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Antioxidant Activity of Aqueous Extract Fractions of Velvet Antler (*Cervus elaphus* Linnaeus)

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ABSTRACT

Velvet antler is believed to have body strengthening, immunomodulatory and anti-aging effects. It is used in Chinese commercial functional foods and nutraceuticals. The antioxidant activity of the aqueous extract of velvet antler (AEVA) from *Cervus elaphus* Linnaeus was evaluated with DPPH-radical scavenging, FRAP, Fe^{2+}-chelating and inhibition of linoleic acid autoxidation assays. AEVA showed antioxidant activity in all four assays. After removal of protein from AEVA, the antioxidant activity was significantly elevated. A semi-preparative HPLC equipped with a C_{18} column was used for further separation of the non-protein components (AEVA-S). Identification of the most active fraction (AEVA-SII) of AEVA-S was accomplished by LC/MS, HPLC and UV/Vis analyses. The HPLC chromatogram showed five main peaks identified as nucleotides (3′-CMP, 2′-CMP, 3′-UMP and 2′-UMP) and hypoxanthine. Nucleotides in AEVA-SII exhibited no free-radical scavenging and ferric-reducing activity. Only UMP exhibited Fe^{2+}-chelating activity which accounted for 34.75% of the total Fe^{2+}-chelating activity of AEVA-SII. The results indicated that other unidentified components with antioxidant activity were present in AEVA-SII.

Key words: velvet antler, nucleotides, antioxidant, HPLC, LC/MS

INTRODUCTION

Velvet antler, the whole cartilaginous antler in a pre-calcified stage, is commonly known as “lu rong” in China. As a renewable resource, it is a mainstay of traditional Chinese medicine (TCM) and secondary only to ginseng in importance. Medicinal uses for velvet antler have been recorded in the Compendium of Materia Medica since the 16th century. It has been used in enriching vital energy, nursing the blood, strengthening the kidney and prolonging life for thousands of years. Velvet antler is reported to have pharmacological activities such as haemotopoietic[(1)], growth-stimulating[(2)], anti-aging[(3)], immunomodulatory and anti-inflammatory effects[(4)].

In China, velvet antler has been used in medicine, nutraceuticals and functional foods. In particular, aqueous decoction is one of its major uses in TCM. The aqueous extract of velvet antler (AEVA) contains proteins, polypeptides, free amino acids, glycosaminoglycans and nucleotides[(5)]. In recent years, there has been an increasing awareness of the benefits of velvet antler. Interest in the discovery of its functional components has risen dramatically. However, the functional components of velvet antler are still not well known.

The antioxidant properties of TCM have generated great interest in researchers. The properties are commonly postulated to play an important role in preventing diseases caused by oxidative stress, such as aging, coronary heart disease and cancer. Antioxidant analysis on the organic extract of velvet antler has been carried out. The extract demonstrated a range of antioxidant-related activities, including the ability to protect carbohydrates from hydroxyl radical-mediated degradation, scavenge hydroxyl radical and inhibit lipid peroxidation[(6)]. However, no research has focused on the antioxidant activity of AEVA and the active components are still not clear.

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The present study focused on two aspects: (1) preparation and evaluation of the antioxidant activity of AEVA, AEVA-S (supernatant of AEVA without protein) and its fractions; (2) identification of the main components in AEVA-S fractions and investigation of their roles in the antioxidant activity of AEVA-S fractions.

MATERIALS AND METHODS

I. *Materials*

Velvet antlers (*Cervus elaphus* Linnaeus) were obtained from male red deer that were bred at the Daxing'an mountain range (Heilongjiang, China). Samples were freeze-dried, homogenized, and stored at 4°C until usage. DPPH, TPTZ, linoleic acid, ferrozine, Trolox and BHT were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cytidylic acid (2′- and 3′- mixture) was purchased from Cambrian Chemicals (London, U.K.). 3′-Uridylic acid was purchased from TCI (Tokyo, Japan). Hypoxanthine was purchased from Ameresco (Solon, Ohio, U.S.A.). HPLC grade acetonitrile was from Mallinckrodt Baker (Phillipsburg, New Jersey, U.S.A.). Other chemicals of analytical grade were purchased from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

II. *Preparation of AEVA*

Submicron powder of velvet antler (50 g) was extracted three times with 1 L of distilled water in boiling-water bath for 2 h. The aqueous extract was obtained by filtering the mixture through Whatman No. 1 filter paper under diminished pressure. The filtrate was then concentrated at reduced pressure. The yield of AEVA was 38.45% (w/w) of the dried sample.

III. *Removal of Protein from AEVA*

Anhydrous ethanol was added to concentrated AEVA solution to obtain a final concentration of 75%. The suspension was left overnight at 4°C, and then centrifuged at 12,000 rpm for 20 min. The supernatant was freeze-dried. The freeze-dried powder was referred to as AEVA-S and stored at 4°C until usage.

IV. *Separation of AEVA-S by Semi-preparative HPLC*

The separation was performed on a HPLC system (LC-10ATvp, Shimadzu, Kyoto, Japan) equipped with two pumps and a diode array detector (DAD). The column used for separation was a Kromasil C_{18} column (10 mm × 250 mm, 5 μm, SE-445 80 Bohus, Sweden). The sample solution was filtered through a 0.22-μm syringe filter before HPLC analysis. Eluent A was 0.1% trifluoroacetic acid (TFA) in acetonitrile and eluent B was 0.1% TFA in water. Samples were loaded onto the column through a 500-μL loop valve. The gradient elution conditions were set as follows: 0 - 30 min, 0 - 5% A; 30 - 40 min, 5 - 50% A; 40 - 50 min, 50% A. The flow rate was set at 3.0 mL/min, with the temperature set at 30°C and the detection was carried out at a wavelength of 260 nm.

V. *Identification of Antioxidants from AEVA-S*

(I) *UV/Vis Spectrum Analysis*

The UV/Vis absorption spectra were obtained at room temperature using a UV spectrometer (GBC, Australia) and 1-cm cuvette. The spectrum was acquired from 200 to 500 nm.

(II) *HPLC/DAD Analysis*

Using the same system as the semi-preparative HPLC, analytical HPLC was performed using a ZORBAX SB-C_{18} column (4.6 mm × 250 mm, 5 μm, Agilent Technologies, U.S.A.). Eluent A was 0.05% formic acid in acetonitrile and eluent B was 0.05% formic acid in water. The elution was started with 100% eluent B. The ratio of eluent A in the system was increased linearly from 0 to 3.5% over 20 min. The flow rate was set at 0.8 mL/min, with the temperature set at 30°C and the detection was carried out at a wavelength of 260 nm. A sample loop of 20 μL was used for the injection.

(III) *HPLC/ESI/MS Analysis*

The Agilent 1100 series LC/MSD Trap used in this study was equipped with an ion trap MS detector with electrospray ionization (ESI) interface. The system used the same column as that in the analytical HPLC analysis. Flow rate was set at 0.8 mL/min, and detection wavelength was set at 260 nm. The flow splitting of the HPLC eluate introduced into the ESI interface was 3 : 1. The ESI voltage was 3.5 kV, and a mass range (*m/z*) of 50 - 1000 was scanned in positive full ion monitoring mode. The nebulizing gas (nitrogen) pressure was set at 35 psi and the drying gas (nitrogen) was held at 8 L/min for ionization.

VI. *Antioxidant Assays*

(I) *DPPH-radical Scavenging Assay*

DPPH-radical scavenging activity was measured by the method of Brand-Williams *et al.*[7]. Briefly, 3.9 mL of DPPH-methanol solution (6×10^{-5} M, about 0.025 mg/mL) was added to 0.1 mL of sample methanol solution. The mixture was shaken and incubated at room temperature for 60 min in the dark, and the decrease in absorbance at 517 nm was determined at the end of incubation period with a spectrophotometer. Trolox was used as positive control. From the obtained values, μmol Trolox equivalents/g sample was calculated for each sample.

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(II) *FRAP Assay*

The ferric-reducing ability of plasma (FRAP) method described by Benzie and Strain[8], was used to measure the ferric ion reducing capacity. Briefly, 3.0 mL of freshly prepared FRAP reagent was warmed to 37°C, 0.1 mL of sample was then added, along with 0.3 mL of H_2O. The mixture was shaken and incubated at 37°C in the water bath for 30 min. Then, the absorbance of the developed reaction mixture was measured at 595 nm. Trolox was used as positive control. The results were expressed as μmol Trolox equivalents/g sample.

Working FRAP reagent was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ solution (dissolved in 40 mM HCl) and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$.

(III) *Metal Chelating Assay*

The Fe^{2+}-chelating ability was determined according to the method of Decker and Welch[9]. The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex. The test sample was mixed with 2 mM $FeCl_2$ and 5 mM ferrozine at a ratio of 50 : 1 : 2 (v/v/v). The mixture was shaken and incubated at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. A lower absorbance of the reaction mixture indicated a higher Fe^{2+}-chelating ability. EDTA was used as a standard metal chelating agent. From the obtained values, μmol EDTA equivalents/g sample was calculated for each sample.

(IV) *Inhibition of Linoleic Acid Autoxidation*

The lipid peroxidation inhibition activity was measured in the linoleic acid system[10]. An aliquot of 1 mL of sample solution, was added to a solution of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL of 0.2 M sodium phosphate buffer (pH 7.0). The mixture was made up to 25 mL with distilled water and incubated at 40°C in the dark. The reaction mixture (0.1 mL) was mixed with 4.7 mL of ethanol (75%), 0.1 mL of ammonium thiocyanate (30%) and 0.1 mL of ferrous chloride (20 mM in 3.5% HCl). After the mixture was stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm. The degree of oxidation was measured every 24 h for 12 days. Butylated hydroxytoluene (BHT, 0.1 mg/mL) and Trolox (0.5 mM) were used as positive controls.

VII. *Statistical Analysis*

Each set of experiments was carried out with three replicates. Data were expressed as means ± S.D. Statistical analysis was performed by the Duncan test at the 95% significance level to express the difference between two groups. $p < 0.05$ was considered statistically significant. The correlation was calculated under Pearson correlation coefficient (2-tailed) in bivariate correlations. Analysis was done with SPSS 13.0 (SPSS, Inc., Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

I. *Antioxidant Activities of AEVA and AEVA-S*

The antioxidant activities of AEVA and AEVA-S may not be attributed to a single mechanism. Therefore, four methods were used to evaluate the antioxidant activities from different aspects: the DPPH-radical scavenging assay was used to evaluate the free-radical scavenging capacity of antioxidants; the FRAP assay was used to reflect the ferric-reducing activity of antioxidants; the metal chelating assay was used to evaluate the Fe^{2+}-chelating ability and demonstrate the inhibitory effect on the generation of radicals (*e.g.* Fenton reaction); the inhibition of linoleic acid autoxidation assay was used to measure the activity of antioxidants to inhibit lipid peroxidation.

The antioxidant activities of AEVA and AEVA-S are shown in Table 1 and Figure 1. From the results, it was obvious that the antioxidant activity of AEVA-S was significantly higher than that of AEVA. The DPPH-radical scavenging activity and reducing power of AEVA-S were 18.66 and 47.64 μmol TE/g, respectively. These two values were 4 and 2.5 times of those of AEVA. AEVA and AEVA-S showed similar Fe^{2+}-chelating abilities, suggesting that both of them were able to capture ferrous before the formation of ferrozine.

In the linoleic acid system, the antioxidant activities of AEVA and AEVA-S were observed and compared with BHT and Trolox (Figure 1). As seen in Figure 1, the linoleic acid autoxidation in the blank was increased gradually over 12 days of oxidative reaction. Lower absorbance at 500 nm indicated higher lipid peroxidation inhibition. Both AEVA and AEVA-S exhibited similar lipid peroxidation inhibitory activities to those of BHT and Trolox. The products of the lipid peroxidation (such as malondialdehyde) could cause damage to proteins and DNA[11]. It was suggested that the compositions of AEVA-S may be beneficial to prevent aging, cardiovascular diseases, cholesterol lowering and other lipid peroxidation processes.

Overall, AEVA-S possessed a higher antioxidant activity than AEVA. The results suggested that the non-protein components were responsible for the antioxidant activity of AEVA. Thus, AEVA-S was selected for further study.

II. *Separation of AEVA-S by Semi-preparative HPLC*

In order to determine the main components responsible for antioxidant activity of AEVA-S, AEVA-S was subjected to fractionation with a semi-preparative HPLC

Table 1. Antioxidant activities of AEVA, AEVA-S and its fractions

Samples	Yield[1] (mg/g)	DPPH-radical scavenging activity (μmol TE[2]/g sample)	Reducing power (μmol TE[2]/g sample)	Fe^{2+}-chelating ability (μmol EE[3]/g sample)
AEVA	--	4.04 ± 0.69^{f}	18.94 ± 0.56^{e}	9.65 ± 0.19^{bc}
AEVA-S	135.27	18.66 ± 0.51^{e}	47.64 ± 0.46^{cd}	9.89 ± 0.28^{b}
AEVA-SI	10.17	21.37 ± 0.01^{d}	46.22 ± 0.76^{d}	9.17 ± 0.37^{c}
AEVA-SII	7.75	35.48 ± 0.06^{a}	86.03 ± 3.94^{b}	11.02 ± 0.27^{a}
AEVA-SIII	6.27	21.33 ± 0.37^{d}	44.72 ± 2.83^{d}	9.75 ± 0.19^{b}
AEVA-SIV	9.75	24.20 ± 0.51^{b}	100.26 ± 4.43^{a}	11.00 ± 0.36^{a}
AEVA-SV	79.74	22.53 ± 0.30^{c}	51.85 ± 3.33^{c}	6.56 ± 0.33^{d}

Data (means ± S.D., n = 3) in the same column followed by a different letter are significantly different ($p < 0.05$). DPPH-radical scavenging activity and reducing power were measured at a concentration of 25 mg/mL, and Fe^{2+}-chelating ability was measured at a concentration of 5 mg/mL.

1. Yield: Extract yield was expressed as mg/g of the dry weight of AEVA.
2. TE: Trolox equivalents.
3. EE: EDTA equivalents.

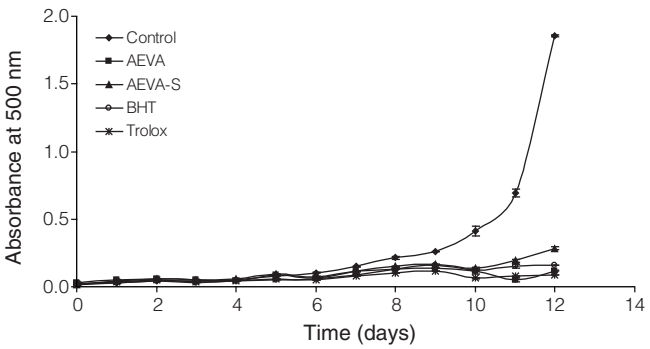

Figure 1. Linoleic acid autoxidation inhibition activities of AEVA and AEVA-S. The concentration of AEVA and AEVA-S was 10 mg/mL. BHT (0.1 mg/mL) and Trolox (0.5 mM) were used as positive controls. Data are presented as means ± S.D. (n = 3).

C_{18} column. As shown in Figure 2, the separated peaks were divided into five fractions (I, II, III, IV and V). The profiles of AEVA-SIV and AEVA-SV were more complicated than the other three fractions. AEVA-SV probably contained more hydrophobic compounds, which were therefore eluted with a high concentration of acetonitrile. The yields of AEVA-S (I-V) are shown in Table 1. They ranged from 2.41 to 30.66 mg/g of the dry weight of velvet antler.

III. *Antioxidant Activities of the Fractions of AEVA-S*

The antioxidant activities of the five fractions of AEVA-S were evaluated by DPPH-radical scavenging, FRAP and metal chelating assays. Table 1 shows the antioxidant activities of AEVA-S (I-V) with different methods. The results varied according to the type of assay used.

In our study, the DPPH-radical scavenging activity was expressed as Trolox equivalents per gram of sample on a dried basis (Table 1). DPPH-radical scavenging activities of AEVA-S and its fractions were in the order: AEVA-SII > AEVA-SIV > AEVA-SV > AEVA-SI ≈ AEVA-SIII > AEVA-S. AEVA-SII exhibited significantly higher DPPH-scavenging activity than the other fractions. The stable radical DPPH has been widely used for the determination of primary antioxidant activity. The effects of the antioxidants on DPPH are based on their ability to donate a hydrogen atom to DPPH[(12)]. The study indicated that AEVA-SII acted as a potent hydrogen donor and could serve as free-radical scavenger, acting possibly as primary antioxidant.

Antioxidants can be explained as reductants. As shown in Table 1, the ferric reducing activities of AEVA-S and its fractions were in the order: AEVA-SIV > AEVA-SII > AEVA-SV > AEVA-S ≈ AEVA-SI > AEVA-SIII. Both AEVA-SII and AEVA-SIV showed approximately 2-fold higher ferric-reducing ability than AEVA-S. In this assay, the AEVA-SII again showed relatively high antioxidant activity among all the fractions. The presence of reductants in AEVA-SII caused the reduction of the Fe^{3+}-TPTZ complex to the blue ferrous (Fe^{2+}) form. The reducing activity of a compound might serve as a significant indicator of its potential antioxidant activity. There was a significant correlation between DPPH and FRAP assay of AEVA and its fractions ($r = 0.791$, $p < 0.05$). The higher the DPPH-scavenging activity, the higher the FRAP activity of the samples.

The chelating activity was measured against Fe^{2+} and reported as EDTA equivalents (Table 1). In this assay, the Fe^{2+}-chelating activities of AEVA-S and its fractions were in the order: AEVA-SII ≈ AEVA-SIV > AEVA-S > AEVA-SIII > AEVA-SI > AEVA-SV. Ferrous ions can

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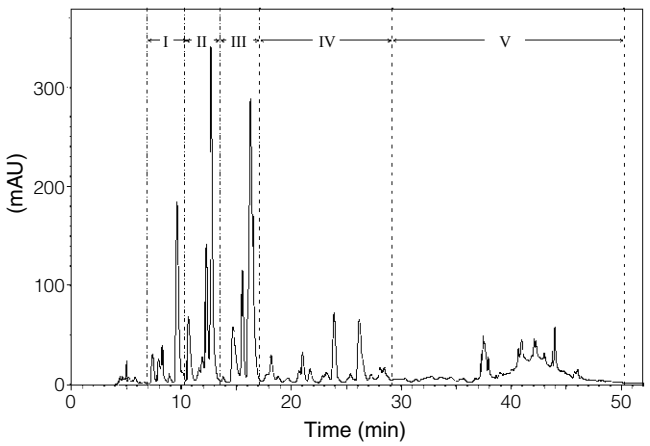

Figure 2. Semi-preparative HPLC chromatogram at 260 nm of AEVA-S. The chromatographic conditions were described in the Materials and Methods section.

stimulate lipid peroxidation by Fenton reaction, and also accelerate peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation[13]. Chelating agents may serve as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ions[14]. Therefore, it was suggested that the Fe^{2+}-chelating effects of AEVA-S and its fractions would be somewhat beneficial to protect against oxidative damage.

According to the results of the antioxidant assays, AEVA-SII exhibited strong antioxidant activities in all three assays, especially in DPPH-radical scavenging and Fe^{2+}-chelating assays. As shown in Figure 3, AEVA-SII produced effective DPPH-radical scavenging, reducing, and Fe^{2+}-chelating activities in a dose-dependent manner. These results suggested that AEVA-SII contained active components responsible for the antioxidant activity.

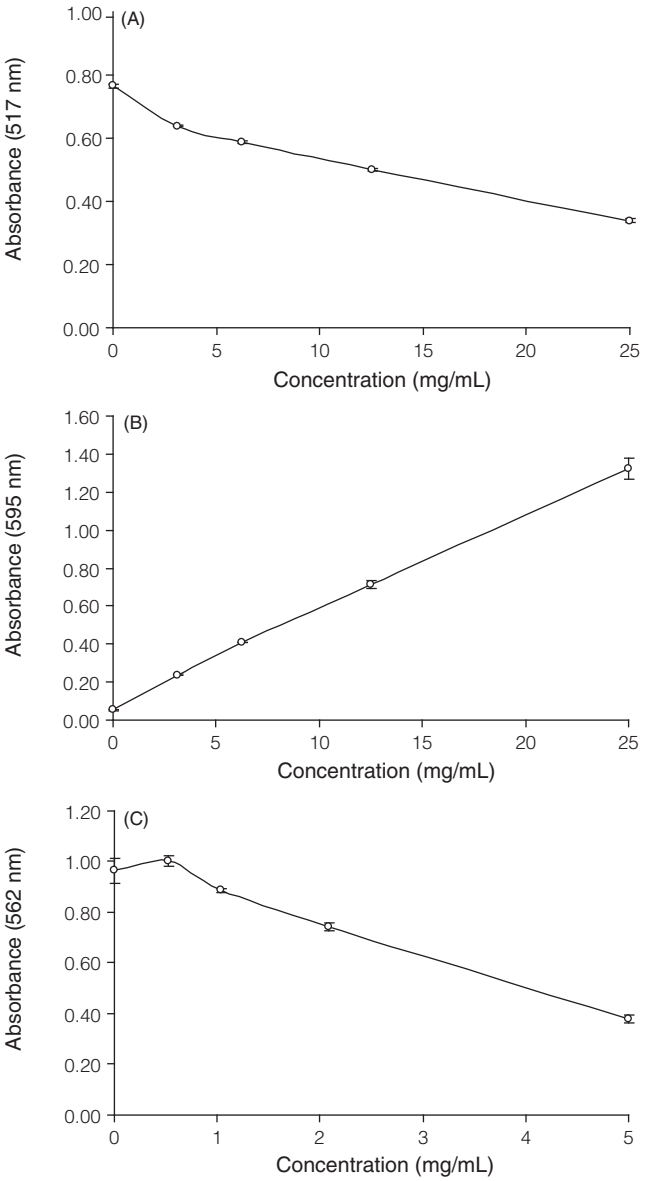

Figure 3. Correlation between AEVA-SII concentrations and absorbance in antioxidant assay. (A) DPPH-radical scavenging activity (r = -0.976, $p < 0.01$), (B) Reducing power (r = 0.999, $p < 0.01$), (C) Fe^{2+}-chelating ability (r = -0.989, $p < 0.01$). Data are presented as means ± S.D. (n = 3).

IV. *Identification of Main Components from AEVA-SII*

HPLC/ESI/MS analysis of AEVA-SII is shown in Figure 4. Using LC/MS, AEVA-SII was separated into five main components, and the total ion current chromatogram of AEVA-SII is shown in Figure 4A. The corresponding (+) ESI-MS spectra of peaks 1-5 from Figure 4A are shown in Figure 4B-4F. Peak 1 gave four main ions at *m/z* 324.0, 346.0, 647.0 and 669.0 (Figure 4B). Data inspection enabled the identification of *m/z* 324.0 and 346.0 as $[M+H]^+$ and $[M+Na]^+$, while *m/z* 647.0 and 669.0 were identified as $[2M+H]^+$ and $[2M+Na]^+$. The molecular weight of peak 1 is thus confirmed to be 323.0 Da. Peak 2, which had a similar mass spectrum as peak 1 (Figure 4C), was considered as the isomer of peak 1. Peak 3 gave one main characteristic $[M+H]^+$ ion at *m/z* 137.0 (Figure 4D). The molecular weight of peak 3 is confirmed to be 136.0 Da. Peak 4 showed six main ions at *m/z* 325.0, 347.0, 369.0, 649.0, 671.0 and 693.0 (Figure 4E). Being similar to peak 1, data inspection enabled the identification of *m/z* 325.0, 347.0 and 369.0 as $[M+H]^+$, $[M+Na]^+$ and $[M+2Na-H]^+$, while *m/z* 649.0, 671.0 and 693.0 were identified as $[2M+H]^+$, $[2M+Na]^+$ and $[2M+2Na-H]^+$. The molecular weight for peak 4 is thus confirmed to be 324.0 Da. Peak 5, which had a similar mass spectrum as peak 4 (Figure 4F), was considered as the isomer of peak 4. The fact that peaks 4 and 5 exhibited $[M+2Na-H]^+$ peaks indicated that a freely exchangeable proton existed on these compounds. The main chemical components of velvet antler are proteins, polypeptides, free amino acids, glycosaminoglycans, fatty acids, cholesterol, phospholipids and

nucleotides[5]. According to the molecular weight of the possible components of velvet antler, peaks 1 and 2 were tentatively determined as CMP; peak 3: hypoxanthine; and peaks 4 and 5: UMP.

HPLC techniques have become the most applicable procedures for the rapid determination of nucleotides[15]. Some monophosphate nucleotides have already been identified by HPLC with ion-pairing reagents coupled to MS[16,17]. In order to further verify the above speculation, the retention times of peaks 1-5 were compared

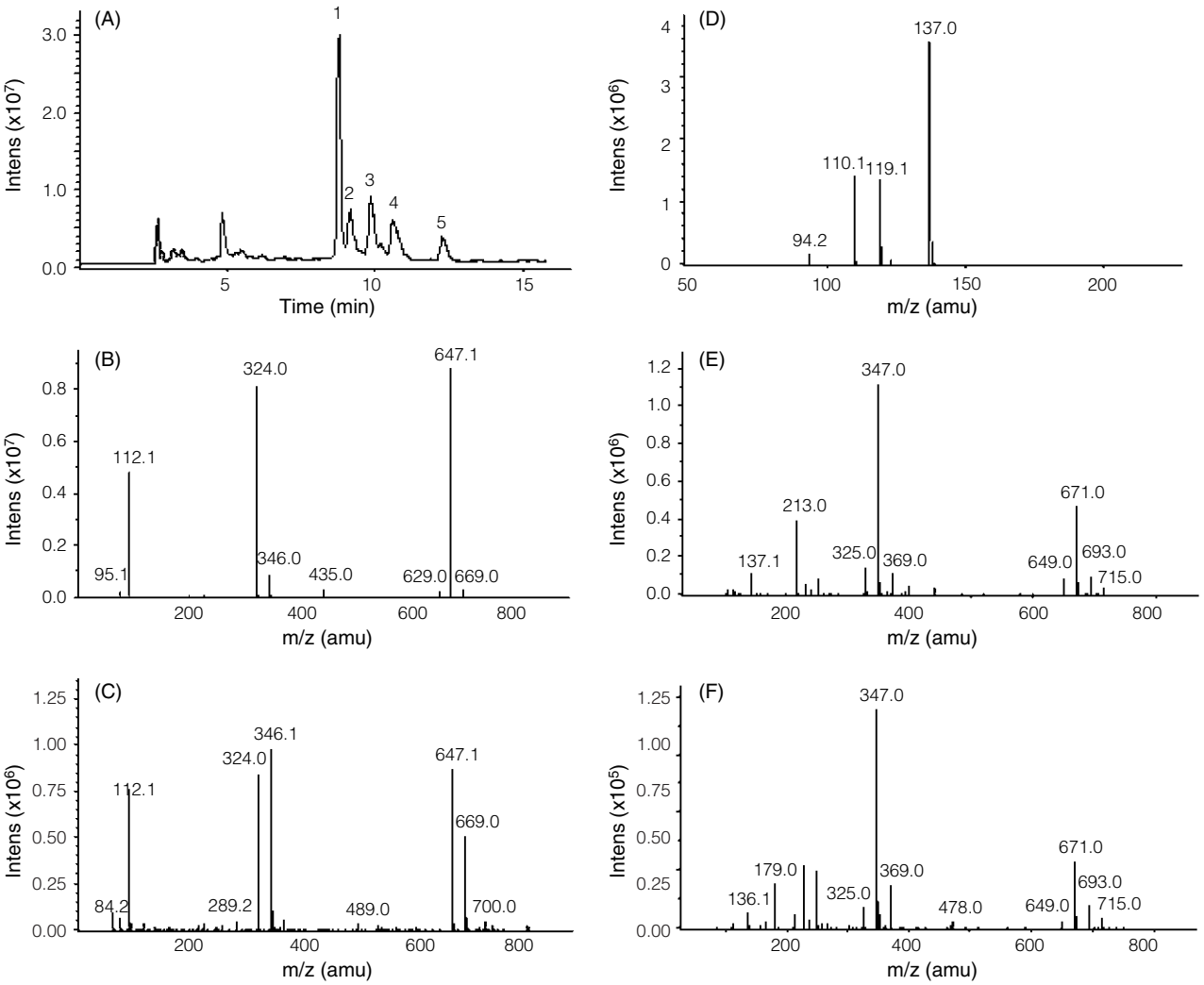

Figure 4. HPLC/ESI/MS spectra of AEVA-SII. The chromatographic conditions and MS parameters were described in the Materials and Methods section. (A) Total ion current chromatogram of AEVA-SII. (B)-(F) The corresponding positive mass spectra of peaks 1-5, respectively.

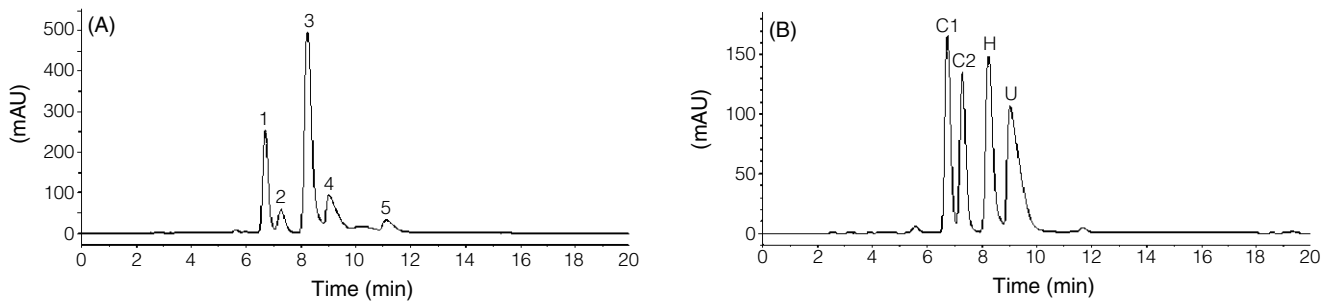

Figure 5. HPLC/DAD chromatograms of AEVA-SII and standards at 260 nm. The chromatographic conditions were described in the Materials and Methods section. (A) HPLC/DAD chromatogram of AEVA-SII. (B) HPLC/DAD chromatogram of standards. C1: 3′-CMP; C2: 2′-CMP; H: Hypoxanthine; U: 3′-UMP.

with nucleotide standards by analytical HPLC analysis. The HPLC chromatograms of the four selected standards (3′-CMP, 2′-CMP, 3′-UMP and hypoxanthine) and AEVA-SII are shown in Figure 5. By comparing AEVA-SII and standard mixture profiles, peaks 1, 2, 3 and 4 had the same retention time with 3′-CMP, 2′-CMP, hypoxanthine and 3′-UMP, respectively. UMP exists as three isomers: 2′-UMP, 3′-UMP and 5′-UMP. In our study, 5′-UMP was eluted earlier than peaks 1-5 (data not shown) and peak 4 was identified as 3′-UMP. Aussenac *et al.*[16] separated 21 reference monophosphate nucleotides by reversed-phase HPLC, and discovered that the separation of the isomers of UMP was in the elution order of 5′-, 3′-, and 2′-UMP. Accordingly, it was suggested that peak 5 in our study was 2′-UMP.

The UV/Vis spectra of peaks from AEVA-SII separated by HPLC (Figure 5) and their corresponding standards are shown in Figure 6. Every compound has its characteristic UV/Vis spectrum. The common purine and pyrimidine bases all absorb in the region of 250 - 280 nm, and the absorption spectra of the nucleosides and nucleotides are similar to those of the free bases[18]. The UV/Vis absorption spectra of all samples were measured in 0.05% formic acid (pH 2.8). By comparing UV/Vis spectra of peaks 1-5 with their corresponding standards, it was found that peaks 1 and 2 shared the same absorption maximum/minimum with CMP (λ_{max} at 278 nm, λ_{min} at 240 nm; Figure 6A, 6B); peak 3: the same with hypoxanthine (λ_{max} at 248 nm, λ_{min} at 220 nm; Figure 6C); and peaks 4 and 5: the same with UMP (λ_{max} at 259 nm, λ_{min} at 230 nm; Figure 6D).

Based on the results of LC/MS, HPLC and UV/Vis analyses, five compounds of AEVA-SII were unequivocally identified as (1) 3′-CMP, (2) 2′-CMP, (3) hypoxanthine, (4) 3′-UMP and (5) 2′-UMP. Quantification was also carried out by integration of the peak areas using external standard calibration. The amount of 3′-CMP, 2′-CMP, hypoxanthine, 3′-UMP and 2′-UMP in AEVA was 0.88, 0.30, 1.29, 1.80 and 0.68 mg/g of dry weight, respectively. Foods in the raw state contain a very low level of free nucleotides. This level increases markedly after heating due to enzymatic breakdown of the endogenous tissue

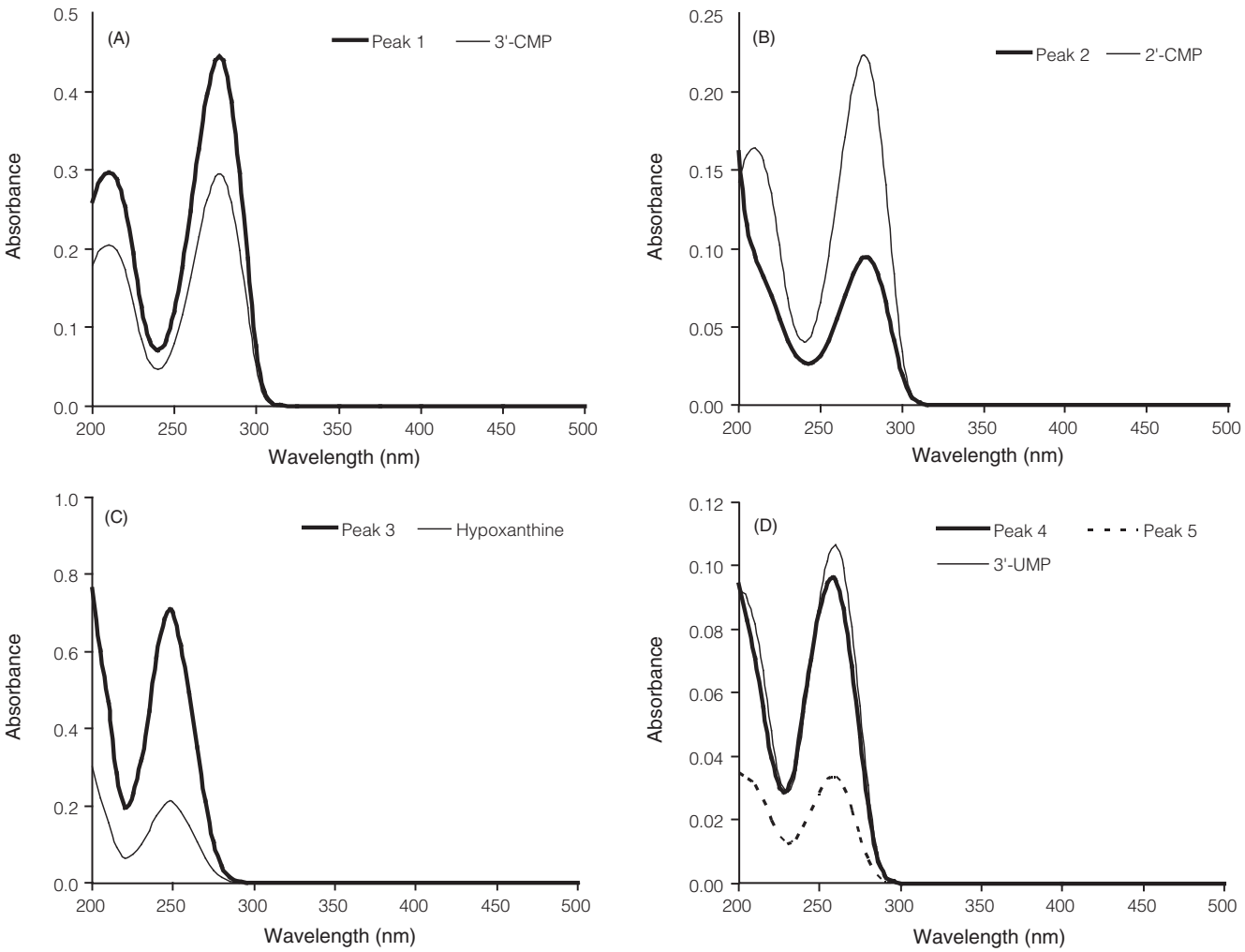

Figure 6. UV/Vis spectra of peaks from AEVA-SII separated by HPLC (Figure 5) and standards. (A) Peak 1, (B) Peak 2, (C) Peak 3, (D) Peaks 4 and 5.

Table 2. Contribution of nucleotides to the total antioxidant activity of AEVA-SII.

Samples	Yield[1] (mg/g)	DPPH-radical scavenging activity (μmol TE[2]/g sample)	Reducing power (μmol TE[2]/g sample)	Fe^{2+}-chelating ability (μmol EE[3]/g sample)	Contribution to total Fe^{2+}-chelating ability[5]	
					(μmol EE[3]/g sample)	(%)
AEVA-SII	7.75	35.48 ± 0.06	86.03 ± 3.94	11.02 ± 0.27		
CMP[4]	1.18	N.A.	N.A.	N.A.	--	--
Hypoxanthine	1.29	N.A.	N.A.	N.A.	--	--
3′-UMP	1.80	N.A.	N.A.	13.27 ± 0.10	3.83 ± 0.23	34.75

Data are presented as means ± S.D. (n = 3). DPPH-radical scavenging activity and reducing power were measured at a concentration of 25 mg/mL, and Fe^{2+}-chelating ability was measured at a concentration of 5 mg/mL. N.A. means no activity.

1. Yield: Extract yield was expressed as mg/g of the dry weight of AEVA.
2. TE: Trolox equivalents.
3. EE: EDTA equivalents.
4. CMP: Cytidylic acid (2′- and 3′- mixture).
5. Contribution of 3′-UMP to Fe^{2+}-chelating ability of AEVA-SII was measured at a concentration of 1.2 mg/mL

RNA[(19)]. Thus, we speculated that relative high amounts of nucleotides in AEVA are due to the degradation of nucleic acids in velvet antler during the decoction process.

V. *Antioxidant Activities of Nucleotides from AEVA-SII*

To evaluate the antioxidant activities of nucleotides from AEVA-SII, the above three antioxidant assays (DPPH-radical scavenging, FRAP and metal chelating assays) were used. Table 2 shows the antioxidant activities of CMP, hypoxanthine and UMP with different methods. They exhibited no DPPH-radical scavenging and ferric reducing activities, both individually and in combination (Table 2). Of the nucleotides (3′-CMP, 2′-CMP, hypoxanthine, 3′-UMP and 2′-UMP) found in AEVA-SII, the combined amount was approximately 64% of AEVA-SII. It was indicated that some other unidentified components existed in AEVA-SII that might have free-radical scavenging activity. To the best of our knowledge, few studies have been reported on the free-radical scavenging activity of nucleotides. However, Wang *et al.*[(20)] reported that nucleotides (5′-AMP, 5′-CMP, 5′-GMP and 5′-UMP) can all effectively scavenge superoxide anion radicals in a luminal-potassium ferricyanide-pyrogallol system, but the antioxidant mechanism was not clarified.

Some researchers found that nucleotides could act as metal-ion chelators[(21,22)]. In our study, CMP and hypoxanthine showed no Fe^{2+}-chelating activity. However, 3′-UMP showed Fe^{2+}-chelating activity. It suggested that 3′-UMP could inhibit oxidant reaction catalyzed by iron-ion. Table 2 shows the calculated antioxidant activities of the individual nucleotides, and their contribution to the total antioxidant activity. The calculated Fe^{2+}-chelating activity of 3′-UMP was only 34.75% of the total measured value. The remaining Fe^{2+}-chelating activity was presumably due to unidentified components.

In brief, AEVA-SII had relatively high free-radical scavenging and Fe^{2+}-chelating activities. However, nucleotides, the main components in AEVA-SII, showed minor contribution to the total antioxidant activity of AEVA-SII. The results suggested that other effective antioxidants in AEVA-SII need to be separated and identified.

CONCLUSIONS

Our present study demonstrated that AEVA, AEVA-S and its fractions had antioxidant activity in different assays *in vitro* including: DPPH-radical scavenging, FRAP, metal chelating and inhibition of linoleic acid autoxidation assays. Nucleotides (3′-CMP, 2′-CMP, 3′-UMP and 2′-UMP) and hypoxanthine were identified from AEVA-SII as its main components by LC/MS, HPLC and UV/Vis analyses. Although UMP exhibited Fe^{2+}-chelating activity, nucleotides in AEVA-SII had minor contribution to the total antioxidant activity of AEVA-SII. Other unidentified components with antioxidant activity might be present in AEVA-SII. Moreover, CMP and UMP in AEVA were firstly reported in velvet antler. To further unravel potentially antioxidant activity of AEVA, other unidentified active components should be further clarified. A scientific research using animal models is necessary to determine their effects in animal systems.

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ABBREVIATIONS

AEVA, aqueous extract of velvet antler;

BHT, butylated hydroxytoluene;

CMP, cytidine monophosphate;

DPPH, 2,2-diphenyl-1-picrylhydrazyl;

Ferrozine, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt; FRAP, ferric reducing antioxidant power;

HPLC, high-performance liquid chromatography;

MS, mass spectrometry;

TPTZ, 2,4,6-tris(2-pyridyl)-s-triazine;

Trolox, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid;

UMP, uridine monophosphate.

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