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Effects of *Dioscorea daemona* Roxb. Stem Extract on the Inflammatory Responses, Antioxidant System and Lipid Levels *in Vivo* and the Production of Inflammatory Mediators in RAW264.7 Cells

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Dioscorea daemona Roxb. (Stem) 추출물이 Rat의 염증반응 및 항산화 체계에 미치는 영향과 RAW264.7 세포주의 염증성 매개물 생성에 미치는 영향

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Abstract

본 연구에서는 다양한 동물 모델을 사용하여 *Dioscorea daemona* Roxb. 줄기 메탄올 추출물(DD)의 항염증 활성을 측정하였으며 DD가 생체내에서 항산화 체계의 변화를 유도할 수 있는지도 살펴보았다. DD를 200 mg/kg 용량으로 3주간 경구투여하였을 때 동물실험모델에서 항염증 및 type IV 알레르기 억제 효과를 나타내었으며 혈청의 Catalase 활성, 지질 과산화, TG 및 HDL cholesterol 수치가 영향을 받았다. DD와 이를 클로로포름과 부탄올로 순차적으로 분획하여 얻은 fraction이 lipopolysaccharide(LPS)로 유도한 RAW264.7 대식세포주의 nitric oxide(NO), prostaglandin E_2(PGE_2), tumor necrosis factor-α(TNF-α), interleukin 6(IL-6)의 생성을 억제하는지도 연구하였다. DD와 그 분획물들은 4~100 μg/mL 농도에서 세포 독성을 나타내지 않고 LPS가 유도한 RAW264.7 세포주의 NO, TNF-α, IL-6 생성을 억제하였다. LPS가 유도한 PGE_2 생성은 DD의 클로로포름 분획에서 유의적으로 감소하였다($p<0.05$). 따라서 *Dioscorea daemona* 추출물은 대식세포의 염증성 매개물의 억제를 통하여 항염증 활성을 나타내는 것으로 사료된다.

Key words : *Dioscorea daemona*, anti-inflammatory activity, antioxidant activity cytokine.

Introduction

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages, mast cells, etc. Macrophages produce reactive oxygen species(ROS) and release various cytokines in response to lipopolysaccharide(LPS). These inflammatory mediators include tumor necrosis factor(TNF-α), interleukin 6(IL-6), nitric oxide(NO) and prostaglandin E_2(PGE_2). The excessively produced ROS can injure cellular biomolecules such as nucleic acids, proteins, carbohydrates, and lipids, causing cellular and tissue damage, which in turn augments the state of inflammation(Hong *et al* 2001). Therefore, compounds that have scavenging activities toward these radicals and/or suppressive activities on lipid peroxidation may thus be expected to have therapeutic potentials for several inflammatory diseases(Trenam *et al* 1992). The potential of macrophages to release cytokines such as TNF-α and IL-6, in response to inflammatory stimuli, has proven a useful measure of the immune status of patients with inflammatory diseases (Zangerle *et al* 1992). After stimulation with LPS many cells, including endothelial cells and macrophages, express the inducible isoforms of COX-2 and iNOS, which are responsible for the production of large amounts of PGE_2 and NO, respectively. As these inducible enzymes are essential components of the inflammatory response and are implicated in the pathogenesis of several inflammatory diseases, the selective modulation of these mediators overproduction might represent a therapeutic goal in different inflammatory pathologies.

Modern research in the field of anti-inflammatory drugs is directed towards developing potent anti-inflammatory compounds with reduction in other major side effects. Drugs of plant origin are used for the treatment of many diseases in

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traditional systems of medicine. Therefore, naturally originated agents with very little side-effects are required to substitute chemical therapeutics. The present study was carried out to determine the anti-inflammatory activity of *Dioscorea daemona* Roxb. stem using different animal models. We further investigated if *Dioscorea daemona* Roxb. stem could induce changes in the indices related to antioxidant defense systems in *in vivo* system. In the course of search for anti-inflammatory agents, the ability of *Dioscorea daemona* Roxb. stem extract and its subfractions (chloroform, butanol, and aqueous fractions) obtained by solvent extraction to separate the components according to their polarities to modulate the production of inflammatory mediators (NO, PGE_2, TNF-α and IL-6) was investigated in mouse macrophage cell line RAW264.7.

Materials and Methods

1. Preparation of Extract and Its Fractions

The stems of *Dioscorea daemona* Roxb. were collected from the Biofarmaka Research Center of Bogor Agricultural Unievrsity(Indonesia), identified by Dr I. Latifah, Dept. Pharmacy. The plant material was shade dried and ground to powder. The powdered material was soaked in 80% methanol for 24 h. On the next day, the methanolic extract was filtered and dried on a rotary evaporator at temperature below 40℃ and the resulting product was designated as MeOH extract. For the purpose of fractionation, MeOH extract dissolved in distilled water was fractionated by successive solvent extraction with chloroform($CHCl_3$) and n-butanol(BuOH). Each fraction was then evaporated to dryness under vacuo. The yields of extract and fractions were as follows (w/w): MeOH extract; 5.0%, $CHCl_3$ fraction; 0.7%, BuOH fraction 1.5%, aqueous (H_2O) fraction 2.6%. All reagents were purchased from Sigma(USA), unless otherwise stated.

2. Animals

Male ICR mice(6 weeks old), BALB/c mice(7 weeks old for type IV anti-allergic assay) and Sprague-Dawley rats(5 week old) were purchased from Jungang Lab Animal Inc (Korea). These animals were maintained under constant temperature (24±2℃) and fed a standard laboratory diet(Jungang Lab Animal Inc, Korea) with water provided *ad libitum*. The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals(National Research Council, National Academy of Science, Bethesda, MD, USA).

3. Anti-inflammatory Activity *In Vivo*

Test samples were prepared by suspending them in 0.5% sodium carboxymethyl cellulose(CMC · Na) solution, immediately before the start of the experiments. Mice were administered orally with *Dioscorea daemona* stem extract(200 mg/kg). Control experiments were performed with the 0.5% CM C · Na suspension of distilled water alone. Indomethacin(10 mg/kg) was used as positive control.

1) Carrageenan-induced Paw Edema Assay

The procedure used to assess anti-inflammatory activity was based on the method used by Winter *et al*(1962). At 1 h after sample administration, edema was induced by injecting in the right hind paw 0.02 mL of 1% -carrageenan in sterile saline. The pad thickness of hind paw was measured with a Dial Thickness Gauge(Mitutoyo, Japan) before and at 1 and 3 h after carrageenan injection, and the differences in the thickness were calculated. The degree of foot-pad swelling was expressed as increase in foot-pad thickness(mm).

2) Arachidonic Acid-induced Ear Edema Assay

Samples were orally administered 1 h prior to the topical application of 2% arachidonic acid dissolved in acetone(0.02 mL/ear) to right ear of mice. The ear thicknesses were measured using a Dial Thickness Gauge before and at 1 and 3 h after arachidonic acid treatment, and the differences in the thickness were calculated. The degree of ear swelling was expressed as increase in ear thickness(mm)(Kim *et al* 1992).

3) Formaldehyde-induced Arthritis Assay

Formaldehyde(2% v/v) solution, 0.02 mL, was injected in the first and third day into the left hind paw just beneath the planter aponeurosis to induce arthritis. Samples were administered orally for seven days(daily) and serum aspartate aminotransferase(AST) and alanine aminotransferase(ALT) activities were measured according to the method described by Reitman & Frankel(1957).

4. Type IV Anti-Allergic Activity

Anti-allergic activity(type IV) were tested by the 2, 4-dinitrofluoro benzene(DNFB)-induced contact hypersensitivity reaction(Ohuchi K 1993). The dorsal skin of the mice was shaved

on the day before the experiment, and the mice were sensitized with 0.02 mL of 0.5% DNFB in acetone-olive oil(4:1) applied to the dorsal skin on days^{-1} and 0. The *Dioscorea daemona* stem extract(200 mg/kg) were administered orally for seven days(daily). After the administration, the mice were challenged on the right ear with 0.25% DNFB in acetone-olive oil(4:1) (0.02 mL/ear). Ear thickness was measured with Dial Thickness Gauge before and 24 h after the DNFB challenge, and the differences in the thickness were calculated. Ciclosporin(0.25 mg/kg) was used as positive control. The degree of ear swelling was expressed as increase in ear thickness(mm).

5. Effects on Antioxidative System and Lipid Levels in Rats

A total of 14 rats were divided into two groups. In the experiment 7 rats were used in each group: saline(Control group); *Dioscorea daemona* stem extract(DD group). Rats were fed one time(*Dioscorea daemona* stem extract 200 mg/kg body weight) per day by intragastric tube. After 21 days of administration, blood was collected from the rats fasted during overnight into heparinized tubes. Samples were immediately centrifuged at 1,500 g for 5 min and the plasma was separated. Superoxide dismutase, catalase, malonedialdehyde, triglyceride, and cholesterol levels were determined in plasma samples.

1) Measurement of Superoxide Dismutase Activity

The activity of superoxide dismutase(SOD) was determined by monitoring the inhibition of the autoxidation of pyrogallol (Marklund & Marklund 1974). At 25°C and 320 nm, the rate of pyrogallol oxidation was recorded with a Shimadzu UV 1201 spectrophotometer(Shimadzu). Activity was expressed as the amount of enzyme that inhibits the oxidation of pyrogallol by 50%, which is equal to 1 unit. The result for SOD activity was expressed as units/mg protein. Protein was determined by the method of Read & Northcole(1981) using bovine serum albumin as a standard.

2) Measurement of Catalase Ativity

Catalase activity was determined according to Beutler E(1973). Briefly, 1mol/L Tris-HCl, 5 mol/L EDTA(pH=8) and 10 mol/L H_2O_2 were added to plasma and the mixture was incubated at 37℃ for 10 min and followed by the change in absorbance of the system at 230 nm. The result for catalase activity was expressed as the mol of H_2O_2 degraded per minute per milligram of protein.

3) Lipid Peroxidation Intermediates

Thiobarbituric acid reactive substances(TBARS) are products of the oxidative degradation of polyunsaturated fatty acids, in particular, malonedialdehyde(MDA). Lipid peroxidation was assayed by the measurement of MDA levels on the base of MDA reacted with thiobarbituric acid at 532 nm, according to Buege & Aust (1978). Briefly, 0.2 mL of serum was mixed with thiobarbituric acid reagent(consisting of 0.375 % thiobarbituric acid and 15% trichloroacetic acid in 0.25 N HCl). The reaction mixture of serum and thiobarbituric acid reagent was placed in boiling water for 15 min, cooled, centrifuged, and then the optical density of the supernatant was recorded at 532 nm. A standard curve was obtained with a known amount of 1,1,3,3-tetraethoxypropane, using the same assay procedure.

4) Triglyceride and Cholesterol Levels

The concentrations of triglyceride(TG), total cholesterol and high density lipoprotein (HDL)-cholesterol in plasma were determined enzymatically using commercial available kit reagents(Boehringer, Mannheim, Germany). Low density lipoprotein(LDL)-cholesterol was calculated by Friedewald formula: LDL cholesterol = total cholesterol HDL cholesterol TG / 5 (Tietz NW 1986).

6. Cell Culture

RAW264.7 cells were cultured in plastic dishes containing Dulbecco's Modified Eagle Medium(DMEM, Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum(FBS, Sigma, St Louis, MO, USA) in a CO_2 incubator (5% CO_2 in air) at 37°C and subcultured every 3 days at a dilution of 1:5 using 0.05% trypsin-0.02% EDTA in Ca^{2+}, Mg^{2+} free phosphate-buffered saline(DPBS). The cells were cultured in 24-well plate (5×10^5) containing DMEM supplemented with 10% FBS for 1 days to become nearly confluent. Then cells were cultured with vehicle or *Dioscorea daemona* stem extract and its fractions in the presence of 10 μg/mL LPS for 24 h. Cell viability was determined using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent(Tubaro *et al* 1996). After culture on 96 well plate, the cells were washed

twice with DPBS and incubated with 110 μL of 0.5 mg/mL MTT for 2 h at 37°C. The medium was discarded and 100 μL dimethylsulfoxide(DMSO) was then added. After 30 min incubation, absorbance at 570 nm was read by using a microplate reader.

7. Measurement of Nitric Oxide Formation by iNOS Activity in Cultured LPS-induced RAW274.7 Cells

For evaluating the inhibitory activity of test materials on iNOS(Tsao *et al* 2002), the cells in 10% FBS-DMEM without phenol red were plated in 24-well plates (5×10^5 cells per mL), and stimulated with 10 μg/mL LPS for 12 h. Then the cells were washed twice with PBS. Test samples were added for an additional 12 h in the absence of LPS. The cell free culture medium was collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reagent(Ding *et al* 1990). Briefly, 150 μL of Griess reagent(0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) were added to 100 μL of each supernatant from sample-treated cells. The plates were incubated for 5 min, and then were read at 570 nm against a standard curve of sodium nitrite. Percent inhibition was expressed as 100×[1−(NO release with sample-spontaneous release)/(NO release without sample-spontaneous release)].

8. Measurement of PGE_2 Formation by COX-2 Activity in Cultured LPS-induced RAW274.7 Cells

For evaluating the inhibitory activity of test materials on COX-2(Hong *et al* 2002), the cells were allowed to adhere for 4 h in the presence of aspirin(500 μM) in culture plate, washed three times with media, and then incubated in the fresh medium with 10 μg/mL LPS. Test materials were simultaneously added to each well. After additional 16 h incubation, the media were removed and analyzed by PGE_2 enzyme immunometric assay kit(R&D System, USA) according to the manufacturer's instructions. The assay is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody. Percent inhibition was expressed as 100×[1−(PGE_2 release with sample-spontaneous release)/(PGE_2 release without sample-spontaneous release)].

9. Cytokine Determination by ELISA

The cells were cultured in 24-well plate (5×10^5) containing DMEM supplemented with 10% FBS for 1 days to become nearly confluent. Then cells were cultured with vehicle or *Dioscorea daemona* stem extract and its fractions in the presence of 10 μg/mL LPS for 48 h. Cell supernatants were assayed for TNF-α and IL-6 using commercially available enzyme-linked immunosorbent assay(ELISA) kits (R&D System, USA) according to the manufacturer's instructions. Percent inhibition was expressed as 100×[1(cytokine release with sample-spontaneous release)/(cytokine release without sample-spontaneous release)].

10. Statistical Analysis

The results are expressed as mean±SEM. Statistical analysis was performed using ANOVA followed by Dunnett's t-test ($p<0.05$). The analysis was performed using SAS statistical software.

Results and Discussion

1. Anti-inflammatory Activity of *Dioscorea daemona* Stem Extract

To assess the anti-inflammatory activity of *Dioscorea daemona* stem, 80% methanol extract was evaluated by three screening protocols widely used for testing the non-steroidal anti-inflammatory drugs (NSAIDs); namely, carrageenan- induced paw edema, arachidonic acid-induced ear edema and formaldehyde-induced arthritis screens. The edema assay was employed as a model for acute inflammation, while the formaldehyde-induced arthritis assay was used as a model for subacute condition. For the acute inflammation assay, *Dioscorea daemona* stem extract was dosed orally at 200 mg/kg 1 h prior to induction of inflammation by carrageenan or arachidonic acid, the anti-inflammatory activity was then calculated 1 and 3 h after induction and summarized in Figs. 1 and 2. Development of edema induced by carrageenan is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology(Ozaki Y 1990). Result in Fig. 1 shows that *Dioscorea daemona* stem extract caused significant($p<0.05$) inhibition of paw edema as compared to the control group after induction. In the beginning of carrageenan injection, there is sudden elevation of paw volume in relation with histamine mediators (Geen KL 1964). After 1 h the inflammation increases gradually and was elevated during the later 3 h. This second phase could

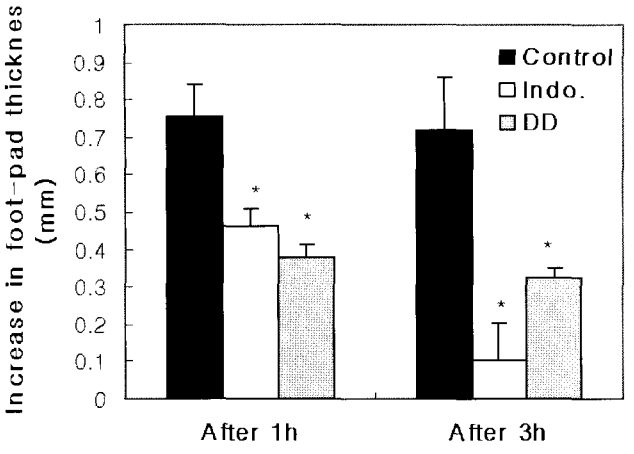

Fig. 1. Effect of *Dioscorea daemona* stem extract on carrageenan-induced paw edema.
DD : *Dioscorea daemona* stem extract (200 mg/kg).
Indo.: Indomethacin (10 mg/kg). Results are mean±SEM. (n=7).
* $p<0.05$ vs. control.

be due to the prostaglandins and kinins liberated, which accompanies leukocyte migration. *Dioscorea daemona* stem used in this study, then, could be anti-inflammatory by inhibi- ting cyclooxygenase pathway, considering that the mechanism involved in the genesis of the carrageenan-induced edema could cause the release of prostaglandins and kinins, among other substances (Garcia *et al* 1973). The anti-inflammatory activity of *Dioscorea daemona* stem extract was also measured on mouse ear using arachidonic acid as the inducer. Arachidonic acid-induced ear edema is a good *in vivo* test useful for evaluating lipoxygenase inhibitors(Young & Young 1989). Result

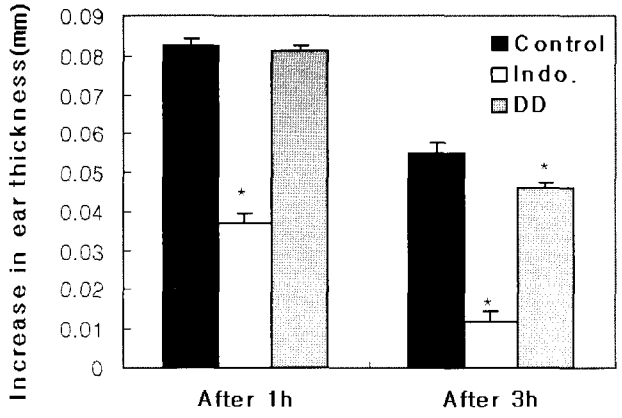

Fig. 2. Effect of *Dioscorea daemona* stem extract on arachidonic acid-induced ear edema.
DD : *Dioscorea daemona* stem extract (200 mg/kg).
Indo.: Indomethacin (10 mg/kg). Results are mean±SEM. (n=7).
* $p<0.05$ vs. control.

in Fig. 2 shows that *Dioscorea daemona* stem extract caused significant($p<0.05$) inhibition of edema as compared to the control group at 3 h after induction. These results suggest that *Dioscorea daemona* stem extract act on both the cyclooxygenase and lipoxygenase pathways.

For the formaldehyde-induced arthritis screen, arthritis was induced by formaldehyde injection in the first and third day and *Dioscorea daemona* stem extract was administered orally at 200 mg/kg daily for seven days. As a result of inflammation, the level of serum transaminase aspartate aminotransferase(AST) and alanine aminotransferase(ALT) enzymes is increased and a decreased level of these enzymes upon administration of the test samples will reflect their anti-inflammatory potentialities. Accordingly, the assessment of the level of AST and ALT enzymes provides a good and simple tool to measure the anti-inflammatory activity of the target compounds(Kataoka *et al* 2002). As shown in Fig. 3, plasma AST activity of group given *Dioscorea daemona* stem extract was not significantly affected, but ALT activity was significantly lower than that of control group($p<0.05$). Developing of adjuvant arthritis is regarded as manifestations of cell-mediated immunity and their suppression suggests immunosuppressant activity(Bani *et al* 2000). As leukocytes play a very important role in the development of acute and chronic inflammation, inhibition of their migration accounts for the anti-inflammatory effect.

We further investigated the effect of *Dioscorea daemona* stem extract on DNFB-induced delayed-type hypersensitivity (type IV allergy). *Dioscorea daemona* stem extract showed

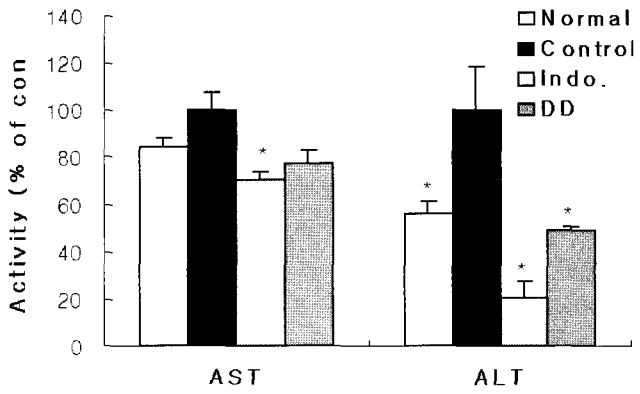

Fig. 3. Anti-inflammatory activity of *Dioscorea daemona* stem extract in formaldehyde-induced arthritis assay.
DD : *Dioscorea daemona* stem extract (200 mg/kg).
Indo.: Indomethacin (10 mg/kg). Results are mean±SEM. (n=7).
* $p<0.05$ vs. control.

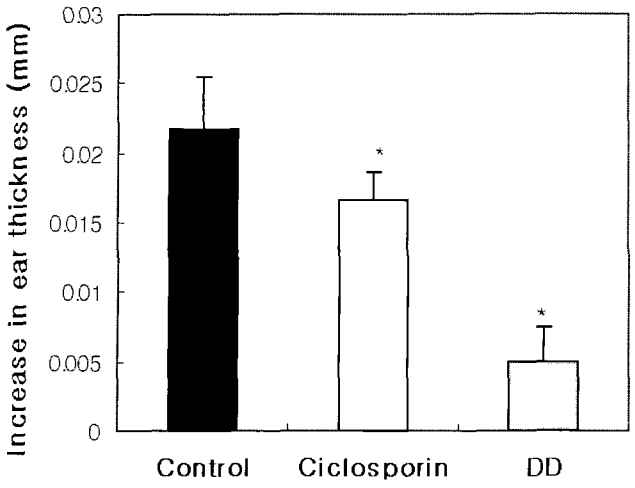

Fig. 4. Effect of *Dioscorea daemona* stem extract on delayed type hypersensitivity in mice.
DD : *Dioscorea daemona* stem extract (200 mg/kg).
Ciclosporin (0.25 mg/kg). Results are mean±SEM. (n=7).
* $p<0.05$ vs. control.

significant($p<0.05$) inhibitory effect on DNFB-induced delayed-type hypersensitivity after oral administration daily for seven days at a dose of 200 mg/kg(Fig. 4), which effect may partly be due to their anti-inflammatory effects. The inhibitory effect on immunologically induced swelling suggests the possible immunosuppressive properties of *Dioscorea daemona* stem. In the inflammatory response, lymphocytes and macrophages invade the inflamed site, and focal reactions such as a granulomatosis and fibrosis occur. In a chronological sequence of reactions, various cytokines, which participate in the pathogenesis of inflammatory reactions, are produced. In contact hypersensitivity, it is thought that a number of cytokines are involved in the initiation and development of the reaction and that the cytokine network is complicated. Therefore, we consider that *Dioscorea daemona* stem extract may be effective at treating contact hypersensitivity, probably attributable to its inhibitory effects on cytokine production.

2. Effect of *Dioscorea daemona* Stem Extract on the Plasma Antioxidant System and Lipid Levels in Rats

To determine if *Dioscorea daemona* stem could induce changes in the indices related to antioxidant defense systems, the effects of *Dioscorea daemona* stem on antioxidant enzymes and lipid levels were examined in *in vivo* system. After 3 weeks of administration with extract(200 mg/kg body weight) to rats, SOD and catalase activities, MDA level, and lipid profiles in plasma were measured(Table 1). Administration of *Dioscorea daemona* stem extract at 200 mg/kg body weight for 3 weeks increased the activity of SOD when compared with control group but had no statistical significance ($p<0.05$). Catalase decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals(Chance *et al* 1952). There was a significant increase in the catalase activity of group administrated with *Dioscorea daemona* stem extract (200 mg/kg), when compared with control group($p<0.05$). Lipid peroxidation has been measured as an index of ROS damage. MDA, measured as TBARS, is the most commonly used marker of lipid peroxidation(Ikuo *et al* 1991). Administration of *Dioscorea daemona* stem extract significantly decreased the concentration of TBARS in plasma($p<0.05$). An increase in the antioxidant enzyme activity and a reduction in the lipid peroxidation by *Dioscorea daemona* stem may result in reducing a number of deleterious effects due to the accumulation of oxygen radicals, which could exert a beneficial action against pathological alterations, especially in inflammatory diseases. Medicinal plant removing ROS is the most effective defense of a living body against diseases(Lin *et al* 1995). Therefore, the natural antioxidants contained in *Dioscorea daemona* stem might contribute towards the total or partial alleviation of some clinical disorders.

Lipoproteins are macromolecules of lipid and protein that transport lipids(including cholesterol and triglycerides) through the vascular and extravascular body fluids. They are involved in a diversity of processes such as immune reactions, coagulation and tissue repair(Harmony JAK 1986). Increase of high-density lipoprotein (HDL)-cholesterol and reduction of triglyceride(TG), total-cholesterol and low-density lipoprotein (LDL)-cholesterol are considered to be anti-coronary artery disease. Table 1 shows the results from rats of the effects of *Dioscorea daemona* stem extract on plasma lipid proflies following administration(200 mg/kg, for 3 weeks). TG level in plasma was significantly reduced in the *Dioscorea daemona* stem group, when compared with control group($p<0.05$). No significant difference between control and experimental groups was found in concentration of total- and LDL-cholesterol, but these lipid parameters tended to decrease in experimental group. HDL-cholesterol significantly increased in the *Dioscorea daemona* stem group. The *Dioscorea daemona* stem extract used in this investigation contain a mixture of natural antioxidants, and the plasma MDA value was significantly decreased after the dietary period, indicating that decrease of

Table 1. Antioxidant enzymes, malondialdehyde and lipid levels in the plasma of rats 3 weeks after oral administration of *Dioscorea daemona* stem extract

	Group	
	Control	DD
Superoxide dismutase(U/mg protein)	10.80± 0.17	14.27± 0.03
Catalase(nmol H_2O_2 degraded/min/mg protein)	10.14± 3.43	30.32± 2.72*
Malondialdehyde(nmol/mL)	1.20± 0.09	0.36± 0.02*
Triglyceride(mg/100 mL)	2.28± 0.05	2.05± 0.11*
Total cholesterol(mg/100 mL)	233.77±25.25	175.97±22.11
LDL cholesterol(mg/100 mL)	231.70±25.22	173.73±21.98
HDL cholesterol(mg/100 mL)	1.61± 0.03	1.83± 0.10*

Results are mean±SEM. (n=7). * p<0.05 vs. control, DD : *Dioscorea daemona* stem extract (200 mg/kg).

peroxidation might influence the lipid metabolism. During the experimental period, there was no overt toxicity, i.e., no significant effects on body and organ(liver and spleen) weights (data not shown). HDL cholesterol, therapeutic strategies aimed at enhancing cholesterol efflux from the arterial wall may be of additional benefit for patients with atherosclerosis. Numerous epidemiological studies have associated HDL with an inverse risk for coronary artery disease(Navab *et al* 1991). In the present study, a daily intake of *Dioscorea daemona* stem extract(200 mg/kg) for 3 weeks resulted in a significant increase in HDL-cholesterol and decrease in plasma MDA and TG; effects which may be beneficial in the prevention of ischemic heart disease.

3. Effects of *Dioscorea daemona* Stem Extract on the Production of Inflammatory Mediators in RAW264.7 Macrophages

In our effort of searching for novel anti-inflammatory agents from *Dioscorea daemona* stem, we fractionated *Dioscorea daemona* stem extract according to polarity and compared their effect on the production of NO, PGE_2 and cytokines (TNF-α and IL-6) released from cultured mouse macrophage cell line RAW264.7. Assay systems for determining of potential suppressors of COX-2 and iNOS expression were conducted in RAW264.7 cells. To assess the effect of *Dioscorea daemona* stem on LPS-induced NO production(for iNOS inhibitors) in RAW264.7 macrophages, cell culture medium was harvested, and the concentration of accumulated nitrite, the oxidative product of NO, was determined by the Griess method. The addition of *Dioscorea daemona* stem extract and its fractions(chloroform, butanol and aqueous fractions) to cells that had been stimulated with LPS for 12 h to induce iNOS activity inhibited the induced iNOS activity as evidenced by nitrite formation(Table 2). Cell viability was > 90% at the concentrations tested as assessed by the MTT assay(data not shown). In this study, *Dioscorea daemona* stem extract and its fractions(chloroform, butanol and aqueous fractions) were evaluated with the LPS-induced PGE_2 accumulation(for COX-2 inhibitors) system in cultured RAW 264.7 cells. As shown in Table 2, chloroform fraction(20 and 100 μg/mL) showed significant inhibitory activity on COX-2, but other fractions did not inhibit COX-2 activity (p<0.05). The ability of chloroform fraction of *Dioscorea daemona* stem to reduce NO and PGE_2 production suggest that *Dioscorea daemona* stem may be an excellent adjunctive therapy in chronic inflammation. It would be of interest to investigate further the active constituents responsible for such activity.

We investigated whether *Dioscorea daemona* stem extract modulates the cytokines productions of RAW264.7 cells. To determine the effect on the production of TNF-α and IL-6, macrophages were treated with various concentrations(4~100 μg/mL) of *Dioscorea daemona* stem extract and its fractions (chloroform, butanol and aqueous fractions) and culture supernatants were assayed for cytokines by ELISA(Table 3). *Dioscorea daemona* stem extract and its fractions significantly decreased the production of TNF-α and IL-6(p<0.05). The macrophage-derived mediators, TNF-α and IL-6, which are used in the present study, are considered to play a key role

Table 2. Inhibitory activity of iNOS and COX-2 by *Dioscorea daemona* stem extract and its fractions

Treatment(μg/mL)		NO production		PGE_2 production	
		μg/10^5 cells	% inh[a)]	ng/10^5 cells	%inh[b)]
Basal(cells alone)		1.92±0.16*	-	0.11±0.01*	-
Control		4.67±0.25	-	1.07±0.10	-
MeOH extract	20	2.61±0.37*	75.1	0.80±0.16	28.4
	100	2.98±0.23*	61.3	0.99±0.05	7.7
$CHCl_3$ fraction	20	1.97±0.30*	98.2	0.40±0.16*	69.8
	100	1.94±0.39*	99.5	0.72±0.11	36.0
BuOH fraction	20	2.17±0.30*	90.8	1.41±0.12	−36.5
	100	2.58±0.24*	75.9	1.02±0.11	4.5
H_2O fraction	20	2.94±0.39*	62.9	1.38±0.23	−32.6
	100	3.56±0.51	40.3	1.33±0.25	−27.6
L-NMMA[c)]	50 μM	2.75±0.23*	70.0	-	-
Celecoxib[d)]	1 μg/mL	-	-	0.13±0.05*	98.0

RAW264.7 cells were treated with 10 μg/mL of LPS only (control) or LPS (10 μg/mL) plus samples. Data shown are mean±SEM. of the results of 6 cultures. Significant differences from control; * $p<0.05$.

[a)] Percent inhibitionof iNOS activity: iNOS activity was determined by the production of NO in cultured LPS-stimulated RAW264.7 cells. [b)] Percent inhibition of COX-2 activity: COX-2 activity was determined by the production of PGE_2 in cultured LPS-stimulated RAW264.7 cells, [c)] L-NMMA (L-NG-monomethyl arginine) was used as positive control for iNOS inhibitor, [d)] Celecoxib was used as positive control for COX-2 inhibitor.

Table 3. Effect of *Dioscorea daemona* stem extract and its fractions on the TNF-α and IL-6 production of RAW264.7 cells

Treatment(μg/mL)		TNF-α production		IL-6 production	
		pg/10^5 cells	% inh[a)]	pg/10^5 cells	%inh[b)]
Basal(cells alone)		2.20±0.07*	-	4.35±0.04*	-
Control		2.70±0.04	-	5.32±0.04	-
MeOH extract	4	2.07±0.03*	126.5	4.50±0.01*	93.2
	20	2.04±0.03*	133.3	4.65±0.04*	76.1
	100	2.10±0.05*	119.7	4.33±0.01*	112.5
$CHCl_3$ fraction	4	2.10±0.03*	119.7	4.53±0.00*	89.2
	20	2.12±0.01*	116.2	3.58±0.00*	197.2
	100	2.27±0.01*	86.5	4.61±0.00*	80.7
BuOH fraction	4	2.40±0.00*	60.3	4.14±0.06*	133.5
	20	2.38±0.00*	64.8	5.53±0.03	−23.9
	100	2.51±0.00*	38.2	4.82±0.02*	56.8
H_2O fraction	4	2.50±0.00*	40.7	3.18±0.01*	243.2
	20	2.31±0.02*	78.9	3.74±0.01*	179.5
	100	2.51±0.00*	38.6	4.39±0.04*	105.1

RAW264.7 cells were treated with 10 μg/mL of LPS only (control) or LPS (10 μg/mL) plus samples. Cytokines concentration was measured in the conditioned medium by an immunoassay. Data shown are mean±SEM. of the results of 6 cultures. Significant differences from control; * $p<0.05$.

in inflammatory and immune responses, based on their occurrence at inflammatory sites and their ability to induce many of the hallmarks in the inflammatory response(Durum & Oppenheim 1989).

In conclusion, results of the present work clearly demonstrate the significant anti-inflammatory activity of *Dioscorea daemona* stem extract and show that catalase activity and the levels of lipid peroxidation, TG and HDL cholesterol are affected by administration of *Dioscorea daemona* stem extract in rat plasma. Moreover, the extract and its fractions from the stem of *Dioscorea daemona* inhibited the production of NO, TNF-α and IL-6 in LPS-activated RAW264.7 macrophages and chloroform fraction of *Dioscorea daemona* stem extract inhibited COX-2 activity. Our results suggest that *Dioscorea daemona* stem may reduce the risk of inflammation-related diseases and further studies are required to investigate the active constituents responsible for such activity.

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