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Oriental Pharmacy and Experimental Medicine 2010 **10(2)**, 51-58
DOI 10.3742/OPEM.2010.10.2.051

Effects of *Vitex rotundifolia* on radical scavenging and nitric oxide production

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Received for publication March 10, 2010; accepted April 14, 2010

SUMMARY

Vitex rotundifolia (*V. rotundifolia*) has been used for treating headache, dizziness, toothache and removal of fever as a traditional medicine in Korea. In the present study, we examined the antioxidant and anti-inflammatory activities of 85% methanol extract of *V. rotundifolia*. In various radical scavenging assays, *V. rotun*difolia exhibited strong scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl free radical, superoxide radical, nitric oxide. To elucidate the anti-inflammatory properties of *V. rotundifolia*, we investigated the inhibition effects of nitric oxide production in IFN-gamma and LPS-stimulated mouse peritoneal macrophages. *V. rotundifolia* suppressed nitric oxide production, iNOS and COX-2 expression dose-dependently through suppression of NF-êB activation without notable cytotoxicity. These findings mean that *V. rotundifolia* may be beneficial in oxidative stress-mediated inflammatory disorders.

Key words: *Vitex rotundifolia*; Antioxidant; Anti-inflammatory

INTRODUCTION

Inflammation is a defense mechanism to minimize the damage by infection or irritation and may be referred to as the innate cascade including various cells and cytokines (Zamora *et al*., 2000). It is characterized by redness, heat, swelling, pain and dysfunction of the organs. Macrophages play a central role in host defense and maintenance as a major immune cell in inflammation since pro-inflammatory mediators such as nitric oxide (NO), prostagladins (PGs) and cytokines are secreted by activated macrophage.

NO produced by one of three kind of NO synthase (NOS) that nNOS (neuronal NOS), endothelial NOS (eNOS), inducible NOS (iNOS) from L-arginine. NO, produced by nNOS and eNOS in nanomolar concentration, play an important role as a neuro-transmitter and vasodilator. However, overproduction of NO, mediated by iNOS, intimately correlated with the pathological conditions in inflammation related diseases (Wang *et al*., 2003). COX (cyclooxy-genase), another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of PGs from arachidonic acid. Levels of PGs increase early in the step of inflammation. Like NOS, COX also exists in both constitutive (COX-1) and inducible (COX-2) forms. It is well known that the COX-1 is a housekeeping protein in most tissues and it catalyzes the synthesis of PGs for normal physiological functions while COX-2 mediates an inflammatory response. It is well

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known that iNOS and COX-2 expression is predominantly regulated by the ubiquitously expressed NF-κB (nuclear factor kappa B) which is required for the inducible expression of genes associated with inflammatory responses. Therefore, attenuation of NF-κB activation may be a reasonable strategy to anti-inflammatory drugs.

During normal aerobic conditions, free radicals such as reactive oxygen species (ROS; $\bullet O_2^-$, •OH, H_2O_2) and reactive nitrogen species (RNS; •NO, HNO_2, $ONOO^-$) generated. Especially the production of ROS and RNS in phagocytic leukocytes such as macrophages, neutrophils, 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).

In Korea, *Vitex rotundifolia* (*V. rotun*difolia) was traditionally used to counteract headache, fever, toothache and eye disease. It is well known that *V. rotundifolia*. has essential oil such as a-pinene, camphene, terpineol, acetylester diterpene alchol, diterpene of labdane type, diterpene of abietane type (Masateru *et al*., 2000) and flavonoid such as casticin and artemetin (Ono *et al*., 1999). *V. rotundifolia* have been found possess anti-leukemic (Ko *et al*., 2000), anti-inflammatory (Zhu *et al*., 1998) and anti-allergic properties (Shin *et al*., 2000). In view of the several reports of *V. rotundifolia* described above, *V. rotundifolia* was proposed to have antioxidant and anti-inflammatory properties. Therefore, in this study we investigate the effect of the *V. rotundifolia* on the radical scavenging and inhibitory effect on inflammatory mediators such as NO, iNOS and COX-2 in rIFN-γ and LPS stimulated murine peritoneal macrophages. To clarify the mechanism of iNOS suppression, we also assessed the effect of *V. rotundifolia* on the activation of NF-κB.

MATERIALS AND METHODS

Preparation of the stem and leaves of *V. rotundifolia*

The plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in March 2005. A voucher specimen (WME008) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. The extract of *V. rotundifolia* was obtained twice from the dried sample (250 g) with 5,000 ml of 85% MeOH under ultrasonification for 2 h. It was evaporated and lyophilized to yield an MeOH extract of *V. rotundifolia* (Yield : 5.35%), which was then stored at -20°C until use.

1, 1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging assay

The scavenging effect of *V. rotundifolia* on DPPH radical was measured using the method of Gyamfi *et al*. (1999) with some modification. A 5 μl aliquot of the different concentrations of *V. rotundifolia* were added to 495 μl of DPPH in absolute ethanol solution (0.25 mM). After incubation for 20 min at room temperature, 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 *V. rotundifolia* was studied using xanthine/xanthine oxidase/ NBT method according to 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 min, 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 sodium dodecyl sulfate (SDS) and the absorbance at 570 nm was measured.

Nitric oxide radical scavenging assay

A 5 μl aliquot of the different concentrations of *V. rotundifolia* 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 was determined and the standard was determined by using sodium nitrite at 540 nm.

Peritoneal macrophage culture

TG-elicited macrophages were harvested 3 - 4 days after i.p. injection of 2.5 ml thioglycolate (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 proliferation, an indicator of cell viability, was analysed 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 *V. rotundifolia*. 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 Griess 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^- concentration 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 prepared 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 (SDS-PAGE) and transferred to nitrocellulose membrane. The membrane was then blocked with 5% skim milk for 2 h at room temperature and then incubated with anti-iNOS (SantaCruz, USA). After washing with phosphate buffered saline (PBS) containing 0.05% tween 20 three times, the blot was incubated with secondary antibody (anti-rabbit IgG, anti-mouse IgG) for 1 h and the antibody specific proteins were visualized by the enhanced chemiluminesence (ECL) detection system according to the recommended procedure (Amersham Corp. Newark, NJ, Germany).

Statistical analysis

All measurement are expressed as the mean ± S.D. of independent experiments. Data between groups were analyzed by a paired students' *t*-test and *P*-values less than 0.01 were considered significant.

RESULTS

Effects of *V. rotundifolia* on cell viability

To determine the effects of *V. rotundifolia* on viability of mouse peritoneal macrophages, the we carried out MTT assay. When we treated the cells were treated with *V. rotundifolia* at the concentrations of 0.01, 0.1, 1 mg/ml, it had no effect on cell viability. The incubation of mouse peritoneal macrophages with *V. rotundifolia* and 10 μg/ml LPS also did not shown any cytotoxicity (Fig. 1).

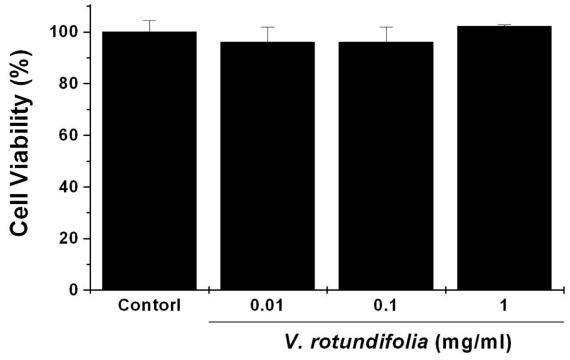

Fig. 1. Effects of *V. rotundifolia* on the viability in rIFN-γ/LPS stimulated peritoneal macrophages. Various concentrations of *V. rotundifolia*-treated peritoneal macrophages (3×10^5 cells/well) were primed for 6 h with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 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.

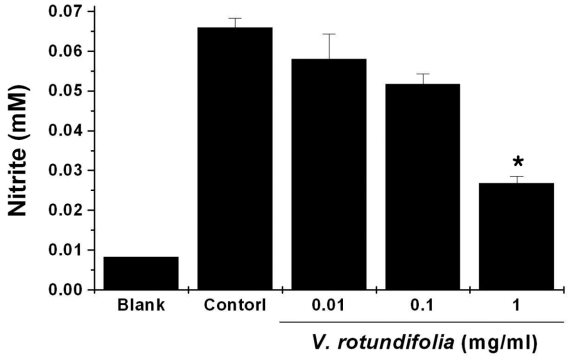

Fig. 2. Effects of *V. rotundifolia* on NO inhibition in rIFN-γ and LPS-stimulated peritoneal macrophages. Peritoenal macrophages (3×10^5 cells/well) were cultured with various concentration *V. rotundifolia*. The peritoneal macrophages were then stimulated with rIFN-γ (20 U/ml) and LPS (10 μg/ml). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO released into the medium is presented as the mean ± S.D. of three independent experiments duplicate in each run *$P < 0.01$ compared to rIFN-γ + LPS.

Inhibition of *V. rotundifolia* on NO production

To determine the effect of *V. rotundifolia* on the production of NO in mouse peritoneal macrophages, nitrite accumulation was measured by the Griess reaction. The author pre-treated the cells in the presence or absence of various concentrations *V. rotundifolia* (0.01, 0.1 and 1 mg/ml). And then stimulated them with rIFN-γ (20 U/ml) and LPS (10 μg/ml). The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants after 48 h treatment. The amount of NO in unstimulated cells was 3.62 ± 0.4 μM. When mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with LPS, NO production was increased about 10 folds (36.2 ± 0.45 μM). *V. rotundifolia* had few effect on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When *V. rotundifolia* was pre-treated in primed cell, *V. rotundifolia* significantly inhibits NO production dose dependently and over 64% ($P < 0.01$) inhibition of NO production was shown at the concentration of 1 mg/ml (Fig. 2). No significant effect on cell viability was observed at a test concentration up to 1 mg/ml *V. rotundifolia* (Fig. 1). Therefore, the inhibitory effect of NO by *V. rotundifolia* was not due to a cytotoxicity on the cells.

Effects of *V. rotundifolia* on expression of iNOS and COX-2

In order to investigate the mechanism of action of *V. rotundifolia* on the inhibition of NO production, Western blotting was performed. We investigate the effect of the *V. rotundifolia* at translational level by western blotting, as shown in Fig. 4, the expression of iNOS and COX-2 protein were markedly increased after rIFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 24 h. This enhanced expression of iNOS protein was significantly reduced by *V. rotundifolia* in a dose-dependent manner (Fig. 3) and *V. rotundifolia* also inhibits expression of COX-2.

Effects of *V. rotundifolia* on activation of NF-κB

NF-κB is activated in cells stimulated with LPS and other inflammatory insults, a process that is related to the transcriptional activation of responsive genes. As shown in Fig. 4, the activation of NF-κB was

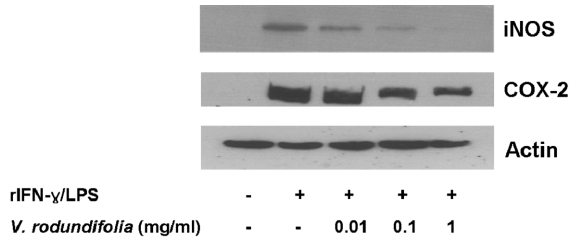

Fig. 3. Effects of *V. rotundifolia* on the expression of iNOS and COX-2 by rIFN-γ/LPS activated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with *V. rotundifolia* and then stimulated for 6 hour with rIFN-γ (20 U/ml). The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 24 h. The protein extracts were prepared; samples were analyzed for iNOS and COX-2 expression by Western blotting as described in the method.

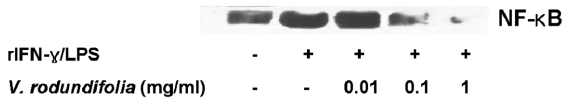

Fig. 4. Effects of *V. rotundifolia* on the activation of NF-κB by rIFN-γ/LPS-stimulated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with *V. rotundifolia*. After 30 min, the cells were stimulated with rIFN-γ (20 U/ml) for 30 min. The peritoneal macrophages were then stimulated with LPS (10 μg/ml) for 2 h. The nuclear extracts were prepared; samples were analyzed by Western blotting as described in the method.

markedly increased after rIFN-γ (20 U/ml) plus LPS (10 μg/ml) challenge for 10 min. This increased expression of NF-κB was significantly reduced by *V. rotundifolia* (Fig. 4).

Antioxidant activities of *V. rotundifolia*

In order to investigate the antioxidant activities of *V. rotundifolia*, we performed three different in vitro assays such as DPPH radical, superoxide anion and nitric oxide scavenging methods. The radical scavenging activity was measured as decolorizing activity following the trapping of the unpaired electron of DPPH (Burda and Oleszec, 2001). *V. rotundifolia* showed relatively high DPPH radical scavenging activity, at the concentration of 1000 μg/ml with 70.1 ± 0.98% inhibition against DPPH radicals. As a control, ascorbic acid was used and % inhibition value obtained was 88.47 ± 0.64% (Table 1). The superoxide radical was generated by the xanthine/xanthine oxidase system. Results from the NBT analysis revealed that *V. rotundifolia* had slight superoxide radical scavenging activity up to 100 μg/ml. However, 1000 μg/ml of *V. rotundifolia*, showed strong superoxide radical scavenging activities similar to ascorbic acid (Table 1). Sodium nitroprusside (SNP) is known to produce nitric oxide and under aerobic conditions, nitric oxide reacts with oxygen to form nitrate and nitrite, which can be determined using Griess reagent. As shown in Table 1, *V. rotundifolia* inhibited nitrite production significantly and the its nitric oxide scavenging capacity was about 89% that of ascorbic acid.

DISCUSSION

In the present study, we evaluated the anti-inflammatory effect on the production of inflammatory

Table 1. Scavenging capacity of *V. rotundifolia* in different free radical scavenging tests

Treatment	Concentration	Radical scavenging capacity[a] (% Inhibition)		
		DPPH	X/XO/NBT	Nitric oxide
Ascorbic acid[b] (mM)	0.01	25.73 ± 6.86	ND	8.66 ± 1.29
	0.1	85.53 ± 0.71	ND	86.28 ± 1.47
	1	85.94 ± 0.75	94.59 ± 4.54	97.97 ± 0.11
V. rotundifolia (μg/ml)	0.01	4.33 ± 3.25	0.85 ± 1.16	1.58 ± 0.95
	0.1	24.29 ± 10.41	25.96 ± 7.51	20.72 ± 1.40
	1	71.98 ± 2.50	94.00 ± 9.60	78.96 ± 0.32

[a]Each value in the table represents the mean ± S.D of three parallel measurements. [b]Ascorbic acid used as positive control.

mediators in IFN-γ and LPS stimulated murine peritoneal macrophages using 85% MeOH extract of *V. rotundifolia*. And we also investigated its antioxidant properties using DPPH radical scavenging assay, superoxide scavenging asaay and nitric oxide radical scavenging assay.

NO is a free radical produced from L-arginine by NO NOS and maintaining diverse physiological homeostasis (Seo *et al*., 2001). However excess of NO cause many inflammatory diseases such as septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases (Thiemermann and Vane, 1990). Therefore, we need to develop anti-inflammatory drug to inhibit overproduction of NO. In this study, we used IFN-γ and LPS stimulated mouse peritoneal macrophage system to assess an inhibitory activities of *V. rotundifolia* on the pro-inflammatory mediators. As shown in Fig. 2 nitrite assay, determined by Griess method, indicated that the inhibition rates of NO production by *V. rotundifolia* were 32%, 75% and 86% at the concentration of 0.01, 0.1, 1 mg/ml respectively. Fig. 1 shows the potent inhibitory action of *V. rotundifolia* on NO production is not due to its cytotoxicity.

It is well known that excess production of NO is mediated by iNOS in activated macrophages. Suppression of iNOS expression levels might be an attractive therapeutic target for the treatment of NO-mediated inflammatory condition. *V. rotundifolia* strongly inhibited the expression of iNOS in a dose dependent manner (Fig. 3). This result explained that the inhibiton effect of *V. rotundifolia* on NO production was due to its suppressive activity on iNOS expression.

COX-2, an another key enzyme in inflammatory cascade, catalyze PGE_2 synthesis from arachidonic acid (Minghetti *et al*., 1998). High levels of PGE_2 take an important part in inflammatory conditions including asthma, rheumatoid arthritis and multiple sclerosis (Fitz Gerald *et al*., 2003). Since COX-2 is related with the synthesis of PGE_2, an inflammatory mediator, inhibitors of COX-2 induction might candidates for the new type of nonsteroidal anti-inflammatory drugs (NSAIDs). The possibility that *V. rotundifolia* might inhibit COX-2 expression was examined and *V. rotundifolia* suppressed the expression of COX-2 dose dependent manner (Fig. 3). Thus, it seems quite reasonable to speculate that *V. rotundifolia* may inhibits PGE_2 production. However, further studies are required to determine whether *V. rotundifolia* is selective inhibitor of COX-2.

iNOS and COX-2 expression is regulated mainly at the transcriptional level and its major transcriptional regulator is the NF-κB, which is also key regulators of a variety of genes involved in immune and inflammatory responese (Xie QW *et al*., 1994). In unstimulated cells, inactive NF-κB is sequestered in the cytoplasm and is linked to the inhibitory IκB protein. However, in active state, following the induction of NF-κB by appropriate extracellular stimulation such as LPS, TNF-α or tissue plasminogen activator, it translocate to the nucleus with phosphorylation, ubiquitation and degradation of IκBα and also acts upon the iNOS gene promoter to activate transcription. In this study, we investigated that the effect of *V. rotundifolia* on the translocation of NF-κB by using Western blotting. The result revealed that translocation of NF-κB to nucleus was attenuated especially at the concentration of 100 mg/ml (Fig. 4). These results indicate that the inhibition by *V. rotundifolia* on of the LPS-stimulated expression of iNOS and COX-2 by blocking of NF-κB activation. It is expected that *V. rotundifolia* on may affect NF-κB inducing kinase or IκB kinase activity. However, this hypothesis requires further investigation.

Free radicals such as ROS and RNS are highly reactive molecules and generated during normal metabolism process under aerobic conditions. They can damage lipids, proteins and DNA with oxidative stress. In immune cells such as macrophages have phagocytic function via secretion of free radical. In inflammatory condition, large amount of free redical is generated and it may cause several chronic inflammatory diseases such as atherosclerosis,

rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (Bonomini *et al*., 2008). Therefore, a radical scavenger is useful for the treat of ROS and RNS mediated inflammatory diseases.

The radical scavenging activity of *V. rotundifolia* was determined from the reduction of absorbance at 520 nm due to scavenging of stable DPPH free radical. *V. rotundifolia* exhibited strong scavenging capacity compared with ascorbic acid (Table 1). We also investigated scavenging effect of *V. rotundifolia* on superoxide anion using X/XO/NBT system. *V. rotundifolia* was able to inhibit the formazan formation from NBT react with superoxide anion generated by xanthine oxidase system in a concentration dependent manner (Table 1). NO radical, a RNS, also can be a reason for oxidative stress (Ding *et al*., 1998). SNP mediated NO radical was reduced dose dependently by the *V. rotundifolia* (Table 1). Superoxide anion may react with NO radical resulting in the formation of peroxynitrite ($ONOO^-$), a powerful oxidant. In the present study, *V. rotundifolia* showed potent radical scavenging activities on superoxide anion and NO radical, and therefore, they may suppress peroxynitrite generation.

The production of NO, iNOS and COX-2 takes an important part of the immune response to many inflammatory stimulti. None the less, excessive overproduction of these mediators is implicated in acute and chronic inflammatory diseases including septic shock, hemorrhagic shock, multiple selerosis, rheumatoid arthritis, ulcerative colitis and atherosclerosis (Bertolini *et al*., 2003). In summary the present results demonstrate that the *V. rotundifolia* inhibits over production of NO in mouse peritoneal macrophages stimulated with LPS. This inhibitory effect was consistent with its down-regulation effect on the expression of iNOS in murine macrophages. Activation of NF-κB is thought play a key role in the LPS-stimulated expression of iNOS. *V. rotundifolia* also suppressed expression of COX-2 release through decreased NF-κB transcriptional factor in a concentration-dependent manner. *V. rotundifolia* has not significant cytotoxicity at the treatment concentration. We also investigated the antioxidant properties of *V. rotundifolia*. The present study clearly revealed that *V. rotundifolia* has strong scavenging activity on DPPH radical, superoxide anion and NO radical. These results strongly suggest that *V. rotundifolia* may represent a potential new source of drugs for the treatment of ROS-mediated chronic inflammatory diseases as an effective immunomodulatory material.

ACKNOWLEDGEMENTS

This work was supported by the research gant from Woosuk University (2010).

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