J. Toxicol. Pub. Health
Vol. 18, No. 2, pp. 117-127, 2002

Saururus chinenesis Extracts Scavenge Reactive Oxygen Species and Modulate Nitric Oxide Production in Raw 264.7 Macrophages

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(Received May 4, 2002)
(Accepted May 31, 2002)

ABSTRACT : *Saururus chinensis Baill has been used in Korean folk medicine for the treatment of various diseases such as edema, jaundice, and furuncle. The components of this plant were extracted into four fractions. Among the four fractions, hexane and ethyl acetate fractions were highly toxic to 3T3 mouse embryo fibroblast and Raw 264.7 mouse macrophage, but n-butanol and residue fractions did not show any toxic effect to those cell lines. n-Butanol and residue fractions exhibited antioxidant effects on hydrogen peroxide, hydroxyl radical, and superoxide anion directly in vitro and in the 3T3 fibroblasts. All the four fractions inhibited lipid peroxidation measured by thiobarbituric acid reactive substances (TBARS) formation. In addition, n-butanol and residue fractions showed inhibitory effects on lipopolysaccharide (LPS)-induced nitric oxide production, and also down-regulated inducible nitric oxide synthase (iNOS) mRNA transcription 6 h after LPS stimulation in Raw 264.7 cells. Only n-butanol fraction, which mainly consists of flavonoids, inhibited NF-κB activation by decreasing IκBa degradation 90 min after LPS stimulation. From the results, it is suggested that this plant could be a good candidate material for drug development based on its antioxidant and/or anti-inflammatory constituents.*

Key Words : *Saururus chinensis, n-Butanol extract, Nitric oxide, Antioxidant, Anti-inflammatory*

I. INTRODUCTION

Saururus chinensis (Lour.) Baill (Saururaceae) has been used in Korean folk medicine for the treatment of various diseases such as edema, jaundice, and furuncle. Various components like lignans, neolignans, aristolactams, and flavonoids have been isolated from the *Saururus* species (Sung and Kim, 2000). In recent works, series of evidences are partly coincident with already known empirical facts that this plant is effective on many diseases, so that this plant could be considered for drug development. 10-aminomethyl-3-hydroxy-4-methoxy-phenanthrene-carboxylic acid lactam, which is isolated from the hexane fraction of the aerial part of this plant, is shown to have a potent cytotoxicity in several kinds of cultured human solid tumor cell lines such as AGS, A549, HCT15, SKOV3, and HEP-3B (Park *et al.*, 1997). Two flavonol glucuronides from the aqueous fraction and three diastereomeric lignans identified as sauchinone, sauchinone A, and 1'-*epi*-sauchinone isolated from the *n*-hexane fraction have been reported to have hepatoprotective activities against CCl_4 (Sung and Kim, 2000). Aqueous, methanol, and butanol fraction of this plant have shown inhibitory effect on the contraction of removed ileum induced by acetylcholine, barium chloride or histamine in mice and guinea pigs (Chung *et al.*, 1987). Methanol and butanol

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Abbreviations: TBARS, thiobarbituric acid reactive substrances; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; NF-κB, nuclear factor-κB; IκB, inhibitor-κB; AP-1, activator protein-1; DMEM, Dulbeccos Modified Eagles Medium; PBS, phosphate buffer saline; MTT, 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, nitroblue tetrazolium; HRP, horseradish peroxidase; SDS, sodium dodesylsulfate; DMSO, dimethylsulfoxide; TBA, thibarbituric acid; TCA, trichloroacetic acid; BHT, Butylated hydroxytoluene; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol tetraacetic acid; RT-PCR, reverse transcription and polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PMSF, phenylmethylsulphonyl fluoride; DTT, dithiothreoitol; ECL, enhanced chemiluminescence; S.D, standard deviation

fractions have also shown anti-histamine activity in mice and diuretic effect in rats (Chung *et al.*, 1987).

Reactive oxygen species (ROS) have been implicated in more than 100 diseases, from malaria and hemorrhagic shock to cancer and acquired immunodeficiency syndrome (Halliwell, 1993), and recently it has been recognized that ROS are widely used as second messengers to propagate proinflammatory or growth-stimulatory signals (Hensley *et al.*, 2000). ROS and reactive nitrogen species are commonly produced during the inflammatory response and involved in the production of inflammatory cytokines (Gossart *et al.*, 1996). NO produced by macrophage or immune response-related cells is critical in inflammatory phenomena, as much as ROS. It has been suggested that a large amount of NO release leads to marked vasodilation and the depressed vascular reactivity to vasoconstrictive agents (Hollenberg *et al.*, 1997; Landin *et al.*, 1994). Furthermore, the cytotoxic effect of NO can also cause multiple organ failure (Hon *et al.*, 1998). Therefore, inhibition of NO production has been proposed as a potential therapy for inflammation-related diseases.

NO is produced endogenously by a family of nitric oxide synthases (NOSs), with a wide range of physiological and pathophysiolocigal actions (Moncada *et al.*, 1991; Nathan and Xie, 1994). The inducible form of NOS (iNOS), expressed in various cell types, including vascular smooth muscle cells, macrophages, hepatocytes, and astrocytes, is induced in response to proinflammatory cytokines and bacterial LPS (Rockey *et al.*, 1998; Nunokawa *et al.*, 1993; Lyons *et al.*, 1992; Geller *et al.*, 1993; Galea *et al.*, 1992). The promoter region of the iNOS gene contains several binding sites for transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1) as well as for various members of the C/EBP, ATF/CREB and Stat family of transcription factors (Lowenstein *et al.*, 1993; Xie *et al.*, 1993; Chartrain *et al.*, 1994). Of these transcription factors, activation of NF-κB has only been shown to mediate the enhanced expression of the iNOS gene and NO production in macrophages exposed to bacterial lipopolysaccharides (LPS) (Mülsch *et al.*, 1993; Sherman *et al.*, 1993; Xie *et al.*,1994), and NF-κB is sensitively activated by ROS (Cho *et al.* 2000a).

In the present study, four kinds of extracted fractions from *Saururus chinensis*: *n*-hexane, ethyl acetate, *n*-butanol and residue (H_2O) were tested for their antioxidant effects against H_2O_2, hydroxyl radical, and superoxide anion, and inhibitory effects on NO production. The results of the present study give important evidences that this plant can be a good candidate for drug development based on its antioxidant and anti-inflammatory effects.

II. MATERIALS AND METHODS

1. Materials

Ferrous sulfate, ferric chloride, H_2O_2, and ethylenediamine tetraacetic acid (EDTA) were purchased from Junsei Chmical Co., Ltd (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum and gentamicin were obtained from Gibco BRL (Grand Island, NY, USA). Cell viability reagent 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Amresco-inc (Ohio, USA). All other chemicals including LPS and nitroblue tetrazolium (NBT) were purchased from Sigma Chemical Co (St. Louis, MO, USA). Anti-IκBα antibody, anti-actin antibody, and horseradish peroxidase (HRP)-conjugated anti rabbit IgG secondary antibody were purchased from Santa Cruz Biotechnology (CA, USA).

2. Extraction

The air-dried, aerial part of *Saururus chinensis* (100 g) was cut into pieces and extracted with 80% methanol stirring for 24 h at room temperature. Removal of the solvent yielded a methanol extract. This methanol extract was then suspended in distilled water (500 m*l*) and partitioned successively with *n*-hexane (500 m*l*×2), ethyl acetate (500 m*l*×2), and *n*-butanol (500 m*l*×2). Each fraction and the residue were concentrated and evaporated into extracts (Jung, 1991; Jung, 1992; Park *et al.*, 1997; Sung and Kim, 2000). Each *Saururus chinensis* extract (hexane, ethyl acetate, *n*-butanol, or the residue fraction) was dissolved in dimethylsulfoxide (DMSO) to the concentration of 80 mg/m*l*, and the DMSO-dissolved extracts were diluted in distilled water or liquid media for the further dilution. Same amount of DMSO was used for

control.

3. Cell culture and cell viability

3T3 mouse embryo fibroblasts and Raw 264.7 mouse macrophages were grown in DMEM containing 10% (v/v) fetal bovine serum and 100 μg/*ml* gentamicin solution at 37°C with 5% CO_2 in a humidified atmosphere. Cells were routinely subcultured in the semi-confluent state. Cell viability was measured by MTT assay. Cells were seeded in 96 well cell culture plates (10^4 cells/well). After attached, cells were treated with the given condition. After incubation for the given time at 37°C, 10 μ*l* of MTT was added for the final concentration to be 0.5 mg/*ml*. After incubation for 2 h in the dark at 37°C, media were removed and 0.1 *ml* of DMSO was added. After 20 min, the absorbance was measured at 570 nm with a Microplate Reader (Bio-Rad, Model 550, Richmond, CA, USA) and the reference absorbance was measured at 655 nm for the control.

4. H_2O_2 scavenging assay

The quantification of H_2O_2 was determined by the method of Baughman *et al.* (Baughman *et al.*, 1986). One hundred μ*l* of H_2O_2 (0.5 mM) was incubated with *Saururus chinensis* (0 to 600 μg/*ml*) for 0.5 h at 37°C. One hundred ml of the mixture was incubated at 37°C for 10 min with 1 *ml* of phosphate buffer saline (PBS) containing 1 unit of horseradish peroxidase (HRP) and 53 mg/*l* of o-dianisidine dihydrochloride. The absorption was measured at 470 nm with DU-7 Spectrophotometer (Beckman, CA, USA).

5. Lipid peroxidation assay

The amount of lipid peroxidation was measured by determination of thiobarbituric acid reactive substances (TBARS) in each sample (Harwig *et al.*, 1993). Cells attached in 60 mm cell culture dish were washed twice with PBS and treated with the given concentration of Fe(II)-EDTA, H_2O_2 and *Saururus chinensis* extracts in 3 *ml* PBS for 1 h at 37°C. Cells were lysed in 75 μ*l* of 20% sodium dodesylsulfate (SDS) solution, and 2 *ml* of the homogenized solution was transferred in a sample tube. After addition of 150 μ*l* of 2% butylated hydroxytoluene (BHT) and 20 μ*l* of 200 mM Ethyleneglycol tetraacetic acid (EGTA) solutions to the mixture, 1 *ml* of thiobarbituric acid (TBA) (0.67% w/w) in 10% (w/w) trichloroacetic acid (TCA) was added. The mixture was heated to 90°C for 20 min, cooled on ice and extracted with 2 *ml* butanol. The organic upper phase was collected for spectrophotometric measurement at 532 nm. The absorbance data were normalized to the control absorbance value of each concentration of *Saururus chinensis* extracts. BHT was added to suppress peroxidation during the experiment (Buege and Aust, 1978). EGTA was also added to inhibit the simple peroxide decomposition in the presence of iron (Esterbauer and Cheeseman, 1990).

6. NBT assay

The amount of superoxide anion was measured by NBT assay. Superoxide anion was generated by preparing mixture of xanthine and xanthine oxidase. The reaction mixtures contained with a final volume of 1 *ml*: 0.2 mM xanthine, 0.1 mM EDTA, 0.1 mM NBT, and 50 mM phosphate buffer at pH 7.8. Reaction was started by adding 20 mU/*ml* of xanthine oxidase and the rate of NBT reduction was measured at 550 nm in a recording spectrophotometer every 20 sec for 3 min at room temperature. Control experiments were performed to determine whether the *Saururus chinensis* extracts themselves directly reduce NBT or inhibit xanthine oxidase. Thus, they were added to solutions containing 0.1 mM NBT in phosphate buffer, and the absorbance at 550 nm was measured. Their actions on xanthine oxidase were tested by measuring uric acid formation under the conditions given in the previous paragraph but with xanthine as substrate and the absorbance was measured at 295 nm.

7. NO production

Accumulated nitrite in culture medium was measured using an automated colorimetric assay based on the Griess reaction (Cho *et al.*, 2000a). Fifty ml of samples was reacted with the Griess reagent (1% sulfanilamide and 0.1% napthylethylenediamine dihydrochloride in 2.5% H_3PO_4) at room temperature for

10 min, and then nitrite concentration was determined by measuring the absorbance at 550 nm in a Microplate Reader. The standard curve was obtained using the known concentration of sodium nitrite. Raw 264.7 cells were pretreated with *Saururus chinensis* extracts or DMSO for 1 h, treated with LPS (500 ng/ml) and incubated at 37°C for 24 h.

8. Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

RNA was isolated using a Tri reagent (Molecular Research Center). The total RNA (1 μg) was reverse-transcribed (RT) into cDNA using reverse transcriptase (Boehrunger-Mannheim) and oligo$(dT)_{15}$ primer by incubating the reaction mixture (20 μl) at 42°C for 90 min followed by heating 75°C for 15 min. The PCR was performed in a 20-μl mixture containing 4 μl of RT product, 1× reaction buffer (TaKaRa), 250 μM dNTP, 10 pM each primer, and 1 unit *Taq* DNA polymerase (TaKaRa) with the following oligonucleotide primers: 5'-CTG CAG CAC TTG GAT CAG GAA CCT-3' and 5'-GGG AGT AGC CTG TGT GCA CCT GGA A-3' for iNOS mRNA. After an initial denaturation for 5 min at 94°C, 31 cycles of amplification (55°C for 1 min, 72°C for 1.5 min, and 94°C for 1 min) were performed followed by a 7-min extension at 72°C. The PCR product from each PCR reaction was electrophoresed in 1% agarose gel and visualized by ethidium bromide staining. The relative amounts of iNOS mRNA were normalized by those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The PCR for GAPDH mRNA was performed in the same condition with the following oligonucleotide primers: 5'-ACC CAG AAG ACT GTG GAT GG-3' and 5'-CTT GCT CAG TGT CCT TGC TG-3'. After an initial denaturation for 5 min at 94°C, 23 cycles of amplification (58°C for 30 sec, 72°C for 1 min, and 94°C for 30 sec) were performed followed by a 7-min extension at 72°C for GAPDH.

9. Western blot analysis

After stimulation, Raw 264.7 cells were collected, washed twice with ice-cold PBS, and with low salt buffer (10 mM HEPES-KOH (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM leupeptin, 1 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM dithiothreoitol (DTT), and 1 μg/ml aprotinin) at indicated time. Then cells were lysed with lysis buffer (0.2% NP-40 in law salt buffer) for 15 min on ice. After incubation, the lysate was clarified by centrifugation at 13,000 rpm for 3 min at 4°C. An aliquot of total protein (30 μg/lane) was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Buckinghamsphere, England). The membrane was stained with 0.1% Ponceau S (Sigma) solution to confirm equal loading and transfer. Immunoblotting was performed using anti-IκBα and HRP-linked anti-rabbit IgG secondary antibody (Santa Cruz Biotech). The immune complexes were detected using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

10. Statistics

Values are expressed as the means±standard deviation (S.D.) of three independent experiments. Student's *t*-test was used to verify the significance of values. In some cases, one-way ANOVA was used and each value of *p* was described for verification of significance.

III. RESULTS

1. Cytotoxicity of *Saururus chinensis* extracts in Raw 264.7 cells and 3T3 cells

The effect of *Saururus chinensis* extracts on cell viability was determined by MTT assay. Raw 264.7 cells [Fig. 1(a)] and 3T3 cells [Fig. 1(b)] were treated with four kinds of extractive fractions. As shown in the Fig. 1, hexane and ethyl acetate fractions showed cytotoxicity to both cell types above 1 μg/ml in a dose-dependent manner, but *n*-butanol and residue fractions did not show any toxicity up to 500 or 600 μg/ml, respectively.

2. H_2O_2 scavenging effects of *Saururus chinensis* extracts

Protective effects of *Saururus chinensis* extracts on

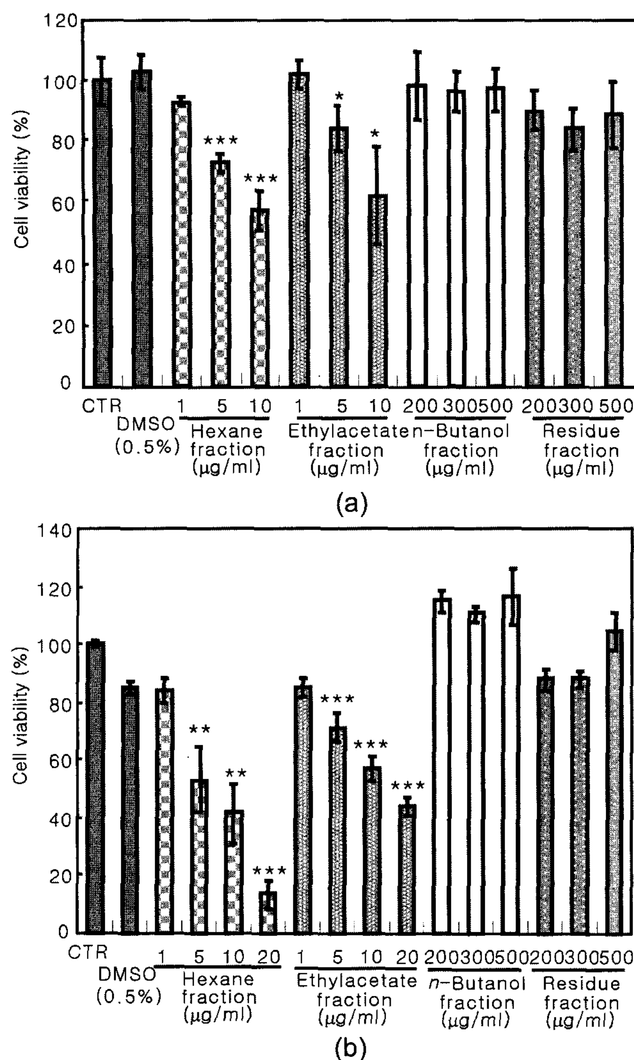

Fig. 1. Cytotoxicity of *Saururus chinensis* extracts. (a) Raw 264.7 macrophages and (b) 3T3 fibroblasts were incubated at 37°C for 24 h with each extract. After incubation, cell viability was measured by MTT assay. Student's *t*-test was used to compare the significance of values as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

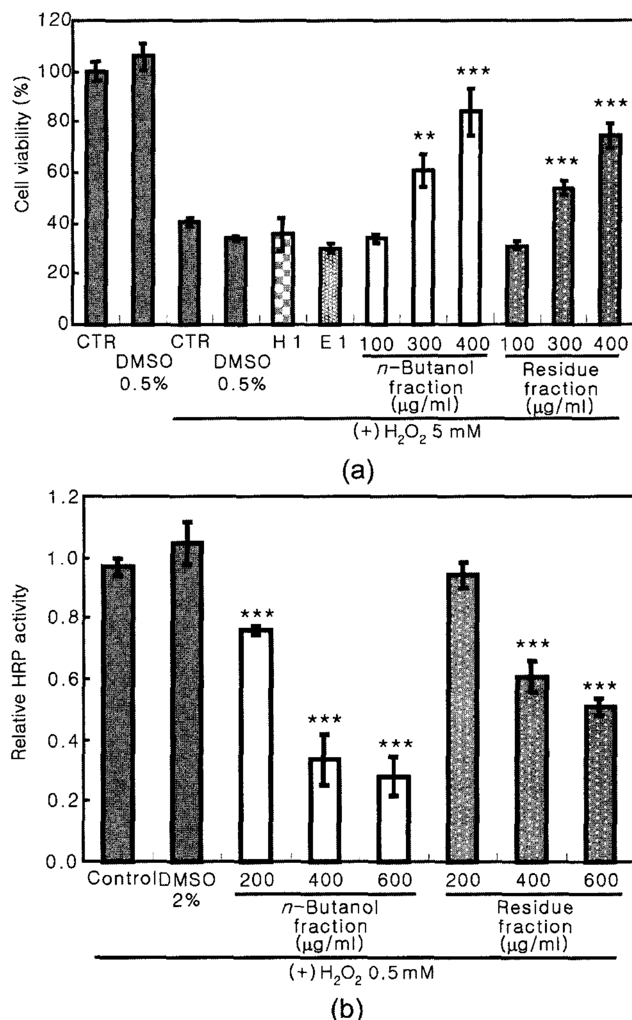

Fig. 2. Protective and scavenging effects of *Saururus chinensis* extracts on H_2O_2 treatment. (a) 3T3 fibroblast cells treated with the extracts and 5 mM H_2O_2 were incubated at 37°C for 1 h and then the cell viability was measured by MTT assay (H: hexane, E: ethyl acetate fraction). (b) 0.5 mM of H_2O_2 was incubated with *n*-butanol and residue fractions (0 to 600 μg/m*l*) for 0.5 h at 37°C. One hundred μ*l* of the mixture was incubated at 37°C for 10 min with 1 m*l* of PBS containing 1 unit of horseradish peroxidase and 53 mg/*l* of o-dianisidine dihydrochloride. Student's *t*-test was used to compare the significance of values, $**p < 0.01$ and $***p < 0.001$.

the toxicity of H_2O_2 were tested in 3T3 cells. After treatment with 5 mM H_2O_2, cell viability was measured by MTT assay. Hexane and ethyl acetate fractions had no effect on cell death induced by 5 mM H_2O_2, but *n*-butanol fractions exhibited cell protective effects at 300 and 400 μg/m*l*, and residue fraction at 300 and 400 μg/m*l*: 60.6±6.5, 84.1±9.3, 53.9±3.0, and 74.5±4.7%, respectively, as survival rate. DMSO (0.5%) showed no effect [Fig. 2(a)]. To examine whether the fractions reacted with H_2O_2 directly, the HRP/o-dianisidine system was used. HRP can convert o-dianisidine to a brown colored molecule by consuming H_2O_2, so the amount of H_2O_2 can be quantified by measuring samples using DU-7 spectrophotometer at 470 nm. One hundred μ*l* of 0.5 mM H_2O_2 were incubated with each fraction of indicated concentration. As shown in Fig. 2(a), *n*-butanol and residue fractions were also effective in this system. *n*-Butanol fraction showed significantly scavenging H_2O_2 at 200, 400, and 600 μg/m*l*, and residue fraction at 400 and 600 μg/m*l*: 0.76±0.01, 0.33±0.09, 0.28±0.06, 0.61±0.05,

and 0.51±0.03, respectively, as relative HRP activity. These results demonstrate that *n*-butanol and residue fraction of *Saururus chinensis* extracts have protective effects on toxicity in 3T3 cells and scavenging activity of H_2O_2.

3. Hydroxyl radical scavenging effects of *Saururus chinensis* extracts

3T3 cells were used for the test of hydroxyl radical

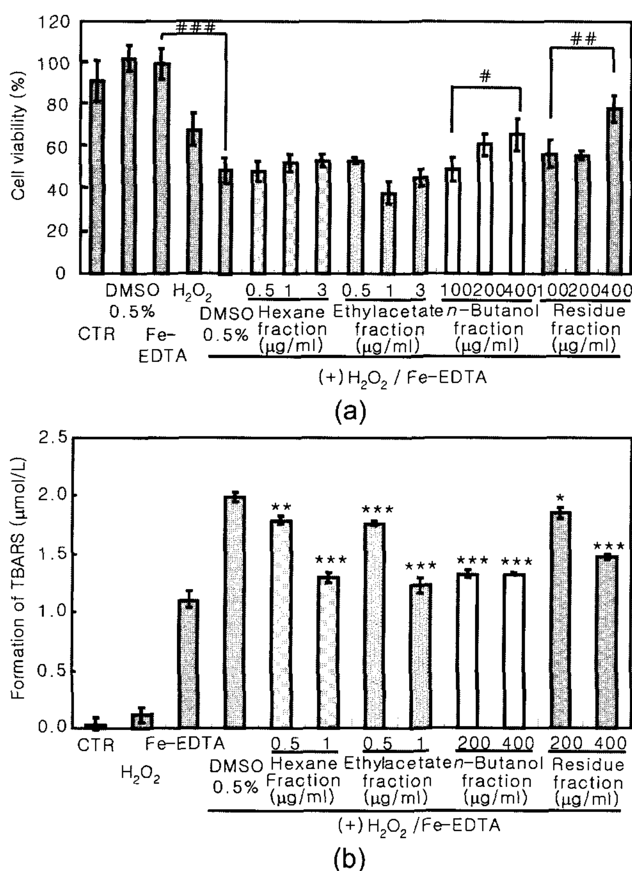

Fig. 3. Hydroxyl radical scavenging effect of *Saururus chinensis* extracts. (a) Protective effects of the extracts on hydroxyl radical induced by Fenton reaction was measured in 3T3 fibroblasts. Cells were incubated at 37°C for 1 h after treatment of extracts, 200 μM Fe-EDTA and 1 mM H_2O_2. After incubation, cell viability was measured by MTT assay. $^{\#}p = 0.02093$ and $^{\#\#}p = 0.00122$ were compared to Fe-EDTA and H_2O_2 treated sample by one-way ANOVA, and $^{\#\#\#}p = 0.00629$ was compared to control sample by one-way ANOVA. (b) Inhibition of lipid peroxidation by the extracts was measured in 3T3 fibroblast cells. Cells were incubated at 37°C for 1 h after treatment of extracts, 400 μM Fe-EDTA and 400 μM H_2O_2. After incubation, the amount of lipid peroxidation in the cells was measured by TBARS assay. Student's *t*-test was used to compare the significance of values, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

scavenging effects of *Saururus chinensis* extracts. As shown in the Fig. 3(a), the 200 μM of Fe-EDTA and 0.5% DMSO had no effect on cell viability but 1 mM of H_2O_2 showed toxicity, as shown in 68.5±7.4% survival rate. Treatment with both 200 μM Fe-EDTA and 1 mM H_2O_2 for the production of hydroxyl radical (Fenton reaction) showed further increased toxicity, as shown in 49.1±5.9% survival rate. Treatment with 200 and 400 μg/*ml* of *n*-butanol fraction, and with 400 μg/*ml* of residue fraction exhibited protective effects by increasing survival rate, 61.3±5.0, 66.4±7.8, and 78.5±6.6%, respectively, but hexane and ethyl acetate fractions had no protective effect [Fig. 3(a)].

The amount of lipid peroxidation in 3T3 cells by Fenton reaction was measured by TBARS assay. Treatment with 400 μM of Fe-EDTA showed a little production of TBARS (1.08±0.09 μmol/*l*) and the treatment with both 400 μM of Fe-EDTA and 400 mM of H_2O_2 showed significantly increased lipid peroxidation (1.99±0.04 μmol/*l*) compared to 400 μM of Fe-EDTA or 400 μM of H_2O_2 alone. The lipid peroxidation induced by the hydroxyl radical was significantly blocked by all four fractions [Fig. 3(b)]. Treatment with 0.5 and 1 μg/*ml* of hexane fraction, with 0.5 and 1 μg/*ml* of ethyl acetate fraction, with 200 and 400 μg/*ml* of *n*-butanol fraction, and with 200 and 400 μg/*ml* of residue fraction showed 1.79±0.03, 1.30±0.04, 1.77±0.01, 1.24±0.06, 1.32±0.04, 1.32±0.01, 1.85±0.04, and 1.47±0.03 mmol/*l* TBARS production, respectively.

4. Superoxide anion scavenging effects of *Saururus chinensis* extracts

NBT assay was used to investigate whether extracted fractions could scavenge the superoxide anion generated by xanthine/xanthine oxidase system. Since *n*-butanol and residue fractions were effective on the toxicity of H_2O_2 and hydroxyl radical, only the two fractions were investigated. Treatment with both fractions decreased the rate of NBT reduction in a dose dependent manner, and that was proven to be effective in scavenging the superoxide anion [Fig. 4(a), (b)]. Since the uric acid formation in the xanthine/xanthine oxidase system was not inhibited by both fractions (data not shown), the generation of the superoxide

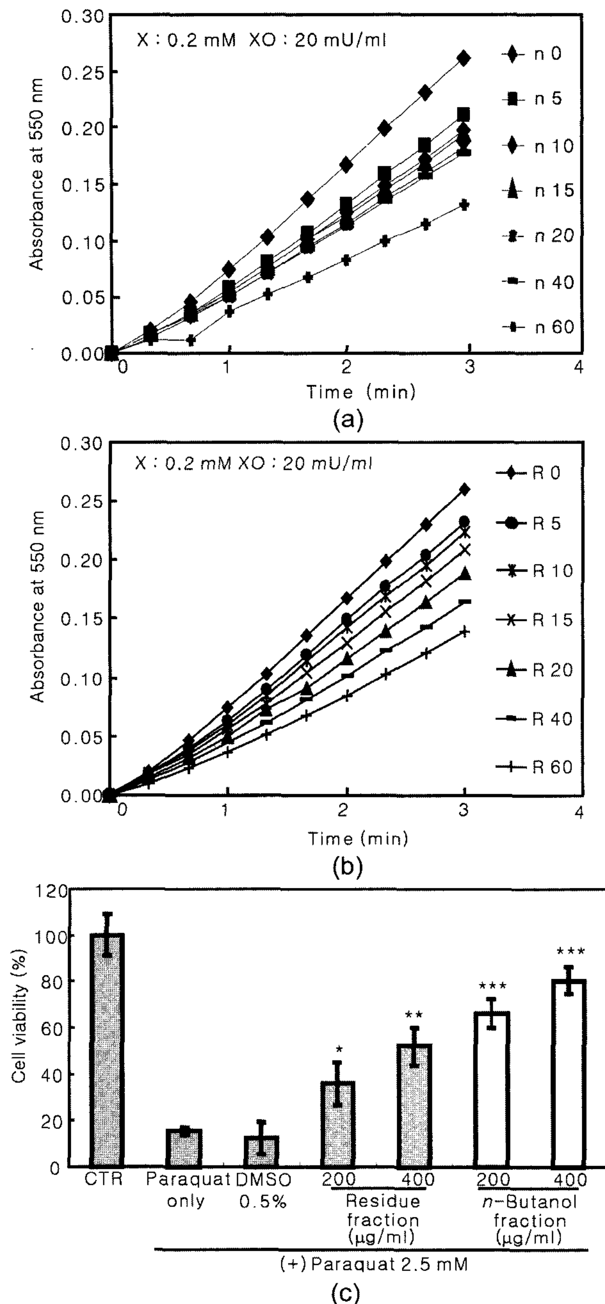

Fig. 4. Superoxide anion scaveging effect of *Saururus chinensis* extracts. Superoxide anion was generated by preparing mixture of 0.2 mM xanthine (X) and 20 mU/ml xanthine oxidase (XO) (a, b) and treatment of paraquat (c). The amount of scavenging superoxide anion by *n*-butanol (a) and residue (b) fraction was determined by measuring the rate of NBT reduction and the samples were measured at 550 nm in a recording spectrophotometer every 20 sec for 3 min. The graphs, both (a) and (b), are representatives of two independently performed experiments (n: *n*-butanol extract, and R: residue). 3T3 fibroblast cells were incubated at 37°C for 18 h after treatment of extracts and paraquat. After incubation, the cell viability was measured by MTT assay. Student's *t*-test was used to compare the significance of values, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

anion itself was not interfered. The NBT reduction by extracted fractions themselves was not detected (data not shown). It is demonstrated that the two fractions have superoxide anion scavenging activity. The two fractions were also tested for the protective effects against paraquat toxicity in 3T3 fibroblasts. Paraquat (2.5 mM) was used for the generation of superoxide anion. After 18 h incubation, cell viability was determined by MTT assay. While treatment with paraquat (2.5 mM) induced cell death and DMSO (0.5%) did not show any protective effect on the induced cell death: 15.4±1.7 and 12.7±9.7% as a survival rate, residue fraction at 200 and 400 μg/ml, and *n*-butanol fraction at 200 and 400 μg/ml showed protective effects from paraquat-induced cell death: 38.3±12.2, 52.0±8.1, 66.1±6.5, and 80.6±6.1%, respectively [Fig. 4(c)].

5. Effects of *Saururus chinensis* extracts on LPS-induced NO production in Raw 264.7 cells

Effects of *Saururus chinensis* extracts on NO production were examined using Raw 264.7 cells. NO production was induced in Raw 264.7 cells by treatment with 500 ng/ml LPS for 24 h (77.4±1.1 pmol/*l*). *n*-Butanol and residue fractions inhibited NO production in the cells in a dose-dependent manner. *n*-Butanol fraction at 100, 200, and 400 μg/ml showed decreased NO production: 52.5±1.2, 44.9±0.3, and 27.0±0.4 pmol/*l*, respectively, and residue fraction at 100, 200, and 400 μg/ml: 44.0±0.3, 32.6±0.8, and 17.0±0.2 pmol/*l*, respectively, but hexane and ethyl acetate fractions did not show any effect [Fig. 5(a)].

Inducible NO synthase (iNOS) is directly involved in NO production, and the regulation of the iNOS mRNA transcription is critical for the inflammatory responses. Because *n*-butanol fraction and residue fraction were effective in NO production [Fig. 5(a)], the two fractions were further investigated on the regulation of iNOS mRNA transcription using RT-PCR. As shown in the Fig. 5(a), (b), the LPS-induced iNOS mRNA transcription was significantly down-regulated by *n*-butanol fraction and residue fraction at 200 or 400 μg/ml. This result is shown that the inhibition of NO production by *Saururus chinensis* extracts is mediated by down-regulation of iNOS mRNA transcription.

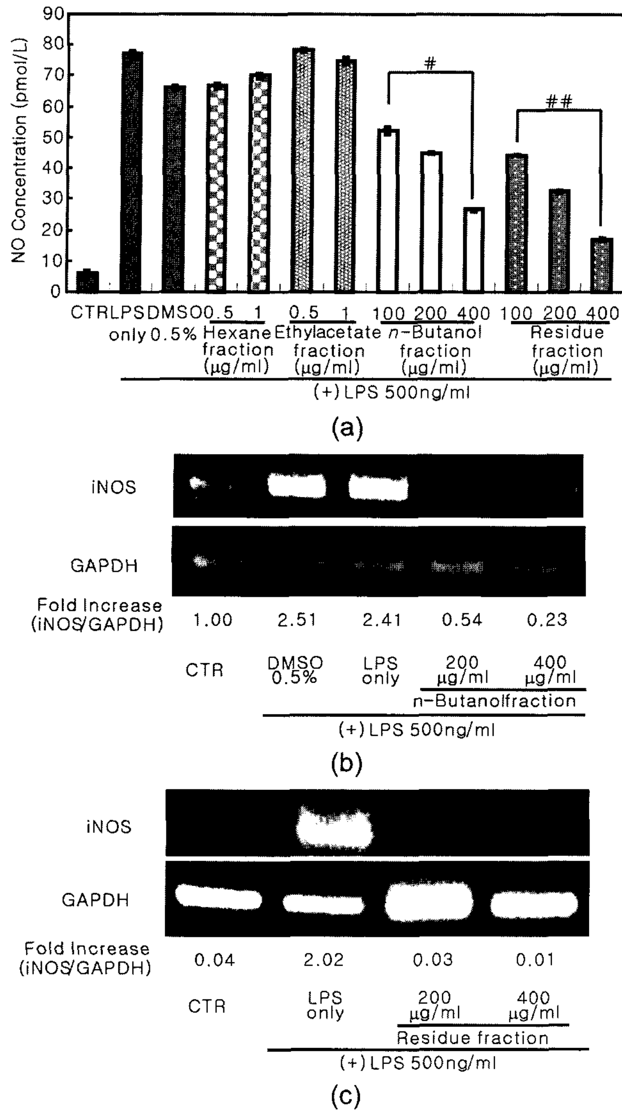

Fig. 5. Inhibition of NO production and iNOS mRNA transcription in Raw 264.7 cells. Raw 264.7 cells were pretreated with *Saururus chinensis* extracts or DMSO for 1h, and then LPS (500 ng/ml) was treated. After the cells were incubated at 37°C for 24 h, 50 μl of media of each sample were used for determination of nitrite by Griess reagent. $^{\#}p = 8.0731\times10^{-12}$ and $^{\#\#}p = 3.39617\times10^{-12}$ were compared to only LPS treated positive control sample by one-way ANOVA (a). Inhibition of iNOS mRNA transcription by *n*-butanol fraction (b) and the residue fraction (c) was also measured in Raw 264.7 cells pretreated with the extracts and then treated with LPS. After the cells were incubated at 37°C for 6 h, total RNAs were isolated and copied into cDNA. The cDNAs were used for determination of mRNA of iNOS or GAPDH by RT-PCR. The figures (b, c) are representatives of two independently performed experiments.

6. Effects of *Saururus chinensis* extracts on LPS-induced IκBα degradation in Raw 264.7 cells

Since the IκB phosphorylation and degradation are closely related to NF-κB activation, IκBα degradation was determined by immunoblotting assay using IκBα antibody. As shown in the Fig. 6, *n*-butanol fraction blocked IκBα degradation induced by LPS, but residue fraction had no effect on IκBα degradation. It is suggested that the down-regulation of the iNOS mRNA transcription by *n*-butanol fraction is mediated by inhibition of IκBα degradation, but not by residue fraction.

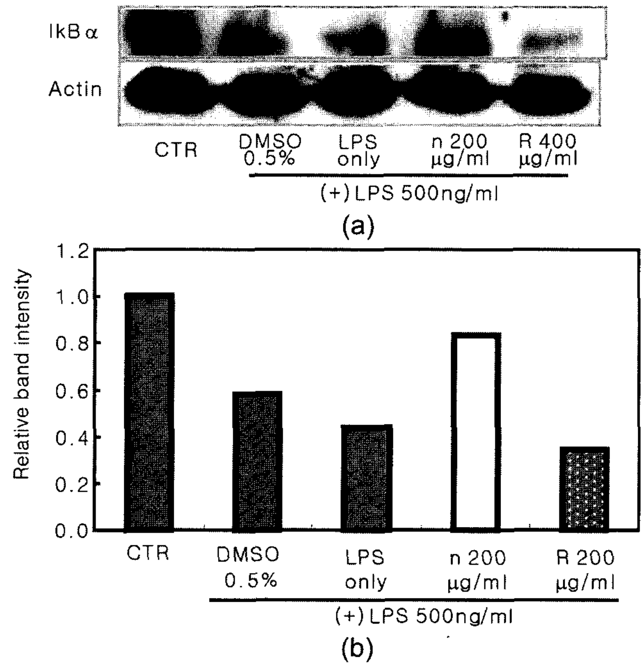

Fig. 6. Inhibition of IκBα degradation by *Saururus chinensis* extracts (n: *n*-butanol extract and R: residue). Cells were pretreated with *Saururus chinensis* extracts or DMSO for 1 h, and then LPS (500 ng/ml) was treated. After the cells were incubated at 37°C for 90 min, the cells were lysed and the lysates (30 μg) were used for immunoblotting of IκBα (a) and relative band intensity of IκBα/actin (b). The figures (a, b) are representatives of two independently performed experiments.

IV. DISCUSSION

In the present study, it was shown that *Saururus chinensis* extracts scavenged ROS and modulated LPS-induced NO production in Raw 264.7 macrophages. Hexane and ethyl acetate fractions had highly toxic effects to 3T3 mouse embryo fibroblast cells and Raw 264.7 mouse macrophage cells, but *n*-butanol and residue fractions had no toxic effect to both cell lines. All the experiments were executed in the range of non-toxic levels of extracts. *n*-Butanol and

residue fractions showed radical scavenging effects against H_2O_2, hydroxyl radical, and superoxide anion. They also showed inhibitory effects on LPS-induced NO production in Raw cells through down-regulation of iNOS transcription. These data demonstrate that some components of these fractions can reduce the oxidative damages induced by reactive oxygen and/or nitrogen species. It also suggests that the antioxidant effects of these fractions participate in modulation of the LPS-induced NO production in the inflammatory responses as shown in our previous experiment (Cho *et al.*, 2000a).

n-Butanol and residue fractions exerted antioxidant properties against H_2O_2, hydroxyl radical, and superoxide anion in the 3T3 cells and in the test tubes. These antioxidant properties of the extracts are derived from their scavenging of ROS. It is suggested that flavonoids are main components having an antioxidant activity. There are many evidences that natural flavonoids show significant antioxidant characteristics (Arts *et al.*, 2002; Bohm *et al.*, 1998; Nair *et al.*, 1998; Noda *et al.*, 1997) and their solubility is similar to that of our *n*-butanol or residue fractions. Further studies for the isolation and purification are underway from these fractions.

All four fractions showed inhibitory effects on the lipid peroxidation by hydroxyl radical in the 3T3 cells. The inhibition of lipid peroxidation by *n*-butanol and residue fractions could be expected by their scavenging and protective effects as shown before, but it is not expected that hexane and ethyl acetate fractions can inhibit lipid peroxidation by hydroxyl radical. It is likely that since hexane and ethyl acetate fractions should be localized to plasma membrane for their hydrophobic property, their scavenging effects appeared only in lipid peroxidation.

Interestingly, various plant flavonoids, which have antioxidant properties, tend to exert also strong anti-inflammatory properties, and this can be a main reason for their clinical application. Based on its antioxidant propery, we further studied the anti-inflammatory effect of *Saururus chinensis*. The involvement of NO in the inflammatory responses was shown by several studies (Cho *et al.*, 2000a, 2000b, 2001; Hollenberg *et al.*, 1997; Hon *et al.*, 1998; Landin *et al.*, 1994), and the inhibition of NO production has been proposed as a potential therapy for inflammation-related diseases. *n*-Butanol and residue fractions but not hexane or ethyl acetate fraction of *Saururus chinensis* exhibited inhibitory effects on NO production induced by LPS in the Raw 264.7 cells. For the detail mechanism, iNOS mRNA transcription level was investigated because NO production in the inflammatory response is caused by iNOS (Cho *et al.*, 2000a, 2000b). As expected, iNOS mRNA transcription level was decreased by treatment with *n*-butanol and residue fractions. This suggests that both two fractions are able to control the NO level by regulating of iNOS mRNA transcription. Since the iNOS mRNA transcription induced by LPS is known to be regulated by NF-κB in the promoter region (Nomura, 2001), and NF-κB has been known as a redox-sensitive transcription factor, the regulation of NF-κB activation was investigated by measuring IκBα degradation. *n*-Butanol fraction but not residue fraction inhibited NF-κB activation, suggesting that the inhibition mechanisms of *n*-butanol and residue fractions on iNOS gene transcription are different. The residue fractions may directly block NF-κB binding activity on DNA promoter region or reduce stability of iNOS mRNA as shown in other researches (Carpenter *et al.*, 2001; Cho *et al.*, 2000a, 2000b, 2001; Ricupero *et al.*, 2001; Kiemer *et al.*, 2001) and those effects may not be mediated by its antioxidant effect, whereas *n*-butanol fraction, which mainly contains flavonoids, seems to regulate the proinflammatory molecule NO through its antioxidant property. These results suggest that *n*-butanol fraction contain antioxidant compounds like flavonoids and further, these compounds are useful to develop anti-inflammatory drugs.

In the present study, the antioxidant and anti-inflammatory properties of *Saururus chinensis* extracts were demonstrated, and it is suggested that this plant could be a candidate material for drug development. Among the four fractions, flavonoid-containing *n*-butanol fraction could exert both antioxidant and anti-inflammatory effects. Further studies are required to isolate and characterize the potent flavonoid from this fraction.

ACKNOWLEDGEMENT

This work was supported by Korea Science and Engineering Foundation Grant (R01-2001-00158). We

thank Dr. Nam Ho Lee, who separated four fractions from *Saururus chinensis* Baill.

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