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Antiinflammatory and antioxidative effects of *Agrimonia pilosa* Ledeb

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SUMMARY

Agrimonia pilosa Ledeb. has long been used for a useful natural agent ameliorating inflammation related symptoms in the folk medicine recipe. This study was performed to investigate effects of *Agrimonia pilosa* Ledeb.(AP) on the expression of inflammation related genes such as the inducible nitric oxide synthase (iNOS) in macrophage cell line, RAW 264.7 cells. The AP (whole plants) was extracted with 80% ethanol and sequentially partitioned with solvents in order to increase polarity. Among the various solvent extracts of AP, the n-butanol (BuOH) fraction showed the most powerful inhibitory ability against nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW 264.7 cells without affecting cell viability. Reverse transcriptase-polymerase chain reaction and Western blot analysis revealed that the BuOH fraction provided a primary inhibitor of the iNOS protein and mRNA expression in LPS-induced RAW 264.7 cells. The DPPH and OH radical scavenging activities of the several fractions of 80% ethanol extracts of AP significantly increased by EtOAC and BuOH fractions. Thus, the present study suggests that the response of a component of the BuOH fraction to NO generation via iNOS expression provide an important clue to elucidate anti-inflammatory mechanism of AP.

Key words: *Agrimonia pilosa*; iNOS, NO; RAW 264.7

INTRODUCTION

Evidence of nitric oxide (NO) synthesis by human skin cells was first reported just over 10 years. Since that time and from a proposed role in non-specific host defense, it is now clear that NO plays a key role in orchestrating the skin's response to external stimuli such as heat, ultraviolet (UV) light, response to infection and wound healing, as well as possibly underlying certain pathological conditions(Cals-Grierson and Ormerod, 2004). Reactive oxygen species (ROS) are generated under various physiological and pathological conditions such as inflammation, aging, and carcinogenesis (Cuda *et al.*, 2002; Dhar *et al.*, 2002; Kurz *et al.*, 2004). An increase in intracellular ROS level, such as superoxide anion, hydrogen peroxide and hydroxyl radical, has been shown to damage tissues and cells via lipid peroxidation and alteration of protein and nucleic acid structure (Berlet and Stadtman, 1997). Some natural anti-oxidant products have been shown to protect cells from oxidative injury. Flavonoids are found in plants, and act as pharmacological active components in medicinal herbs. Flavonoids such as quercetin, catechin, kaempferol are better anti-oxidants than the antioxidant Vitamin C and Vitamin E (Noroozi *et al.*, 1998). A number of natural plants used in

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traditional anti-inflammatory medicines have been shown to inhibit pro-inflammatory genes (Setty and Sigal, 2005). *Agrimonia pilosa* Ledeb. (AP) has been used as an anti-inflammatory agent in Chinese herbal medicines (Kang, 1992). Previous studies have shown that APL possesses anti-bacterial and hepatoprotective activities (Park *et al*., 2004; Kwon *et al*., 2005). The pharmacological activities of AP are primarily due to such phenolic compounds as agrimonolide, agrimonolide-6-O-glucose, catechin, querectin, rutin, etc (Kasai *et al*., 1992; Xu *et al*., 2005). To gain better insight into the anti-inflammatory activity, in this study, we conducted *in vitro* analysis and investigated the effects of AP on such macrophage-mediated inflammatory phenomena as NO release, functional activation of adhesion molecules and oxidative stresses in order to understand its anti-inflammatory properties.

MATERIALS AND METHODS

Chemicals

2, 2-Diphenyl-1-picrylhydrazyil radical (DPPH), nitroblue tetrazolium (NBT), Folin & Ciocalteu's phenol reagent, 3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, quercetin, phenyl-methylsulfonylfluoride (PMSF) and LPS were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies(Gaithersburg, MD, USA). COX-2, iNOS and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunoassay kit used for the determination of Nitric oxide was obtained from Assay Designs Inc (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO, USA).

Extract and fraction of extract

Agrimonia pilosa Ledeb. AP was purchased from Omniherb Co. (Yeongcheon, Republic of Korea). AP was ground into 50-mesh sieve powder. One hundred grams of AP was extracted twice by sonication for 30 min with 80% ethanol and then was filtered. The filtrate was combined and concentrated in a vacuum evaporator.

AP Ethanol extract suspended in distilled water was re-extracted with diethyl ether, $CHCl_3$, EtOAC and BuOH in order to increase polarity. The dehydrated fractionation were dissolved in dimethyl sulfoxide (DMSO) to a regular concentration and then the activity was measured.

HPLC analysis

In order to identify and to determine the constituents in the BtOH fraction of AP, the HPLC analysis was performed. The HPLC system consisted of a Waters 717 plus autosampler, Waters 1,525 Binary HPLC pump, Waters 2,996 Photodiode Array Detector (PDA) and MilleniumTM 3.2 software (Millipore Corporation, Milford, MA, USA) with Phenomenex Gemini C18 110A column (4.6 mm $\leftrightarrow$ 150 mm, 5 μm). The mobile phase consisted of water(A) and acetonitrile(B)(50:50). The gradient was 22% B (0-16 min), 40% B (16-50 min) and 22% B (50-52 min) at flow rate of 1.0 ml/min. The injected volume was 10 μl.

Total phenolic and flavonoid contents

Total phenolic contents in the extracts of AP were determined using Folin-Ciocalteu's reagent according to the method of Singleton and Lamuela-Raventos (1999) with some modifications. One milliliter of diluted extracts and 1.0 ml of diluted Folin-Ciocalteu's reagent were mixed. After 3 min, 1.0 ml of 10% sodium carbonate was added. After 1 h of reaction, the concentration of total phenolic contents was measured by reading absorbance at 760 nm, and the reading was compensated in accordance with the scale of standard gallic acid. Total flavonid contents in samples were determined by the method of Woisky and Salatino(Woisky and Salatino, 1998). 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$-ethanol

solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as Quercetin (Karmpferol) from a calibration curve.

Antioxidant activity

The antioxidant activities of the extracts and of the standards were assessed on the basis of the radical scavenging effect of the stable DPPH free radical. 0.1 ml samples with varying in concentrations, were added to 0.1 ml of DPPH (0.1 mM) in methanol solution in a 96-well microplate. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 545 nm using ELISA microplate reader (Bio Rad Laboratories Inc., California, USA, Model 550) (Brand-Williams *et al*., 1995).

The Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-deoxyribose oxidation by hydroxyl radical(Chung *et al*., 1997). The reaction mixture consisted of 0.30 ml of 0.02 M sodium phosphate buffer (pH 7.0), 0.15 ml of 10 mM 2□deoxyribose, 0.15 ml of 10 mM $FeSO_4$, 0.15 ml of 10 mM EDTA, 0.15 ml of 10 mM H_2O_2, 0.525 ml of H_2O and 0.075 ml of sample solutions. The reaction mixture was incubated at 37°C for 2 h and 0.75 ml of 2.8% trichloroacetic acid and 0.75 ml of 1.0% TBA were added to test tubes and boiled for 20 min. After cooling the mixture, absorbance was measured at 520 nm. The radical scavenging activity was calculated employing the following equation.

Radical scavenging activity (%)
$= [1 - (A_{sample}/A_{control})] \times 100$

Cell culture

RAW 264.7, a mouse macrophage cell line, was obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM medium supplemented with 2 mM glutaminne, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin) and 10% heat-inactivated fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO_2.

MTS cytotoxicity assay

The cell viability assay was performed by using the MTS and an electron coupling reagent, phenazine methosulfate (PMS). The samples were dissolved in DMSO and diluted in cell culture medium. The final concentration of DMSO of the cell was 0.1%. In control experiments, this concentration did not show any effects on the measured parameters. The cells were cultured in 96-well plate at concentration of 5×10^5 cells/well. After 24 h of preconditioning, culture medium was aspirated and cells were treated with various concentrations of sample for 24 h. Cells were then exposed to 100 μl samples diluted by H_2O_2 or DMSO and had been cultured for 24 h at 37°C. Subsequently, 10 μl of MTS dye (1 mg/ml) was added to culture and further incubated for 2 h at 37°C. Index of cell viability was calculated by determined water-soluble formazan absorbance at 595 nm with an ELISA micro plate reader (Bio Rad Laboratories Inc., California, USA, Model 550).

Nitrite assay

The cells were cultured in 96-well plate at concentration of 5×10^4 cells/well. After 24 h of preconditioning, culture medium was evaporated and the cells were treated with various concentration of sample and were stimulated with LPS 1 μg/ml for 18 h.

The nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction. Briefly, 100 μl of each supernatant from each well was transferred to another 96 well tissue culture media. Then, 20 μl of griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water) and distilled water 130 μl were added. This mixture was incubated at room temperature for 10 min. The absorbance of the mixture at 540 nm was determined with an ELISA plate reader (Bio Rad Laboratories Inc., California, USA, Model 550).

ROS production

Oxidative damage of cells was carried out using hydrogen peroxide and oxidative stress of cells was determined by using the fluorescent probe DCHF-DA(Oyama *et al*., 1994; Ferretti *et al*., 2003). The molecule DCHF-DA is freely permeable to cells. After incorporation into cells, DCHF-DA is converted into the fluorescent 2,7-dichlorofluorescein (DCF) by oxidative substances. Therefore, DCHF-DA indicates the intracellular production of redox-active substances and it has been widely used to investigate oxidative damage in intact cells. Briefly, Cells (5×10^4 cells/well) were pretreated with samples of various concentration for 24 h. After 24 h of preconditioning, culture medium was aspirated and added 100 μl of various concentration samples diluted with DMSO and then was cultured for 1 h. Subsequently, the cells were stimulated with 10 μl LPS (1 μg/ml) for 18 h.

At the end of the oxidation treatment, cells were incubated with 50 μM of fluorescent probe DCHF-DA for 30 min at 37°C. The degree of fluorescence, corresponding to intracellular ROS, was determined using Fluoroscan Ascent FL (Labsytems Inc., Helsinki, Finland, Type 374) (490 nm excitation and 526 nm emission wavelengths).

Western blot analysis

iNOS (120 KDa) and COX-2 (74 KDa) protein were tranfered at a 7.5% SDS-PAGE gell. After 18 h (iNOS or COX-2) of incubation, cells were washed twice with ice-cold phosphated-buffered saline (PBS) and lysated directly in the plate wells after removal of media. The cell lysates were obtained by centrifugation at 13,000 × g for 15 min at 4°C. Protein concentrations were determined by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (50 μg) of the cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a poly vinylidene difluride membrane. The membrane was then blocked in PBS-0.1% Tween 20 (PBST) containing 1% skim milk and 1% BSA for 1 h at room temperature. Thereafter, the membrane was incubated for overnight at 4°C with a 1:1000 dilution of monoclonal anti-iNOS or anti-COX-2 antibodies. The membrane was then washed three times with PBST and further incubated with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Subsequently, blots were extensively washed again three times with PBST and then developed by enhanced chemiluminescence (ECL).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

After washing the harvested cells by PBS, cells were centrifuged at 4, cells were lysing by the addition of 1 ml TRIzole (Invitrogen, Grand Island NY, USA) and the cell lysate was incubated for 5 min with 0.2 ml of chloroform. The tubes were shaken vigorously and centrifuged at 12,000 × g for 10 min. The upper phase was tranfsered to a fresh tube and RNA was precipitated with the addition of 0.5 ml isopropanol. The RNA pellet was washed by 75% ethanol and then air-dried and solved in diethylpyrocarbonate (DEPC) water solution. The residual genomic DNA was removed by adding RNase-free DNase (Promega Co., Madison WI, USA) 2 unit at 37°C for 30 min. Then the same volume of the phenol and chloroform solution (1:1, v/v) was added. The upper phase was left alone by the addtion of the two times of the amount of the ethanol of the previous treatment and the 0.1 times of the amount of the 3 M sodium acetate (pH.5.2) of the previous treatment at -70°C for over 2 h. After centrifuging at 12,000 × g at 4°C for 10 min, the sediment was washed by 70% ethanol and was dried at room temperature. Then the dried sediment resolved in DEPC water solution. After quantification of the purified RNA quantified by Gene Quant Pro (Amersham Biosciences, NJ, USA), the integrity and the accurate quantification was reconfirmed by transferation in agarose electrophoresis with ethidium bromide. 4 μg of

random hexamer (Amersham Biosciences, NJ, USA) and DEPC water solution were added in 1 μg of purified RNA, and the mixture was incubated at 65°C for 10min, and cooled in ice for 2 min. Subsequently with the addition of 5 × first strand buffer, 0.1M DTT, 10 mM dNTP, 200 unit M-MLV reverse trancriptase (Invitrogen, Carlsbad CA, USA) and DEPC water solution, total volume of the mixture was 30 μl. The reaction mixture was then incubated at 42°C for 1 h. Reheating at 95°C for 5 min, cDNA was transcripted. Primers for iNOS, COX-2 and rat GAPDH mRNA (used as a control for total RNA content for each sample) were shown Table 1 (Fort *et al*., 1985; Lin and Lin, 1997; Shin *et al*., 2004).

PCR amplification was performed in a reaction volume of 30 μl containing 2.5 μl of the appropriate cDNA, 25 μl of each set of primers, 1× PCR buffer, 2.5 mM $MgCl_2$ and 2.5 units of Taq. DNA polymerase (Takara-korea, Seoul, Republic of Korea). PCR conditions were at 95°C for 1 min, at 50°C for 1 min and at 72°C for 1 min. After amplification, 2 μl of the PCR productswere subjected to a 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis

The results are expressed by the mean of ± S.E.M. or the mean ± S.D. The statistical comparisons between the groups were made by ANOVA followed by Duncan's multiple range test using the SAS. Differences were statistically significant for $P < 0.05$.

RESULTS

Yields of the several solvent fractions

Yields of each fraction were BuOH (5.0%), water (4.0%), ether (2.2%), $CHCl_3$ (0.79%) and EtOAC (0.44%) (Table 2).

Table 2. Yields of several solvent fractions from the 80% ethanol of AP

Fractions	Yields (g/100 g of powder, dry weight)
Diethyl ether	2.25
Chloroform	0.79
Ethyl acetate	0.44
n-Butanol	5.00
Water	4.01
Total	12.49

HPLC analysis

The HPLC analysis was used to identify and to determine the constituents in BuOH fraction of AP. 3 spectra of peaks were found and their maximum absorbance were 274, 271 and 277 nm (Fig. 2).

Total phenolic and flavonoid contents

Total phenolic contents in the extracts were determined using Folin & Ciocalteu's reagent. Phenolic contents in 80 % ethanol extract was 15.81 g per dried powder 100 g. Phenolic contents

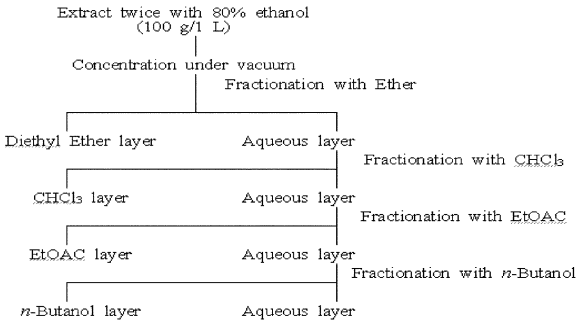

Fig. 1. Flow sheet for fractionation by several solvent.

Table 1. Primers for iNOS, COX-2 and Rat GAPDH mRNA

	Sense	Anti-sense
iNOS	5′-AATGGCAACATCAGGTCGGCCATCACT-3′	5′-GCTGTGTGTCACAG AAGTCTCGAACTC-3′
COX-2	5′-GGAGAGACTATCAAGATAGT-3′	5′-ATGGTCAGTAGACTTTTACA-3′
GAPDH mRNA	5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′	5′-CATGTAGGCCATG AGGTCCACCAC-3′

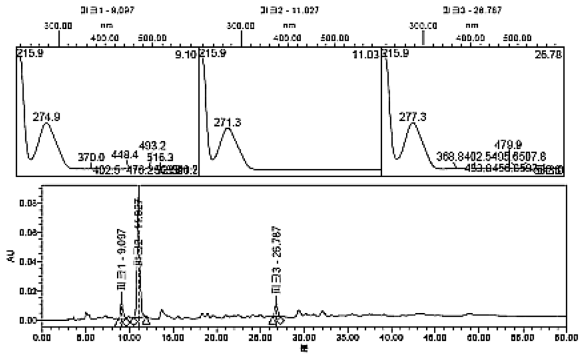

Fig. 2. HPLC chromatogram of BuOH fraction from AP.

in each fraction were BuOH (27.4 g/100 g), EtOAC (22.1 g/100 g), Water (7.3 g/100 g), Ether (5.13 g/100 g) and Chloroform (4.4 g/100 g) (Table 3).

In the current study, the total amount of flavonoid measured by the colorimetric method was 1.76 g/100 g in the BuOH fraction.

Although the amount of flavonoid contents in the $CHCl_3$ fractions was also shown as much as in the BuOH fraction, it was not actually the real flavonoidal substance judging from the finding that the colorimetric method at 420 nm showed higher absorbance value on the measurement of materials such as Chlorophyll or other coloring matters.

Antioxdant activities of the fractions

The DPPH and OH radical scavenging activities of the several fractions of 80% ethanol extracts of AP significantly increased by EtOAC and BuOH fractions. The DPPH radical scavenging activity significantly increased by EtOAC and BuOH was 15.6 μg/ml and 15.3 μg/ml. Hydroxyl radical scavenging activity fractions significantly decreased by BuOH, EtOAC and Water fraction was 30.9, 48.1 and 73.2 μg/ml respectively. Which suggest that EtOAC and BuOH fractions exert potent anti-oxidative activity considering with the result more phenolic and flaovonoid contents were included in EtOAC and BuOH fractions together. Therefore, BuOH and EtOAC fractions were adopted for the present study.

Cytotoxicity

The cytotoxicity of the various concentrations of EtOAC and BuOH fractions in RAW 264.7 cells was measured through MTS Assay. Little cytotoxic effect was presented in EtOAC and BuOH fractions at concentrations ranging from 2.5 - 100 μg/ml (Fig. 3).

NO assay and ROS production

Current study demonstrated that BuOH and EtOAC fractions inhibit ROS generation as well as NO generation dose-dependently in RAW 264.7 cells. NO generation by BuOH and EtOAC fraction was significantly decreased at concentrations more than 10 μg/ml respectively. ROS production By BuOH and EtOAC fraction was significantly decreased at concentrations more than 25 and 50

Table 3. Total phenolic and flavonoid contents of the 80% ethanol extract and the several solvent fractions from AP

	Total phenolics (g/100 g of extract, dry weight)	Total flavonoids (g/100 g of extract, dry weight)
80% EtOH	15.81 ± 0.77	1.44 ± 0.13
Diethyl ether	5.13 ± 0.13	0.65 ± 0.47
$CHCl_3$	4.48 ± 0.10	1.41 ± 0.17
EtOAC	22.16 ± 1.22	0.70 ± 0.38
n-BuOH	27.47 ± 0.60	1.76 ± 0.38
Water	7.37 ± 1.02	0.68 ± 0.39

The ethanol extract from AP suspended in the distilled water was extracted with diethylether, $CHCl_3$, EtOAC and BuOH in order to increase polarity. Each value is the mean ± S.E.M. Mean values with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple-range test.

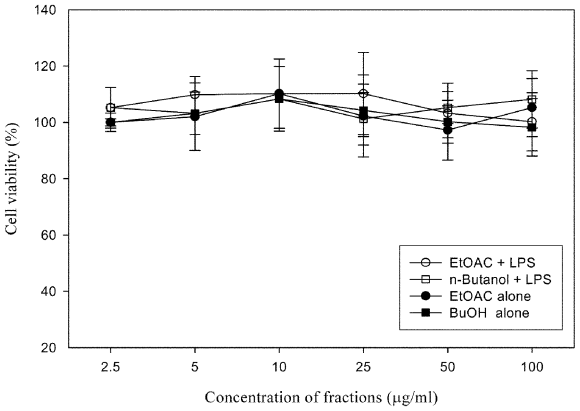

Fig. 3. Effects of EtOAC and BuOH fractions from AP on cell viability (A), nitrite (B) and ROS (C) production in LPS-induced RAW 264.7 cells. RAW 264.7 cells were treated with LPS (open) or without LPS (solid) for up to 24 h. *P < 0.05 compared with LPS alone.

μg/ml respectively. NO and ROS generation was significantly decreased by over 25 μg/ml of BuOH, and by over 50 μg/ml of EtOAC respectively.

iNOS and COX-2 protein expression

In order to investigate whether the EtOAC fraction and the BuOH fraction of AP can inhibit LPS-indeuced iNOS and COX-2 protein expressions in RAW 264.7 cells, we evaluated their expressions by the western blot analysis. As shown in Fig. 4, the expression of iNOS decreased, but the level of COX-2 was unchanged. The expression rate of iNOS significantly decreased by 50, 100 μg/ml BuOH and EtOAC fraction was 19, 62 and 32, 81% (P < 0.05).

iNOS and COX-2 mRNA expression

The RT-PCR analysis of the mRNA levels of iNOS and COX-2 was performed in order to provide an estimate of the relative levels of expression of these genes in treatment with the BuOH fraction. In the present study, the level of iNOS in the control set as 100%. The level of iNOS was significantly decreased by treatment with the BuOH fraction. In contrast, the level of COX-2 did not show statistically significant change (Fig. 6).

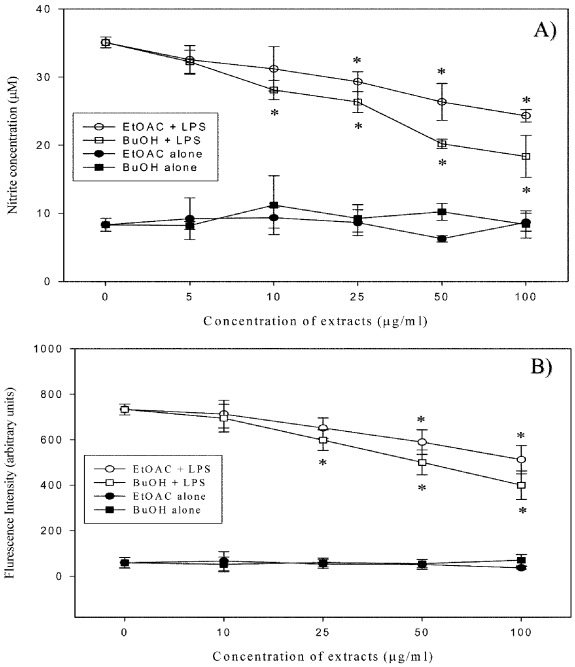

Fig. 4. Effects of EtOAC and BuOH fractions from AP on nitrite (A) and ROS (B) production in LPS-induced RAW 264.7 cells. RAW 264.7 cells were treated with LPS (open) or without LPS(solid) for up to 1 h. *P < 0.05 compared with LPS alone.

In this result, the BuOH fraction significantly decreased the level of iNOS protein and iNOS mRNA whereas it did not actually have influence upon COX-2 and COX-2 mRNA expression.

DISCUSSION

NO is a locally synthesized messenger with many physiological and pathological activities (Ormerod *et al.*, 1998). NO is produced from L-arginine by constitutive and inducible nitric oxide synthase in various mammalian cells and tissues. iNOS is induced by either LPS or cytokines including TNF-α and IFN-γ in macrophages (Ignarro *et al.*, 1988; Alderton *et al.*, 2001). Expression of iNOS is closely related with the upregulation of NF-κB, whose sites have been identified in the promoter region of iNOS gene (Cho *et al.*, 2005). NF-κB, an inducible transcription factor, is activated in

Table 4. Radical scavenging activities of the 80% ethanol extract and the several solvent fractions from AP

	$DPPH^{\bullet}$(μg/ml)	$OH^{\bullet}$(μg/ml)
80% EtOH	25.02 ± 1.71[b]	87.68 ± 8.21[c]
Diethyl ether	>100	>200
$CHCl_3$	>100	>200
EtOAC	15.63 ± 1.10[a]	48.17 ± 2.21[a]
n-BuOH	15.31 ± 0.05[a]	30.93 ± 3.21[b]
Water	35.21 ± 1.21[c]	73.27 ± 9.25[c]

The ethanol extract from AP suspended in distilled water was extracted with diethylether, $CHCl_3$, EtOAC and BuOH in order to increase polarity. Each value is the mean ± S.E.M. Mean values with different superscript letters are significantly different ($P < 0.05$) by Duncan's multiple-range test.

response to various extracellular stimuli, including cytokines, LPS, and oxidative stress (Cho *et al*., 2005). Especially, Oxidative stress induced inflammation is mediated by NF-κB activation, MAP kinases and affect a wide variety of cellular signaling processes leading to generation of inflammatory mediators. The latter allows expression of pro-inflammatory genes such as IL-1b, IL-8, TNF-α and iNOS (Schoonbroodt and Piette, 2000; Rahman *et al*., 2006).

AP has been used as an anti-inflammatory agent in Chinese herbal medicines (Kang, 1992). Previous studies have shown that AP possesses anti-bacterial, hepatoprotective, antibacterial, free radical scavenging activity, antitumor, antihemostatic and antiplatelet activities (Kang, 1992; Murayama *et al*., 1992; Copland *et al*., 2003; Park *et al*., 2004; Kwon *et al*., 2005). The pharmacological activities of AP are primarily due to such phenolic compounds as agrimonolide, agrimonolide-6-O-glucose, catechin, querectin and rutin etc (Kang, 1992; Kasai *et al*., 1992; Murayama *et al*., 1992; Copland *et al*., 2003; Park *et al*., 2004; Kwon *et al*., 2005; Xu *et al*., 2005). Despite its famous legacy, the pharmacological effects of AP have not been fully explored. In this study, to gain better insight into the anti-inflammatory activity, we performed *in vitro* analysis and investigated the effects of AP on such macrophage-mediated inflammatory phenomena as NO release, functional activation of adhesion molecules and oxidative stresses in order to understand its anti-inflammatory properties.

Flavonoids exert a wide range of therapeutic activities such as anticarcinogenic, anti-inflammatory, antiviral, cytotoxic, cytostatic and antioxidant properties (Isao *et al*., 1988). These activities were flavonoids modulating cellular signaling processes such as NF-κB activation, scavenge effect of ROS directly or via glutathione peroxidase activity and as a consequence regulate inflammatory genes. However, recent data suggest that flavonoids can work as modifiers of signal transduction pathways to elicit their beneficial effects. It also has been reported that APL contains catechin class of flavonoids (Raso *et al*., 2001; Phytochemical Database, 2002). In the current study, the total amount of flavonoid measured by the coloration was 1.76 g/100 g in the BuOH fraction. In the $CHCl_3$ fraction, the amount of flavonoid contents appeared to be higher value, but the value can not be considered as the real substance of flavoniod, since the colorimetric method at 420 nm shows higher absorbance value on the measurement of materials such as Chlorophyll or other coloring matters. The DPPH radical scavenging activity significantly increased by EtOAC and BuOH was 15.6 μg/ml and 15.3 μg/ml. hydroxyl radical scavenging activity fractions significantly decreased by BuOH, EtOAC and water fraction was 30.9, 48.1 and 73.2 μg/ml respectively. Which suggest that EtOAC and BuOH fractions exert potent anti-oxidative activity considering with the result more phenolic and flaovonoid contents were included in EtOAC and BuOH fractions together. Therefore, BuOH and EtOAC fractions were adopted for the present study.

NO, a short-lived gas and highly reactive free radical is produced by monocytes and macrophages upon exposure to LPS. Physiological production of NO plays an important role in host defense such as ROS or ROI(Wang and Smart, 1999; Boscá

et al., 2005). Overproduction of NO and its metabolites has been implicated in the pathogenesis of conditions such as bacterial sepsis and inflammation. Large amount of NO led to the formation of a strong oxidant, the peroxynitrite anion (Siebra *et al*., 2006). The proposed cytotoxic properties of peroxynitrite inhibition of cellular metabolic pathways and signal transduction mechanisms and DNA strand breaks (Tsao *et al*., 2005). In this study, the increasing scale of NO production by the treatment with LPS decreased by the treatment with two fractions of AP in the order of the concentration, as illustrated in Fig. 4. Especially, the 10 μg/ml extract of BuOH fraction inhibited the NO production effectively in the LPS-induced RAW 264.7 cells.

The fact that ROS palys a key role in inflammation was widely recognized, thus determined in LPS-induced macrophage RAW 264.7 cells (Flohe *et al*., 1997). Exposuring of RAW 264.7 cells to LPS strikingly stimulated the accumulation of intrecelluar ROS. This accumulation of ROS could have malicious effects directly on the cell, causing lipid peroxidation, leading to apoptotic cell death and production of pro-inflammatory mediators (Flohe *et al*., 1997; Allen and Tresini, 2000; Chen *et al*., 2000; Tsao *et al*., 2005). In the present study, The ROS production in treatment with the two fractions in LPS-induced RAW 264.7 cells was decreased and the decreased ROI production and the decreased NO production showed the identical trend. Phenolic materials - phenolic acid, poly phenol and flavonoid etc- are reported to possess the antioxidant activity, which could allow them to inhibit NF-κB activity by reducing ROS (Flohe *et al*., 1997; Allen and Tresini, 2000; Chen *et al*., 2000; Bremner and Heinrich, 2002; Tsao *et al*., 2005).

Among the known up-regulator proteins of NO and PGE2, iNOS and COX-2 were activated through the expressions of MAPKs and Jak-Stats pathway (Chen *et al*., 2000; Bremner and Heinrich, 2002). In the present study, we determined the expressions of iNOS, COX-2, iNOS mRNA and COX-2 mRNA in the treatment with the EtOAC and BuOH fractions of AP in LPS-induced RAW 264.7 cells. As shown in Fig. 5, the expression of iNOS decreased, but the level of COX-2 was unchanged. The expression rate of iNOS significantly decreased by 50, 100 μg/ml BuOH and EtOAC fraction was 19, 62 and 32, 81% ($P < 0.05$). This result shows that the BuOH fraction is more effective than the EtOAC fraction in inhibiting the expression of iNOS. This results were same to the report of Hong *et al.* (2002). The level of iNOS mRNA was significantly decreased by treatment with the BuOH fraction. In contrast, the level of COX-2 mRNA did not show any statistically significant change (Fig. 6). In this result, the BuOH fraction significantly decreased the level of iNOS protein and iNOS mRNA, showing the inhibitory effect of NO synthesis. While it did not actually have influence upon COX-2 and COX-2 mRNA expression.

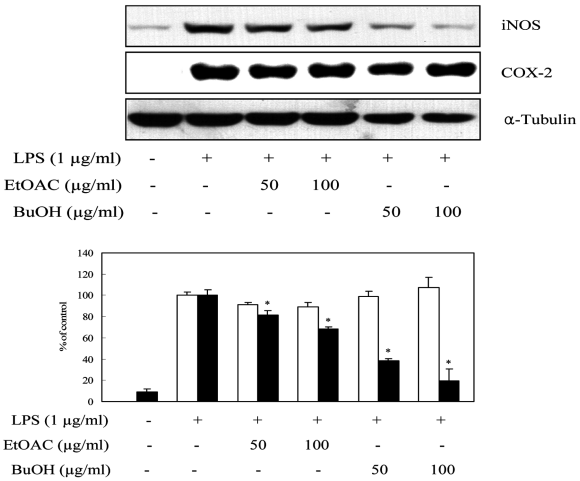

Fig. 5. Effects of the EtOAC and BuOH fractions on iNOS and COX-2 protein expression in LPS-induced RAW 264.7 cells. Cells were pretreated with different concentrations of EtOAC and BuOH fractions for 1 h before LPS treatment (1 μg/ml), and the cells further incubated for 18 h. The protein levels of iNOS and α-tubulin were determined by Western blot analysis and the ratio of immunointensity between the iNOS and the α-tubulin calculated. Each bar represents the means ± SD from three independent experiments. *$P < 0.05$ compared with LPS alone.

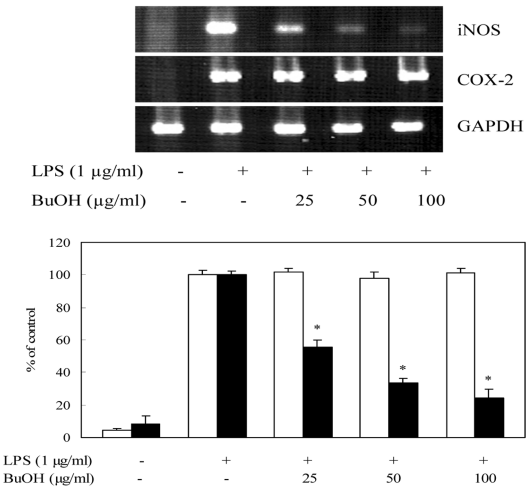

Fig. 6. Effects of the BuOH fraction on iNOS and COX-2 mRNA expression in LPS-induced RAW 264.7 cells. Cells were pretreated with different concentrations of the BuOH fraction for 1 h before LPS treatment (1 μg/mL), and the cells further incubated for 18 h. Total mRNAs were prepared from the cell pellets using TRIzol. The relative levels of mRNA were assessed by RT-PCR. Each bar represents the mean ± SD from three independent experiments. *P < 0.05 compared with LPS alone.

The HPLC analysis was used to identify and to determine the constituents in BuOH fraction of AP. 3 spectra of peak were found and their maximum absorbance were 274, 271 and 277 nm (Fig. 2). The constituents separated from the BuOH extract of natural plants were mainly belong to the saponin and lignan classes. Thus, the similar characteristics in terms of the spectra peaks can be viewed as the indication of the presence of constituents of saponin or lignan classes at the condition of the HPLC analysis and the maximum absorbance. This 3 peaks materials showed to possess the anti-inflammatory effect. The current study leaves the analysis of the structure such as FAB-MS, MNR and IR for further research.

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