

Anti-oxidant activity with inhibition of osteoclastogenesis by *Atractylodes Rhizoma* extract

S.H. LEE, S.J. KIM, S.-J. KIM

Department of Pharmacology and Toxicology, School of Dentistry, Kyung Hee University, Seoul, Korea

Abstract. – BACKGROUND AND OBJECTIVES: *Atractylodes Rhizoma* is commonly used in Korean folk medicine for digestion, diuresis, sedation, antibacterial agent. The present study was performed to explore the antioxidant capacities of methanolic extract of *Atractylodes Rhizoma*. In addition, its effect on the expression of inducible nitric oxide synthase (iNOS) in response to lipopolysaccharides (LPS) and osteoclastogenesis in macrophage cells was also tested.

MATERIALS AND METHODS: The anti-oxidative activities were tested by measuring free radical scavenging activity (DPPH, NO) and reducing power. The potential mechanism of anti-oxidative action of *Atractylodes Rhizoma* extract was determined by performing Western blot analysis for iNOS expression in LPS-stimulated RAW 264.7 cells. The RAW cells were induced to osteoclastic cells by RANKL and LPS and the *Atractylodes Rhizoma* extract was tested to determine whether it inhibits osteoclastogenesis of the cells.

RESULTS: The extract exerted significant NO and DPPH radical scavenging activity and it had dramatic reducing power. Induction of iNOS and NO by LPS was significantly inhibited by the extract, suggesting that the *Atractylodes Rhizoma* extract inhibits nitric oxide (NO) production by suppressing iNOS expression. Strikingly, the *Atractylodes Rhizoma* extract significantly inhibited the osteoclastic differentiation of RAW cells. The *Atractylodes Rhizoma* extract contains significant amount of anti-oxidant components including phenolics, flavonoids and anthocyanins.

CONCLUSIONS: These results suggest that methanolic extract of *Atractylodes Rhizoma* exerts remarkable anti-oxidant activity potentially via inhibiting free radicals and iNOS induction, those could lead to the inhibition of osteoclastogenesis.

Key Words:

Antioxidant, Osteoclastogenesis, *Atractylodes rhizoma*, iNOS, Nitric Oxide.

lodes lancea DC., *Atractylodes chinensis* KOIDZUMI, and *Atractylodes koreana* NAKAI. It is widely used in the oriental medicine for digestion, diuresis, sedation, antibacterial agent¹. In addition, it has been reported that water extract of *Atractylodes Rhizoma* has anti-arthritis action². It contains β -eudesmol, furaldehyde, atractyrodin, atractylodinol, atractylosides A-I and acetyl atractylodinol, etc³.

Many detrimental diseases such as periodontal disease, metabolic bone diseases, diabetes mellitus, atherosclerosis, neurodegenerative diseases are associated with the increase of reactive oxygen species (ROS), leading to impose oxidative stress in the body. It has been widely accepted that the overproduction of free radicals more than their scavenging by anti-oxidative defense system in the body, plays an essential role in the damaging cellular components such as lipid, protein, carbohydrate and DNA⁴⁻⁷. Reactive oxygen species (ROS) contain superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^\bullet)⁸, and they are produced during various cellular aerobic metabolism and as by-products of many enzyme reactions including prostaglandin synthesis by the mitochondrial respiratory chain. Nitric oxide (NO) is produced by inducible nitric oxide synthetase (iNOS) in the mitochondrial respiratory chain and is associated with the production of reactive nitrogen species (RNS)⁹, which is responsible for the excessive production of lipid peroxidation. Considering the toxic effects of ROS in the cells, removing free radicals (active oxygen) are essential to protect cells against oxidative damages. Maintaining functional antioxidant system and sufficient supply of antioxidant molecules are important in preventing the production and scavenging ROS (reactive oxygen species) which are essential risk factors in the onsets of oxidative stress-associated diseases¹⁰. iNOS is a key enzyme responsible for the production of nitric oxide (NO) that plays an im-

Introduction

Atractylodes Rhizoma is the dried root parts of *Atractylodes japonica* KOIDZUMI, *Atracty-*

portant role in the oxidative stress, inflammation, bone metabolism and vascular tone¹⁰. In this study, we sought to determine if 70% methanol extract of *Atractylodes Rhizoma* has anti-oxidative activities associated iNOS system. It has been also tested whether the *Atractylodes Rhizoma* extract could exert any effect on the osteoclastogenesis.

Materials and Methods

Preparation of Plant Extracts

Authentic samples of *Atractylodes Rhizoma* were purchased from Kyung-Dong Oriental Market in Seoul. They were authenticated by Emeritus Professor Chang-Soo Yook, Department of Oriental Pharmacy, Kyung Hee University, Seoul, KOREA. A voucher specimen (No. 00-14) was deposited at the Herbarium of the Department of Pharmacology, School of Dentistry, Kyung Hee University, Seoul, KOREA. They were collected during early autumn season. *Atractylodes Rhizoma* (100 g) was cut into small pieces and extracted with 70% methanol (300 mL) for 3 h three times. The resulting methanol extract was concentrated by rotary evaporator and dried by freeze-dryer.

Reagents and Materials

The iNOS and GAPDH (glyceraldehydephosphatedehydrogenase) antibodies were purchased from Cell Signaling and Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA), respectively. The ECL kit was bought from Amersham Co (Little Chalfant, UK). Other reagents were bought from Sigma Co (St Louis, MO, USA). All cell culture media were purchased from Gibco Co (Grand Island, NY, USA).

Scavenging of DPPH (2,2-diphenyl-1-picrylhydrazyl)

DPPH radical scavenging activity was measured by evaluating the ability to remove DPPH under the principle of reduction reactions of DPPH radical solutions in the presence of hydrogen-donating antioxidants¹¹. Briefly, *Atractylodes Rhizoma* extract dissolved in 1 ml MeOH was mixed with 1 ml DPPH solution at 23°C and optical density was measured in 30 minutes at 515 nm.

Scavenging of NO

NO generated from sodium nitroprusside was measured by using the Greiss reagent¹². The ex-

tract was added to 0.2 ml of nitroprusside (10 mM) and 1.8 ml of phosphate buffer (pH 7.4). The reaction mixture was allowed to incubate at 37°C for 3h. 1 ml aliquot of the reaction mixture was diluted with 0.5 ml of Greiss reagent and subjected to measure absorbance at 540 nm by using spectrophotometer.

Measurement of Oxidation of Deoxyribose

The experiment was carried out as described by Halliwell et al¹³. The reaction mixture (1.4 mL) containing extracts (0.2 mL), deoxyribose (6 mM) (Fluka, St Louis, MO, USA), H₂O₂ (3 mM), FeCl₃ (400 μM), ethylenediaminetetraacetic acid (400 μM), and ascorbic acid (400 μM) in phosphate buffer (20 mM, pH 7.4) was incubated at 37°C for 1 h. The extent of deoxyribose degradation was measured by the use of the thiobarbituric acid method. One milliliter of thiobarbituric acid (1%, w/v) and 1.0 mL of trichloroacetic acid (2.8%, w/v) were added to the mixture, and allowed to be heated in a water bath at 90 °C for 20 min. The absorbance of the mixture was read spectrophotometrically at 532 nm.

Determination of Reducing Power

The reducing power of *Atractylodes Rhizoma* extract was measured using the Oyaizu's method¹⁴. 2.5 ml of *Atractylodes Rhizoma* extract (0.2M phosphate buffer, pH 6.6) was put into 2.5 ml of potassium ferricyanide (10 mg/ml) solution and made to react for 15 minutes at 30°C. 2.5 ml of Trichloroacetic acid (100 mg/ml) was put into the reactant and mixed up and 2.5 ml of the mixture was again mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride (1.0 mg/ml) and optical density was measured at 700 nm.

Cell Culture

Murine RAW 264.7 macrophage cells were cultured in DMEM: Dulbecco's modified Eagle's Medium (Gibco BRL, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum in 5% humidified CO₂ atmosphere at 37°C.

Measurement of Nitric Oxide (NO)

Raw cells were cultured with DMEM and 10% FBS (Fetal Bovine Serum). NO was measured by measuring the amount of nitric oxide (NO) in the cell supernatant as nitrite and nitrate. The safe form of nitrite after being reduced to nitrate was measured using the Greiss reagent (Sigma, St Louis, MO, USA). 2×10⁶ Raw cells

were put into a 6 well plate and washed two times with phosphate buffered saline (PBS) when the confluence was 80% and then cultured for at least 24 hours and the samples were made into the final concentrations of 10, 1.0, 0.1 mg/ml for experiments. Four hours later, LPS (Lipolysaccharide) of the final concentration 1 mg/ml was put into all wells except for the well for the control group to stimulate the samples. The amounts of NO generated were measured by gathering the supernatant around 18 hours later, having them react with the Greiss reagent for 10 minutes after shading and then measuring the optical density at 540 nm.

Measurement of iNOS Expression by Western Blotting

When the cells reached confluence, the DMEM culture medium was removed and replaced by the EMEM (eagle's minimal essential medium) culture medium which is a serum-free culture medium and then the cells were treated with *Atractylodes Rhizoma* extracts and cultured for 24 hrs. The cells were washed two times with PBS and scraped into a buffer containing 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM EDTA, 30 mM NaF, 0.1 mM Na₃VO₄, 1% triton X-100, 0.5% NP-40, 1 µg/ml leupeptin, 1 µg/ml aprotinin. Then, the cells were disrupted by passing them through a 1 ml tuberculin syringe five times. The cell lysate was subjected to centrifugation at 10,000 x g for 10 min and the supernatant was used for Western blot analysis. The protein content of the soluble fraction was assessed by the method of Bradford¹⁵. Protein (50 mg/lane) was electrophoretically separated in 10% polyacrylamide gels containing SDS (sodium dodecyl sulphate). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) was carried out for 1 h at 100 V (constant) as described by Towbin et al¹⁶. The filter papers were preincubated for 1 h at 23°C with PBS containing 0.1% Tween 20 and 5% skim milk and washed with PBS containing 0.1% Tween 20 three times for 10 min each. Followed by the blots were probed with primary antibody directed against iNOS (1:1,000) and GAPDH (1:1,000) for 2 h at room temperature or overnight at 4°C diluted in blocking buffer. The blots were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1,000 for iNOS and GAPDH) for 1 h at room temperature and washed with PBS containing Tween 20 three times for 10 min each. The detection of immobi-

lized specific antigens was carried out by electrochemiluminescence (ECL) (NEN). The images analysed using Image J software.

Determination of Osteoclastic Differentiation

2×10³ Raw cells were put into a 6 well plate and washed two times with PBS when the confluence was 80% and then cultured for at least 24 hours and the samples were made into the final concentrations of 10, 1.0, 0.1 mg/ml for experiments. Four hours later, RANKL (Receptor activator of nuclear factor kappa B ligand) (50 ng/ml) was put into all wells except for the well for the control group for 72 hrs and LPS of the final concentration 1 mg/ml was added to stimulate osteoclastic differentiation. After 6-7 days, TRAP (telomeric repeat amplification protocol) assay was performed according to the manufacturer's instruction to count multinucleated osteoclastic cell numbers (Sigma Cat # 387A).

Component Analysis (Anthocyanin, Phenolics, Flavonoids)

Measurement of Total Phenolics

The total phenolics content was measured using the Folin-Ciocalteu procedure at 725 nm¹⁷. Gallic acid was used as a standard for phenolic compounds and the phenolic concentration was calculated by using a gallic acid standard calibration curve. The total phenolics content was expressed as the gallic acid equivalent (mg gallic acid/g extract).

Measurement of Total Flavonoids

The total flavonoids existing was measured using the method of Miliuskasa et al¹⁸ and was expressed as the rutin equivalent (mg rutin acid/g extract) using rutin as a standard flavonoid. 1 ml of *Atractylodes Rhizoma* extract was mixed with aluminum thichloride in ethanol (20 mg/ml) and diluted to 25 ml. After incubation for 40 minutes at 20°C, the optical density was measured at 415 nm.

Measurement of Total Anthocyanin

The total anthocyanin was measured using color reactions¹⁹. *Atractylodes Rhizoma* extract was dissolved in 1 ml of acetate buffer (25 mM, pH 4.5) and the optical density was measured at 520 nm. The content of anthocyanin was expressed as kouromanin equivalent (mg Kouromanin/g extract).

Statistical Analysis

All data were expressed as mean \pm SEM. Statistical analysis was performed using the Graph-Pad Prism 5 with one-way ANOVA followed by Tukey's multiple comparison test. $p < 0.05$ was considered as significant.

Results

DPPH free radicals were decreased by approximately 37% at 0.01 mg/ml of *Atractylodes Rhizoma* extract and about 41% at 0.1 mg/ml as compared to the control. DPPH free radicals were decreased by 48% at 0.1 mg taurine (Figure 1).

Nitric Oxide scavenging activity was also measured. *Atractylodes Rhizoma* extract had the ability to scavenge NO by 31% and 29% as compared to control at 0.01 mg/ml and 0.1 mg/ml, respectively. Taurine exerted only 14% decrease of NO levels at 1 mg/ml (Figure 2).

The deoxyribose oxidation was suppressed by 18% by the *Atractylodes Rhizoma* extract at the concentration of 10 mg/ml (Figure 3); however, there was little effect observed at 0.1 mg/ml and 1 mg/ml,

Regarding reducing power of *Atractylodes Rhizoma* extract, it had the reducing power of 13.5-fold as compared to control at 0.1 mg/ml, respectively, whereas taurine at 0.1mg/ml have the reducing power of 1.8-fold over control, respectively (Figure 4).

The amount of nitric oxide production markedly increased by 2.5-fold over control when the Raw cells were treated with LPS (lipopolysaccharide) to activate the macrophages whereas when the cells

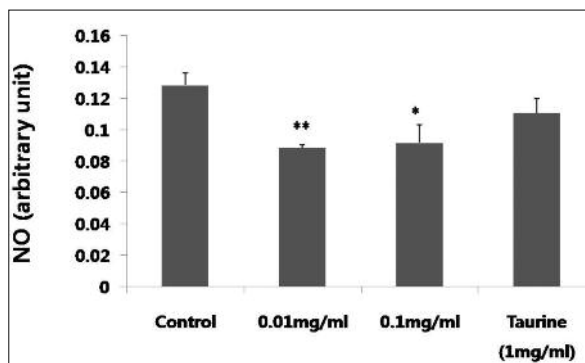


Figure 1. DPPH Scavenging Activity by *Atractylodes Rhizoma* extract. DPPH scavenging activity of *Atractylodes Rhizoma* extracts was measured as described in the Materials and Methods. All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the control group at the levels of $*p < 0.05$ and $**p < 0.01$.

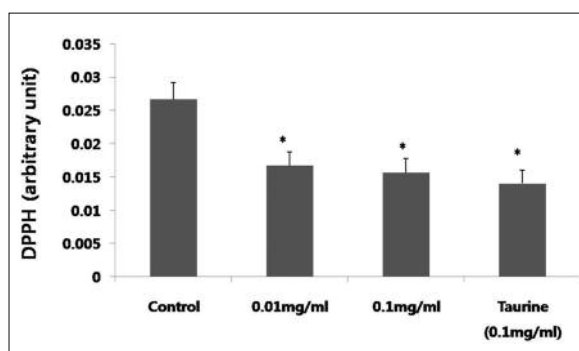


Figure 2. NO Scavenging Activity by *Atractylodes Rhizoma* extract. NO scavenging activity of *Atractylodes Rhizoma* extracts was measured as described in the Materials and Methods. All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the control group at the levels of $*p < 0.05$.

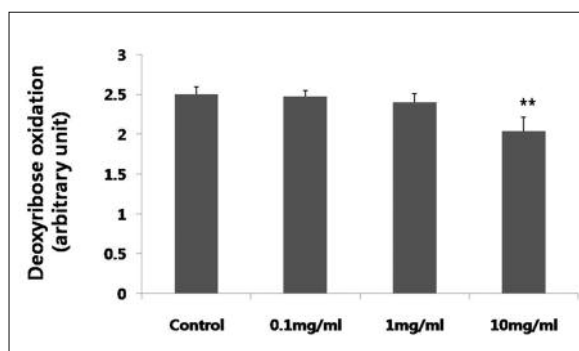


Figure 3. Effect of *Atractylodes Rhizoma* extract deoxyribose oxidation. Effect of *Atractylodes Rhizoma* extract on the deoxyribose oxidation was measured as described in the Materials and Methods. All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the control group at the levels of $**p < 0.01$.

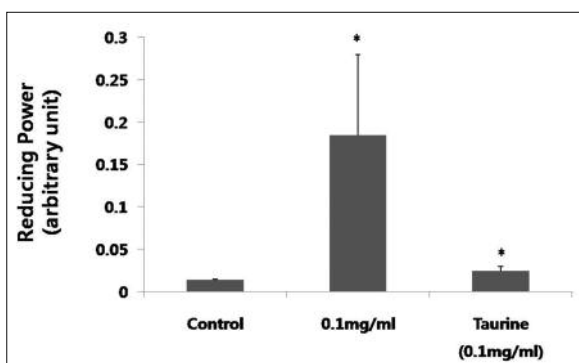


Figure 4. Reducing Power by *Atractylodes Rhizoma* extract. The reducing power of *Atractylodes Rhizoma* extracts was measured by the Oyaizu's method as described in the Materials and Methods. All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the control group at the levels of $*p < 0.05$.

were pretreated with the *Atractylodes Rhizoma* extract, NO production was significantly decreased by 29% and 51% in response to 0.01 mg/ml and 0.1mg/ml extract, respectively, as compared to the LPS-induced stimulation (Figure 5).

To elucidate mechanisms of antioxidant actions by the *Atractylodes Rhizoma* extract, the expression of iNOS which is a key enzyme for the generation of NO was examined by Western blot analysis using a specific iNOS antibody. LPS increased iNOS expression by 9.3-fold over control. On the other hand, when the cells were pretreated with the *Atractylodes Rhizoma* extract, iNOS expression levels were markedly decreased by 53% and 72% in response to 0.01 mg/ml, and 0.1 mg/ml, respectively as compared to the LPS-induced stimulation (Figure 6).

When COX-2 (cyclooxygenase-2) was also explored to see if it is a target of the *Atractylodes Rhizoma* extract, it did not cause any significant effect on COX-2 expression.

When the *Atractylodes Rhizoma* extract was tested on the osteoclastogenesis, it inhibited RANKL induced osteoclastic differentiation of the Raw cells by 23%, and 57% at the concentrations of 0.01 mg/ml and 0.1 mg/ml, respectively.

When antioxidant components present in the 70% methanolic extract of *AtractylodesRhizoma* were analyzed, total phenolics, total flavonoids and total anthocyanin were determined as 0.027 mg/g, 4.978 mg/g and 0.107 mg/g, respectively (Table I)

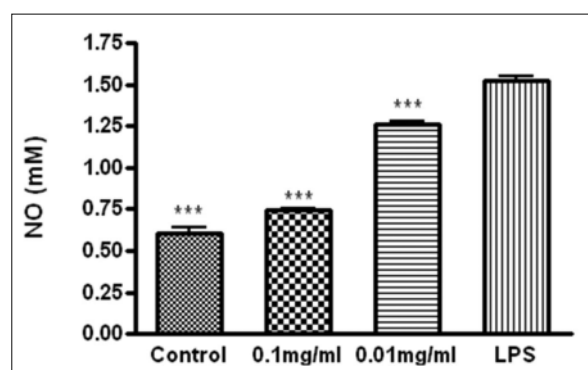


Figure 5. Effect of *Atractylodes Rhizoma* extract on NO production induced by LPS. Raw 264.7 cells were pretreated with *Atractylodes Rhizoma* extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for four hours and then treated with LPS 10 mg/ml for 18-24 hours and the amount of NO was measured using the Greiss reagent. All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the LPS-treated group at the levels of *** $p < 0.001$.

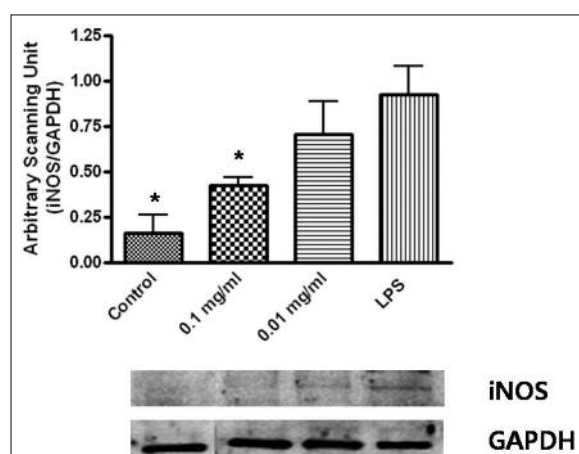


Figure 6. Effect of *Atractylodes Rhizoma* extract on LPS-induced iNOS induction. Raw 264.7 cells were pretreated with *Atractylodes Rhizoma* extracts at the concentrations of 0.01 and 0.1 for four hours and then treated with LPS 10 mg/ml for 18-24 hours and then Western blot analysis with a specific antibody against iNOS and GAPDH as a internal control as described in the Materials and Methods (Lower). The blots were subjected to scanning densitometry and expressed as a ratio of iNOS to GAPDH (Upper). All the measured values were shown as means \pm SEM of three experiments and showed significant differences from those of the LPS-treated group at the levels of * $p < 0.05$.

Discussion

A significant body of evidence highlights the key role of oxidative stress in the pathogenesis of various diseases such as cancer, aging, Alzheimer's disease, atherosclerosis, diabetes mellitus, vascular disease, Parkinson diseases and periodontal diseases. In the present study, we provide evidence that *Atractylodes Rhizoma* ex-

Table I. Antioxidant component of *Atractylodes Rhizoma* extract. To elucidate the antioxidant components of *Atractylodes Rhizoma* extract, the contents of total phenolics, total flavonoids and total anthocyanin were analyzed as described in the Materials and Methods. All the measured values were indicated as means \pm SEM of three experiments.

Components	Mean \pm SEM
Flavonoids (μ g rutin/mg extract)	4.9780 \pm 0.5427
Phenolics (μ g gallic acid/mg extract)	0.0265 \pm 0.0027
Anthocyanins (μ g kuromanin/mg extract)	0.1067 \pm 0.01157

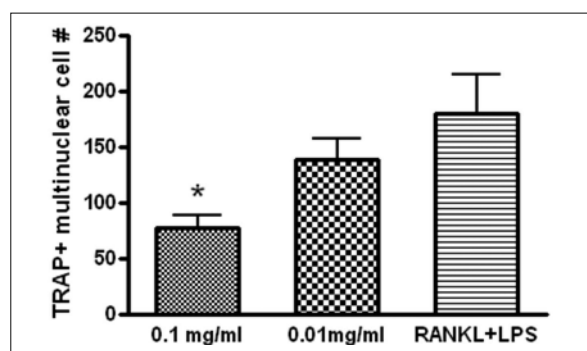


Figure 7. Effect of *Atractylodes Rhizoma* extract on osteoclastogenesis. Raw 264.7 cells were pretreated with *Atractylodes Rhizoma* extracts at the concentrations of 0.01 and 0.1 for four hours and then treated with LPS and RANKL and TRAP assay was performed as described in Materials and Methods. The data shown are the mean values \pm SEM of three experiments and showed significant differences from those of the RANKL+LPS-treated group at the levels of $*p < 0.05$.

tract has the ability to inhibit iNOS induction and DNA oxidation and possibly thereby preventing osteoclastogenesis.

ROS produced by abnormal cellular metabolism could induce oxidative stress in the body, promoting many detrimental diseases and aging^{20,21}. Oxidative stress interferes protein synthesis, lipid integrity and DNA function, eventually leading to serious cell damages. The DPPH free radical removing action of the 70% methanolic *Atractylodes Rhizoma* was clearly observed in a dose-dependent manner and its activity at 1 mg/ml was stronger than that of Vitamin C. Its effect on the NO scavenging was also significantly observed and was much higher than that of Vitamin C. Strikingly, the *Atractylodes Rhizoma* extract had the ability to inhibit deoxyribose oxidation. These results suggest that the *Atractylodes Rhizoma* extract has significant free radical scavenging activity as well as protective action against DNA oxidation.

Oxidative stress also induces DNA damage by the modulation of DNA damaging response²². The protein p53 is regulated by redox equilibrium and it trans-activates key redox active enzymes such as glutathione peroxidase, nitric oxide synthase and cyclooxygenase²³. Interestingly, it has been suggested that DNA damage itself could lead to ROS generation²⁴. Thus, the *Atractylodes Rhizoma* extract's inhibitory action against DNA oxidation could be useful for DNA damage itself as well as ROS generation. Moreover, the *Atractylodes Rhizoma* extract has re-

markable reducing power activity. Thus, it can not only remove various kinds of free radicals but also suppress the DNA oxidation. The strong reducing power of the extract will be beneficial in removing toxic peroxides generated by oxidizing reactions.

Nitric oxide (NO) is generated by the iNOS enzyme that is induced by inflammation. NO exerts various biological functions such as regulation of vascular permeability, leukocyte migration and inflammation²⁵⁻²⁷. Interestingly, iNOS enzyme presents in osteoblast and osteoclast cells and plays an important role in mediating bone development and alveolar bone loss²⁸. We have identified that *Atractylodes Rhizoma* extract has the ability to reduce NO generated by LPS in the Raw cells with the marked inhibition of iNOS induction. Since iNOS plays an essential role in promoting osteoclast function²⁹, it is reasonable to expect the *Atractylodes Rhizoma* extract could affect osteoclastic differentiation. In fact, the *Atractylodes Rhizoma* extract caused significant inhibition in the osteoclastic differentiation of Raw cells. These results strongly suggest that inhibition of iNOS induction by the the *Atractylodes Rhizoma* extract could lead to the inhibition of osteoclastogenesis. Thus, anti-oxidative actions of the *Atractylodes Rhizoma* extract could be useful in the prevention and treatment of various oxidative stress-related diseases including periodontal disease, alveolar bone loss, diabetes mellitus, cancer, Alzheimer's disease, and chronic temporomandibular diseases. Taking into consideration that plant extract containing phenolics could exert anti-oxidant activity, the rich content of phenolics including anthocyanins and flavonoids present in the *Atractylodes Rhizoma* extract could be a contributor to the iNOS inhibitory and anti-osteoclastogenic actions of the *Atractylodes Rhizoma* extract.

Conclusions

We propose that the antioxidant activities of the *Atractylodes Rhizoma* extract could be applied to the prevention and treatment of various kinds of diseases triggered by oxidative stress.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

- 1) Cheong, Bo-seop and Shin, Min-gyo, *Iconographical Folk Medicine Encyclopedia*, Younglim Co., Ltd, 1999; pp. 795-796.
- 2) SAKURAI T, YAMADA H, SAITO K, KANO Y. Enzyme inhibitory activities and sesquiterpene compounds in *atractylodes* rhizome. *Biol Pharmac Bull* 1993; 16: 142-145.
- 3) KIM CM, SHIN MK, AHN DG, LEE KS. *Chungyak Dae-sajun*, vol. 8. Jungdam Publisher, Seoul, pp. 3969-3976, 1997.
- 4) JOHANSEN JS, HARRIS AK, RYCHLY DJ, ERGUL A. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. *Cardiovasc Diabetol* 2005; 4: 5.
- 5) FRIDLAND LE, PHILIPSON LH. Reactive species and early manifestation of insulin resistance in type 2 diabetes. *Diabetes Obes Metab* 2006; 8: 136-145.
- 6) CARTA S, CASTELLAN P, DELFINO L, TASSI S, VENE R, RUBARTELLI A. DAMPs and inflammatory processes: the role of redox in the different outcomes. *J Leukoc Biol* 2009; 86: 549-555.
- 7) BEATTY S, KOH H, PHIL M, HENSON D, BOULTON M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 2000; 45: 115-134.
- 8) FRIDOVICH I. 1978. The biology of oxygen radicals. *Science* 1978; 201: 332-340.
- 9) POYTON RO, BALL KA, CASTELLO PR. Mitochondrial generation of free radicals and hypoxic signaling. *Trends Endocrinol Metab* 2009; 20: 332-340.
- 10) CONFORTI F, MENICINI F. Phenolic compounds from plants as nitric oxide production inhibitors. *Curr Med Chem* 2001; 18: 1137-1145.
- 11) BRAND-WILLIAMS W, CUVELIER ME, BERSET C. Use of a free radical method to evaluate antioxidant activity. *Lebensm-Wiss Technol* 1995; 28: 25-30.
- 12) YEN GC, LAI HH, CHOU HY. Nitric oxide-scavenging and antioxidant effects of *Urariacrinite* root. *Food Chem* 2001; 74: 471-478.
- 13) HALLIWELL B, GUTTERIDGE JM. Formation of thiobarbituric-acid reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Lett* 1981; 15: 347-352.
- 14) OYAZU M. Studies on products of browning reaction: Antioxidative activities of products of browning reaction prepared from glucosamine. *Jap J Nutr* 1986; 44: 307-314.
- 15) BRADFORD MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-254.
- 16) TOWBIN H, STAHELIN J, GORDON J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci USA* 1979; 76: 4350-4354.
- 17) SINGLETON VL, ROSSI JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic reagents. *Am J Enol Vitic* 1965; 16: 144-158.
- 18) MILIAUSKASA G, VENSKUTONIS PR, VAN BEEK TA. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem* 2004; 85: 231-237.
- 19) PICCOLELLA S, FIORENTINO A, PACIFICO S, D'ABROSCA B, UZZO P, MONACO P. Antioxidant properties of sour cherries (*Prunus cerasus* L.): Role of colorless phytochemicals from the methanolic extract of ripe fruits. *J Agric Food Chem* 2008; 56: 1928-1935.
- 20) KIM BJ, KIM JH, KIM HP, HEO MY. Biological screening of 100 plant extracts for cosmetic use (II): anti-oxidative activity and free radical scavenging activity. *Int J Cosmet Sci* 1997; 19: 299-307.
- 21) EVEREKLIOGLU C, ER H, DOGANAY S, CEKME M, TURKOZ Y, OTLU B, OZEROL E. Nitric oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration. *Doc Ophthalmol* 2003; 106: 129-136.
- 22) SINGH RP, DHANALAKSHMI S, ATARWAL R. Phytochemicals as cell cycle modulators—a less toxic approach in halting human cancers. *Cell Cycle* 2002; 1: 156-161.
- 23) MEPLAN C, RICHARD MJ, HAINAUT P. Redox signaling and transition metals in the control of the p53 pathway. *Biochem Pharmacol* 2000; 59: 25-33.
- 24) SALMON TB, EVERT BA, SONG BW, DOETSCH PW. Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. *Nucleic Acid Res* 2004; 32: 3712-3723.
- 25) VAN'T HOF RJ, RALSTON SH. Nitric oxide and bone. *Immunology* 2001; 103: 255-261.
- 26) NATHAN C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992; 6: 3051-3064.
- 27) LEFER AM. Nitric oxide: nature's naturally occurring leukocytic inhibitor. *Circulation* 1997; 95: 553-554.
- 28) GYURKO R, SHOJI H, BATTAGLINO RA, BOUSTANY G, GIBSON III FC, GENCO CA, STASHENKO P, VAN DYKE TE. Inducible nitric oxide synthase mediates bone development and *P. gingivalis*-induced alveolar bone loss. *Bone* 2005; 36: 472-479.
- 29) LEE SK, HUANG H, LEE SW, KIM KH, KIM KK, KIM HM. Involvement of iNOS-dependent NO production in the stimulation of osteoclast survival by TNF- α . *Exp Cell Res* 2004; 298: 359-368.