Research Article

First Evaluation of the Biologically Active Substances and Antioxidant Potential of Regrowth Velvet Antler by means of Multiple Biochemical Assays

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Received 31 May 2015; Accepted 2 July 2015

Academic Editor: Patricia Valentao

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We investigated the biologically active substances contained in RVA (regrowth velvet antler) by comparing the composition of biologically active substances and antioxidant potential of different antler segments. RVA was subjected to extraction using DW (distilled water). RVA was divided into 3 segments: T-RVA (top RVA), M-RVA (middle RVA), and B-RVA (base RVA). The T-RVA section possessed the greatest amounts of uronic acid (36.251 mg/g), sulfated GAGs (sulfated glycosaminoglycans) (555.76 mg/g), sialic acid (111.276 mg/g), uridine (0.957 mg/g), uracil (1.084 mg/g), and hypoxanthine (1.2631 mg/g). In addition, the T-RVA section possessed the strongest antioxidant capacity as determined by DPPH, H₂O₂ (hydrogen peroxide), hydroxyl, and ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonate) radical scavenging activity as well as FRAP (ferric reducing antioxidant power) and ORAC (oxygen radical absorbance capacity). The values of those were 53.44, 23.09, 34.12, 60.31, and 35.81 TE/ μ M at 1 mg/mL and 113.57 TE/ μ M at 20 μ g/mL. These results indicate that the T-RVA section possesses the greatest amount of biologically active substances and highest antioxidant potential. This is the first report on the biologically active substances and antioxidant potential of RVA.

1. Introduction

Velvet antler consists of the cartilaginous, prequalified antlers of moose, elk, and sika deer, which regrow yearly. The growth of deer antlers is one of the fastest types of tissue growth in mammals. Growing antlers contain nerves and blood vessels and are covered with a hairy, skin covering tissue commonly known as “velvet” [1]. 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 [2]. 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 RVA. Although

studies have been conducted on the chemical composition of RVA [3, 4], there have been no comprehensive reports on the composition of biologically active substances and antioxidant potential of RVA.

Numerous studies have demonstrated that free radicals are generated by oxidative damage to biomolecules such as lipids, nucleic acids, proteins, and carbohydrates [5–7]. Overproduction of free radicals and reactive oxygen species 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 [8]. Many medical reports and clinical observations convincingly show that disease-resistance can be conferred by enhancing antioxidative processes [9–14].

Therefore, antioxidant supplementation could prevent or inhibit oxidative stress induced by ROS. Antioxidants terminate free radical chain reactions by removing free radical intermediates while inhibiting other oxidation reactions. Because of the clinical potential of antioxidants, significant interest has been focusing on the development of natural antioxidants that are safe and effective.

In this study, RVA was subjected to extraction by DW to allow determination of its constituent biologically active substances, including uronic acid, sulfated GAGs, sialic acid, uracil, hypoxanthine, and uridine. In addition, the antioxidant activities of RVA were determined by assessing DPPH, H_2O_2 , hydroxyl, and ABTS radical scavenging activity as well as FRAP and ORAC.

2. Materials and Methods

2.1. 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, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), FL, and AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of Samples. 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 DW and subjected to extraction in boiling DW for 1 h. The RVA extracts were filtered (0.25 μ m pore size) and lyophilized (yields: T-RVA, 3.87%; M-RVA, 3.61%; B-RVA, 2.66%) in a freeze dryer for 5 days.

2.3. Analysis of Bioactive Compounds

2.3.1. Uronic Acid. Uronic acid content was determined by the carbazole reaction [15]. Briefly, a 50 μ L serial dilution of the standards or samples was placed in a 96-well plate, after which 200 μ L 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 μ L 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.

2.3.2. Sulfated GAGs. GAGs content was determined by the DMB (dimethylmethylene blue) dye binding method [16]. 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.

2.3.3. Sialic Acid. Sialic acid content was determined based on the procedures described by Matsuno and Suzuki [17]. All solutions were precooled in an ice bath. Sodium periodate

solution (10 mM, 20 μ L) was added to 200 μ L 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 μ L of 50 mM sodium thiosulfate solution. Next, 500 μ L of 4.0 M ammonium acetate (pH 7.5) and 400 μ L 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.

2.3.4. Uracil, Hypoxanthine, and Uridine. Uracil, hypoxanthine, and uridine were determined as described previously [18]. 1 mg of the DW 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 (Kyoto, Japan) and RI (refractive index) detector (Lab Alliance, model 500). The separation was conducted on a ZORBAX Eclipse Plus C18 column (4.6 $\times$ 150 mm, 5 μ m, 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 μ m cellulose ester membrane before injection into the HPLC system. Detection was performed at a wavelength of 254 nm.

2.4. Antioxidant Activity

2.4.1. DPPH Radical Scavenging Activity. The DPPH scavenging activity of each antler extract was measured according to a slightly modified version of the method of Blois [19]. DPPH solutions (1.5×10^{-4} M, 100 μ L) were mixed with and without each extract (100 μ L), after which the mixtures were incubated at room temperature for 30 min. After standing for 30 min, absorbance was recorded at 540 nm using a microplate reader. The scavenging activity was calculated as a percentage using the following equation:

$$\text{Inhibition (\%)} = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100, \quad (1)$$

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.

2.4.2. Hydrogen Peroxide Radical Scavenging Activity. Hydrogen peroxide scavenging activity was determined according to the method of Muller [20]. A 100 μ L of 0.1 M phosphate buffer (pH 5.0) was mixed with each extract in a 96-microwell plate. A 20 μ L of hydrogen peroxide was added to the mixture and then incubated at 37°C for 5 min. After the incubation, 30 μ L of 1.25 mM ABTS and 30 μ L 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 (1).

2.4.3. Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of each antler extract was determined according to the method of Chung et al. [21]. Hydroxyl radicals were generated by the Fenton reaction in the presence of FeSO₄. A reaction mixture containing 0.1 mL of 10 mM FeSO₄, 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 to the reaction mixture to reach a total volume of 0.9 mL. Subsequently, 0.1 mL of 10 mM H₂O₂ was added to the reaction mixture, which was incubated at 37°C for 4 h. After incubation, 0.5 mL of 2.8% TCA and 1.0% TBA were added to each mixture, after which each mixture was placed in a boiling water bath for 10 min. Absorbance was measured at 532 nm. Hydroxyl radical scavenging activity was calculated as a percentage using (1).

2.4.4. ABTS Radical Scavenging Activity. The ABTS scavenging activity of each antler extract was assessed following the method of Arnao et al. [22]. Stock solutions included ABTS^{•+} solution and potassium persulfate solutions. A working solution was prepared by mixing the 2 stock solutions in equal quantities and allowing them to react for 12 h. The working solution was diluted with fresh ABTS^{•+} solution and mixed with or without each extract. After incubation for 2 h, the absorbance of each solution was recorded at 735 nm. The scavenging activity was calculated as a percentage using (1).

2.4.5. FRAP Assay. The FRAP assay was performed according to the method of Benzie and Strain [23]. Fresh working solution was prepared by mixing acetate buffer, TPTZ solution, and FeCl₃·6H₂O solution and warmed at 37°C before use. Each extract was allowed to react with the FRAP solution in a dark room at room temperature for 30 min. The absorbance of the colored product was measured at 595 nm. Scavenging activity was calculated as a percentage using (1).

2.4.6. ORAC Assay. For ORAC assay, the method of Ou et al. was used with some slightly modification [24]. The working solution of FL and AAPH radical was prepared daily. Sample, blank, or standard was 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/mg) were calculated by applying the following formula:

$$\text{ORAC } (\mu\text{M TE}) = \frac{(C_{\text{Trolox}} \times (\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}) \times k)}{(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})}, \quad (2)$$

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 spreadsheet (Microsoft, Washington, USA):

$$\text{AUC} = \left(0.5 + \frac{f_5}{f_0} + \frac{f_{10}}{f_0} + \dots + f_n + \frac{5}{f_0} \right) \times 5, \quad (3)$$

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

2.5. Statistical Analysis. The results shown are summaries of the data from at least 3 experiments. All data are presented as mean ± SEM (standard error of the mean). Statistical analyses were performed using SAS statistical software (SAS Institute, Inc., Cary, NC, USA). Treatment effects were analyzed using one-way ANOVA followed by Dunnett's multiple range test. Results of $P < 0.05$ indicated statistical significance.

3. Results and Discussion

3.1. Bioactive Composition. The biologically active substances contained in the 3 RVA segments, including uronic acid, sulfated GAGs, sialic acid, uridine, uracil, and hypoxanthine, are listed in Tables 1 and 2.

The uronic acid content, sulfated GAGs 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 DW extract of the T-RVA section contained 36.25 mg/g uronic acid, 555.76 mg/g sulfated GAGs, and 111.28 mg/g sialic acid (Table 1). The DW extract of the RVA contained 0.957 mg/g uridine, 1.084 mg/g uracil, and 1.263 mg/g hypoxanthine (Table 2).

Uronic acid has been reported to improve circulation and decrease stroke risk [25]; therefore, our chemical analyses indicate that the DW extract of T-RVA might possess similar activities. Sulfated GAGs, particularly CS (chondroitin sulfate), are of particular interest to physicians and pharmacists. Sulfated GAGs are composed of units of amino sugar, including D-glucosamine and D-galactosamine, and bond with core proteins to form proteoglycans. Cartilage proteoglycans regulate water retention and are integral to the differentiation and proliferation of chondrocytes. The most prominent sulfated GAG in velvet antler tissue is chondroitin sulfate [26]. Sialic acid is a water soluble component that was efficiently extracted by DW and showed significant accumulation in the T-RVA section. Our findings are similar to those of a previous report [27], which showed that the T-RVA, or "wax piece," contains sialic acid levels higher than those of other antler regions. Uracil is a primary mediator of MAO (monoamine oxidase) inhibition by velvet antler extract [28]. Our data indicate that the T-RVA section may contribute the majority of the inhibitory effect on MAO activity produced by velvet antler. In a report by Wang et al. [29] aimed at identifying the active compound in velvet antler responsible for inhibiting MAO-B activity, the author suggested that the main antiaging compound in velvet antler is hypoxanthine. Zhou et al. [28] showed that uridine was

TABLE 1: Sulfated GAGs, sialic acid, and uronic acid contents of regrowth velvet antler extracts.

	T-RVA	M-RVA	B-RVA
	mg/g	mg/g	mg/g
Sulfated GAGs	555.76 ± 12.48	369.30 ± 19.81	228.98 ± 24.42
Sialic acid	111.28 ± 4.27	79.50 ± 5.20	72.96 ± 1.50
Uronic acid	36.25 ± 2.96	25.90 ± 2.29	23.11 ± 2.40

TABLE 2: Hypoxanthine, uridine, and uracil contents of regrowth velvet antler extracts.

	T-RVA	M-RVA	B-RVA
	mg/g	mg/g	mg/g
Hypoxanthine	1.08 ± 0.03	1.00 ± 0.01	0.92 ± 0.01
Uridine	1.26 ± 0.03	1.14 ± 0.04	1.06 ± 0.03
Uracil	0.96 ± 0.08	0.092 ± 0.03	0.845 ± 0.04

responsible for 34.75% of the Fe^{2+} -chelating activity of velvet antler. Therefore, the DW extract of T-RVA is expected to show strong antioxidant activity due to its abundance of uridine. Zhou and Li [18] investigated the amounts of uridine, uracil, and hypoxanthine from ethanol extracted velvet antler, and the values were 3.7, 3.6 and 3.9 mg/g, respectively. Their values were higher than ours. This may be attributed to extraction method.

3.2. Antioxidant Activity. The antioxidant activities of RVA may not be attributed to a single mechanism. Therefore, 6 methods were used to evaluate different aspects of the antioxidant activities of RVA.

The antioxidant activities of the DW extracts of RVA were evaluated by assessing DPPH, H_2O_2 , ABTS, and hydroxyl radical scavenging activity. In addition, FRAP and ORAC were estimated.

The antioxidant activity of the DW 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 highest for the T-RVA section ($53.44 \mu\text{M TE/mg}$, IC_{50} 0.853 mg/mL) and lowest for the B-RVA section (Figure 1). H_2O_2 ($32.20 \mu\text{M TE/mg}$; Figure 2) and ABTS ($60.31 \mu\text{M TE/mg}$; Figure 3) radical scavenging activities were also highest for the T-RVA section. The hydroxyl radical scavenging activity was highest for the T-RVA section ($23.09 \mu\text{M TE/mg}$), whereas the activities of M-RVA and B-RVA were similar (Figure 4). The T-RVA section was the most effective section in the FRAP assay ($35.81 \mu\text{M TE/mg}$), whereas the activities of B-RVA and M-RVA were similar (Figure 5). In the ORAC assay, $1,000 \text{ mg/mL}$ T-RVA showed excellent activity ($121.58 \mu\text{M TE/mg}$) (Figure 6).

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 [30]. In a report by Lee and Chung [31], the DPPH radical scavenging

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. Light gray square, $125 \mu\text{g/mL}$; gray square, $250 \mu\text{g/mL}$; dark grey square, $500 \mu\text{g/mL}$; black square, $1,000 \mu\text{g/mL}$.

FIGURE 2: The effect of RVA on H_2O_2 radical scavenging activity. ^{a-i}Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range tests. Light gray square, $125 \mu\text{g/mL}$; gray square, $250 \mu\text{g/mL}$; dark grey square, $500 \mu\text{g/mL}$; black square, $1,000 \mu\text{g/mL}$.

FIGURE 3: The effect of RVA on hydroxyl radical scavenging activity. ^{a-g}Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range tests. Light gray square, $125 \mu\text{g/mL}$; gray square, $250 \mu\text{g/mL}$; dark grey square, $500 \mu\text{g/mL}$; black square, $1,000 \mu\text{g/mL}$.

FIGURE 4: The effect of RVA on ABYS radical scavenging activity. ^{a–g}Values not sharing a common letter are significantly different at $P < 0.05$ by Dunnett's multiple range tests. Light gray square, 125 $\mu\text{g/mL}$; gray square, 250 $\mu\text{g/mL}$; dark grey square, 500 $\mu\text{g/mL}$; black square, 1,000 $\mu\text{g/mL}$.

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. Light gray square, 125 $\mu\text{g/mL}$; gray square, 250 $\mu\text{g/mL}$; dark grey square, 500 $\mu\text{g/mL}$; black square, 1,000 $\mu\text{g/mL}$.

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. Light gray square, 125 $\mu\text{g/mL}$; gray square, 250 $\mu\text{g/mL}$; dark grey square, 500 $\mu\text{g/mL}$; black square, 1,000 $\mu\text{g/mL}$.

activity of velvet antler extract obtained from the upper section was reported to be 67.1% at an extract concentration of 100 mg/mL, which was lower than the activity measured in our analysis. H_2O_2 is a reactive nonradical and a clinically important compound due to its ability to penetrate biological membranes. H_2O_2 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 of H_2O_2 scavenging by velvet antler produced results similar to those reported by Je et al. [30]. 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. [32]. In addition, our analysis of ABTS radical scavenging activity by RVA identified activity higher than that reported by Zhao et al. [33]. 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 [34]. Zhao et al. [33] reported activity of $85.8 \pm 0.02\%$ by 5 mg/mL velvet antler extract in the FRAP assay, which was lower than the activity measured in our analysis. The ORAC assay has been applied extensively to evaluate the antioxidant activities of fruits, vegetables, leaves, stems, herbs, and spices. As a result, the ORAC assay is commonly mentioned in scientific publications and health food publications [35]. However, the antioxidant activity of RVA has not been evaluated using the ORAC assay. Therefore, this is the first report of an assessment of the antioxidant activity of RVA using the ORAC assay. ORAC value of gallic acid was shown 161 ± 4.8 by Zulueta et al. [36], which was higher than the activity of RVA found in our study.

4. Conclusions

In the present study, we provided the first comprehensive evaluation of the biologically active substances of RVA and the antioxidant potential of different RVA segments. Future studies are required to further elucidate the other biological activities of the T-RVA, M-RVA, and B-RVA sections and the biological mechanisms underlying their effects.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This paper was supported by Konkuk University in 2015.

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