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

Hoon Jeon[1,2,*]

[1]*College of Pharmacy, Woosuk University, Jeonbuk 565-701, Republic of Korea*
[2]*Center for Healthcare Technology Development, Jeonbuk 561-756, Republic of Korea*

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 lipopoloysaccharide (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 (cNOS) 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 polyrhiza 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-

*Author for correspondence
Tel: +82-63-290-1577; E-mail: hoonj6343@hanmail.net

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, Woosuk 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 ºC until use.

1,1-diphenyl-2-picrylhydrasyl (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 ºC for 5 minutes, the reaction was initiated by adding 1 mL of xanthine oxidase (0.05 U/mL) and incubation at 37 ºC 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% sulfanilamide, 0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride, 2.5% H_3PO_4). 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 (3×10^5 cells/well) incubated for 3 h at 37 ºC in an atmosphere of 5% CO_2, 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-(3,4-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 (3×10^5 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. NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 M of NO_2^-. 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 chemiluminesence 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.

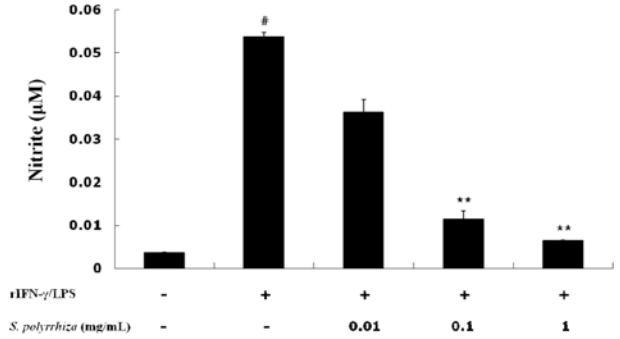

Fig. 1. Effects of *S. polyrrhiza* on NO inhibition in rIFN-γ and LPS-stimulated peritoneal macrophages. NO release was measured by the Griess method (nitrite). NO released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicate in each run; # and ** represent statistically differences from control group and rIFN-γ/LPS treated group respectively ($^{\#}p < 0.001$, $^{**}p < 0.001$).

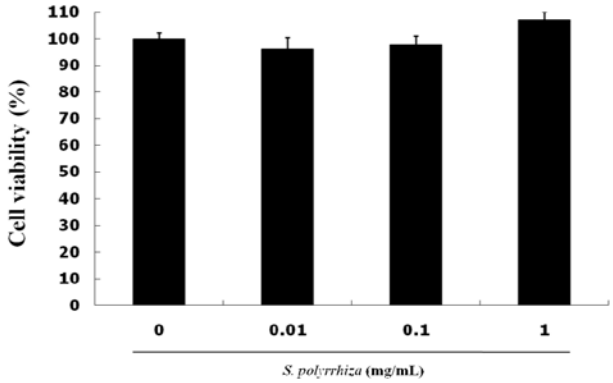

Fig. 2. Effects of *S. polyrrhiza* on the viability in mouse peritoneal macrophages. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means ± S.E.M. of three independent experiments duplicate in each run.

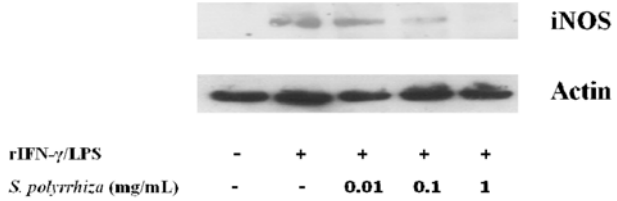

Fig. 3. Effects of *S. polyrrhiza* on the expression of iNOS in rIFN-γ and LPS-stimulated peritoneal macrophages. The protein extracts were prepared; samples were analyzed for iNOS expression by western blotting as described in the method.

Results and Discussion

Nitric oxide (NO) is a free radical produced from L-arginine by NO synthase (NOS) and maintaining diverse physiological homeostasis (Seo *et al*., 2001). However excess NO cause many inflammatory diseases such as septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases (Thiemermann and Vane, 1990). Therefore, to avoid overproduction of NO, the use of exogenous modulators becomes necessary. In this study, we used IFN-γ and LPS stimulated mouse peritoneal macrophage system to assess an inhibitory activities of *S. polyrrhiza* on the pro-inflammatory mediators. As shown in Fig. 1. nitrite assay, determined by Griess method, indicated that the inhibition rates of NO production by *S. polyrrhiza* were 35.1%, 84.5%, and 94.4%, at the concentration of 0.01, 0.1, 1 mg/mL respectively. Fig. 2 shows the potent inhibitory action of *S. polyrrhiza* on NO production is not due to cytotoxicity.

NO produced by one of three kinds of NOS that neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS). nNOS and eNOS were critical to normal physiology and thus, inhibition of these enzymes caused damage. In the contrary, the level of iNOS playing a crucial role of excess production of NO in activated macrophages. Therefore, suppression of NO production via inhibition of iNOS expression levels might be an attractive therapeutic target for the treatment of inflammation (Singh *et al.*, 2000). Thus, we evaluated the effect of *S. polyrrhiza* on iNOS expression using western blotting analysis to confirm the mechanism on the regulation of NO production. According to the Fig. 3. *S. polyrrhiza* suppressed iNOS induction significantly without changes in the levels of β-actin, a control, and this result suggest that the inhibition of NO production by *S. polyrrhiza* was caused by a down-regulation of the iNOS protein level.

Cyclooxygenase-2 (COX-2), another key mediator in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins E_2 (PGE_2) from arachidonic acid. Levels of PGE_2 increase early in the course of the inflammation (Wallace, 1999). Since, COX-2 is induced by stimulation in inflammatory cells, inhibitors of COX-2 induction might candidates for the new type of nonsteroidal anti-inflammatory drugs (NSAIDs). Based on the data in Fig. 4. *S. polyrrhiza* slightly suppressed the COX-2 expression but it is not so good as an effect on iNOS level.

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are highly reactive molecules and can damage lipids, proteins and DNA with oxidative stress (Halliwell, 1994). In addition to promoting direct toxicity, ROS may also initiate and amplify inflammation via the upregulation of several genes involved in the inflammatory response through nuclear

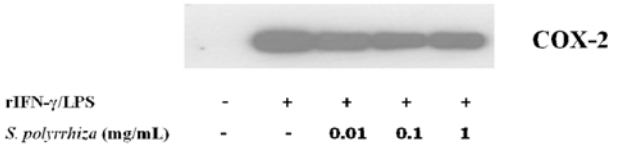

Fig. 4. Effects of *S. polyrrhiza* on the expression of COX-2 in rIFN-γ and LPS-stimulated peritoneal macrophages. The protein extracts were prepared; samples were analyzed for COX-2 expression by western blotting as described in the method.

factor-kappa B (NF-κB) pathway (Conner and Grisham, 1996; Watt, 1979). Therefore, it is becoming apparent that chronic inflammatory diseases such as atherosclerosis, rheumatoid arthritis, inflammatory bowel disease (IBD) are directly or indirectly mediated by ROS (Bonomini *et al*., 2008; Kaplan *et al*., 2007).

Thus, antioxidants, a free radical scavenger can contribute for ROS mediated-inflammatory diseases, at least in part inhibiting NF-κB activation (Schreck *et al.,* 1992). The radical scavenging activity of *S. polyrrhiza* was determined from the reduction of absorbance at 520 nm due to scavenging of stable DPPH free radical. *S. polyrrhiza* exhibited strong scavenging capacity compared with ascorbic acid (Fig. 5).

We also investigated scavenging effect of *S. polyrrhiza* on superoxide anion using X/XO/NBT system. *S. polyrrhiza* was able to inhibit the formazan formation from NBT react with superoxide anion ($\bullet O_2^-$) generated by xanthine oxidase system in a concentration dependent manner. This result suggests that *S. polyrrhiza* has potent scavenging capacity on superoxide anions (Fig. 5). In Haber-Weiss reaction, superoxide anion reacts with H_2O_2 to form hydroxyl radical (•OH) which is a more toxic radical leading to aging and chronic diseases. According to the report by Wang *et al*. (Wang *et al.,* 2007), the total flavonoid from *S. polyrrhiza* increased ablility of scavenging H_2O_2 and SOD, CAT, GPX activities. Thus, *S. polyrrhiza* may be relevant in the prevention of the formation of hydroxyl radical.

Moreover, the scavenging effect of *S. polyrrhiza* on NO radical was also carried out. As shown in Fig 5. *S. polyrrhiza* exhibited significant SNP-induced NO radical scavenging activity. This observation implied that inhibitory effect of *S. polyrrhiza* on NO production in IFN-γ and LPS stimulated macrophages has a dual mechanism involving directly scavenging of NO radical and indirectly down-regulation of iNOS expression. In addition, NO reacts rapidly and voluntarily with superoxide anion to form a peroxynitrite anion ($ONOO^-$). Peroxynitrite is more toxic than NO or superoxide anion

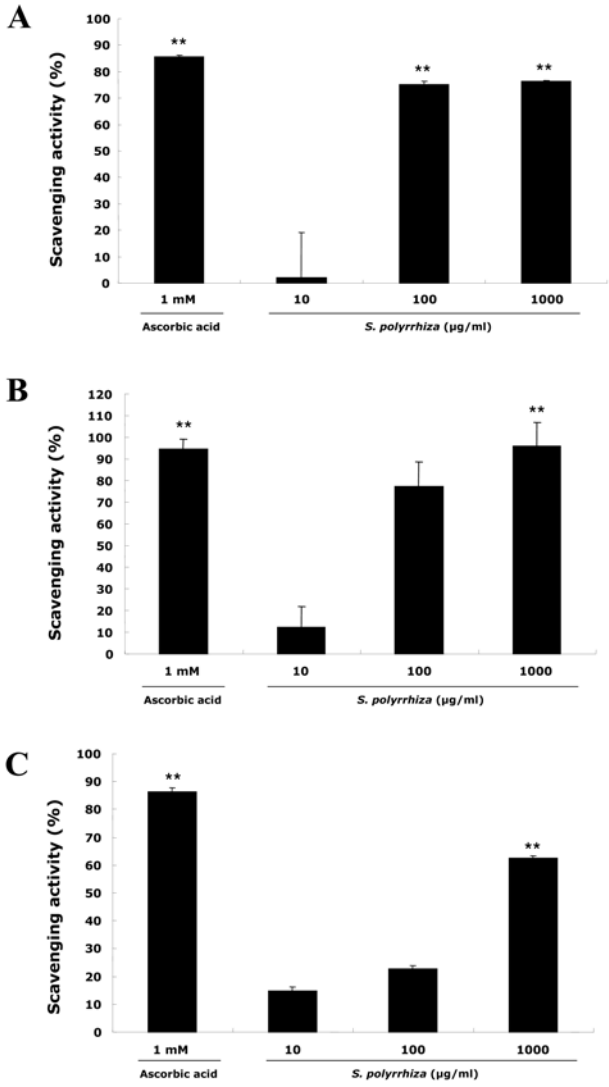

Fig. 5. Radical scavenging activities of *S. polyrrhiza* on the DPPH radical (A), superoxide anion (B) and NO radical (C). The radical scavenging activities are presented as the mean ± S.E.M. of three independent experiments duplicate in each run; ** represent statistically differences from vehicle treated group (**$p < 0.001$).

by causing damage of protein (Van der Vliet *et al.,* 1995) or nucleic acid (Yermilov *et al.,* 1995). In this study, *S. polyrrhiza* showed potent scavenging activity both on the NO radical and superoxide anion and these data suggest that *S. polyrrhiza* may decrease the peroxynitrite formation.

In conclusion, *S. polyrrhiza* showed strong inhibitory effect on NO production by attenuating of iNOS expression and slight inhibitory activity on COX-2 expression in IFN-γ and LPS stimulated mouse peritoneal macrophages. *S. polyrrhiza* also possesses a strong scavenging capacity on DPPH radicals, superoxide anions, nitric oxide and these results may contribute to the prevention of the more

toxic radicals like hydroxyl radical and peroxynitrite formation. Therefore, the present study clearly revealed that *S. polyrrhiza* has potent antioxidant and anti-inflammatory effects and may hold great promise for use in ROS-mediated chronic inflammatory diseases including atherosclerosis, rheumatoid arthritis as an effective immunomodulatory material. However, further study, an *in vivo* experiment, is required to demonstrate the efficiency of the obtained data.

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