Lonicera hypoglauca Inhibits Xanthine Oxidase and Reduces Serum Uric Acid in Mice

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Abstract
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Xanthine oxidase (XOD) catalyzes the oxidation of hypoxanthine to xanthine and then to uric acid, and is a key enzyme in the pathogenesis of hyperuricemia. The ability of extracts of *Lonicera hypoglauca* (Caprifoliaceae) to inhibit XOD was investigated in this study. An ethanol extract (LH-crude) of the leaves of *L. hypoglauca* and its derived EtOAc soluble sub-fractions (LH-EA) significantly inhibited XOD activity, with IC_{50} values for LH-crude and LH-EA of 48.8 and 35.2 µg/mL. Moreover, LH-EA reduced serum urate levels *in vivo* in a potassium oxonate-induced hyperuricemic mouse model, by 70.1% and 93.7% of the hyperuricemic untreated group at doses of 300 and 500 mg/kg of LH-EA, respectively. Finally, we used bioactivity-guided fractionation to isolate a new bisflavonoid, loniceraflavone, which showed significant inhibition of XOD ($IC_{50} = 0.85$ µg/mL). These results suggest that *L. hypoglauca* and its extracts may have a considerable potential for development as an anti-hyperuricemia agent for clinical application.

Abbreviations
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LH-crude: EtOH extract of *L. hypoglauca*
LH-EA: ethyl acetate (EtOAc)-soluble fraction of *L. hypoglauca*
LH-water: H_2O-soluble fraction of *L. hypoglauca*
PO: potassium oxonate
TCM: traditional Chinese medicine
XOD: xanthine oxidase

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Introduction
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Traditional Chinese medicine (TCM) has been used extensively to prevent and cure human disease for thousands of years. Recently, the potential of certain lead compounds from plants used in TCM has caught the attention of the scientific community in the Western world [1]. In TCM, "Jinyinhua" is well-known for the treatment of what is known as "affection by exopathogenic wind-heat", or the early stages of epidemic febrile disease [2], as well as urinary disorders, fever and headache [3]. The plant sources of Jinyinhua are the flower and bud of Asian *Lonicera* species, usually *L. japonica* Thunb., *L. hypoglauca* Miq., *L. confusa* DC., and/or *L. dasystyla* Rehd. (Caprifoliaceae). Although both *L. hypoglauca* and *L. japonica* are widely used as Jinyinhua in TCM, studies of the phytochemistry and bioactivity of Jinyinhua have mostly focused on *L. japonica* (Japanese honeysuckle), while the cognate *L. hypoglauca*, an endemic rattan that grows at middle elevations in Taiwan's hardwood forest [4], has barely been studied. In this study, we evaluated the xanthine oxidase (XOD) inhibition activity of extracts from *L. hypoglauca in vitro* and examined its effects *in vivo* in a mouse model of hyperuricemia. Moreover, we also isolated a new bisflavonoid, loniceraflavone (**1**), with significant XOD inhibition activity.

Materials and Methods
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General experimental procedures
Extracts were chromatographed on silica gel (70–230 mesh, ASTM; Merck) and purified with a semi-preparative normal phase HPLC column (Luna 5 µm silica, 250 × 10 mm; Phenomenex) on an Agilent 1100 HPLC system; melting points were obtained with Yanagimoto micromelting point apparatus and are uncorrected. The specific rotation was measured with a Jasco DIP-180 digital polarimeter. IR spectra were measured with a

Perkin-Elmer 983 G spectrophotometer. ^{1}H-, ^{13}C-, and 2D NMR spectra were measured with a Varian Mercury-400 spectrophotometer. Electron impact mass spectra were obtained on a JEOL JMS-HX 300 mass spectrometer.

Plant material

Leaves and stems of *L. hypoglauca* were collected in March 2006 from the Da-kang area of the Taichung County in central Taiwan. The species were identified by Prof. Y.-H. Tseng (Department of Forestry, National Chung-Hsing University), and voucher specimens (*YHT001* [TCF]) were deposited at the Herbarium of the Department of Forestry, National Chung-Hsing University, Taiwan.

Extraction and isolation

Air-dried leaves of *L. hypoglauca* (5.0 kg) were extracted with ethanol (EtOH, 25 L × 2) at ambient temperature for 5 days, and concentrated under vacuum to yield 732.2 g of EtOH extract (LH-crude). A fraction of this EtOH extract (200.0 g) was partitioned between EtOAc-H$_2$O to give 60.8 g of the EtOAc-soluble fraction (LH-EA) and the H$_2$O-soluble fraction (LH-water). The LH-EA fraction displayed potent XOD inhibition activity, and was further chromatographed over silica gel (8 × 120 cm, 70–230 mesh; Merck) eluted with pure *n*-hexane initially, followed by an *n*-hexane : EtOAc step gradient (95 : 5; 90 : 10; 85 : 15; 80 : 20; 70 : 30; 60 : 40; 50 : 50; 40 : 60; 0 : 100, each 2 L) to produce 20 fractions, LH-EA-1 to -20. LH-EA-17 exhibited the strongest XOD inhibition activity, therefore, part of LH-EA-17 (8.0 g) was further separated by HPLC using a C18 column (Luna 5 µm silica, 250 × 10 mm; Phenomenex) with *n*-hexane : EtOAc : dichloromethane = 26 : 37 : 37 as eluant at a flow rate of 3.0 mL/min to obtain 1.2 g of compound **1** (retention time = 15.4 min). The structure of **1** was spectroscopically determined by 1D and 2D NMR and other techniques. The purity of **1** was estimated from the ^{1}H-NMR spectrum and HPLC analysis to be greater than 99.5%.

Loniceraflavone (**1**): White amorphous powder; m. p. 178–182 °C; $[\alpha]_D^{24}$: + 2.8 (*c* 0.75, acetone); UV (MeOH): λ_{max} = 320, 278, 250, 228 nm; IR (KBr): ν_{max} = 3 470, 2 923, 2 853, 1 650, 1 610, 1 572, 1 472, 1 221, 1 148 cm^{-1}; ^{1}H NMR (400 MHz, acetone-d_6): δ = 12.77, 12.55 (1H each, s, Ph-O<u>H</u>), 7.45 (2H, d, *J* = 8.8 Hz, H-2‴, -6‴), 7.22 (2H, d, *J* = 8.8 Hz, H-2′, -6′), 6.92 (1H, d, *J* = 8.8 Hz, H-3‴, -5‴), 6.83 (2H, d, *J* = 8.8 Hz, H-3′, -5′), 6.64 (1H, s, H-8″), 6.29 (1H, d, *J* = 2.4 Hz, H-6), 6.21 (1H, d, *J* = 2.4 Hz, H-8), 5.67 (1H, dd, *J* = 13.2, 3.0 Hz, H-2″), 3.44 (1H, dd, *J* = 17.4, 13.2 Hz, H-3″), 2.96 (1H, dd, *J* = 17.4, 3.0 Hz, H-3″); ^{13}C NMR (100 MHz, acetone-d_6): δ_C = 43.7 (C-3″), 80.3 (C-2″), 89.9 (C-3), 94.6 (C-6), 100.2 (C-8), 101.1 (C-8″), 103.5 (C-10), 107.5 (C-10″), 115.8 (C-3′, -5′), 116.2 (C-3‴, -5‴), 116.5 (C-6″), 122.1 (C-1′), 129.2 (C-2‴, -6‴), 130.1 (C-1‴), 132.5 (C-2′, -6′), 156.2 (C-9), 156.7 (C-9″), 157.9 (C-4′), 158.9 (C-4‴), 162.4 (C-5″), 162.6 (C-7″), 163.1 (C-5), 164.5 (C-7), 166.5 (C-2), 184.2 (C-4), 199.5 (C-4″); FAB-MS: *m/z* = 541 [M + H]$^+$; HR-FAB-MS: *m/z* = 541.1 125 [M + H]$^+$ (calcd. for C$_{30}$H$_{21}$O$_{10}$: 541.1 128).

HPLC/DAD analysis

HPLC analysis of crude and EtOAc fractions was performed on an Agilent 1100 system equipped with a diode array detector (DAD) model G1315B, and an analytical C18 column (Phenomenex Luna; 5 µm, 150 × 4.6 mm). The injection volume was 5 µL, with a gradient of 5% to 100% MeOH in water, at 0.8 mL/min over 30 min. UV-VIS detection was carried out at 320 nm. Quantification was based on the measured integration area of the peak of interest, compared to a calibration value from a solution in MeOH of compound **1** between 0.1–1.0 mg/mL.

Xanthine oxidase inhibition *in vitro* assay

The reaction mixture for the XOD inhibition assay consisted of 400 µL of 200 mM sodium pyrophosphate buffer (pH 7.5), 200 µL of 0.6 mM xanthine, 20 µL of sample solution dissolved in distilled water or 1% dimethyl sulfoxide (DMSO), and 200 µL xanthine oxidase (0.1 U). A solution in 1% DMSO was used for those samples not soluble in distilled water. The increased UV absorption at 295 nm indicated the formation of uric acid [5,6]. All determinations were performed in triplicate. XOD inhibition assays examined LH-crude, LH-EA, and LH-water fractions at doses of 500, 250, 100, 50, and 10 µg/mL, and loniceraflavone and allopurinol (reference compound, purchased from Sigma-Aldrich; purity > 99.0%) at concentrations of 100, 50, 25, 10, 5, 3, 2, 1 µg/mL. The XOD inhibition was calculated as a percentage, (%) = (1 – b/a) × 100, where "a" is the change in absorbance per min without the sample, and "b" is the change in absorbance per min with the sample. The concentration of samples required to inhibit 50% of XOD activity (IC$_{50}$) were estimated from the % inhibition versus concentration plot using a linear regression algorithm.

Determination of hypouricemic effects in mouse model of hyperuricemia

Experiments were performed with the approval of the local Institutional Ethics Committee, and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [7]. Three-week-old male ICR mice (25–28 g) were purchased from BioLASCO and the 10 mice in each group were housed in a plastic cage. Mice were housed under the following conditions: temperature 25 ± 2 °C, relative humidity 55 ± 5%, light 06 : 00–18 : 00 h, fed with rodent diet (LabDiet 500 l rodent diet; Purina Mills, LLC) and water *ad libitum*, and were allowed one week to adapt to the environment before testing. The animals were transferred to the laboratory at least 1 h before induction of hyperuricemia by potassium oxonate (PO), a uricase inhibitor [8]. The method used to examine the hyperuricemic effects of LH-EA was as described previously [9–11] with slight modifications. Mice were divided into five groups (*n* = 10): "normal" (no treatment) control group, "PO", "PO + LH-EA300", "PO + LH-EA500", and "PO + allopurinol". All PO groups were injected intraperitoneally with PO at a dosage of 250 mg/kg 1 h before drug administration to increase serum urate levels. One hour later, the mice in the "PO + LH-EA300" and "PO + LH-EA500" groups were orally administrated 300 mg/kg and 500 mg/kg of LH-EA; the mice in the "PO + allopurinol" group were orally administrated 10 mg/kg allopurinol; the mice in the "PO" and "normal" group were administrated saline only. Two hours after PO-induced action, whole blood samples were collected from mice. The blood was allowed to clot for 1 h at ambient temperature and then centrifuged at 3 000 rpm for 5 min to obtain the serum. The serum was stored at − 20 °C until assayed. The uric acid level was determined by the phosphotungstic acid method, as previously described [14].

Statistical analysis

Data are expressed means ± SE. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences (* $p < 0.05$ and ** $p < 0.01$) between the control (untreated) and treated mice were analyzed by Dunnett's test.

Results

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In the present study, XOD inhibitory activity of *L. hypoglauca* extracts was evaluated using a spectrophotometric method (○ **Table 1**). Both LH-crude and LH-EA inhibited XOD, with 50% inhibition concentration values (IC$_{50}$ values) of 48.8 and 35.2 µg/mL, respectively. LH-water did not show significant XOD inhibition. The most inhibitory fraction, LH-EA, was then examined for any potential effects on serum uric acid levels in oxonate-induced hyperuricemic mice (○ **Fig. 1**). The initial serum uric acid level in mice was 1.84 ± 0.26 mg/dL. Intraperitoneal injection of PO caused a significant increase in serum uric acid, with a maximum serum uric acid level of 5.52 ± 0.68 mg/dL, 3 h after injection. After oral administration of LH-EA at doses of 300 and 500 mg/kg in PO-induced hyperuricemic mice, the serum uric acid values fell to 2.94 ± 0.44 (a reduction of 70.1% from untreated PO mice) and 2.07 ± 0.15 mg/dL (a reduction of 93.7%), respectively. Allopurinol, at a dose of 10 mg/kg, reduced the serum uric acid of mice to 1.92 ± 0.02 mg/dL.

These *in vitro* and *in vivo* studies suggest that LH-EA possesses significant XOD inhibitory activity and can efficiently reduce serum urate levels in a potassium oxonate-induced hyperuricemic mouse model. To identify the compound responsible for XOD inhibition, LH-EA was further separated into 20 fractions (LH-EA-1 to −20) by chromatography. The derived 20 sub-fractions were continuously evaluated for their XOD inhibitory activity using the same *in vitro* method. The fraction LH-EA-17 exhibited the strongest XOD inhibition activity, so it was purified by HPLC to obtain compound **1**.

Compound **1** was obtained as a white powder. Its molecular formula was determined to be $C_{30}H_{20}O_{10}$ by high resolution fast atom bombardment mass spectrometry (HR-FAB-MS). The IR spectrum exhibited bands at 3470 and 1650 cm^{-1} due to a hydroxy and a conjugated carbonyl group. The ^{1}H- and ^{13}C-NMR spectra of **1** established the presence of six hydroxy groups, 11 aromatic carbons, and one ABX system. Two of the six hydroxy protons resonated at δ = 12.55 and 12.77 indicating the presence of two chelated hydroxy groups at the 5 and 5″ positions, respectively. The remaining four non-chelated hydroxy carbons at δ = 164.5, 162.6, 163.1, and 162.4 were assigned to the 7, 7″, 5, and 5″ positions, respectively. The ^{1}H-NMR spectrum of **1** showed the presence of an ABX coupling system with signals at δ = 5.67 (dd, *J* = 13.2, 3.0 Hz, H-2″), 3.44 (dd, *J* = 17.4, 13.2 Hz, H-3″), and 2.96 (dd, *J* = 17.4, 3.0 Hz, H-3″) indicating the partial structure of a flavonoid. Two *meta*-coupled protons H-6 and H-8 at δ = 6.29 and 6.21 (each 1H, d, *J* = 2.4 Hz), and the proton at δ = 6.64 (H-8″) were confirmed by an HMBC experiment. Two AA'XX' coupling systems with signals at δ = 7.22 (2H, d, *J* = 8.8 Hz, H-2′, -6′), 6.83 (2H, d, *J* = 8.8 Hz, H-3′, -5′), 7.45 (2H, d, *J* = 8.8 Hz, H-2‴, -6‴), and 6.92 (2H, d, *J* = 8.8 Hz, H-3‴, -5‴) were also found. The above data excluded the possibility of a linkage between the two flavone moieties at C-6, C-8, C-2′, C-3′, C-5′, C-6′, C-2″, C-3″, C-2‴, C-3‴, C-5‴, and C-6‴. To confirm the possible linkage position of the two flavonoid units, an HMBC spectrum was obtained. The resonance at δ = 6.64 (H-8″) correlated with C-10″ (δ$_C$ = 107.5) on the HMBC spectrum indicating that C-6″ was the position where two flavonoid units were linked together. The olefinic carbons (δ$_C$ = 89.9 and 166.5) were assigned to C-3 and C-2, which was again supported by HMBC where a cross-peak was observed between H-2′ and C-2. This result suggested that C-3 was another connecting position for the two flavones. The observed long-range carbon proton coupling between H-2‴

Table 1 Xanthine oxidase (XOD) inhibitory activity of extracts from *Lonicera hypoglauca*.

	IC$_{50}$ of XOD inhibition (µg/mL)
LH-crude	48.8 ± 0.3
LH-EA	35.2 ± 0.4
LH-water	> 200
Allopurinol[a]	0.6 ± 0.0

[a] Allopurinol was used as a reference compound in this assay.
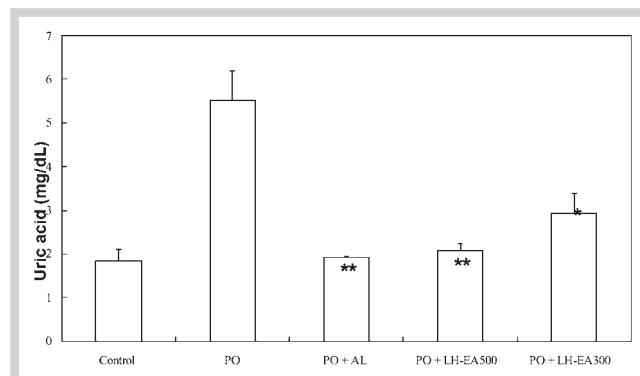
Fig. 1 Effects of LH-EA and allopurinol on urate levels in serum of hyperuricemic mice pretreated with potassium oxonate (PO). Control: untreated mice; PO: PO only; PO + LH-EA500: 500 mg/kg LH-EA after PO; PO + LH-EA300: 300 mg/kg LH-EA after PO; PO + allopurinol: 10 mg/kg allopurinol after PO. The data are representative of 10 animals and expressed as mean ± S.E. * $p < 0.05$ compared with PO-only group; ** $p < 0.01$ compared with PO-only group.
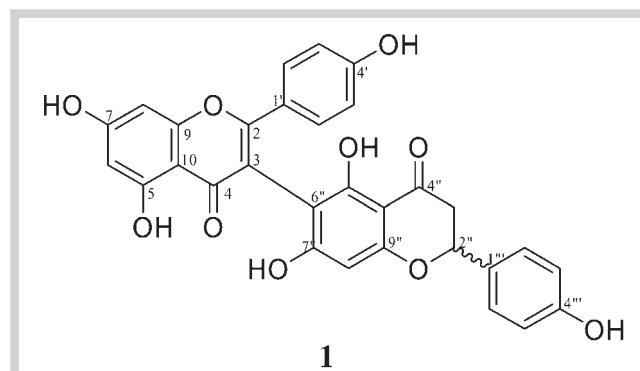
Fig. 2 Structure of loniceraflavone.

and C-2″ provided further proof of the above interpretation. These data indicated that **1** was a bisflavonoid having a C-3−C-6″ interflavonoid linkage but the configuration of 2″ has not been solved in this study. Based on the above deduction, **1** was designated to be a new compound 5,5″,7,7″-tetrahydroxy-2,2″-di(*para*-hydroxyphenyl)-2″,3″-dihydro[3,6″]bichromenyl-4,4″-dione, and was assigned the name loniceraflavone (○ **Fig. 2**). Assignment of the ^{1}H- and ^{13}C-NMR spectrum of **1** was based on HSQC, HMBC, COSY, and NOESY spectra. The concentrations of **1** in LH-crude and LH-EA were determined by HPLC (○ **Fig. 3**) to be 45.6 ± 6.2 and 150.2 ± 14.5 mg/g, respectively, compared to a reference calibration using 99.5% pure **1**. The IC$_{50}$ for *in vitro*
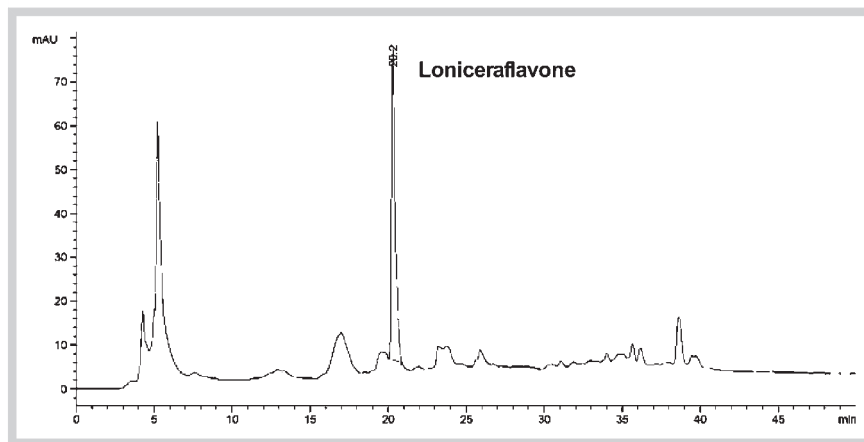
Fig. 3 HPLC chromatogram of the ethyl acetate (EtOAc)-soluble fraction of *L. hypoglauca*. The EtOAc fraction was further separated by HPLC on a Phenomenex Luna C18(2) column (5 µm, 150 × 4.6 mm) with a H_2O : MeOH solvent system. Gradient: 0–5 min, 50 % MeOH (isocratic); 5–39 min, 50–100 % MeOH (linear gradient); flow rate: 0.8 mL/min; detector wavelength: 254 nm. The loniceraflavone was eluted at 20.2 min.

XOD inhibition by loniceraflavone and allopurinol were 0.85 and 0.40 µg/mL, respectively.

Discussion

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Overproduction or underexcretion of uric acid leads to hyperuricemia, which is present in 5–30 % of the general population and is an increasing global concern. Hyperuricemia is considered an important risk factor for gout [12]. Xanthine oxidase (XOD) catalyses the oxidation of hypoxanthine and xanthine to uric acid [13]. XOD inhibitors could block the biosynthesis of uric acid from purine, which is one of the possible therapeutic approaches for hyperuricemia [10, 11]. Among the XOD inhibitors, allopurinol has been the most commonly used in past decades [15], although it is associated with a number of side effects, e.g., hepatitis, nephropathy, allergic reaction and 6-mercaptopurine toxicity [5, 11]. Therefore, there is an urgent need to develop new XOD inhibitors, particularly from less toxic natural sources. Zhao and his co-workers investigated the hypouricemic effects of cassia oil from *C. cassia* using a similar animal model to ours [9]. Cassia oil demonstrated good inhibition of XOD, and significantly reduced the serum uric acid level in hyperuricemic mice at a dose of 450 mg/kg or more. In a previous study, we showed that the essential oil from *Cinnamomum osmophloeum* is a strong XOD inhibitor, with an IC_{50} of 16.3 ± 0.2 µg/mL. The IC_{50} of the major compound, cinnamaldehyde, was 8.4 µg/mL [6]. Unno and his coworkers reported that two active compounds, valoneic acid dilactone ($IC_{50} = 2.5$ µM) and ellagic acid ($IC_{50} = 71.5$ µM) were the principal XOD inhibitory components of *Lagerstroemia speciosa* leaves [10]. In this study, we showed that the ethanol extract and an EA fraction of *L. hypoglauca* also exhibited a significant anti-hyperuricemia effect. The active principle in *L. hypoglauca*, named loniceraflavone, was then isolated from LH-EA by bioactivity guided fractionation.

Loniceraflavone is a new bisflavonoid, which demonstrates a significant XOD inhibitory activity, with an IC_{50} of 0.85 µg/mL. Based on the results presented here, we conclude that the EA extract of *L. hypoglauca* and loniceraflavone are potent inhibitors of XOD, with considerable potential for development as a clinical anti-hyperuricemic agent.

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