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The methanol extract of *Euonymus laxiflorus*, *Rubia lanceolata* and *Gardenia jasminoides* inhibits xanthine oxidase and reduce serum uric acid level in rats

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ABSTRACT

Chinese herbal medicinal plants, *Euonymus laxiflorus* (EL), *Rubia lanceolata* (RL) and *Gardenia jasminoides* (GJ), have been used wildly to treat arthritis and gout in Taiwan for decades. To understand the beneficial effects of these three plants, their xanthine oxidase (XO) inhibitory activity *in vitro* and hypouricaemic activity *in vivo* were investigated. Our results suggested that methanol extracts were better than water extracts for inhibition of XO activity and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, except the water extract of GJ, which exhibited the strongest radical scavenging effect. In animal study, the serum urate level was significantly decreased after oral administration of higher dose (0.39 g/kg) methanol extract of the mixture of three plants (ERG). In addition, methanol extract of ERG reduced the pain reaction time in the second phase of formalin induced pain. The results provide useful information on the pharmacological activities of these plants for the potential in treating hyperuricemia.

1. Introduction

Gout is a common metabolic disorder with a worldwide distribution and continues to be a major health problem. Hyperuricemia, which is associated with gout, results from the overproduction or underexcretion of uric acid in the body. The deposition of urate crystals in the joints and kidneys causes inflammation as well as gouty arthritis and uric acid nephrolithiasis (Kramer and Curhan, 2002; Tomita et al., 2000). More importantly, uric acid is not only related to an increased risk of gout, but also to cardiovascular disorder, nephrolithiasis and diabetes (Colvine et al., 2008; Corrado et al., 2006). It is widely accepted that the control of the uric acid may be considered in the prevention and treatment of these diseases.

Xanthine oxidase (XO), which is present in significant concentration only in liver and intestine, oxidizes hypoxanthine and xanthine to uric acid in the purine catabolic pathway. Treatment and prevention of gout entail to reduce serum uric acid concentration in the body. XO inhibitors are available to block the synthesis of uric acid in the body, as well as anti-inflammatory agents to relieve the symptoms of the disease (Sarkozi, 1996). Allopurinol is the only inhibitor of the enzyme in clinical use, but its use can result in a number of adverse side effects, such as allergic and hypersensitivity reactions, nephropathy, and enhancement of 6-mercaptopurine toxicity (Fels and Sundy, 2008). Thus, the development of new hypouricemic agents of greater effectiveness and safety is highly desirable.

The ability of XO to produce reactive oxygen species (ROS) has led to widespread interest in the enzyme as an initiator of tissue

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damage in a range of pathological states (Bonomini et al., 2008). Hydrogen peroxide (H_2O_2) and superoxide anion radicals ($O_2^{\cdot-}$) can interact in the presence of certain transition metal ions to yield a highly reactive oxidizing species, the hydroxyl radical ($^{\cdot}OH$). Continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body. Free radicals have been regarded as a fundamental cause of different kinds of diseases, including aging, coronary heart disease, inflammation, stroke, diabetes mellitus, rheumatic disease, liver disorders, renal failure and cancer (Bulkley, 1983; Cheng et al., 2003; Dormandy, 1983).

The use of natural products is gaining renewed interest in connection with the treatment of some diseases. Scientists have turned to explore the potent XO inhibitor from a wide variety of traditional folk medicines. To identify potential XO inhibitory agents from natural sources, we have tested 3 selected medicinal plants, which are used by the indigenous people in Taiwan for the treatment of gout. *Gardenia jasminoides* is widely used in several Asian countries as a natural colorant, and has been used in Chinese traditional medicine for its cholagogue, sedative, diuretic, antiphlogistic, homeostatic, and antipyretic effects (Jung et al., 2008; Luo et al., 2007). *Rubia lanceolata* is endemic in Taiwan, and is a perennial climbing herb that grows throughout the island and distributes in forests at middle to high altitudes (Kuo et al., 1995). The root of *Rubia* species is an important Chinese folk medicine which possesses circulation-promoting, expectorant, against cough properties and antitumor activities. The aim of the present study was to investigate the potential of selected medicinal plants to inhibit XO and to act as an antioxidant and free radical scavenging material and examine for their phenolic contents *in vitro*. Moreover, the extract was investigated for its effects on elimination of uric acid in urine *in vivo*. Screening of the extracts for the XO inhibitory activity followed by their potential to reduce the serum urate level may play a crucial role in identifying a potent chemical entity for treating gout and related inflammatory disorders.

2. Materials and methods

2.1. Chemicals and reagents

Xanthine, XO, DPPH (2,2-diphenyl-1-picrylhydrazyl), vitamin E (α-tocopherol), Folin–Ciocalteu reagent, chlorogenic acid, gallic acid, allopurinol, and oxonate (oxonic acid potassium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMSO (dimethyl sulfoxide) and methanol were purchased from Tedia Co. (St. Fairfield, OH, USA). All other chemicals were of analytical reagent grade. Allopurinol was dissolved initially in DMSO (at 0.25% v/v) followed by dilution in PBS solution (phosphate-buffer saline; pH 7.5) to desired concentrations before the use.

2.2. Preparation of Euonymus laxiflorus, Rubia lanceolata and Gardenia jasminoides extracts

Euonymus laxiflorus (EL), *R. lanceolata* (RL) and *G. jasminoides* (GJ) were collected from local Chinese herb markets during the month of April, and identified by one of authors, Prof. Jih-Jung Chen. The voucher specimens have been deposited at the herbarium of Graduate Institute of Pharmaceutical Technology, Tajen University. The voucher specimen number of *E. laxiflorus* is 74279, *R. lanceolata* is 72491, and *G. jasminoides* is 054093, respectively. After being dried and cut up, each and mix of three plants (ERG) were extracted by macerating and refluxing them in methanol and water for 3 h. The ERG is a mixture of equal amounts of three medicinal plants. The extracts respectively are EL/H_2O, RL/H_2O, GJ/H_2O, ERG/H_2O, EL/MeOH, RL/MeOH, GJ/MeOH and ERG/MeOH. Those extracts were filtered for removal of peel particles. After solvent removal in a rotary evaporator (Eyelan-N, Tokyo Rikakikai Co. Ltd., Japan), the extracts of methanol and water obtained were assessed for their biological activities.

2.3. Analysis of total phenolic content

The total phenolic content of the aqueous and methanol extracts of EL, RL, GJ and ERG were analyzed according to the Folin–Ciocalteu method (Cliffe et al., 1994). The reaction solution consisting of 0.2 ml of the extracts, 1 ml of 7.5% Na_2CO_3 reagent and 1 ml of the Folin–Ciocalteu stock reagent (10X) were well mixed and incubated at room temperature in dark for 30 min. The standards were prepared in the same way. The mixture absorbance was measured spectrophotometrically at wavelength 765 nm. The total phenol content was expressed in milligrams of gallic acid or chlorogenic acid equivalents per gram of those extracts.

2.4. DPPH free radical-scavenging assay

Quantitative measurement of radical scavenging properties was carried out in a 96-well plate assay. The free radical-scavenging activity of the aqueous and methanol extracts of EL, RL, GJ and ERG on the DPPH radical was assessed using the method described previously (Hu et al., 2004), with some modifications. A stock solution (1 mg/ml) of each extract was prepared and diluted with methanol to various concentrations. An aliquot of 50 μL of each dilution was transferred into a 96-well microplate (NUNC, Roskilde, Denmark). A working solution of DPPH (250 μM) in methanol was freshly prepared and then an aliquot of 150 μL was added to each well. After incubation for 30 min, the quenching at an absorbance of 517 nm was measured on an ELISA reader (ThermoLabsystems, Cheshire, UK). Each dilution was performed at least in triplicate. Free radical-scavenging activities of test samples and the positive control (Vit. E) were expressed in terms of IC_{50} values, which is the concentration of a sample required to decrease the absorbance at 517 nm by 50% compared to the control response.

2.5. Assay of XO inhibitory activity

The inhibitory effect on XO was assayed spectrophotometrically at 295 nm under aerobic condition (Kong et al., 2000; Nguyen et al., 2004; Unno et al., 2004) with modification by using 96-well plates. The reaction solution consisting of 50 μL test solution dissolved in nanopure water or DMSO, 35 μL PBS (50 mM, pH 7.5), and 30 μL XO (0.1 units/mL in 50 mM PBS, pH 7.5) was prepared immediately before use. DMSO was used for the samples not dissolvable in distilled water. One unit of XO is defined as the amount of enzyme required to produce 1 μmol of uric acid/min at 25 °C. The reaction solution was well mixed and incubated at 25 °C for 15 min. The reaction was initiated by the addition of 60 μL xanthine solution (150 mM) in the same buffer (substrate solution), and incubated for 30 min at the same temperature. The reaction was stopped by adding 25 μL of 1 N HCl, and the absorbance at 290 nm was measured with a Universal Microplate Spectrophotometer SpectraMax190 (Molecular Devices Corporation, Sunnyvale, California, USA). The blank and positive control groups were prepared in the same way, but each group was added 50 μL PBS and allopurinol without test solution.

The XO inhibitory activity was expressed as the percentage inhibition of XO calculated as % Inhibition = $(1 - B/A)$, where A (ΔA_{blank} with enzyme − ΔA_{blank} without enzyme) and B (ΔA_{test} with enzyme − ΔA_{test} without enzyme) are the activities of the enzyme without and with test solution. The crude extracts were dissolved initially in DMSO followed by dilution with PBS, and the final concentration of DMSO was less than 0.25%.

2.6. In vivo studies

The methanol extract of the mix of *E. laxiflorus*, *R. lanceolata* and *G. jasminoides* (ERG/MeOH) produced significantly higher *in vitro* XO inhibitory activity than those of water extract. Thus we selected ERG/MeOH for the screening of uric acid clearance via hyperuricemic rat model *in vivo*.

2.6.1. Preparation of the hyperuricemic rat model

Wistar rats (10 weeks old, 230–280 g) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) following China Medical University Institutional Animals Ethics Committee clearance (99-151-N). The rats were housed in stainless steel wire-bottomed cages and acclimated under laboratory conditions (19–23 °C, humidity of 60%, and 12 h light/12 h dark cycle) for at least one week before each study. Rats were fasted before administration of test substances. The hyperuricemic rat model was prepared by intraperitoneal (i.p.) administration of oxonate (250 mg/kg single dose) according to the method described previously (Yonetani et al., 1980). Oxonate for administration was suspended in 0.5% carboxymethylcellulose (CMC) aqueous solution.

2.6.2. Hypouricaemic activity study

Hyperuricemic rats (i.p. oxonate 250 mg/kg) were assigned to this experiment, six groups of rats (n = 8–10 per group): Group 1 was control group of normal rat. This group was only administered with vehicle 0.5% CMC solution. In group 2, the animals only received 0.39 g/kg ERG/MeOH. Group 3 was intraperitonially administered with potassium oxonate (250 mg/kg) and served as hyperuricemic control. Groups 4 and 5 received the oral treatment with 0.13 g/kg and 0.39 g/kg ERG/MeOH in the hyperuricemic rats, respectively. Group 6 received oral treatment with allopurinol 10 mg/kg as positive control. Each sample was dissolved in 0.5% CMC solution. Blood from orbital vascular plexus was collected at the different times (0, 60, 90, 120, 180, and 270 min). The blood was allowed to clot and serum separated. The serum was separated immediately after the blood collection. The serum urate level was determined using the method described previously (Nguyen et al., 2005; Basah et al., 2011).