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Anti-inflammatory activity of Korean thistle *Cirsium maackii* and its major flavonoid, luteolin 5-*O*-glucoside

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ABSTRACT

The anti-inflammatory activity of whole *Cirsium maackii* (family Compositae) plants and of its major flavonoid, luteolin 5-*O*-glucoside, was evaluated for their ability to inhibit lipopolysaccharide (LPS)-induced nitric oxide (NO) production, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) protein expression, and *tert*-butylhydroperoxide (*t*-BHP)-induced reactive oxygen species (ROS) generation in RAW 264.7 murine macrophage cells. The methanolic extract of *C. maackii* showed strong anti-inflammatory activity, and was thus fractionated with several solvents. The ethyl acetate-soluble fraction, exhibiting the highest anti-inflammatory activity potential, was further to yield a major flavonoid, luteolin 5-*O*-glucoside. We found that luteolin 5-*O*-glucoside, at a non-toxic concentration, inhibited LPS-induced NO production and *t*-BHP-induced ROS generation in a dose-dependent manner in RAW 264.7 cells. It also suppressed the expression of iNOS and COX-2 in LPS-stimulated macrophages. Furthermore, the efficacies of the methanolic extract of *C. maackii* in inhibiting both NO and ROS were attributed to its flavonoid content by HPLC analysis. These results indicated that *C. maackii* whole plants and its flavonoids inhibit the expression of iNOS and COX-2 in through the inhibition of ROS generation, and therefore can be considered as a useful therapeutic and preventive approach for the treatment of various inflammatory and oxidative stress-related diseases.

1. Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in cells or tissues under physiological and pathological conditions. Excessive ROS and RNS result in tissue damage and vascular leakage, common to conditions such as septicaemia, rheumatoid arthritis, and inflammatory bowel disease, through the activation of inflammatory proteins (Darley-Usmar et al., 1995; Rahman et al., 2006). Moreover, the interaction between ROS and RNS can also lead to the production of highly reactive species such as peroxynitrite, a product of nitric oxide (NO) and superoxide (Ischiropoulos et al., 1992). NO is primarily produced by three isoforms of NO synthase (NOS) enzymes that are encoded on separate chromosomes by separate genes in humans and other organisms (Napoli and Ignarro, 2001; Napoli et al., 2006). Among them, neuronal NOS and endothelial NOS are expressed constitutively, while inducible NOS (iNOS) is expressed in response to interferon-γ, lipopolysaccharide (LPS), and a variety of pro-inflammatory cytokines (Szabo, 1995). In addition to iNOS, macrophages will overproduce inducible cyclooxygenase (COX-2), which is involved in the transformation of arachidonic acid to prostaglandin (PG) H_2, a precursor of PGE_2, prostacyclin, and thromboxan H_2 upon endotoxin stimulation. Enhanced expression of iNOS and COX-2 proteins is associated with an inflammatory response. iNOS and COX-2 play pivotal roles in immunity against infectious agents by producing an excess amount of NO and PGE_2, respectively. These enzymes have attracted attention for

Abbreviations: AMT, 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride; COX-2, cyclooxygenase-2; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorodihydrofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DHR 123, dihydrorhodamine 123; DMEM, Dulbecco's modified eagle's medium; DMSO-d_6, deuterated dimethylsulfoxide; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTPA, diethylenetriaminepentaacetic acid; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HRP, horseradish peroxidase; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoli um bromide; NO, nitric oxide; NOS, nitric oxide synthase; PG, prostaglandin; PVDF, polyvinylidene fluoride; RNS, reactive nitrogen species; ROS, reactive oxygen species; *t*-BHP, *tert*-butylhydroperoxide; TLC, thin layer chromatography; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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their detrimental roles in inflammation-related disease (Yun et al., 1996). Therefore, the inhibition of iNOS and COX-2 expression might be one of the mechanisms by which plants such as *Cirsium maackii*, a variety of Korean thistle, act as anti-inflammatory agents (Hu and Kitts, 2004).

Anti-inflammatory flavonoids derived from botanical sources have been found to possess the bioactivities capable of suppressing the expressions of inflammatory proteins and cytokines through the removal of both ROS and RNS by their antioxidant activities (Heim et al., 2002; Hu and Kitts, 2003, 2004). The extracts from different parts of *C. maackii*, particularly the flavonoid-rich ethyl acetate fraction, exhibited strong antioxidant activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), peroxynitrite, hydroxyl radicals, and total ROS (Jung et al., 2009). In particular, the larger the quantity of two key flavonoids, luteolin and its 5-*O*-glucoside, in the extract and fractions from various thistle parts, the more effective the intensity of the antioxidant activities in the peroxynitrite, hydroxyl radical, and total ROS assays, supporting the findings that these two major flavonoids might be employed as key candidate compounds for further characterization in the antioxidant potential of *C. maackii* (Jung et al., 2009).

The objective of this study was to evaluate the effect of the methanolic extract of the whole plant of *C. maackii* as well as its different solvent-soluble fractions, together with the isolated compound from the most active fraction, on the anti-inflammatory potential through the inhibition of NO and ROS using murine macrophage RAW 264.7 cells activated by bacterial LPS or *tert*-butylhydroperoxide (*t*-BHP). Since *C. maackii* whole plant extract contains a relatively rich source of luteolin 5-*O*-glucoside, we also employed this compound to evaluate its activity on iNOS and COX-2 as well as its inhibitory activity against NO and ROS.

2. Materials and methods

2.1. General experimental procedures

Uncorrected melting points were measured on a melting point apparatus (Mitamura-Riken Kogyo Inc., Tokyo, Japan). All ^{1}H and ^{13}C NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts were referenced to the respective residual solvent peaks (2.50 ppm for ^{1}H NMR and 39.5 ppm for ^{13}C NMR). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm) and RP-18 F_{254s} plates (5 × 10 cm, Merck), using 50% H_2SO_4 as a spray reagent. All solvents for column chromatography were of reagent grade and acquired from commercial sources.

2.2. Chemicals

LPS from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), *t*-BHP, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), diethylenetriaminepentaacetic acid (DTPA), dihydrorhodamine 123 (DHR 123), ethylenediaminetetraacetic acid (EDTA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fetal bovine serum (FBS), and antibiotics were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA), and Dulbecco's Modified Eagle's Medium (DMEM) from Hyclone (Logan, Utah, USA). Various primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Supersignal® West Pico Chemiluminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All chemicals and solvents used were purchased from E. Merck, Fluka, and Sigma–Aldrich Co., unless stated otherwise.

2.3. Plant materials

Whole plants of *C. maackii* were collected in Ulsan, Republic of Korea, in August 2007. Plants were authenticated by Dr. Y. Kadota, a specialist in the area of *Cirsium* taxonomy, at the Department of Botany, National Museum of Nature and Science in Tsukuba, Japan. Voucher specimens of whole plants were registered and deposited in the herbarium of the National Museum of Nature and Science in Tsukuba, Japan as well as the Department of Food Science and Nutrition, Pukyong National University (Professor J.S. Choi).

2.4. Extraction, fractionation, and isolation of C. maackii whole plants

The dried whole plants of *C. maackii* (2.0 kg) were refluxed with MeOH for 3 h (20 L × 3). The total filtrate was then concentrated to dryness *in vacuo* at 40 °C in order to render the MeOH extract (450.0 g). This extract was suspended in distilled H_2O and then successively partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH to yield the CH_2Cl_2 (125 g), EtOAc (72 g), and *n*-BuOH (58.5 g) fractions, respectively, as well as the H_2O residue (188 g). The EtOAc fraction (72.0 g) was repeatedly recrystallized from MeOH to yield compound **1** (8.23 g). This compound was identified and characterized as luteolin 5-*O*-glucoside by spectroscopic methods, including ^{1}H and ^{13}C NMR as well as by comparisons with spectral published data and TLC. The spectral data of luteolin 5-*O*-glucoside are can be obtained from the corresponding authors. The structure is shown in Fig. 1.

Luteolin 5-*O*-β-D-glucopyranoside (**1**): ^{1}H NMR (400 MHz, DMSO-d_6) δ: 10.97, 9.83, 9.39 (aromatic OH), 7.38 (1H, dd, *J* = 9.0, 2.0 Hz, H-6′), 7.36 (1H, d, *J* = 2.0 Hz, H-2′), 6.88 (1H, d, *J* = 9.0 Hz, H-5′), 6.79 (1H, d, *J* = 2.2 Hz, H-8), 6.70 (1H, d, *J* = 2.2 Hz, H-6), 6.55 (1H, s, H-3), 4.71 (1H, d, *J* = 7.3 Hz, Glucose H-1), 3.76 (1H, dd, *J* = 13.0, 2.1 Hz, Glucose H-6a), 3.55 (1H, m, Glucose H-5), 3.54 (1H, m, Glucose H-6b), 3.49 (1H, s, Glucose H-3), 3.30 (1H, s, Glucose H-4), 3.20 (1H, s, Glucose H-2). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 176.95 (C-4), 162.59 (C-2), 161.37 (C-7), 158.68 (C-5), 158.32 (C-9), 149.26 (C-4′), 145.69 (C-3′), 121.52 (C-6′), 118.56 (C-1′), 116.01 (C-5′), 113.12 (C-2′), 108.25 (C-10), 105.70 (C-3), 104.52 (Glc C-1), 104.33 (C-6), 98.20 (C-8), 77.56 (Glc C-3), 75.62 (Glc C-5), 73.65 (Glc C-2), 69.70 (Glc C-4), 60.86 (Glc C-6).

2.5. HPLC quantitative analysis

Reverse-phase HPLC was performed on a JASCO HPLC system (Tokyo, Japan), consisting of a PU-1580 Intelligent HPLC pump, LG-1580-04 quaternary gradient unit, UV-1575 intelligent UV/vis detector, PG-1580-54 four-line degasser, and CO-1560 intelligent column thermostat. The BORWIN chromatographic system (Le Fontanil, France) was used for HPLC data analysis. Chromatographic separation was accomplished on a Phenomenex C18 reverse-phase column (Phenomenex, 4.6 × 250 cm, 5 μm) at 25 °C, and monitored at 340 nm. A linear gradient solvent system consisted of 0.5% phosphoric acid in water (solvent A) and 100% methanol (solvent B) and was adjusted from 75% (solvent A): 25% (solvent B) to 0% (solvent A) and 100% (solvent B) over 60 min at a flow rate of 0.5 ml/min. For preparation of stock solutions, plant extracts and ten flavonoids were dissolved in 100% MeOH at concentrations of 4 and 2 mg/ml, respectively. After filtration through a centrifugal filter device (0.45 μm, Millipore Co., Bedford, MA, USA), 10 μL of each sample was injected. Kaempferol was used as the internal standard. The retention time of **1** was approximately 28.20 min. The calibration curve of **1** was drawn using five standards at concentrations ranging from 10 to 500 μg/ml. The regression equation was calculated in the form of $y = ax + b$, where *y* and *x* correspond to the peak area and concentration, respectively. The regression equation and correlation coefficient (r^2) of **1** are as follows: $y = 25267x + 831222$, $r^2 = 0.9959$. The relative quantity of **1** in the MeOH extract (mg/g of the extract) was calculated from this equation.

2.6. Cell culture

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with humidified air containing 5% CO_2.

2.7. Assay for cell viability

Cell viability was assessed using the MTT assay as described previously (Mossman, 1983). To summarize, RAW 264.7 cells were seeded into a 96-well plate at a density of 1.0 × 10^4 cells per well and incubated at 37 °C for 24 h. The cells were

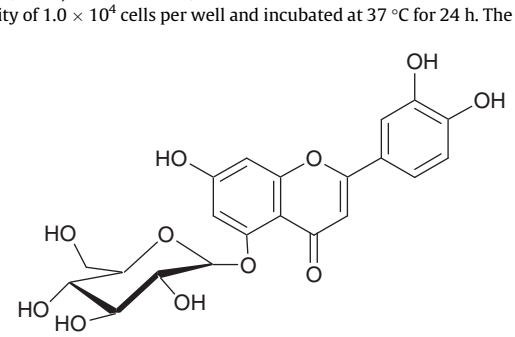

Fig. 1. Structure of luteolin 5-*O*-glucoside.