

Anti-inflammatory and radical scavenging properties of *Verbena officinalis*

Hwan Ki Shim, Seong Yeol Kim, Borim Kim, Jae Park Cho, Yae Jeong Park, Won Geun Ji, Dong Seok Cha and Hoon Jeon*

Department of Oriental Pharmacy, College of Pharmacy, Woosuk University, Chonbuk, Republic of Korea

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SUMMARY

Verbena officinalis (Verbenaceae) has been used as herbal medicine or health supplement in both Western and Eastern countries for centuries. In the present study, we examined the anti-inflammatory and antioxidant activities of the methylene chloride fraction of *V. officinalis* (VMC). To elucidate the anti-inflammatory properties of VMC, we investigated the inhibition effects of nitric oxide production in interferon-gamma (IFN- γ) and lipopolysaccharide-stimulated mouse peritoneal macrophages. VMC suppressed nitric oxide production, inducible nitric oxide synthase and cyclooxygenase-2 expression dose-dependently without notable cytotoxicity. In various radical scavenging assays, VMC exhibited strong scavenging effect on DPPH radical, superoxide radical, nitric oxide radical and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) radical. VMC also showed potent reducing power. These findings strongly suggest that VMC may be beneficial in oxidative stress-mediated inflammatory disorders.

Key words: *Verbena officinalis*; 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). Many types of autoimmune diseases and allergies such as asthma, rheumatoid arthritis and multiple sclerosis are an example of excessive inflammatory response (Rakel and Rindfleisch, 2005). During the inflammatory condition, macrophages play a central role as a major immune cell since pro-inflammatory mediators such as nitric oxide (NO), prostaglandins (PGs) and cytokines are secreted by activated

macrophage.

NO produced by one of three kind of NO synthase (NOS) that neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS) from L-arginine. NO, produced by nNOS and eNOS in nanomolar concentration, play an important role as a neurotransmitter and vasodilator. However, iNOS-mediated excessive NO production intimately correlated with the pathological conditions in inflammatory diseases (Wang *et al.*, 2003). Cyclooxygenase (COX), 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

*Correspondence: Hoon Jeon, Department of Oriental Pharmacy, College of Pharmacy, Woosuk University, Chonbuk, Republic of Korea. Tel: +82-63-290-1577; Fax: +82-63-290-1576; E-mail: hoonj6343@hanmail.net

of PGs for normal physiological functions while COX-2 mediates an inflammatory response. Therefore, attenuation of COX-2 expression may be a reasonable strategy to anti-inflammatory drugs.

During normal aerobic conditions, free radicals such as reactive oxygen species (ROS; $\cdot\text{O}_2$, $\cdot\text{OH}$, H_2O_2) and reactive nitrogen species (RNS; $\cdot\text{NO}$, 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, *V. officinalis* was traditionally used to counteract acute dysentery, enteritis, amenorrhea and depression. It is well known that *V. officinalis* has iridoids (Makboul, 1986), verbascosides (Hansel and Kallmann, 1986), flavonoids (Calvo *et al.*, 1997), triterpenic acids and sterols (Deepak and Handa, 2000). *V. officinalis* have been found possess anti-inflammatory (Calvo *et al.*, 1998; Deepak and Handa, 2000), antifungal (Casanova *et al.*, 2008) and anti-bacterial (Hernández *et al.*, 2000) properties. However, despite its wide use with many interesting medicinal properties, the pharmacological and biological studies of the plant have not been sufficiently revealed. Therefore, in this study we investigated the effect of the methylene chloride fraction of *V. officinalis* (VMC) 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.

MATERIALS AND METHODS

Preparation of VMC

The plant materials were purchased from Wansanyakupsa (Jeonju, South Korea) in April 2009. A voucher specimen (WME058) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University. The extract of *V.*

officinalis was obtained twice from the dried sample (600 g) with 12,000 ml of 100% MeOH under ultrasonification for 2 h (Yield : 2.83%). It was evaporated and then subjected to successive solvent partitioning to give *n*-hexane (Yield : 1.14%), methylene chloride (Yield : 0.16%), EtOAc (Yield : 0.20%) and *n*-BuOH (Yield : 0.83%) soluble fractions. Each fractions were lyophilized and then stored at -20°C . The preliminary experiments showed that among the four fractions of *V. officinalis*, the methylene chloride fraction (VMC) has the most potent anti-inflammatory activity, and therefore, further studies were conducted using VMC.

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

The scavenging effect of VMC 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 VMC 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 VMC 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 VMC 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% H₃PO₄). The absorbance was determined and the standard was determined by using sodium nitrite at 540 nm.

Trolox equivalent antioxidant capacity (TEAC)

The experiments were carried out using a modified ABTS decolorisation assay (Obón, *et al.*, 2005) and it involves the generation of ABTS radical chromophore by the oxidation of ABTS with potassium persulfate. The ABTS radical cation was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate and allowing the mixture to stable for at least 16 h in the dark at room temperature before use. The ABTS radical solution was diluted to an absorbance of 0.70 ± 0.1 at 734 nm with distilled water. Absorbance was measured 3 min after the initial mixing of different samples with ABTS radical solution. Trolox, was used as a reference standard.

Reducing Power

The reducing power of VMC was determined according to the method of Athukorala *et al.* (2006). Samples were mixed with phosphate buffer (2.5 ml, 200 mM, pH 6.6) and potassium ferricyanide (K₃Fe(CN)₆; 2.5 ml, 30 mM). The mixture was incubated at 50°C for 20 min. A 2.5 ml trichloroacetic acid (600 mM) was added to the mixture, which was then centrifuged for 10 min at 12,000 rpm. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 6 mM), and the absorbance was measured at 700 nm in a spectrophotometer.

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 × 10⁵ cells/well) incubated for 3 h at 37°C in an atmosphere of 5% CO₂, 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 × 10⁵ cells/well) were cultured with various concentrations of VMC. The cells were then stimulated with rIFN-γ (100 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. NO₂⁻ concentration was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 9 M of NO₂⁻. 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 chemiluminescence (ECL) detection system according to the recommended procedure (Amersham Corp. Newark, NJ, Germany).

Densitometric and statistical analysis

All measurement are expressed as the mean \pm S.D. of independent experiments. Data between groups were analyzed by a paired Students' *t*-test and *p*-values less than 0.001 were considered significant. Intensity of the bands obtained from Western blotting were estimated with ImageQuantTL (GE Healthcare, Sweden) and the values were expressed as mean \pm standard error.

RESULTS

Effects of VMC on cell viability

To determine the effects of VMC on the viability of mouse peritoneal macrophages, we carried out MTT assay. When we treated the cells with VMC at the concentrations of 125, 250, 500 $\mu\text{g/ml}$, it had no effect on cell viability (Fig. 1). The incubation of mouse peritoneal macrophages with VMC and 10 $\mu\text{g/ml}$ LPS also did not shown any cytotoxicity (Data not shown).

Inhibition of VMC on NO production

To determine the effect of VMC on the production of NO in mouse peritoneal macrophages, nitrite accumulation was measured by the Griess reaction. We pre-treated the cells in the presence or absence of various concentrations VMC (125, 250, 500 $\mu\text{g/ml}$). And then stimulated them with rIFN- γ (100 U/ml) and LPS (10 $\mu\text{g/ml}$). The resultant NO production was determined by detecting nitrite concentrations

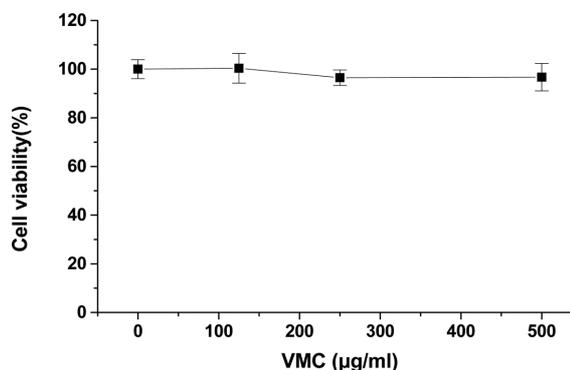


Fig. 1. Effects of VMC on the viability in rIFN- γ /LPS stimulated peritoneal macrophages. Various concentrations of VMC-treated peritoneal macrophages (3×10^5 cells/well) were primed for 6 hours with rIFN- γ (100 U/ml). The peritoneal macrophages were then stimulated with LPS (10 $\mu\text{g/ml}$) for 24 h. Cell viability was evaluated by MTT colorimetric assay as described in the method. The results are expressed as means \pm S.D. of three independent experiments duplicate in each run.

in the cell supernatants after 48 h treatment. The amount of NO in unstimulated cells was 2.75 ± 0.17 μM . When mouse peritoneal macrophages were primed for 6 hours with murine rIFN- γ and then treated with LPS, NO production was increased about 17 folds (47.7 ± 0.24 μM). VMC had few effect

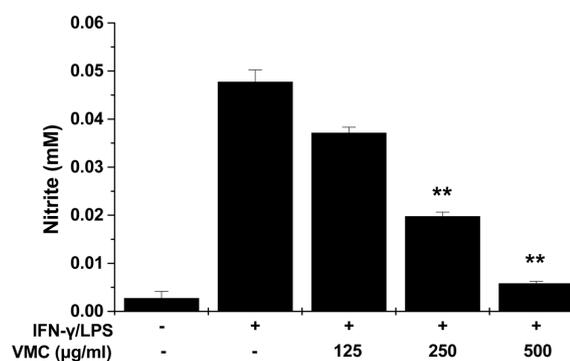


Fig. 2. Effects of VMC on NO inhibition in rIFN- γ and LPS-stimulated peritoneal macrophages. Peritoneal macrophages (3×10^5 cells/well) were cultured with various concentration VMC. The peritoneal macrophages were then stimulated with rIFN- γ (100 U/ml) and LPS (10 $\mu\text{g/ml}$). After 48 h of culture, NO release was measured by the Griess method (nitrite). NO released into the medium is presented as the mean \pm S.D. of three independent experiments duplicate in each run $**p < 0.001$ compared to rIFN- γ + LPS.

on NO production in resting mouse peritoneal macrophages compared to non-primed conditions. When VMC was pre-treated in primed cell, VMC significantly inhibits NO production dose dependently and over 85% ($p < 0.001$) inhibition of NO production was shown at the concentration of 500 $\mu\text{g/ml}$ (Fig. 2). Since no significant effect on cell viability was observed at a test concentration up to 500 $\mu\text{g/ml}$ VMC (Fig. 1), the inhibitory effect of NO by VMC was not due to a cytotoxicity on the cells.

Effects of VMC on expression of iNOS and COX-2

In order to investigate the mechanism of action of VMC on the inhibition of NO production, Western blotting was performed. We investigate the effect of the VMC at translational level by western blotting, as shown in Fig. 3, the expression of iNOS and COX-2 protein were markedly increased after

rIFN- γ (100 U/ml) plus LPS (10 $\mu\text{g/ml}$) challenge for 24 hours. This enhanced expression of iNOS protein was significantly reduced by VMC in a dose-dependent manner (Fig. 3) and VMC also inhibits expression of COX-2.

Antioxidant activities of VMC

In order to investigate the antioxidant activities of VMC, we performed five different *in vitro* assays such as DPPH radical, superoxide anion, nitric oxide radical, ABTS radical scavenging methods and reducing power. The DPPH radical scavenging activity was measured as decolorizing activity following the trapping of the unpaired electron of DPPH (Burda and Oleszcz, 2001). VMC showed DPPH radical scavenging activity, at the concentration of 500 $\mu\text{g/ml}$ with $41.62 \pm 2.75\%$ inhibition against DPPH radicals. As a control, ascorbic acid was used

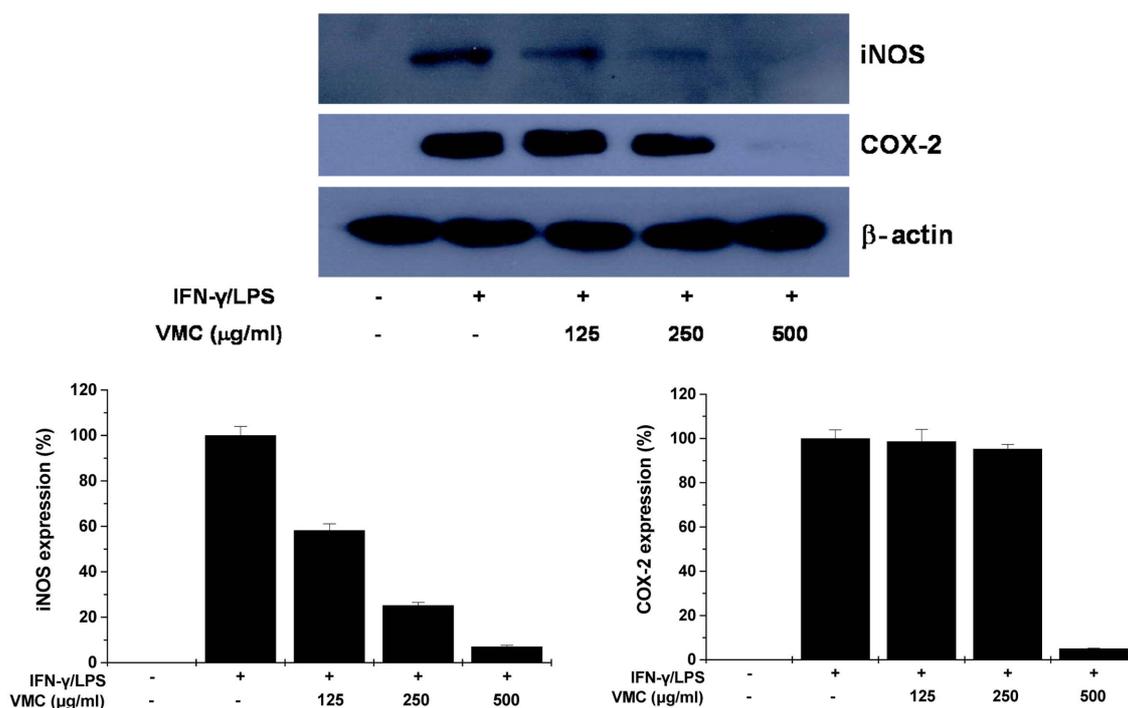


Fig. 3. Effects of VMC on the expression of iNOS and COX-2 by rIFN- γ /LPS activated peritoneal macrophages. Peritoneal macrophages (5×10^6 cells/well) were pretreated with VMC and then stimulated for 6 h with rIFN- γ (100 U/ml). The peritoneal macrophages were then stimulated with LPS (10 $\mu\text{g/ml}$) 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.

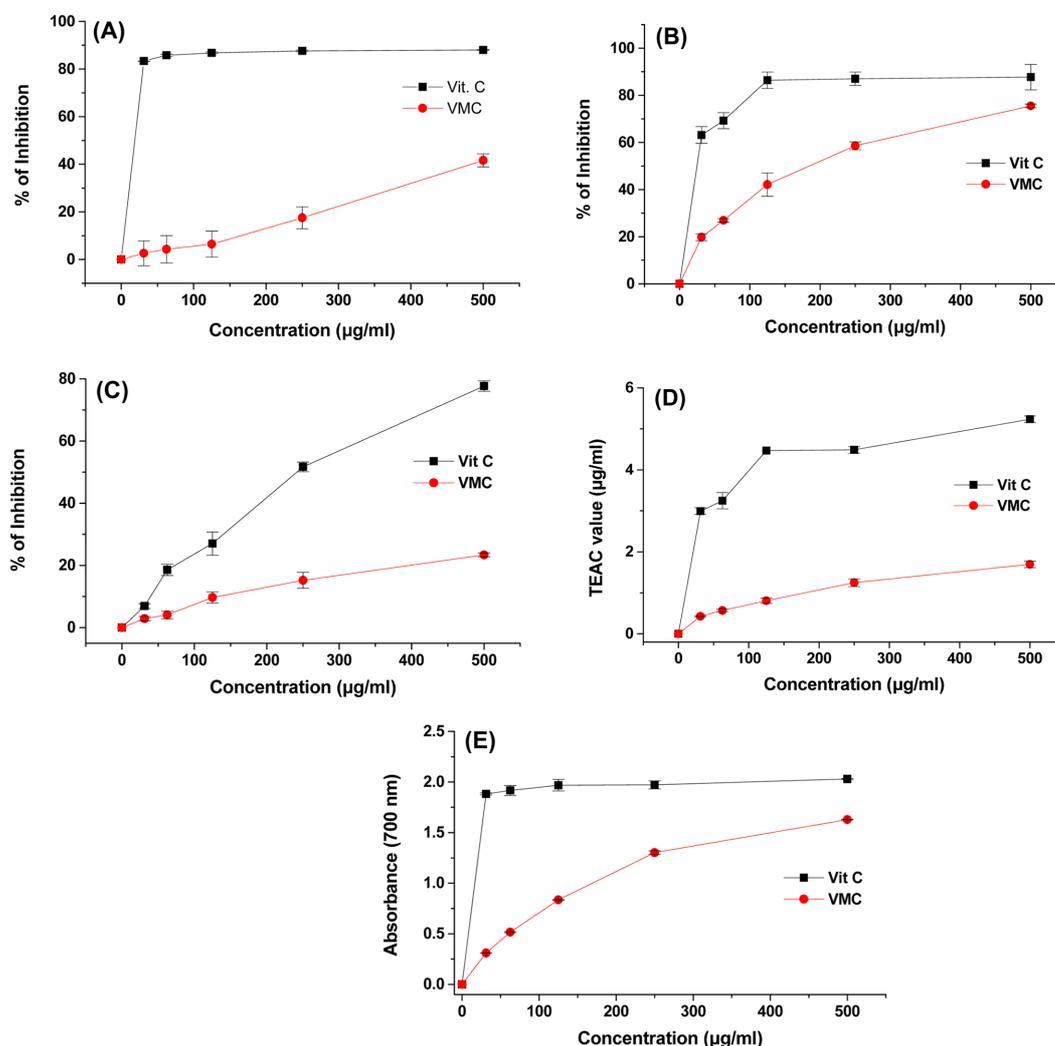


Fig. 4. Antioxidant capacity of VMC. Radical scavenging activity on various radicals such as DPPH (A), superoxide anion (B), Nitric Oxide (C), ABTS (D) and reducing power (E) of VMC were evaluated. The values of DPPH, superoxide anion and Nitric Oxide radical scavenging activities are % inhibition compared with vehicle treated group. ABTS radical scavenging activity is presented as Trolox equivalent values ($\mu\text{g/ml}$). The values of reducing power are absorbances at 700 nm. Ascorbic acid used as a positive control. Results are expressed as means \pm S.D. of three independent experiments duplicate in each run.

and % inhibition value obtained was $88.01 \pm 0.18\%$ (Fig. 4A). The superoxide radical was generated by the xanthine/xanthine oxidase system. Results from the NBT analysis revealed that $500 \mu\text{g/ml}$ of VMC, showed strong superoxide radical scavenging activities similar to ascorbic acid (Fig. 4B). 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 Fig. 4C, VMC showed relatively low nitric oxide antiradical activity. The ABTS radical assay is also frequently used to determine the radical scavenging activity. VMC exhibited high antiradical effect on ABTS radical ($1.69 \mu\text{g/ml}$ TEAC at $500 \mu\text{g/ml}$) compared to ascorbic acid (Fig. 4D). The reducing capacity implies that the sample can reduce the oxidized intermediates as an electron

donor, and many reports suggest that there is a strong correlation between antioxidant activity and reducing power. The reducing capacity was visualized by formation of Prussian blue complex at 700 nm. Higher absorbance of the reaction mixture indicates better reducing capacity. Fig. 4E. demonstrates the concentration-dependent curve of reducing power in the presence of VMC.

DISCUSSION

In the present study, we evaluated the anti-inflammatory effect of VMC on the production of inflammatory mediators in IFN- γ and LPS stimulated murine peritoneal macrophages. And we also investigated its antioxidant properties using various radical scavenging assay.

Nitric oxide (NO) is a free radical produced from L-arginine by NO synthase (NOS) and maintaining diverse physiological homeostasis (Seo *et al.*, 2001). Nevertheless, excessive NO production can cause many inflammatory diseases such as septic shock, neurologic disorders, rheumatoid arthritis and autoimmune diseases (Thiemermann and Vane., 1990). Therefore, there are growing interest in developing anti-inflammatory drug to inhibit overproduction of NO. In this study, we used IFN- γ and LPS-stimulated mouse peritoneal macrophage system to assess the inhibitory activity of VMC 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 VMC were 22.1%, 58.5% and 87.7% at the concentration of 125, 250 and 500 $\mu\text{g}/\text{ml}$ respectively. The cell viability data shows the potent inhibitory action of VMC on NO production is not due to its cytotoxicity (Fig. 1).

It is well known that excess production of NO is mediated by iNOS in activated macrophages. Therefore, attenuation of iNOS expression levels might be an attractive therapeutic target for the treatment of NO-mediated inflammatory condition. As shown in Fig. 3, VMC strongly inhibited the

expression of iNOS in a dose dependent manner. This result explained that the inhibition effect of VMC on NO production was due to its suppressive activity on iNOS expression.

COX-2, an another key enzyme in inflammatory cascade, catalyze PGE₂ synthesis from arachidonic acid (Minghetti *et al.*, 1998). High levels of PGE₂ take an important part in inflammatory conditions including asthma, rheumatoid arthritis and multiple sclerosis (FitzGerald, 2003). In this study, the possibility that VMC might inhibit COX-2 expression was examined and VMC suppressed the expression of COX-2 dose dependent manner (Fig. 3). Thus, it seems quite reasonable to speculate that VMC may inhibits PGE₂ production. However, further studies are required to determine whether VMC is a selective COX-2 inhibitor.

The previous report showed the anti-inflammatory effect of the 50% methanolic extract of *V. officinalis* using carrageenan-induced paw edema model (Calvo, 2006), and this finding implies the possibility that VMC may suppress the pro-inflammatory mediators. Here in our study, we have shown that VMC exerts its anti-inflammatory effects by inhibition of pro-inflammatory mediators such as NO, iNOS and COX-2. In addition, Calvo (2006) also revealed that *V. officinalis* significantly suppressed second phase of formalin test which is known as inflammatory pain that can be down-regulated by anti-inflammatory drug. In this study, VMC showed attenuated COX-2 expression, and therefore, it could be assumed that anti-nociceptive activity of *V. officinalis* in second phase may be due to its anti-inflammatory potential, at least in part.

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

In the present study, we investigated the radical scavenging activity and reducing power of VMC using various radicals. The values obtained with DPPH assay, X/XO/NBT assay, Nitric oxide radical assay and ABTS radical assay showed that VMC has strong radical scavenging and potent reducing power properties as a natural ROS scavenger. These findings strongly indicate that VMC have potent antioxidant potential and are in agreement with previous reports (Casanova *et al.*, 2008).

In summary the present results demonstrate that VMC 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. VMC also suppressed expression of COX-2 release in a concentration-dependent manner. We also investigated the antioxidant properties of VMC. The present study clearly revealed that VMC has strong scavenging activity and reducing power. These results strongly suggest that VMC could be an useful therapeutic agent for the treatment of ROS-mediated chronic inflammatory diseases as an effective immunomodulatory material.

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