ORIGINAL ARTICLES

Department of Veterinary Physiology[1], College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea, Laboratory of Physiology & Signaling[2], College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea, School of Biotechnology and Bioengineering[3], Kangwon National University, Chuncheon, Republic of Korea, College of Biomedical Science and Engineering[4], and Regional Research Center, Inje University, Gimhae, College of Veterinary Medicine[5], Konkuk University, Seoul, Republic of Korea

In vitro anti-oxidative and anti-inflammatory effects of solvent-extracted fractions from *Suaeda asparagoides*

Jong Min Park[1], Sung Dae Kim[2], Whi Min Lee[2], Jae Youl Cho[3], Hwa Jin Park[4], Tae Wan Kim[2], Nong-Hoon Choe[5], Sang Keun Kim[1], Man Hee Rhee[2]

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Man Hee Rhee, Laboratory of Veterinary Physiology & Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Republic of Korea
rheemh@knu.ac.kr

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Suaeda asparagoides Miq. (Chenopodiaceae: *S. asparagoides*) is a salt-marsh plant that has long been prescribed in traditional Oriental medicine for the treatment of hypertension and hepatitis. In order to elucidate the pharmacological mechanisms of the herb, we conducted an examination of the anti-oxidative and anti-inflammatory properties of solvent-extracts of *S. asparagoides*. All of the solvent fractions showed potent anti-oxidative effects, as assessed using a radical generation assay system (xanthine oxidase assay) and an electron-donating activity system (DPPH [2,2-diphenyl-l-picrylhydrazyl radical] assay), with IC_{50} values ranging from 9 to 42 μg/ml. In agreement with this pattern, the total phenolic contents were widely distributed in the various solvent fractions, and ranged from 36.5 to 50.3 mg/g of dry weight. All of the solvent fractions significantly suppressed NO production in RAW264.7 cells induced by lipopolysaccharide (LPS, 0.1 μg/ml) and of the fractions, only the chloroform (CHC) fraction completely blocked the expression of inducible NO synthase (iNOS). Additionally, the hexane (HEX) and CHC fractions suppressed the mRNA expression of granulocyte/macrophage colony-stimulating factor (GM-CSF) and monocyte chemoattractant protein 1 (MCP-1), respectively, in the LPS-stimulated RAW264.7 cells. Therefore, these results suggest that the pharmacological action of *S. asparagoides* is due to its potent anti-oxidative effects and anti-inflammatory effects, and that therefore it can be applied to other diseases caused by oxidative stress and inflammation, such as cardiovascular diseases.

1. Introduction

Several salt-marsh plants such as *S. asparagoides, Salicornia herbacea*, and *Calystegia soldanella* (Ihm and Lee 1986; Lee et al. 2002), are widely distributed in Korean estuaries (Ihm and Lee 1986; Kim et al. 2004; Lee et al. 2004; Salvemini et al. 1993; Tori et al. 2000). Pharmacological and chemical studies of these plants have recently been conducted (Lee et al. 2004; Lee et al. 2005). The *S. asparagoides* plant is traditionally employed in several medicinal remedies for illnesses such as anti-hypertensive, anti-hepatitis, and the removal of intestinal waste, although the mechanisms of its pharmacological activity have not been fully examined.

Free radicals such as ROS (e.g. superoxide anion radical, hydroxyl radical, singlet oxygen, hydrogen peroxide) and peroxynitrite are highly reactive molecules that are generated predominantly during cellular respiration and normal metabolism. An imbalance between the cellular production of free radicals and the ability of cells to defend against them is referred to as oxidative stress, which is implicated as a potential contributor to lipid peroxidation. Oxidative stress, however, can damage many targets other than lipids, including proteins, DNA and small molecules. Antioxidants are of interest to biologists and clinicians because they help to protect the human body against damage by reactive free radicals found in cancer, atherosclerosis, and aging (Halliwell et al. 1995; Mates et al. 1999). There are many reports that natural products and their derivatives have efficient anti-oxidative characteristics, such as anticancer, hypolipidemic, anti-aging, and anti-inflammatory activities (Aruoma et al. 1997; Cho et al. 2006; Halliwell and Aruoma 1991; Hogg 1998; Mates et al. 1999).

Chronic and acute inflammation are multiple-stage processes that are mediated by activated inflammatory or immune cells (Guzik et al. 2003). Macrophages play a central role in managing immunopathological phenomena such as the over-production of pro-inflammatory cytokines and inflammatory mediators (i.e. ROS, NO and prostaglandin E_2 [PGE_2]) (Lundberg 2003; Walsh 2003). In the case of oxidative stress, NO and ROS affect virtually every stage in

the development of inflammation. Macrophages mediate the inflammatory process through the release of chemokines (e.g. MIP-1α and MCP-1) and cytokines (e.g. TNF-α, IL-1β and IL-6). Indeed, a number of inflammatory stimuli such as lipopolysaccharide (LPS) and pro-inflammatory cytokines activate immune cells to up-regulate such inflammatory states (Gallucci et al. 1998) and therefore, they are important for the development of new anti-inflammatory drugs and for determining the potential of molecular anti-inflammatory mechanisms. In addition, the use of natural products as anti-oxidants and anti-inflammatory agents has a long history that began with folk medicine, and through the years those products have been incorporated into traditional and allopathic medicine.

Therefore, in this study, first we determined whether the solvent-extracted fractions of *S. asparagoides* displayed anti-oxidant activity and if they inhibited LPS-induced NO production in RAW264.7 cells. Furthermore, we examined the modulation of solvent-extracted fractions of *S. asparagoides* in the expression of pro-inflammatory chemokines and cytokines in LPS-stimulated RAW264.7 cells.

2. Investigations and results

2.1. *S. asparagoides exhibits potent anti-oxidative effects*

In order to compare the anti-oxidant capacities of ethanol, hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous fractions of *S. asparagoides*, first, we measured the anti-oxidant activity of each extract of the plant by xanthine oxidase assay and DPPH assay.

According to the xanthine oxide assay (a radical generation system) (Fig. 1), the radical scavenging effect of the ethyl acetate fraction (IC_{50} = 12.3 μg/ml) was much stronger than those of the ethanol fraction (19.3 μg/ml), hexane fraction (18.9 μg/ml), chloroform fraction (26.4 μg/ml), and *n*-butanol fraction (39.8 μg/ml). The anti-oxidant activity of the aqueous fraction of the plant, however, was negligible. The standard compound, ascorbic acid, also displayed scavenging activity, showing an IC_{50} value of 180 μM (data not shown). In order to confirm the radical scavenging effect of *S. asparagoides*, a DPPH assay (for electron-donating activities) was also employed. As shown

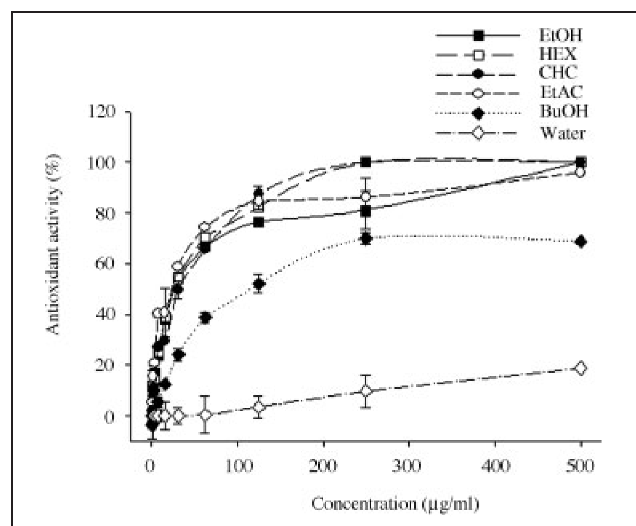

Fig. 1: The antioxidant activity of the solvent extracts of *S. asparagoides* in xanthine oxidase assay. Phosphate buffer and various solvent extracts of *S. asparagoides* were mixed. After adding xanthine and xanthine oxidase, the difference of the absorbance at 295 nm was monitored for 3 min and the enzyme activity was calculated as described in "Experimental"

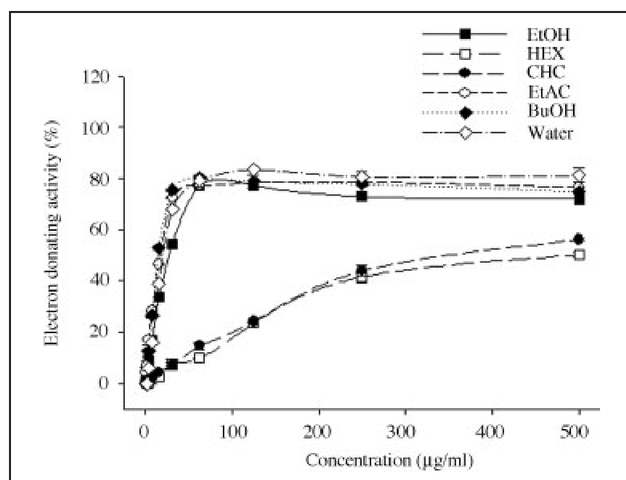

Fig. 2: The antioxidant activities of the solvent extracts of *S. asparagoides* in a DPPH assay. The acetate buffer and various concentrations of the solvent extracts of *S. asparagoides* were mixed. After adding an ethanolic DPPH solution, the absorbance was monitored at 595 nm and enzyme activity was calculated as described in "Experimental"

in Fig. 2, the *n*-butanol extract of *S. asparagoides* also highly scavenged the radical generation, exhibiting an IC_{50} value of 9.0 μg/ml. The radical scavenging activities of the ethyl acetate and ethanol fractions were less than that of the *n*-butanol fraction from *S. asparagoides* (i.e. showing IC_{50} values of 16.5 and 17.8 μg/ml, respectively). In contrast to the results for the xanthine oxide assay, however, the chloroform fraction and the hexane fraction of *S. asparagoides* scavenged the radical generation with IC_{50} values of 63.0 and 80.8 μg/ml. Unlike the results for the xanthine oxidase assay, in the DPPH assay, the EDA of the aqueous fraction was superior to that of ascorbic acid, a natural oxidant material (exhibiting an IC_{50} value of 17.3 μg/ml versus 42.8 μg/ml).

2.2. *S. asparagoides extracts suppress NO production and iNOS expression stimulated by LPS*

Since most anti-oxidants such as quercetin and caffeic acid show modulatory effects on LPS-mediated cytokine production and on the release of inflammatory mediators such as NO and PGE_2 (da Cunha et al. 2004; Gerhauser et al. 2003; Saha et al. 2004), we tested whether a solvent

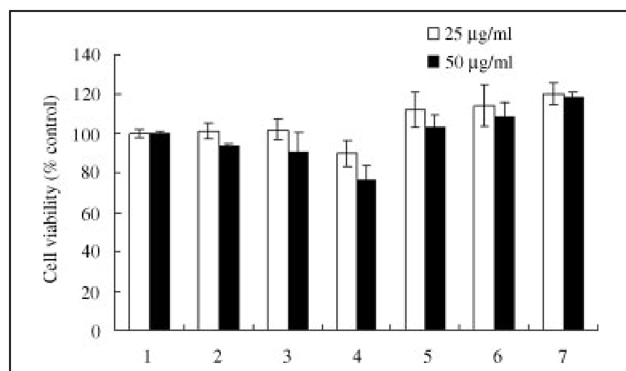

Fig. 3: The effects of solvent extracts of *S. asparagoides* on the cytotoxicity of RAW264.7 cells (1×10^6 cells/ml). Cell viability was determined using a MTT assay as described in "Experimental". RAW 264.7 cells (1×10^6 cells/ml) were incubated with various extracts of S. asparagoides. Means ± SEM were calculated from three independent experiments that were performed in triplicate. 1 Basal; 2 LPS; 3 ethanol extract; 4 hexane extract; 5 chloroform extract; 6 ethyl acetate extract; 7 *n*-butanol extract; 8 aqueous extract

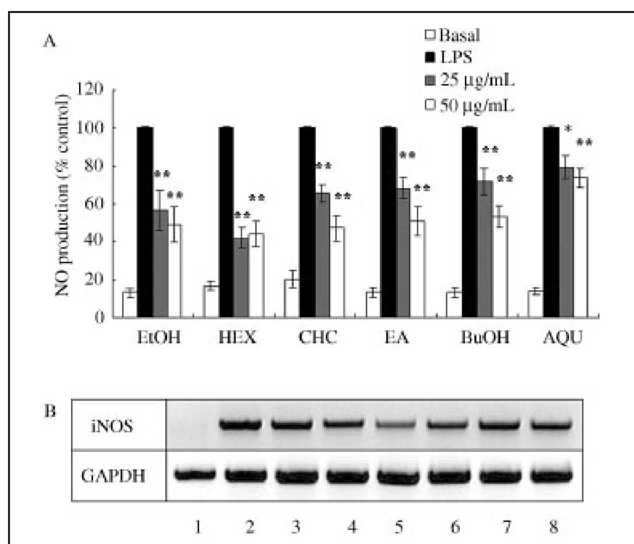

Fig. 4: The effects of solvent extracts of *S. asparagoides* on the production of NO and on the mRNA expression of iNOS in LPS-activated RAW264.7 cells (1×10^6 cells/ml). RAW 264.7 cells (1×10^6 cells/ml) were stimulated by a LPS (0.1 μg/ml) and incubated with ethanol (EtOH), hexane (HEX), chloroform (CHC), ethyl acetate (EA), *n*-butanol (BuOH), and aqueous fractions (AQU) of *S. asparagoides*. 1 Basal; 2 vehicle + LPS; 3 LPS + 50 μg/ml of ethanol extract; 4 LPS + 50 μg/ml of hexane extract; 5 LPS + 50 μg/ml of chloroform extract; 6 LPS + 50 μg/ml of ethyl acetate extract; 7 LPS + 50 μg/ml of *n*-butanol extract; 8 LPS + 50 μg/ml of aqueous extract. $^{*}P < 0.05$, $^{**}P < 0.01$

extract of *S. asparagoides* was capable of suppressing LPS-induced NO production in RAW264.7 cells. We first determined the cytotoxicity of each fraction of *S. asparagoides* to RAW264.7 cells. The pretreatment of unstimulated RAW264.7 cell lines, with each fraction prepared from *S. asparagoides* for 24 h, did not significantly affect cell viability (Fig. 3). Figure 4 shows that all of the solvent fractions of *S. asparagoides* dose-dependently suppressed NO production in the RAW 264.7 cells stimulated by 0.1 μg/ml of LPS. The extent of the inhibition in LPS-induced NO production was prominent in the hexane and ethanol fractions, implying that the non-polar components of *S. asparagoides*, through the modulation of NO production, might play a major role in anti-inflammatory activity. Next, we examined whether each solvent extract of *S. asparagoides* (50 μg/ml) modulated the expression of iNOS, an inducible enzyme that produces NO in response to LPS. All of the solvent extracts suppressed the expression of iNOS to a certain degree.

2.3. *S. asparagoides extracts modulate the expression of pro-inflammatory cytokines stimulated by LPS*

TNF-α, GM-CSF, and MCP-1 are known to be pro-inflammatory cytokines, exhibiting a multitude of biological activities that are linked to the immunopathology of acute or chronic inflammatory diseases such as septic shock and rheumatoid arthritis, as well as autoimmune diseases (Charo and Taubman 2004; Eigler et al. 1997; Hamilton and Anderson 2004). Therefore, we addressed whether solvent extracts of *S. asparagoides* modulated the mRNA expression of TNF-α, GM-CSF, and MCP-1. Figure 5 shows that the solvent extracts did not have any inhibitory effect on the expression of TNF-α stimulated by 0.1 μg/ml LPS. However, the hexane extract of the plant significantly suppressed the expression of GM-CSF mRNA. In addition, the hexane and the chloroform extracts of *S. asparagoides* strikingly blocked the MCP-1 mRNA in LPS-stimulated

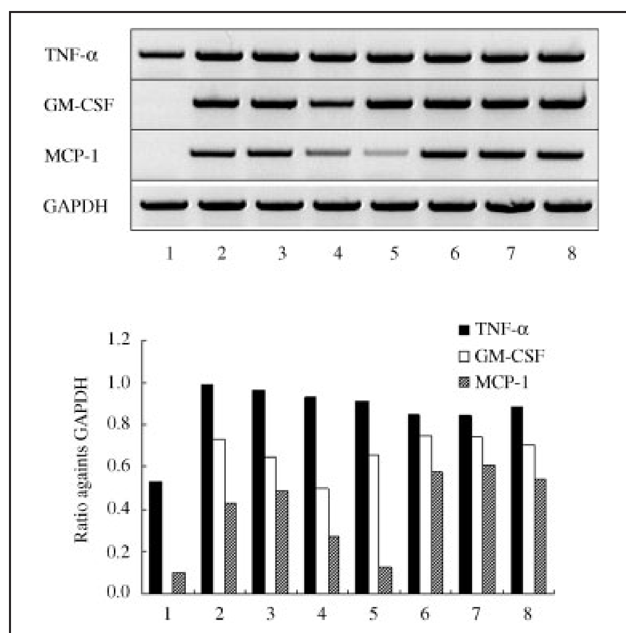

Fig. 5: The effects of various extracts of *S. asparagoides* on the mRNA expression of TNF-α, GM-CSF, and MCP-1 in LPS-activated RAW264.7 cells. RAW 264.7 cells (1×10^6 cells/ml) were stimulated by a LPS (0.1 μg/ml) and incubated with ethanol, hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous extracts of *S. asparagoides*. 1 Basal; 2 vehicle + LPS; 3 LPS + 50 μg/ml of ethanol extract; 4 LPS + 50 μg/ml of hexane extract; 5 LPS + 50 μg/ml of chloroform extract; 6 LPS + 50 μg/ml of ethyl acetate extract; 7 LPS + 50 μg/ml of *n*-butanol extract; 8 LPS + 50 μg/ml of aqueous extract

macrophages, suggesting that there are GM-CSF- and MCP-1-specific components in these two fractions.

3. Discussion

This study demonstrates that solvent-extracted fractions of *S. asparagoides*, which have been used by Koreans in the treatment of hypertension and hepatitis from time immemorial, exert anti-oxidative and anti-inflammatory effects *in vitro*. In order to understand the pharmacological mechanism involved, various solvent fractions (ethanol, hexane, chloroform, ethyl acetate, butanol, and water), which were prepared from the leaves of the plant, were tested using DPPH and xanthine oxidase assays and LPS-activated macrophage cells. The different anti-oxidative screening assay systems showed different pharmacological profiles of the radical scavenging activity. In the xanthine oxidase assay, the hexane, chloroform, and ethyl acetate fractions (probably including more non-polar components) of the herbs had more potent anti-oxidant effects than the *n*-butanol and aqueous fractions (probably including polar components); in the DPPH assay, however, the radical scavenging activity of ethanol, ethyl acetate, *n*-butanol, and the aqueous fractions of *S. asparagoides* were much stronger than those of the hexane and chloroform fractions. This seems to imply that there are some anti-oxidative functions in *S. asparagoides* that display distinct chemical properties and that these components might play an important role in protecting against oxidative stress. Considering that quercetin is a powerful anti-oxidative flavonoid in *Opuntia humifusa* Raf, exhibiting IC_{50} values of 97.5 nM (in the xanthine oxidase assay) and 23.5 μM (in the DPPH assay) (Cho et al. 2006), the potential anti-oxidative compounds in the hexane and chloroform fractions (IC_{50} [in the xanthine oxidase assay]: 18.9 and 26.4 μg/ml; IC_{50} [in the

Table 1: Total phenolic contents of solvent-extracted fractions of S. asparagoides

Gene	Primer sequence		Fragment size (bp)
GAPDH	F	5′-CACTCACGGCAAATTCAACGGC-3′	505
	R	5′-CCTTGGCAGCACCAGTGGATGCAGG-3′	
iNOS	F	5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′	496
	R	5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′	
TNF-α	F	5′-TTGACCTCAGCGCTGAGTTG-3′	364
	R	5′-CCTGTAGCCCACGTCGTAGC-3′	
GM-CSF	F	5′-CAGGATGAGGACATGAGCACC-3′	350
	R	5′-CTCTGCAGACTCAAACTCCAC-3′	
MCP-1	F	5′-TTGACCTCAGCGCTGAGTTG-3′	425
	R	5′-CCTGTAGCCCACGTCGTAGC-3′	

DPPH assay]: 80.8 and 63.0 μg/ml) might be of other than flavonoid-type structure.

Regardless of the difference of scavenging effects according to the two assay systems, our results suggest that *S. asparagoides* includes various anti-oxidative components that are widely distributed and distinct in chemical properties. Indeed, the assay results regarding the total phenolic compounds, major anti-oxidative components with many physiological and pharmacological functions (Shahidi and Wanasundara 1992), also support this conclusion. Thus, each solvent fraction contained a similar amount of phenolic compounds, even though that of the hexane fraction was less than the others (Table 1).

There are many reports that conclude that the majority of naturally-occurring phenolics have anti-oxidative and anti-inflammatory properties (Shen et al. 2002; Wang et al. 2003). Therefore, we examined, using RAW264.7 cells, the modulatory role of the *S. asparagoides* solvent fractions relative to anti-inflammatory activity. Pretreatment of unstimulated RAW264.7 cells with each fraction for 24 h, did not significantly affect cell viability (Fig. 5). It is noteworthy that the *n*-butanol and aqueous fractions of the plant rather stimulated cell viability, suggesting that these extracts might contain proliferation-stimulating components remaining to be further chemically characterized.

Another remarkable feature of the *S. asparagoides* solvent extracts was the inhibition of NO production. The stimulation of macrophages with bacterial endotoxins such as LPS leads to the release of NO and cytokines. NO, synthesized by iNOS, is involved in the physiological regulation of many cell functions and also in the pathological onsets of several diseases including sepsis, atherosclerosis, multiple sclerosis, and arthritis (Bosca et al. 2005; Cho et al. 2006; Hogg 1998; Guzik et al. 2003; Kim et al. 2004). Diverse plant-derived materials therefore have been investigated to develop effective NO inhibitors for the treatment of NO-mediated diseases (Bae et al. 2005; Kim et al. 2004; Lee et al. 2005; Lo et al. 2002; Shen et al. 2002). In addition, based on the fact that most anti-oxidants such as quercetin and *N*-actyl-L-cysteine show a modulatory effect on LPS-mediated cytokine production and release inflammatory mediators such as NO and PGE_2 (Gerhauser et al. 2003; Saha et al. 2004), we examined whether the solvent extract of *S. asparagoides* was capable of suppressing LPS-induced NO production in murine macrophage RAW264.7 cells. We found that all of the solvent fractions of *S. asparagoides* dose-dependently suppressed the production of NO in RAW264.7 cells stimulated by 0.1 μg/ml of LPS. The extent of the inhibition in LPS-induced NO production was prominent in the hexane and ethanol fractions, implying that the more non-polar components of *S. asparagoides* might, through the modulation of NO production, play a major role in the plant's anti-inflammatory activity. In addition to NO's inflammatory role, pro-inflammatory molecules such as cytokines, mostly synthesized by activated NF-κB, are known to act as major inflammation inducers (Lo et al. 2002). Therefore, we examined whether solvent extracts blocked the expression of pro-inflammatory cytokines such as TNF-α, GM-CSF, and MCP-1.

TNF-α, GM-CSF, and MCP-1 are known to be pro-inflammatory cytokines that possess a multitude of biological activities linked to the immunopathology of acute or chronic inflammatory diseases such as septic shock and rheumatoid arthritis, as well as autoimmune diseases (Charo and Taubman 2004; Eigler et al. 1997; Hamilton and Anderson 2004). Therefore, we addressed whether solvent-extracts of *S. asparagoides* modulated the expression of TNF-α, GM-CSF, and MCP-1. Figure 5 shows that there was no inhibitory effect on the LPS-induced mRNA expression of TNF-α by any of the solvent fractions. The hexane extract of the plant, however, significantly suppressed the mRNA expression of GM-CSF. GM-CSF, as the name implies, was initially defined by its ability to generate, as a result of proliferation and differentiation, both granulocyte and macrophage colonies from precursor cells (Hamilton and Anderson 2004). There is increasing evidence regarding the key role of GM-CSF in inflammatory and autoimmune diseases (Hamilton 2002; Hamilton and Anderson 2004). In addition, the hexane and chloroform extracts of *S. asparagoides* strikingly blocked the MCP-1 mRNA induced by the LPS stimulation. Numerous studies have demonstrated that activation of NF-κB is essential for the induction of GM-CSF (Kim et al. 2005), TNF-α (Tse et al. 2005), and MCP-1 (Kim et al. 2004), in that the degradation of IκB is a crucial step in NF-κB activation (Collins and Cybulsky 2001). However, the solvent extracts of *S. asparagoides* did not modulate TNF-α signaling, but did influence pro-inflammatory chemokines such as GM-CSF and MCP-1, suggesting that the blocking of these cytokines by solvent extracts might not be involved at the NF-κB level. Moreover, MCP-1 appears to play an early and important role in the recruitment of monocytes for atherosclerotic lesions as well as in the formation of intimal hyperplasia after arterial injury (Charo and Taubman 2004; Rajasekaran et al. 2005). Therefore, the inhibition of MCP-1 mRNA's expression by the hexane fraction might imply that the plant can be used in treating cardiovascular diseases such as atherosclerosis.

In conclusion, we found that all of the solvent fractions showed potent anti-oxidative effects as assessed by xanthine oxidase assay and DPPH assay, with IC_{50} values ranging from 9 to 42 μg/ml. In agreement with this pat-

tern, the total phenolic contents were widely distributed in various solvent fractions ranging from 36.5 to 50.3 mg. All of the solvent fractions significantly suppressed the LPS-induced NO production in RAW264.7 cells, and of them, only the chloroform (CHC) fraction completely blocked the expression of inducible NO synthase (iNOS). Additionally, the hexane and chroroform fractions suppressed the mRNA expression of GM-CSF and MCP-1 in LPS-stimulated RAW264.7 cells. Therefore, these results suggest that the pharmacological action of *S. asparagoides* is due to its potent anti-oxidative and anti-inflammatory effects, and that therefore it can be applied to other diseases caused by oxidative stress and inflammation, such as cardiovascular diseases.

4. Experimental

4.1. Materials

Ascorbate, dimethylsulfoxide (DMSO), DPPH, LPS, Griess's reagent, and xanthine oxidase were obtained from Sigma Co (St. Louis, MO). Xanthine was acquired from Merck Co. (Milwaukee, WI). All of the other reagents were of reagent grade.

4.2. Solvent extraction

S. asparagoides was collected from the province of Gunsan (Korea) in May, 2004. A voucher specimen bearing the number PLSA-1212 is deposited in the herbarium of our laboratory. Powder from the leaves (100 g) of *S. asparagoides* was extracted overnight with 0.7 L of ethanol. The ethanol extract subsequently was filtered through filter paper (Whatman No. 3) and centrifuged at 5,000 × g for 10 min. The filtrate was evaporated in a Rotavapor (yield 3.8 g [3.8%]). The ethanol extract was successively extracted except for 0.62 g, a suitable amount for an ethanol fraction. The extraction was conducted using hexane, chloroform, ethyl acetate, *n*-butanol and water, and the yields were 23.8%, 1.7%, 6.4%, 13.7%, and 54.4%, respectively. The crude extracts were stored at −20 °C until use.

4.3. Determination of total phenolic contents

The total phenolic contents were estimated following the Folin-Ciocalteu colorimetric method (Cai et al. 2005). Briefly, the appropriate dilutions of *S. asparagoides* extracts were oxidized with 0.5 N Folin-Ciocalteu reagent, and then the reaction was neutralized with saturated sodium carbonate (75 g/L). The absorbance was read at 725 nm by spectrophotometry (Kontron Italy) after incubation for 1 h at room temperature. The results were expressed as milligrams of gallic acid equivalent (mg of GAE) per g of dry weight.

4.4. Radical scavenging activity

A DPPH assay measured hydrogen-atom-donating (of one electron) activity and hence provided an evaluation of anti-oxidant activity due to free radical scavenging. DPPH, a purple-colored stable free radical, is reduced to yellow-colored diphenylpicryl hydrazine. The Blios method with slight modifications was used in this experiment (Blois 1958). A fresh batch of a radical stock solution was prepared daily. The EDA described the difference in the absorbance between the mixture and the control solution, expressed in a percentage: EDA (%) = (the absorbance of the control − the absorbance of the mixture)/the absorbance of the control × 100.

4.5. Assay for inhibition of xanthine oxidase activity

The activity of xanthine oxidase with xanthine, as a substrate, was measured spectrophotometrically according to Noro et al. (1983), but with the following modifications. The final concentration of xanthine oxidase was 250 μU/ml in a 0.1 mM phosphate buffer (pH 7.4). Xanthine and xanthine oxidase were mixed in a cuvette with either the compound being tested or the vehicles. The difference of the absorbance was measured to 295 nm for 3 min and the enzyme activity was calculated with references: (the activity of control − the activity of the mixture)/(the activity of control) × 100.

Table 2: The sequences of primers and fragment sizes of the investigated genes

Fractions	Total phenolic content (mg of GAE/g of DW)
EtOH	47.4 ± 0.2
HEX	46.4 ± 0.1
CHC	36.5 ± 0.1
EA	43.7 ± 0.5
BuOH	50.3 ± 1.4
AQU	48.4 ± 1.6

4.6. Cell culture

RAW264.7 cells were maintained in RPMI supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 5% FBS. The cells were grown at 37 °C and in humidified, 5% CO_2 air.

4.7. Measurement of nitrite

In order to determine the NO concentration, nitrite (NO_2^-) was measured using Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), as described previously (Cho et al. 2006; Hong et al. 2003). The concentrations of nitrite were calculated from a regression analysis, using serial dilutions of sodium nitrite as a standard. The percentage inhibition was calculated based on the ability of the extracts to inhibit NO formation by cells, as compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as no inhibition (0 %).

4.8. MTT assay for the measurements of cell proliferation

A cell proliferation assay was also performed to exclude the possibility that the observed NO inhibition was, due to its cytotoxic effect, false positive. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously (Cho et al. 2006; Hong et al. 2003).

4.9. Extraction of total RNA

The total RNAs from the LPS treated-RAW264.7 cells were prepared by adding Easy blue Reagent (InTron Biotechnology Co. Seoul), according to the manufacturer's instructions. The total RNA solution was stored at −70 °C until use.

4.10. Semi-quantitative RT-PCR amplification

Semi-quantitative RT reactions were carried out using RT premix (Bioneer Co. Korea). Briefly, the total RNAs (2 μg) were incubated with oligo-dT_{18} at 70 °C for 5 min and cooled on ice for 3 min. The reaction mixture was incubated for 90 min after the addition of RT premix at 42.5 °C. The reactions were terminated at 95 °C for 5 min in order to inactivate the reverse transcriptase. The PCR reaction was further conducted using a PCR premix (Bioneer Co. Korea), with an appropriate sense and anti-sense primer, under the following incubation conditions: a denaturation time of 45 s at 94 °C, an annealing time of 45 s at 55 to 60 °C, an extension time of 45 s at 72 °C, and a final extension time of 10 min at 72 °C at the end of the cycles. The primers (Bioneer Co. Korea) used in this experiment are indicated in Table 2 (F: forward, R: reverse).

4.11. Statistical analysis

A one-way and a two-way ANOVA were used to determine the statistical significance of the differences between the values for the various experimental and control groups. The data were expressed as means ± standard errors (SEM) in triplicates. P values of 0.05 or less were considered to be statistically significant.

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