Inhibition of inducible nitric oxide synthase and osteoclastic differentiation by *Atractylodis Rhizoma Alba* extract

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ABSTRACT

Background: *Atractylodis Rhizoma Alba* (ARA) has been used in Korean folk medicine for constipation, dizziness, and anticancer agent. In the present study, we performed to test whether the methanolic extract of ARA has antioxidant and antiosteoclastogenesis activity in RAW 264.7 macrophage cells. **Materials and Methods:** Antioxidant capacities were tested by measuring free radical scavenging activity, nitric oxide (NO) levels, reducing power, and inducible nitric oxide synthase (iNOS) expression in response to lipopolysaccharides (LPS). Antiosteoclastogenesis activity was evaluated by performing tartrate-resistant acid phosphatase assay in RAW 264.7 macrophage cells. **Results:** The extract exerted significant 1,1-diphenyl-2-picrylhydrazyl and NO radical scavenging activity, and it exerted dramatic reducing power. Induction of iNOS and NO by LPS in RAW 264.7 cells was significantly inhibited by the extract, suggesting that the ARA extract inhibits NO production by suppressing iNOS expression. Strikingly, the ARA extracts substantially inhibited the receptor activator of NF-κB ligand-induced osteclastic differentiation of LPS-activated RAW 264.7 cells. The ARA extract contains a significant amount of antioxidant components, including phenolics, flavonoids and anthocyanins. **Conclusion:** These results suggest that the methanolic extract of ARA exerts significant antioxidant activities potentially via inhibiting free radicals and iNOS induction, thereby leading to the inhibition of osteoclastogenesis.

Key words: Antioxidant, *Atractylodis Rhizoma Alba*, inducible nitric oxide synthase, nitric oxide, osteolastogenesis, receptor activator of NF-κB ligand

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INTRODUCTION

Atractylodis Rhizoma Alba (ARA) is the dried rhizome of *Atractylodes macrocephala* Koidzumi, *Atractylodes japonica* Koidzumi ex Kitam and *Atractylodes ovata* Koidzumi, and belonging to the Composite family. It is widely distributed in Asian countries such as Korea, China and Japan,[1] and many medicinal prescriptions contain it as the principle drug.[2] It has been used in the oriental medicine for constipation, dizziness and anticancer agent.[3] In addition, it has been reported that ARA has antiulcer action and adipogenic differentiating activity.[4,5] Phytochemical investigations have revealed a number of chemical components such as sesquiterpenes, sesquiterpene glycosides, polyacetylenes, monoterpene glycosides, aromatic glycosides, sucrose esters, and steroids were isolated.[6-9] The main constituents from ARA are atractylon, atractylodin, and atractylenolides.[10,11]

Many detrimental diseases are associated with the increase of reactive oxygen and nitrogen species (RONS), leading to imposing oxidative stress in the body, which can initiate tissue damages. Overproduction of free radicals more than their scavenging capacity of the antioxidative defense system in the cellular components plays a pivotal role in the damaging lipid, protein, carbohydrate and DNA, ultimately causing cell death.[12] Reactive oxygen species (ROS) contains superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^•$), and they are produced during various cellular aerobic metabolism and as by-products of many enzyme reactions.[12,13] Nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is associated with the production of reactive nitrogen species,[14] which are responsible for the excessive production of lipid and protein oxidation processes.[12] Functional antioxidant system and sufficient supply of antioxidant molecules are critical in scavenging

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RONS. Novel nutraceuticals/drugs with anti-iNOS activity will be of great value for the management of the oxidative stress, inflammation, bone metabolism and vascular tone.[15]

Multinucleated osteoclasts are responsible for bone resorption and excessive bone resorption by osteoclasts could cause an imbalance in bone remodeling, leading to the bone lytic diseases and cancer metastasis to the bone.[16,17] Thus, understanding the molecular processes of osteoclast formation and identifying potential pharmacological interventions are critical for the prevention and treatment of abnormal bone loss.[18] In recent times, many herbal plants with antioxidant and antiinflammatory activity have been shown to suppress osteoclast differentiation through inhibition of specific signaling pathways.[18] The pharmacological action of ARA on the osteoclast formation has not yet been fully determined.[19] In this study, we sought to determine if 70% methanol extracts of ARA have antioxidative activities associated iNOS system. It has been also tested whether the ARA extracts could exert any effect on the osteoclastogenesis using a receptor activator of NF-κB ligand (RANKL)/lipopolysaccharide (LPS)-induced bone resorption model in RAW 264.7 macrophage cells.

MATERIALS AND METHODS

Preparation of plant extracts

Authentic samples of ARA 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, the Republic of Korea. A voucher specimen (No. 00-14) was deposited in the herbarium of the Department of Pharmacology, School of Dentistry, Kyung Hee University, Seoul, the Republic of Korea. They were collected during the early autumn season. ARA (100 g) was cut into small pieces and extracted 3 times with 70% methanol (300 ml) for 3 h. The resulting methanol extracts was concentrated by rotary evaporator and dried by a freeze-dryer.

Reagents and materials

The iNOS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Cell Signaling and Santa Cruz Biotechnology Co., respectively. The ECL kit was bought from Amersham Co. Other reagents were bought from Sigma Co. All cell culture media were purchased from Gibco Co.

Atractylenolide II was kindly provided by Prof. Jinwoong Kim at College of Pharmacy, Seoul National University, the Republic of Korea.

Scavenging of 1,1-diphenyl-2-picrylhydrazyl radicals

1,1-diphenyl-2-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 antioxidants.[20] Briefly, ARA extracts dissolved in 1 ml of MeOH was mixed with 1 ml of $DPPH^{\bullet}$ solution at 23°C and an optical density were measured in 30 min at 515 nm.

Scavenging of nitric oxide radicals

Nitric oxide generated from sodium nitroprusside (SNP) was measured by using the Griess reagent.[21] The extract was added to 0.2 ml of SNP (10 mM) and 1.8 ml of phosphate buffer (pH 7.4). The reaction mixture was allowed to incubate at 37°C for 3 h. A volume of 1 ml aliquots of the reaction mixture were mixed with 0.5 ml of Griess reagent and subjected to measure absorbance at 540 nm by using a spectrophotometer.

Measurement of oxidation of deoxyribose

The experiment was carried out as described by Halliwell and Gutteridge.[22] The reaction mixture (1.4 ml) containing extracts (0.2 ml), deoxyribose (6 mM), H_2O_2 (3 mM), $FeCl_3$ (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 using the thiobarbituric acid (TBA) method. One milliliter of TBA (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 ARA extract was measured using the Oyaizu's method.[23] 2.5 ml of ARA extract in 0.2 M phosphate buffer (pH 6.6) was added to 2.5 ml of potassium ferricyanide (10 mg/ml) solution and made to react for 15 min 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 the optical density was measured at 700 nm.

Cell culture

Murine RAW 264.7 macrophage cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco BRL, Grand Island, NY) with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, NY) in 5% humidified CO_2 atmosphere at 37°C.

Measurement of nitric oxide

RAW 264.7 cells were cultured with DMEM and 10% FBS. NO was measured by measuring the amount in the

cell supernatant as nitrite and nitrate. The safest form of nitrite after being reduced to nitrate was measured using the Griess reagent (Sigma, USA). The cells (2×10^6) were seeded into 6 well plate and washed 2 times with phosphate buffered saline (PBS) when the confluence was approximately 80% and then cultured for at least 24 h, and the ARA extract were made into the final concentrations of 10, 1.0 and 0.1 mg/ml for experiments. Four hours later, LPS of the final concentration 10 μg/ml was added 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 h later, having them react with the Greiss reagent for 10 min in darkness and then measuring the optical density at 540 nm.

Measurement of inducible nitric oxide synthase expression by western blotting

When the cells reached confluence, the DMEM culture medium was removed and replaced by the serum-free DMEM medium, and subsequently, the cells were treated with ARA extracts and cultured for 24 h. The cells were washed 2 times with PBS and scraped into a buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 30 mM NaF, 0.1 mM Na_3VO_4, 1% Triton X-100, 0.5% NP-40, 1 μg/ml leupetin, 1 μg/ml aprotinin after that, the cells were disrupted by passing them through a 1 ml tuberculin syringe 5 times. The cell lyaste was subjected to centrifugation at 10,000 ×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 Bradford.[24] Protein (50 μg/lane) was electrophoretically separated in 10% polyacrylamide gels containing sodium dodecyl sulfate and transferred to nitrocellulose membranes (Schleicher and Schuell) for 1 h at 100 V (constant) as described by Towbin *et al.*[25] The membranes were blocked 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 3 times for 10 min each. Followed by, the blots were probed with primary antibody directed against iNOS (1:1000) and GAPDH (1:1000) for 2 h at room temperature or overnight at 4°C diluted in blocking buffer. The blots were then incubated with horseradish peroxidase-conjugated antirabbit IgG (1:1000 for iNOS and GAPDH) for 1 h at room temperature and washed with PBS containing Tween 20 3 times for 10 min each. The detection of immobilized specific antigens was carried out by ECL. The images were analyzed using ImageJ software (NIH, USA).

Determination of osteoclastic differentiation

RAW 264.7 cells (2×10^3) were seeded into 6 well plate and washed 2 times with PBS when the confluence was 80% and then cultured for at least 24 h and the ARA extract were made into the final concentrations of 10, 1.0 and 0.1 mg/ml for experiments. Four hours later, RANKL (50 ng/ml) was put into all wells for 72 h and LPS of the final concentration 10 μg/ml was added to stimulate osteoclastic differentiation. After 6~7 days, tartrate-resistant acid phosphatase (TRAP) 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 phenolic content was measured using the Folin–Ciocalteu procedure at 725 nm.[26] 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 was measured using the method of Miliauskas *et al.*[27] and was expressed as the rutin equivalent (mg rutin acid/g extract) using rutin as a standard flavonoid. 1.0 ml of ARA extract was mixed with aluminum thiochloride in ethanol (20 mg/ml) and diluted to 25 ml. After incubation for 40 min at 20°C, the optical density was measured at 415 nm.

Measurement of total anthocyanin

The total anthocyanin was measured using color reactions.[28] ARA extracts were dissolved in 1.0 ml of acetate buffer (25 mM, pH 4.5), and an optical density was measured at 520 nm. The content of anthocyanin was expressed as kuromanin equivalent (mg Kuromanin/g extract).

High-performance liquid chromatography analysis

Atractylodis Rhizoma Alba extract was analyzed by high-performance liquid chromatography (HPLC) (Nova-Pak® C18, 60Å 4uM, 3.9 × 150 mm). A mobile phase system (50:50 of Acetonitrile: Water) was applied at a flow rate of 1 ml/min for 35 min, and the absorption peaks were detected at 236 nm and compared with a standard marker molecule, Atractylenolide II. The HPLC profile was quantified using its integrated area [Figure 1].

Statistical analysis

All data were expressed as mean ± standard error of the mean. Statistical analysis was performed using the GraphPad Prism 4 (GraphPad software, Inc., La Jolla, CA, USA) 4 with one-way ANOVA followed by Tukey's multiple comparison test and $P < 0.05$ was considered to be significant.

RESULTS

1,1-diphenyl-2-picrylhydrazyl free radicals were decreased by 37% at 0.01 mg/ml of ARA extract and about 41% at 0.1 mg/ml as compared to the control, whereas taurine at 0.1 mg decreased DPPH free radicals by 48% [Figure 2]. The ARA extract also scavenged NO by 31% and 29% as compared to control at 0.01 and 0.1 mg/ml, respectively. However, taurine exerted only a 14% decrease of NO levels at 1 mg/ml [Figure 3].

The deoxyribose oxidation was suppressed by 18% by the ARA extract at the concentration of 10 mg/ml [Figure 4]; however, there was little effect observed at 0.1 and 1 mg/ml. Regarding reducing power of the ARA extract, it had the reducing power of 13.5-fold when compared to control at 0.1 mg/ml, whereas taurine at 0.1 mg/ml had the reducing power of 1.8-fold over control [Figure 5]. The amount of NO produced increased by 2.5-fold over control when the RAW 264.7 cells were treated with LPS to activate the macrophages. When the cells were pretreated with the ARA extract, NO production was significantly decreased by 29% and 51% in response to 0.01 and 0.1 mg/ml extract, respectively as compared to the LPS-induced stimulation [Figure 6].

To elucidate potential mechanisms of antioxidative action by the ARA extract, the expression of iNOS which are a key enzyme for the generation of NO was explored. LPS increased iNOS expression by 9.3-fold over control. On the other hand, when the cells were pretreated with the ARA extract, iNOS expression levels were decreased by 53% and 72% in response to 0.01 and 0.1 mg/ml, respectively when compared to the LPS-induced stimulation [Figure 7]. Cyclooxygenase-2 (COX-2) was also tested to see if it is a target of the ARA extract. It did not cause any significant effect on the COX-2 expression (data not shown). When the ARA extract was tested on the osteoclastogenesis, it inhibited RANKL induced osteoclastic differentiation of the RAW 264.7 cells by 23%, and 57% at the concentrations of 0.01 and 0.1 mg/ml, respectively [Figure 8].

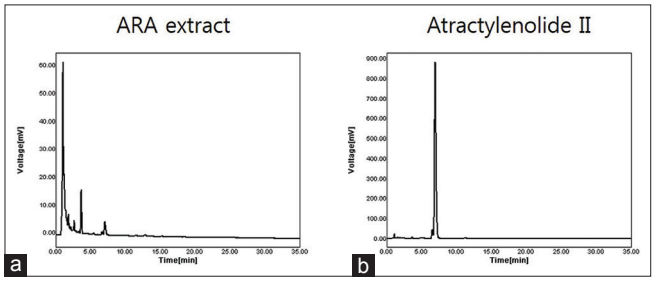

Figure 1: Representative high-performance liquid chromatography chromatogram of the 70% methanolic extracts of *Atractylodis Rhizoma Alba* (a) and the standard atractylenolide II (b). Atractylenolide II was detected at around 6.97 min in this system

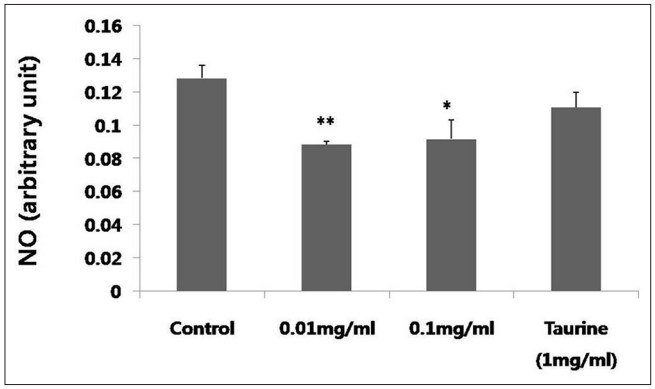

Figure 3: Nitric oxide (NO) scavenging activity by *Atractylodis Rhizoma Alba* (ARA) extracts. NO scavenging activity of ARA extracts was measured as described in the methods. All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the control group at the levels of $*P < 0.05$ and $***P < 0.001$

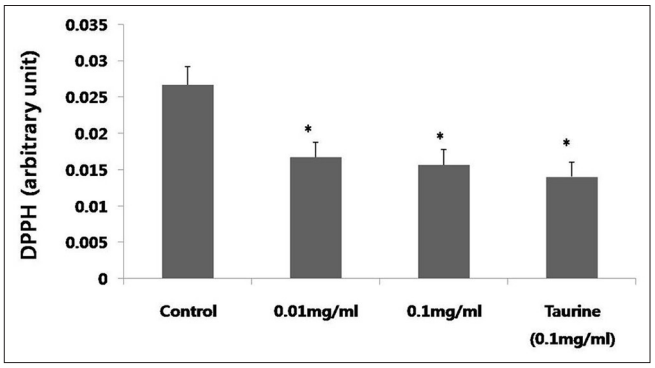

Figure 2: 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity by *Atractylodis Rhizoma Alba* extracts. The DPPH scavenging activity of *Atractylodis Rhizoma* extracts was measured as described in the methods. All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the control group at the levels of $*P < 0.05$ and $**P < 0.01$

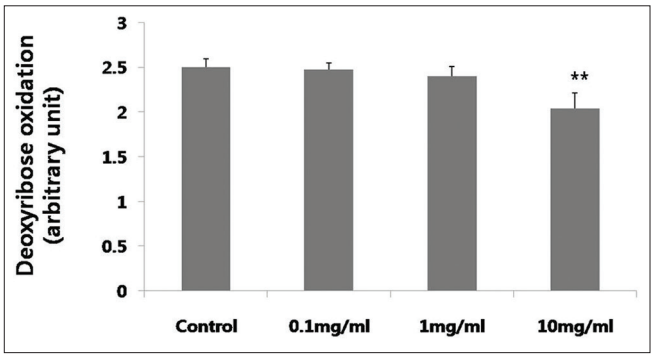

Figure 4: Effect of *Atractylodis Rhizoma Alba* (ARA) extracts on the deoxyribose oxidation. Effect of ARA extracts on the deoxyribose oxidation was measured as described in the methods. All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the control group at the levels of $**P < 0.01$

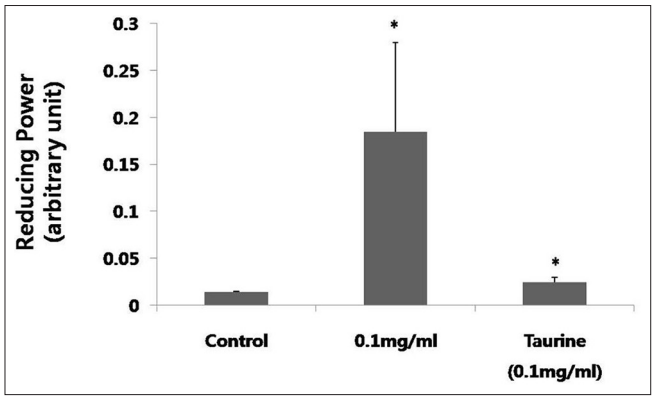

Figure 5: Reducing power by *Atractylodis Rhizoma Alba* (ARA) extracts. The reducing power of ARA extracts was measured by the Oyaizu's method as described in the methods. All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the control group at the levels of $^{**}P < 0.01$ and $^{***}P < 0.001$

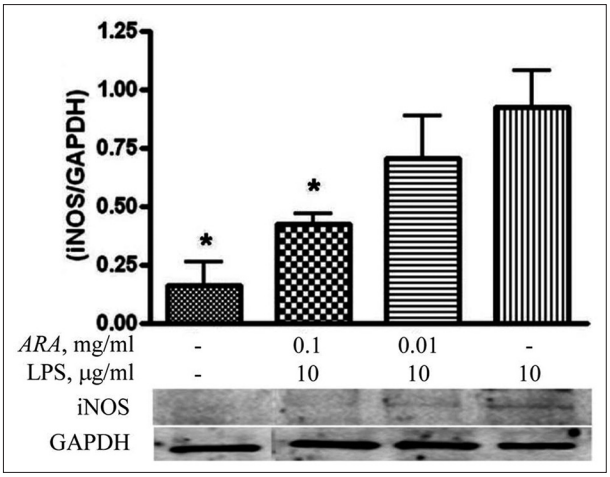

Figure 7: Effect of *Atractylodis Rhizoma Alba* (ARA) extracts on lipopolysaccharides (LPS)-induced inducible nitric oxide synthase (iNOS) induction. RAW 264.7 cells were pretreated with ARA extracts at the concentrations of 0.01 and 0.1 mg/ml for 4 h and then treated with LPS (10 μg/ml) for 18-24 h and then Western blot analysis as described in the methods (B). The blots were subjected to densitometry and expressed as a ratio of iNOS to glyceraldehyde 3-phosphate dehydrogenase (A). All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the LPS-treated group at the levels of $^{*}P < 0.05$

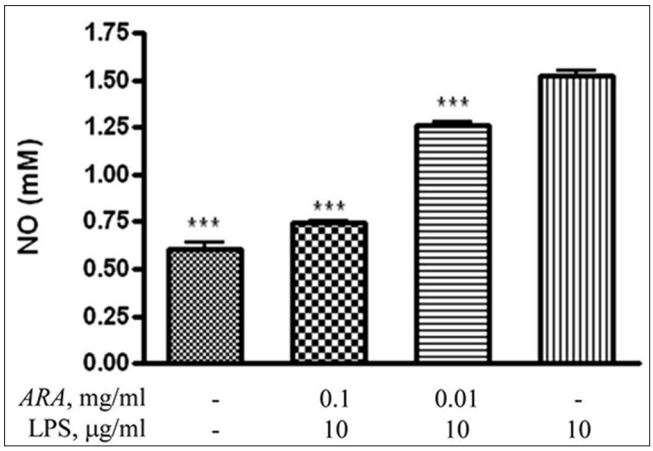

Figure 6: Effect of *Atractylodis Rhizoma Alba* (ARA) extracts on nitric oxide (NO) production induced by lipopolysaccharides (LPS). RAW 264.7 cells were pretreated with ARA extracts at the concentrations of 0.01, 0.1 and 1.0 mg/ml for 4 h and then treated with LPS (10 μg/ml) for 18-24 h and the amount of NO was measured using the Griess reagent. All the measured values were shown as means ± standard error of the mean of three experiments and showed significant differences from those of the LPS-treated group at the levels of $^{***}P < 0.001$

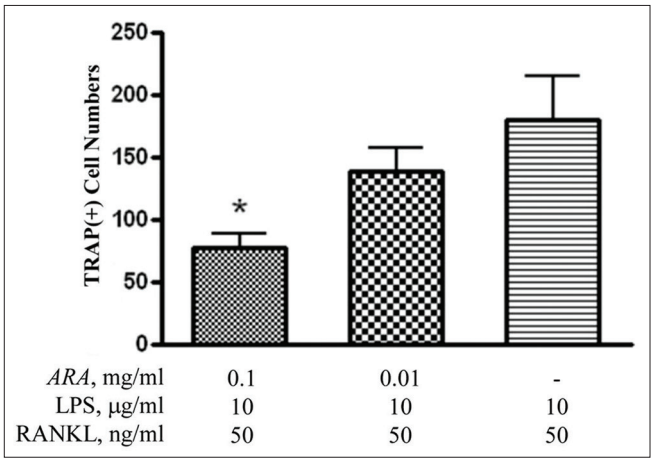

Figure 8: Effect of Atractylodis Rhizoma Alba (ARA) extracts on osteoclastogenesis. RAW 264.7 cells were pretreated with ARA extracts at the concentrations of 0.01 and 0.1 mg/ml for 4 h and then treated with RANKL + lipopolysaccharides (LPS). The TRAP assay was performed as described in Materials and Methods. The data shown are the mean values ± standard error of the mean of three experiments and showed significant differences from those of the RANKL + LPS-treated group at the levels of $^{*}P < 0.05$

The antioxidant components are present in the 70% methanolic extract of ARA was analyzed. The total phenolics, total flavonoids and total anthocyanin contents were as 0.027, 4.978 and 0.107 mg/g, respectively [Table 1]. The HPLC profile of the ARA extract was obtained [Figure 1a]: A peak at retention time 6.97 min was identified as atractylenolide II [Figure 1b]. The total content of atractylenolide II in the ARA extract was 0.25% (w/w).

DISCUSSION

Oxidative stress is one of the crucial factors in the development of various diseases such as cancer, aging, Alzheimer's disease, atherosclerosis, diabetes, vascular disease, Parkinson's disease and periodontal diseases. However, new materials or strategies are still being developed for the prevention and cure of the oxidative stress-mediated diseases. We propose that the ARA extract

Table 1: Antioxidant component of ARA extract

Components	Mean±SEM
Flavonoids (mg rutin/g extract)	4.9780±0.5427
Phenolics (mg gallic acid/g extract)	0.0265±0.0027
Anthocyanins (mg kuromanin/g extract)	0.1067±0.0116

To elucidate the antioxidant components of ARA extract, the contents of total phenolics, total flavonoids and total anthocyanin were analyzed as described in the methods. All the measured values were indicated as means±SEM of three experiments. SEM: Standard error of the mean; ARA: *Atractylodis Rhizoma Alba*

can inhibit the iNOS induction and DNA oxidation and thereby preventing osteoclastogenesis.

Reactive oxygen species produced by abnormal cellular metabolism could induce oxidative stress in the body, promoting many detrimental diseases and aging.[29,30] Oxidative stress interferes protein synthesis, lipid integrity and DNA function, eventually leading to serious cell damages. The antioxidant activities of the ARA extract were investigated in terms of their free-radical scavenging and reducing capacity. The DPPH free radical removing action of the ARA extract was clearly observed in a dose-dependent manner and its activity at 1 mg/ml was stronger than that of taurine. The DPPH radical scavenging by the ARA extract may be a reaction between its antioxidant molecules and radical by hydrogen-donation. Its effect on the NO scavenging was also significantly observed and was much higher than that of taurine.

Strikingly, the ARA extract had the ability to inhibit the deoxyribose oxidation. These results suggest that the ARA extract has significant free radical scavenging activity as well as a protective action against DNA oxidation by hydroxyl radicals. Interestingly, it has been suggested that DNA damage itself could lead to ROS generation.[31] Thus, the ARA extract's inhibitory action on hydroxyl radicals could be beneficial against DNA damage itself as well as other ROS generation. Moreover, the ARA extract has remarkable reducing power activity. Thus, it cannot 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.

Macrophages are the primary immune cells; however, aberrant activation of macrophages has been reported to play pathogenic roles in inflammatory disorders.[32] It has been previously identified that *A. japonica* Koidzumi inhibits pro-inflammatory cytokine in human mast cell, suggesting its potential antioxidative activity.[33] NO exhibits various biological functions such as regulation of vascular permeability, leukocyte migration and inflammation.[34-36] Interestingly, iNOS enzyme, responsible for the production of NO, presents in osteoblast and osteoclast cells and plays a fundamental role in mediating bone development and alveolar bone loss.[37] Macrophage activation and NO production are necessary for proper defense mechanism; strong activation of macrophages causes the releases of inordinate cytokines and mediators, which has been implicated in many pathophysiological conditions. We have identified that the LPS activation of macrophages produces an excessive amount of NO via iNOS protein. The ARA extract significantly inhibited LPS-stimulated excessive production of NO with the suppression of iNOS. Considering that iNOS play an essential role in promoting osteoclast function,[38] it is reasonable to expect the ARA extract could affect osteoclastic differentiation.

The differentiation of osteoclasts requires RANKL, a member of the tumor necrosis factor.[39] RANKL, produced by osteoblasts/stromal cells, binds to the receptor activator of NF-κB on the preosteoclast and stimulates differentiation into a mature osteoclast.[40,41] The differentiation process includes inducible expression of TRAP.[39] In the present study, the RANKL plus LPS treatment caused a significant increase in TRAP-positive multinucleated cells when the Raw cells were stained for TRAP. However, the ARA extracts significantly inhibited the osteoclastic differentiation induced by RANKL and LPS as evidenced by a decrease in multinucleated TRAP-positive cells. These results strongly suggest that inhibition of osteoclastogenesis by the ARA extract might be due to its antioxidant activity.

CONCLUSION

Taken together, antioxidative actions of the ARA extract could be useful in the prevention and treatment of various oxidative stress-related diseases, including periodontal disease, alveolar bone loss, diabetes, cancer, Alzheimer's disease, and chronic temporomandibular diseases. The remarkable content of phenolics including anthocyanin's and flavonoids present in the ARA extract could play an important role in the inhibition of osteoclastogenesis by regulating iNOS expression and intracellular ROS generation. Thus, we propose that the antioxidant and free radical scavenging activities of the ARA extract could be useful in the prevention and treatment of various kinds of diseases triggered by oxidative stress.

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