a key enzyme in the biosynthetic pathway of uric acid. The effect of MC heartwood extract on renal transporters, which contribute to the reabsorption of uric acid, is unknown and will be the focus of our next study.

There is a hypothesis that enhanced XOD expression and activity increases reactive oxygen species (ROS) production.[28] Moreover, uric acid is believed to be a pro-oxidant and its enhancement by XOD activity generates superoxide anion that can rapidly react with nitric oxide to form the cytotoxic oxidant, peroxynitrite, leading to endothelial dysfunction, atherosclerosis, and cardiovascular diseases.[10] It has been shown that plant-derived antioxidants, such as flavonoids, alkaloids, essential oils, phenolic compounds, tannins, iridoid glucosides, and coumarins, have anti-gout potential.[10] In accordance with a previous study,[6] we found that MC heartwood extract demonstrated antioxidant activity in the three evaluated assays. Several studies reported positive correlations of phenolic and flavonoid content with XOD inhibitory activity, uricosuric effects, and anti-inflammatory effects.[10,29] High phenolic and flavonoid contents were observed in the MC heartwood extract, which is supported by previously reported findings.[6] These results indicated that the flavonoid and phenolic contents may contribute to the XOD inhibitory activity of the extract.

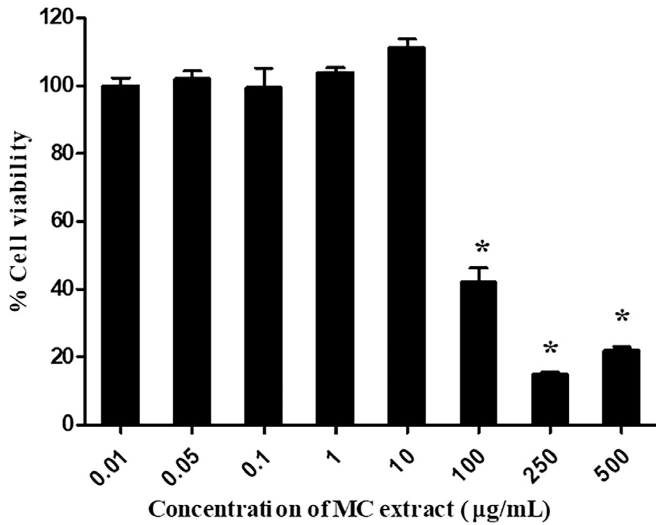

Fig. 4. Effect of *Maclura cochinchinensis* (MC) heartwood extract on mouse uric acid parameters. (a) Plasma uric acid concentrations in potassium oxonate (PO)-induced hyperuricemic mice. (b) Rate of uric acid production in liver homogenates from mice. Data are expressed as the means ± SEM (n = 5–6). # and * indicate a statistically significant difference at $p < 0.05$ when compared with normal control mice and untreated-hyperuricemic mice, respectively.

The deposition of urate crystals can stimulate the generation of inflammatory cytokines (e.g., TNF-α, IL-1β, and IL-6), which are important initiation factors in the inflammatory process of gout. In addition to having XOD inhibitory and antioxidant activities, MC heartwood extract was found to possess a notable anti-inflammatory effect in LPS-induced RAW 264.7 cells. In these cells, MC heartwood extract significantly attenuated the mRNA expression of TNF-α, TGF-β1, iNOS, and COX-2, which are pivotal mediators of inflammatory responses. These inhibitory effects were equal to those of dexamethasone. The inhibition of ROS production and providing a radical scavenging effect during oxidative stress are possible mechanisms of the anti-inflammatory effect of MC heartwood extract. However, the exact mechanism requires further investigation. In addition, the effects of the extract on *in vivo* inflammatory models, such as carrageenan-induced paw edema, should be assessed.

Through HPLC analysis, morin was identified as the major biochemical compound in MC heartwood extract. This result is consistent with that of a previous report.[3] Recent evidence has demonstrated that morin exhibits hypouricemic effects by two mechanisms: acting as a uricosuric agent and inhibiting xanthine oxidase activity.[9,30] Moreover, its antioxidant and anti-inflammatory activities have been demonstrated.[7,31] Since MC heartwood extract contains morin as a major active compound and

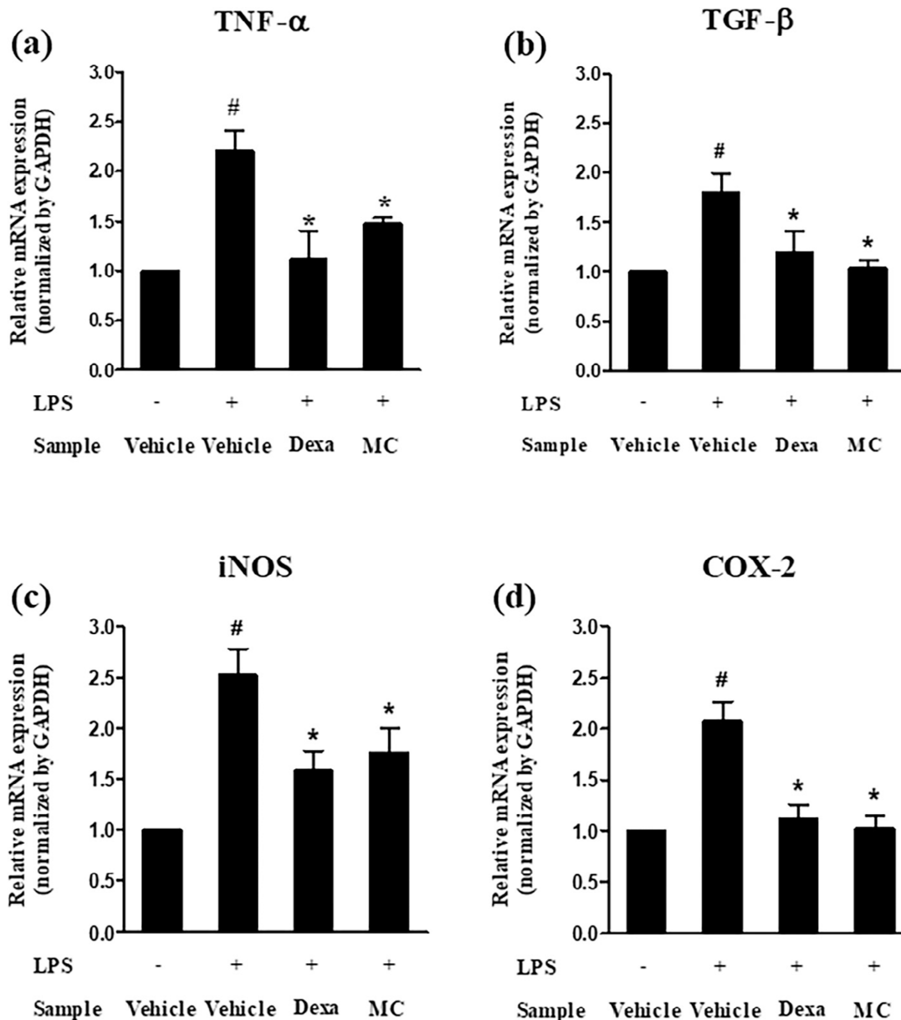

Fig. 5. The effect of *Maclura cochinchinensis* (MC) heartwood extract on cytotoxicity of RAW 264.7 macrophage cells. RAW 264.7 cells were treated with MC heartwood extracts (0.01–500 µg/mL) for 24 h. Cell viability was quantified, expressed as a percentage of untreated cells, and shown as the means ± SEM (n = 4). *$p < 0.05$ versus group.

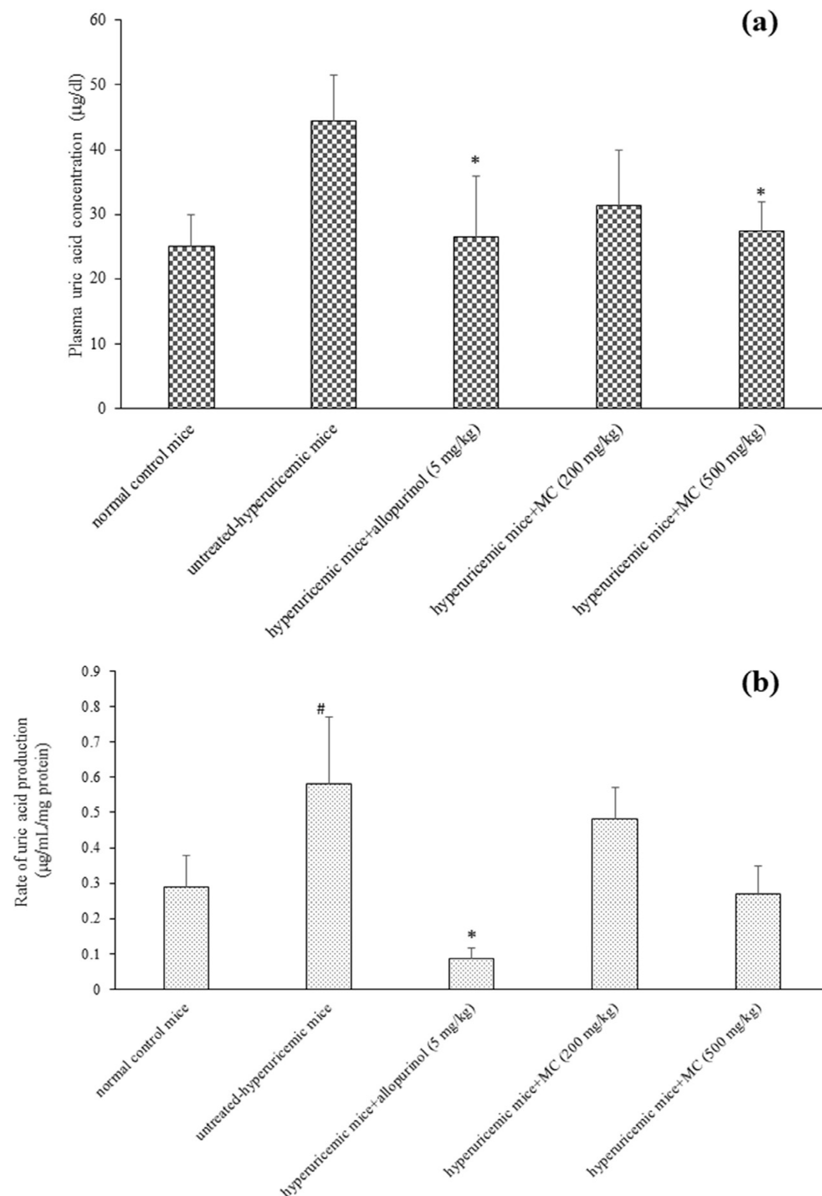

Fig. 6. Effects of *Maclura cochinchinensis (*MC*)* heartwood extract on mRNA expression of TNF-α, TGF-β1, iNOS, and COX-2 in LPS-induced RAW 264.7 macrophage cells. The relative mRNA levels of (a) TNF-α, (b) TGF-β1, (c) iNOS, and (d) COX-2 were quantified, normalized to the corresponding GAPDH level, and expressed as fold over vehicle group. Data are expressed as the means ± SEM four independent experiments. [#] and [*] indicate a statistically significant difference at $p < 0.05$ when compared with vehicle and vehicle + LPS, respectively.

its pharmacological effects observed in the present study are in accordance with those of morin, we therefore propose that morin is at least partially responsible for the pharmacological activities of MC heartwood extract. Taken together, we concluded that MC extract contained the combinations of several bioactive phenolics and flavonoids including morin, which contributed to XOD inhibitory activity, uricosuric effects, and anti-inflammatory effects, and could exhibit the favorable effects for anti-hyperuricemia than only single active compound.

MC is a traditionally used medicinal plant in Thailand. The safety of MC heartwood extract has been reported.[1] The LC_{50} values of the chloroform, ethyl acetate, and methanol extracts against *Artemia salina* (brine shrimp) were 112.2, 69.2, and 131.8 ppm, respectively (unpublished data). No toxicity of morin, as an active compound, was observed in rats at doses of 16 g/kg and 10 g/kg via gastric intubation and subcutaneous injection, respectively. In addition, no mortality or abnormalities were observed when rats were fed up to 5% (w/w) morin in the diet for 13 weeks.[32]

To the best of our knowledge, this is the first report of MC heartwood extract as a hypouricemic agent by inhibition of hepatic XOD activity. Considering that it also possesses free radical scavenging and anti-inflammatory activities, we believe that MC heartwood extract could be developed as a promising candidate to treat hyperuricemia. A further study to examine the effect of MC heartwood extract on renal transporters and clinical efficacy and safety in hyperuricemic patients is needed in the future.

Conflicts of interest

We declare that we do not have any conflicts of interest related to this work.

Acknowledgements

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References

1. Mokkhasmit M, Swatdimongkol K, Satrawaha P. Study on toxicity of Thai medicinal plants. *Bull Dept Med Sci*. 1971;12:36–65.
2. Kummee S, Intaraksa N. Antimicrobial activity of *Desmos chinensis* leaf and *Maclura cochinchinensis* wood extracts. *Songklanakarin J Sci Technol*. 2008;30:635–639.
3. Bunyapraphatsara N, Dechsree S, Yoosook C, Herunsalee A, Panpisutchai Y. Anti-herpes simplex virus component isolated from *Maclura cochinchinensis*. *Phytomedicine*. 2000;6:421–424.
4. Sithisarn P, Rojsanga P, Sithisarn P, Kongkiatpaiboon S. Antioxidant activity and antibacterial effects on clinical isolated *Streptococcus suis* and *Staphylococcus intermedius* of extracts from several parts of *Cladogynos orientalis* and their phytochemical screenings. *Evid Based Complement Alternat Med*. 2015;2015:908242.
5. Ministry of Public Health. *National List of Essential Medicines (List of Herbal Medicinal Products)*. 2013. Thailand).
6. Swargiary A, Ronghang B. Screening of phytochemical constituents, antioxidant and antibacterial properties of methanolic bark extracts of *Maclura cochinchinensis* (Lour.) corner. *Int J Pharma Bio Sci*. 2013;4:P449–P459.
7. Zheng ZP, Zhu Q, Fan CL, Tan HY, Wan M. Phenolic tyrosinase inhibitors from the stems of *Cudrania cochinchinensis*. *Food Funct*. 2011;2:259–264.
8. Chen Y, Li Y, Xu H, Li G, Ma Y, Pang YJ. Morin mitigates oxidative stress, apoptosis and inflammation in cerebral ischemic rats. *Afr J Tradit, Complementary Altern Med*. 2017;14:348–355.
9. Yu Z, Fong WP, Cheng CH. The dual actions of morin (3,5,7,2',4'-pentahydroxyflavone) as a hypouricemic agent: uricosuric effect and xanthine oxidase inhibitory activity. *J Pharmacol Exp Ther*. 2006;316:169–175.
10. Gliozzi M, Malara N, Muscoli S, Mollace V. The treatment of hyperuricemia. *Int J Cardiol*. 2016;213:23–2237.
11. Lupton GP, Odom RB. The allopurinol hypersensitivity syndrome. *J Am Acad Dermatol*. 1979;1:3653–3674.
12. Perez-Ruiz F, Becker MA. Inflammation: a possible mechanism for a causative role of hyperuricemia/gout in cardiovascular disease. *Curr Med Res Opin*. 2015;31(Suppl 2):9–14.
13. Ghorbani A, Esmaeilizadeh M. Pharmacological properties of *Salvia officinalis* and its components. *J Tradit Complement Med*. 2017;7:433–440.
14. Kongkiatpaiboon K, Tungsukruthai P, Sriyakool K, Pansuksan K, Tunsirikongkon A, Pandith H. Determination of morin in *Maclura cochinchinensis* heartwood by HPLC. *J Chromatogr Sci*. 2017;55:346–350.
15. Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. *Ind Crops Prod*. 2013;44:566–571.
16. Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Hawkins Byrne D. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Compos Anal*. 2006;19:669–675.
17. Stanković MS. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. *Kragujevac J Sci*. 2011;33:63–72.
18. Sungthong B, Manok S, Sato H, Sato VH. Effects of *Aquilaria Crassna* on xanthine oxidase activity *in vitro* and hyperuricemic mice. *Indian J Pharm Sci*. 2016;78:537–542.
19. Sato VH, Sungthong B, Nuamnaichati N, et al. Pharmacological effects of Chatuphalatika in hyperuricemia of gout. *Pharm Biol*. 2018;56:76–85.
20. Shiina T, Kawasaki A, Nagao T, Kurose H. Interaction with beta-arrestin determines the difference in internalization behavior between beta1 -and beta2-adrenergic receptors. *J Biol Chem*. 2000;275:29082–29090.
21. Kosem N, Han YH, Moongkarndi P. Antioxidant and cytoprotective activities of methanolic extract from *Garcinia mangostana* hulls. *Sci Asia*. 2007;33:283–292.
22. Mukwevho E, Kohn TA, Lang D, Nyatia E, Smith J, Ojuka EO. Caffeine induces hyperacetylation of histones at the MEF2 site on the Glut4 promoter and increases MEF2A binding to the site via a CaMK-dependent mechanism. *Am J Physiol Endocrinol Metab*. 2008;294:E582–E588.
23. Han MS, Kim EH, Lee J. Inhibition of lipopolysaccharide-induced expression of nitric oxide and cyclooxygenase by aqueous of *Aconitum Pseudoleave* var. erectum in RAW264.7 macrophages. *Korean J Orient Physiol Pathol*. 2008;22:683, 678.
24. Jiang H, Deng CS, Zhang M, Xia J. Curcumin-attenuated trinitrobenzene sulphonic acid induces chronic colitis by inhibiting expression of cyclooxygenase-2. *World J Gastroenterol*. 2006;12:3848–3853.
25. Xiong W, Frasch SC, Thomas SM, Bratton DL, Henson PM. Induction of TGF-β1 synthesis by macrophages in response to apoptotic cells requires activation of the scavenger receptor CD36. *PLoS One*. 2013;8. e72772.
26. Vongsak B, Kongkiatpaiboon S, Jaisamut S, Machana S, Pattarapanich C. *In vitro* alpha glucosidase inhibition and free-radical scavenging activity of propolis from Thai stingless bees in mangosteen orchard. *Rev Bras Farmacogn*. 2015;25:445–450.
27. Kongkiatpaiboon S, Chewchinda S, Vongsak B. Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. *Rev Bras Farmacogn*. 2018;28:145–150.
28. Berry CE, Hare JM. Xanthine oxidoreductase and cardiovascular disease: molecular mechanisms and pathophysiological implications. *J Physiol*. 2004;555:589–606.
29. Huang J, Wang S, Zhu M, Chen J, Zhu X. Effects of Genistein, Apigenin, Quercetin, Rutin and Astilbin on serum uric acid levels and xanthine oxidase activities in normal and hyperuricemic mice. *Food Chem Toxicol*. 2011;49:1943–1947.
30. Shi YW, Wang CP, Wang X, et al. Uricosuric and nephroprotective properties of Ramulus Mori ethanol extract in hyperuricemic mice. *J Ethnopharmacol*. 2012;143:896–904.
31. Sultana F, Neog MK, Rasool M. Targeted delivery of morin, a dietary bioflavanol encapsulated mannosylated liposomes to the macrophages of adjuvant-induced arthritis rats inhibits inflammatory immune response and osteoclastogenesis. *Eur J Pharm Biopharm*. 2017;115:229–242.
32. Cho YM, Onodera H, Ueda M, Imai T, Hirose M. A 13-week subchronic toxicity study of dietary administered morin in F344 rats. *Food Chem Toxicol*. 2006;44:891–897.