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ARTICLE

Composition of Biologically Active Substances and Antioxidant Activity of New Zealand Deer Velvet Antler Extracts

Jae-Young Je, Pyo-Jam Park[1], Eun-Kyung Kim[1], Hyun-A Kim, Dong Hwan Lim[2], Byong-Tae Jeon[3], and Chang-Bum Ahn*

School of Food Technology and Nutrition, Chonnam National University, Yeosu 550-749, Korea
[1]Department of Biotechnology, Kunkuk University, Chungju 380-701, Korea
[2]Shin Hung Pharm. Co., Yeosu 550-230, Korea
[3]Korean Nokyong Research Center, Konkuk University, Chungju 380-701, Korea

Abstract

Deer velvet antler was subjected to the extraction process using boiling water at three different temperatures (100, 110 and 120°C) and 70% ethanol solution. Functional components such as uronic acid, sulfated-glycosaminoglycans (sulfated-GAGs) and sialic acid in the extracts were analyzed, and their antioxidant activities were investigated using several *in vitro* models. Uronic acid and sulfated-GAGs content of each extract significantly decreased with increasing extraction temperature ($p<0.05$), while the residues obtained from the upper and middle part of the antler had a higher uronic acid content than the residues obtained from the base section. Sialic acid contents were highest in compounds extracted at 110°C, followed by 120 and 100°C. The 70% ethanol extracts also had a high levels of uronic acid content, but not for sulfated-GAGs and sialic acid. All extracts showed good antioxidant ability in a dose-dependant manner, with the 100°C residue exhibiting the strongest activity compared to the 110 and 120°C extracts. In relation to the hydroxyl radical scavenging activity and reduction power, the 70% ethanol extract exhibited the strongest activity. Furthermore, the velvet antler extracts inhibited apoptosis in hydrogen peroxide-induced PC-12 cells.

Key words: velvet antler, uronic acid, sulfated-GAGs, sialic acid, antioxidant activity

Introduction

Velvet antler, the unossified antler of *Cervus elaphus* (*Cervidae*) is the fastest growing mammalian tissue, and is widely used within Asia as an alternative oriental medicine to treat various diseases, including cardiovascular, gynaecological, immunological and blood cancers in oriental medicine (Huang, 1997; Kim and Lim, 1999). In Korea, it is generally referred to as '*Nokyong*' and is one of the most popular Korean traditional medicines, the benefits of which have been supported by extensive *in vivo* and *in vitro* studies (Kim, 1994; Suttie *et al*., 1994). Numerous studies have demonstrated that velvet antler extracts possess anti-inflammatory effects, anti-stress effects, and anti-aging effects (Sunwoo *et al*., 19972; Takikawa *et al*., 1972; Wang *et al*., 1988a, 1988b; Zhang *et al*., 1992). However, little is known about composition of biologically active substances such as uronic acid, sulfated-glycosaminoglycans (sulfated-GAGs) and sialic acid within velvet antler extracts, and the relative differences according to different extraction methods. Furthermore, there is also a paucity of data on the antioxidant activity of extracts.

Currently, there is great interest in finding antioxidant substances from natural sources, as it is increasingly becoming evident that oxidative cell damage is caused by free radicals and reactive oxygen species (ROS), mostly generated endogenously (Abraham *et al*., 2002). Uncontrolled generated ROS are very unstable, and react rapidly with other groups or substances including DNA, membrane lipids and proteins in the body, which is believed to be involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative, gastric ulcers, ischemic reperfusion, arthritis and inflammatory conditions (Butterfield *et al*., 2002; Debashis *et al*., 1997; Sussman and Bulkley, 1990; Vajragupta *et al*., 2000).

In this study, velvet antler was subjected to extraction

*Corresponding author : Chang-Bum Ahn, School of Food Technology and Nutrition, Chonnam National University, Yeosu 550-749, Korea. Tel: 82-61-659-3411, Fax: 82-61-659-3419, E-mail: a321@chonnam.ac.kr

using hot water at three different temperatures and also 70% ethanol solution, in order to quantify biologically active substances such as uronic acid, sulfated-GAGs, and sialic acid, and antioxidant activities of the extracts were investigated against DPPH, hydroxyl radical, hydrogen peroxide scavenging and reducing power. In addition, neuroprotective effect of the extracts against hydrogen peroxide-induced cytotoxicity in PC-12 cells was evaluated.

Materials and Methods

Chemicals

All chemicals including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, gallic acid, ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu's phenol reagent, hydrogen peroxide, 2,2-azino-bis(3-ethylbenzthiazoline)-6-sulfonic acid (ABTS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), galacturonic acid, carbazole, 1,9-dimethylmethylene blue, chondroitin 4-sulfate, *N*-acetylneuraminic acid, periodic acid, peroxidase, potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals and reagents used were of analytical grade commercially available.

Preparation of velvet antler extracts

Red deer antler (*Cervus elaphus*), from New Zealand, was donated from Shin Hung Pharm. Co. (Yeosu, Korea). The antler was divided into two sections (mixture of upper and middle section and base section), with a 60 g portion being used for each experiment. The first extraction process was conducted with hot water at 100°C for 1 h by autoclaving. After filtering, the residue was subjected to re-extraction at 110°C for 1 h, and then filtered. Finally, the residue from the extraction at 110°C was subjected to re-extraction at 120°C for 2 h, repeated twice, and then supernatant was collected by filtering. The justification for this is that water at 100°C may not be hot enough to completely extract all functional components. A 60 g portion of the antler was also subjected to extraction using 70% ethanol solution for 2 h and repeated three times. Therefore, we designated the extracts as NUM100 (upper and middle section extract of New Zealand velvet antler at 100°C), NUM110 (upper and middle section extract of New Zealand velvet antler at 110°C), NUM120 (upper and middle section extract of New Zealand velvet antler at 120°C), NUME (upper and middle section extract of New Zealand velvet antler by 70% ethanol), NB100 (base section extract of New Zealand velvet antler at 100°C), NB110 (base section extract of New Zealand velvet antler at 110°C), NB120 (base section extract of New Zealand velvet antler at 120°C) and NBE (base section extract of New Zealand velvet antler by 70% ethanol). All recovered extracts were lyophilized on a freeze dryer for 5 days.

Determination of uronic acid, sulfated-GAGs and silalic acid of velvet antler extracts

Uronic acid content was determined by the carbazole reaction. Briefly, a 50 μL serial dilution of standard or sample grade 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 mim at 100°C in an oven. After cooling at room temperature for 15 min, 50 μL of 0.125% carbazole in absolute ethanol were 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 (EL×808^{TM}, BioTek, VT, USA) at a wavelength of 550 nm (Cesaretti *et al*., 2003).

Sulfated-glycosaminoglycans (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 to 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 M equi/L 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 periodate solution at 37°C for 30 min. After addition of 0.25 mL sodium thiosulfate (0.32 M) and the tubes are shaken until a yellow-brown color disappears. The reaction was completed by addition of 1.25 mL TBA (0.1 M) and then 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.

DPPH scavenging activity

DPPH scavenging activity of various antler extracts was measured according to a slightly modified 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 by microplate reader and the percentage of scavenging activity was calculated using the following equation:

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

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 517 nm.

Hydroxyl radical scavenging activity

Deoxyribose non site-specific hydroxyl radical scavenging activity of various antler extracts was determined according to the method of Chung *et al*. (1997). 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:

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

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

Hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was determined according to the method of Müller (1985). A 100 μL of 0.1 M phosphate buffer (pH 5.0) was mixed with each extracts 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:

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

where $A_{control}$ is absorbance of reaction mixture without sample and A_{sample} is absorbance of reaction mixture with sample at 405 nm.

Reducing power

The reducing power of antler extracts 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 potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min, after which 0.5 mL TCA (10%) was added to the mixture and centrifuged at 1,036 g for 10 min. From the upper layer of solution, 0.5 mL solution was mixed with 0.5 mL distilled water and 0.1 mL $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Cell culture and treatment

PC-12 cells used in this study were obtained from the American Type Culture Collection, and used for no more than 10-12 passages. Growth medium consisted of DMEM supplemented with 10% heat-activated FBS, 10 U/mL penicillin, and 100 μg/mL streptomycin, and maintained at 37°C under a humidified atmosphere with 5% CO_2. 1×10^5 cells/mL were seeded onto 96-well culture plates in complete medium and DMEM with 10% FBS. Experiments continued 24 h after cells were seeded. A 1 mM H_2O_2, freshly prepared prior to experiment, and the extracts with different concentrations were added to the medium.

MTT assay

When cells reached 70-80% confluence, the extracts with desired concentration was transferred to the wells, followed by 24 h of incubation. Culture media were aspirated, after which 200 μL of MTT dye solution (0.5 mg/mL) was added to each well. After 4 h of incubation, the media was aspirated and purple color crystals dissolved with DMSO. The absorbance in each well was measured at 540 nm using Genios Multifunction microplate reader (Tecan, UK).

Flow cytometric analysis

Cells were collected and washed with ice-cold PBS, suspended in ethanol with 0.5% Tween-20, and left for 24 h at 4°C. Fixed cells were harvested by centrifugation at 1,000 *g* and resuspended in 1 mL PBS containing 50 μg/mL propidium iodide (PI) and 50 μg/mL RNAase A, followed by incubation at 37°C for 30 min. Analysis of apoptotic cell death was performed by measuring hypodiploid DNA contents using a flow cytometer (FACS-caliber,

Becton Dickinson, NJ). The cells in sub-G1 population were considered as apoptotic, while the percentage of each cell cycle phase was determined.

Statistics

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 a one-way ANOVA followed by Tukey's or Duncan's test. *P*-value of less than 0.05 was considered significant.

Results and Discussion

Generally, the upper, middle and base sections of velvet antler are specifically used to treat different diseases such as anemia, arthritis, hypercholesterolemia, and cancer, or to promote health as traditional oriental tonics (Sunwoo *et al*., 1995). In Korea, the consumers generally believed that upper and middle sections are good quality for various diseases. Therefore, in this study, we prepared velvet antler extracts from a mixture of upper and middle sections, and also from the base section, using different water temperatures and 70% ethanol solution.

Analysis of biologically active substances is summarized in Table 1. Uronic acid content of each extract significantly decreased with increasing temperature ($p<0.05$), while NUM exhibited higher uronic acid content than that of NB. These results indicate that uronic acid can be effectively extracted at 100°C. In the case of NUME and NBE, uronic acid content was higher than all other residues. This indicates that 70% ethanol can better extract uronic acid than heat. Sulfated-GAGs content of NB and 70% ethanol extracts significantly decreased with increasing extraction temperature ($p<0.05$). Sulfated-GAGs content in NUM was higher than that of NB. In contrast, NUME and NBE showed lower overall sulfated-GAGs content. Sialic acid content was highest in residue extracted at 110°C followed by 120 and 100°C. However, no sialic acid was observed in residue extracted with 70% ethanol. This indicates that sialic acid extraction was dependant on extraction temperature and solvent.

There is little information available on the biological active substances of velvet antler extract. However, a limited number of studies have been published. Scott and Hughes (1981) reported that uronic acid concentration in deer antler was 300 μg/g. Sunwoo *et al*. (1995) reported that uronic acid content of velvet antler was highest in the upper section. However, their uronic acid value was much lower than ours. The difference could be due to the fact that we analyzed uronic acid content from the extracts, while these authors examined the antler itself. The presence of complement-activating proteoglycan containing chondroitin sulfate in antlers of *Cervus nippon* Temminck has also been reported (Zhao *et al*., 1992). This indicates a potentially important source of carbohydrate. Furthermore, oral administration of chondroitin sulfate has been shown to reduce pain in osteoarthritis patients (Paroli *et al*., 1991). Jhon *et al*. (1999) reported that the amount of gangliosides, the sialic acid-containing glycosphingolipids, is often used by Asian folk medicine practitioners for quality evaluation. Our results showed that sialic acid content was highest in residue extracted at 110°C, while the extraction yield was depend on temperature and solvent.

Antioxidant activity of velvet antler extracts were evaluated by looking at scavenging ability toward DPPH, hydroxyl radical, hydrogen peroxide, and reduction power. Fig. 1 depicts DPPH radical scavenging activity of extracts, which appears augmented with increasing concentrations. Residue extracted at 100°C exhibited the highest scavenging activity, while activity decreased with increasing extraction temperature. The 70% ethanol extracts showed DPPH radical scavenging activity which was similar to

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

Extracts		Contents (mg/g)			
		100°C	110°C	120°C	70% ethanol
NUM section	Uronic acid	61.10±1.90[a]	39.28±1.40[b]	18.08±1.10[c]	96.12±5.50[d]
	Sulfated-GAGs	41.04±2.30[a]	38.32±1.87[a]	26.80±3.56[b]	12.66±1.20[c]
	Sialic acid	1.92±0.62[a]	7.73±1.35[b]	5.78±0.66[c]	ND[1)]
NB section	Uronic acid	44.49±1.60[a]	25.86±3.60[b]	15.95±3.20[c]	103.85±2.70[d]
	Sulfated-GAGs	23.04±2.01[a]	18.78±1.59[b]	15.08±1.66[c]	2.83±0.58[d]
	Sialic acid	3.71±0.86[a]	8.22±0.74[b]	4.93±0.99[c]	ND

[a-d]The values with different subscripts indicate significant difference ($p<0.05$).
[1)]ND: Not detected.

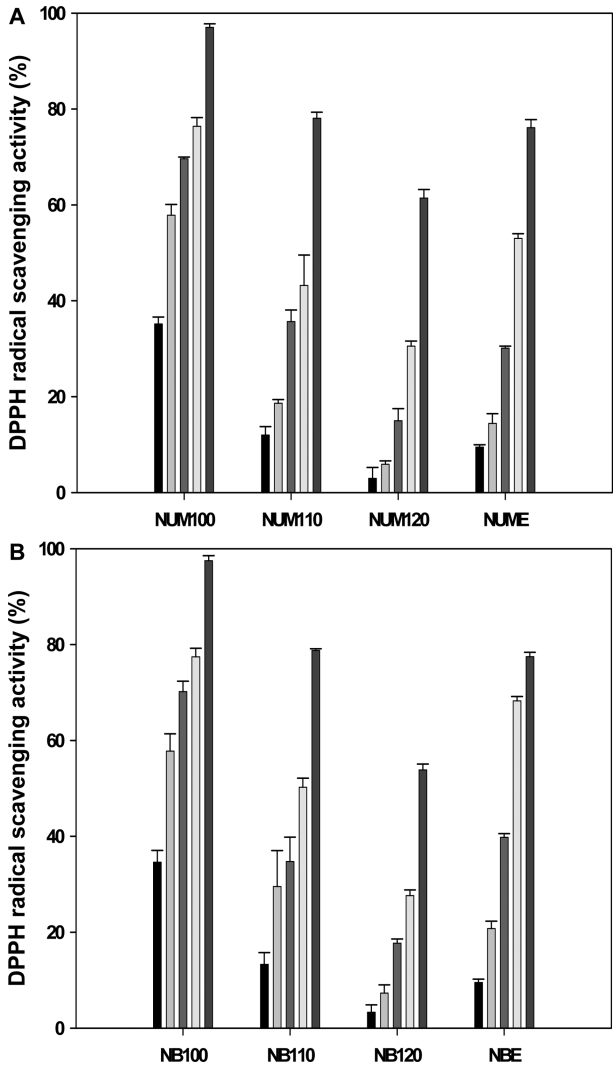

Fig. 1. DPPH radical scavenging activities of deer velvet antler extracts (A) upper and middle section extract and (B) base section extract. Results were presented as means±SE of three determinations. ■, 125 μg/mL; ■, 250 μg/mL; ■, 500 μg/mL; ■, 1,000 μg/mL; ■, 2,000 μg/mL.

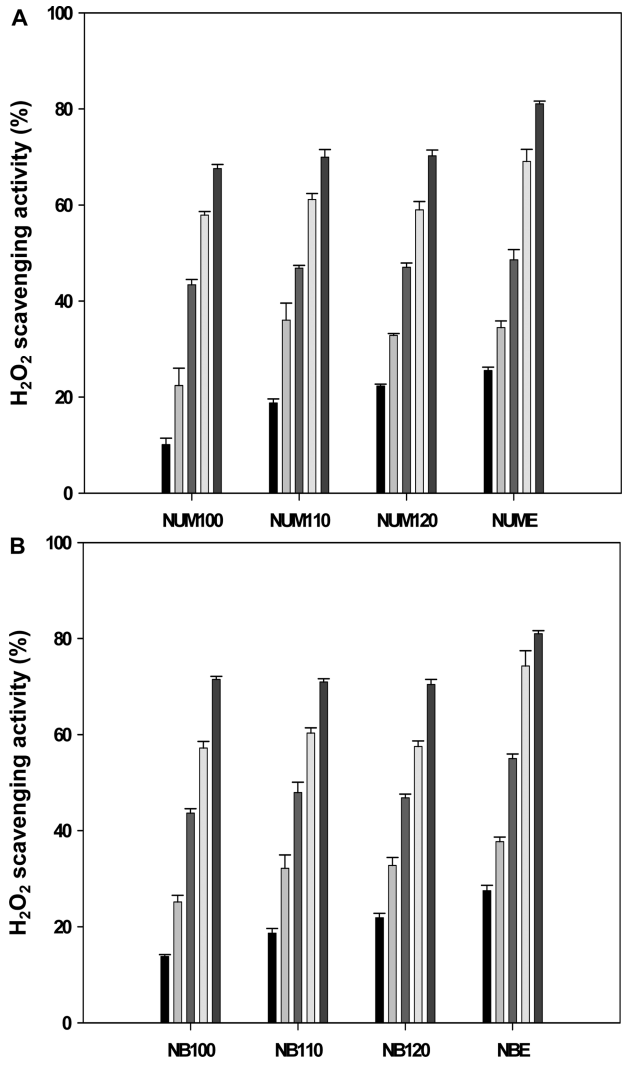

Fig. 2. Hydrogen peroxide scavenging activities of the water extracts (A) upper and middle section extract and (B) base section extract. Results were presented as means± SE of three determinations. ■, 250 μg/mL; ■, 500 μg/mL; ■, 1,000 μg/mL; ■, 2,000 μg/mL; ■, 4,000 μg/mL.

the residue extracted at 110°C. No significant difference (p>0.05) was found between the upper and middle mixture and base section extracts. Hydrogen peroxide scavenging activity of the extracts is shown in Fig. 2. Residues extracted using the three different temperatures displayed similar activities, while the 70% ethanol extracts showed comparatively higher activity. Hydroxyl radical scavenging activity is displayed in Fig. 3. Scavenging activity was related with extraction temperature, as activity increased with increasing concentrations. The 70% ethanol extracts showed excellent hydroxyl radical scavenging activity, recorded around 85% at concentration of 0.25 mg/mL. Reduction power of the extracts is depicted in Fig. 4. Results show that the 70% ethanol extracts possessed higher reducing power than those of temperature extracts. Among them, residue extracted at 100°C exhibited the highest reducing power in a dose-dependant manner. BHA and BHT were also tested as reference compounds, in which the antioxidant activities of the extracts were lower than those of BHA or BHT (data not shown).

The protective effect of velvet antler extracts on hydrogen peroxide-induced PC-12 cell damage was investigated. In order to avoid cytotoxic interference of velvet antler extracts at high concentrations, the influence of velvet antler extracts on cell viability of PC-12 cells was determined using MTT assay. According to the results, velvet antler extracts did not show any cytotoxic effect to

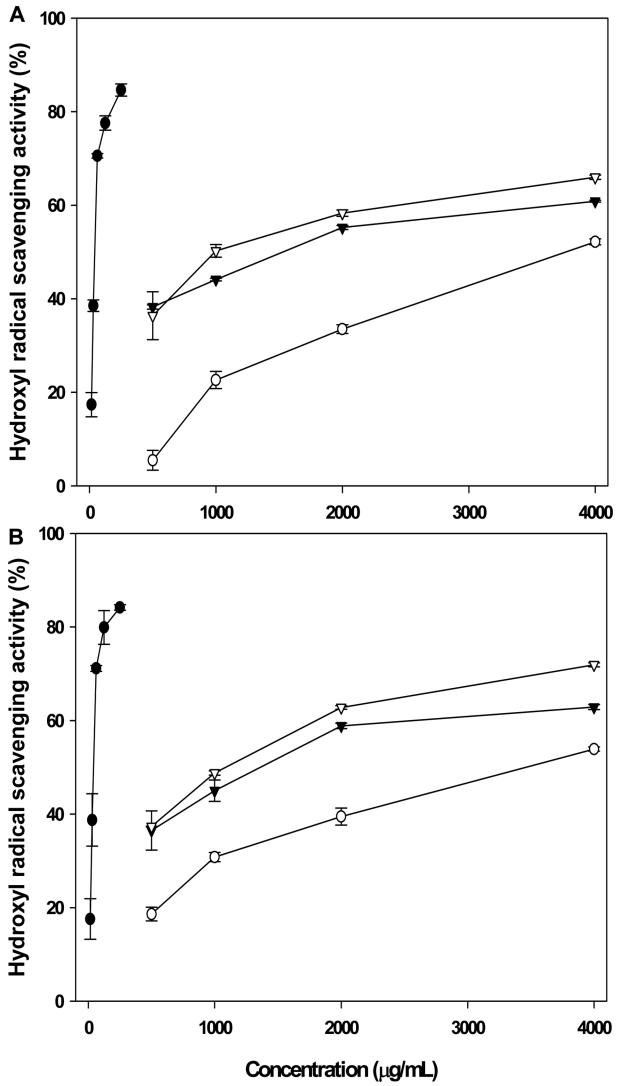

Fig. 3. Hydroxyl radical scavenging activities of the water extracts (A) upper and middle section extract and (B) base section extract. Results were presented as means± SE of three determinations. ●, NUME; ○, NUM100; ▼, NUM110; ▽, NUM120.

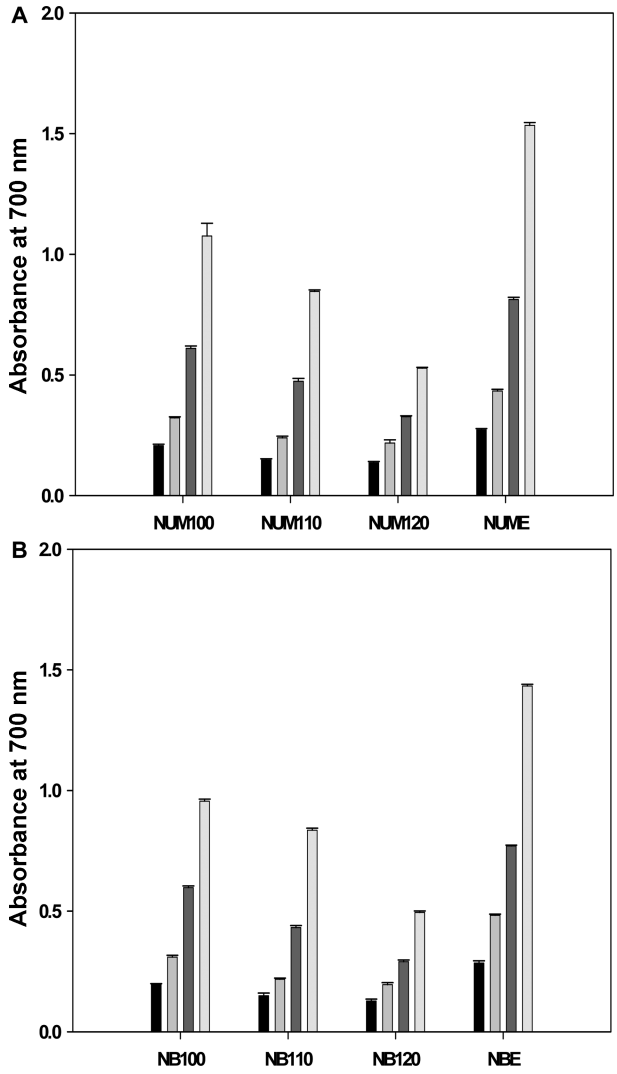

Fig. 4. Reducing power of the water extracts (A) upper and middle section extract and (B) base section extract. Results were presented as means±SE of three determinations. ■, 500 μg/mL; ■, 1,000 μg/mL; ■, 2,000 μg/mL; ■, 4,000 μg/mL.

PC-12 cells at the tested concentrations. As shown in Fig. 5, in the presence of hydrogen peroxide (no extracts treatment), the percentage of apoptotic cells observed were 34.2%, while velvet antler extracts treated groups exhibited decreasing apoptotic cell concentrations. This indicates that velvet antler extracts significantly protected neuronal cells against hydrogen peroxide-induced oxidative damage.

It is well known that velvet antler have various biological activities which make them a good candidate as functional food ingredients and nutraceuticals. However, little is known about their antioxidant activity. In this study, we demonstrated that velvet antler extracts exhibited great reduction power and antioxidant activity against DPPH, hydroxyl, and hydrogen peroxide. DPPH is a stable free radical donor which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Therefore, DPPH is often used as a substrate to evaluate antioxidant activity. All extracts exhibited DPPH scavenging activities in a dose-dependant manner, with residues extracted at 100°C showing the highest scavenging activity. This may be attributed to active compounds extracted at 100°C. However, scavenging activity was also evident in residues extracted at over 100°C, indicating the presence of active compounds at different extractions temperatures. In comparison with the 70% ethanol extracts, water extracts possessed higher scavenging activity. This indicates that water extraction is a more effective method compared to extraction with organic solvents. DPPH rad-

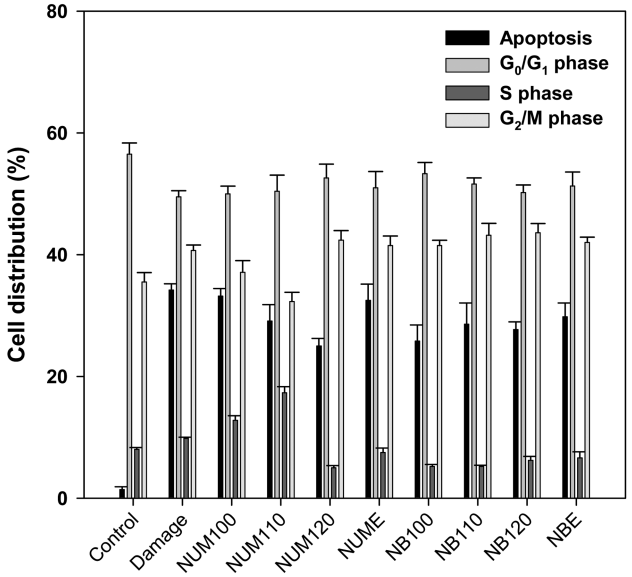

Fig. 5. Cell death and cell cycle of PC-12 after treating with velvet antler extracts, prior to hydrogen peroxide treatment. The cells were treated with velvet antler extracts at 1.0 mg/mL concentrations prior 10 mM hydrogen peroxide for 24 h. Results are overall mean±SE from three independent experiments.

ical scavenging activity of velvet antler extract obtained from the upper section has been reported to be 67.1% at 100 mg/mL (Lee and Chung, 2007), which was lower than our analysis. Hydrogen peroxide, a reactive nonradical, is an important compound as it can penetrate biological membranes. Although hydrogen peroxide itself is not very reactive, it may convert into more reactive species such as singlet oxygen and hydroxyl radicals, which is formed by fenton reaction, subsequently initiating lipid peroxidation or be toxic to cells. The measurement of hydrogen peroxide scavenging activity is known to be a useful method in determining the ability of antioxidant to decrease the level of prooxidants such as hydrogen peroxide (Czochra and Widensk, 2002). Our results showed that water extracts scavenged hydrogen peroxide in a dose-dependant manner, but no significant difference was observed. On the other hand, the 70% ethanol extracts exhibited higher scavenging potential than those of water extracts. Among the ROS, hydroxyl radical showed the strongest chemical reactivity, which can easily react with amino acids, DNA and membrane components. Therefore, removal of hydroxyl radicals is probably one of the most effective defensive mechanisms against various diseases. In this study, the 70% ethanol extracts exhibited strong hydroxyl radical scavenging activity whereas water extracts showed weak scavenging activity compared. This indicates that the 70% ethanol extracts contained more potent electron donor compounds than water extracts. Reduction power, for example, generally means that reductones in samples are able to reduce ferric (III) to ferrous (II) in a redox-linked colorimetric reaction. This involves single electron transfer in antioxidant components. Our results clearly showed that water and 70% ethanol extracts possessed effective reducing power, augmenting with increasing concentrations.

We have demonstrated that velvet antler subjected to extraction by heat and 70% ethanol had different levels of biologically active substances. Furthermore, the extracts exhibited excellent reduction power and antioxidant activities toward DPPH, hydroxyl radical and hydrogen peroxide. However, further studies are required in order to identify individual antioxidant compounds.

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