

Antioxidant and Anti-inflammatory Activities of *Equisetum hyemale*

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Abstract – *Equisetum hyemale* L. has been prescribed widely as a traditional medicine for the treatment of inflammatory diseases such as rheumatoid arthritis, conjunctivitis, pyelonephritis. In order to identification the mechanism, we examined an antioxidant and anti-inflammatory activity of 85% methanol extract of *E. hyemale*. In this study *E. hyemale* exhibited strong scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, superoxide radical, and nitric oxide. To elucidate the anti-inflammatory properties of *E. hyemale*, we investigated the inhibition effects of nitric oxide and IL-6 by *E. hyemale* in IFN-gamma and LPS-stimulated mouse peritoneal macrophages. *E. hyemale* suppressed nitric oxide, IL-6 production and iNOS expression dose-dependently without notable cytotoxic activity. These data suggest that *E. hyemale* might be useful in inflammatory diseases by inhibiting the free radicals and inflammatory mediators.

Keywords – *Equisetum hyemale*, Antioxidant, Anti-inflammatory

Introduction

Inflammation is characterized by redness, heat, swelling, pain and dysfunction of the organs. It is a complex physiological and pathological process mediated by cytokines produced by various cells including neutrophils, macrophages, mast cells, platelets (Saha *et al.*, 2004). Many types of autoimmune diseases and allergies such as asthma, rheumatoid arthritis and multiple sclerosis are example of excessive inflammatory responses (Rakel and Rindfleisch, 2005).

Macrophages play a central role in host defense and maintenance as a major immune cell in inflammation, since they are concerned in not only natural immunity but specific acquired immunity. Lipopolysaccharide (LPS) is a component of the outer cell membrane of gram-negative bacteria. It is an endotoxin, which induces septic shock and stimulates the production of inflammatory mediators such as nitric oxide (NO), interleukins, prostanoids and leukotrienes (Chen *et al.*, 2005; Erridge *et al.*, 2002; Hewett *et al.*, 1993). The stimulation of macrophages with LPS also induces expression of the inducible isoform of nitric oxide synthase (iNOS) (Cao *et al.*, 2006).

Moreover, the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in phagocytic leukocytes, such as macrophages, neutrophils and mono-

cytes, is one of the important process in inflammation (D'Acquisto *et al.*, 2002). Therefore, antioxidant may provide a therapeutic approach in cellular injury and dysfunction observed in inflammatory disorders (Conner and Grisham, 1996).

Since ROS, NO production, related enzymes, pro-inflammatory cytokines might cause inflammatory damage, many studies about inflammation focused to find materials which selective modulate these free radicals and inflammatory mediators from traditional plant-derived medicines (Lee *et al.*, 2005).

The dried stem of *Equisetum hyemale* L. (Equisetaceae) is an oriental drug, which have been used in therapy to counteract the problems associated with inflammatory-mediated eye diseases. The chemical constituents and biological activities of *E. hyemale* remain unknown. However, there are some reports about allied plants, *Equisetum arvense* L. called field horsetail. The major types chemical constituent, flavonoid glycosides (Syrchina *et al.*, 1980), phenolic acid (Syrchina *et al.*, 1978), sterol (D'Agostino *et al.*, 1984) and brassinosteroid (Takatsuto *et al.*, 1990), were analyzed from *E. arvense* and have been found to possess anti-hyperlipemia (Xu *et al.*, 1993), antioxidant, antimicrobial, genotoxicity (Milovanoviæ *et al.*, 2007), antinociceptive and anti-inflammatory activities (Do Monte *et al.*, 2004). In view of the several reports of *E. arvense* described above, *E. hyemale* was also proposed to have antioxidant and anti-inflammatory properties.

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Thus, the aim of this work is to investigate radical scavenging effect and inhibition effect of the *E. hyemale* against NO and cytokines such as IL-6 in LPS stimulated mouse peritoneal macrophages. For clear the evident mechanism of NO suppression, we also assessed the effect of *E. hyemale* on the expression level of iNOS.

Experimental

Preparation of the stem and leaves of *E. hyemale* –

The plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in March 2006. A voucher specimen (WME039) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained twice from the dried sample (250 g) with 6,000 mL of 85% MeOH under ultrasonification for 2 h. It was evaporated and lyophilized to yield an MeOH extract of *E. hyemale* (Yield : 0.95%), which was then stored at -20°C until use.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay – The scavenging effect of *E. hyemale* 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 *E. hyemale* 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 *E. hyemale* 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 *E. hyemale* 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-naphthyl)-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 macro-

phages were harvested 34 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°C 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 *E. hyemale*. The cells were then stimulated with rIFN- γ (20 U/mL). After 6 h, the cells were finally treated with LPS (10 $\mu\text{g}/\text{mL}$). 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.

Assay of cytokine release – Peritoneal macrophages (3×10^5 cells/well) were treated with various with concentrations of *E. hyemale*. The cells were then stimulated rIFN- γ (20 U/mL) plus LPS (10 $\mu\text{g}/\text{mL}$) and incubated for 24 h. IL-6 in supernatants from the cells (3×10^5 cells/mL, culture medium DMEM with 10% FBS) were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer's protocol. Absorption of the avidin-horseradish peroxidase color reaction was measured at 405 nm and compared with serial dilutions of mouse IL-6 recombinant as a standard.

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