

양제엽(羊蹄葉) 메탄올 추출물의 항산화 및 항염증 효과

조현진, 윤현정, 박선동*

동국대학교 한의과대학 방제학교실

Anti-oxidative and anti-inflammatory effects of aerial parts of *Rumex japonicus* Houtt. in RAW 264.7 cells

Hyun-Jin Cho, Hyun-Jeong Yun, Sun-Dong Park*

Dept. of Prescriptionology, College of Oriental Medicine, Dongguk University.

ABSTRACT

Objectives : The aerial parts of *Rumex japonicus* Houtt. (RF) is used by traditional clinics to treat parasite infection in East Asia. This study aims a verification of anti-oxidative and anti-inflammatory effects of RF methanol extract.

Methods : Anti-oxidative effects of RF were measured by scavenging activities of DPPH, superoxide, nitric oxide (NO) and peroxynitrite radicals. And also scavenging activities of anti-oxidation in lipopolysaccharide (LPS)-treated RAW 264.7 cells were measured. The inhibitory effects against the production of inflammatory mediators including NO, prostaglandin E₂ (PGE₂), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and the translocation of nuclear factor (NF)- κ B in LPS-stimulated RAW 264.7 cells by RF were tested.

Results : RF scavenged DPPH, superoxide, NO and peroxynitrite radicals, and RF (at 200 μ g/ml) reduced the inflammatory mediators definitely.

Conclusions : These results indicate that RF may be a potential drug source for oxidative stress related inflammatory diseases.

Key words : The aerial parts of *Rumex japonicus* Houtt. (RF), Anti-inflammation, Anti-oxidation, RAW264.7 cell Introduction

INTRODUCTION

Restricted inflammation is inevitable for cell or organ restoration but long-term lasting inflammation become a issue because it occur wide variety of conditions including type 2 diabetes, cardiovascular and neurodegenerative diseases, cancer, obesity, asthma, and ageing¹⁻². To cure these diseases, many new drugs were developed. Although single pure chemical compounds can be potent therapeutic agents, poor compliances, limited applications, and potential withdrawal from the market sometimes happen by various and severe side effects. On these score,

consumer prefer natural product based medicines more than chemically synthesized drugs because it may have fewer side effects and be similarly or more effective than new chemical entities³.

In the process of inflammatory response, several characteristic situation occurred that include depletion of local nutrients, increase demand for oxygen (O₂) and increase generation of reactive oxygen and nitrogen species (ROS/RNS). The increased synthesis ROS/RNS are common hallmark of inflammatory diseases and these acts as a versatile mediator of several aspects of inflammation that induced Diabetes, Alzheimer and etc⁴⁻⁶. So the natural products that

*교신저자 : 박선동. 경북 경주시 석장동 707, 동국대학교 한의과대학 방제학교실.
· Tel : 054-770-2371, · E-mail : sundong@dongguk.ac.kr.
· 접수 : 2011년 2월 7일 · 수정 : 2011년 3월 6일 · 채택 : 2011년 3월 10일

have anti oxidative effects can be a candidate for new anti-inflammation medicine.

Rumex japonicus HOUTT. is a perennial herb with erect stems 40–100 cm long. Its leaves are long-petioled 10–25 cm long and 4–10 cm wide. It grows in wet meadows along ditches in the low lands of Korea, Japan and China⁷⁾.

The roots and aerial parts of *R. japonicus* has used as a traditional medicine in east asia, and it has been used for the treatment of heat, phlegm, jaundice, constipation, scabies, and uterine hemorrhage⁸⁾. *R. japonicus* has been found to possess various biological and pharmacological activities including anti-oxidative and anti-bacterial activity^{9–10)}, anti-fungal activity¹¹⁾ and inhibitory effects on atopic dermatitis-like skin lesions¹²⁾. The aerial parts of *R. japonicus* (RF) that include flowers, stems, and leaves has been used rarely than the roots in Korea, but the leaves include the flavonoid quercitrin that be ascertained to anti-oxidant¹³⁾, 24-nor-Ursane Type Triterpenoids exist in stems¹⁴⁾ while the major anthraquinones, emodin, chrysophanol, and their glucosides has been identified from the roots¹⁵⁾.

These previous studies shows that RF have different constituent in comparison with the roots and anti-oxidative effect of aerial parts was verified partially, the definition of inhibitory effect on inflammation via ROS and RNS by RF is necessary. Also aerial parts outmeasure in contrast with roots, so when perform additional studies and industrialization, investigator can acquire samples more easily.

So to define the activity of RF on anti-oxidative and anti-inflammatory effects, methanol extract of it was prepared. And RF scavenging activities of DPPH radical, superoxide anion (O_2^-), nitric oxide (NO) and peroxynitrite ($ONOO^-$) were measured in vitro. MTS assay was performed for measurement of RAW 264.7 cell viability. As a result, prefer concentration was determined. The intracellular oxidation production that induced by lipopolysaccharide (LPS) inhibition effect of RF was tested. Nitrite, PGE_2 was measured as described in Material and Methods, and also inflammatory cytokines, IL-1 β , IL-6 and TNF- α levels were evaluated. Using western blotting, the suppression of COX-2, iNOS, NF κ B activation and I κ B α phosphorylation that induced by RF were tested.

Materials and Methods

1. Materials

1) Preparation and Extraction of RF

RF was collected from riverside of Hyung-san river (Naenam myun, Gyeongju, Republic of Korea) in July 2010, and identified by professor Sun-Dong Park, Department of Prescriptionology, Dongguk University, Gyeongju City, Republic of Korea.

According to the method that is described in Fig1, methanol extract of RF was prepared. In brief, the fresh aerial parts (400 g) were cut into small parts and placed in methanol of 5 L at room temperature. The methanol was acquired after 48 hours, and the processes were repeated once more. The acquired methanol was filtered through wattman paper.

Subsequently, acquisition was concentrated by using rotary evaporator and then freeze dried. In consequence, 17.92 g of methanol extract of RF was gained. For the bioassay test, samples were dissolved in dimethyl sulfoxide (DMSO) and filtered through a 0.20 μ m filter.

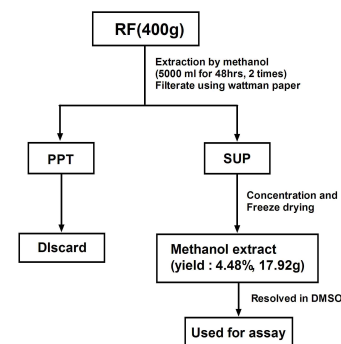


Fig.1. Extraction procedures of RF.

2) Chemicals and Reagents

Dimethyl sulfoxide (DMSO) was purchased from Amresco (Solon, USA), methanol was purchased from Samchun chemicals (Pyungtack city, Republic of Korea),

1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA), hypoxanthine, nitro blue tetrazolium (NBT), xanthine oxidase, sodium nitroprusside (SNP), 4,5-diaminofluorecein DAF-2, Diethylenetriaminepenta-acetic acid (DTPA), 6-carboxy-2',7'-dichlorofluorescein diacetate (DCF-DA), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), lipopolysaccharide (LPS), CAPS, tween 20, sodium phosphate, sodium chloride, potassium chloride were purchased from Sigma-Aldrich (St. Louis, USA).

Dihydrorhodamine 123 (DHR 123) was purchased from Molecular Probes (Eugene, USA).

Peroxyntirite was purchased from Cayman Chemical Co. (Ann Arbor, USA).

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), streptomycin-penicillin, Nuclear and cytoplasmic protein extraction reagents and enzyme-linked immunosorbent assay (ELISA) kits for

tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , enhanced chemiluminescence detection kit were purchased from Thermo scientific (Rockford, USA).

Sodium Dodesyl Sulfate (SDS), Acrylamide, Bis and protein assay reagent were purchased from Bio-Rad (Hercules, USA).

Tris base and The CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (MTS) kit and Griess Reagent System were purchased from Promega (Madison, USA).

Prostaglandin E₂ immunoassay kit was purchased from R&D Systems (Minneapolis, USA).

Immunosorbent assay (ELISA) kit for IL-6 was purchased from BD Biosciences (San Diego, USA).

Antibodies against COX-2 (Cyclooxygenase-2), β -actin, p-I κ B α (phospho inhibitory kappa B alpha) and NF- κ B (Nuclear factor kappa B) p65, PARP (Poly (ADP-ribose) polymerase) were purchased from Cell Signaling Technology (Beverly, USA).

Antibody against iNOS (inducible nitric oxide synthase), anti-rabbit and anti-mouse secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).

2. Methods

1) Free radical scavenging activities

1-1) DPPH scavenging activity

DPPH radical scavenging activity of RF extract was determined by the method according to Gyamfi et al, with a slight modification¹⁶⁾. Briefly, different concentrations of RF extract (50 μ l) were mixed with 1 ml of 0.1 mM DPPH-ethanol solution and 450 μ l of 50 mM Tris-HCl buffer (pH7.4). The mixture was shaken and incubated during 30 mins at room temperature. The absorbance was measured at 517 nm and DPPH radical scavenging activity was calculated as follows : % inhibition = [(absorbance of control - absorbance of sample) / absorbance of control] \times 100.

1-2) Superoxide anion scavenging activity

Scavenging activity of RF extract on superoxide radical (O₂⁻) was evaluated by the method of Gotoh and Niki¹⁷⁾. The RF extract of different concentrations were added to a reaction solution containing 100 μ l of 30 mM EDTA (pH 7.4), 10 μ l of 30 mM hypoxanthine and 200 μ l of 1.42 mM NBT. After incubation during 3 mins at room temperature, 100 μ l of 0.5 U/ml xanthine oxidase was added and then the final volume was supplemented with 50 mM phosphate buffer (pH 7.4) up to 3 ml. After the solution was incubated during 20 mins at room temperature, the absorbance was measured at 560 nm.

1-3) Nitric Oxide scavenging activity To measure nitric oxide scavenging activity, the method of Nagata et al. was used¹⁸⁾. First, 1 mg of DAF-2 was dissolved in 0.55 mL of DMSO, which was diluted with 50 mM phosphate buffer (1:400, v/v) to prepare DAF-2 solution. Various concentrations of RF extract (10 μ l) were mixed with 130 μ l of 50 mM phosphate buffer (pH 7.4), 10 μ l of 40 mM SNP and 50 μ l of DAF-2 solution. After incubation at room temperature during 10 min, The fluorescence signal caused by the reaction of nitric oxide and DAF-2 was measured by the fluorescence microplate reader (SPECTRA MAX GEMINI EM, Molecular Devices Corp., USA) in a wavelength of 495 nm excitation and 515 nm emission.

1-4) Peroxynitrite scavenging activity

The peroxynitrite scavenging activity of RF extract was determined by the method of Kooy et al. with a slight modification¹⁹⁾. Briefly, 10 μ l of the RF extract of different concentrations were mixed with 175.8 μ l of rhodamine buffer (50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride and 5 mM potassium chloride) containing 4 μ l of 5 mM DTPA and 0.2 μ l of 5 mM DHR 123. The reaction was started by adding 10 μ l of 10 μ M peroxynitrite. After 10 mins at room temperature, fluorescent intensity of the mixture was monitored at excitation and emission wavelengths of 480 and 530 nm. The scavenging effect of extract was expressed as the percent inhibition of DHR 123 oxidation.

All experiments were carried out in triplicate and the free radical scavenging activities of RF extract were expressed as IC50. The IC50 value was defined as the final concentration (in μ g/ml) of samples that inhibits 50% of the free radical creation.

2) Cell culture

RAW 264.7, the mouse macrophage cell line, was obtained from the American Type Culture Collection (Rockville, USA). RAW 264.7 cells were maintained in a monolayer culture in DMEM supplemented with 10% fetal bovine serum and 1% antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin) at 37 \circ C in a humidified atmosphere containing 5% CO₂.

3) Assessment of cell viability

Cell viability was measured with a Cell Titer 96 colorimetric assay using an MTS tetrazolium compound according to the manufacturer's instructions. In brief, RAW 264.7 cells were seeded into 96-well plates, and then various concentrations of RF that diluted with

DMEM were treated at 96-well plates during 18 hours. After that, the assays were performed by adding 20 μ l of MTS reagent/well and then incubating them during 4 hours. And then the absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay reader (Opsys MR, USA). The results were expressed as percentage changes from the basal conditions using 3–5 culture wells for each experimental condition.

4) DCF-DA assay

In an attempt to determine antioxidant effect of RF extract in a cell model, 6-carboxy-2',7'-dichlorofluorescein diacetate (DCF-DA) assay was carried out^{20–21}. Cells were seeded into 96-well culture plates 1 day before the experiment. On the day of the experiment, RF extract was added to wells with or without LPS and incubated during 18 hours. After incubation, 10 μ M DCF-DA diluted in PBS buffer was added. After 45 mins, cells were washed twice with PBS buffer. The fluorescent intensity was measured at excitation and emission wavelengths of 485 and 535 nm using a fluorescence microplate reader. The cells, which were incubated without any inducers or samples, served as control and the increase of fluorescent intensity was compared with that of control. An increasing rate was expressed as fold of control.

5) Preparation of samples for western blot and measurement of Nitrite, PGE₂ and Cytokine production

RAW 264.7 cells that cultured at 60mm petri dish were pretreated with RF extract or AEBSF during 1 hr, and then treated by 100 ng/ml LPS. After that cells incubated during 18 hrs in CO₂ incubator.

After these process, the culture media were collected to measure Nitrite, PGE₂ and cytokine production and cells were washed three times with ice-cold PBS and then harvested.

6) Measurement of Nitrite, PGE₂ and Cytokine production

The nitrite production was measured using the Griess reagent system²². PGE₂, TNF- α , IL-1 β and IL-6 production were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Samples that used in these experiments are prepared as described in fifth clause.

7) Western blot analysis

RAW 264.7 cell samples were prepared as described in fifth clause. And then the cells were lysed with

RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na₃VO₄, 2 mM EDTA, 1% NP-40, and various protease inhibitors) or nuclear and cytoplasmic extraction reagents. Nuclear and cytoplasmic extraction reagents were treated according to the manufacturer's instructions.

Next, the samples were centrifuged at 12,000 G during 15 mins at 4 \circ C, after which the supernatants (hereafter referred to as the cell extracts) were collected. The protein concentrations of the cell extracts were then measured using Bradford reagent. Next, the cell extracts were separated by 8~10% SDS-PAGE and then electrophoretically transferred onto nitrocellulose membranes (Whatman GmbH, Germany). And then the membranes were blocked with 5% skim milk in Tris buffered saline (TBS) and subsequently incubated with the primary antibodies in TBS containing 3% skim milk. Antibody-antigen complexes were then detected using goat anti-rabbit or anti-mouse IgG-HRP conjugated antibodies and an enhanced chemiluminescence detection kit.

8) Statistics

All data shown represent the means \pm SEM from at least three independent experiments. Differences among groups were evaluated by one-way ANOVA, followed by Duncan's multiple range tests using the GraphPad Prism 4.0 software. A $p < 0.05$ was considered to indicate statistical significance. The band intensity obtained by western blot assay, was analysed using GelDoc-It BioImaging System.

Results

1. Free radical scavenging activity

To evaluate anti-oxidative effects of RF, several free radical scavenging activity assessments were accomplished.

Figure 2A show the scavenging activity of RF against DPPH radical. DPPH radical was dose-dependently decreased by RF extract treatment and the IC₅₀ numerical value was 11.23 \pm 0.17 μ g/ml.

And Figure 2B show that scavenging activity of superoxide anion. Superoxide anion statistically and significantly decreased by RF extract in contrast with control group. IC₅₀ value was 16.10 \pm 0.07 μ g/ml.

Next, DAF-2 assay was performed to measure nitric oxide scavenging activity. Nitric oxide was dose-dependently decreased and IC₅₀ value was 1.35 \pm 0.16 μ g/ml (Figure 2C).

Last, RF extract brought out decline of

peroxynitrite effectively. Figure 2D show reduction of peroxynitrite decidedly ($IC_{50}=2.09\pm 0.14 \mu\text{g/ml}$).

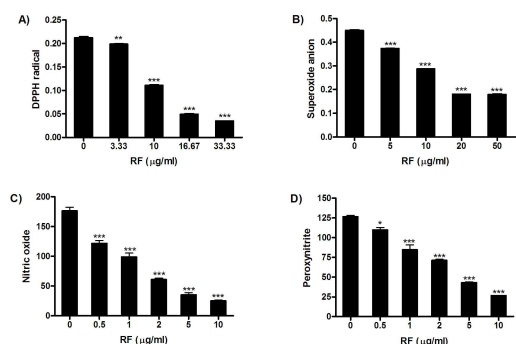


Fig.2. Scavenging activities of RF extract on various free radicals. These results show the numerical value that measured by absorbance or wavelength as previously described in Materials. And each value represents the mean of three separate experiments. A) Scavenging activity on DPPH radical. B) Scavenging activity on superoxide anion. C) Scavenging activity on nitric oxide. D) Scavenging activity on peroxynitrite. Significantly different from control (*); *: P<0.05, **: P<0.01, ***: P<0.001

2. Cell viability of RF against RAW 264.7 cells

To progress more studies that contain measurement of intracellular ROS, nitrite, PGE_2 and etc, determination of cell viability of RF against mouse macrophage RAW 264,7 cells. For this measurement, various concentrations of RF treated at RAW 264,7 cells. As shown in figure 3, Treatment of 200 $\mu\text{g/ml}$ RF had cytotoxicity that occurred 20% below cell death (cell viability : 82.47 %). Therefore 50, 100 and 200 $\mu\text{g/ml}$ RF were chosen to be used for all subsequent experiments.

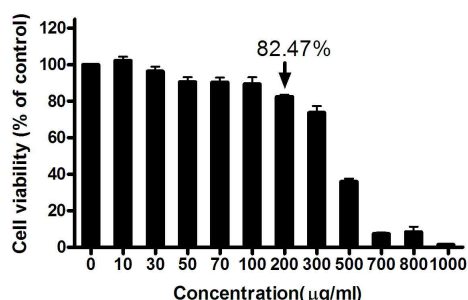


Fig.3. Effect of RF on the cell viability of RAW 264.7 cells. RAW 264.7 cells were treated with various concentrations (0, 10, 30, 50, 70, 100, 200, 300, 500, 700, 800, 1000 $\mu\text{g/ml}$) of RF during 18 hours. Cell viability was measured by MTS assay as described in materials and methods. Data represent the mean the mean \pm SEM with three separate triplicate experiments.

3. Inhibition of LPS-induced ROS production in RAW 264.7 cells by RF.

This test was executed to evaluate that depression of ROS production that induced by lipopolysaccharide

(LPS). Test was conducted as described at column of DCF-DA assay within Material and Methods. As shown in figure 4, groups with 100 ng/ml LPS treatment show significant increase to compare with control group that without LPS treatment. And ROS production that induced by LPS was effectively and dose-dependently declined with RF treatment. Treatment of 0.3 mM AEBSF was used as positive control.

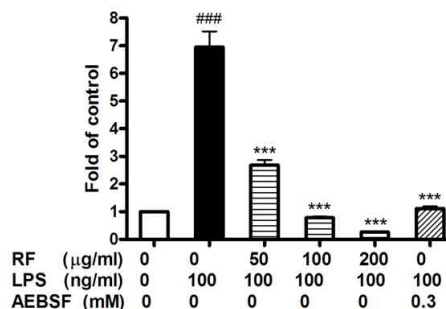


Fig.4. Inhibitory effect of RF on ROS production in RAW 264.7 cells induced by LPS. RAW 264.7 cells were preincubated with 50, 100 and 200 $\mu\text{g/ml}$ of RF during 18 hrs, after that 100 ng/ml LPS was treated to induce ROS production. The increase of DCF fluorescence was calculated as increasing fold of control. Data represent the means \pm SEM. Significantly different from control (#) or LPS alone (*); ###, ***: P < 0.001.

4. Inhibition of LPS-induced NO and PGE_2 production in RAW 264.7 cells by RF

Griess reagent system as described in Material and Methods was used to evaluate NO inhibition effect of RF. For this examination, three concentrations (50, 100, 200 $\mu\text{g/ml}$) of RF were treated to RAW 264.7 cells. Also 0.3 mM AEBSF was used as positive control. As shown in Figure 5A, NO production increased by LPS 100 ng/ml treatment validly. But RF 50 $\mu\text{g/ml}$ was treated, NO production was declined as 4.64%, RF 100 $\mu\text{g/ml}$ suppressed as 19.83 % and RF 200 $\mu\text{g/ml}$ suppressed NO production as 87.34 %. Synthetically, RF methanol extract have inhibition effect of NO that induced by LPS in RAW 264.7 cells.

As next experiment, PGE_2 level in RAW 264.7 cells was measured. This experiment also performed as previously described RF, AEBSF and LPS concentrations in first paragraph. LPS grew PGE_2 production articulately in RAW 264.7 cells, however RF treatment led to decrease in the levels of LPS-induced PGE_2 production (Fig 5C). AEBSF, the positive control was showed low effect on PGE_2 inhibition (4.81%), but 200 $\mu\text{g/ml}$ of RF inhibited PGE_2 of 52.81 % (Fig 5D).

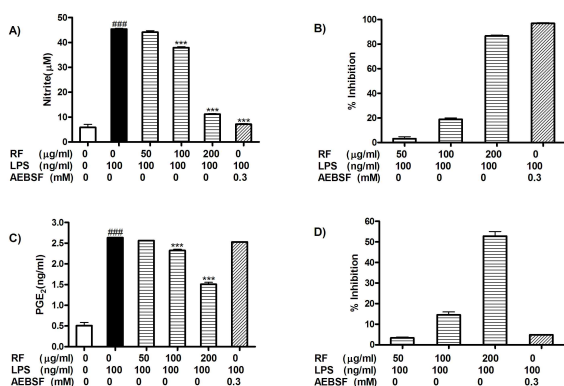


Fig.5. Inhibition of LPS-induced NO and PGE₂ production by RF. RAW 264.7 cells were preincubated with 50, 100 and 200 µg/ml of RF during 1 hr and then treated with 100 ng/ml of LPS during 18 hrs. A) NO production was measured by Griess Reagent System. B) Inhibition % of LPS-induced NO by RF. C) PGE₂ production was measured by ELISA as described in Materials and Methods. D) Inhibition % of LPS-induced PGE₂ by RF. Data represent the means ± SEM. Significantly different from control (#) or LPS alone (*); ###,***: P < 0.001.

5. Inhibition of LPS-induced IL-1β, IL-6 and TNF-α production in RAW 264.7 cells by RF

To evaluate inhibition effects of LPS-induced cytokines, samples were prepared as described in Material and Methods and RF concentration were same as shown in fig.6.

The production of inflammatory cytokines, including IL-1β (A), IL-6 (B) and TNF-α (C), were increased after exposure to LPS as shown in figure 6. However, RF treatment led to dose-dependently decrease of IL-1β production (A), that were observed in cells that had been treated with LPS. Figure 6B and 6C obviously show that LPS-induced IL-6 and TNF-α were inhibited by treatment of RF 200 µg/ml. So RF can be considered as prominent IL-1β inhibitor, and in high-dose, RF has inhibitory effects of IL-6 and TNF-α.

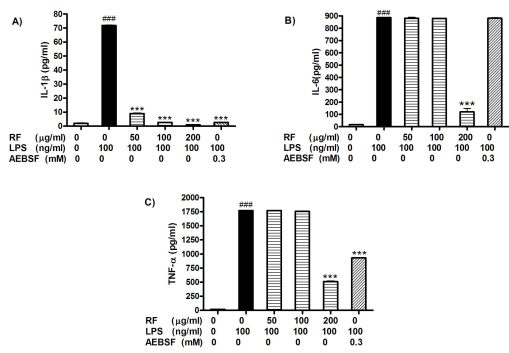


Fig.6. Inhibition of LPS-induced TNF-α, IL-1β and IL-6 by RF. The IL-1β, IL-6 and TNF-α production were measured by ELISA as described in materials and methods. Data represent the means ± SEM. Significantly different from control (#) or LPS alone (*); ###,***: P < 0.001.

6. Inhibition of LPS-induced COX-2 and iNOS expression in RAW 264.7 cells by RF

To determine whether the inhibitory effects of RF on the productions of NO and PGE₂ were related to changes in the expressions of COX-2 and iNOS, RF was pretreated to RAW 264.7 cells with 50, 100 and 200 µg/ml during 1 hr and then stimulated them with 100 ng/ml LPS during 18 hrs and the expression levels of COX-2 and iNOS were determined by western blotting.

The expression levels of COX-2 and iNOS were obviously up-regulated in response to 100 ng/ml LPS, but RF inhibited specifically the expression levels of COX-2 and iNOS, as shown in figure 7A and 7B. For more accurate certification, relative band intensities of COX-2 and iNOS per β-actin were calculated, and that results mentioned in lower side of figure 7. The sample that treated with 200 µg/ml RF had especially excellent inhibitory effects of COX-2 and iNOS. AEBSF, the positive control, also show inhibitory effects but sample that treated by RF 200 µg/ml was better than that.

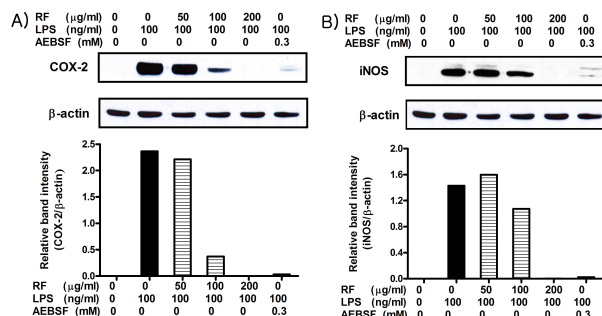


Fig.7. Inhibition of LPS-induced COX-2 and iNOS expression by RF. RAW 264.7 cells were preincubated with RF (50, 100 and 200 µg/ml) during 1 hr and then treated with 100 ng/ml of LPS during 18 hrs. The expression levels of COX-2 and iNOS were determined by western blotting as described in Materials and Methods and the density of band was calculated by GelDoc-It BioImaging System. β-actin levels were used as internal markers for loading variation.

7. Inhibition of LPS-induced nuclear translocation of the NF-κ B p65 subunit as well as degradation of p-Iκ Bα by RF

To investigate that whether RF have inhibitory effect on translocation of LPS-induced p65, a major component of NF-κ B activated by LPS in macrophage, Western blot analysis was performed.

RAW 264.7 cells were treated with 50, 100 and 200 µg/ml of RF and 0.3mM AEBSF during 18 hrs and then stimulated them with 100 ng/ml LPS during 1 hr and the expression levels of NF-κ B p65 in the nucleus were measured.

In consequence, Amount of NF- κ B p65 in the nucleus was significantly increased when exposure to LPS, but RF inhibited nuclear translocation of NF- κ B p65 that induced by LPS in a dose-dependent manner (Fig. 8A).

And also, to confirm RF could affect phosphorylation of I κ B α , RAW 264.7 cells were treated with same concentration of RF and AEBSF. And the expression levels of p-I κ B α were determined by Western blotting as described in Material and Methods.

As a result, RF inhibited LPS-mediated phosphorylation of I κ B α , while the sample that only treated with LPS caused obvious increase on the levels of p-I κ B α (Fig. 8B).

These results suggested that RF can intercept LPS-induced nuclear translocation of p65 by diminution of I κ B α phosphorylation.

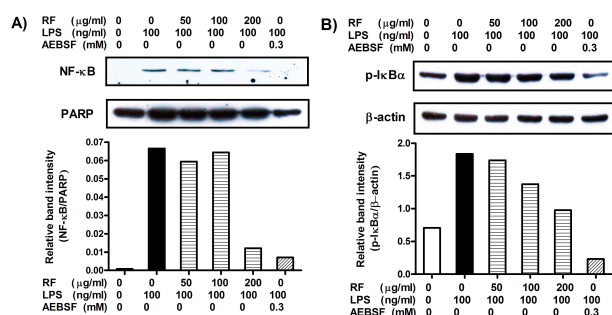


Fig.8. Effect of RF on LPS-induced nuclear translocation of NF- κ B and on the phosphorylation of I κ B α . The samples were prepared as described in Material and Methods and Results. The amount of NF- κ B p65 in the nucleus, and the levels of p-I κ B α in the cytosol were checked by Western blotting. The density of band was calculated by GelDoc-It Biolmaging System. PARP, β -actin levels were used as internal markers for loading variation.

Discussion

As described in Introduction, inflammation is a hot issue in the field of biotechnology. Because of inflammation is related to variety kind of diseases, such as rheumatoid arthritis, type 2 diabetes and atherosclerosis, several dozens control solution of inflammation were developed. Traditional oriental medicine (TOM, sometimes called traditional chinese medicine) can be a database for anti-inflammation drugs, indeed several TOM and it based substances such as *Scutellaria baicalensis* Georgi²³⁾, *Rheum tanguticum*²⁴⁾ and *Coptis chinensis* Franch²⁵⁾ were ascertained that have anti-inflammatory effect. RF was used traditionally in east asia for treatment of parasite infection²⁶⁾, but anti-inflammatory effect of it wasn't revealed. The anti-oxidative effect of RF was

reported previously⁷⁾. We already knew that some herbs that have anti-oxidative effect also have anti-inflammatory effect as mentioned in introduction, so a hypothesis that RF may had anti-inflammatory effect was formed. To examine the hypothesis, several tests carried out.

Oxidative stress that induced by reactive oxygen and nitrogen species play a major role in many diseases including cancer, liver disease, Alzheimer's disease, aging, arthritis, inflammation, diabetes, Parkinson's disease, atherosclerosis, and AIDS²⁷⁾. Oxygen play a key element in many processes of the living creature including cellular metabolism, but oxygen can react with DNA, proteins, and other cellular components and provoke some problems²⁸⁾. Oxygen become reactive oxygen species in the process of metabolism. ROS are a family of molecules, including molecular oxygen and its derivatives, produced in all aerobic cells. Many ROS possess unpaired electrons and thus are free radicals. These include superoxide anion (O₂⁻), hydroxyl radical (HO⁻), nitric oxide (NO⁻) and lipid radicals. Other ROS, such as hydrogen peroxide (H₂O₂), peroxyxynitrite (ONOO⁻), and hypochlorous acid (HOCl) are not free radicals, but have potent oxidizing effects that emerge to oxidative stress²⁹⁾.

To evaluate the anti-oxidative effects of RF, inhibitory effects of four type ROS/RNS were measured. First, DPPH radical scavenging activity of RF was tested. The DPPH assay is popular in natural antioxidant studies, because of it's simplicity and high sensitiveness. This assay is based on the theory that a hydrogen donor is an antioxidant and the anti-oxidative effect can be measured by the proportional decline of DPPH. The color turns from purple to yellow when the DPPH radicals were arised by hydrogen absorption from an antioxidant³⁰⁾. RF scavenged DPPH radical dose-dependently as shown in Fig 2A.

Next, RF induced scavenging activity of superoxide anion, nitric oxide, and peroxyxynitrite were measured. Superoxide is ordinary controlled by superoxide dismutase (SOD) that degrades superoxide to hydrogen peroxide (H₂O₂), which is then converted to H₂O and O₂ by other enzymes. But, on inflammation situation, superoxide is produced excessively more than the capacity of the endogenous SOD enzyme defence system. And excessive superoxide destroy the biological activity of nitric oxide. Nitric oxide has some physiological function via the activation of cyclooxygenase and subsequent release of beneficial and anti-inflammatory prostaglandins in low concentrations (nM). Therefore nitric oxide is a

essential element for maintenance of blood vessel tone, inhibition of platelet adhesion/aggregation and cytoprotection in numerous organs including heart, intestine and kidney. But when nitric oxide interact with superoxide, it lose physiological abilities³¹⁻³². The high concentrations ($\leq 1 \mu\text{M}$) and reaction with superoxide of NO generate a variety of reactive nitrogen species (RNS) that include peroxynitrite (ONOO^-). These produced ROS and RNS cause damage to all types of cellular biomolecules, and finally induce inflammation³³. Figure 2B, C, D show the inhibitory effects of superoxide anion, nitric oxide and peroxynitrite that induced by RF. RF suppressed these ROS and RNS effectively.

As a next step, RF induced cell viability in mouse macrophage RAW 264.7 cells was evaluated. RAW 264.7 cells shown 80 % over cell viability within 200 $\mu\text{g}/\text{ml}$ of RF. So further studies were practiced within that concentration. And then LPS induced intracellular ROS production was tested. AEBSF was used as positive control. AEBSF is a stable, water-soluble and relatively nontoxic compound, possessing NADPH oxidase suppressor. So it used for the design of anti-inflammatory drugs frequently³⁴. 200 $\mu\text{g}/\text{ml}$ RF suppressed ROS production effectively more than AEBSF 0.3 mM. LPS-induced NO and PGE_2 were measured, too. PGE_2 is a arachidonate-derived eicosanoid and exert immunosuppressive action via T cell suppression. So, to control inflammation, reduction of PGE_2 level is need³⁵⁻³⁶. RF inhibited LPS-induced NO and PGE_2 level in high concentration (Fig 5).

Next, the suppression of COX-2 and iNOS were tested by western blotting. COX-2 is an enzyme that catalyze the transformation of arachidonic acid into prostaglandin, and it is produced by macrophage when stimulation occurred by LPS, $\text{TNF-}\alpha$, $\text{IL-1}\beta$, IL-6 , and etc. Overexpression of COX-2 has been demonstrated in different animal models of inflammation and tumors³⁷. And then, nitric oxide synthase (NOS) gene has three isoforms: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) and iNOS synthesize nitric oxide that operate as described before. iNOS is absent in resting cells, but it's expressed in response to proinflammatory stimuli such as cytokines. Expressed iNOS cause many inflammatory diseases, therefore iNOS has to be regulated very tightly³⁸. Hence, to control inflammation, suppression of COX-2 and iNOS is a key element. As shown in figure 7, LPS induced COX-2 and iNOS expansion definitely. In concentration 200 $\mu\text{g}/\text{ml}$, RF inhibited COX-2 and

iNOS expression more than AEBSF. Also, the inflammatory cytokines ($\text{IL-1}\beta$, IL-6 , $\text{TNF-}\alpha$) that stimulate COX-2 and iNOS were tested as described. As shown in figure 6, RF treatment was valid to repress level of inflammatory cytokines that induced by LPS in high concentration.

$\text{NF-}\kappa\text{B}$ is a transcription factor that plays a critical role in inflammatory response. It is present in the cytoplasm, binding to the inhibitory protein $\text{I}\kappa\text{B}$ when the cells have no stimulus. But the cells are exposed to the stimulant such as LPS, $\text{I}\kappa\text{B}$ is phosphorylated and liberates $\text{NF-}\kappa\text{B}$, resulting in $\text{NF-}\kappa\text{B}$ translocation into the nucleus. Nuclear $\text{NF-}\kappa\text{B}$ then binds to the promoters of pro-inflammatory mediators, resulting in the induction of their gene expression include iNOS and COX-2³⁹⁻⁴⁰. $\text{NF-}\kappa\text{B}$ expression was evaluated by western blotting. As a result, high concentration of RF suppressed $\text{NF-}\kappa\text{B}$ expression in nucleus and the phosphorylation of $\text{I}\kappa\text{B}\alpha$ in cytosol was dose-dependently decreased (Fig 8). These results show that the anti-inflammation effects of RF were induced by suppression of $\text{NF-}\kappa\text{B}$ pathway.

In conclusion, RF has anti-oxidative and anti-inflammatory effects and it's a potential drug source for oxidative stress related inflammatory diseases such as rheumatoid arthritis, atherosclerosis, and many other diseases.

Acknowledgements

This study was supported by Dongguk University Research Fund.

References

1. Scrivo R, Vasile M, Bartosiewicz I, Valesini G. Inflammation as "common soil" of the multifactorial diseases. *Autoimmun Rev.* 2010 ; Epub ahead of print.
2. Xie W, Du L. Diabetes is an inflammatory disease: evidences from traditional Chinese Medicines. *Diabetes Obes Metab.* 2010 ; Epub ahead of print.
3. Gossiau A, Li S, Ho CT, Chen KY, Rawson NE. The importance of natural product characterization in studies of their anti-inflammatory activity. *Mol Nutr Food Res.* 2011 ; 55(1) : 74-82.
4. Olson N, van der Vliet A. Interactions between nitric oxide and hypoxia-inducible factor signaling pathways in inflammatory disease. *Nitric Oxide.* 2011 ; Epub ahead of print

5. Marchesi VT. Alzheimer's dementia begins as a disease of small blood vessels, damaged by oxidative-induced inflammation and dysregulated amyloid metabolism: implications for early detection and therapy. *FASEB J.* 2011 ; 25(1) : 5-13.
6. Mendoza-Núñez VM, Rosado-Pérez J, Santiago-Osorio E, Ortiz R, Sánchez-Rodríguez MA, Galván-Duarte RE. Aging Linked to Type 2 Diabetes Increases Oxidative Stress and Chronic Inflammation. *Rejuvenation Res.* 2011 ; Epub ahead of print.
7. Elzaawely AA, Xuan TD, Tawata S. Antioxidant and antibacterial activities of *Rumex japonicus* HOUTT. Aerial parts. *Biol Pharm Bull.* 2005 ; 28(12) : 2225-30.
8. Jang DS, Kim JM, Kim J, Yoo JL, Kim YS, Kim JS. Effects of compounds isolated from the fruits of *Rumex japonicus* on the protein glycation. *Chem Biodivers.* 2008 ; 5(12) : 2718-23.
9. Elzaawely AA, Xuan TD, Tawata S. Antioxidant and antibacterial activities of *Rumex japonicus* HOUTT. Aerial parts. *Biol Pharm Bull.* 2005 ; 28(12) : 2225-30.
10. Park YK. Studies on the anti-oxidative effects of *Rumexis Radix*. *Kor. J. Herbology.* 2003 ; 18(4) : 269-277.
11. Cho KH, Kim HS. A study of antifungal activity with *Rumex Japonicus* Houttuyn. *The Korean journal of Dermatology.* 1980 ; 18(5) : 383-389.
12. Lee HS, Kim SK, Han JB, Choi HM, Park JH, Kim EC, Choi MS, An HJ, Um JY, Kim HM, Min BI. Inhibitory effects of *Rumex japonicus* Houtt. on the development of atopic dermatitis-like skin lesions in NC/Nga mice. *Br J Dermatol.* 2006 ; 155(1) : 33-8.
13. Kumari A, Yadav SK, Pakade YB, Kumar V, Singh B, Chaudhary A, Yadav SC. Nanoencapsulation and characterization of *Albizia chinensis* isolated antioxidant quercitrin on PLA nanoparticles. *Colloids Surf B Biointerfaces.* 2011 ; 82(1) : 224-32.
14. Jang DS, Kim JM, Kim JH, Kim JS. 24-nor-Ursane type triterpenoids from the stems of *Rumex japonicus*. *Chem Pharm Bull (Tokyo).* 2005 ; 53(12) : 1594-6.
15. Koyama J, Morita I, Kawanishi K, Tagahara K, Kobayashi N. Capillary electrophoresis for simultaneous determination of emodin, chrysophanol, and their 8-beta-D-glucosides. *Chem Pharm Bull (Tokyo).* 2003 ; 51(4) : 418-20.
16. Gyamfi MA, Yonamine M, Aniya Y. Free-radical scavenging action of medicinal herbs from Ghana. *Gen Pharmacol.* 1999 ; 32 : 661-7.
17. Gotoh N, Niki E. Rates of interactions of superoxide with vitamin E, vitamin C and related compounds as measured by chemiluminescence. *Biochim Biophys Acta.* 1992 ; 1115 : 201-7.
18. Nagata N, Momose K, Ishida Y. Inhibitory effects of catecholamines and anti-oxidants on the fluorescence reaction of 4,5-diaminofluorescein, DAF-2, a novel indicator of nitric oxide. *J Biochem.* 1999 ; 125 : 658-61.
19. Kooy, N.W., Royall, J.A. Ischiropulos, H. and Beckman, J.S. Peroxynitrite-mediated oxidation of dihydrorhodamine 123. *Free Radical Biol. Med.* 1994 ; 16 : 149-56.
20. Rong Y, Geng Z, Lau BH. Ginko biloba attenuates oxidative stress in macrophages and endothelial cells. *Free Radic Biol Med.* 1996 ; 20 : 121-7.
21. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radic Biol Med.* 1999 ; 27 : 612-6.
22. Wang S, Chen Y, He D, He L, Yang Y, Chen J, Wang X. Inhibition of vascular smooth muscle cell proliferation by serum from rats treated orally with *Gastrodia* and *Uncaria* decoction, a traditional Chinese formulation. *J Ethnopharmacol.* 2007 ; 114(3) : 458-62.
23. Yoon SB, Lee YJ, Park SK, Kim HC, Bae H, Kim HM, Ko SG, Choi HY, Oh MS, Park W. Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. *J Ethnopharmacol.* 2009 ; 125(2) : 286-90.
24. Liu L, Guo Z, Lv Z, Sun Y, Cao W, Zhang R, Liu Z, Li C, Cao S, Mei Q. The beneficial effect of *Rheum tanguticum* polysaccharide on protecting against diarrhea, colonic inflammation and ulceration in rats with TNBS-induced colitis: the role of macrophage mannose receptor in inflammation and immune response. *Int Immunopharmacol.* 2008 ; 8(11) : 1481-92.
25. Kim JM, Jung HA, Choi JS, Min BS, Lee NG. Comparative analysis of the anti-inflammatory activity of Huang-lian extracts in lipopolysaccharide-stimulated RAW264.7 murine macrophage-like cells using oligonucleotide microarrays. *Arch Pharm Res.* 2010 ; 33(8) : 1149-57.
26. Heo J. Dongeubogam. Seoul, Bubinsa. 1999 ; p1955.
27. Moon JK, Shibamoto T. Antioxidant assays for plant and food components. *J Agric Food Chem.* 2009 ; 57(5) : 1655-66.
28. Kryston TB, Georgiev AB, Pissis P, Georgakilas

- AG. Role of Oxidative stress and DNA Damage in Human Carcinogenesis. *Mutat Res.* 2011 ; Epub ahead of print.
29. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res.* 2000 ; 87(10) : 840-4.
30. Moon JK, Shibamoto T. Antioxidant assays for plant and food components. *J Agric Food Chem.* 2009 ; 57(5) : 1655-66.
31. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circ Res.* 2010 ; 107(9) : 1058-70.
32. Salvemini D, Doyle TM, Cuzzocrea S. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. *Biochem Soc Trans.* 2006 ; 34(5) : 965-70.
33. Lonkar P, Dedon PC. Reactive species and DNA damage in chronic inflammation: Reconciling chemical mechanisms and biological fates. *Int J Cancer.* 2010 ; Epub ahead of print.
34. Diatchuk V, Lotan O, Koshkin V, Wikstroem P, Pick E. Inhibition of NADPH oxidase activation by 4-(2-aminoethyl)-benzenesulfonyl fluoride and related compounds. *J Biol Chem.* 1997 ; 272(20) : 13292-301.
35. Sakata D, Yao C, Narumiya S. Prostaglandin E2, an immunoactivator. *J Pharmacol Sci.* 2010 ; 112(1) : 1-5.
36. Bannenberg GL. Therapeutic applicability of anti-inflammatory and proresolving polyunsaturated fatty acid-derived lipid mediators. *Scientific World Journal.* 2010 ; 10 : 676-712.
37. Pratheeshkumar P, Kuttan G. Cardiospermum halicacabum inhibits Cyclophosphamide Induced Immunosuppression and Oxidative Stress in Mice and also Regulates iNOS and COX-2 Gene Expression in LPS Stimulated Macrophages. *Asian Pac J Cancer Prev.* 2011 ; 11(5) : 1245-52.
38. Qidwai T, Jamal F. Inducible nitric oxide synthase (iNOS) gene polymorphism and disease prevalence. *Scand J Immunol.* 2010 ; 72(5) : 375-87.
39. Choi RJ, Shin EM, Jung HA, Choi JS, Kim YS. Inhibitory effects of kaurenoic acid from *Aralia continentalis* on LPS-induced inflammatory response in RAW264.7 macrophages. *Phytomedicine.* 2011 ; Epub ahead of print.
40. Bonvissuto G, Minutoli L, Morgia G, Bitto A, Polito F, Irrera N, Marini H, Squadrito F, Altavilla D. Effect of *Serenoa repens*, lycopen, and selenium on proinflammatory $I\kappa B-\alpha$ phenotype activation: an *in vitro* and *in vivo* comparison study. *Urology.* 2011 ; 77(1) : 248.e9-16.