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Investigation of Bioactive Compounds and Free Scavenging Activity of Ethanol Extracts from Sika Deer (*Cervus nippon*) Antler.

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ABSTRACT

Regrowth velvet antler (RVA) of sika deer (*Cervus nippon*) was subjected to the extraction process using 70% ethanol solution. RVA was divided into 3 segments: top RVA (T-RVA), middle RVA (M-RVA), and base RVA (B-RVA). Biologically active substances such as uronic acid, sulfated-glycosaminoglycans (GAGs), sialic acid, uridine, uracil and hypoxanthine in the extract was analyzed, and their antioxidant activities were investigated using multiple biochemical assays from three segments. The T-RVA section possessed the greatest amounts of uronic acid (97.04 mg/g), GAGs (832.14 mg/g), sialic acid (26.16 mg/g), uridine (5.77 mg/g), uracil (4.73 mg/g), and hypoxanthine (3.80 mg/g). In addition, the antioxidant activities were estimated. The TE values of DPPH, hydrogen peroxide (H_2O_2), hydroxyl, ABTS (2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate) radical scavenging activity, and ferric reducing antioxidant power (FRAP) from T-RVA were 48.781, 30.688, 42.637, 72.074 and 29.112 TE/μM at 1 mg/ml, respectively. The TE value of oxygen radical absorbance capacity (ORAC) was 129.573 TE/μM at 20 μg/ml. These results indicate that the T-RVA section possesses the greatest amount of biologically active substances and the highest antioxidant potential. This is the first report on the bioactive components and antioxidant potential of RVA ethanol extract.

Keywords: regrowth velvet antler, bioactive components, antioxidant potential

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INTRODUCTION

Velvet antler is a widely used traditional Asian medicine that has been used clinically in East Asia for millennia to treat various diseases and as a tonic for more than 2000 years [1]. It is believed to have many health benefits such as energy and growth enhancement [2], immunomodulatory function, anti-inflammatory properties [1,3], haemotopoietic effect [4], anti-aging effect [5], chronic wound healing effect, blood pressure modulation and cholesterol reduction [6]. It grows at a very fast rate of up to 2 cm/day in some species and is a renewable resource. During the optimum regeneration period, velvet antler is removed and processed with no harm to the animal. In China, velvet antler is generally harvested twice per year. The first velvet antler harvest occurs after 40-45 days of growth, while a second harvest occurs after 50–55 days of regrowth, at which point the harvested velvet antler is known as regrowth velvet antler (RVA). Although studies have been conducted on the chemical composition of RVA, there have been no comprehensive reports on the composition of biologically active substances and antioxidant potential of RVA [7,8].

A lot of studies have demonstrated that free radicals are generated by oxidative damage to biomolecules such as nucleic acids, carbohydrates, proteins, and lipids [9-11]. Oxidative stress plays a crucial role in a number of disease outbreaks and progression such as cancer, diabetes mellitus, and neurodegenerative and inflammatory diseases [12,13]. Numerous medical reports and clinical observations convincingly show that disease-resistance can be conferred by enhancing antioxidative processes [14-19]. Therefore, antioxidant supplementation could prevent or inhibit oxidative stress induced by free radicals. Antioxidants terminate free radical chain reactions by removing free radical intermediates, while inhibiting other oxidation reactions. On account of the clinical potential of antioxidants, significant interest has been focused on the development of natural antioxidants that are safe and effective.

In the present work, therefore, RVA was determined its constituent biologically active substances, including uronic acid, GAGs, sialic acid, uracil, hypoxanthine, and uridine. In addition, the antioxidant activities of RVA were determined by assessing DPPH, H_2O_2, hydroxyl, ABTS radical scavenging activity, FRAP and ORAC.

MATERIALS AND METHODS

Materials

Seven specimens of Sika deer (*Cervus nippon*) RVA were collected at the same farm (Fanrong farm, China). Carbazole, sodium tetraborate, dimethylmethylene blue, glycine, sodium thiosulfate, acetoacetanilide, uracil, hypoxanthine, uridine, DPPH, ABTS, potassium persulfate, FL (fluorscein), and 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chemical composition

Velvet antlers were analyzed for ash, crude protein (CP), and ether extracts (EE) by the method of the Association of Official Analytical Chemists [20].

Preparation of extract

The RVA specimens were divided into 3 sections; T-RVA, M-RVA, and B-RVA, lyophilized, and homogenized with a grinder. Next, 10 g of each segment was added to 100 ml of 70% of ethanol and subjected to extraction for 2 h and repeated three times the same process. The RVA extracts were filtered (0.25-μm pore size) and lyophilized (yields: T-RVA, 4.01%; M-RVA, 3.57%; B-RVA, 2.38%) in a freeze dryer for 5 days.

Bioactive components

Uronic acid

Uronic acid content was determined by the carbazole reaction [21]. Briefly, a 50 μl serial dilution of the standards or samples was placed in a 96-well plate, after which 200 μL of 25 mM sodium tetraborate in sulfuric acid was added to each well. 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 in a microplate reader at a wavelength of 550 nm.

Sulfated-GAGs

GAGs content was determined by the dimethylmethylene blue (DMB) dye binding method [22]. Briefly, the color reagent was prepared by dissolving 0.008 g of DMB in a solution containing 1.185 g NaCl, 1.520 g glycine, 0.47 ml HCl (12 M), and 500 ml DW. Each sample was mixed into 1 ml of color reagent and the absorbance was read immediately at 525 nm.

Sialic acid

Sialic acid content was determined based on the procedures described by Matsuno and Suzuki [23]. All solutions were precooled in an ice bath. Sodium periodate solution (10 mM, 20 µl) was added to 200 µl of a glycoconjugate sample in a 15-ml polypropylene test tube. The solution was chilled in an ice bath for 45 min. The reaction was terminated by the addition of 100 µl of 50 mM sodium thiosulfate solution. Next, 500 µL of 4.0 M ammonium acetate (pH 7.5) and 400 µl of ethanolic solution of 100 mM acetoacetanilide were added to the solution, which was left standing for 10 min at room temperature. The fluorescence intensity of the solution was measured at 471 nm with an excitation wavelength of 388 nm.

Uracil，hypoxanthine, uridine

Uracil, hypoxanthine, and uridine were determined as described previously [24]. One mg of the Ethanol extract was dissolved in 1 ml of 3% methanol solution, after which 1 ml of the resulting solution was filtered for HPLC analysis. The analysis was performed on an HPLC system equipped with an isocratic pump and refractive index (RI) detector (Shimadzu Corporation, Kyoto, Japan). The separation was conducted on a ZORBAX Eclipse Plus C18 column (4.6 × 150 mm, 5 µm, Agilent Technologies, USA). The mobile phase was 0.07% acetic acid methanol-water (3:97, v/v; pH 3.5) at a flow-rate of 1.0 ml/min. A series of standards of uracil, hypoxanthine, and uridine in the range of 0.625–40.00 ppm were prepared in the mobile phase. Quantification was carried out by integration of the peak areas using external standard calibration. A linear response with a correlation coefficient of 0.999 (n=6) was obtained for the standards. For all experiments, the extracts and standards were filtered through a 0.45-µm cellulose ester membrane before injection into the HPLC system. Detection was performed at a wavelength of 254 nm.

Antioxidant activity

DPPH radical scavenging activity

DPPH scavenging activity of various antler extracts was measured according to a slightly modified method of Blois [25]. 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 540 nm by microplate reader and the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 540 nm.

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller [26]. A 100 µL of 0.1 M phosphate buffer (pH 5.0) was mixed with each extract in a 96 microwell plate. A 20 µl of hydrogen peroxide was added to the mixture, and then incubated 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, and then incubated at 37 °C for 10 min. The absorbance was recorded at 405 nm by microplate reader and the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ was the absorbance of the reaction mixture without an RVA sample and A_{sample} was the absorbance of the reaction mixture with an RVA sample.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of various antler extracts was determined according to the method of Chung et al [27]. Hydroxyl radical was generated by Fenton reaction in the presence of $FeSO_4$. A reaction mixture containing 0.1 ml of 10 mM $FeSO_4$, 10 mM EDTA and 10 mM 2-deoxyribose was mixed with 0.1 ml of the extract solution, after which 0.1 M phosphate buffer (pH 7.4) was added into the reaction mixture until the total volume reached to 0.9 ml. Subsequently, 0.1 ml of 10 mM H_2O_2 was finally added to the reaction mixture and incubated at 37°C for 4 h. After incubation, each 0.5 ml of 2.8% TCA and 1.0% TBA were added to the mixture, following which the mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm and the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 532 nm.

ABTS radical scavenging activity

The procedure of ABTS scavenging activity was followed the method of Arnao et al [28]. Stock solutions included $ABTS^{\bullet+}$ solution and potassium persulfate solutions. Working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h. The solution was then diluted with fresh $ABTS^{\bullet+}$ solution and mixed with or without each extract. After incubation for 2 h, the absorbance was recorded at 735 nm, the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 735 nm.

FRAP assay

FRAP assay was done according to Benzie and Strain [29]. The fresh working solution was prepared by mixing acetate buffer, TPTZ solution and $FeCl_3 \bullet 6H_2O$ solution and then warmed at 37°C before using. Each extract was allowed to react with FRAP solution at dark room and room temperature for 30 min. Readings of the colored product were then taken at 595 nm and the percentage of scavenging activity was calculated using the following equation:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample})/A_{control} \times 100$$

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 595 nm.

ORAC assay

For ORAC assay, the method of Ou et al was used with some slightly modification [30]. The working solution of FL and AAPH radical were prepared daily. Sample, blank or standard were placed in 96 microwell plate, and the plate was heated to 37°C for 15 min prior to the addition of AAPH. The fluorescence was measured immediately after the AAPH addition and measurements with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm were taken every 5 min until the relative fluorescence intensity was less than 5% of the value of the initial reading.

The ORAC values, expressed as µM trolox equivalents (µM TE) were calculated by applying the following formula:

$$\text{ORAC (µM TE)} = (C_{Trolox} \times (AUC_{sample} - AUC_{blank}) \times k)/(AUC_{sample} - AUC_{blank})$$

where C_{Trolox} is the concentration of Trolox (20 µM), k is the sample dilution factor, and AUC is the area below the fluorescence decay curve of the sample, blank, and trolox, respectively, calculated by applying the following formula in a Microsoft Excel spread-sheet (Microsoft, Washington, USA).

$$AUC = (0.5 + f_5/f_0 + f_{10}/f_0 + \dots + f_{n+5}/f_0) \times 5$$

where f_0 is the initial fluorescence and f_n is the fluorescence at time n.

Statistical analysis

The experiments shown are, in fact, summaries of the data sourced from at least three experiments. All of data are presented using the mean±SE. Statistical analyses were performed using SAS statistical software (SAS Institute, Cray, NC, USA). Treatment effects were analyzed using one-way analysis of variance, followed by Dunnett's multiple range tests. The results $p<0.05$ was used to indicate significance.

RESULTS AND DISCUSSION

Chemical composition

The contents of ash, CP and EE in RVA are given in Table 1. The ash content was the lowest in the T-RVA ($p<0.05$) and has a significant difference between the M-RVA and B-RVA (16.74% < 22.4% < 31.43%). In contrast, the content of CP was the highest in the top section ($p<0.05$) and has a significant difference between the (55.15% > 45.83% > 44.96%). The content of EE was the highest in the top section ($p<0.05$) and has a significant difference between the middle and base section (6.4% > 5.7% > 4.25%).

Table 1: Contents of crude ash, crude protein and ether extract of regrowth velvet antler extracts

	T-RVA	M-RVA	B-RVA
	...................................DM%...................................		
Ash	16.74±0.63[c]	22.40±1.53[b]	31.43±0.75[a]
CP	55.15±1.85[a]	45.83±0.09[b]	44.96±1.26[c]
EE	6.40±0.55[a]	5.76±0.24[b]	4.25±0.24[c]

[a-c]Mean with different superscripts in the same column are different ($p<0.05$).

Bioactive components

The biologically active substances contained in the 3 RVA segments, including uronic acid, sulfated-GAGs, sialic acid, uridine, uracil, and hypoxanthine, are listed in Table 2.

Table 2: Sulfate-GAGs, sialic acid , uronic acid, uridine, uracil and Hypoxanthin,contents of regrowth velvet antler extracts

			mg/g
	Top	Middle	Base
Sulfate-GAGs	832.14±8.47[a]	646.69±7.03[b]	500.75±6.05[c]
Sialic acid	26.16±0.26[a]	22.94±0.16[b]	11.30±0.83[c]
Uronic acid	97.04±4.44[a]	77.01±3.65[b]	57.79±3.14[c]
Uridine	5.77±0.09[a]	4.83±0.06[b]	2.05±0.05[c]
Uracil	4.73±0.06[a]	3.70±0.06[b]	3.00±0.06[c]
Hypoxanthine	3.80±0.08[a]	2.60±0.04[b]	2.14±0.05[c]

[a-c]Mean with different superscripts in the same column are different ($p<0.05$).

The uronic acid content, sulfated-GAG content, and sialic acid content of the T-RVA and M-RVA sections were significantly greater than those of the B-RVA section (p<0.05). The extract of the T-RVA section contained 97.04 mg/g of uronic acid, 832.14 mg/g of sulfated-GAGs, and 26.16 mg/g of sialic acid, 5.77 mg/g of uridine, 4.73 mg/g of uracil, and 3.80 mg/g of hypoxanthine, respectively (Table 2).

Antioxidant activity

The antioxidant activity of the extract of T-RVA was significantly better than those of the M-RVA and B-RVA sections (*p*<0.05) and appeared to be dose-dependent. The DPPH radical scavenging activity was the highest for the T-RVA section (48.78 µM TE/mg) and the lowest for the B-RVA section (Fig 1). The H_2O_2 (30.69 µM TE/mg; Fig 2) and hydroxyl (42.64 µM TE/mg; Fig 3) radical scavenging activities were also the highest for the T-RVA section. The T-RVA exhibited the highest ABTS radical scavenging activity (72.07 µM TE/mg), whereas the activities of M-RVA and B-RVA were similar (Fig 4). The T-RVA was the most effective section in the FRAP assay (29.11 µM TE/mg), whereas the activities of B-RVA and M-RVA were similar (Fig 5). In the ORAC assay, 1,000 µg/ml of T-RVA showed the excellent activity (129.57 µM TE/mg) (Fig 6).
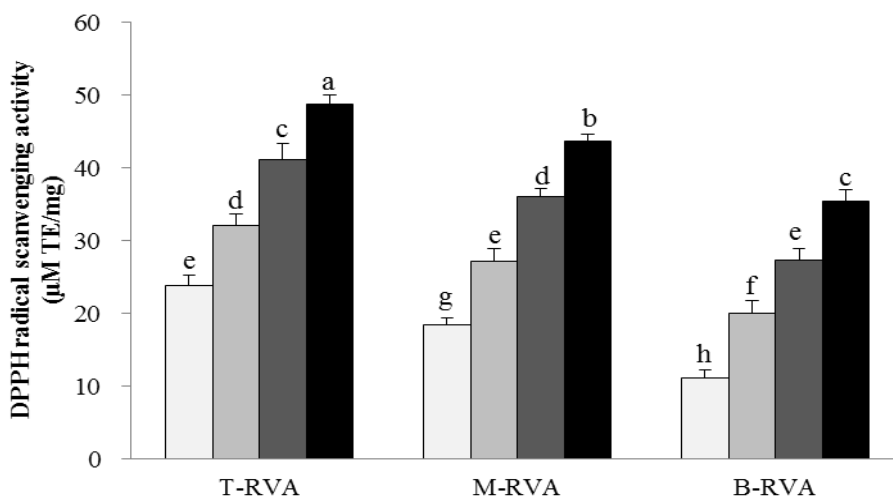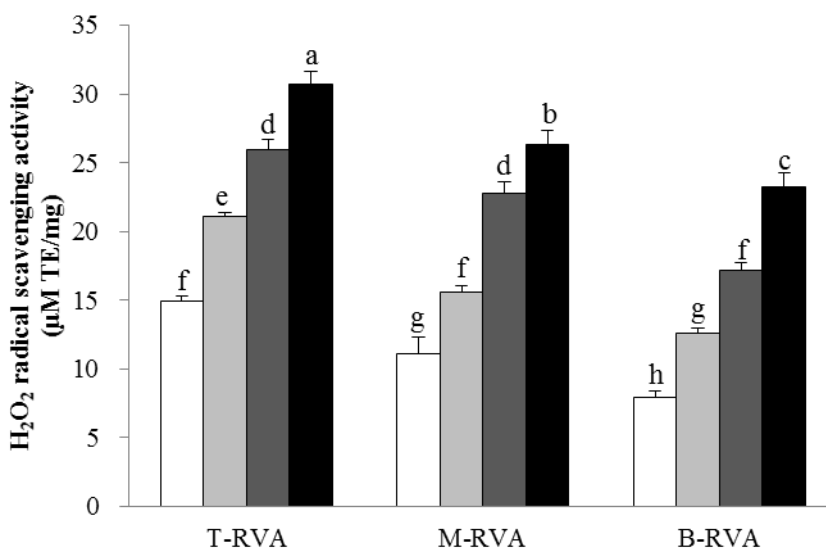

Figure 1: The effect of RVA on DPPH radical scavenging activity. [a-i]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ☐ , 125 µg/ml; ▨ , 250 µg/ml; ▨ , 500 µg/ml; ■ , 1,000 µg/ml.

Figure 2: The effect of RVA onH_2O_2radical scavenging activity. [a-i]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ☐ , 125 µg/ml; ▨ , 250 µg/ml; ▨ , 500 µg/ml; ■ , 1,000 µg/ml.
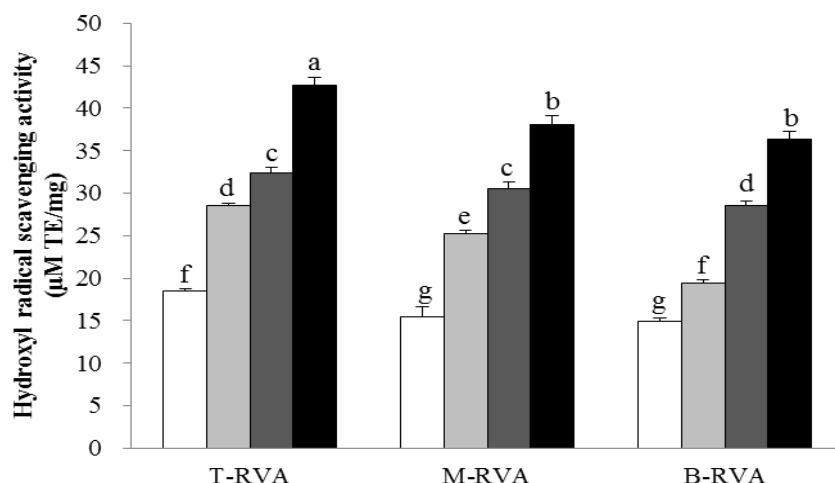
Figure 3: The effect of RVA onHydroxyl radical scavenging activity. [a-g]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ☐ , 125 µg/ml; ▨ , 250 µg/ml; ▨ , 500 µg/ml; ■ , 1,000 µg/ml.
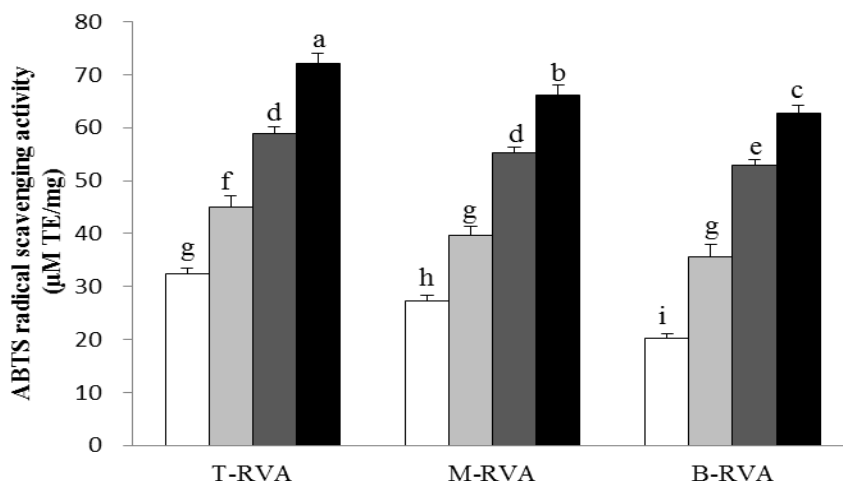

Figure 4: The effect of RVA onABYS radical scavenging activity. [a-g]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ☐ , 125 µg/ml; ▨ , 250 µg/ml; ▨ , 500 µg/ml; ■ , 1,000 µg/ml.
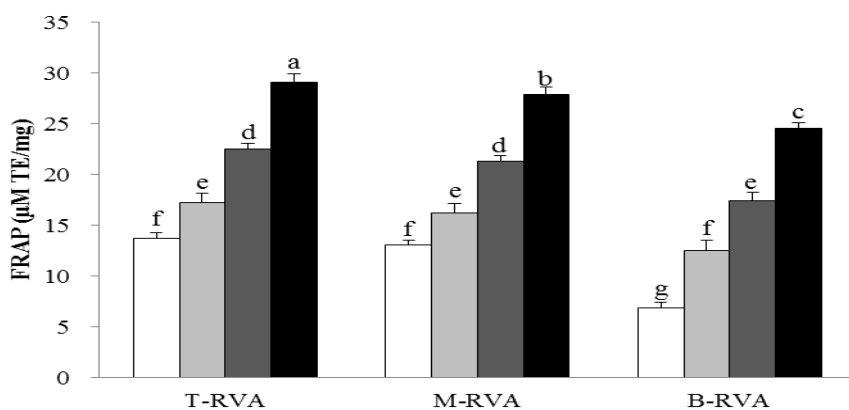

Figure 5: The effect of RVA on FRAP assay. [a-g]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ☐ , 125 µg /ml; ▨ , 250 µg/ml; ▨ , 500 µg/ml; ■ , 1,000 µg/ml.
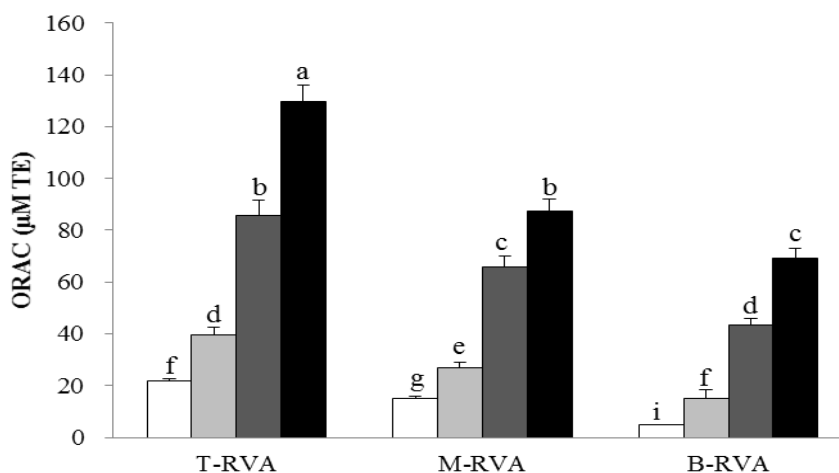
Figure 6: The effect of RVA on ORAC. [a-g]Values not sharing a common letter are significantly different at *p*<0.05 by Dunnett's multiple range tests. ▢ , 125 µg /ml; ▢ , 250 µg/ml; ▧ , 500 µg/ml; ■ , 1,000 µg/ml.

Jeon et al reported the approximate composition of velvet antler; the content of protein decreased and the content of ash increased from the top section to the base section [31], and which is perfect matched our results shown above (Table 1). GAGs, particularly chondroitin sulfate (CS), are of particular interest to physicians and pharmacists. GAGs are composed of units of amino sugar, including D-glucosamine and D-galactosamine, and bond to core proteins to form proteoglycans. Cartilage proteoglycans regulate water retention and are integral to the differentiation and proliferation of chondrocytes. The most prominent GAGs in velvet antler tissue are chondroitin sulfate [32]. Sialic acid is a water soluble component that was efficiently extracted by Ethanol and showed significant accumulation in the T-RVA section. Je et al reported the GAGs and sialic acid contents of whole deer velvet antler were 41.04 mg/g and 1.92 mg/g [33], and the values were much lower than ours (Table 2). This may be attributed to different extraction method. Uronic acid has been reported to improve circulation and decrease stroke risk [34]; therefore, our chemical analyses indicate that the Ethanol extract of T-RVA might possess similar activities. Scott and Hughes reported that uronic acid concentration in whole deer velvet antler was 300 µg/g [35], and the value was much lower than ours (97 mg/ml, Table 2). Zhao et al investigated the amounts of uridine, uracil, and hypoxanthine from ethanol extracted velvet antler [36]. Uracil is a primary mediator of monoamine oxidase (MAO) inhibition by velvet antler extract [3]. Our data indicate that the T-RVA section, which contains the highest amount of uridine, may contribute the majority of the inhibitory effect on MAO activity produced by regrowth velvet antler. They also showed that uridine was responsible for 34.75% of the Fe^{2+}-chelating activity of velvet antler [37]. Therefore, the ethanol extract of T-RVA is expected to show strong antioxidant activity due to its abundance of uridine (5.77 mg/ml). The content of uridine and uracil is lower than ours. They suggested that the main anti-aging compound of velvet antler was hypoxanthine, and the hypoxanthine value (3.9 mg/ml) of theirs was similar with ours (3.8 mg/ml) [24].

DPPH radical scavenging activity is often used as a method of evaluating antioxidant activity. DPPH is a stable radical that accepts an electron and/or hydrogen radical from donor molecules to form a stable diamagnetic molecule. Therefore, the extracts of velvet antler may have provided an electron and/or hydrogen radical to neutralize DPPH [38]. In a report by Lee and Chuang, the DPPH radical scavenging activity of velvet antler extract obtained from the top section was reported to be 67.1% at an extract concentration of 100 mg/ml [39]. Meanwhile, our T-RVA exhibited 48.7% DPPH radical activity at 1 mg/ml (Fig 1). Therefore we could assume that T-RVA possessed higher DPPH radical scavenging activity than Lee and Chuang's top section velvet antler. Hydrogen peroxide is a reactive non-radical and a clinically important compound due to its ability to penetrate biological membranes. Hydrogen peroxide can be converted into more reactive species, such as singlet oxygen and hydroxyl radicals, thereby causing lipid peroxidation or toxicity to cells. Therefore, scavenging of hydrogen peroxide can decrease prooxidants levels. Our analysis showed that T-RVA possessed the highest activity, and the TE value was 30.69 µM TE/mg (Fig 2). Hydroxyl radicals are extremely reactive and easily react with amino acids, DNA, and membrane components. In this study, the hydroxyl radical scavenging activity of RVA was higher than that of velvet antler as reported by Je et al (Fig 3) [33]. In addition, our analysis

of ABTS radical scavenging activity by RVA identified higher than that reported by Zhao et al (Fig 4) [37]. The FRAP assay treats the antioxidants contained in the samples as reductants in a redox-linked colorimetric reaction, allowing assessment of the reducing power of antioxidants [40]. Zhao et al reported the activity of 85.8% by 5 mg/ml of velvet antler extract in the FARP assay [37], which was lower than the activity measured in our analysis (Fig 5). Recently, the ORAC assay has been applied extensively to evaluate the antioxidant activities of fruits, vegetables, leaves, stems, herbs, and spices. The ORAC assay is commonly mentioned in scientific publications and health food publications [41]. However, the antioxidant activity of RVA has not been evaluated using the ORAC assay. Especially important, since this is the first time evaluation of ORAC from RVA Ethanol extract was performed and it gives basic data for further study. In a recent study using the ORAC assay to test the antioxidant activity of gallic acid, Ana et al reported activity of 161 μM TE/mg [42], which was higher than the activity of RVA found in our study (Fig 6). These results show that RVA possesses bioactive components and antioxidant activity.

CONCLUSIONS

From theses multiple chemical and biological in vitro investigations, it was first proved that the amount of bioactive components and free radical scavenging activity of RVA Ethanol extract. This proof indicates the possibility for RVA Ethanol extract to be used as a new source of natural antioxidants. Future studies are required to further elucidation of the other biological activities of the RVA and the biological mechanisms underlying their effects.

ACKNOWLEDGMENT

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