Natural Product Sciences
14(4) : 239-243 (2008)

Antioxidant and Anti-inflammatory Activities of *Equisetum hyemale*

Eun Young Park[1] and Hoon Jeon*

College of Pharmacy, Woosuk University, Chonbuk, Korea
[1]Department of Rehabilitation, College of Alternative Medicine, Jeonju University, Chonbuk, Korea

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 monocytes, 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.

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

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-picrylhydrasyl (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-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 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 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.

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 μg/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

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 0.01 were considered significant.

Results and Discussion

Free radicals such as ROS ($\bullet O_2^-$, •OH, H_2O_2) are highly reactive molecules and generated during normal metabolism process under aerobic conditions. Since they can damage lipids, proteins and DNA with oxidative stress, the body has developed several endogeneous defence mechanism. However, an imbalance of prooxidants and antioxidants in the organism can cause tissue damage and cell death may result (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 (Watt, 1979) and 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 are useful targets for ROS mediated-inflammatory diseases.

The radical scavenging activity of *E. hyemale* was determined from the reduction of absorbance at 520 nm due to scavenging of stable DPPH free radical. *E. hyemale* exhibited strong scavenging capacity compared with ascorbic acid (Table 1). We also investigated scavenging effect of *E. hyemale* on superoxide anion using X/XO/NBT system. In the present study, *E. hyemale* showed significant inhibitory effects on formazan formation from NBT react with superoxide anion ($\bullet O_2^-$) generated by xanthine oxidase system. This result suggests that *E. hyemale* has potent scavenging capacity on superoxide anions (Table 1).

The values obtained with DPPH assay and X/XO/NBT assay showed that *E. hyemale* has antioxidant properties as natural ROS scavenger and this result is supported by several reports about the antioxidant activities of *E. arvense*, an allied plants of *E. hyemale*. Oh *et al.* noted that onitin and luteolin from *E. arvense* showed strong superoxide and DPPH radical scavenging effects (Oh *et al*., 2004) and it is also demonstrated that inhibitory activity for superoxide anion and hydroxyl radical formation (Oka *et al*., 2007).

Nitric oxide (NO) is a free radical produced from L-arginine by NOS and has diverse physiological roles and contributes to the immune defense against viruses, bacteria and other parasites (Seo *et al*., 2001). However, large amount of NO act as toxic radical and can cause tissue and cell damage. In the present study, the scavenging effect of *E. hyemale* on NO was carried out. As shown Table 1, 5 mM SNP produced 160 μM of NO and *E. hyemale* in the range of 10 to 1000 μg/mL showed dose-dependent NO scavenging activity.

In macrophage, over production of NO is recognized to play a central role in the pathogenesis of inflammation and result in septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases (Thiemermann and Vane, 1990). Therefore, to avoid NO production, the use

Table 1. Scavenging capacity of *E. hyemale* in different free radical scavenging tests

Treatment	Concentration	Radical scavenging capacity[a] (% Inhibition)		
		DPPH	X/XO/NBT	Nitric oxide
Ascorbic acid[b] (μM)	10	25.73 ± 6.86	–	8.66 ± 1.29
	100	85.53 ± 0.71	–	86.28 ± 1.47
	1000	85.94 ± 0.75	94.59 ± 4.54	97.97 ± 0.11
E. hyemale (μg/mL)	10	4.33 ± 3.25	0.85 ± 1.16	1.58 ± 0.95
	100	24.29 ± 10.41	25.96 ± 7.51	20.72 ± 1.40
	1000	71.98 ± 2.50	94.00 ± 9.60	78.96 ± 0.32

(–) Means not done
[a] Each value in the table represents the mean ± S.D of three parallel measurements
[b] Ascorbic acid used as positive control

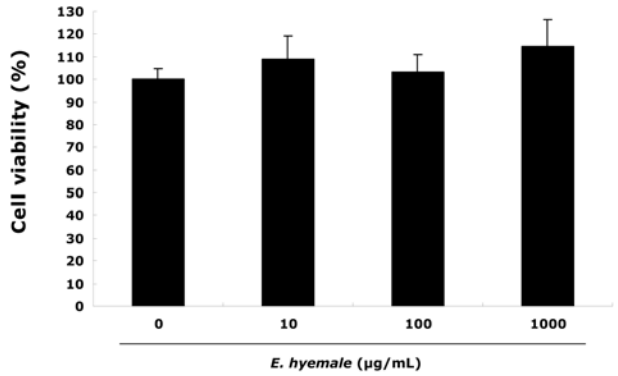

Fig. 1. Effects of *E. hyemale* on the viability in mouse peritoneal macrophages. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means ± S.E.M. of three independent experiments duplicate in each run.

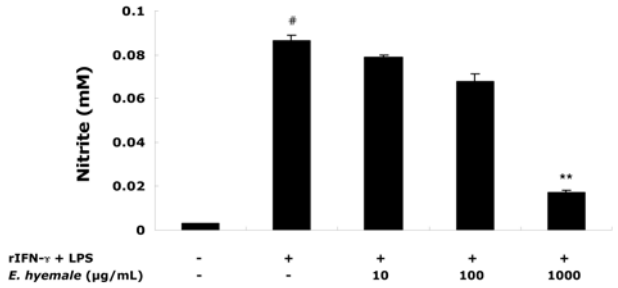

Fig. 2. Effects of *E. hyemale* on NO inhibition in rIFN-γ and LPS-stimulated peritoneal macrophages. NO release was measured by the Griess method (nitrite). NO released into the medium is presented as the mean ± S.E.M. of three independent experiments duplicate in each run; # and ** represent statistically differences from control group and rIFN-γ/LPS treated group respectively ($^{\#}p < 0.001$, $^{**}p < 0.001$).

of exogenous modulators becomes necessary.

At this point of view, we also evaluated the effect of *E. hyemale* on NO production in IFN-γ and LPS stimulated mouse peritoneal macrophages. In this study, IFN-γ and LPS-induced NO production was inhibited dose-dependently by *E. hyemale* without notable cytotoxicity (Fig. 1, 2).

NO produced by one of three kinds of NO synthase (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. Thus the possibility that *E. hyemale* might inhibit iNOS expression was examined and *E. hyemale* suppressed the expression of iNOS significantly (Fig. 3).

Cytokines are the physiological messengers of the

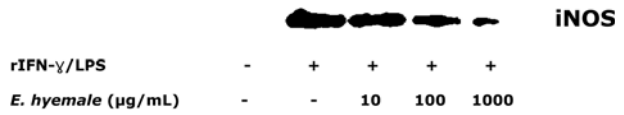

Fig. 3. Effects of *E. hyemale* on the expression of iNOS in rIFN-γ and LPS-stimulated peritoneal macrophages. The protein extracts were prepared; samples were analyzed for iNOS expression by western blotting as described in the method.

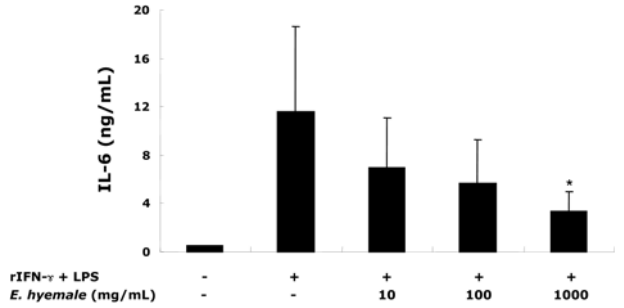

Fig. 4. Effects of *E. hyemale* on IL-6 production in rIFN-γ and LPS-stimulated peritoneal macrophages. The amount of IL-6 secretion in mouse peritoneal macrophages was measured by ELISA method. Data represent mean ± S.E.M. ** represent statistically differences from rIFN-γ /LPS treated group ($^{*}p < 0.01$).

inflammatory response and some of the principal molecules involved inflammation is interleukin-6 (IL-6). It is reported that IL-6 is considered to play an important role in inflammatory and immune response, based on their occurrence at inflammatory sites and their ability to induce many hallmarks in the inflammatory response (Park *et al*., 2002). We investigated the inhibitory effect of *E. hyemale* on IL-6 production in mouse peritoneal macrophages. rIFN-γ/LPS activated macrophages secreted IL-6 and *E. hyemale* showed inhibitory effect on IL-6 secretion (Fig. 4).

Similar to its antioxidant properties, Oka *et al.* (Oka *et al*., 2007) demonstrated *in vivo* anti-nociceptive and anti-inflammatory effect of *E. arvense*, and this finding implies the possibility that *E. hyemale* may suppress the pro-inflammatory mediators. Here in our study, we have shown that *E. hyemale* exerts its anti-inflammatory effects by inhibition of pro-inflammatory mediators such as NO, iNOS and IL-6.

In summary, the production of ROS and pro-inflammatory mediators like NO, iNOS and IL-6 takes an important part of the immune response to many inflammatory stimuli. Nonetheless, excessive overproduction of these ROS and mediators are implicated in acute and chronic inflammatory diseases including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis and atherosclerosis (Bertolini *et al*., 2001). The present study clearly revealed that *E. hyemale*

had strong scavenging capacity on DPPH radicals, superoxide anions and nitric oxide and *E. hyemale* also showed inhibitory effect on pro-inflammatory mediators such as NO, iNOS and IL-6. In conclusion, these results establish that *E. hyemale* has potent antioxidant and anti-inflammatory effects, and may hold great promise for use in ROS-mediated chronic inflammatory diseases as an effective immunomodulatory material.

References

Bertolini, A., Ottani, A., and Sandrini, M., Dual acting anti-inflammatory drugs: a reappraisal. *Pharmacol. Res.* **44**, 437-450 (2001).

Bonomini, F., Tengattini, S., Fabiano, A., Bianchi, R., and Rezzani, R., Atherosclerosis and oxidative stress. *Histol Histopathol.* **23**, 381-390 (2008).

Cao, X.Y., Dong, M., Shen, J.Z., Wu, B.B., Wu, P.M., Du, X.D., Wang, Z., Qi, Y.T., and Li, B.Y., Tilmicosin and tylosin have anti-inflammatory properties via modulation of COX-2 and iNOS gene expression and production of cytokines in LPS-induced macrophages and monocytes. *Int. J. Antimicrob. Agents* **27**, 431-438 (2006).

Chen, Y.J., Hsu, K.W., Tssai, J.N., Hung, C.H., Kuo, T.C., and Chen, Y.L., Involvement of protein kinase C in the inhibition of lipopoly-saccharide-induced nitiric oxide production by thapsigargin in RAW 264.7 macrophages. *Int. J. Biochem. Cell Biol.* **37**, 2574-2585 (2005).

Conner, E.M. and Grisham, M.B., Inflammation, free radicals, and antioxidants. *Nutrition.* **12**, 274-277 (1996).

D'Acquisto, F., May, M.J., and Ghosh, S., Inhibition of nuclear factor kappa B (NF-B): an emerging theme in anti-inflammatory therapies. *Mol. Interv.* **2**, 22-35 (2002).

D'Agostino, M., Dini, A., Pizza, C., Senatore, F., and Aquino, R., Sterols from *Equisetum arvense*. *Boll-Soc. Ital. Biol. Sper.* **60**, 2241-2245 (1984).

Do Monte, F.H., dos Santos, JG. Jr., Russi, M., Lanziotti, V.M., Leal, L.K., Cunha, GM., Antinociceptive and anti-inflammatory properties of the hydroalcoholic extract of stems from *Equisetum arvense L.* in mice. *Pharmacol. Res.* **49**, 239-243 (2004).

Erridge, C., Bennett-Guerrero, E., and Poxton, I.R., Structure and function of lipopolysaccharides. *Microbes Infect.* **4**, 837-851 (2002).

Gyamfi, M.A., Yonamine, M., and Aniya, Y., Free-radical scavenging action of medicinal herbs from Ghana: Thonningia sanguinea on experimentally-induced liver injuries. *Gen. Pharmacol.* **32**, 661-667 (1999).

Halliwell, B., Free radicals, antioxidants, and human disease: curiosity, cause, or consequence. *Lancet.* **344**, 721-724 (1994).

Hewett, J.A. and Roth, R.A., Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol. Rev.* **45**, 382-411 (1993).

Ibrahim, H.R., Hoq, M.I., and Aoki, T., Ovotransferrin possesses SOD-like superoxide anion scavenging activity that is promoted by copper and manganese binding. *Int. J. Biol. Macromol.* **41**, 631-640 (2007).

Kaplan, M., Mutlu, E.A., Benson, M., Fields, J.Z., Banan, A., and Keshavarzian, A., Use of herbal preparations in the treatment of oxidant-mediated inflammatory disorders. *Complement Ther. Med.* **15**, 207-216 (2007).

Lee, Y.S., Han, O.K., Park, C.W., Yang, C.H., Jeon, T.W., Yoo, W.K., Kim, S.H., and Kim, H.J., Pro-inflammatory cytokine gene expression and nitric oxide regulation of aqueous extracted *Astragali radix* in RAW 264.7 macrophage cells. *J. Ethnopharmacol.* **100**, 289-294 (2005).

Milovanović, V., Radulović, N., Todorović, Z., Stanković, M., and Stojanović, G., Antioxidant, antimicrobial and genotoxicity screening of hydro-alcoholic extracts of five serbian Equisetum species. *Plant Foods Hum. Nutr.* **62**, 113-119 (2007).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).

Oh, H., Kim, D.H., Cho, J.H., and Kim, Y.C., Hepatoprotective and free radical scavenging activities of phenolic petrosins and flavonoids isolated from *Equisetum arvense*. *J. Ethnopharmacol.* **95**, 421-424 (2004).

Oka, M., Tachibana, M., Noda, K., Inoue, N., Tanaka, M., and Kuwabara, K., Relevance of anti-reactive oxygen species activity to anti-inflammatory activity of components of eviprostat, a phytotherapeutic agent for benign prostatic hyperplasia. *Phytomedicine* **14**, 465-472 (2007).

Park, S.J., Lee, S.C., Hong, S.H., and Kim, H.M., Degradation of IkappaBalpha in activated RAW264.7 cells is blocked by the phosphatidylinositol 3-kinase inhibitor LY294002. *Cell Biol. Toxicol.* **18**, 121-130 (2002).

Rakel, D.P. and Rindfleisch, A., Inflammation: nutritional, botanical, and mind-body influences. *South Med. J.* **98**, 303-310 (2005).

Saha, K., Lajis, N.H., Israf, D.A., Hamzah, A.S., Khozirah, S., Khamis, S., and Syahida, A., Evaluation of antioxidant and nitric oxide inhibitory activities of selected Malaysian medicinal plants. *J. Ethnopharmacol.* **92**, 263-267 (2004).

Seo, W.G., Pae, H.O., Oh, G.S., Chai, K.Y., Kwon, T.O., Yun, Y.G., Kim, N.Y., and Chung, H.T., Inhibitory effects of methanol extract of *Cyperus rotundus* rhizomes on nitric oxide and superoxide productions by murine macrophage cell line, RAW 264.7 cells. *J. Ethnopharmacol.* **76**, 59-64 (2001).

Syrchina, A.I., Voronkov, M.G., Tyukavkina, N.A., and Babkin, V.A., Phenolic acids and flavonoids of sporogenous stems of *Equisetum arvense*. *Khim. Prir. Soedin.* **6**, 803-804 (1978).

Syrchina, A.I., Zapesochnaya, G.G., Tyukavkina, N.A., and Voronkov, M.G., Glycosides of *Equisetum arvense* flavones. *Khim. Prir. Soedin.* **3**, 413-414 (1980).

Takatsuto, S., Abe, H., and Gamoh, K., Evidence for brassino-steroids of *Equisetum arvense L. Agric. Biol. Chem.* **54**, 1057-1059 (1990).

Thiemermann, C. and Vane, J., Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. *Eur. J. Pharmacol.* **182**, 591-595 (1990).

Watt, J.G., The price of factor VIII. *Lancet.* **2**, 301 (1979).

Xu, C.F., Bian, X.Y., Qu, S.M., You, L.H., Qi, Z.M., Cheng, W., Liu, X.J., Liu, W.Z., and Ren, S.J., Effect of *Equisetum hyemale* on experimental hyperlipemia in rats and its toxic test. *Zhongguo Zhong Yao Za Zhi* **18**, 52-53 (1993).

(Accepted December 8, 2008)