Natural Product Sciences
18(3) : 141-146 (2012)

Anti-inflammatory Activity of *Veronica peregrina*

Hoon Jeon*

College of Pharmacy, Woosuk University, Jeonbuk 565-701, Republic of Korea

Abstract – *Veronica peregrina* (Scrophylariaceae) has been widely used as a Korean traditional medicine for the treatment of various pathological conditions including infection, hemorrhage and gastric ulcer. In the current study, we investigated the inhibitory effect of methanolic extracts of *V. Peregrina* (VPM) on the LPS-mediated nitric oxide (NO) production in mouse (C57BL/6) peritoneal macrophages. NO production was significantly down-regulated by the treatment of VPM dose dependently. To evaluate the mechanism of the inhibitory action of VPM on NO production, we performed iNOS enzyme activity assay and checked the change of inducible nitric oxide synthase (iNOS) levels by Western blotting. Although VPM did not affect iNOS enzyme activity, iNOS protein expression was attenuated by VPM indicating VPM inhibits NO production via suppression of iNOS enzyme expression. In addition, VPM attenuated the expression of another pro-inflammatory mediator such as cyclooxygenase-2 (COX-2) in a dose dependent manner. We also found that VPM can reduce trypsin-induced paw edema in mice. Based on this study, we suggest that *V. peregrina* may be beneficial in diseases which related to macrophage-mediated inflammatory disorders.
Keywords – *Veronica peregine*, Inflammation, Nitric oxide, Edema

Introduction

Inflammation is defined the main response of living tissue to outer stimuli or local injury, and leads to dysfunction of tissue structure and organs (Lawrence *et al*., 2002). It is a complex cascades which are regulated by various cytokines, nitric oxide (NO) and prostaglandins (PGs) produced by immune cells such as neutrophils, macrophages and mast cells (Fujiwara and Kobaashi, 2005; Stephanie *et al*., 2010; Saha *et al*., 2004). Although physiological activity of inflammation may provide a distinct benefit to the organism, excessive inflammatory responses has been implicated in the pathogenesis of many disease processes including rheumatoid arthritis, asthma and cancer (Rakel and Rindfleisch, 2005). Macrophages play a central role in innate and adaptive immunity and act as a mediator of the inflammatory responses (Audrey and Siamon, 2009). Macrophage can be activated by different inflammatory stimuli such as inflammatory cytokines and bacterial endotoxic lipopoly-saccharide (LPS) results in providing inflammatory mediators such as NO, inducible isoform of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

NO participates in various physiological functions such as vasodilation, neurotransmission, inflammation (Brown, 1999; György *et al*., 2007). NO is synthesized by the one of three different kind of NO synthetases (NOS) including neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) (Bredt, 1999). Among them, iNOS is expressed in response to several inflammatory mediators and produces large amount of NO which can cause normal tissue damage during inflammatory response (Kim *et al*., 1999; Van't Hof and Ralston, 2001). Like NOS, cyclooxygenase (COX) has two isoforms such as COX-1 and COX-2. While COX-1 is expressed at a constant level and involved in normal physiologic functions, the inducible isoform, COX-2 is responsible for the production of large amounts of pro-inflammatory prostaglandins (PGs) at the inflammatory site (Park et al., 2004). Several studies demonstrated that overproduction of PGE_2 caused by COX-2 is a key role in NO prodcution. (Chang *et al*., 2006).

Veronica peregrina (Scrophylariaceae) which is widely distributed through Korea and Japan has been used as a traditional medicine for the treatment of various diseases such as hemorrhage, leucorrhoea and pain. Previous phytochemical studies of this plant have shown the presence of chrysoeriol, diosmetin, 4-hydroxybenzoic acid, apigenin and caffeic acid methylester (Ahn *et al*., 2011). *V. peregrina* has been found to possess antioxidant properties (Ahn *et al*.,

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

2011; Kwak *et al*., 2009). However, nobody reported anti-inflammatory activity of *V. peregrina* until now.

Using LPS-stimulated murine peritoneal macrophage model, we validate the anti-inflammatory action of VPM by checking NO production, iNOS and COX-2 expression. In addition, we also confirmed VPM's anti-inflammatory activities *in vivo* with trypsin-induced paw edema test in mice.

Experimental

Reagents – Murine recombinant IFN-γ was purchased from Pharmingen (Munchen, Germany). LPS, sodium nitrite and Nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO, USA). Thioglycollate (TG), Phosphate buffered saline (PBS) and Bovine serum albumin (BSA) was purchased from Gibco Laboratories (Detroit, MI, USA). 0.4 μm syringe filter and tissue culture plates of 96 wells, 6 wells, 24 wells and 60-mm, 100-mm diameter dishes were purchased from Nunc (Naperville, IL, USA). DMEM containing L-arginine (84 mg/l), fetal bovine serum (FBS) and other tissue culture reagents were purchased from Life Technologies (Grand Island, NY, USA).

Animals – Male C57BL/6 (6 weeks old) and ICR (6 weeks old) mice were purchased from Damul Science Co. (Daejeon, South Korea). All animals were housed at 22 ± 1 °C with a 12 h light/dark cycle maintaining humidity at 50% and fed a standard pellet diet with tap water *ad libitum.*

Preparation of *V. peregrine* – The plant materials were collected in June 2011 at Wanju, Jeonbuk, South Korea and identified by Prof. Dae Keun Kim, College of pharmacy, Woosuk university. A voucher specimen (WME 080) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. An extract was obtained three times from the dried sample (1000 g) with 16,000 mL of 100% MeOH under ultrasonication for 3 h. The solutions were filtered and the solvents were evaporated in the rotary vacuum evaporator. The evaporated samples were lyophilized and then stored at –20 °C until use.

Peritoneal macrophage culture – TG-elicited macrophages were harvested 3 days after i.p. injection of 2.5 mL TG to the mice and isolated. Using 8 mL of PBS 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 PBS 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 570 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 VPM (125, 250 and 500 μg/mL). 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. And Nitro-L-arginine methyl ester (L-NAME) was used as a reference drug.

Measurement of iNOS enzyme activity – To determine whether VPM affected the iNOS enzyme activity, iNOS enzyme activity was conducted as described by Israf with minor modification (Israf *et al*., 2006). Cells were induced to produce iNOS over a 12 h period with rIFN-γ (20 U/mL) and LPS (10 μg/mL). After 12 h the medium was discarded and changed. Then, cells were treated with various concentrations of VPM, and incubated another 12 h. Supernatants were removed and levels of nitrite were determined using the Griess reagant as describe previously.

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% BSA solution for 3 h at room temperature and then incubated with anti-iNOS, COX-2 (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-mouse, anti-rabbit) 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).

Trypsin-induced paw edema – Edema was induced in the right-hind paw by a 30 μl intraplantar (i.pl.) injection of trypsin (30 μg/paw, prepared in saline). The left paw received 30 μL of saline and it was used as the control. Edema was measured with a plethysmometer (LE7500, Panlab, Spain), at 1 h after injection of trypsin. Edema was expressed as the difference between the right and left paws.

Densitometric and statistical analysis – All measurement are expressed as the mean ± S.D. 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. Intensity of the bands obtained from Western blotting was estimated with Image Quant TL (GE Healthcare, Sweden) and the values were expressed as mean ± standard error.

Results and Discussion

Veronica peregrina (Scrophylariaceae) is a well-known oriental medicine which has been used for lots of diseases with diverse pharmacological effects. Nevertheless, previous reports with *V. peregrina* are still not enough to understand how this plant works on inflammatory diseases. Thus, the present study was designed to validate the anti-inflammatory activities of methanolic extracts of *V. peregrina* (VPM) using *in vitro* and *in vivo* experiments.

Using IFN-γ and LPS-induced murine peritoneal macrophage model, we evaluated VPM's anti-inflammatory properties. In macrophage, nitric oxide (NO) is synthesized from L-arginine and contributes to the immune defense against viruses, bacteria and other parasites (Bredt, 1999; Seo *et al*., 2001). However, large amount of NO act as a toxic radical and can cause tissue and cell damage results in rheumatoid arthritis, gastritis, autoimmune diseases (Ponchel *et al*., 2002; Sakagami *et al*., 1997; Thiemermann and Vane, 1990). Therefore, down-regulation of NO production can be one of the desirable ways to avoid excess inflammatory responses. We pretreated the macrophages with various concentration of VPM (125, 250, 500 μg/mL), and then, stimulated with IFN-γ (20 U/mL) and LPS (10 μg/mL). After 48 h incubation, the cell supernatants were collected and added to Griess Reagent to determine nitrite concentration. As shown in Fig. 2, LPS-induced NO production was inhibited dose-dependently by VPM. Interestingly, high concentration of VPM (500 μg/mL) perfectly suppressed NO production compared to L-NAME, a positive control (Fig. 2). At the treatment concentration, no notable cytotoxicity was detected suggesting VPM's inhibitory action was not due to change in cell viability (Fig. 1).

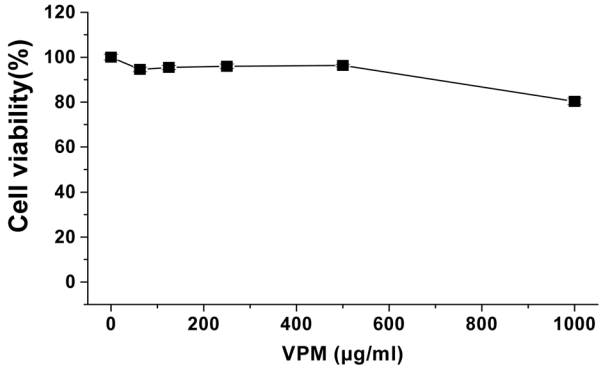

Fig. 1. Effects of VPM on the viability in IFN-γ/LPS-treated peritoneal macrophages. Various concentrations of VPM-treated peritoneal macrophages (3×10^5 cells/well) were primed for 6h with IFN-γ (20 U/mL). The peritoneal macrophages were then stimulated with LPS (10 μg/mL) for 24 h. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means ± S.D. of three independent experiments duplicate in each run.

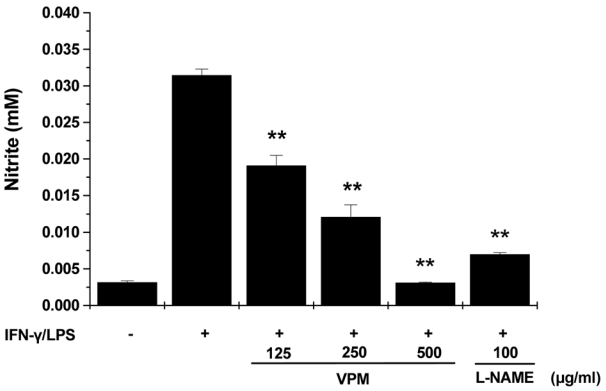

Fig. 2. Dose-dependent inhibition effects of VPM on NO production in IFN-γ/LPS-treated peritoneal macrophages. Peritoenal macrophages (3×10^5 cells/well) were cultured with various concentration of VPM. The peritoneal macrophages were then stimulated with IFN-γ (20 U/mL) and LPS (10 μg/mL). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO (nitrite) released into the medium is presented as the mean ± S.D. of three independent experiments duplicate in each run; $^{**}p < 0.001$ compared to IFN-γ + LPS.

Since inducible nitric oxide synthase (iNOS) playing a crucial role of excess production of NO in activated macrophages, we checked two possible case, inhibition of enzyme activity and attenuation of enzyme expression, respectively. To verify whether or not VPM could suppress the iNOS enzyme activities, we performed iNOS enzyme activity assay. Fig. 3A shows that the iNOS enzyme activity was inhibited by L-NAME, a well-known iNOS inhibitor, while VPM did not change the NO production by iNOS. Next, we checked whether iNOS expression

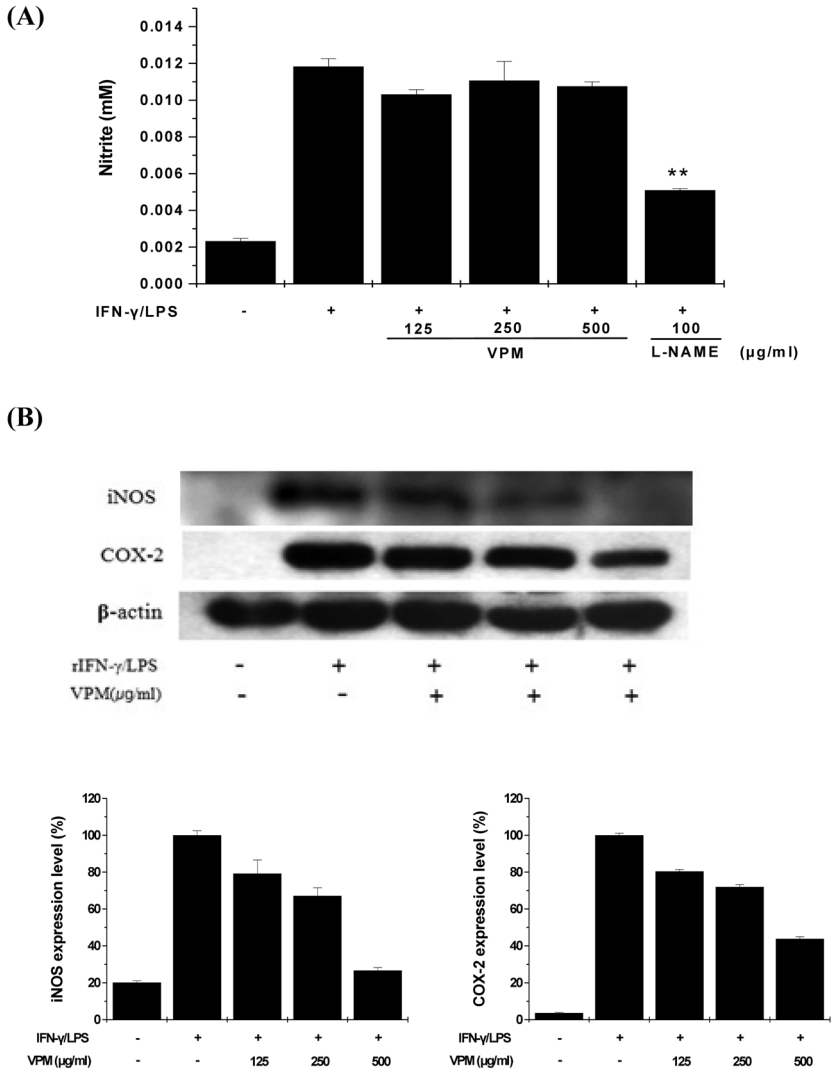

Fig. 3. Effects of VPM on iNOS enzyme activity and expressions of iNOS and COX-2. iNOS enzyme activity was measured by stimulating peritoneal macrophages (3×10^5 cells/well) with IFN-γ (20 U/mL) for 6 h, then stimulated with LPS (10 μg/mL). After 12 h, various concentrations of VPM were treated and incubated for another 12 h. The supernatant were obtained and measured by the Griess method as described previously. iNOS and COX-2 expression were measured as follows. Peritoneal macrophages (5×10^6 cells/well) were pretreated with VPM and then stimulated for 6 h with IFN-γ (20 U/mL). The peritoneal macrophages were then stimulated with LPS (10 μg/mL) for 24 h. The protein extracts were prepared and samples were analyzed for iNOS and COX-2 expression by Western blotting as described in the method. Determined expression of iNOS and COX-2 was subsequently quantified by densitometric analysis with that of IFN-γ/LPS treated control being 100% as shown just below the gel data.

could be changed by VPM treatment, and VPM successfully down-regulated iNOS protein expression (Fig. 3B). These results indicate that VPM's NO inhibitory effects might be due to attenuation of iNOS protein level, not by alteration of enzyme activity or direct scavenging of NO radical.

Cyclooxygenase-2 (COX-2), another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. Since, COX-2 is induced by stimulation in inflammatory cells, COX-2 inhibitors might be candidates for the new type of nonsteroidal anti-inflammatory drugs (NSAIDs). We documented the increased production of COX-2 protein by macrophages exposed to IFN-γ and LPS (Fig. 3B). IFN-γ and LPS in combination with VPM led to a reduction in COX-2 expression (Fig. 3B). From this result, we could speculate that VPM inhibits PGE_2 production.

We further confirmed VPM's anti-inflammatory activity using *in vivo* model such as trypsin-induced paw edema

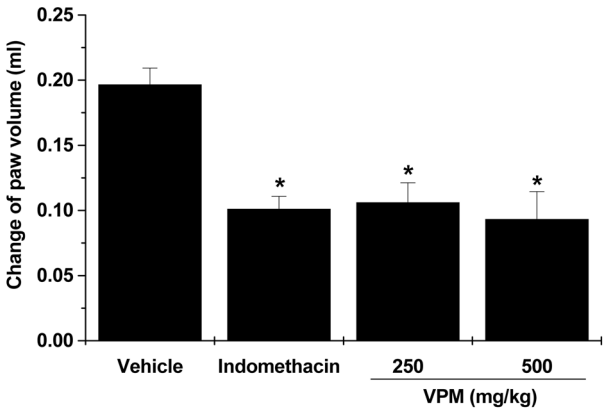

Fig. 4. Effects of VPM on trypsin-induced paw edema in mice. Mice were pre-treated distilled water, Indomethacin, VPM before injected trypsin solution. After 1 h, the trypsin solution was injected in a volume of 30 μL subplantarly. Trypsin was dissolved in sterile 0.9% saline. The size of edema was assessed by measuring the volume of the hindpaw immediately before and 1 h after the agonist injection. Data show the mean ± S.E.M. (n = 8 - 12). *$p <$ 0.01 compared to the control group.

in mice. Subplantar injection of trypsin, a proteinase-activated receptor2 (PAR2) agonist, induces an increase in vascular permeability as well as in the marked infiltration of granulocytes (Kawabata *et al*., 1998). In this study, VPM reduced the paw volume about 50% at 500 mg/kg concentration and indomethacin, the reference drug also showed similar reduction (Fig. 4). Paszcuk *et al*. (2008) reported that the edematogenic response is likely related to the production of COX metabolites, especially COX-2. Therefore, VPM might block trypsin-induced paw edema through inhibition of COX-2 expression, at least in part.

In conclusion, VPM inhibited NO production via attenuation of iNOS expression in the IFN-γ and LPS-induced murine peritoneal macrophages. VPM also suppressed COX-2 expression in a dose dependent manner. Not only in vitro study, but VPM exerts its anti-inflammatory activities in the in vivo paw edema model. These results establish that VPM has potent anti-inflammatory effects and may hold great promise for the usage on inflammatory diseases as an effective immune-suppressive agent.

Acknowledgement

This work was supported by the Leaders in INdustry-university Cooperation (LINK) of Woosuk University.

References

Ahn, D.R., Lee, S.I., and Yang, JH., Superoxide Radical Scavengers from the Whole Plant of *Veronica peregrina*. *Nat. Product Sci.* **17**, 142-146 (2011).

Audrey, V. and Siamon, G., Alternative activation of macrophages: Immune function and cellular biology. *Immunobiology* **214**, 630-641 (2009).

Bredt, D.S., Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radicical. Res.* **31**, 577-596 (1999).

Brown, C.G., Nitric oxide and mitochondrial respiration. *Biochim. Biophys. Acta.* **1411**, 351-369 (1999).

Chang, Y.C., Li, P.C., Chen, B.C., Chang, M.S., Wang, J.L., Chiu, W.T., and Lin, C.H., Lipoteichoic acid-induced nitric oxide synthase expression in RAW 264.7 macrophages is mediated by cyclooxygenase-2, prostaglandin E2, protein kinase A, p38 MAPK, and nuclear factor-kappa B pathways. *Cell. Signal.* **18**, 1235-1243 (2006).

Fujiwara, N. and Kobaashi, K., Macrophages in inflammation. *Curr. Drug Targets Inflamm. Allergy* **4**, 281-286 (2005).

György, N., Joanna, M.C., Edit, I.B., Claire, L.G., and Andrew, P.C., Nitric oxide, chronic inflammation and autoimmunity. *Immunology Letters* **111**, 1-5 (2007).

Israf, D.A., Khaizurin, T.A., Syahida, A., Lajis, N.H., and Khozirah, S., Cardamonin inhibits COX and iNOS expression via inhibition of p65 NF-kappa B nuclear translocation and I-kappa B phosphorylation in RAW 264.7 macrophage cells. *Mol. Immunol*. **44**, 673-679 (2006).

Kawabata, A., Kuroda, R., Minami, T., Kataoka, K., and Taneda, M., Increased vascular permeability by a specific agonist of protease-activated receptor-2 in rat hindpaw. Br. *J. Pharmacol*. **125**, 419-422 (1998).

Kim, H.K., Cheon, B.S., Kim, Y.H., Kim, S.Y., and Kim, H.P., Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. *Biochem. Pharmacol*. **58**, 759-765 (1999).

Kwak, J.H., Kim, H.J., and Lee, K.H., Antioxidative Iridoid Glycosides and Phenolic Compounds from *Veronica peregrina*. *Arch. Pharmacal Res.* **32**, 207-213 (2009).

Lawrence, T., Willoughby, D.A., and Gilroy, D.W., Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat. Rev. Immunol.* **10**, 787-795 (2002).

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

Park, E.J., Min, H.Y., Ahn, Y.H., Bae, C.M., Pyee, J.H., and Lee, S.K., Synthesis and inhibitory effects of pinosylvin derivatives on prostaglandin E2 production in lipopolysaccharide-induced mouse macrophage cells. *Bioorg. Mad. Chem. Lett.* **14**, 5895-5898 (2004).

Paszcuk, A.F., Quintão, N.L., Fernandes, E.S., Juliano, L., Chapman, K., Andrade-Gordon, P., Campos, M.M., Vergnolle, N., and Calixto, J.B., Mechanisms underlying the nociceptive and inflammatory responses induced by trypsin in the mouse paw. *Eur. J. Pharmacol*. **581**, 204-215 (2008).

Ponchel, F., Morgan, A.W., Bingham, S.J., Quinn, M., Buch, M., Verburg, R.J., Henwood, J., Douglas, S.H., Masurel, A., Conaghan, P., Gesinde, M., Taylor, J., Markham, A.F., Emery, P., van Laar, J.M., and Isaacs, J.D., Dysregulated lymphocyte proliferation and differentiation in patients with rheumatoid arthritis. *Blood* **100**, 4550-4556 (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).

Sakagami, T., Vella, J., Dixon, M.F., O'Rourke, J., Radcliff, F., Sutton, P., Shimoyama, T., Beagley, K., and Lee, A., The endotoxin of Helicobacter pylori is a modulator of host-dependent gastritis. *Infect*

Immun. **65**, 3310-3316 (1997).
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).
Stephanie, L., Andreas, W., and Bernhard, B., Neuromediators in inflammation-a macrophage/nerve connection. *Immunobiology* **215**, 674-684 (2010).
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).
Van't, Hof, R.J. and Ralston, S.H., Nitric oxide and bone. *Immunology* **103**, 255-261 (2001).

Received March 9, 2012
Revised May 3, 2012
Accepted May 7, 2012