Anti-inflammatory and Radical Scavenging Effects of *Spirodela polyrrhiza*

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Abstract – Anti-inflammatory and anti-oxidant effect of *Spirodela polyrrhiza* (Lemnaceae), a widely used traditional medicinal plant were investigated. In macrophages nitric oxide (NO) is released as an inflammatory mediator and has been proposed to be an important modulator of many pathophysiological conditions including inflammation. 85% MeOH extracts of *S. polyrrhiza* (0.01, 0.1, 1 mg/mL) suppressed nitric oxide production in interferone-γ (IFN-γ) and lipopolysaccharide (LPS)-stimulated macrophages. It also attenuated the expression of inflammatory enzymes like inducible NOS (iNOS) and cyclooxygenase-2 (COX-2) as assessed by immunoblotting with specific antibodies. Moreover, the values obtained with DPPH radical, superoxide anion and NO radical scavenging assay showed that *S. polyrrhiza* has potent antioxidant properties as a natural ROS scavenger. The results of the present study suggest the potential use of *S. polyrrhiza* in the treatment of ROS-mediated chronic inflammatory diseases such as atherosclerosis and rheumatoid arthritis.

Keywords – *Spirodela polyrrhiza*, Nitric Oxide, iNOS, COX-2, Antioxidant

Introduction

Inflammation is a complex pathophysiological process mediated by various cells including macrophages, neutrophils, mast cells and platelets (Saha *et al.*, 2004). Macrophages concerned in not only natural immunity but specific acquired immunity and play a crucial role in inflammation as a major immune cell. Under inflammatory conditions, macrophages may generate large amount of nitric oxide (NO) and superoxide anion.

NO, a toxic free radical, is synthesized from L-arginine, by a family of nitric oxide synthase (NOS). Nanomolar concentration of NO generation by constituent NOS (eNOS) act as neurotransmitter and vasodilator (Nakagawa and Yokozawa, 2002). On the other hand, inducible NOS (iNOS) mediated mass NO production has been reported to cause cytotoxicity and tissue damage (Kim *et al.*, 1999). Moreover, the generation of free radicals including superoxide anion in macrophages is one of the important process in inflammation (D’Acquisto *et al.*, 2002). Therefore, radical scavenging activity may be a therapeutic target in cellular injury and dysfunction observed in inflammatory disorders (Conner and Grisham, 1996).

Cyclooxygenase-2 (COX-2), an inducible enzyme, catalyze of PGE$_2$ from arachidonic acid (Surh *et al.*, 2001). Several reports demonstrated that overproduction of PGE$_2$ by COX-2 are in close connection with NO generation (Chang *et al.*, 2006). Thus, COX-2 mediated PGE$_2$ production take important part in the process of inflammation (Park *et al.*, 2004).

*Spirodela polyrrhiza* Schleider (Lemnaceae) is an aquatic plant called ‘duckweed’ which is distributed throughout Korea and China. The whole plant of *S. polyrrhiza* is an oriental drug, used in therapy to counteract the diseases as a cold, edema, acute nephritis and urticaria. Previous phytochemical studies of this plant have shown the presence of sterol, anthocyanin and flavonoid such as vitexin, orientin, cynaroside (Wallace 1975; Suh *et al.*, 1969; Harborne 1986). Pharmacological studies has been indicated that *S. polyrrhiza* has anticoagulant (Choi *et al.*, 2001), gastroprotective (Khasina *et al.*, 2003), immunomodulatory (Ovodova *et al.*, 2000) and inhibitory activity on immediate hypersensitivity (Kim *et al.*, 2004).

In view of the several reports described above, *S. polyrrhiza* was proposed to have immune-suppressive properties. However, the anti-inflammatory activity of *S. polyrrhiza* in IFN-γ and LPS-stimulated macrophage is still unclear. Thus, in this study, the radical scavenging activity and inhibitory effect of *S. polyrrhiza* on pro-

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inflammatory mediators like NO, iNOS and COX-2 were investigated.

**Experimental**

**Preparation of the stem and leaves of S. polyrrhiza** – The plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in April 2007. A voucher specimen (WME048) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woonsuk University. An extract was obtained twice from the dried sample (150 g) with 7,000 mL of 85% MeOH under ultrasonification for 2 h. It was evaporated and lyophilized to yield an MeOH extract of S. polyrrhiza (Yield : 9.91%), which was then stored at –20°C until use.

**1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay** – The scavenging effect of S. polyrrhiza on DPPH radical was measured according to the method of Gyamfi et al. (Gyamfi et al., 1999) with some modification. A 5 µL aliquot of the different concentrations of S. polyrrhiza were added to 495 µL of DPPH in absolute ethanol solution (0.25 mM). After incubation for 20 min, the absorbance of each solution was determined at 520 nm using microplate reader (GENios, Tecan).

**Superoxide scavenging by NBT method** – The superoxide scavenging ability of S. polyrrhiza was studied by xanthine/xanthine oxidase/NBT method according to Ibrahim et al. (Ibrahim et al., 2007) with some modification. The reaction mixture contained 0.5 mL of 1.6 mM xanthine, 0.48 mM NBT in 10 mM phosphate buffer (pH 8.0). After pre-incubation at 37°C for 5 minutes, the reaction was initiated by adding 1 mL of xanthine oxidase (0.05 U/mL) and incubation at 37°C for 20 min. The reaction was stopped by adding 1 mL of 69 mM SDS, and the absorbance at 570 nm was measured.

**Nitric oxide radical scavenging assay** – A 5 µL aliquot of the different concentrations of S. polyrrhiza were added to 495 µL of sodium nitroprusside solution (5 mM). After incubation at room temperature for 150 min, 100 µL aliquots were removed from reaction mixture and incubated with an equal volume of Griess reagent (1% sulfinilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, 2.5% H₃PO₄). The absorbance at 540 nm was determined and the standard was determined by using sodium nitrite.

**Peritoneal macrophage culture** – TG-elicited macrophages were harvested 3–4 days after i.p. injection of 2.5 mL TG to the mice and isolated. Using 8 mL of HBSS containing 10 U/mL heparin, peritoneal lavage was performed. Then, the cells were distributed in DMEM, which was supplemented with 10% heat-inactivated FBS, in 24-well tissue culture plates (3 × 10⁵ cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO₂, washed three times with HBSS to remove non-adherent cells and equilibrated with DMEM that contained 10% FBS before treatment.

**MTT assay** – Cell respiration, an indicator of cell viability, was performed by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, as described by Mosmann (Mosmann, 1983). The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density (OD) at 540 nm using an automated microplate reader (GENios, Tecan, Austria).

**Assay of nitrite concentration** – Peritoneal macrophages (3 × 10⁵ cells/well) were cultured with various concentrations of S. polyrrhiza. The cells were then stimulated with rIFN-γ (20 U/mL). After 6 h, the cells were finally treated with LPS (10 µg/mL). NO synthesis in cell cultures was measured by a microplate assay method. To measure nitrite, 100 µL aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent at room temperature for 10 min. The absorbance at 540 nm was determined by an automatic microplate reader. NO₃⁻ was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 M of NO₂⁻. This value was determined in each experiment and subtracted from the value obtained from the medium with peritoneal macrophages.

**Western blot analysis** – Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk for 2 h at room temperature and then incubated with anti-iNOS (SantaCruz, CA, USA). After washing in with phosphate buffered saline (PBS) containing 0.05% tween 20 three times, the blot was incubated with secondary antibody (anti-rabbit, anti-mouse) for 1 h and the antibody specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp. Newark, NJ, Germany).

**Statistical analysis** – All measurement are expressed as the mean ± S.E.M. of independent experiments. Data between groups were analyzed by a paired Student’s t-test and P-values less than 0.001 were considered significant.