

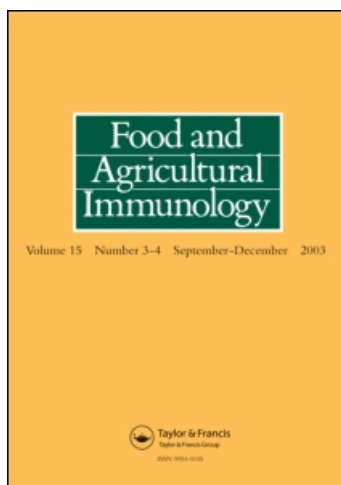
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Food and Agricultural Immunology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t713422299>

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First published on: 10 March 2011

To cite this Article Lee, J. H. , Kang, B. S. , Hwang, K. H. and Kim, G. H.(2011) 'Evaluation for anti-inflammatory effects of *Siegesbeckia glabrescens* extract *in vitro*', Food and Agricultural Immunology,, First published on: 10 March 2011 (iFirst)

To link to this Article: DOI: 10.1080/09540105.2010.549210

URL: <http://dx.doi.org/10.1080/09540105.2010.549210>

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Evaluation for anti-inflammatory effects of *Siegesbeckia glabrescens* extract *in vitro*

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(Received 19 September 2010; final version received 14 December 2010)

The aim of this study is to elucidate the anti-inflammatory effects of *Siegesbeckia glabrescens* extract *in vitro*. The *n*-butanol fraction (100 µg/ml) of *S. glabrescens* had an approximately 92% inhibitory effect on nitrite production from lipopolysaccharide (LPS)-induced macrophage cells, whereas other fractions showed relatively low nitric oxide (NO) inhibition. The *n*-butanol and ethyl acetate fractions of *S. glabrescens* showed significant chemical NO quenching to approximately 39–46% in the cell-free system, and showed the decreases of inducible nitric oxide synthase (iNOS) transcription potently. The hexane, *n*-butanol, and ethyl acetate fractions of *S. glabrescens* showed approximately 90–96% inhibition of prostaglandin E₂ (PGE₂) synthesis. The ethyl acetate, *n*-butanol, and aqueous fractions of *S. glabrescens* showed a potent inhibition of LPS-induced cyclooxygenase-2 (COX-2) mRNA transcription. All *S. glabrescens* extract-fractions showed significant decreases of IL-1β biosynthesis to 69.7–97.0%, as compared to LPS-induced IL-1β production in macrophage cells.

Keywords: *Siegesbeckia glabrescens*; anti-inflammation; antioxidant activity; PGE₂; COX-2

Introduction

Siegesbeckia orientalis Linné ssp. *glabrescens* (Mak.) kitamura (*S. glabrescens*, Compositae family), well known as 'Hi-Chum', is an annual herb growing in Korea (Kim, Lim, & Ryu, 2008). The aerial parts of *S. glabrescens* have been used as a traditional medicine to treat rheumatoid arthritis (RA), asthma, paralysis, allergic disorders, intoxication and brain stroke (Kim et al., 2008). It has been reported that the extract of *S. glabrescens* exhibits antioxidative, antiallergic, antihypertensive, antitumor and anti-inflammatory activities, and *S. glabrescens* is involved in aiding osteoporotic fractures, quadriplegia, paralysis and hemiplegia (Jun, Choi, & Shin, 2006; Kang, Lee, & Kim, 1997; Kim et al., 2008). The extract of *S. glabrescens* was reported to have an inhibitory effect on mast cell-mediated anaphylactic reactions (Kang et al., 1997; Kim et al., 2001).

Nitric oxide (NO), in the pathological responses, is involved in various physiological and pathophysiological responses. Especially in inflammatory symptoms, the production of NO is catalysed by the inducible NO synthase (iNOS) in the

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host or isolated cells stimulated by bacteria or bacterial products such as lipopolysaccharide (LPS) and/or inflammatory cytokines (e.g. TNF- α , IL-1 β , IFN- γ). Uncontrolled over-production of NO in inflammation sites for the long-term causes chronic inflammation, asthma, neurodegenerative diseases, multiple sclerosis, arthritis and cancers (Lala & Chakraborty, 2001). Therefore, NO was considered as a target of intensive research and drug development for anti-inflammation. In addition, the prostaglandin E₂ (PGE₂), one of the inflammatory mediators, is mainly synthesised from arachidonic acid (AA) by cyclooxygenase-2 (COX-2), and contributes to vasodilation, pain and fever (Lu & Wahl, 2005). The inhibition of prostaglandin (PG) synthesis causes the suppression of symptoms of inflammation. Though COX-2 is expressed at low levels normally, it is strongly induced by proinflammatory agents, including LPS, tumour promoters and growth factors in inflammation responses. Over-produced COX-2 results in inflammation, carcinogenesis, cardiovascular diseases and Alzheimer's disease (Hata & Breyer, 2004), and PGE₂ acts as a potent vasodilator that is present at high concentrations in inflammation sites. Also, the proinflammatory cytokines, such as interleukin-1 β (IL-1 β) and interleukin-6 (IL-6), modulate the expression of COX-2 through nuclear factor-kappa B (NF- κ B), which is implicated in gene expression in human neuroblastoma cells and tracheal smooth muscle (Fiebich, Mueksch, Boehringer, & Hull, 2000). However, the anti-inflammatory mechanism concerning proinflammatory cytokines is clearly still unknown.

In addition, little is known about anti-inflammatory activities of *S. glabrescens* extracts using macrophage cells. This study describes the effects of *S. glabrescens* extract for an anti-inflammatory activity, related to the suppression of NO, PGE₂ and production of cytokines from LPS-induced macrophage cells.

Materials and methods

Chemicals and reagents

Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), heparin and LPS (*Escherichia coli* serotype 0127:B8) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The cell culture medium and reagents, such as the Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin-EDTA, were obtained from GIBCO (Invitrogen Inc., NY, USA). Ethanol and other reagents were used as first grade reagents.

Plant material and the preparation of fractions

Siegesbeckia glabrescens (*S. glabrescens*) was purchased at a medicinal plants market in Seoul, Korea, in March 2009, authenticated by Dr K.W. Park, Korea National Arboretum, Korea, and deposited as the voucher specimen (NP20-085) at the Plant Resources Research Institute, Duksung Women's University, Seoul, Korea.

S. glabrescens was washed, dried by freeze drier and crushed. Crushed *S. glabrescens* was extracted with 70% methanol (in water) for seven days at room temperature. Extracts were filtered through defatted cotton, and were concentrated by evaporator under reduced pressure. The methanolic extract procedures were

repeated three times, and the filtered solutions were collected and concentrated. The methanol extracts were dissolved in distilled water before being fractionated sequentially with hexane, chloroform, ethyl acetate, *n*-butanol and water at room temperature. *S. glabrescens* extract-fractions were resolved in DMSO to 100 mg/ml, stored at -20°C , and used as a stock.

Determination of total phenolic and total flavonoid contents

The total phenolic content was determined according to the Folin-Ciocalteu method with some modification (Amin, Norazaidah, & Hainida, 2006). *S. glabrescens* extracts were dissolved in 1 ml of 1N Folin-Ciocalteu reagent. The mixture was allowed to stand for min, after which 2 ml of 20% Na_2CO_3 was added. After 90 min incubation at room temperature, the mixture was centrifuged for eight min, and the absorbance of the supernatant was measured at 765 nm on a spectrophotometer (SpectraMax M2, Molecular Devices Inc., USA). The total phenolic content of the extracts was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight.

Total flavonoid content analysis was performed using a modified colorimetric Dowd method (Zhishen, Mengcheng, & Jianming, 1999). A proper amount of extracts was added to a test tube containing 1.25 ml of distilled water, after which 75 μl of 5% NaNO_2 was added and the mixture was allowed to stand for five min. Then, 0.15 ml of 10% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was added. After six min, 0.5 ml of 1 M NaOH was added, and the mixture was diluted with 0.275 ml distilled water. The absorbance of the mixture at 510 nm was measured immediately using a UV-spectrophotometer (SpectraMax M2, Molecular Devices Inc., USA). The total flavonoid content of extracts was expressed as milligrams of catechin equivalents (CE) per gram of dry weight.

Free radical scavenging activity

The scavenging effect of *S. glabrescens* extract-fractions on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were monitored according to the following procedure (Lee, Chang, & Kim, 2009). A 0.2 ml of methanolic solution containing extract-fractions was mixed with 4 ml of methanol, and a methanolic solution of DPPH (1 mmol/l, 0.5 ml) was added. The mixture was vortexed for 15 sec, left to stand at room temperature for 30 min, and the absorbance was read at 517 nm using UV-spectrophotometry (Agilent Technologies Inc., CA, USA).

Reducing power

The reducing power of *S. glabrescens* extract-fractions was determined by Fe^{3+} reduction (Lee et al., 2009). *S. glabrescens* extract-fractions were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $\text{K}_3\text{Fe}(\text{CN})_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at $2,090 \times g$ for 10 min. A 2.5 ml of supernatant layer was added to 2.5 ml of distilled water and 0.5 ml of 0.1% FeCl_3 . The absorbance of the mixture was measured at 700 nm using UV-spectrophotometry (Agilent Technologies Inc., CA, USA).

Cell culture

Murine macrophage cells (RAW 264.7) were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in DMEM containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 µg/ml) in a 5% CO₂ humidified incubator at 37°C. They were used at passage numbers 10–20 for this study.

Determination of cell viability

Cell viability was examined using an MTT assay (Lee et al., 2009). Macrophage cells were plated at a density of 1×10^5 cells/well in 96-well tissue culture plate (Corning Inc., USA), and incubated at 37°C for 3–4 h. Plated cells were treated with indicated concentrations of *S. glabrescens* extract-fractions. After 24 h incubation, MTT was added to all wells at a concentration of 0.5 mg/ml, and incubated for 4 h at 37°C. After discarding all medium from the plates, 100 µl of dimethyl sulphoxide (DMSO) was added to all wells. The plates were placed for five min at room temperature and shaken, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by a UV-spectrophotometric plate reader (SpectraMax M2, Molecular Devices Inc., USA). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Nitrite assay

Macrophage cells were plated at a density of 2×10^5 cells/well in a 96-well culture plate and incubated for 3–4 h in a 5% CO₂ humidified incubator at 37°C. Plated cells were treated with LPS (1 µg/ml) for stimulation of nitrite production and the indicated concentrations of *S. glabrescens* extract-fractions, followed by 24 h incubation. LPS-stimulated nitrite production from macrophage cells was measured by the Griess reaction (Taechowisan, Wanbanjob, Tuntiwachwuttikul, & Liu, 2009, 2010).

Extracellular NO scavenging assay

Extracellular NO radical scavenging activity was measured by the modified protocol (Babu, Shylesh, & Padikkala, 2001). Sodium nitroprusside, a chemical NO donor, generates NO when it interacts with oxygen in an aqueous solution at physiological pH, and NO is measured by the Griess reaction. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM in PBS) and *S. glabrescens* extract-fractions were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture and 0.5 ml of the Griess reagent were mixed. The absorbance of the chromophore formed was evaluated at 540 nm.

Measurements of inflammatory mediators

Macrophage cells were cultured in 24-well culture plates. After reaching confluence, LPS-induced cells were treated with *S. glabrescens* extract-fractions, and then incubated in a humidified incubator at 37°C for 48 h. The supernatants were

collected, subdivided into portions and frozen at -74°C . The productions of PGE_2 , interleukin- 1β (IL- 1β) and interleukin-6 (IL-6) were measured by ELISA according to the manufacturer's instructions on a monoclonal antibody-based mouse cytokines microplate strip (Quantikine[®], R&D system, Minneapolis, MN, USA).

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

For the determination of the transcription level for the iNOS and COX-2 gene, the LPS-stimulated macrophage cells were incubated with *S. glabrescens* extract-fractions for 24 h in a 5% CO_2 humidified incubator at 37°C . N^G -monomethyl-L-arginine monoacetate (NMMA) (10 μM) and celecoxib (20 μM) were used as positive controls for selective inhibitors against iNOS and COX-2, respectively. Total RNA was isolated from macrophage cells using the RNeasy kit (Qiagen Inc. Valencia, CA) after treatment with *S. glabrescens* extract-fractions. RT-PCR was performed using the One-Step RT-PCR kit (Qiagen Corp.) and primers at a final concentration of 1 μM . For PCR of iNOS and COX-2 in murine macrophage cells, the primers were used as followed (Ahn et al., 2005): mouse iNOS forward primer: 5'-CCCTTCCGAAGTTTCTGGCAGC-3', mouse iNOS reverse primer: 5'-GGCTGTCAGAGCCTCGTGGCTT-3', mouse COX-2 forward primer: 5'-GGA-GAGACTATCAAGATAGTGATC-3', mouse COX-2 reverse primer: 5'-ATGGT-CAGTAGACTTTTACAGCTC-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, the house-keeping gene, for murine macrophage cells were used to determine PCR efficiency and the quantitation; mouse GAPDH forward primer: 5'-TGAAGGTCGGTGTGAACGGATTTGGC-3', mouse GAPDH reverse primer: 5'-CATGTAGGCCATGAGGTCCACCAC-3'. For cDNA synthesis and predenaturation, one cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. The PCR reactions of iNOS and COX-2 gene were cycled 25 times between 94°C (for denaturation) for 45 s; 60°C (for annealing) for iNOS, 55°C (for annealing) for COX-2 and GAPDH for one min; and 72°C (for extension) for two min. And the last cycle was performed for the final extension at 72°C for 10 min using a Bio-Rad thermal cycler (MJ Mini, Bio-Rad Inc., USA). After amplification, portions of the PCR reactions were electrophoresed on 1% agarose gel and visualised by ethidium bromide staining and UV irradiation. Transcriptional change was calculated using an electrophoresis image quantification program (Bio-Rad Inc., USA). To analyse the results of semi-quantitative RT-PCR, gel images were scanned, and the relative intensities of iNOS and COX-2 bands were determined as ratios of the intensity to GAPDH using Bio-Rad's imaging software for Windows (Bio-Rad Inc., USA).

Statistical analysis

All results were expressed as mean $\pm$ standard deviation (SD), and were analysed using one-way analysis of variance (ANOVA) and Dunnett's multiple comparison test for individual comparisons. Results were considered statistically significant when $p < 0.05$.

Results and discussion

Total phenolic and flavonoid contents of *S. glabrescens* extract-fractions

The hexane and *n*-butanol fractions of *S. glabrescens* had the highest values for the total phenolic contents (298.9 and 316.1 mg GAE/g dw, respectively), whereas the chloroform, ethyl acetate and aqueous fractions had a small total phenolic content (33.5, 86.4 and 31.8 mg GAE/g dw, respectively; Figure 1). In addition, the *n*-butanol and ethyl acetate fractions contained relatively much more total flavonoids (147.9±4.0 and 44.6±0.9 mg, respectively) than other fractions. The chloroform fraction had the highest total flavonoids portion (87.9%) in total phenolic contents, as compared with other fractions (7.7–51.2%). One of the markers for antioxidant activity of plant extracts is to determine the total amount of phenolic compounds. Phenolic compounds exist in plants with diverse structures and molecular weights. The phenolic hydroxyl group of phenolic compounds is available to bind easily to macromolecules, i.e. proteins. The phenolic compounds play an important role for antioxidant, anticancer, anti-inflammatory and antimicrobial activity (Kosmider & Osiecka, 2004).

Antioxidant activities of *S. glabrescens* extract-fractions

All *S. glabrescens* extract-fractions had hydrogen-donating activity to DPPH radicals in a concentration-dependent manner (Figure 2A). Potent DPPH radical scavenging activity was found in the *n*-butanol and ethyl acetate fractions of *S. glabrescens*, with 21.7 and 97.3 µg/ml of IC₅₀, respectively. Otherwise, the hexane, chloroform and aqueous fractions showed comparatively weak free radical scavenging activity (IC₅₀ > 300 µg/ml). Additionally, *S. glabrescens* extract showed a great reducing power in the *n*-butanol and ethyl acetate fractions. These results were totally consistent with the DPPH radical scavenging results. The reducing power of the *n*-butanol and ethyl acetate fractions was increased significantly in a concentration-dependent manner, whereas other fractions showed only a small increase in reducing

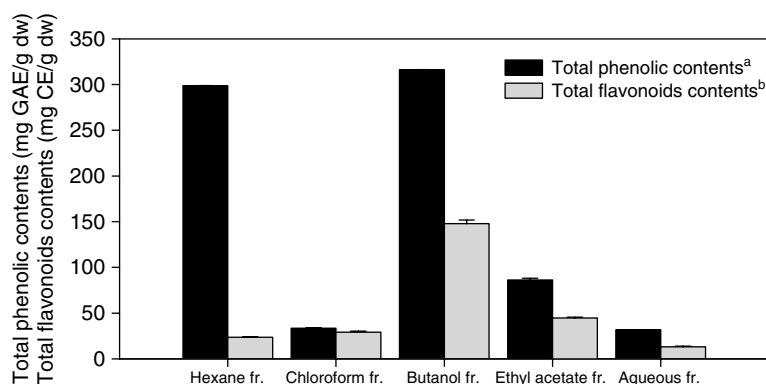


Figure 1. Total phenolic and total flavonoids contents of *S. glabrescens* extract-fractions.

^aTotal phenolic contents are expressed as GAE; milligrams of gallic acid per gram of dried extract). ^bTotal flavonoid contents are expressed as catechin equivalents (CE; milligrams of catechin per gram of dried extract). Error bars have been omitted when the standard deviation was less than the size of the symbol.

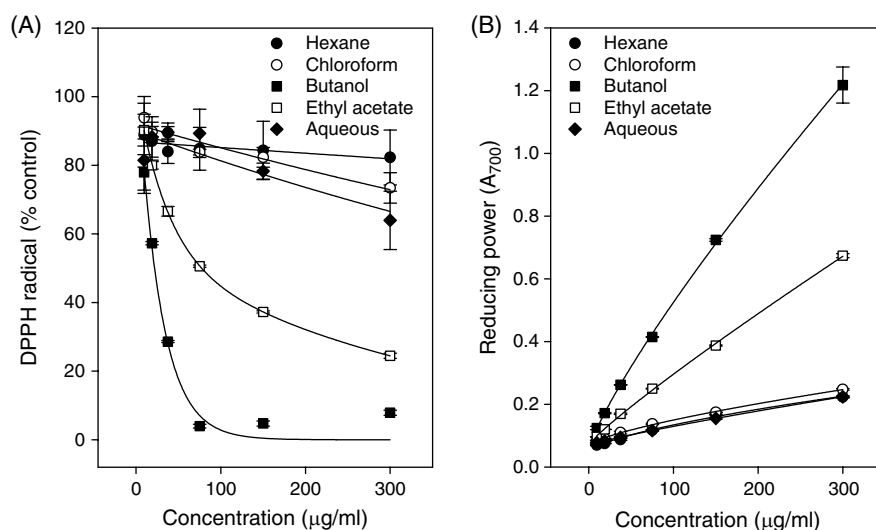


Figure 2. Antioxidant activities of *S. glabrescens* extract-fractions. (A) Scavenging effect on DPPH radicals. A 0.2 ml of methanolic solution containing extract-fractions was mixed with 4 ml of methanol, and DPPH (1 mmol/l, 0.5 ml) was added and left to stand at room temperature for 30 min, and the absorbance read at 517 nm. (B) Reducing power of different amounts of *S. glabrescens* extract-fractions. *S. glabrescens* extract-fractions were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min. After that, 2.5 ml of 10% trichloroacetic acid was added and centrifuged. A 2.5 ml of supernatant layer was added to 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. The absorbance was measured at 700 nm. Error bars have been omitted when the standard deviation was less than the size of the symbol.

power, as shown in Figure 2B. The phenolic compounds present in plant extracts are associated with antioxidant activity. Their hydrogen-donating property is responsible for the inhibition of free radicals derived from DPPH. The *n*-butanol and ethyl acetate fractions of *S. glabrescens* with high total flavonoid contents were determined to have potent DPPH radical scavenging activity and reducing power. There seemed to be a good correlation between the total flavonoid contents and the antioxidant activity of the *S. glabrescens* extracts in this report.

In many pathological responses, the cell and tissue injury associated with acute and chronic inflammation is due to the toxicity of reactive oxygen species (ROS) generated and released by activated phagocytes (Leirisalo-Repo, Paimela, Koskimies, & Repo, 1993). Free radical scavenging activity and reducing power play a critical role in quenching superoxide anions and maintaining the cellular redox homeostasis against harmful oxidants and free radicals in cells.

In inflammatory sites in RA, the cellular reducing power might support the quenching of superoxide anions secreted from cells inside the synovial membrane and prevent the inflammatory symptoms, caused by harmful radical compounds, in joint cells. The antioxidant activity of plant extracts indicates their potential as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

Viability of murine macrophage cells by *S. glabrescens* extract-fractions

The *n*-butanol and aqueous fractions of *S. glabrescens* did not affect cell viability within the tested concentrations ($IC_{50} > 100 \mu\text{g/ml}$), whereas the hexane, chloroform, and ethyl acetate fractions of *S. glabrescens* showed a significant cytotoxicity of macrophage cells at more than 6.25 ($IC_{50} = 9.5 \mu\text{g/ml}$), 25 ($IC_{50} = 39.8 \mu\text{g/ml}$) and 12.5 $\mu\text{g/ml}$ ($IC_{50} = 23.4 \mu\text{g/ml}$), respectively (Table 1). Based on this result, 0–100 $\mu\text{g/ml}$ of the *n*-butanol and aqueous fractions of *S. glabrescens* was used for further inhibitory studies on NO, PGE_2 and cytokine production by LPS-stimulated macrophage cells.

Effect of *S. glabrescens* extract-fractions on NO production from LPS-induced macrophage cells

To investigate the anti-inflammatory effect of *S. glabrescens* extract-fractions, we examined whether *S. glabrescens* extract-fractions could modulate NO synthesis in LPS-stimulated murine macrophages cells (Figure 3). All *S. glabrescens* extract-fractions had partially inhibited LPS-induced nitrite production in a dose-dependent manner in the tested concentration range (0–100 $\mu\text{g/ml}$). We observed LPS-stimulated nitrite production from macrophage cells, as compared to the basal. Also, L-NMMA (10 μM), which is one of the selective NOS blockers, inhibited nitrite production significantly (approximately 76.3%) as a positive control. Among *S. glabrescens* extract-fractions, the *n*-butanol fraction (100 $\mu\text{g/ml}$) of *S. glabrescens* had an approximately 92% ($IC_{50} = 35.7 \mu\text{g/ml}$) inhibitory effect on nitrite production by LPS-induced macrophage cells. This was superior to the inhibitory effect of NMMA as a positive control. Additionally, other fractions of *S. glabrescens* showed relatively low inhibition (approximately 36–56%, $IC_{50} > 100 \mu\text{g/ml}$) of NO production by LPS-induced macrophage cells. But due to the cytotoxicities, it was not expected to have NO inhibition over 50% for the hexane, chloroform and ethyl acetate fractions of *S. glabrescens*.

NO, an important inflammatory mediator, is associated with the pathophysiology of inflammatory joint disease and plays a key role in cartilage catabolism mediated by inflammation. NO may represent key regulatory molecules in the inflammatory process of RA. Also, NO either up-regulates or down-regulates cyclooxygenase (COX) activity, while PGs can affect iNOS activity (Salvemini, 1997). This finding shows that the amount of LPS-induced nitrite in macrophage cells is reduced by adding *S. glabrescens* extract-fractions and, in turn, might affect the NO-mediated inflammatory responses.

Effects of *S. glabrescens* extract-fractions on chemical scavenging of NO and the suppression of iNOS mRNA transcription from LPS-induced macrophage cells

The inhibition of nitrite production by *S. glabrescens* extract-fractions, as shown in Figure 3, can be explained by two possibilities. One explanation is the chemical quenching of NO by *S. glabrescens* extract-fractions. To demonstrate this possibility, the chemical quenching of NO by *S. glabrescens* extract-fractions was investigated in the cell-free system. Sodium nitroprusside, which is a chemical NO donor, produces nitrite ions in an aqueous solution at physiological pH. As shown in Figure 4A, the

Table 1. Viabilities of murine macrophage cells by *S. glabrescens* extract-fractions.

Fraction ($\mu\text{g/ml}$)	% Control					
	3.125	6.25	12.5	25	50	100
Hexane fraction	95.6 ± 3.1	90.0 ± 3.8	$25.2 \pm 36.6^*$	$5.7 \pm 0.7^*$	$7.5 \pm 1.3^*$	$15.3 \pm 2.2^*$
Chloroform fraction	93.0 ± 2.1	103.4 ± 5.2	110.8 ± 5.0	115.9 ± 5.2	$27.0 \pm 1.7^*$	$8.0 \pm 0.5^*$
<i>n</i> -Butanol fraction	105.4 ± 8.7	107.5 ± 7.0	105.5 ± 10.9	115.5 ± 5.1	131.9 ± 5.3	138.5 ± 2.8
Ethyl acetate fraction	102.0 ± 6.6	99.5 ± 4.8	105.2 ± 2.8	$47.7 \pm 4.0^*$	$9.2 \pm 1.0^*$	$9.1 \pm 0.5^*$
Aqueous fraction	96.5 ± 6.1	100.4 ± 9.1	105.2 ± 2.7	110.2 ± 3.0	119.8 ± 4.4	140.0 ± 4.8

* $p < 0.05$ vs. control (untreated group). Control value was $100.2 \pm 2.5\%$.

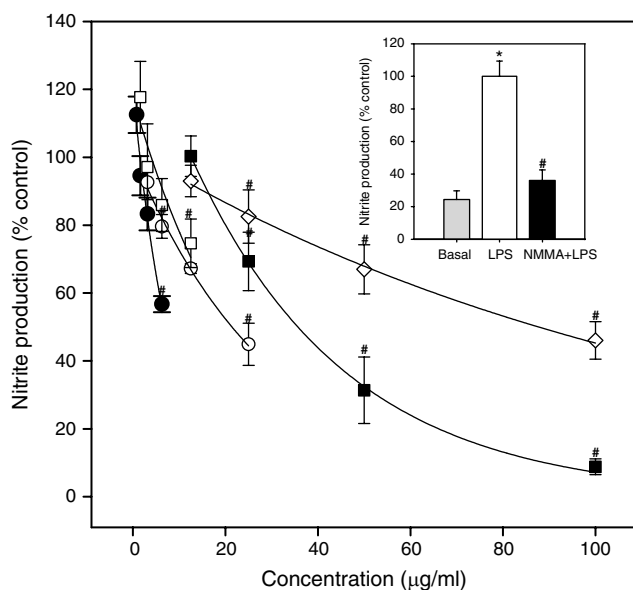


Figure 3. Effects of *S. glabrescens* extract-fractions on nitric oxide production from lipopolysaccharide (LPS)-stimulated murine macrophage cells. * $p < 0.05$ vs. control, # $p < 0.05$ vs. LPS-treated group. NMMA and LPS were used in 10 μ M and 1 μ g/ml, respectively. ●: Hexane fraction; ○: Chloroform fraction; ■: *n*-Butanol fraction; □: Ethyl acetate fraction; ◇: Aqueous fraction.

n-butanol and ethyl acetate fractions of *S. glabrescens* showed significant chemical NO quenching to approximately 39–46% in the cell-free system. Other fractions of *S. glabrescens*, except for the aqueous fraction, showed mild chemical NO quenching properties to 28–37%. Haemoglobin (Hb), a NO scavenger, scavenged approximately 67.9% of NO. From this result, the inhibition of NO production by *S. glabrescens* extract-fractions, except for the aqueous fraction, in LPS-induced macrophage cells is expected to be partially due to chemical quenching of NO, which was produced by the iNOS reaction.

The other explanation is the suppression of iNOS mRNA transcription by *S. glabrescens* extract-fractions. We observed a significant up-regulation of iNOS mRNA transcription in LPS-stimulated macrophage cells (Figure 4B). NMMA, a selective iNOS inhibitor, suppressed approximately 55.1% of up-regulated iNOS gene transcription. All *S. glabrescens* fractions inhibited iNOS mRNA transcription by LPS-induced macrophage cells in a concentration-dependent manner. Especially the *n*-butanol and ethyl acetate fractions of *S. glabrescens* showed the decreases of iNOS transcription to approximately 71.8% ($IC_{50} = 48.1$ μ g/ml) and 61.5% ($IC_{50} = 8.24$ μ g/ml) at concentrations of 100 and 12.5 μ g/ml, respectively. Taken together, the inhibition of NO production by *S. glabrescens* extract-fractions might be due to chemical scavenging of NO and the suppression of iNOS transcriptional level in LPS-induced macrophage cells.

In this paper, the inhibition of NO production and iNOS transcription by *S. glabrescens* extract is in agreement with the anti-inflammatory activity of

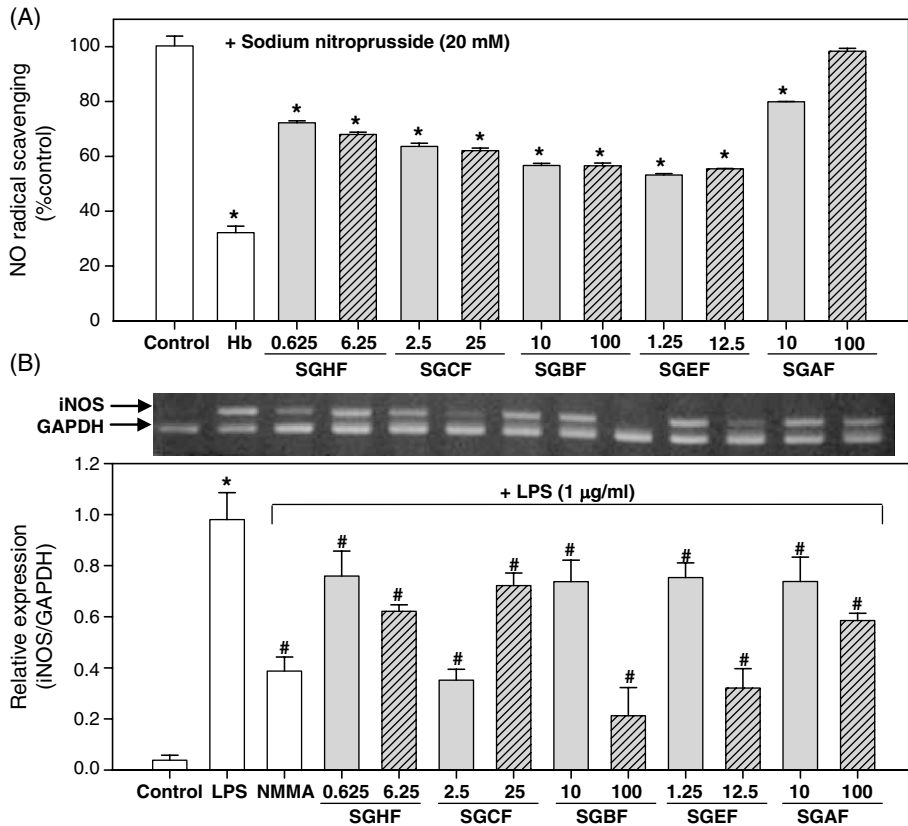


Figure 4. Effects of *S. glabrescens* extract-fractions on the chemical nitric oxide (NO) quenching and the suppression of iNOS mRNA transcription. (A) Chemical NO scavenging of *S. glabrescens* extract-fractions in the cell-free system. * $p < 0.05$ vs. control (treated only with sodium prusside, 20 μ M). (B) Suppression of iNOS mRNA transcription by *S. glabrescens* extract-fractions in lipopolysaccharide (LPS)-induced macrophage cells. NMMA (10 μ M) was used as a positive control. The intensity of PCR product bands was quantitated by scanning densitometry and standardised to equivalent GAPDH mRNA levels. Data were expressed as mean $\pm$ SD of three independent experiments. * $p < 0.05$ vs. control, # $p < 0.05$ vs. LPS-treated group. Hb: haemoglobin (5 μ M); SGHF, *S. glabrescens* hexane fraction; SGCF, *S. glabrescens* chloroform fraction; SGBF, *S. glabrescens* *n*-butanol fraction; SGEF, *S. glabrescens* ethyl acetate fraction; SGAF, *S. glabrescens* aqueous fraction. Error bars have been omitted when the standard deviation was less than the size of the symbol.

flavonoids derived from *S. glabrescens*, reported by Kim et al. (2008). Otherwise, in carcinogenesis, *S. glabrescens* extract induced NO production and iNOS transcription, and caused apoptosis in vascular smooth muscle cells on the contrary (Jun, Shin, Son, & Shin, 2004; Lee et al., 2006). This result suggests that the inhibitory effect of *S. glabrescens* extract-fractions on NO production was caused by both the chemical NO quenching and the regulation of iNOS and did not occur as a result of cell viability.

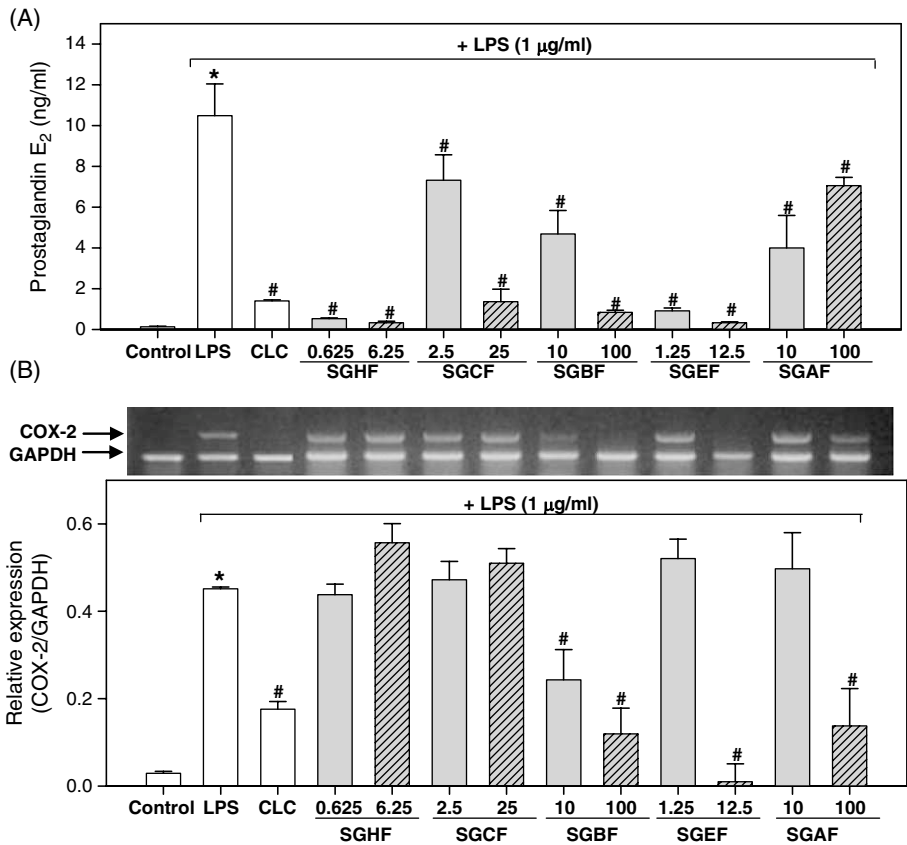


Figure 5. Effects of *S. glabrescens* extract-fractions on the production of prostaglandin E₂ (PGE₂) and cyclooxygenase-2 (COX-2) transcription. (A) Effect of *S. glabrescens* extract-fractions on the production of PGE₂ from lipopolysaccharide (LPS)-stimulated macrophage cells. (B) Suppressive effect of *S. glabrescens* extract-fractions on COX-2 mRNA transcription in LPS-stimulated macrophage cells. Celecoxib (20 µg/ml) was used as a positive control. The intensity of PCR product bands was quantitated by scanning densitometry and standardised to equivalent GAPDH mRNA levels. Data were expressed as mean $\pm$ SD of three independent experiments. * p < 0.05 vs. control, # p < 0.05 vs. LPS-treated group. CLC, celecoxib; SGHF, *S. glabrescens* hexane fraction; SGCF, *S. glabrescens* chloroform fraction; SGBF, *S. glabrescens* *n*-butanol fraction; SGEF, *S. glabrescens* ethyl acetate fraction; SGAF, *S. glabrescens* aqueous fraction. Error bars have been omitted when the standard deviation was less than the size of the symbol.

Effects of *S. glabrescens* extract-fractions on the production of PGE₂ and the suppression of COX-2 mRNA transcription in LPS-induced macrophage cells

The biosynthesis of PGE₂, which is a typical inflammatory and RA mediator, was monitored in LPS-induced macrophage cells (Figure 5A). LPS stimulated significantly PGE₂ production by macrophage cells, and celecoxib (20 µM), which is a selective COX-2 inhibitor, inhibited approximately 87.5% of PGE₂ synthesis. The hexane, *n*-butanol and ethyl acetate fractions of *S. glabrescens* showed approximately 90–96% inhibition of PGE₂ synthesis, which was superior to the effect of celecoxib.

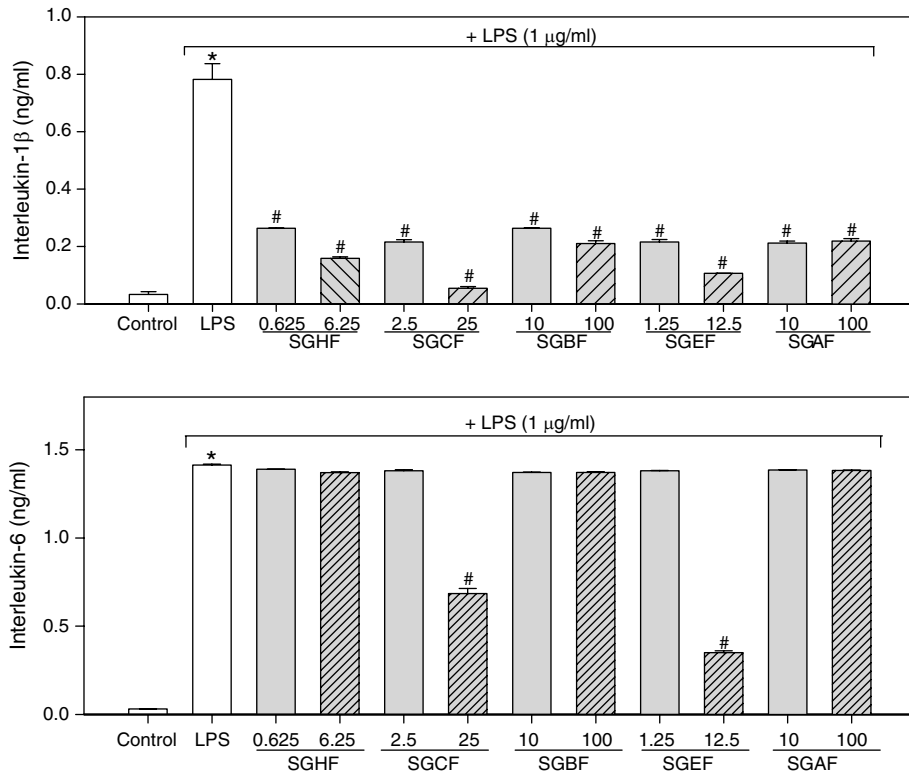


Figure 6. Effects of *S. glabrescens* extract-fractions on the production of IL-1 β and IL-6 in lipopolysaccharide (LPS)-stimulated macrophage cells. * $p < 0.05$ vs. control, # $p < 0.05$ vs. LPS-treated group. SGHF, *S. glabrescens* hexane fraction; SGCF, *S. glabrescens* chloroform fraction; SGBF, *S. glabrescens* *n*-butanol fraction; SGEF, *S. glabrescens* ethyl acetate fraction; SGAF, *S. glabrescens* aqueous fraction. Error bars have been omitted when the standard deviation was less than the size of the symbol.

The synthesis of PGE₂ is regulated by COX-2 in acute inflammatory responses. Therefore, the effect of *S. glabrescens* extract-fractions on the COX-2 mRNA transcriptional level was investigated in LPS-induced macrophage cells. The gene transcription of COX-2, one of the important enzymes in inflammatory pathogenesis, was almost not observed in basal RAW 264.7 cells. However, LPS increased COX-2 gene transcriptional levels significantly in macrophage cells, and COX-2-transcription was suppressed by celecoxib (20 μ M, 65.4%), a selective inhibitor, depicted in Figure 5B. The ethyl acetate fraction of *S. glabrescens* showed a potent inhibition of LPS-induced COX-2 mRNA transcription below the basal expression level. The *n*-butanol and aqueous fractions of *S. glabrescens* (100 μ g/ml) inhibited approximately 76.9% and 71.2% of the COX-2 mRNA transcription, respectively, superior to the effect of celecoxib.

COX, the rate-limiting enzyme in the conversion of AA to PGs, exists as two isoforms. COX-1 is constitutively produced and detectable in most human tissues. In contrast, COX-2, normally expressed at low levels, is strongly induced by inflammatory stimuli, including LPS, tumour promoters and growth factors. At

inflammatory sites, such as human atherosclerotic lesions, high levels of COX-2 have been found compared with low levels in unaffected arteries (Gorgoni, Caivano, Arizmendi, & Poli, 2001). COX-2 is expressed in many cells, including fibroblasts and macrophages, with inflammatory stimulators and mediates the release of large quantities of proinflammatory PGs at the site of inflammation. COX-2 and NO represent key regulatory molecules in the inflammatory process in RA. Many studies have demonstrated that some inducible enzyme (COX and iNOS)/cytokines and their reaction products are involved in chronic inflammatory disease. Additionally, the reduction of writhing responses in mice is probably related to the reduced synthesis of the inflammatory mediators by inhibition of cyclooxygenases and/or lipoxygenases (Ojewole, 2006), and in RA, it is known that the third phase of the edema induced by carrageenan, in which the edema reaches its highest volume, is characterised by the presence of PGs (Spector & Willoughby, 1963).

This report elucidated that the potential analgesic and anti-rheumatoid arthritis activities of *S. glabrescens* extract-fractions are due to the inhibition of PGE₂ synthesis through the suppression of COX-2 mRNA transcription, and raises the possibility that *S. glabrescens* extract-fractions may retard the inflammatory process in rheumatoid synovium by suppression of PGE₂ synthesis and COX-2 mRNA transcription.

Effect of S. glabrescens extract-fractions on the production of inflammatory cytokines

The levels of IL-1 β and IL-6 are remarkably elevated by adding LPS to RAW 264.7 cells (Figure 6). All *S. glabrescens* extract-fractions showed significant decreases of IL-1 β biosynthesis to 69.7–97.0%, as compared to LPS-induced IL-1 β production in macrophage cells. Especially the chloroform and ethyl acetate fractions of *S. glabrescens* suppressed both IL-1 β and IL-6 levels remarkably in a concentration-dependent manner. In addition, the chloroform and ethyl acetate fractions of *S. glabrescens* showed a suppressive effect on IL-6 production significantly, to 52.4% and 72.7%, respectively, whereas the hexane, *n*-butanol and aqueous fractions of *S. glabrescens* did not affect IL-6 biosynthesis at all.

Once activated, the alveolar macrophages can release different inflammatory mediators, such as IL-1 β (Friedlander, Hilbert, Wu, Finegan, & Rich, 1994). IL-1 β up-regulates metalloproteinases and fibroblast proliferation, features that are closely associated with the chronic inflammation and structural changes observed in patients (Sasaki et al., 2000). This cytokine is a potent inflammatory mediator, stimulating chemokine production, recruiting leukocytes to the site of injury and inducing the synthesis of TNF- α and IL-6 (Barnes, Chung, & Page, 1998). This result suggests that *S. glabrescens* extract-fractions might have cartilage-protecting effects by decreasing inflammatory cytokines such as IL-1 β and IL-6, and may be helpful in the relief of RA.

Conclusions

In the present study, it is concluded that *S. glabrescens* extract-fractions suppressed NO and PGE₂ production via the suppression of iNOS and COX-2 mRNA transcription, and of IL-1 β and IL-6 biosynthesis from LPS-stimulated macrophage cells. Taken together, *S. glabrescens* extract-fractions may be useful as a functional

food material and an alternative medicine for the relief and retardation of immunological inflammatory responses.

Acknowledgements

This work was supported by the Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0029692).

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