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Antioxidant, Anti−acetylcholinesterase and Composition of Biochemical Components of Russian Deer Velvet Antler Extracts

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Abstract

Russian deer velvet antlers were divided into three parts and subjected to a extraction process using hot water at 100, 110, and 120°C or an extraction with 70% ethanol. Each extract was analyzed for its biochemical components, including uronic acid, sulfated-glycosaminoglycans (sulfated-GAGs), and sialic acid, and the antioxidant and anti-acetylcholinesterase activities were investigated. Different levels of uronic acid and sulfated-GAGs were observed in the extracts according to the water temperature used for the extraction, and contents decreased with increasing extraction temperature. The upper layer of each extract showed high amounts of uronic acid and sulfated-GAGs, followed by the middle and base layers. Ethanol extraction was more effective for recovering uronic acid than sulfated-GAGs. Sialic acid content was the highest in the 110°C extracts but was not observed in the ethanol extracts. Velvet antler extracts showed strong antioxidant activities against DPPH and hydrogen peroxide as well as strong reducing power in a dose-dependent manner. However, the antioxidant activities were different in each layer and according to the extraction method. Additionally, velvet antler extracts exhibited inhibitory activity against acetylcholinesterase, which is associated with Alzheimer's disease, in a dose-dependent manner. These results suggest that velvet antler extracts are useful as a functional food ingredient and/or a pharmaceutical.

Key words: velvet antler, uronic acid, sulfated-glycosaminoglycans, sialic acid, antioxidant activity, anti-acetylcholinesterase

Introduction

Overproduction of free radicals and reactive oxygen species (ROS) is believed to be associated with cellular and tissue pathogenesis, which leads to several chronic diseases such as cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases (Butterfield *et al.*, 2002; Pryor and Ahn, 1982). It is also believed that ROS can oxidize biomacromolecules such as DNA, proteins, membrane lipids, and vital molecules. To prevent or slow down the oxidative stress induced by ROS, supplementation of antioxidants may be useful. Therefore, great interest has been focused on the development of natural antioxidants that are safe, non-toxic, and effective.

Acetylcholinesterase (AChE) is an oligomeric enzyme

that attaches to the neuromuscular junction, which catalyzes the cleavage of neurotransmitter acetylcholine to choline and acetate. Based on the cholinergic hypothesis, loss of cholinergic function due to a deficiency in neurotransmitter acetylcholine is the only evidential finding responsible for the cognitive characteristics of Alzheimer's disease (AD). Therefore, AChE inhibitors are widely used in AD patients to inhibit AChE activity, thereby activating the central cholinergic system and alleviating cognitive deficits.

In Korea, unossified horn of *Cervus elaphus* (*Cervidae*) is generally referred to as '*Nokyong*' and is one of the most popular Korean traditional medicines, the benefits of which are supported by extensive *in vivo* and *in vitro* studies (Kim, 1994; Suttie *et al.*, 1994). Recently, the beneficial effects of *C. elaphus* with regard to its anti-inflammatory, anti-stress, anti-aging, and antioxidant activities were studied (Je *et al.*, 2010; Sunwoo *et al.*, 1997; Takikawa *et al.*, 1972; Wang *et al.*, 1988a; Wang *et al.*, 1988b; Zhang *et al.*, 1992). In our previous report, we

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reported the contents of biologically active materials such as uronic acid, sulfated-glycosaminoglycans (sulfated-GAGs), and sialic acid in New Zealand deer velvet antler extracts under various conditions as well as their antioxidant effects on various model systems (Je *et al.*, 2010). These compounds are generally considered for quality evaluation of velvet antler, and in particular, sulfated-glycosaminoglycans had been shown to reduce pain in osteoarthritis patients (Paroli *et al.*, 1991). However, there is very little information on Russian deer velvet antler in this regard.

In this study, Russian deer velvet antler was divided into three parts, which were then subjected to extraction using hot water at three different temperatures and also 70% ethanol solution. The biochemical compositions of the extracts were analyzed, and their antioxidant and anti-acetylcholinesterase activities were evaluated.

Materials and Methods

Chemicals

All chemicals, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu's phenol reagent, hydrogen peroxide, 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), acetylcholinesterase (electric eel), acetylthiocholine, galacturonic acid, carbazole, 1,9-dimethylmethylene blue, chondroitin 4-sulfate, N-acetylneuraminic acid, periodic acid, peroxidase, and potassium ferricyanide, were purchased from Sigma Chemical Co. (USA). Other chemicals and reagents used were of analytical grade.

Preparation of velvet antler extracts

Russian red deer velvet antler (*C. elaphus*) was donated by Shin Hung Pharm. Co. (Yeosu, Korea). The antler was divided into three parts (upper (RU), middle (RM), and base part (RB)), with a 60 g portion being used for each experiment (Fig. 1). The first extraction process was conducted with hot water at 100°C for 1 h by autoclaving (MAC-601, Tokyo Rikakikai Co., Ltd., Japan) (designated as RU100, RM100, and RB100). After filtration, the residue was subjected to re-extraction at 110°C for 1 h, followed by filtration (designated as RU110, RM110, and RB110). Finally, the residue from the extraction at 110°C was subjected to re-extraction at 120°C for 2 h, repeated twice, and the supernatant was collected by filtering (RU120, RM120, and RB120). The justification for this is that water at 100°C may not be hot enough to completely extract all of the functional components. A 60 g
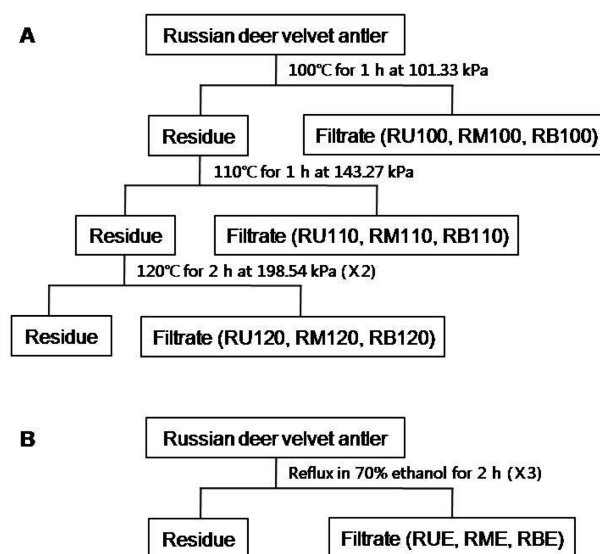

Fig. 1. Schematic diagram for the extraction of Russian deer velvet antler. (A), water extraction; (B), 70% ethanol extraction.

portion of the antler was also subjected to extraction using 70% ethanol solution for 2 h, which was repeated three times (designated as RUE, RME, and RBE). All recovered extracts were lyophilized on a freeze dryer (FD8508, Ilshin Co., Ltd., Korea) for 5 d.

Analysis of uronic acid, sulfated-GAGs, and sialic acid contents

Uronic acid content was determined by the carbazole reaction. Briefly, a 50 μL serial dilution of standard or sample was placed in a 96-well plate, after which a 200 μL solution of 25 mM sodium tetraborate in sulfuric acid was added. The plate was heated for 10 min at 100°C in an oven. After cooling at room temperature for 15 min, 50 μL of 0.125% carbazole in absolute ethanol was carefully added. After heating at 100°C for 10 min in an oven and cooling at room temperature for 15 min, the plate was read using a microplate reader (ELx 808[TM], BioTek, VT, USA) at a wavelength of 550 nm (Cesaretti *et al.*, 2003).

Sulfated-GAGs content was determined by the dimethylmethylene blue (DMB) dye binding method (Farndale *et al.*, 1986). Briefly, the color reagent was prepared by dissolving 0.008 g of DMB in a solution containing 1.185 g of NaCl, 1.520 g of glycine, 0.47 mL of HCl (12 M), and 500 mL of distilled water. Each sample was mixed with 1 mL of color reagent, and the absorbance was read immediately at 525 nm.

Sialic acid content was determined by the method of Warren (1959) with slight modification. Briefly, samples were hydrolyzed in 0.1 N H_2SO_4 in a final volume of 1.0

mL for 1 h at 80°C. Both standard and sample were incubated with 1 mL of periodate solution at 37°C for 30 min. After addition of 0.25 mL of sodium thiosulfate (0.32 M), the tubes are shaken until the yellow-brown color disappeared. The reaction was completed by the addition of 1.25 mL of thiobarbituric acid (0.1 M), after which the tubes were heated at 100°C for 15 min and cooled to room temperature. The product was extracted with acidic butanol, and optical density was determined at 549 nm.

Assay for antioxidant activity

DPPH scavenging activity was measured according to the method of Blois (1958). DPPH solution ($1.5×10^{-4}$ M, 100 μL) was mixed with and without each extract (100 μL), after which the mixture was incubated at room temperature for 30 min. After standing for 30 min, absorbance was recorded at 517 nm using a microplate reader.

Hydrogen peroxide scavenging activity was determined according to the method of Müller (1985). Briefly, 100 μL of 0.1 M phosphate buffer (pH 5.0) was mixed with each extract in a 96-well plate. Then, 20 μL of hydrogen peroxide was added to the mixture, followed by incubation at 37°C for 5 min. After the incubation, 30 μL of 1.25 mM ABTS and 30 μL of peroxidase (1 unit/mL) were added to the mixture, followed by incubation at 37°C for 10 min. The absorbance was recorded at 405 nm using a microplate reader.

The reducing power was determined according to the method of Oyaizu (1986). Each of the extracts was mixed with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min, followed by addition of 0.5 mL of TCA (10%) to the mixture and centrifugation at 1,036 g for 10 min. From the upper layer of the solution, 0.5 mL of solution was mixed with 0.5 mL of distilled water and 0.1 mL of $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Assay for inhibition of acetylcholinesterase

Acetylcholinesterase inhibition assay was conducted via the spectrophotometric method developed by Ellman *et al*. (1961) with slight modification. Acetylthiocholine chloride was employed as the substrate to assay for inhibition of acetylcholinesterase. The reaction mixture contained 140 μL of 100 mM sodium phosphate buffer (pH 8.0), 20 μL of test sample solution, and 20 μL of acetylcholinesterase (0.36 U/mL), which were mixed and incubated for 15 min at room temperature. The reactions were

then initiated via the addition of 10 μL of 5-5'-thiobis-2-nitrobenzoic acid (0.5 mM) and 10 μL of acetylthiocholine chloride (0.6 mM). Hydrolysis of acetylthiocholine chloride was monitored at 412 nm for 15 min based on the formation of yellow 5-thio-2-nitrobenzoate anion, which resulted from the reaction of 5-5'-thiobis-2-nitrobenzoic acid with thiocholine, by the enzymatic hydrolysis of acetylthiocholine chloride.

Statistical analysis

Data were evaluated for statistical significance using the SPSS package for Windows (Version 14.0). Values were expressed as mean±standard error (SE). The mean values were compared using one-way ANOVA followed by Duncan's test. *p*-value of less than 0.05 was considered significant.

Results

Biochemical components such as uronic acid, sulfated-GAGs, and sialic acid were analyzed in each extract, and the results are shown in Table 1. Uronic acid contents of the hot water extracts significantly decreased with increasing temperature ($p < 0.05$), and the extract obtained from the upper part possessed significantly higher uronic acid content than those of the extracts obtained from the middle and base parts. On the other hand, the uronic acid contents in each part of the 70% ethanol extracts were significantly higher than those of the hot water extracts. Further, uronic acid content was the highest in the upper part, followed by the middle and base parts. Sulfated-GAGs contents of each hot water extract decreased with increasing temperature, and the extract obtained from the upper part showed a higher level of sulfated-GAGs than those of the extracts obtained from the middle and base parts. No significant difference was observed between the extracts from the middle and base parts. Furthermore, 70% ethanol extracts of each part showed lower sulfated-GAGs contents compared to those of the hot water extracts. Sialic acid contents of each hot water extract showed a different pattern compared to the contents of uronic acid and sulfated-GAGs. Overall, sialic acid content was highest in the upper part, and extracts at 110°C showed the highest content. However, no sialic acid was observed in the 70% ethanol extracts.

Antioxidant activities of Russian deer velvet antler extracts were evaluated based on scavenging of DPPH, hydrogen peroxide, and reducing power. As shown in Fig. 2, velvet antler extracts obtained from each part

Table 1. Uronic acid, sulfated-GAGs, and sialic acid contents of Russian deer velvet antler extracts

Extracts		Contents (μg/mg)			
		100°C water	110°C water	120°C water	70% ethanol
Upper part	Uronic acid	59.72±1.44[a]	43.62±0.72[b]	12.16±1.56[c]	112.34±1.18[d]
	Sulfated-GAGs	67.96±3.20[a]	51.20±3.50[b]	38.80±2.70[c]	2.46±0.60[d]
	Sialic acid	2.61±0.12[a]	9.07±0.46[b]	5.90±0.39[c]	ND
Middle part	Uronic acid	37.29±1.35[a]	27.28±1.44[b]	15. 15±1.81[c]	106.03±1.50[d]
	Sulfated-GAGs	17.12±2.05[a]	15.26±2.31[a]	10.26±1.50[b]	2.83±0.60[c]
	Sialic acid	6.02±0.34[a]	6.51±0.42[a]	2.37±0.31[b]	ND
Base part	Uronic acid	33.69±0.87[a]	20.84±1.29[b]	10.02±0.74[a]	91.43±2.01[d]
	Sulfated-GAGs	18.04±1.80[a]	14.70±1.90[b]	8.76±1.40[c]	2.46±0.60[d]
	Sialic acid	4.32±0.25[a]	4.56±0.38[a]	2.61±0.28[b]	ND

[a-d] The values with different superscripts indicate a significant difference ($p<0.05$).
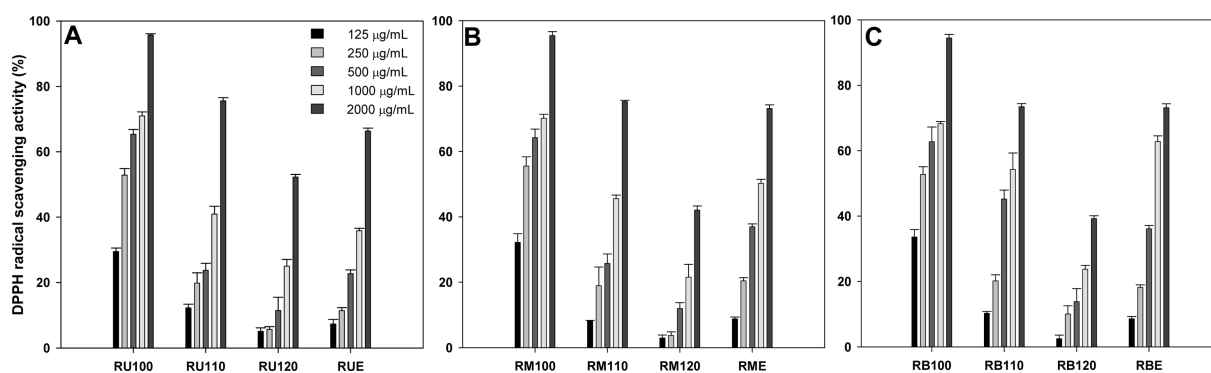Values are mean±SE (n=3).
ND: not detected

Fig. 2. DPPH radical scavenging activities of Russian deer velvet antler extracts. (A), Upper part extracts with hot water at 100°C, 110°C, and 120°C and 70% ethanol; (B), Middle part extracts with hot water at 100°C, 110°C, and 120°C and 70% ethanol; (C); Base part extracts with hot water at 100°C, 110°C, and 120°C and 70% ethanol. Results are presented as means±SE of three determinations.

effectively quenched DPPH radical in a dose-dependent manner. The DPPH scavenging activity decreased with increasing temperature, and RU100, RM100, and RB100, which were extracted at 100°C from each part, exhibited the highest scavenging activities. The 70% ethanol RUE, RME, and RBE also showed DPPH radical scavenging activities in a dose-dependent manner, whereas their scavenging activities were lower than those of RU100, RM100, and RB100, respectively. Fig. 3 depicts hydrogen peroxide scavenging activity, which appeared to be augmented at increasing concentrations. All hot water extracts of velvet antler displayed similar activities, whereas the 70% ethanol extracts showed comparatively higher activity than those of the hot water extracts. The levels of reducing power of the velvet antler extracts are shown in Fig. 4. Dose-dependent augmentation of reducing power was observed, and the 70% ethanol extracts (RUE, RME, and RBE) showed comparatively higher reducing power compared to those of the hot water

extracts. In addition, the upper part extracts (RU100, RU110, and RU120) possessed higher reducing power than those of the middle (RM100, RM110, and RM120) and base part extracts (RB100, RB110, and RB120).

Acetylcholinesterase (AChE) inhibitory activities of velvet antler extracts were investigated using Ellman assay, and the results are depicted in Fig. 5. All of the extracts inhibited AChE activity in a dose-dependent manner, and the extracts at 100°C in each part, including RU100, RM100, and RB100, showed potent anti-AChE activities. On the other hand, RUE, RME, and RBE also inhibited AChE activity, and the levels of inhibition were higher and/or similar compared to those of RU100, RM100, and RB100.

Discussion

Generally, velvet antler can be divided into four parts, the tip, upper, middle, and base part, and is used to treat
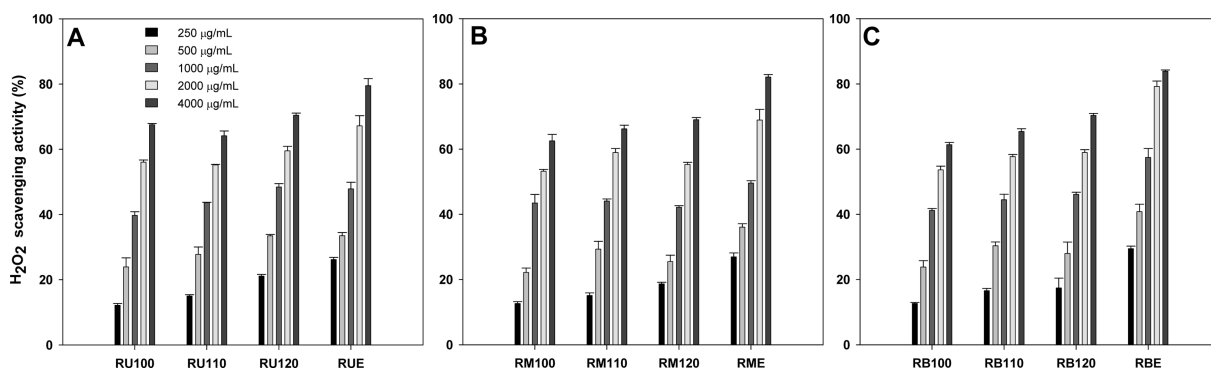
Fig. 3. Hydrogen peroxide scavenging activities of Russian deer velvet antler extracts. (A), Upper part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (B), Middle part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (C), Base part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol. Results are presented as means±SE of three determinations.
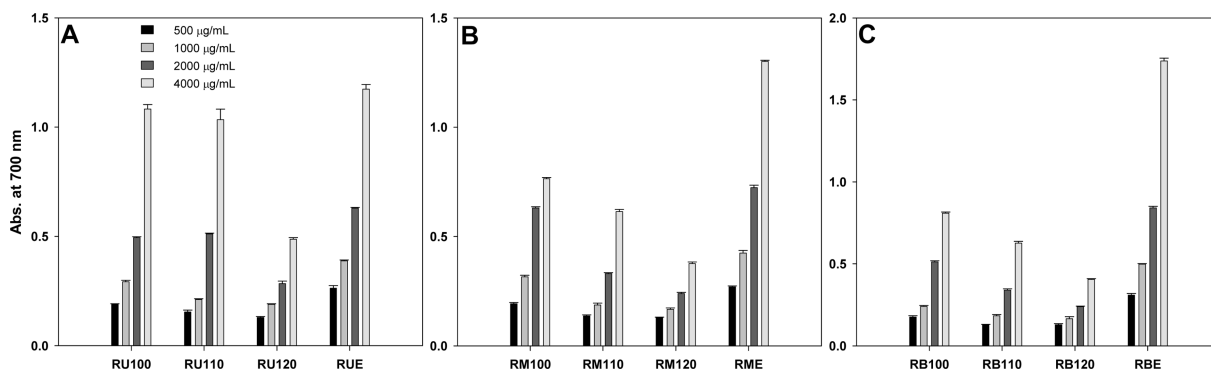

Fig. 4. Reducing power of Russian deer velvet antler extracts. (A), Upper part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (B), Middle part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (C), Base part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol. Results are presented as means±SE of three determinations.

Fig. 5. Acetylcholinesterase inhibitory activity of Russian deer velvet antler extracts. (A), Upper part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (B), Middle part extracts with hot water at 100ºC, 110ºC, and 120ºC and 70% ethanol; (C), Base part extracts with hot water at 100ºC, 110ºC and 120ºC and 70% ethanol. Results are presented as means±SE of three determinations.
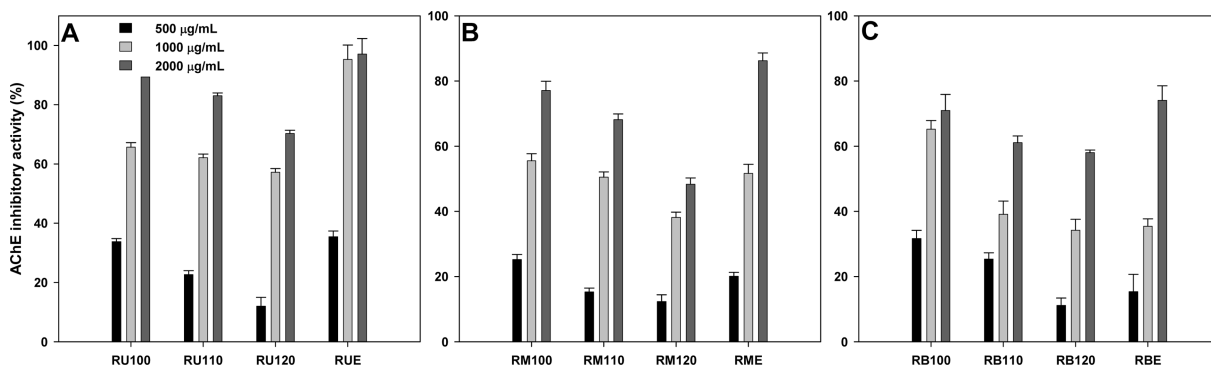
various diseases such as anemia, arthritis, hypercholesterolemia, and cancer or to promote health as a traditional oriental tonic (Fennessy, 1991; Kang *et al*., 2006; Sunwoo *et al*., 1995). Therefore, antler parts are a useful ingredient for developing new food products and/or

nutraceuticals capable of modulating the physiology of human's body and protecting humman against diseases. However, the upper part normally includes the tip part since the tip part is of very small quantity compared to other parts. In Korea, consumers generally believe that the

upper parts of antler root are the most effective for treating various diseases. This stimulated our curiosity to evaluate the antioxidant effects of antler root in different *in vitro* model systems using artificial induction of free radicals or ROS and to determine its biochemical contents of uronic acid, sulfated-GAGs, and sialic acid.

In Korea, velvet antler is generally subjected to extraction as a traditional medical practice (1 atm, 100°C). To determine whether or not this practice is sufficient for the complete extraction of functional components from velvet antler, we used the residue from the second and third extraction process described in the Materials and Methods part. We also investigated the levels of biochemical components as well as the antioxidant activities of velvet antler extracts using different extraction solvents. For this, extraction of velvet antler was performed using an autoclave machine at different temperatures.

Table 1 displays the biochemical composition of the velvet antler extracts. Based on these results, all biochemical components determined in this study were not completely extracted at 100°C. Considerable amounts of uronic acid and sulfated-GAGs were detected at high temperature compared to extracts at 100°C. Furthermore, high temperature was needed to extract sialic acid. These results indicate that traditional medical practice could not fully extract the functional components. The uronic acid, sulfated-GAGs, and sialic acid contents of the ethanol extracts showed different patterns compared to the hot water extracts. Ethanol was more appropriate for extraction of uronic acids, as its content was equal to the sum of the hot water extracts. However, sulfated-GAGs and sialic acid were not extracted by ethanol.

To verify the beneficial bioactivity of the velvet antler extracts, we tried to examine the antioxidant activities *in vitro* using several model systems. Fig. 1 shows the DPPH scavenging activities of the extracts. All extracts, including the upper, middle, and base parts, exhibited good scavenging activity, and the activity was dependent on the extract temperature. It is well known that DPPH is a stable radical and accepts an electron and/or hydrogen radical from donors to become a stable diamagnetic molecule. Therefore, the extracts of velvet antler could have provided an electron and/or hydrogen radical. The RU100, RM100, and RB100 extracts at 100°C showed good scavenging ability compared to those of the RU110, RM110, and RB110 and RU120, RM120, and RB120 extracts. This result indicates that a considerable amount of antioxidant compounds were extracted at 100°C, but also a high amount of antioxidant compounds existed in the residue.

Therefore, the optimal extraction conditions of velvet antler include high temperature and pressure. Ethanol extracts also exhibited lower levels of DPPH scavenging activity than that of the hot water extracts. However, the activities of the parts exhibited no significant difference. Hydrogen peroxide is a reactive non-radical and an important compound as it can penetrate biological membranes. Hydrogen peroxide can also be converted into more reactive species, such as singlet oxygen and hydroxyl radicals, thereby causing lipid peroxidation or toxicity to cells. Therefore, it is important to scavenge hydrogen peroxide to decrease the level of prooxidants. As shown in Fig. 2, velvet antler extracts showed higher hydrogen peroxide scavenging capacity, and the activities of all the extracts were similar. This result also indicates that considerable antioxidant content existed in the residue. Measurement of reducing power was carried out to evaluate the content reductones, which are capable of reducing ferric (III) iron to ferrous (II) in a redox-linked colorimetric reaction, by single electron transfer from the antioxidant components. As shown in Fig. 3, velvet antler extracts exhibited strong reducing power. Among the hot water extracts, reducing power decreased with increasing extraction temperature, whereas the 70% ethanol extracts (RUE, RME, and RBE) exhibited higher reducing power than those of the hot water extracts.

We also carried out anti-AChE activity assay. The efficacy of cholinergic therapies in treating AD supports the cholinergic hypothesis and validates this neurotransmitter system as a therapeutic target (Andreani *et al*., 2008). Therefore, AChE inhibitors currently being used in clinical trials along with some chemically synthesized inhibitors appear to give rise to severe side effects such as nausea, vomiting, bradycardia, anorexia, and sweating (Giacobini, 2004; Jia *et al*., 2009). Therefore, naturally occurring AChE inhibitors without side effects have earned much attention for treatment of AD. Velvet antler is a naturally occurring product and has long been used as a medicinal treatment. In this study, we provided evidence that velvet antler extracts showed anti-AChE activity with different levels of inhibition activity using different extraction methods.

From the present results, the extraction of biochemical components from Russian deer velvet antler was dependent on each biochemical component. For the effective extraction, both water with different temperature and ethanol extraction methods may be needed to combine to extract the biochemical components including uronic acid, sulfated-GAGs, and sialic acid. In addition, all

extracts showed good antioxidant and anti-AChE activity, however, *in vivo* experiments are needed to clarify its effectiveness.

Acknowledgment

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