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Article

Inhibitory Effects of an Ethanol Extract of *Artemisia capillaris* on Inflammatory Mediators from LPS-Stimulated RAW 264.7 Cells

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Artemisia capillaris **is a perennial herb that belongs to the family *Campositae spp,* and has been utilized in Korea for the treatment of abdominal pain, hepatitis, chronic liver disease, and coughing. The anti-inflammatory activity of the ethanol extract of *A. capillaris* (EtEAC) was investigated in this study. EtEAC did not affect the viability of various cells in a concentration range of 0-200 μg/mL. The DPPH radical scavenging ability of 10 μg/mL of EtEAC was comparable to that of 10 μg/mL of catechin. EtEAC exerted an anti-inflammatory effect on the mRNA expression levels of typical inflammatory cytokines (IL-6, IL1β) and cyclooxygenase-2 in LPS-stimulated RAW 264.7 cells. Additionally, EtEAC reduced nitric oxide (NO) production via the downregulation of inducible nitric oxide synthase transcription, rather than by scavenging NO. EtEAC exerted its anti-inflammatory effects by suppressing the activation of p38 and ERK at 200 μg/mL in LPS-stimulated RAW 264.7 cells.**

Key words: anti-oxidant, *Artemisia capillaris*, COX-2, ERK, inflammation, iNOS, p38

Inflammatory mediators, including reactive oxygen species (ROS) and cytokines, contribute to the inflammatory cascade in modulating the immune system of inflammatory disease [Kupeli and Yesilada, 2007]. Macrophages perform a pivotal function in host defenses against infection and cancer. The activation of macrophages by stimuli, such as lipopolysaccharides (LPS) and interferon (IFN)-α have been well established as an effective stimulus in the activation of macrophages to secrete pro-inflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, and secondary mediators, such as nitric oxide (NO) [Linton and Fazio, 2003]. NO participates in a variety of chemical reactions and has been associated with various diseases including chronic inflammation, autoimmune diseases, and cancer [Szekanecz and Koch, 2007].

Bioactive compounds from plant sources are commercially promoted as nutraceuticals, and have been demonstrated to reduce the incidence of these diseases [Hermans *et al*., 2007]. Currently, there is a great deal of interest in newer bioactive molecules derived from nature with potential health-promoting effects [Yesilada, 2005]. *Artemisia capillaris,* one of the herbal plants belonging to genus *Compositae,* is widely distributed throughout China, Japan, Korea, and Europe. Traditionally, *Artemisia* spp. (*A. mongolia, A. vulgaricus, A. capillaris*, and *A. annua*) have been utilized for the treatment of a variety of symptoms, such as cancer, chronic hepatitis, anorexia, diabetes, and chronic ulcers. Recently, the ethanol extract of *A. capillaris* has been shown to evidence high levels of anti-oxidant activity [Lee *et al.,* 2000] and contained several other bioactive flavonoids including scoparone, capillarisin, cirsimaritin, genkwanin, and rhamnocitrin [Wang *et al.,* 1994].

Scoparone is a major component isolated from the shoot of *A. capillaris* Tumb. Several studies demonstrated that scoparone exhibited free radical scarvenging, immunosuppressive and vasodilator activities [Huang *et al*., 1992; Rakotoarison *et al*., 2003]. Also, scoparone inhibited PMA-induced IL-8 and MCP-1 production through suppression of NF-κB activation [Jang *et al*., 2006] and inhibited NOS in insulinoma cells [Kim *et al*., 2007]. Capillarisin, one of the components of *A.*

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capillaris extracts, inhibited the activation of NF-κB activation [Lee *et al.,* 2008]. High anti-oxidant activity may scavenge inflammatory mediators such as ROS or NO: however, the anti-inflammatory effect of *A. capillaris* has not been extensively studied. Here, we show that the extract of *A. capillaris* contains very high anti-oxidant activity *in vivo* and suppresses LPS-induced NO and IL-6 production by inhibiting the activation of ERK and p38MAPK in macrophages.

Materials and Methods

Materials. All of the plastic materials were purchased from Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, fetal bovine serum (FBS), penicillin, streptomycin, and Trizol were purchased from Invitrogen Life Technologies Inc. (Grand Island, NY). Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Amresco Inc. (Solon, OH). Anti-GAPDH, -p-pERK, and -p-p38 antibodies were purchased from Cell Signaling (Danvers, MA). Anti-iNOS antibody was purchased from Santa Cruz (CA). The Bradford Protein Assay Kit and polyvinylidene fluoride (PVDF) membranes were purchased from Bio-Rad (Hercules, CA). All other chemicals and reagents were of analytical grade.

Plant materials and extraction procedures. Dried *A. capillaris* was purchased from a market in Jeju city. Dried *A. capillaris* (50 g) was pulverized using a milling machine and extracted with 90% ethanol by stirring for 2 days at room temperature (RT). The extract was filtered, concentrated with a vacuum rotary evaporator under reduced pressure, and lyophilized (3.81 g). The extract powder(s) was dissolved in Ethanol:PBS (1:1) and diluted with phosphate buffered saline (PBS, pH 7.4) to give the final concentrations.

Cell culture. HeLa, MCF-7, Hep-G2, A549, and RAW264.7 cells obtained from the Korea Cell Line Bank (Seoul, Korea) were maintained at 37°C in a humidified atmosphere under 5% CO_2 in RPMI 1640 or DMEM (Invitrogen Gibco BRL, Gaithersburg, MD) containing 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Primary human dermal fibroblasts were obtained from the foreskins of healthy volunteers, age 5-13 y. The skin was minced, followed by incubation with collagenase (1 mg/mL in DMEM) for 1-2 h at 37°C. The collagenase was then removed by washing with DMEM. The isolated cells were allowed to attach on plastic plates and cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. After 5 and 15 passages, the fibroblasts were utilized for experimentation.

Cell viability assay. The effects of the *A. capilliaris* ethanol extracts on the viability of various cell lines were determined via an MTT-based assay [Carimichael *et al.*, 1987]. In brief, the exponential-phase cells were collected and transferred to a microtiter plate (10^4-10^5 cells per well). The cells were then incubated for one day in the presence of various concentrations of the extracts. After incubation, 0.1 mg of MTT was added to each well, and the cells were incubated for 4 h at 37°C. The plates were then centrifuged for 20 min at 1,500 rpm at RT and the medium was carefully removed. DMSO (150 μL) was then added to each well to dissolve the formazan crystals. The plates were then immediately read at 540 nm on a Sunrise microplate reader (Tecan, Salzburg, Austria).

Nitrite analysis. Nitrite (NO_2) contents in the media obtained from the cell cultures and the exudates prepared from the air pouches were determined based on the Griess reaction [Sherman *et al.*, 1993]. The samples (100 μL) were allowed to react with 100 μL Griess reagent for 10 min at room temperature, and then NO_2 concentration was determined by measurements of absorbance at 540 nm. The calibration curve was constructed using the known concentrations of sodium nitrite.

Determination of intracellular reactive oxygen species (ROS). Cellular ROS were quantified via dichlorofluorescein (DCFH) assays [Wang and Joseph, 1999], in which the esterified form of 2',7'-dichoro-fluoresceindiacetate (DCFH-DA) diffuses through the cell membrane and is enzymatically deacetylated by intracellular esterase. The resultant compound, DCFH, can react with ROS to yield an oxidized fluorescent compound, dichlorofluorescein (DCF).

Determination of intracellular ROS in HepG2 cells. HepG2 cells were pretreated with *A. capilliaris* for 30 min, then washed twice and incubated for 30 min with 1 mM H_2O_2. DCFH-diacetate was added to the culture plates at a final concentration of 12 μM and incubated for 30 min at 37°C. DCF fluorescence was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Genios multiwell fluorescence plate reader (GENios, Tecan, Salzburg, Austria).

Determination of intracellular ROS in RAW264.7 cells. After preincubation with *A. capilliaris* for 1 h, the 1×10^6 RAW264.7 cells were treated for 24 h with 1 μg/

mL of LPS. The treated cells were incubated for 30 min with 5 μM DCFH-DA at 37°C, then immediately analyzed via flow cytometry (FACSCalibur, BD, USA)

Determination of free radical scavenging activity using ESR spectrometry DPPH radical scavenging. DPPH radical scavenging activity was measured using the method described by Nanjo *et al.* [1996]. A 30 μL aliquot of each sample in PBS (or PBS itself as a control) was added to 30 μL of DPPH (60 μM) in ethanol solution. After 10 s of vigorous mixing, the solutions were transferred into 50 μL Teflon capillary tubes and fitted into the cavity of the ESR spectrometer. The spin adduct was measured on an ESR spectrometer exactly 2 min later. The measurement conditions were as follows: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 5 mW, gain 6.3×10^5, and temperature 297 K. The radical-scavenging activity of the *A. capilliaris* extracts was calculated by scavenging rate= $[(h_0-h_x)/h_0]\times100\%$, where h_0 and h_x are the ESR signal intensities of samples in the absence and presence of the extracts, respectively.

Hydroxyl radical scavenging. Hydroxyl radicals were generated by the Fenton reaction, and reacted rapidly with nitrone spin trap DMPO: the resultant DMPO-OH adducts could be detected with an ESR spectrometer [Rosen and Rauckman, 1984]. The ESR spectrum was recorded 2.5 min after mixing in a phosphate buffer solution (pH 7.4) with 20 μL of 0.3 M DMPO, 20 μL of 10 mM $FeSO_4$, 20 μL of 10 mM H_2O_2 20 using JES-FA electron spin resonance spectrometer (JEOL, Tokyo, Japan) set to the following conditions: central field 3475 G, modulation frequency 100 kHz, modulation amplitude 2 G, microwave power 1 mW, gain 6.3×10^5, and temperature 298 K. The radical-scavenging activity of the extracts of *A. capilliaris* was calculated via the scavenging rate=$[(h_0-h_x)/h_0]\times100\%$, where h_0 and h_x are the ESR signal intensities of samples in the absence and presence of the extracts, respectively.

Polymerase chain reaction for iNOS, COX-2, IL-6, IL1-β, and TNF-α. Total RNA was extracted using Trizol (Biostar Biologic technology Co. Ltd. USA.) in accordance with the manufacturer's instructions. The total RNA was then reverse-transcribed into cDNA. The PCR system contained 2 μL cDNA, 5 μL 10×buffer, 5 μL 25 mmol/L $MgCl_2$, 1 μL 10 mmol/L dNTP, 1 μL 20 pmol/μL target gene sense and anti-sense primer, 1 μL 20 pmol/μL β-actin primer pair, and 3 units of Taq DNA polymerase. PCR was conducted as follows: initial denaturation at 95°C for 5 min, 30 amplification cycles (denaturation at 94°C for 45 s, annealing at 55°C for 30 s, extension at 72°C for 1 min) and a final extension at 72°C for 7 min.

Immunoblot analysis. After treatment, the cells were collected and washed twice in cold PBS. The cells were then lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 25 μg/mL leupeptin) and maintained on ice for 30 min. The lysates were then centrifuged at 12,000×*g* at 4°C for 20 min; the supernatants were stored at −70°C until use. The protein concentration was determined via the Bradford method. Aliquots of the lysates (30-50 μg of protein) were separated by 8-12% SDS-PAGE and transferred onto PVDF membranes using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% methanol [v/v]). After blocking with 5% nonfat dried milk, the membrane was incubated for 2 h with primary antibodies followed by 30 min with secondary antibodies in milk containing Tris-buffered saline (TBS) and 0.5% Tween. Anti-GAPDH, -ppERK, -pp38, and -iNOS antibodies were used at a dilution of 1:1,000 as the primary antibodies with horseradish peroxidase-conjugated goat anti-human IgG (Vector Laboratories, Burlingame, CA, USA) at a 1:5,000 dilution was used as the secondary antibody. The membrane was then exposed to X-ray film. Protein bands were detected using a WEST-ZOL® plus Western Blot Detection System (iNtRON, Gyeonggi-do, Korea).

Results and Discussion

Since the methanol extract of *A. capillaris* was reported to evidence anticancer activity *in vitro* [Jung *et al.*, 2008], we tested the cytotoxicity of EtEAC via MTT assays in a variety of cell lines. At concentrations of up to 200 μg/mL, the EtEAC-treated cells did not evidence any proliferation-inhibitory effects as compared to untreated fibroblast cells (Fig. 1). In particular, the RAW264.7 mouse macrophage cell line evidenced viability greater than 97% at a concentration of 200 mg/mL. A previous study showed that a MeOH extract of *A. capillaris* caused a 67% inhibition of growth in NCI-N87 stomach cancer cells and a 48% inhibition of growth in an HT-29 colon cancer cell line [Jung *et al.*, 2008].

The antioxidant activity and radical scavenging ability of EtEAC have been well documented [Jo *et al.,* 2004; Jung *et al.,* 2008]. However, because we wished to compare its radical scavenging ability and anti-inflammatory effects, DPPH radical and OH radical scavenging abilities were tested *in vitro* using ESR, and fluorescence detection for ROS scavenging ability *in vivo* was conducted in H_2O_2-treated cells using DCFDA. As is shown in Fig. 2A, the *in vitro* DPPH radical scavenging ability of 10 μg/mL of EtEAC was quite high, and was

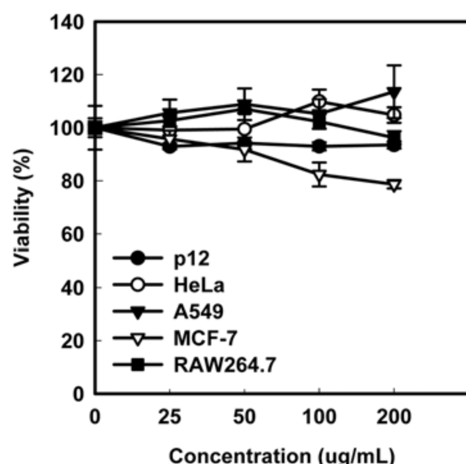

Fig. 1. Effects of EtEAC on cytotoxicity in various cell lines. Levels of cell viability were measured using MTT assay and expressed as a percentage of counts relative to untreated control cells. Values are mean±SD (n=4). ●-fibroblast passage 12, ○-HeLa, ▼-A549, ▽-MCF-7, ■-RAW264.7

comparable to the single positive control compound (10 μg/mL of catechin). Since the DPPH radical is quite sensitive and the hydroxyl radical less so in ESR, the amount needed to elicit these effects varies. However, the hydroxyl radical scavenging ability was also very high, because at the same concentration, EtEAC exerted effects similar to those of the single positive compound (Fig. 2B). The *in vitro* ESR technique is quite useful in addressing the radical quenching activity of the compound itself, but the quenching or scavenging activities of compounds may not be the same *in vivo* because the compounds can be metabolized in cells or the compounds may not be able to localize to the correct compartments. In HepG2 hepatocarcinoma cells, intracellular ROS was generated by overnight treatment with H_2O_2, and the *in vivo* scavenging ability of EtEAC was detected with DCFDA (Fig. 2C). As compared to the strong antioxidant N-acetylcystein (2 mM), EtEAC (500 μg/mL) exhibited about half of the ROS scavenging ability *in vivo*.

Since attempts were undertaken to inhibit intracellular ROS production in order to limit inflammatory responses [Lafeber *et al.*, 1999; van den Worm *et al.*, 2001] and EtEAC exhibited very high ROS scavenging ability, we evaluated the anti-inflammatory effects using LPS-stimulated RAW 264.7 cells. The mRNA expression levels of typical inflammatory cytokines (IL-6, IL1β and TNFα) and COX-2 were measured via RT-PCR after LPS stimulation with or without EtEAC. As shown in Fig. 3, at high dosages (above 100 μg/mL), mRNA expression levels were reduced in the cases of IL-6, IL1β and COX-2, but no effects were observed in the case of TNFα.

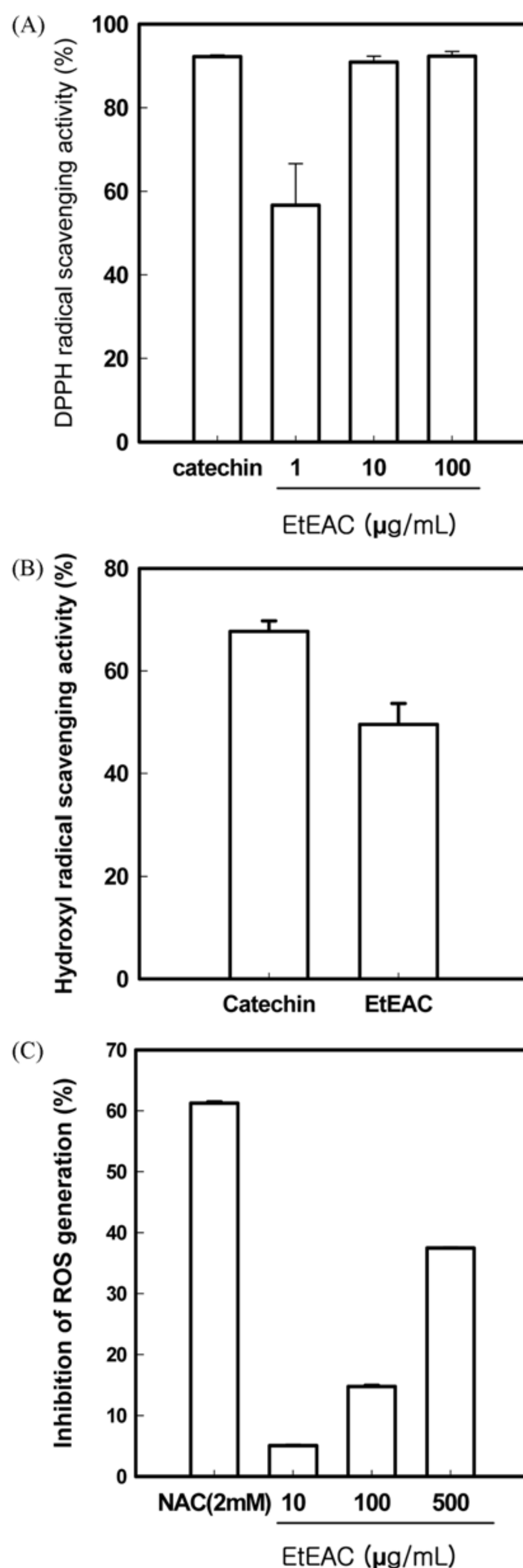

Fig. 2. Antioxidant effects of EtEAC. Data showed the percent of DPPH radical scavenging activity (A), hydroxyl radical scavenging activity (B) and intracellular reactive oxygen species (ROS) generation induced by H_2O_2 in HepG2 cells (C) of EtEAC. A described amount of catechin or N- acetylcystein was used as a positive control in each experiment. Data are expressed as mean±SD.

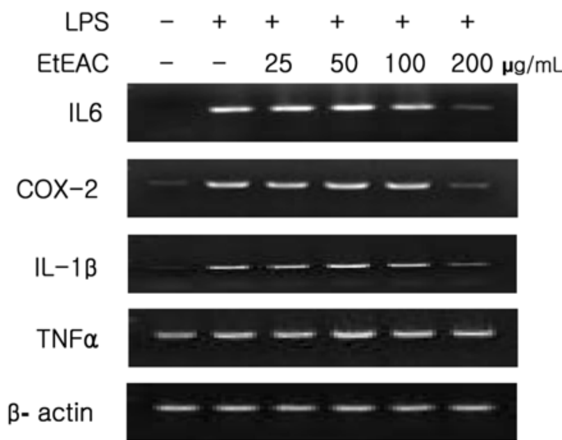

Fig. 3. Effect of EtEAC on LPS-induced production of proinflammatory cytokines in RAW264.7 cells by RT-PCR. Levels of each mRNA of IL6, Cox-2, IL-1β, and TNF-α was determined as described in Methods.

During the course of the inflammatory response, large quantities of NO were produced by iNOS. The effects of EtEAC on NO formation and iNOS induction were evaluated in LPS-stimulated RAW 264.7 cells. The NO production levels were elevated markedly via the addition of LPS to RAW 264.7 cells. EtEAC suppressed NO production to basal levels at a concentration of 200 μg/mL and this effect occurred in a concentration-dependent manner. However, high doses (100 and 200 μg/mL) of catechin, which is known as an anti-oxidant agent, did not suppress NO production (Fig. 4A). The mRNA and protein levels of iNOS were suppressed markedly by EtEAC treatment in LPS-stimulated RAW 264.7 cells (Fig. 4B and C).

Because catechin did not affect NO production, we assessed *in vivo* ROS production in LPS-stimulated RAW 264.7 cells using DCFDA (Fig. 5). LPS alone induces large quantities of ROS *in vivo;* however, 100 μg/mL of catechin or EtEAC was shown to scavenge most of the produced ROS. These results demonstrate that EtEAC evidences excellent ROS-scavenging ability, and simultaneously exerts an anti-inflammatory effect, unlike catechin. These results suggested that NO production was not suppressed as the result of the scavenging ability of EtEAC, but was rather due to the alteration of other inflammation-associated signaling pathways, because catechin reduces ROS production to a level similar to that of EtEAC but cannot suppress NO production. In LPS-stimulated RAW cells, LPS activates TLR4 and can trigger several downstream signals, including ERK, JNK, p38 or NFκB signals, to regulate transcriptions of IL-1, IL-6, IL-8, TNF-α, iNOS, etc. We analyzed the activation of ERK, JNK, and p38 MAPK via Western blotting using phosphorylation-specific antibodies. As is shown in Fig.

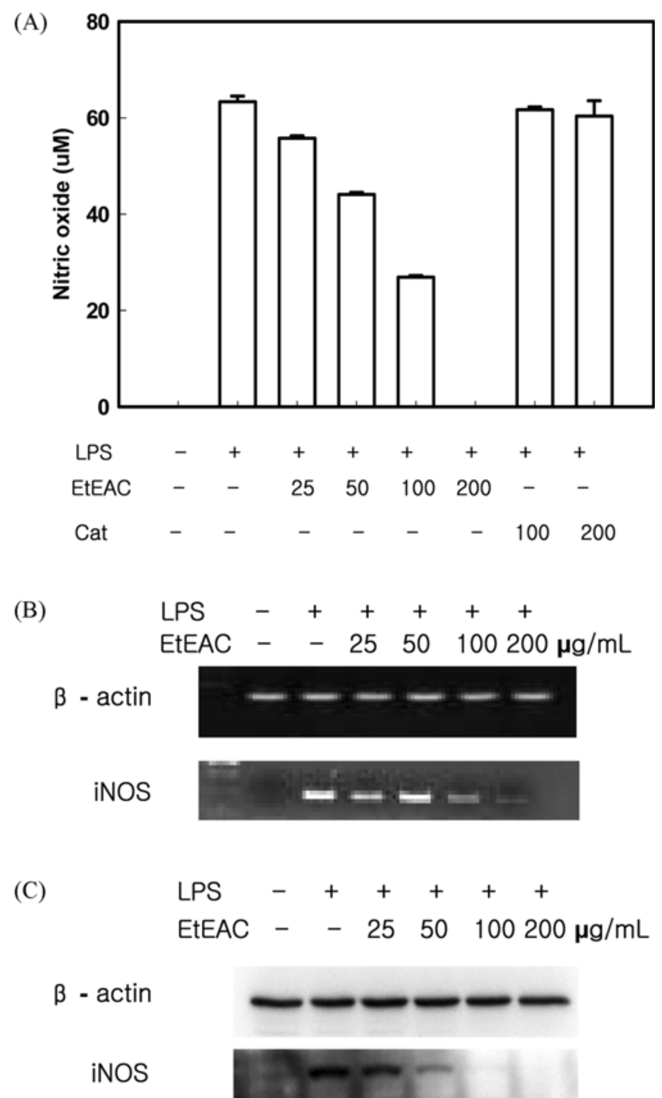

Fig. 4. Effects of EtEAC on LPS-induced nitric oxide generation and iNOS mRNA expression in RAW 264.7 Cells. The cells were treated with LPS alone or LPS plus different concentrations (25, 50,100 or 200 μg/mL) of EtEAC for 24 h. (A) RAW 264.7 macrophages were treated with LPS (1 μg/mL) in the presence or absence of EtEAC for 24 h. The mRNA level (B) and protein level (C) of iNOS was examined by RT-PCR and western blot analysis. RAW 264.7 macrophages were treated with LPS (1 μg/mL) in the presence or absence of EtEAC for 24 h. Images are representative of three independent experiments that shows similar results.

6, the phosphorylation of p38 MAPK is affected principally by EtEAC treatment in a dose-dependent manner. EtEAC appears to affect ERK phosphorylation to a lesser degree than p38. The phosphorylated form of JNK was not detected.

The purpose of inflammatory responses is to deactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair. Nitric oxide may perform

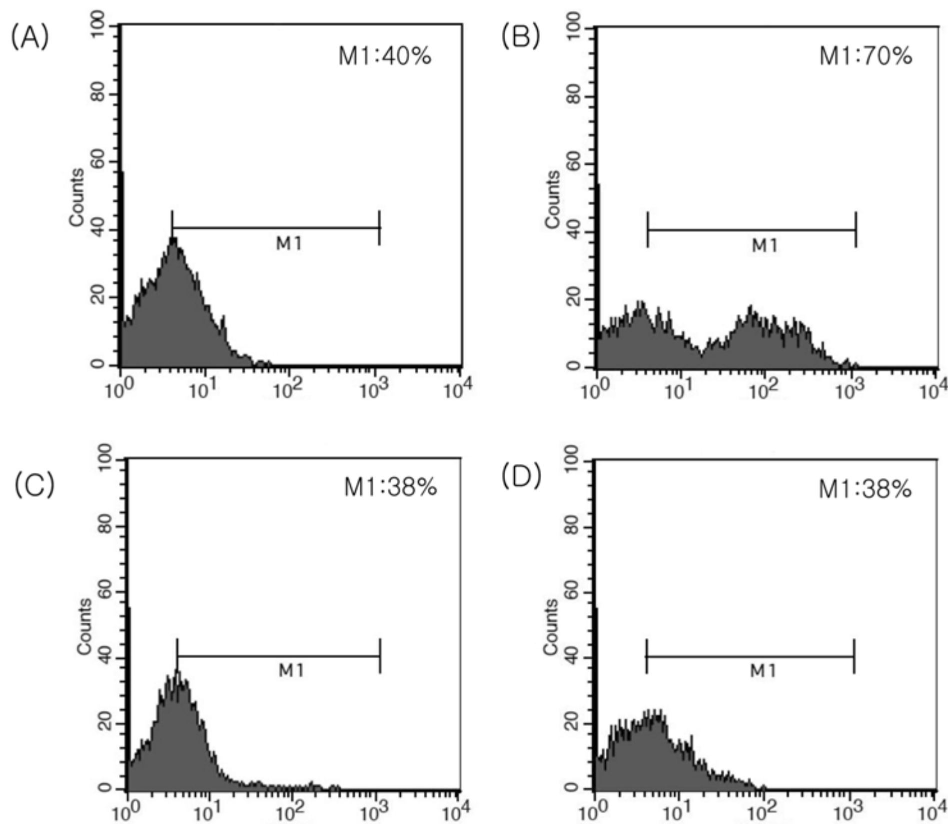

Fig. 5. Detection of the intracellular ROS generation in the RAW264.7 cells by a flow cytometer. Carboxy-H2DCFDA fluorescent signals were measured after incubation with 100 μg/mL EtEAC for 16 h with or without LPS (1 μg/mL) by flow cytometer. (A) none, (B) LPS, (C) LPS+EtEAC, and (D) LPS+catechin (100 μg/mL)

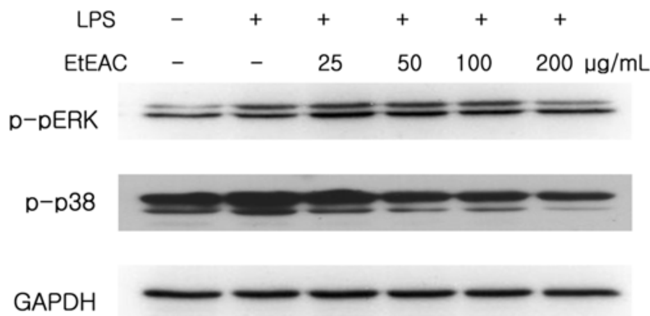

Fig. 6. Effect of EtEAC on MAP kinase activation in LPS-stimulated RAW 264.7 cells. The cells were treated with LPS alone or LPS plus different concentrations (25, 50,100 or 200 μg/mL) of EtEAC for 24 h. The cellular proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were probed with the indicated primary antibodies then with horseradish peroxidase conjugated goat anti-rabbit or mouse IgG. GAPDH was used as an internal control.

regulatory roles at virtually every stage of the development of inflammation. Over the course of an inflammatory response, large quantities of NO formed by immunologically or chemically activated macrophages kill microorganisms and nitrosylate macromolecules. Within a few seconds, NO is oxidized to nitrites or nitrates. Large quantities of "inflammatory NO" from myeloid cells are usually generated side-by-side with large quantities of the superoxide anion (O_2^-). These two can form peroxynitrite (ONOO-) [Channon and Guzik, 2002; Guzik *et al.*, 2002], which mediates the cytotoxic effects of NO, which include DNA damage, LDL oxidation, isoprostane formation, tyrosine nitration, the inhibition of aconitase and mitochondrial respiration [Ischiropoulos and al-Mehdi, 1995]. The discovery of this reaction raises new possibilities as to the possible therapeutic uses of compounds or mixtures that can scavenge ROS without influencing NO production. The toxic properties of nitric oxide are critically important in the pathogenesis of septic shock [Parratt, 1997]. NO overexpression induced by iNOS during septicemia has been suggested to be responsible for irreversible arterial hypotension, vasoplegia (loss of responses to noradrenaline), lactic acidosis, necrosis, and apoptosis [Parratt, 1997]. However, it is important to remember that NO generated by iNOS can be of benefit in terms of the host defense reaction, by contributing to microbial killing.

ROS production is very important to the modulation of inflammatory reactions. The major ROS produced within

the cell are superoxide anion, hydrogen peroxide, and hydroxyl radical [Salvemini *et al*., 2003]. The extracellular release of large quantities of superoxide, produced as a respiratory burst in leukocytes, is an important pathogen-killing mechanism, and also results in endothelial damage, resulting in increased vascular permeability as well as cell death [Tiidus, 1998]. Accordingly, attempts were undertaken to inhibit intracellular ROS production in order to limit inflammatory responses. Apocynin, an inhibitor of NAD(P)H oxidase activation, has been successfully used to limit inflammation in an animal model of rheumatoid arthritis [Lafeber *et al*., 1999; van den Worm *et al*., 2001), while the decoy peptide, which prevents the association of NAD(P)H oxidase subunits, was shown to be effective in atherosclerosis-associated inflammation [Rey *et al*., 2001]. In conclusion, EtEAC contains compound(s) that may be capable of reducing NO production and inflammatory cytokine production via alternating inflammatory signals and, at the same time, evidenced very high ROS scavenging effects. These results suggest that EtEAC could be a good raw material for the development of drugs useful for the treatment of chronic inflammatory diseases.

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