



The Anti-inflammatory Effect and DPPH Free Radical Scavenging Capability of *Rhizoma drynariae* Aqueous Extract

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골쇄보 열수추출물 약침액의 항염증반응 및 DPPH 라디칼 소거능 활성에 대한 연구

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Objectives : This study is to investigate the effects of *Rhizoma drynariae* aqueous extract(RDA) on cell cytotoxicity, Nitric Oxide (NO) and Prostaglandin E₂(PGE₂) production and 1,1-diphenyl-2-picryl ghdrazyl(DPPH) free radical scavenging capability. **Methods :** Cell cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay. The production of NO was measured by Griess assay. The production of PGE₂ was measured by immunoassay. And, the anti-oxidant activity was measured by the DPPH method. **Results :** Cell cytotoxicity in 50, 100, 200 and 400 μg/ml RDA did not increase significantly compared to the RDA untreated group. RDA(200 μg/ml and 400 μg/ml) inhibited NO and PGE₂ production in lipopolysaccharide-stimulated RAW 264.7 cells. RDA had high DPPH free radical scavenging capability. **Conclusions :** This study indicates that RDA inhibits NO and PGE₂ production in lipopolysaccharide-stimulated RAW 264.7 cells and improve DPPH free radical scavenging capability. RDA may have an anti-inflammation effect and an anti-oxidant activity.

Key words : anti-inflammation, anti-oxidant activity, Nitric Oxide, Prostaglandin E₂, *Rhizoma drynariae*

Introduction

Inflammation is a local, protective response of the immune system. Excessive inflammatory responses can be harmful, as in diseases such as rheumatoid arthritis, Alzheimer's disease and septic shock syndrome¹. Lipopolysaccharide(LPS), a component of the cell wall of Gram-negative bacteria, stimulates macrophages to produce pro-inflammatory mediators such as tumor necrosis factor alpha, interleukin-6, and inducible

nitric oxide synthase, which trigger a cascade responsible for the inflammatory response².

Of these, nitric oxide(NO) is endogenously generated from L-arginine by nitric oxide synthase(NOS) and plays an important role in the regulation of many physiological processes³. In addition, prostaglandins(PGs) are key inflammatory mediator, converted from arachidonic acid by cyclooxygenase⁴. Antioxidants can protect against the damage caused by free radicals that have been implicated in the etiology of large

Received November 24, 2015, Revised December 10, 2015, Accepted December 14, 2015

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This paper was supported by the Semyung University Research Grant of 2013.

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number of major diseases(arterosclerosis, cancer, cardiac diseases, aging, brain diseases etc.)⁵⁾.

Rhizoma drynariae(*R. drynariae*) is made from the dried roots of *Drynaria fortunei* of the polypodiaceae family. It is bitter taste, providing inner warmth, and has meridian tropism for the liver and kidney. It can tone the kidney, improve blood circulation, reduce bleeding, and mediate injury healing. It is consistently used in the clinical treatment of kidney disease and bone injury⁶⁾. There are many reports of modern pharmacological research on *R. drynariae*, mainly focusing on the treatment of bone disorders and fractures^{7,8)}. *R. drynariae* is also known to be effective for the treatment of inflammation, hyperlipidemia, arteriosclerosis, rheumatism, and gynecological diseases such as osteoporosis and bone resorption in oriental medicine⁹⁾. Liu et al¹⁰⁾ have also shown that *R. drynariae* has an antioxidant effect on rat osteoblasts from hydrogen peroxide-induced death.

Although many studies have been previously reported about the medical effects of *R. drynariae*, the mechanism of the anti-inflammatory responses and anti-oxidant effects has not been elucidated. In this study, we investigated the anti-inflammatory and the anti-oxidant effects of *R. drynariae* using by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay, nitric oxide(NO) and prostaglandin E₂(PGE₂) production and 1,1-diphenyl-2-picryl hydrazyl (DPPH) assay.

Materials and Methods

1. Cell culture

Cells of the murine macrophage RAW 264.7 were purchased from the Korean Cell Line Bank(KCLB, Seoul, Korea). Cells were cultured in Dulbecco's Modified Eagle Medium(DMEM)(Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum(FBS)(Gibco BRL) at 37°C in 5% CO₂, 95% O₂ in a humidified cell incubator. Cells were plated in culture dish (Corning Incorporated, Corning, NY, USA) at a density of 1×10⁶ cells per dish, and the media was changed once every 2 days.

2. Preparation of extract

To obtain the water extract of *R. drynariae*, 200 g of *R. drynariae* was added to distilled water, and extraction was performed by heating at 80°C concentrated with a rotary evaporator and lyophilized. The resulting powder, weighing 30 g, was dissolved in saline.

3. MTT cytotoxicity assay

Cell viability was determined by the MTT assay kit using as per the manufactures protocol. Cells were cultured in 96 well plates. Experimental groups are exposed to *R. drynariae* aqueous extract(RDA) at final concentrations of 50, 100, 200 and 400 μg/ml for 24 hrs, and saline of an equal volume was added to untreated group. Ten ml of the MTT labeling reagent was then added to each well, and the plates were incubated for 4 hrs. After the cells were incubated in 100 ml of the solubilization solution for 12 hrs, the absorbance was measured with a microtiter plate reader(Bio-Tek, Winooski, VT, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density(O.D.) was calculated as the result of the subtraction of the absorbance at the reference wavelength from that of the test wavelength. Percent viability was calculated as(O.D. of drug-treated sample/control O.D.)×100.

4. NO assay

The concentration of NO in the culture supernatants was determined by measuring nitrite, a major stable product of NO, using the Griess reagent. Briefly, Cells were plated onto 24-well plates and pretreated with the various concentrations of RDA 1 hr prior to stimulation with 1 μg/ml of LPS for 24 hrs. Supernatant samples were mixed with equal volume of Griess reagent(1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) and the incubated at room temperature for 10 min. The absorbance was measured at 540 nm on a microplate reader (Thermo electron corporation, Marietta, OH).

5. Measurement of PGE₂

Enzyme-linked immunosorbent assay(ELISA) kit(R&D Systems,

Minneapolis, MN, USA) was used to measure PGE₂ production according to the manufacturer's instructions. Cells were plated in 24-well plates and pretreated with the indicated concentrations of RDA 1 hr prior to stimulation with 1 μg/ml LPS for 24 hrs. One hundred microliters of culture media were collected for the determination of PGE₂ concentrations by ELISA according to the manufacturer's instructions.

6. Assessment of DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to previous studies¹¹⁾ with a few modifications. Briefly, 2 mL of 0.2 mM methanolic solution of DPPH radicals were added to 2 mL of water-solution of RDA at various concentrations. The absorbance of the mixture was measured at 517 nm after 30 min of incubation at 37°C in the dark. Ascorbic acid was used as the control and distilled water as the blank. The scavenging effect was calculated according to the following equation: Scavenging rate%=(1-A_s/A₀)×100%, where A_s is the absorbance obtained for a sample and A₀, the absorbance of the blank.

7. Statistical analysis

Statistical analysis was performed using Student's *t*-test (SPSS 11.0 software) and the results were expressed as mean±S.E.M. Differences were considered significant for *p*<0.05.

Results

1. MTT assay for cell viability

In order to find out the concentration at which the cytotoxic effect of RDA on the RAW 264.7 cell line become evident, cells were cultured with RDA at final concentrations of 50 μg/ml, 100 μg/ml, 200 μg/ml, and 400 μg/ml for 24 hrs, and MTT assays were carried out, with cells cultured in RDA-free media as the control. The viabilities of cells incubated with RDA at concentrations of 50 μg/ml, 100 μg/ml, 200 μg/ml and 400 μg/ml were 94.48±4.78%, 94.09±5.21%, 92.53±7.68% and 92.82±5.51% of the control value(100.00±1.92%) respectively. As shown in Fig. 1, RDA, at concentrations 0~400 μg/ml, showed no obvious cytotoxicity on RAW264.7 cells.

2. Measurement of NO production

To determine the effects of RDA on NO production in RAW 264.7 cells, the cells were pre-incubated with various concentrations of RDA for 1 hr and then stimulated with 1 μg/ml of LPS for 24 hrs. The control group was not treated with LPS or RDA. Supernatant from cell culture media was collected, and NO levels were determined with the Griess assay. RDA was found to inhibit LPS-induced NO productions in a dose-dependent manner. The NO production of RDA at concentrations of 0 μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml

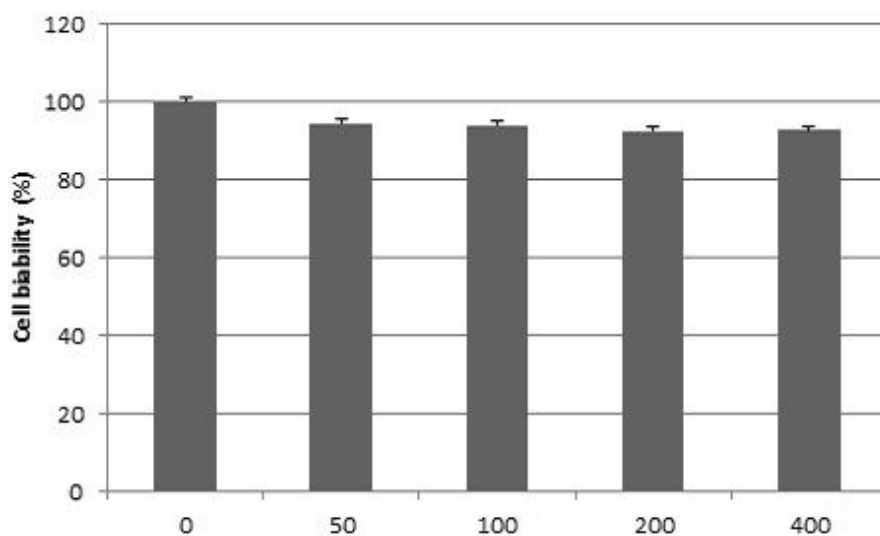


Fig. 1. Cytotoxic effects of *Rhizoma drynariae* aqueous extract(RDA). RAW 264.7 cells were incubated with RDA at various concentrations (0, 50, 100, 200 and 400 μg/ml) prior to the determination of cellular viability through MTT assay. Results were represented as mean±standard error.

and 400 $\mu\text{g/ml}$ were $13.52 \pm 0.83\%$, $97.60 \pm 5.30\%$, $94.85 \pm 5.46\%$, $76.28 \pm 4.88\%$ and $66.42 \pm 5.23\%$ of the control value ($100 \pm 5.06\%$) respectively. RDA significantly inhibited NO production at concentrations of 200 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ (Fig. 2).

3. Measurement of PGE₂ production

To determine the effects of RDA on PGE₂ production in RAW 264.7 cells, the cells were pre-incubated with various concentrations of RDA for 1 hr and then stimulated with 1 $\mu\text{g/ml}$ of LPS for 24 hrs. The control group was not treated with LPS or RDA. Supernatant from cell culture media was collected, and PGE₂ levels were determined with the EIA kit. RDA was found to inhibit LPS-induced PGE₂ productions in a dose-dependent manner. The PGE₂ production of RDA at concentrations of 0 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ were $4.70 \pm 0.64\%$, $100.96 \pm 5.82\%$, $98.36 \pm 7.31\%$, $84.88 \pm 3.76\%$ and $82.55 \pm 6.06\%$ of the control value ($100.00 \pm 6.56\%$) respectively. RDA significantly inhibited PGE₂ production at concentrations of at 200 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ (Fig. 3).

4. Measurement of DPPH radical scavenging activity

Scavenging of DPPH radicals is the basis of a common antioxidant assay. Antioxidants can protect against the damage caused by free radicals that have been implicated in

the etiology of large number of major diseases. RDA displayed concentration dependent radical scavenging effects. RDA were $4.76 \pm 1.88\%$, $24.43 \pm 3.14\%$, $61.86 \pm 2.15\%$ and $77.03 \pm 4.64\%$ at concentrations of 50 $\mu\text{g/ml}$, 100 $\mu\text{g/ml}$, 200 $\mu\text{g/ml}$ and 400 $\mu\text{g/ml}$ (Fig. 4).

Discussion

The purpose of this study is to investigate the effects of RDA on the production of NO and PGE₂ induced by LPS-stimulated RAW 264.7 cells and DPPH free radical scavenging capability.

Inflammation is a complex process regulated by a variety of immune cells and effector molecules. NO, PGE₂, and pro-inflammatory cytokines are important mediators of macrophage-mediated inflammation^{12,13}. Therefore, the inhibition of these mediators with pharmacological modulators may be an effective therapeutic strategy for preventing inflammatory reactions and diseases¹⁴.

Macrophages play critical roles in immune reactions, allergy, and inflammation¹⁵. These cells induce inflammatory reactions, and initiate and maintain specific immune responses by releasing different types of cytokines. LPS, a component of the gram-negative bacterial cell wall, has often been used in inflammatory response because it can activate

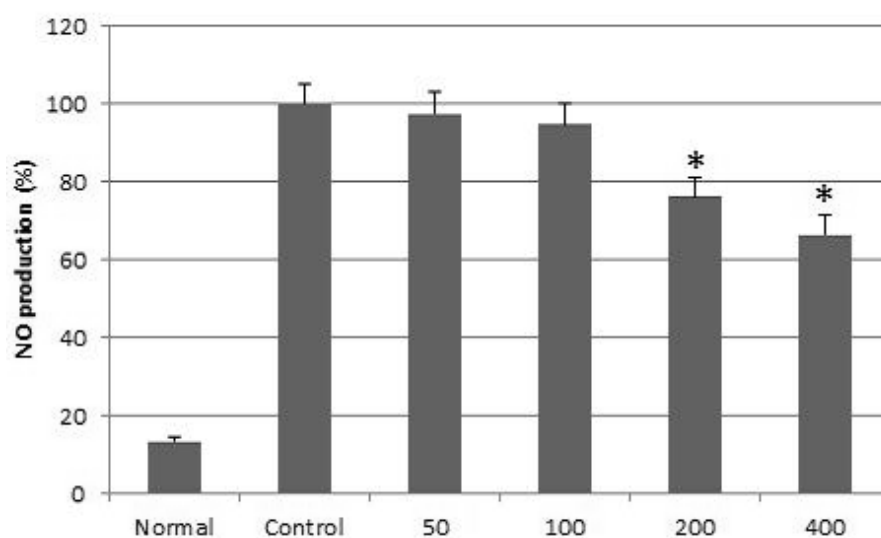


Fig. 2. Effects of *Rhizoma drynariae* aqueous extract(RDA) on the nitric oxide(NO) production in RAW 264.7 cells. Normal, untreated group : Control, treated with LPS(1 $\mu\text{g/ml}$) ; 50~400, treated with LPS and RDA(50~400 $\mu\text{g/ml}$). *represents $p < 0.05$ compared to the Control group.

macrophages¹⁶.

In general, NO plays an important role in the antitumour, antiviral replication and other diseases^{17,18}, the overproduction of NO is harmful to the host, leading to rheumatoid arthritis¹⁹ and allograft rejection²⁰. NO production from macrophages can be induced by inflammatory cytokines or bacterial products, including LPS, IFN-gamma, or TNF-alpha²¹.

PGE₂ is considered the one of the strongest inflammatory mediators in inflammatory response. It was transformed from arachidonic acid via the cyclooxygenase-2 (COX-2) catalytic reaction. Nonsteroidal anti-inflammatory drugs (NSAIDs),

which were used widely in current clinical, play their anti-pyretic, anti-inflammatory and analgesic effects through the inhibition of COX activity and the reduction of inflammatory mediator production such as PGE₂²².

Oxidative stress is an important factor in the genesis of most pathologies, ranging from cancer to cardiovascular and degenerative diseases²³. In order to protect the body against the consequences of oxidative stress, an efficacious approach consists in improving the anti-oxidant nutrition. Anti-oxidants from natural sources have a higher bioavailability and therefore higher protective efficacy than synthetic anti-oxidants²⁴.

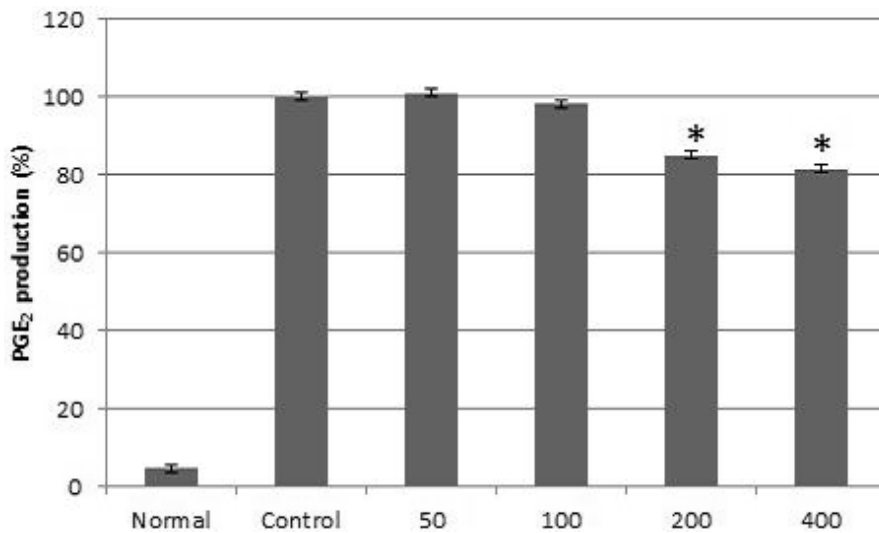


Fig. 3. Effects of *Rhizoma drynariae* aqueous extract(RDA) on the prostaglandin E₂(PGE₂) production in RAW 264.7 cells.

Normal, untreated group ; Control, treated with LPS(1 μg/ml) ; 50~400, treated with LPS and RDA(50~400 μg/ml). *represents $p < 0.05$ compared to the Control group.

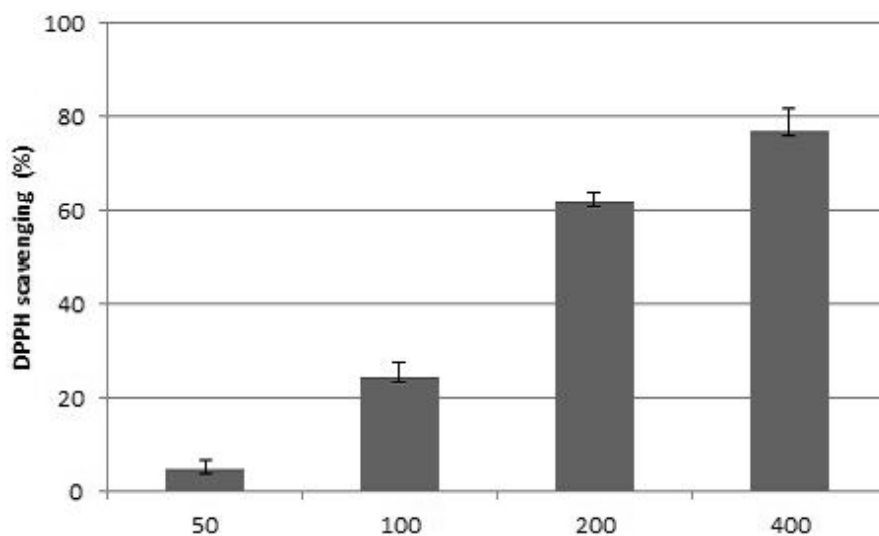


Fig. 4. DPPH free radical scavenging capability of *Rhizoma drynariae* aqueous extract(RDA).

The absorbance of the RDA (50~400 μg/ml) was measured at 517 nm. Results were represented as mean±standard error.

Free radicals are chemical species with one or two unpaired electrons in their outermost layer, which can be created in a multiple ways. They can be exogenic (e.g. ultraviolet radiation, pollution, infections, tobacco) or endogenic. A lack of anti-oxidant or an overproduction in free radicals can lead to an imbalance between the oxidant and anti-oxidant system. One of the most significant factors in the production of free radicals is oxidative stress²⁵⁻²⁸. Oxidative stress is involved in several illnesses, including diabetes^{29,30}, atherosclerosis, Alzheimer's disease, Parkinson's disease, glaucoma and age-related macular degeneration^{31,32}. The provision of anti-oxidants through diet or herb-medicine is a simple means to reduce the development of illnesses brought on by oxidative stress^{33,34}.

RDA has effects on reinforcing kidney, improving circulation, reduce bleeding, air wound healing, etc. In the clinical setting, RDA is consistently used for the treatment of kidney dysfunction as well as traumatic fractures. In the ancient monographs of orthopedics and traumatology, RDA had a very high frequency of prescription. Under these influences, modern pharmacological studies mainly focus on the treatment of bone fractures and bone diseases^{35,36}. Lee et al³⁷ reported that RDA inhibited proliferation of mitogen (phytohaemagglutinin) and antigen (purified protein derivative)-stimulated human peripheral blood mononuclear cells. In addition, RDA inhibited growth of several cell lines of mouse and human origin. Current Korean Medical treatments are not only confined to acupuncture, moxibustion, and herbal medication. Rather, various other treatments have been developed and are already being used clinically³⁸. Therefore, it may be useful for the development of aqua-acupuncture.

Currently, there are few reports on the effects of RDA on the inflammatory disease. In our study, we investigated that RDA inhibits the production of NO and PGE₂ induced by LPS-stimulated RAW 264.7 cells and improve DPPH free radical scavenging capability. RDA may have an anti-inflammation effect and anti-oxidant activity. Therefore, we believe that RDA may be useful for the patients with several inflammation diseases.

Acknowledgement

This paper was supported by the Semyung University Research Grant of 2013.

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국문초록

목적 : 한의학에서 골절이나 신장질환에 사용되어 왔던 골쇄보열수추출물 약침액이 항염증 및 항산화활성에 대한 효능이 있는지를 연구하고자 한다. **방법** : 골쇄보열수추출물 약침액이 세포독성에 미치는 영향을 관찰하기 위하여 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) assay를 실시하였다. 골쇄보열수추출물 약침액이 항염증효능에 미치는 영향을 관찰하기 위하여 lipopolysaccharide(LPS)로 유도된 RAW264.7 대식세포에서의 산화질소(NO) 및 프로스타글란딘(PGE₂) 생성 억제력을 관찰하였다. 또한 골쇄보열수추출물 약침액이 항산화활성에 미치는 영향을 관찰하기 위하여 1,1-diphenyl-2-picrylhydrazyl(DPPH) radical 소거능을 관찰하였다. **결과** : 골쇄보열수추출물 약침액은 50~400 µg/ml 농도에서 세포독성이 나타나지 않았다. 골쇄보열수추출물 약침액은 200 및 400 µg/ml 농도에서 LPS로 유도된 NO 및 PGE₂ 생성을 통계학적으로 유의하게 감소시켰다. 골쇄보추출물은 50~400 µg/ml 농도에서 DPPH 소거능을 관찰한 결과 농도의존적으로 활성화되는 것을 관찰하였다. **결론** : 골쇄보열수추출물 약침액은 항염증 및 항산화 효과가 있을 것으로 사료된다.