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Antioxidant and anti-inflammatory activities of *Lonicera japonica* Thunb. var. *sempervillosa* Hayata flower bud extracts prepared by water, ethanol and supercritical fluid extraction techniques

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ABSTRACT

Lonicera japonica Thunberg (*LJ*) has long been used as an antipyretic, anti-inflammatory and anti-infectious agent in East Asia. The subspecies *L. japonica* Thunb. var. *sempervillosa* Hayata (*LJv*) is a variant that mainly grows in Taiwan. This study examined the antioxidant and anti-inflammatory activities of the extracts from the flower buds of these two species. The extracts were obtained by three extraction methods: water extraction, ethanol extraction, and supercritical-CO_2 fluid extraction (SFE). The antioxidant activities of dry *LJ* (dLJ) extracts were superior to those of *LJv* extracts. Water extracts possessed higher activities than that prepared by ethanol or SFE. The total polyphenols content, total flavonoids content, and the amount of chlorogenic acid and luteolin-7-*O*-glucoside were all higher in the water extracts compared to the other two. The SFE extracts of these two species all exhibited excellent anti-inflammatory activities. Although the water and ethanol extracts of dLJ extracts had higher anti-inflammatory activity than that of *LJv* extracts, the SFE extracts prepared from fresh *LJv* flower buds (fLJv) exhibited the highest activity among all extracts. The SFE effectively isolates the bioactive components of *L. japonica* and can obtain the *L. japonica* extracts with high anti-inflammatory activity.

1. Introduction

Lonicera japonica Thunb. (*LJ*), belonging to the Caprifoliaceae family, is known as Japanese honeysuckle, Jin Yin Hua and Ren Dong. It is a traditional Chinese medicine endowing with antidote, diuretic, tonic, antipyretic, anti-inflammatory and anti-infectious activities. It has been widely used in treating exopathogenic wind-heat, furuncles, epidemic febrile diseases, carbuncles, sores and some infectious diseases. It is also applied to treat chronic enteritis, pneumonia, acute tonsillitis, nephritis, acute mastitis, leptospirosis in some folk prescriptions. Recent uses include the prevention and treatment of human and animal viruses, such as SARS coronavirus and swine H1N1 flu virus (Shang et al., 2011). Several pharmacological studies have shown that *LJ* and its active ingredients possess wide bioactivities, such as antioxidant, anti-inflammatory, antibacterial, antiviral, blood fat reducing, antipyretic and antiendotoxin (Shang et al., 2011). Therefore, in addition to its folk medicinal uses, *LJ* is also used in health food and cosmetics.

More than 150 chemical compounds have been isolated from *LJ*. The major compositions are essential oils, flavones, saponins, iridoids, and organic acids. Chlorogenic acid, luteolin, luteolin-7-*O*-glucoside and essential oils have good pharmacological effects and are believed to be the active ingredients of *LJ* (Ikeda et al., 1994;

Abbreviations: COX, cyclooxygenase; dLJ, dry flower buds of *Lonicera japonica* Thunberg; dLJv, dry flower buds of *L. japonica* Thunb. var. *sempervillosa* Hayata; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ERK, extracellular signal-regulated kinases; fLJv, fresh flower buds of *L. japonica* Thunb. var. *sempervillosa* Hayata; HPX, hypoxanthine; iNOS, inducible nitric oxide synthase; *LJ*, *L. japonica* Thunberg; *LJv*, *L. japonica* Thunb. var. *sempervillosa* Hayata; LPS, lipopolysaccharide; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT, nitroblue tetrazolium; L-NMMA, N^G-monomethyl-L-arginine acetate; PAR2, proteinase activated receptor 2; ROS, reactive oxygen species; RT, retention time; SFE, supercritical-CO_2 fluid extraction; SOD, superoxide; TFC, total flavonoids content; TPC, total polyphenols content; XOD, xanthine oxidase.

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Wang, 2010). Different plant parts, including flowers, flower buds, leaves and whole plant, contain different ingredients and have different activities. Among them, flowers and flower buds of *LJ* are the most frequently used plant parts in Taiwan. However, differences in habitat, harvest time, extraction methods, and flower preparation (fresh *versus* dry) also cause variations in chemical compositions and the quality of *LJ* extract (Shang et al., 2011).

L. japonica Thunb. var. *sempervillosa* Hayata (*LJv*, Mao Rong Tong) is a variant subspecies of *LJ* that mainly grows in Pingtung County and Taitung County, Taiwan (Hayata, 1919). The plant *LJv* resembles *LJ* except for its leaves, which are thinly hairy, densely villose beneath, and its flowers spread a special fresh fragrance. To the best of our knowledge, this study is the first to examine the bioactivities of *LJv*.

The *L. japonica* is usually extracted with water or organic solvents, such as methanol or ethanol. One alternative to traditional extraction by organic solvents is supercritical fluid extraction (SFE). The most commonly used supercritical fluid is CO_2 because of its favorable operating temperature and pressure for extracting thermolabile ingredients. Supercritical CO_2 is also non-toxic, non-flammable, widely available, chemically inert, and has low viscosity, low surface tension, high diffusivity and favorable density (Wang, 2011; Quitain et al., 2013). Compared to conventional organic solvent extraction methods, the advantages of SFE are its non-toxicity and high separation selectivity. Especially, the CO_2 gas used in SFE operation can be easily recycled to avoid the concern of greenhouse effects. Therefore, the use of SFE for extracting natural products is rapidly increasing (King, 2014; Uddin et al., 2015; Yen et al., 2015).

This work compared extracts from dry and fresh flower buds of *LJv* and the dry flower buds of *LJ*. The fresh flower buds of *LJ* were not included because the fresh samples produced at the same area and the same season as the dry flower buds of *LJ* were difficult to purchase throughout the experiment duration. These extracts were prepared with water, ethanol, and supercritical-CO_2 fluid. Their antioxidant, anti-inflammatory activities and their chemical compositions were then analyzed.

2. Materials and methods

2.1. Chemicals and plant materials

(+)-Catechin, chlorogenic acid, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), *Escherichia coli* lipopolysaccharide (LPS), Folin-Ciocalteu reagent, gallic acid, hypoxanthine (HPX), luteolin-7-*O*-glucoside, N^G-monomethyl-L-arginine acetate (L-NMMA), nitroblue tetrazolium (NBT), penicillin, streptomycin, xanthine oxidase (XOD) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco (Grand Island, New York, USA). All chemicals used in this study were of reagent or higher grade.

The reference dry flower buds of *LJ* (dLJ) were purchased from Henan, China. Flower buds of *LJv* (fLJv) freshly harvested from Jhutian Township, Pingtung (Taiwan) were obtained from a local farmer, Mr. Hong Ying. Some of these fresh flower buds were air-dried at 40 °C for 24 h, and then stored at −20 °C (designated as "dLJv"). All samples were harvested in April. The morphological characteristics of *LJv* were compared with those of a sample authenticated by Prof. Den-En Shieh (Department of Food Science and Technology, Tajen University of Technology, Pingtung, Taiwan). A DNA analysis of the plant materials was performed as described in our previous study (Chiou et al., 2007). The similarity of ITS2 sequences between *LJv* and *LJ* exceeded 99%, indicating that these two materials are indistinguishable at the species level.

2.2. Preparation of LJv and LJ extracts

The ethanol and water extracts of dLJv or dLJ were prepared by crushing or drenching 300 g of flower buds in 1800 ml ethanol (50, 75 or 95%) or water for 1 day. After repeating the extraction procedure three times, medicinal gauze was used to filter out insoluble debris. The filtrates were collected and concentrated with a vacuum evaporator. The extract samples were then dried in a freeze-dryer.

The ethanol and water extracts of fLJv were prepared by crushing 800 g of fresh flower buds and drenching them in 4800 ml ethanol (50, 75 or 95%) or water for one day, and repeated this operation for three times. The other operations were the same as above.

The supercritical-CO_2 fluid extracts were prepared by processing the crushed flower buds (1 kg each) in the supercritical fluid extractor (5 l/1000 bar R&D unit, Natex, Ternitz, Austria). The dynamical extractions were performed at 150, 250 and 350 bar at 45 °C for 2 h. The extract samples were dried in a freeze-dryer.

2.3. Determination of antioxidant activity

2.3.1. Determination of scavenging activity on DPPH radicals

The scavenging activity against DPPH free radical was measured using the method of Hsu et al. (2005). In brief, 0.25 ml of 0.5 M DPPH ethanolic solution was mixed with 1.0 ml extract solution in an eppendorf tube. The absorbance at 517 nm was measured after keeping the solution in the dark for 30 min. The control was the measurement using ethanol to replace the extract sample in the reaction solution. The blank was measured by using ethanol to replace DPPH in the solution. After conducting the scavenging activity measurements under different concentrations of samples, the IC_{50} value, i.e. the concentration of sample that causes 50% inhibition, was estimated from the plot of scavenging activity against the sample concentration.

2.3.2. Determination of scavenging activity on superoxide anions

Superoxide anion radicals were generated in a HPX-XOD system by HPX oxidation and assayed by NBT reduction (Hsu et al., 2005). The solutions of NBT (300 μM), HPX (1.1 mM) and XOD (1.67 IU/ml) were prepared separately in a 0.1 M sodium phosphate buffer (pH 7.4). The HPX (760 μl) was mixed with 100 μl NBT and 100 μl extract solution in a 1-ml cuvette. Next, 40 μl XOD was added. The decrease in absorbance at 560 nm was measured every 15 s for 6 min. The rate of decreasing, designated as R_1, was estimated by enzyme kinetic function of the spectrophotometer (Ultrospec 2100 pro, GE Healthcare, Amersham Place, UK). The control was the measurement using phosphate buffer to replace the extract sample in the reaction solution, and the decreasing rate of the absorbance is designated as R_0. The $O_2{\cdot}^-$ scavening activity was determined by the following equation:

$$O_2{\cdot}^- \text{scaveningactivity}(\%) = (R_0 - R_1)/R_0 \times 100\%.$$

2.4. Determination of total polyphenols content

The total amount of phenolic compounds of each extract was determined according to the method of Zielinski and Kozlowska (2000). A 0.15 ml aliquot of the diluted extract solution was mixed with 0.75 ml of 0.2 N Folin-Ciocalteu reagent and 0.6 ml of 7.5% sodium carbonate solution. The mixture was allowed to stand at room temperature for 30 min and then measured at 765 nm with a spectrophotometer. A calibration curve was constructed using gal-