

## Anti-inflammatory Activity of *Stevia rebaudiana* in LPS-induced RAW 264.7 Cells

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### Abstract

*Stevia rebaudiana* (SR) is an herb used traditionally as a sweetener in Paraguay and Brazil, whose use is spreading to other countries, such as Japan, Korea and China. In addition to its low calorie sweet taste, SR appears to have other beneficial properties, such as hypotensive capabilities and inflammation reduction. To identify the bioactive natural constituents exerting anti-inflammatory activities, we examined the EtOAc fraction of SR. In the inflammatory mediator inhibitory assay from lipopolysaccharide (LPS)-activated macrophages, the EtOAc fraction significantly, and dose dependently, inhibited the enhanced production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression. We also found that treatment of cells with the EtOAc fraction significantly inhibited LPS-stimulated nuclear factor- $\kappa$ B (NF- $\kappa$ B) reporter gene expression. Such inhibition of NF- $\kappa$ B was closely associated with the inhibition of interleukin-6 (IL-6) and the monocyte chemoattractant protein-1 (MCP-1). Therefore, we suggest that SR has the potential for development as a functional food for the treatment of immune diseases, such as rheumatoid arthritis and lupus.

**Key words:** *Stevia rebaudiana*, lipopolysaccharide, nitric oxide, inducible nitric oxide synthase, nuclear factor kappa B

### INTRODUCTION

Macrophages play an important role in the host defense against infection with pathogens. Macrophages can kill pathogens directly by phagocytosis and indirectly via the secretion of various pro-inflammatory mediators such as bioactive lipids, reactive oxygen and nitrogen species, cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) (1). Overproduction of the inflammatory mediators by activated macrophages has been implicated in the pathophysiology of many inflammatory diseases, including rheumatoid arthritis, atherosclerosis, chronic hepatitis, and pulmonary fibrosis (2,3).

These pro-inflammatory mediators are primarily regulated at the level of gene transcription through the activities of several transcription factors. One of the most ubiquitous transcription factors is nuclear factor  $\kappa$ B (NF- $\kappa$ B). NF- $\kappa$ B is activated by numerous stimuli such as lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall, and interferon (IFN)- $\gamma$  (4,5). Therefore, an NF- $\kappa$ B inhibitor may be useful in the development of therapeutic drugs to control the inflammation associated with human diseases.

*Stevia rebaudiana* (SR) is an herbaceous perennial

plant indigenous to Paraguay and Brazil where its leaves have been used by the local Guarani Indians as natural sweetener for hundreds of years. This plant is of worldwide importance today because its leaves are used as non-nutritive high potency sweetener, primarily in Japan, Korea, China and South America. Several recent reports have documented that SR has beneficial effects on human health including hypoglycemic and hypotensive effects, and as a source of antioxidant (6-8). In addition, major compounds such as steviol and stevioside inhibit the production of cytokine and NO in THP-1 cells (9). However, the biological activities and mechanisms of SR during inflammation of macrophages are largely unknown.

Therefore, we evaluated the pharmacologic effects of the EtOAc fraction from SR on the production of inflammatory mediators and regulation of transcription in LPS-stimulated mouse macrophages.

### MATERIALS AND METHODS

#### Reagents

*Stevia rebaudiana* was purchased from Duk Hyun Dang (Seoul, Korea). Griess reagent, protease inhibitor cocktail and phenylmethylsulfonyl fluoride chemicals

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were from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Hyclone (Logan, UT, USA). Ez-cytox cell viability kit was from DAEIL Lab (Seoul, Korea). ELISA kit was from R&D System (Minneapolis, MN, USA). RNeasy mini kit was from Qiagen (Valencia, CA, USA). PCR primers were from Bioneer (Seoul, Korea). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

#### Cell culture

RAW 264.7 macrophage cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C with 5% carbon dioxide.

#### Extraction and isolation

The dried leaves of SR (2 kg) were extracted with hot water (85°C) under reflux. The extract was filtered through filter paper. The concentrated water extract (270 g) was partitioned using ethyl acetate (EtOAc), butanol (BuOH) and water. The fractions were redissolved in DMSO to 100 mg/mL and used.

#### Cell viability

RAW 264.7 cells were cultured in a 96-well plate at 37°C for 24 hr. After 24 hr the EtOAc fraction was added to each well according to the concentration and then incubated at 37°C for 24 hr. After the incubation period 10 µL of EZ-cytox cell viability assay kit solution was added into each well and incubated at 37°C, 5% CO<sub>2</sub> for 4 hr. The extent of the reduction to formazan within the cells was quantified by measuring the absorbance at 480 nm using an ELISA reader. The reference wavelength was 650 nm.

#### Nitrite assay

RAW 264.7 cells were cultured in a 96-well plate and incubated with medium containing various concentrations of the EtOAc fraction in the presence or absence of LPS (1 µg/mL) for 18 hr. Equal volumes of culture supernatant and Griess reagent were mixed and incubated for 15 min at room temperature. The absorbance was measured at 570 nm on a spectrophotometer and referred to a nitrite standard curve to determine the nitrite concentration in the supernatants.

#### Measurement of IL-6 and MCP-1 by ELISA

The level of IL-6 and MCP-1 in the supernatants from macrophage cultures was determined by ELISA kit. RAW 264.7 cells were incubated with LPS (1 µg/mL) in the presence of different concentrations of the EtOAc fraction for 4 hr. The supernatants were collected and stored at -80°C before analysis.

#### Quantitative real-time PCR

RAW 264.7 cells were incubated with LPS (1 µg/mL) in the presence of different concentrations of the EtOAc fraction for 18 hr. Total RNA was extracted from cultured cells using an RNeasy mini kit according to the manufacturer's instructions. Reverse transcription of the total RNA was performed using Advantage RT-for-PCR kit. Real-time quantitative PCR was performed using a Chromo4 real-time PCR detection system employing iTaq<sup>TM</sup> SYBR<sup>R</sup> Green supermix as the dsDNA-specific binding dye for continuous fluorescence monitoring. All reactions were performed in triplicate to confirm reproducibility. The amount of mRNA in each sample was normalized using that of the mean β-actin levels. Primer sequences were as follows: iNOS, forward primer 5'-TCCTACACCACACCAAAGTGTGTGC-3', reverse primer 5'-CTCCAATCTCTGCCTATCCGTCTC-3'; β-actin, forward primer 5'-TGAGAGGGAAATCGTGCGTGAC-3', reverse primer 5'-GCTCGTTGCCAATAGTGATGACC-3'.

#### Western blotting

RAW 264.7 cells were incubated with LPS (1 µg/mL) in the presence of different concentrations of the EtOAc fraction. After 18 hr the treated and control cells were harvested for analysis. The cells were lysed with NP-40 cell lysis buffer containing a protease inhibitor cocktail and phenylmethylsulfonyl fluoride for 30 min on ice. Insoluble debris was removed by centrifugation, and the protein concentration was determined using the Bio-Rad protein assay reagent. Cell lysates were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with a solution of 0.05% Tween 20/Tris-buffered saline that contained 5% nonfat dry milk for 1 hr at room temperature. After incubation overnight at 4°C with the rabbit polyclonal anti-iNOS/NOS type II antibody and rabbit polyclonal anti β-tubulin antibody the membranes were incubated with goat anti-rabbit IgG HRP-conjugated secondary antibody for 1 hr at room temperature. Immunodetection was carried out using enhanced chemiluminescence. The relative band densities were determined by densitometry using image acquisition and analysis software.

#### Luciferase assay for NF-κB activity

To determine promoter activity we used a dual-luciferase assay system. Briefly, RAW 264.7 cells were cultured in 6-well plates overnight and transiently co-transfected with NF-κB-Luc vector and pRL-TK vector using Lipofectamine. After transfection the EtOAc fraction was added for 2 hr and the cells were treated with LPS

(1  $\mu\text{g/mL}$ ) for 24 hr. The firefly and Renilla luciferase activities in cell lysates were measured using a luminometer. Relative luciferase activities were calculated by normalizing NF- $\kappa\text{B}$ -Luc-driven firefly activity to Renilla luciferase activity.

#### Data analysis

Significance between pairs of mean values was determined by the Student's t-test. A  $p < 0.05$  was considered significantly for all the analyses.

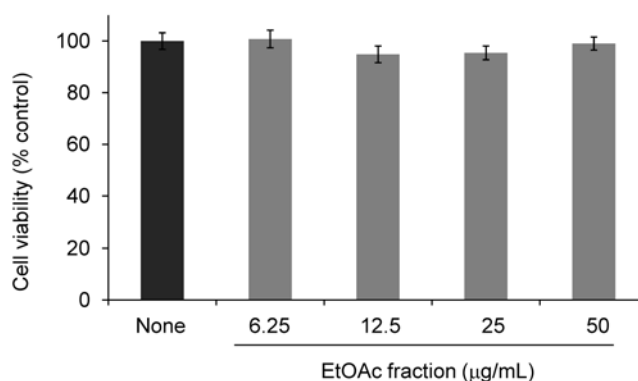
## RESULTS AND DISCUSSION

### Cytotoxicity of the EtOAc fraction for RAW 264.7 cells

The cytotoxicity of the EtOAc fraction was examined to determine the effective concentration for treatment. Exposure to 50  $\mu\text{g/mL}$  of the EtOAc fraction for 24 hr did not affect the viability of the RAW 264.7 cells (Fig. 1). These data indicate that the EtOAc fraction does not affect the viability of RAW 264.7 cells at a concentration lower than 50  $\mu\text{g/mL}$ .

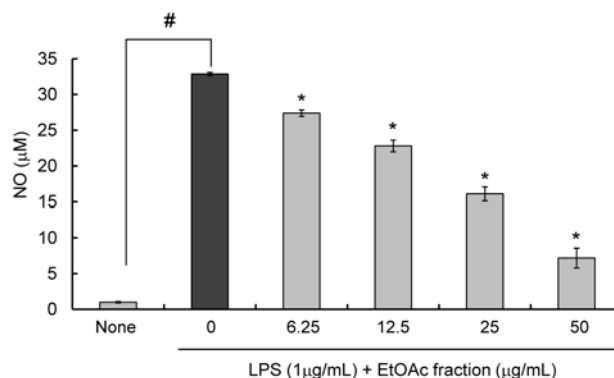
### Inhibition of the EtOAc fraction on LPS-induced NO production by suppressing iNOS expression

Nitric oxide (NO) is a pluripotent signaling molecule synthesized by a family of nitric oxide synthase isoforms (NOS) found in most tissues (10,11). NO possesses numerous biologic properties in many cell types ranging from bactericidal effects of macrophages, signal transduction during inflammation, cytoprotection, vasodilation, and regulation of apoptosis, to long term potentiation in neural networks. There are three distinct NOS isoforms that enzymatically produce NO from L-arginine. These isoforms are identified as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). The expression of iNOS is induced in a wide range of cell types during inflammation. For this reason, we evaluated the effect of the EtOAc, BuOH,

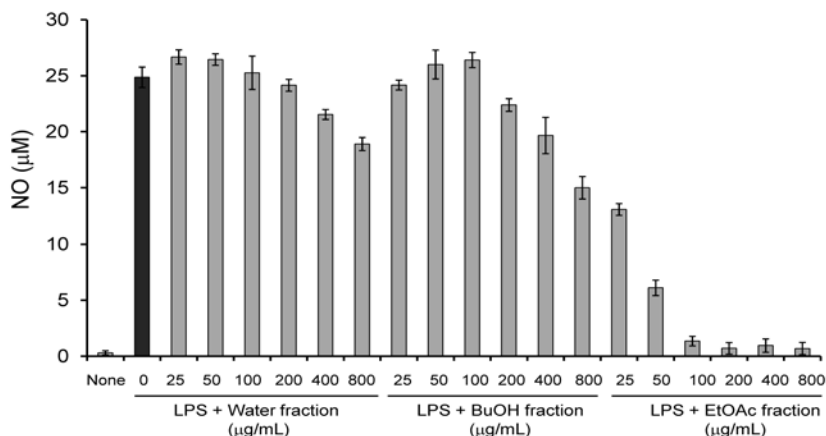


**Fig. 1.** Cytotoxicity of the EtOAc fraction on RAW 264.7 cells. RAW 264.7 cells were treated with various concentrations of the EtOAc fraction for 24 hr.

and water fractions on LPS-induced NO. Unstimulated RAW 264.7 cells secreted basal levels of NO, while LPS stimulation resulted in an increase in NO production (Fig. 2 and Fig. 3). As shown Fig. 2, the inhibitory activity of the EtOAc fractions was higher than others.



**Fig. 3.** Effects of the EtOAc fraction on LPS-induced NO production. RAW 264.7 cells were treated with various concentrations of the EtOAc fraction for 2 hr prior to the addition of LPS (1  $\mu\text{g/mL}$ ), and the cells were further incubated for 18 hr. Data are shown the mean  $\pm$  SD ( $n=4$ ). # $p < 0.001$  versus a media alone-treated group. \* $p < 0.001$  versus LPS alone-treated group.



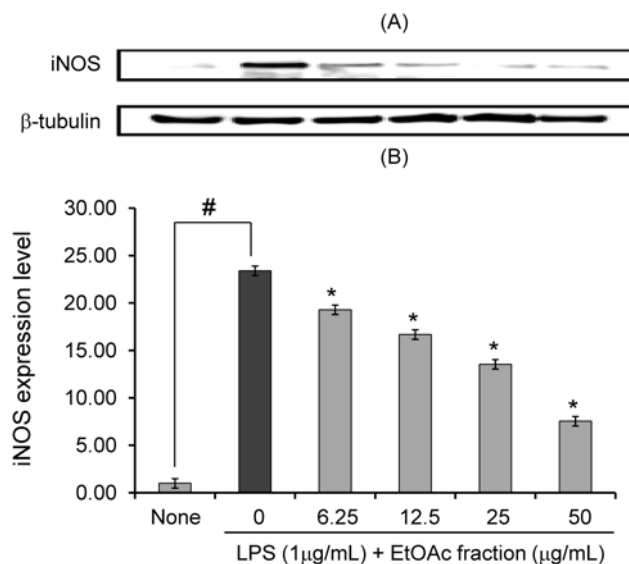
**Fig. 2.** Effects of the water, BuOH, and EtOAc fractions on LPS-induced NO production. RAW 264.7 cells were treated with various concentration of the water, BuOH, and EtOAc fractions for 2 hr prior to the addition of LPS (1  $\mu\text{g/mL}$ ), and the cells were further incubated for 18 hr. Data are shown the mean  $\pm$  SD ( $n=4$ ).

Therefore, the EtOAc fraction was chosen for further investigation. The EtOAc fraction significantly inhibited the production of LPS-induced NO in a concentration-dependent manner with an  $IC_{50}$  value of 29.4  $\mu\text{g/mL}$  (Fig. 3).

To further understand the effects of the EtOAc fraction on iNOS expression levels, RT-PCR and Western blot analyses were performed to determine the mRNA and protein expression levels at 18 hr after LPS stimulation. Untreated cells expressed undetectable levels of iNOS mRNA and protein, and the EtOAc fraction attenuated mRNA and protein levels of LPS-induced iNOS in RAW264.7 cells (Fig. 4). This implies that the EtOAc fraction inhibited NO production at the transcriptional level of the iNOS gene in LPS-stimulated RAW264.7 cells.

#### Effect of the EtOAc fraction on LPS-induced NF- $\kappa$ B activation

A search for common pathways involved in the regulated induction of diverse inflammatory gene production has focused on transcriptional control mechanisms and has identified NF- $\kappa$ B as a likely converging point of various immune and inflammatory responses (12). NF- $\kappa$ B can be activated in cells stimulated with LPS or other inflammatory insults to stimulate transcription. Therefore, we performed reporter gene analysis using luciferase reporter plasmids containing minimal NF- $\kappa$ B binding se-

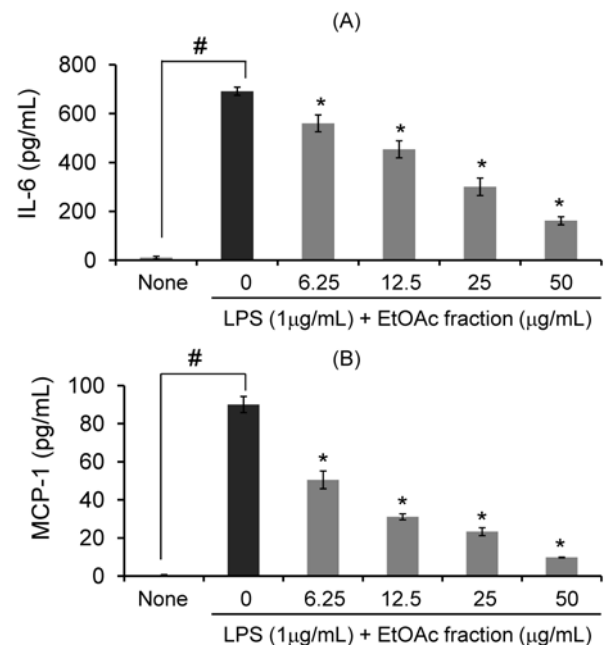


**Fig. 4.** Effects of the EtOAc fraction on LPS-induced iNOS expression levels. (A) Western blotting, (B) Quantitative real time PCR. RAW 264.7 cells were treated with various concentration of the EtOAc fraction for 2 hr prior to the addition of LPS (1  $\mu\text{g/mL}$ ), and the cells were further incubated for 18 hr. Data are shown the mean  $\pm$  SD (n=3). #p<0.001 versus a media alone-treated group. \*p<0.001 versus LPS alone-treated group.

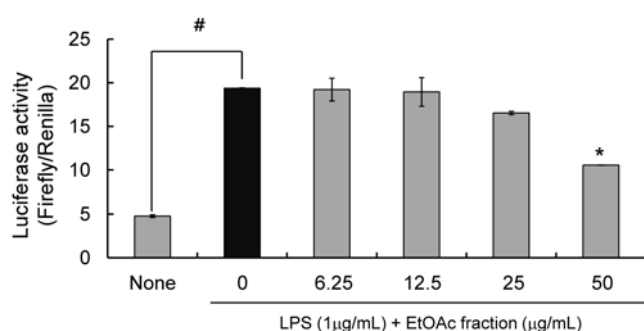
quences to determine if the EtOAc fraction inhibited NF- $\kappa$ B reporter activation. RAW264.7 cells were transfected with pNF- $\kappa$ B-Luci vector and pRL-TK vector using lipofectamine. It was found that incubation of transfected RAW 264.7 cells with LPS (1  $\mu\text{g/mL}$ ) for 24 hr increased luciferase activity by 4.1-fold compared with that of the control group. Concurrent treatment of the cells with 6.25, 12.5, 25 and 50  $\mu\text{g/mL}$  of the EtOAc fraction suppressed promoter activity by 99.4, 97.09, 85.4 and 54.4%, respectively (p<0.05) (Fig. 5). Therefore, the EtOAc fraction suppressed NO formation by blockade of NF- $\kappa$ B activation. Boonkaewwan et al. (13) reported that stevioside is a major compound in SR inhibited NF- $\kappa$ B activation in T84 cells. However, the EtOAc fraction contained a small amount of stevioside by HPLC spectrum analysis of the EtOAc fraction (data not shown). Based on this result, we hypothesized that another major compound of the EtOAc fraction likely participates in blocking NF- $\kappa$ B activity on RAW 264.7 cells.

#### Effect of the EtOAc fraction on an LPS-induced pro-inflammatory cytokine and chemokine

We already confirmed that the EtOAc fraction suppressed the NF- $\kappa$ B activity; therefore, we want to know whether EtOAc fraction could also regulate the pro-



**Fig. 5.** Effects of the EtOAc fraction on NF- $\kappa$ B-dependent reporter gene activity. RAW 264.7 macrophage cells were treated with various concentration of the EtOAc fraction for 2 hr prior to the addition of LPS (1  $\mu\text{g/mL}$ ), and were further incubated for 24 hr. Data are shown the mean  $\pm$  SD (n=3). #p<0.001 versus a media alone-treated group. \*p<0.001 versus LPS alone-treated group.



**Fig. 6.** Effects of the EtOAc fraction on LPS-induced production of pro-inflammatory cytokines and chemokines in RAW 264.7 cells by ELISA analysis. (A) IL-6, (B) MCP-1. Cells were treated with various concentration of the EtOAc fraction for 2 hr prior to the addition of LPS (1 µg/mL), and were further incubated for 4 hr. Data are shown the mean ± SD (n=3). #p<0.001 versus a media alone-treated group. \*p<0.001 versus LPS alone-treated group.

duction of an inflammatory mediator along with NF-κB. IL-6 plays an important role in the homeostasis of the immune and hematopoietic systems, in addition to its physiological effects upon the nervous and endocrine system and bone metabolism (14). However, IL-6 production is rapidly increased in acute inflammatory responses associated with infection, injury, trauma, and other stresses. As such, a dysregulated high-level production of IL-6 could induce an undesirable inflammatory state. MCP-1, the primary chemokine responsible for the recruitment of monocytes to sites of active inflammation, is involved in the change from rolling to adhesion of monocytes in the early stage of inflammation (15). After migration into the sub-endothelial space, monocytes differentiate into macrophages that secrete MCP-1, which recruits and activates additional monocytes (16). Untreated cells had no effect on the production of IL-6 and MCP-1 in RAW 264.7 cells (Fig. 6). IL-6 and MCP-1 levels were increased in the culture media of LPS-stimulated RAW 264.7 cells. The EtOAc fraction significantly blocked the production of IL-6 by 84% and MCP-1 by 90% at a concentration of 50 µg/mL. As a result, the EtOAc fraction suppressed the production of IL-6 and MCP-1 by inhibiting NF-κB activation in LPS-induced RAW 264.7 cells.

In conclusion, the EtOAc fraction of SR significantly blocked NO, IL-6, and MCP-1 production through NF-κB transcription modulation in activated macrophage cells, indicating that the EtOAc fraction of SR has anti-inflammatory activities and could have potential as a functional food.

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#### REFERENCES

- Nathan C. 1992. Nitric oxide: discovery and impact on clinical medicine. *FASEB J* 92: 164-169.
- Szekanecz Z, Koch E. 2007. Macrophages and their products in rheumatoid arthritis. *Curr Opin Rheumatol* 19: 289-295.
- Mörmann M, Thederan M, Nackchbandi I. 2008. Lipopolysaccharides (LPS) induce the differentiation of human monocytes to osteoclasts in a tumour necrosis factor (TNF) alpha-dependent manner: a link between infection and pathological bone resorption. *Mol Immunol* 45: 3330-3337.
- Baeuerle A, Henkel T. 1994. Function and activation of NF-κB in the immune system. *Annu Rev Immunol* 12: 141-179.
- Baldwin R. 1996. The NF-κB and IκB Proteins: new discoveries and insights. *Annu Rev Immunol* 14: 649-683.
- Jeppesen PB, Gregersen S, Alstrup KK, Hermansen K. 2002. Stevioside induces antihyperglycaemic, insulinotropic and glucagonostatic effects *in vivo*: studies in the diabetic Goto-Kakizaki (GK) rats. *Phytomedicine* 9: 9-14.
- Chan P, Tomlinson B, Chen Y, Liu J, Hsieh M, Cheng J. 2002. A double-blind placebo controlled study of the effectiveness and tolerability of oral stevioside in human hypertension. *Clin Pharmacol* 50: 215-220.
- Ghanta S, Banerjee A, Poddar A, Chattopadhyay S. 2007. Oxidative DNA damage preventive activity and anti-oxidant potential of *Stevia rebaudiana* (Bertoni) Bertoni, a natural sweetener. *J Agric Food Chem* 55: 10962-10967.
- Chaiwat B, Chaiwat T, Molvibha V. 2006. Anti-inflammatory and immunomodulatory activities of stevioside and its metabolite steviol on THP-1 cells. *J Agric Food Chem* 54: 785-789.
- Clancy RM, Amin AR. 1998. The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* 41: 1141-1151.
- Salas M, Gironella M, Salasa M. 2002. Nitric oxide supplementation ameliorates dextran sulfate sodium-induced colitis in mice. *Lab Invest* 82: 597-607.
- Sun Z, Andersson R. 2002. NF-kappaB activation and inhibition: a review. *Shock* 18: 99-106.
- Boonkaewwan C, Ao M, Toskulkaeo C, Rao MC. 2008. Specific immunomodulatory and secretory activities of stevioside and steviol in intestinal cells. *J Agric Food Chem* 56: 3777-3784.
- Bosca L, Zeini M, Traves G, Hortelano S. 2005. Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicology* 208: 249-258.
- Gerszten RE, Garcia-Zepeda EA, Lim YC, Yoshida M. 1999. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow condition. *Nature* 398: 718-723.
- Charo IF, Taubman MB. 2004. Chemokines in the pathogenesis of vascular disease. *Circ Res* 95: 858-866.

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