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Article

Anti-inflammatory Effects of Cowpea (*Vigna sinensis* K.) Seed Extracts and Its Bioactive Compounds

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Anti-inflammatory effects of methanol extract and solvent fractions of cowpea (*Vigna sinensis* K.; VS) seeds and the isolated compounds were evaluated. Ethyl acetate and *n*-butanol fractions of VS seeds were found to strongly inhibit nitric oxide (NO) production, and inducible nitric oxide synthase (iNOS) mRNA and protein expressions in lipopolysaccharides (LPS)-stimulated RAW264.7 macrophage cells. Compounds inhibiting NO production in RAW264.7 cells were isolated and identified via successive partitioning and spectroscopic characterization. The active compounds included oleanolic acid, linolenic acid (LnA), linoleic acid (LA), 7-ketositosterol, stigmasterol-glucose (glc), and soyasaponin 1. Among the isolated compounds, LnA and LA were found to inhibit NO production significantly. Contents of LnA and LA in VS seeds were 2.034 and 1.162 mg/g on dry weight basis, respectively. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis show inhibitory effects of these compounds. LnA and LA repressed mRNA and protein levels of iNOS in LPS-stimulated cells. LA suppressed the production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in LPS-induced macrophage cells. Thus, VS was found to exhibit strong anti-inflammatory activity, which may be partly attributed to the polyunsaturated fatty acids such as LnA and LA.

Key words: inducible nitric oxide synthase, inflammation, linoleic acid, linolenic acid, *Vigna sinensis* K.

Legume seeds have been utilized as a good source of proteins, carbohydrates, fiber, and microelements in the human diet all over the world, and they have been known to exert beneficial and protective effects with regards to cardiovascular diseases, hypercholesterolemia, and cancers. It is believed that these beneficial health effects are mainly attributed to flavonoids and proteins [Ren *et al*., 2003; McCue and Shetty, 2004]. There are hundreds of non-isoflavonoid phytochemical components in legume seeds, including triterpenoids, saponins, and phytic acid [Kang *et al*., 2010]. It has been well documented that consumption of foods rich in omega 3 (n-3) fatty acids is beneficial for persons with cardiovascular disease and rhematioid arthritis [James and Cleland, 1997; Simopoulos, 1997].

Vigna sinensis K. (VS) is a black-eyed cultivar of cowpea [Doblado *et al*., 2005; Dueñas *et al*., 2005], which is traditionally referred to as Dongbu bean in Korea [Cui *et al*., 2010]. Cowpea is one of the most important food grain legume crops in the semi-arid tropics covering Asia, Africa, southern Europe, and Central and South America, and it is widely grown as a multipurpose crop that is utilized not only for human food and medicine, but also for animal feed [Sprent *et al*., 2010]. Proteins and extracts from various *Vigna* species have been reported to exhibit anti-mitogenic, antiviral, antifungal activities [Ye and Ng, 2001], and anticarcinogenic activity [Joanitti *et al*., 2010]. The identification and analysis of the phytochemical constituents of VS seeds and their biological activities have been limited so far, although there have been some reports on the isolation and analysis of flavonoids and phytochemical compositions

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from the cowpea extracts [Gaydou *et al*., 1983; Cai *et al*., 2003; Chang and Wong, 2004].

Recently, interests in the identification of anti-inflammatory activities of plants for development of natural therapeutic agents or functional foods have been increasing. Our laboratory is involved in screening for anti-inflammatory activities of extracts from leaves and seeds of edible plants, and seeds of VS have been found to possess one of the strongest anti-inflammatory activities among the tested plant extracts. In the present study, the anti-inflammatory activities of different solvent fractions of VS seeds were evaluated, and the bioactive compounds in LPS-stimulated RAW264.7 macrophage cells were identified and analyzed. Moreover, the effects of VS extracts and polyunsaturated fatty acids such as LnA and LA on the productions of NO and pro-inflammatory cytokines were also examined.

Materials and Methods

Extraction and fractionation. VS seeds (100 g) were extracted with 80% aqueous methanol (MeOH, 200 mL×3) at room temperature. The concentrated MeOH extracts (VSM) were suspended in H_2O (300 mL), and then extracted successively with ethyl acetate (EtOAc, 300 mL×3) and *n*-butanol (*n*-BuOH, 300 mL×3), and concentrated to afford the residues of EtOAc fraction (VSE), *n*-BuOH fraction (VSB), and H_2O fraction (VSH), respectively.

Isolation of active compounds from VS seeds. VS seeds (9 kg) were extracted with 80% aqueous MeOH (20 L×3) three times at room temperature. The concentrated MeOH extracts were suspended in H_2O (3 L), extracted with *n*-BuOH (2.8 L×3), and further concentrated. The repeated silica gel and octadecyl silica gel (ODS) column chromatographic separations for the *n*-BuOH fraction (60 g) led to isolation of LnA, LA, oleanolic acid, 7-ketositosterol, stigmasterol-glc, and soyasaponin 1 [Cui *et al*., 2010].

Gas-chromatographic (GC) analysis of LnA and LA. VS seeds (2 g) were refluxed in *n*-hexane (20 mL) and 1 M KOH/MeOH (5 mL) at 90°C for 3 h. The reaction solution was filtered through 8-μm filter paper (Whatman, Little Chalfont, UK). The filtrate was then treated with cation exchange resin (DOWEX® 50WX4-400, Sigma-Aldrich Co., St. Louis, MO) for 30 min in a stirrer, and filtered through 8-μm filter paper. The filtrate was vacuum-evaporated. The purified LnA (2 mg) and LA (2 mg) were esterified through the addition of 1 mL methanolic BF_3 at 80°C for 30 min. The obtained fatty acid methyl esters were suspended in H_2O (1 mL), extracted with *n*-hexane (1 mL), and concentrated. Analysis was carried out using a GC-14B series gas chromatograph equipped with a flame ionization detector (Shimadzu, Tyoko, Japan) as described by Parashar *et al*. [2010]. Chromatographic analysis was performed using a DB-5 capillary column with 30-m, 0.25-mm inner diameter (ID), and 0.53-mm film-thickness (J & W Scientific, Folsom, CA). Helium was used as the carrier gas at a flow rate of 1.1 mL/min. GC conditions of fatty acid methyl esters were applied as follows: the oven was heated at 180°C and held for 2 min, increased to 200°C at a rate of 10°C/min and held for 10 min, and then increased to 220°C at 5°C/min. The fatty acid methyl esters of sample solution (3 μL) were injected with a 3 : 1 split ratio. The injector and the detector were maintained at 250°C [Tong *et al*., 2007].

Cell culture. Murine macrophage RAW264.7 cells (Korean Cell Line Bank, Seoul, Korea) were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM, WelGENE, Inc., Daegu, Korea) containing 10% fetal bovine serum (FBS, WelGENE Inc.), 2 mM glutamate, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO_2. Cells were incubated with 1 μg/mL LPS (Sigma-Aldrich Co.) along with various concentrations of extracts for 24 h as indicated.

Measurements of NO and cell viability. NO content was determined by measuring the amount of nitrite, a stable oxidized product, in the cell culture supernatants as previously described [Lee *et al*., 2007]. To test the inhibitory effect of single compounds on NO synthesis, RAW264.7 cells (1×10^4 cells/well) were grown in serum-free medium on 96-well cell culture plates for 12-18 h and treated with 1 μg/mL LPS in the presence of various concentrations of extracts for 24 h. Then 100 μL cell culture supernatant was mixed with 100 μL Griess reagent (Sigma-Aldrich Co.) in a new 96-well plate, and the absorption was read at 550 nm with a spectrophotometer (EL800 universal microplate reader, Bio-Tek Instruments, Inc., Winooski, Vermont). Concentrations of the nitrite were determined by comparison with a sodium nitrite standard curve. The cells remaining after the Griess assay were used to determine cell viability by the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT, Sigma-Aldrich Co.)-based colorimetric assay. Each well was added with 10 μL of 5 μg/mL MTT solutions, and the cells were re-incubated in a 37°C, 5% CO_2 incubator in darkness. After 30-60 min, culture supernatants were removed, and each well was added with 100 μL DMSO (Sigma-Aldrich Co.). The absorption was read at 550 nm with a spectrophotometer (Bio-Tek Instruments, Inc.).

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was prepared from

RAW264.7 cells with an RNA-Bee (Tel-Test, Inc., Friendswood, TA) RNA isolation kit following the manufacturer's guide. The following primers were used for PCR amplification: iNOS, 5'-TCTTCGAAATCCCA CCTGAC-3' (forward) and 5'-CCATGATGGTCACATT CTGC-3' (reverse); IL-1β, 5'-GAAGCTGT GGCAGCT ACCTATGTCT-3' (forward) and 5'-CTCTGCTTGTGAG GTGCTGATGTAC-3' (reverse); IL-6, 5'-ATGAAGTTC CTCTCTGCAAGAGACT-3' (forward) and 5'-CACTA GGTTTGCCGAGTAGATCTC-3' (reverse); TNF-α, 5'-TTGACCTCAGCGCTGAGTTG-3' (forward) and 5'-CCTGTAGCCCACGTCGTAGC-3' (reverse). mRNA levels of β-actin, 5'-GTGGGCCGCCCTAGGCACCAG-3' (forward) and 5'-GGAGGAAGAGGATGCGGCAGT-3' (reverse), were measured as an internal control. The cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 23 to 28 amplification cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s.

Western blot analysis. RAW264.7 cells were lysed with RIPA Buffer (BIOSESANG, Inc., Seoul, Korea) containing proteinase inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were quantified with a Bradford Reagent (BIOSESANG, Inc.) protein assay kit. The proteins (40 μg/lane) were resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transfer to nitro-cellulose membrane (Pall Corporation, Washington, NY). Transferred proteins were blocked with 5% non-fat dry milk in tris buffered saline (TBS), Rabbit anti-iNOS (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as primary antibody and peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology, Inc.) was used as secondary antibody. The membranes were developed with an enhanced chemiluminescence western blotting detection reagent from Amersham™ (GE Healthcare, Little Chalfont, UK), and exposed to X-ray film (Agfa HealthCare NV, Mortsel, Belgium) for 1-10 min.

ELISA assays. Murine RAW264.7 peritoneal macrophage cells were cultured in 48-well plates for 24 h. They were then washed with phosphate buffered saline (PBS) and treated with various concentrations of LA with LPS for 24 h. Supernatants were collected, and levels of IL-1β, IL-6, and TNF-α released into the culture supernatants were measured using ELISA kits (all from Bender MedSystems, Inc., San Diego, CA), according to the manufacturers' recommendations.

Statistical analysis. Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way Analysis of Variance (ANOVA) test, followed by Student's t-test.

Results

Effects of VS extracts on LPS-activated NO production. To determine the effects of the MeOH extract and the solvent fractions on NO production, mouse macrophage RAW264.7 cells were incubated with 1 μg/mL of LPS for 24 h in the presence of various concentrations (0-100 μg/mL) of the MeOH extract and its solvent fractions of VS seeds. The NO production, measured as the stable nitrite metabolite of NO, increased to about 24 μM in LPS-stimulated RAW264.7 cells, which is about seven-fold higher than that in the control cells without LPS. Among the VSM and the subsequent solvent fractions, VSE and VSB most strongly inhibited the LPS-stimulated production of NO (Fig. 1A), whereas VSH showed no significant inhibition of NO production in LPS-stimulated cells up to 100 μg/mL. Cell viabilities were measured under the same conditions and were determined to be greater than 80% for the concentrations used in the present study (Fig. 1B). These results indicate that the inhibition of NO production is not attributable to cell death.

Effects of VS extracts on iNOS mRNA and protein expression in LPS-stimulated cells. To investigate whether VS extracts inhibited NO production at the gene level, the expression levels of iNOS mRNA and protein in LPS-stimulated RAW264.7 cells in the presence of various concentrations of VS extracts were measured for 24 h. RT-PCR analysis indicated that VSE and VSB strongly inhibited expression of iNOS mRNA without affecting the levels of β-actin mRNA, a house-keeping gene (Fig. 1C). Western blot analysis showed that the amount of the 130-kDa iNOS protein was increased by LPS, and the increased iNOS protein level was significantly reduced by VSM of VS seeds and strongly by treatment with the VSE or VSB, whereas the iNOS protein was not reduced by VSH (Fig. 1D). These results were consistent with the effects of VS extract and fractions on NO production.

Isolation and analysis of the active compounds from VS. VS seeds were extracted with 80% MeOH, and the extracts were successively partitioned with *n*-BuOH and H_2O. Repeated SiO_2 and ODS c.c. for the *n*-BuOH fraction afforded several purified compounds, such as oleanolic acid, LnA, LA, 7-ketositosterol, stigmasterol-glc, and soyasaponin 1. Their chemical structures were determined on the basis of spectroscopic data including NMR, mass spectroscopy, and IR [Cui *et al*., 2010]. Among the isolated compounds, the stereostructures of LnA [(Z,Z,Z)-9,12,15-octadecatrienoic acid] and LA [(Z,Z)-9,12-octadecadienoic acid] were analyzed by gas-chromatography-mass spectrometry (GC-MS) and identified

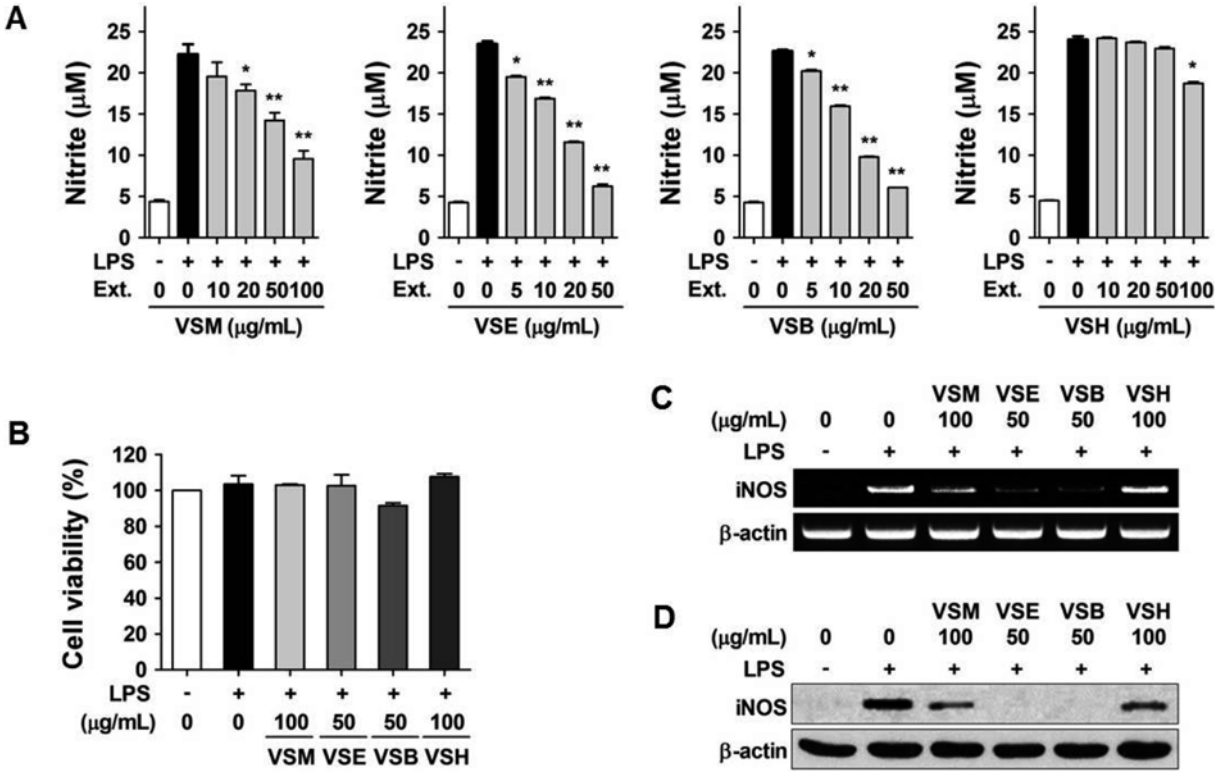

Fig. 1. Effects of MeOH extract and different solvent fractions of VS seeds on LPS-activated NO production, iNOS expression, and cell viability. (A) RAW264.7 cells were treated with LPS (1 μg/mL) in the presence of various concentrations of VSM extract, VSE, VSB, and VSH fractions of VS seeds for 24 h. Nitrite levels in the culture media of LPS-stimulated cells were measured by the Griess reaction. The data are means ± SD of at least three independent experiments with triplicate samples (*p <0.05, **p <0.01 versus LPS alone). (B) Cell viability was measured using the MTT assay. (C) The levels of iNOS mRNAs were determined by RT-PCR analysis. β-Actin mRNA was used as an internal control. (D) Levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS. The blot was rehybridized with antibody against β-actin to verify the equal loading of proteins.

by the aid of mass spectra library (online National Institute of Standards and Technology, NIST). The compounds were analyzed for their inhibitory activities of NO production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with 1 μg/mL of LPS for 24 h, in the presence of various concentrations (0, 10, and 50 μM) of each compound, among which oleanolic acid, a tetracyclic triterpene, and fatty acids such as LnA and LA exhibited strong inhibitory effects on NO production (Fig. 2). Analysis of other compounds demonstrated that 7-ketositosterol, stigmasterol-glc, and soyasaponin 1 have weak inhibitory activities compared to those of oleanolic acid, LnA, and LA. The cell viability and cytotoxicity of the isolated compounds were also evaluated, and results showed that all compounds examined did not affect the viability of RAW264.7 cells at the concentrations used in the present study (Data not shown). The anti-inflammatory activities of oleanolic acid and LnA have been previously reported [Ren and Chung, 2007; Puangpraphant and de Mejia, 2009].

Quantitation of LnA and LA in hydrolytic *n*-hexane extracts. The contents of LnA and LA in VS extracts of VS seeds were determined by gas chromatography. Because fatty acids generally occur in plant cell as glyceride rather than free form, the seeds were treated by alkaline hydrolysis to liberate the fatty acids from the glyceride. Contents of methyl LnA and methyl LA in hydrolytic *n*-hexane extracts were determined using gas chromatography. The results from the GC chromatograms indicated that hydrolytic and normal *n*-hexane extracts contained LnA and LA as identified by comparison of their retention time values with those of standards. Using a standard curve, the amounts of LnA and LA in the VS extracts were calculated to be 2.034 and 1.162 mg/g extract, respectively.

Inhibitory effects of LA on LPS-activated iNOS mRNA and protein expression levels. LnA and phytochemicals such as oleanolic acids, 7-ketositosterol, stigmasterol-glc, and soyasaponin 1 have been previously isolated from various plants, and they were shown to

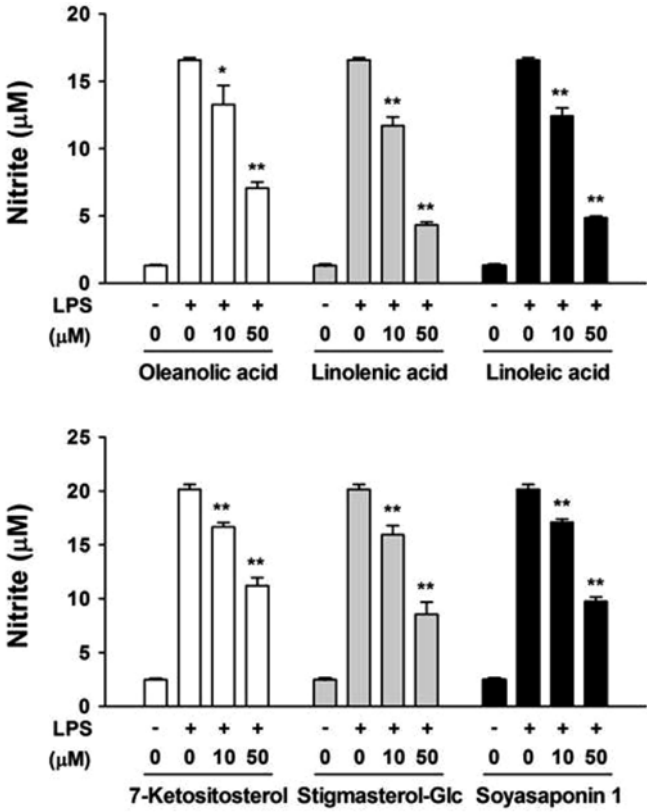

Fig. 2. Effects of the isolated compounds from VS seeds on LPS-induced NO production. RAW264.7 cells were treated with LPS (1 μg/mL) in the absence or presence of each compounds from VS seeds for 24 h. Nitrite levels in the culture media of LPS-stimulated cells were measured by the Griess reaction. Data shown are the means ± SD (n=3). *$p <$ 0.05 or **$p <$0.01 compared to treatment with LPS alone.

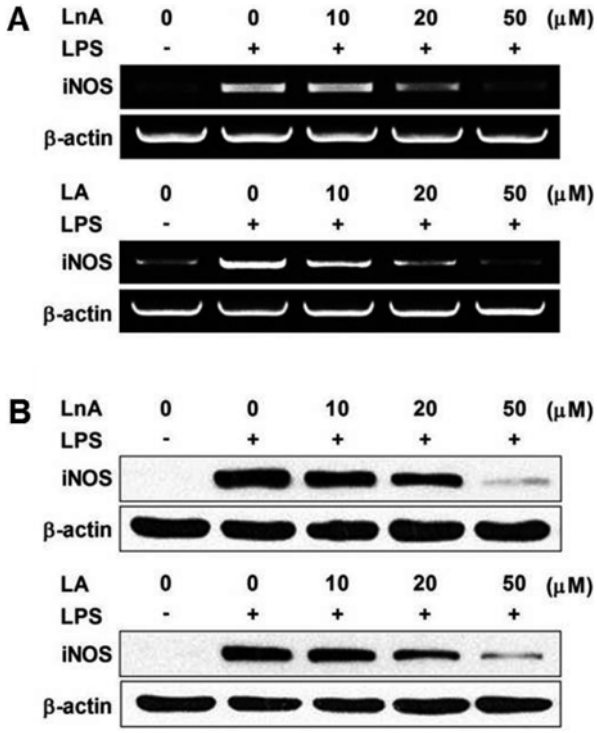

Fig. 3. Effects of LnA and LA on LPS-activated iNOS mRNA and protein expression levels. RAW264.7 cells were treated with LPS (1 μg/mL) in the presence of various concentrations of LnA or LA for 24 h. (A) Levels of iNOS mRNAs were determined by RT-PCR analysis. β-Actin mRNA was used as an internal control. (B) Levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS. The blot was rehybridized with antibody against β-actin to verify the equal loading of proteins.

exert inhibitory activities [Ren and Chung, 2007; Koschutnig *et al*., 2009; Puangpraphant and de Mejia, 2009; Reutrakul *et al*., 2010; Zha *et al*., 2011]. Thus, focus was placed on the analysis of anti-inflammatory activity of LA. Effects of LA on iNOS gene expression level in LPS-stimulated cells was investigated and compared to those of LnA. Furthermore, iNOS mRNA and protein levels were measured in RAW264.7 cells treated with 1 μg/mL LPS for 24 h in the presence of various concentrations of LA or LnA. RT-PCR and Western blot analyses indicated that LA caused dose-dependent reductions in iNOS mRNA and protein levels (Fig. 3). The results revealed that inhibitory effects of LA on iNOS expression were comparable to those of LnA in LPS-stimulated RAW264.7 macrophage cells.

Inhibitory effects of LA on LPS-induced expressions of pro-inflammatory cytokines. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are also induced as part of the inflammatory process in LPS-stimulated RAW264.7 cells. Therefore, the effects of LA were investigated on the expressions of IL-1β, IL-6, and TNF-α in RAW264.7 macrophage cells treated with 1 μg/mL LPS for 24 h in the presence of various concentrations of LA. The anti-inflammatory activities of VSB and LA were evaluated, and the results indicated that LA as well as VSB inhibited the expressions of IL-1β, IL-6, and TNF-α mRNAs in a concentration-dependent manner in LPS-stimulated cells, without affecting the expression of the control gene β-actin (Fig. 4). Secreted pro-inflammatory cytokine levels were measured in the supernatant of LPS-stimulated cells treated with various concentrations of LA. The results of the ELISAs are shown in Fig. 5. The cytokine levels of IL-1β, IL-6, and TNF-α in the supernatants of the stimulated cells were reduced by LA in dose-dependent manners.

Discussion

Cowpeas (*V. Sinensis* K.) are recognized as a source of

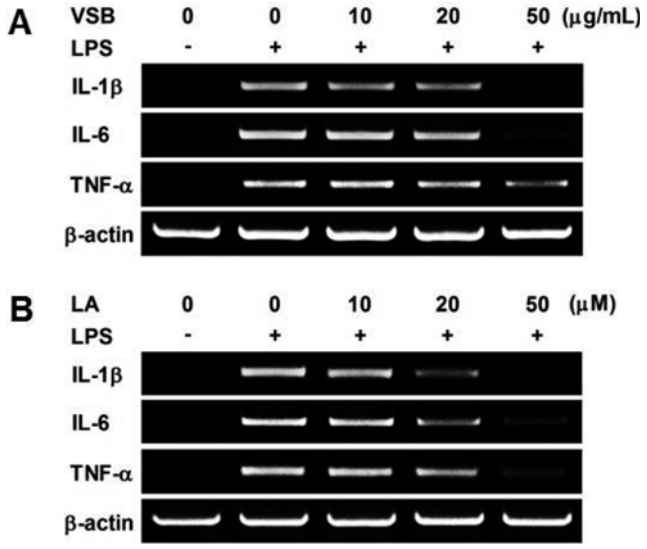

Fig. 4. Effects of VSB and LA on LPS-induced expression of pro-inflammatory cytokines mRNA levels. RAW264.7 cells were treated with LPS (1 μg/mL) in the presence of various concentrations of VSB or LA for 24 h. VSB (A) and LA (B) were reduced in IL-1β, IL-6, and TNF-α mRNA levels in a concentration-dependent manner in LPS-stimulated cells, without affecting the mRNA expression of β-actin, the housekeeping gene.

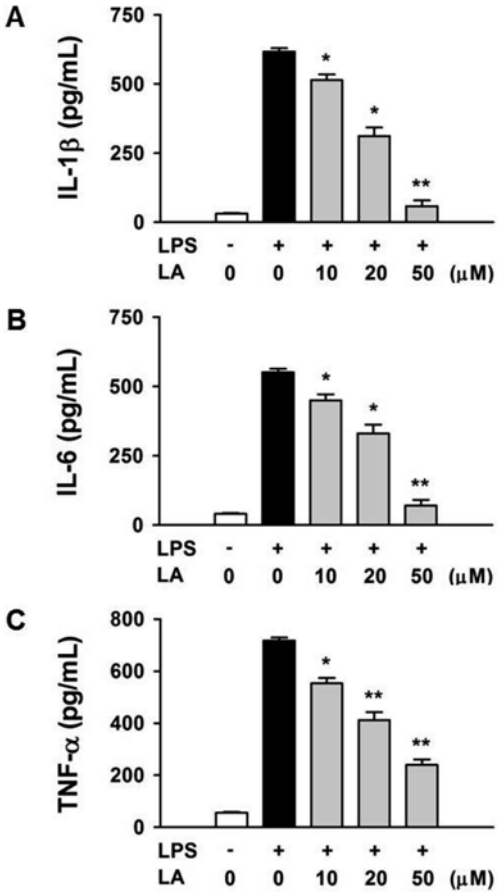

Fig. 5. Effects of LA on LPS-stimulated expression of pro-inflammatory cytokines. RAW264.7 cells were treated with LPS (1 μg/mL) in the presence of various concentrations of LA for 24 h. The secreted pro-inflammatory cytokines levels of IL-1β (A), IL-6 (B), and TNF-α (C) in the supernatants were determined by ELISA analysis. Data are shown as the means ± SD (n=3). $*p<0.05$ or $**p<0.01$ compared to treatment with LPS alone.

proteins and other nutrients, and are consumed especially in developing countries. In addition, legume seeds contain a variety of bioactive compounds, which include isoflavones, phenolic acids, saponins, phytic acid, and phytosterols. Unlike bioactive compounds from soybeans, which have been extensively investigated, bioactive compounds of cowpeas have received limited attention. The objective of the present study was to investigate the anti-inflammatory effects of the extract and the solvent fractions of VS seeds, and identify the bioactive compounds in LPS-stimulated RAW264.7 macrophage cells. Our results showed that the ethyl acetate and *n*-BuOH fractions of VS seeds have important anti-inflammatory effects on macrophage cell culture systems. Moreover, a number of phytochemicals were isolated as active components for anti-inflammatory activity in VS seeds. Investigation of present study verified that LnA, LA, and oleanolic acids exhibit significant anti-inflammatory activities, whereas phytochemicals such as 7-ketositosterol, stigmasterol-glc, and soyasaponin 1 show relatively weak inhibitory activities, which are consistent with the observations of other investigators [Koschutnig *et al*., 2009; Puangpraphant and de Mejia, 2009; Reutrakul *et al*., 2010; Zha *et al*., 2011].

TNF-α, IL-1β, and IL-6 are produced mainly from activated macrophages, and TNF-α and IL-1β are pivotal mediators of the acute inflammatory response to Gram-negative bacteria and other bacterial infections. IL-6 also plays important roles in both innate and adaptive immunities and is responsible for many inflammatory responses. Here, we observed that VS seed extracts could remarkably suppress the production of inflammatory cytokines, including TNF-α, IL-1β, and IL-6 in LPS-stimulated RAW264.7 cells. Furthermore, the VS extract could inhibit mRNA expressions of IL-1β, IL-6, and TNF-α probably at the transcriptional level.

An examination of the cell viability in the presence of different solvent extracts of VS in RAW264.7 cells indicated that the concentrations of these extracts used in the present study did not affect the viability of RAW264.7 cells. Furthermore, the compounds were found to not affect the viability of the macrophage cells, indicating that they did not cause cytotoxicity in the cells under the conditions used in the present study. Thus, the inhibitory

effects are not attributable to cytotoxic effects.

Legume seeds contain high contents of oils, and the seed lipids are rich in unsaturated fatty acid. The concentrations of LnA and LA in VS seeds were found to be 2.034 and 1.162 mg/g, respectively, according to GC analysis, showing almost two-fold more LnA than LA in VS seeds, indicating that these values are nutritionally desirable. LnA (18:3ω3) is a precursor for the formation of eicosapentaenoic acid (20:5ω3), which can displace arachidonic acid (20:4ω6) and reduce the production of prostaglandins. Dietary α-linolenic acid has been reported to modulate some of the inflammatory response in experimental animal models and clinical trials [Chavali *et al*., 1998]. A number of investigators have also reported the effects of dietary fatty acids on expression and production of inflammatory cytokines [Weaver *et al*., 2009; Ambrozova *et al*., 2010]. Ren and Chung [2007] reported that LnA repressed iNOS expression via nuclear factor-κB and mitogen-activated protein kinase pathways. In recent years, ω3 fatty acids such as α-linolenic acid have been suggested to have many pharmacological effects on the treatment of arthritis and cardiovascular diseases such as heart attack, hypercholesterolemia, and hypertension [James and Cleland, 1997; Simopoulos, 1997; 2008], and possess a strong neuroprotective effect [Lauritzen *et al*., 2000].

LA (18:2ω6) is the most abundant polyunsaturated fatty acid identified in most legume seeds, making up 40 to 50% of total lipids [Siddhuraju *et al*., 2000; Ryan *et al*., 2007]. LA inhibited iNOS gene expression at both mRNA and protein levels and reduced the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in LPS-stimulated RAW264.7 cells. Previous reports showed that LA metabolites suppressed pro-inflammatory mediator expression in LPS-stimulated macrophage and 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced inflammation in ears and skin of mice [Murakami *et al*., 2005; Yasuda *et al*., 2009], which appear to be correlated with our observations that LA inhibited NO production and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. However, studies by others showed that LA or LnA increased the expression of iNOS and cyclooxygenase-2 genes and the production of prostaglandin E_2 and NO in retinal pigment epithelial cells (RPE) and that LA can induce oxidative stress in RPE cells, consequently leading to cellular damage [Akeo *et al*., 1996; Fang *et al*., 2007; 2009]. These discrepancies may be explained partly by different conditions of experiments, in that macrophage cells or RPE were either stimulated or non-stimulated with LPS or TPA.

In conclusion, the present study demonstrated *V. sinensis* extract exhibits strong anti-inflammatory activity and contains a variety of non-flavonoid phytochemicals and polyunsaturated fatty acids with higher amounts in LnA, suggesting that VS seeds may be utilized as good sources of functional foods for protection against inflammatory diseases.

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