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ICR 마우스 모델을 이용한 녹용 추출물의 생화학적 평가 및 급성 경구 독성을 포함한 세포 독성 효과

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Biochemical Assessment of Deer Velvet Antler Extract and its Cytotoxic Effect including Acute Oral Toxicity using an ICR Mice Model

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ABSTRACT - Velvet antler is widely used as a traditional medicine, and numerous studies have demonstrated its tremendous nutritional and medicinal values including immunity-enhancing effects. This study aimed to investigate different deer velvet extracts (Sample 1: raw extract, Sample 2: dried extract, and Sample 3: freeze-dried extract) for proximate composition, uronic acid, sulfated glycosaminoglycan, sialic acid, collagen levels, and chemical components using ultra-performance liquid chromatography-quadrupole-time-of-light mass spectrometry. In addition, we evaluated the cytotoxic effect of the deer velvet extracts on BV2 microglia, HT22 hippocampal cells, HaCaT keratinocytes, and RAW264.7 macrophages using the cell viability MTT assay. Furthermore, we evaluated acute toxicity of the deer velvet extracts at different doses (0, 500, 1000, and 2000 mg/kg) administered orally to both male and female ICR mice for 14 d (five mice per group). After treatment, we evaluated general toxicity, survival rate, body weight changes, mortality, clinical signs, and necropsy findings in the experimental mice based on OECD guidelines. The results suggested that in vitro treatment with the evaluated extracts had no cytotoxic effect in HaCaT keratinocytes cells, whereas Sample-2 had a cytotoxic effect at 500 and 1000 μg/mL on HT22 hippocampal cells and RAW264.7 macrophages. Sample 3 was also cytotoxic at concentrations of 500 and 1000 μg/mL to RAW264.7 and BV2 microglial cells. However, the mice treated in vivo with the velvet extracts at doses of 500–2000 mg/kg BW showed no clinical signs, mortality, or necropsy findings, indicating that the LD50 is higher than this dosage. These findings indicate that there were no toxicological abnormalities connected with the deer velvet extract treatment in mice. However, further human and animal studies are needed before sufficient safety information is available to justify its use in humans.

Key words: Velvet antler extract, Acute toxicity, ICR mice, Bioactive compounds, Cytotoxicity

The use of natural products is expanding worldwide, and there has been interest in non-lethal deer antler animal medicine[1,2]. Commercial deer antler farming has become a valuable product for the western dietary supplement market in recent decades[3]. According to recently published reports, global annual production of velvet antler (VA) reaches 1,300 tons, and there is a continuing increase in demand for it[4,5]. For over a millennium, Asian nations have utilized, a well-known traditional oriental medicine, to treat a range of ailments, including immune modulation[6,7]. The most commonly farmed animal species in Korea for VA exploitation are elk and deer (*Cervus elaphus*), where VA is recognized as one of the most well-known forms of traditional Korean medicine[8,9]. As a result, scientists are

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actively investigating the properties of novel bioactive compounds derived from VA as well as VA extraction techniques[4,5].

Recent studies have isolated various bioactive compounds from deer antlers, such as carbohydrates, polysaccharides, mucopolysaccharides, peptides, lipids, phospholipids, nucleotides, glycoproteins, glycolipids, cholesterol, cholesterol esters, omega-3 fatty acids, hexosamines, uronic acids, sialic acids, prostaglandins, hypoxanthine, collagens, glycosaminoglycan (GAG), prostaglandins, trace elements, and mineral elements (Ca, Zn, and Pb), in varying concentrations[2-4,10-13]. All of these bioactive compounds are heavily utilized by the pharmaceutical industry to produce drugs and dietary supplements that aid in the treatment of disorders associated with lifestyle choices[4,14,15].

According to current research, bioactive compounds and chemical compositions have been proven to be important nourishing and healthcare products with high nutritional and medicinal values; moreover, velvet antler extracts have certain curative effects against breast cancer[16], prostate cancer[17], acute kidney injury[18], physical decline[19], sexual dysfunction[20], neuronal damage[21], cardiac fibrosis[14], vascular disease[22], osteoporosis[19], hepatocyte injury[23], and so on. According to findings from modern medical research, deer antler velvet can help strengthen the immune system, enhance athletic performance, speed up muscle recovery[24,25], improving liver function mitigating skeletal weakness symptoms, and reduce inflammation[20,26].

Deer velvet should be avoided in cases of liver and renal disease, according to certain clinical case reports[27]. In Traditional Chinese Medicine (TCM), deer velvet is used extremely sparingly, especially in certain pathological states. Furthermore, consumers ought to be better informed about the bioactivities and safety of velvet antler extract since the harmful effects of deer velvet are not widely recognized[27]. To date, there has been limited research conducted to assess the biosafety of velvet extracts in terms of potential acute toxicity before establishing their efficacy. Thus, in order to ascertain the likelihood of human safety when consuming Korean deer velvet, especially with regard to its use as a dietary supplement, we suggested assessing the acute toxicities of the velvet in mice.

Here, we examine the proximate composition (moisture, crude protein, crude fat, crude ash, and carbohydrate) of VA extracts and identify any potentially dangerous substances, and other bioactive compounds. Moreover, the cytotoxic effect was analyzed by cell viability with MTT assay *in vitro* study. In addition, to find the potential toxicity of deer antler extracts, acute and subacute toxicity study was analyzed by oral administration at doses of 0, 500, 1000, and 2000 mg/kg for 14 days using ICR mouse models. The general toxicity, body weight changes, mortality, clinical signs, and necropsy findings are observed.

Materials and Methods

Deer velvet powder

The Korea Rabbit and Deer Agricultural Cooperative Federation (Seoul, Korea) provided the deer velvet antlers. The tissues from the antlers were kept at 4°C until needed. The velvet antlers were cut into small pieces, and each 4 kg of velvet material was suspended in 12 L of purified water and thoroughly mixed to remove impurities, this process was repeated five times. Then the antlers were extracted by an electric extractor (Gyeongseo ENP, Incheon, Korea) at 95°C for 12 hours using purified water, and the residual part by filtered with 120 mesh. The filtrate was concentrated under a reduced presser, then the concentrated sample was freeze-dried and the yield of velvet extract was stored at 4°C for *in vitro* and *in vivo* experiments. The experimental protocol used for collecting deer velvet antler extracts (raw, dried, and freeze-dried) method was approved by the Animal Ethics Committee with approval number: IAC2022-1319 for raw extract, approval number: IAC2022-1320 for dry extract and the approval number: IAC2022-1325 for freeze-dried extract and followed by the animal protection act no. 16977.

General ingredients

Using techniques developed by the Association of Official Analytical Chemists[28], the crude protein, crude fat, carbs, and total ash content of deer antler samples were measured. In brief, the total ash content was ascertained by incinerating at 500°C in a muffle furnace for six hours, in accordance with the Association of Official Analytical Chemists method, and the moisture content was assessed using the 105°C atmospheric pressure heating drying method in a dry oven[28]. The crude protein was calculated by quantifying the total amount using an element analyzer (DKSH Holding Ltd. Zürich, Switzerland). The Soxhlet extraction method was used to analyze the crude fat in accordance with AOAC guidelines[28]. By deducting the values of moisture, ash, crude fat, and crude protein from 100%, carbohydrates were found. The uronic acid content of the antler was analyzed according to the method of Cesaretti et al.[29]. The sulphated-GAGs levels were determined in antler extracts using microtitre plate adaptation of a dimethyl methylene blue assay according to Farndale et al.[30]. The sialic acid levels of the antler were determined according to the method of Warren et al.[31], as explained by Jeon et al.[32]. The Kolar et al.[33] method was used to determine the collagen content of antlers.

Heavy metals and residual pesticides analysis in velvet-extracted samples

Four types of heavy metals (Cd, Pb, As, and Hg) were analyzed following the Agricultural Products Quality Control Act (Ministry of Food and Drug Administration Notice 2021-27), and three types of velvet antler extracts were analyzed using iCAP7400 ICP-OES Duo (Thermo Fisher Scientific Inc. Waltham, MA, USA)[34].

We analyzed 464 residual pesticides (526 including residual pesticide compounds) according to the working manual of the 464 residual pesticides multi-component analysis method of the National Agricultural Products Quality Management Service. The velvet-extracted samples residual pesticides were analyzed using Agilent GC-MS/MS 7000D (Agilent Technologies, Inc., Santa Clara, CA, USA)[35].

Bioactive compounds analysis using HPLC and UPLC/QTOF-HRMS method

All three extracts were prepared at 5 mg/mL concentrations using 50% MeOH and filtered with a 0.2 m membrane filter to conduct HPLC analysis of three types of deer antlers. A SHIMADZU Prominence LC-20A HPLC instrument (Shimadzu Corporation, Kyoto, Japan) outfitted with a YMC-Pack ODS-A column (4.6 mm × 250 mm; YMC Co., Ltd., Kyoto, Japan) was used to perform the chromatographic analysis. Three kinds of extracts were analyzed at 254 nm for 40 minutes with YMC-Pack ODS-A Column at 25°C according to Xue et al.[36].

Following their dissolution in distilled water, the 1g velvet-extracted samples were filtered through a 0.45 μm filter membrane (Merck Millipore, Seoul, Korea). The protocol of Zhang et al.[12] was followed in order to separate and detect the bioactive compounds in the velvet antler extracts using an ultra-performance liquid chromatography-tandem mass spectroscopy (UPLC-Q-TOF-MS/MS, Xevo TQ-5, Waters, Milford, MA, USA). A high-resolution tandem mass spectrometer SYNAPT G2 Si HDMS QTOF (Waters) identified the compounds that eluted from the column in both positive and negative ion modes[8].

Evaluation of cytotoxicity

The cytotoxicity of velvet antler extracts (sample 1-3) against HT22 hippocampal, BV2 microglia, RAW264.7 macrophages, and HaCaT keratinocytes cells was measured through MTT assay by treating cells at three concentrations of 250, 500, and 1000 μg/mL for 24 h. The cells were stained for an additional three hours at 37°C in the dark after the addition of the MTT working solution. After that, the supernatant was discarded and the formazan crystals were dissolved with 150 mL of dimethyl sulphoxide (DMSO). The absorbance was measured in a microplate reader at 540 nm[37].

Deer velvet antler samples *in vivo* treatment for analyzing acute toxicity

Animals and experimental design

Our experimental animals were male and female five-week-old ICR mice from Gwangmyeong Bio Co., Ltd. (Gwangju, Korea). The Chonnam National University Guidelines for Care and Use of Laboratory Animals were followed when housing the animals. Prior to the experiments, they spent a week being acclimated in a room with humidity controlled at 55±5% and a temperature of 22±2°C over a 12-hour light/dark cycle. In the experimental design, we maintained 10 groups of male and 10 groups of female ICR mice for acute toxicity test following the guidelines of OECD[38]. In each group, five male or female mice per cage were maintained with free access to food and water. Each velvet extract sample (samples 1-3) was administered orally once a day (09:00–10:00) for 14 days with the dose of 0, 500, 1000, and 2000 mg/kg body weight for both male and female mice after 4 hours of fasting. The Animal Ethics Committee of Chonnam National University approved the experimental protocols used in this study (CNU IACUC-YS-2020-9).

Toxicity studies

The Organization for Economic Co-operation and Development (OECD) Guidelines 425 and 407 served as the foundation for acute toxicity studies[38]. The general symptoms of the experimental mouse were observed periodically during the first 0.5, 1, 2, 3, and 4 hours after administering each velvet extract, and then once a day for 14 days. The weight changes were analyzed on 0, 7, and 14th day after administration. After 14 days of oral administration of the antler extracts, we observed the mortality, clinical signs, and necropsy findings of experimental mice based on OECD guidelines[38].

Microbiological analysis of velvet extract samples

The entire microbiological analysis was performed using commercially available 3M^{TM} petrifilm^{TM} plates (3M Microbiology Products, St. Paul, MN, USA), in accordance with the manufacturer's instructions. Using a sterilized sample bag (3M^{TM} Sample Bag), 10 g of velvet extract samples were homogenized for two minutes with 100 mL of 0.85% physiological saline. Subsequently, 3M^{TM} Petri film plates were used to culture 1 mL of the homogenized sample suspension at 35±1°C for 24 to 48 hours[39]. The majority of bacterial species generated different colonies with varying hues. The number of colonies on a petrifilm was counted after the red-colored colonies were developed on the film, which is visible to the unaided eye. After

multiplying the average number of red-colored colonies by the dilution factor for the total number of microorganisms, the result was expressed as log cfu/g. As directed by the manufacturer, the 3M™ petrifilm™ plate counting method was utilized to measure *Escherichia coli* (*E. coli*) using the same protocol. *E. coli* bacteria were indicated by the colonies of visible blue color on the petrifilm, which were encircled by trapped gas.

Statistical analysis

The means±SEM are used to express the data. The IBM SPSS statistic version 21.0 (Statistical Package for Social, SPSS Inc., Chicago IL, USA) was used for all statistical analyses. Tukey's multiple comparison test was conducted after one-way analysis of variance (ANOVA) was used to evaluate the data. At $P<0.05$, differences were deemed significant.

Results and discussion

Chemical composition and mineral contents of raw, dried, and freeze-dried antler extracts

In general ingredients, moisture content was significantly higher in dried antlers (3.18%) than in raw and freeze-dried samples. Ash, carbohydrates, and crude fat contents were highest in raw antler, followed by freeze-dried and dried antler samples. The crude protein contents are high in dried antler (88.21%), followed by freeze-dried (84.9%) and raw antler (78.79%) samples respectively. Brown[40] also suggested that white-tailed antler deer antler extracts also contain 80% of proteins. While, ash content was lowest in dried (6.83%), followed by freeze-dried (8.39 %) and raw antlers (9.12 %) respectively as shown in (Fig. 1).

Uronic acid, sulfated-GAGs, sialic acid, and collagen contents

Velvet antler contains varying amounts of various proteins, omega-3 fatty acids, collagens, glycosaminoglycan (GAG), and minerals[41,42]. However, the antler portion examined, the antler developmental stage, and the species of deer all affect the chemical composition, specifically the concentrations of GAG, sialic acid, uronic acid, and collagens[9,43,44]. In this study, we analyzed the chemical compositions such as uronic acid, sialic acid, sulfated-GAGs, and collagen levels were analyzed in raw, dried, and freeze-dried extract samples, and the results are shown in Table 1. The content of uronic acid was significantly higher in freeze-dried antler extract (56.38 μg/mg) than in raw extract (47.05 μg/mg) and dry sample extract (49.31 μg/mg). The sulphated-GAG levels were significantly higher in freeze-dried extract (21.74 μg/mg) and dry extract (18.93 μg/mg) than in raw extract (6.96 μg/mg). The primary antler GAG component, chondroitin sulfate, makes up 88% of the total uronic acid content. It may also be a significant carbohydrate and functions as an ion exchanger in the formation of endochondral bone[45-47]. The sialic acid contents in raw extract were significantly the highest at 1.36 mg/g, followed by dry (0.84 mg/

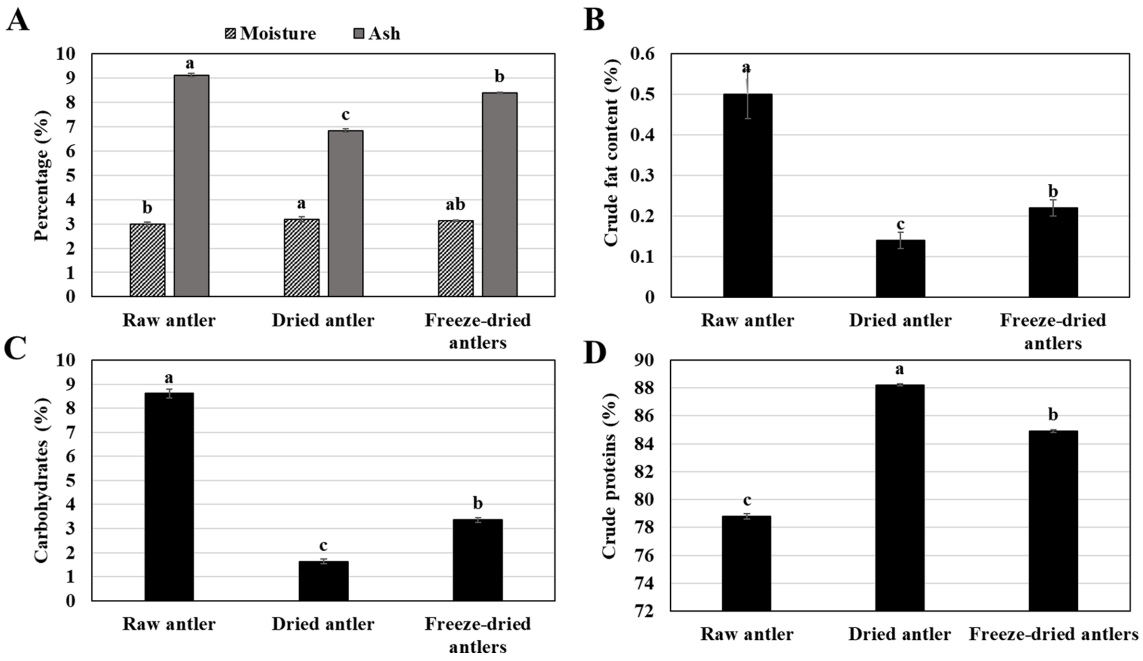

Fig. 1. Proximate composition (%) of deer velvet antler extracts. Values represent mean ± standard deviation (SD) (n = 3). Letters a, b and c in in each bar graph represents significant differences ($P < 0.05$).

Table 1. Deer velvet antler extracts chemical compositions such as uronic acid, sulfated-GAGs, sialic acid, and collagen contents of deer antler extracts

Chemical compositions	Raw antler	Dried antler	Freeze-dried antlers
Uronic acid (μg/mg)	47.05±2.3[b]	49.31±1.8[b]	56.38±2.1[a]
Sulfated-GAGs (μg/mg)	6.96±1.2[b]	18.93±2.01[a]	21.74±2.2[a]
Sialic acid (mg/g)	1.36±0.1[a]	0.84±0.08[b]	0.52±0.06[c]
Collagen (mg/100 g)	46.27±2.3[c]	151.18±2.1[a]	125.37±1.8[b]

Values represent mean ± standard deviation (SD) (n=3). Different superscript letters within each row indicate significant differences (P<0.05).

g) and freeze-dried extract (0.52 mg/g).

As an organic component, collagen is thought to be the main protein responsible for supporting the mineralized tissue structure and giving the tissue mechanical strength[46,48]. The collagen contents in the raw antler extract were 46.27 mg/100 g, the dry antler extract was 151.18 mg/100 g, and the freeze-dried rust extract was 125.37 mg/100 g are shown in Table 1. Collagen content was found to be higher in the later stage of antler development compared to the early and middle stages, according to Jeon et al.[44]. However, our observation results clarified that the collagen, uronic acid, and sulphated-GAG were higher in dry antler extracts than in raw extracts, while sialic acid was higher in raw antler extracts than in dry extracts.

Bioactive compounds analysis

The quality of different types of antlers can vary greatly, and their composition is incredibly complex. It can be broadly separated into three categories: organic components, inorganic elements, and bioactive ingredients. We analyzed the bioactive compounds by HPLC analysis at 254 nm for 40 minutes using a YMC-Pack ODS-A column and compared the differences in the components of raw, dried, and freeze-dried antler extracts. When comparing the HPLC analysis results, we identify a common peak for each specificity in the three types of antler extracts. In the raw antler fraction, after 6 to 8 minutes and 16 to 18 minutes, the peak was largely divided into two zones (Fig. 2). It was confirmed that the difference in the components of the extracts in raw extracts is greater than that in dried and freeze-dried antler extracts. Then we intend to conduct an additional separation study by LC-MS analysis for raw antler extract.

A high diversity of bioactive substances was revealed by the UPLC-QTOF-HRMS analysis results of the positive and negative modes for the MS/MS analysis, as shown in (Fig. 3). A detailed list of annotated metabolites can be found in Table 2 together with their molecular formulas. Similarly, Salek et al.[49] identified around 898 compounds according to

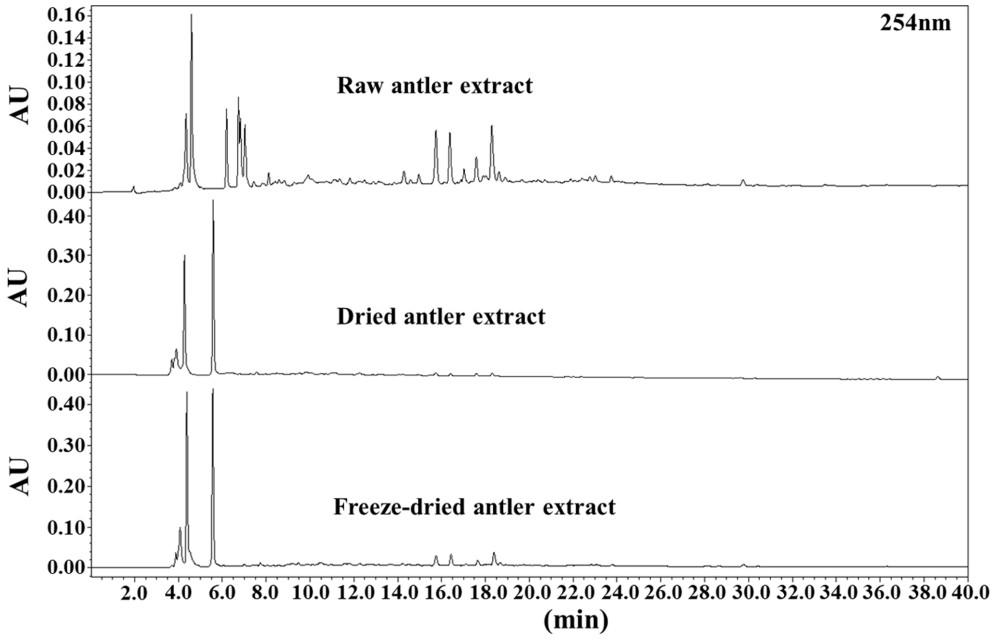

Fig. 2. HPLC analysis results in deer antler extract at 254 nm for 40 min.

an HRMS-based approach using various samples. For the first time, the bioactive components in the raw velvet extract sample were identified and compared in this study, and it can be concluded that all of the velvet extracts under investigation are highly bioactive. It is also mentioned that the various forms of velvet antler extract may have distinct medicinal and pharmaceutical qualities due to differences in the bioactive components. Furthermore, the bioactive compounds found in this study may have potential uses as candidate biomarkers to distinguish between the quality of velvet antlers from various countries of origin.

In the pharmaceutical industry, all of these bioactive substances are widely used to create medications and dietary supplements that help treat disorders linked to an unhealthy lifestyle[14,15]. To quantify these potential biomarkers in the velvet antler extracts, more thorough research is required. The chemical components of velvet antler are commonly linked to its anti-osteoporosis[50,51], anti-fatigue[15,52], anti-inflammatory[53], and anti-cancer[54] effects, all of which have been shown in velvet antler experiments. The present study's identification of the chemical components of velvet antlers may make it easier to evaluate the substance's bioactivity and functional mechanism in the future.

Analysis of residual pesticides and heavy metal concentrations in velvet antler extracts

Velvet antlers contain pesticide residues along with other potentially harmful substances. Recent research status

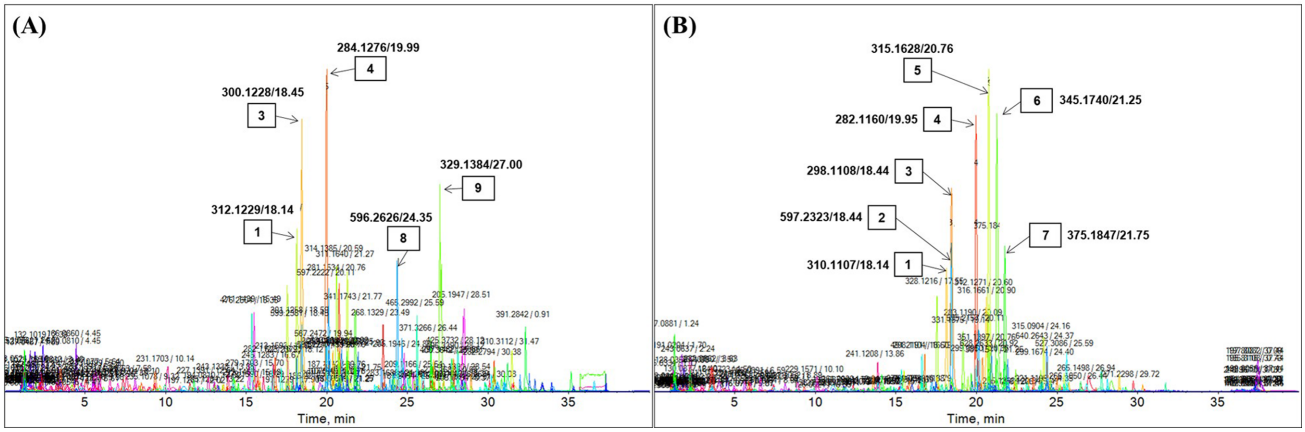

Fig. 3. (A) Positive and (B) negative mode of UPLC-QTOF-HRMS results in raw deer antler extract

Table 2. Identified the bioactive compounds from raw velvet antler extract

S.No	Retention time (min)	Identified bioactive compounds	Observed (m/z) (+)	Observed (m/z) (-)
1	18.14	*N*-trans-Feruloyloctopamine or (S)-Nandigerine	312.123 $[M+H-H_2O]^+$	310.108 $[M-H-H_2O]^-$
2	18.44	*N*-Acetoxymethyl flindersine	300.123 $[M+H]^+$	298.108 $[M-H]^-$
3	18.45	Piplartine	300.123 $[M+H-H_2O]^+$	298.111 $[M-H-H_2O]^-$
4	19.99	Anaxagoreine or Tapentadol *O*-sulfate	284.128 $[M+H]^+$	282.116 $[M-H]^-$
5	20.76	heminordihydroguaiaretic acid	339.157 $[M+Na]^+$	315.16 $[M-H]^-$
6	21.25	Cinncassiol C2 or Verimol G	347.185 $[M+H]^+$	345.174 $[M-H]^-$
7	21.75	18-Oxocortisol	377.196 $[M+H]^+$	375.181 $[M-H]^-$
8	24.35	Taurocholic acid 3-sulfate	596.256 $[M+H]^+$	-
9	27.00	3-Hydroxy-7,2',3'-trimethoxyflavone	329.102 $[M+H]^+$	-

Table 3. Contents of pesticide and heavy metal concentrations (Cd, Pb, As, and Hg) in deer antler extract (mg/kg)

Toxic elements	Elements	Raw antler	Dried antler	Freeze-dried antlers
Pesticides	Cypermethrin	0.074±0.005[b]	0.098±0.004[a]	0.044±0.011[c]
Heavy metals	Cd	0.08±0.005[NS]	0.10±0.006	0.10±0.017
	Pb	N.D	N.D	N.D
	As	0.33±0.079	N.D	0.14±0.032
	Hg	N.D	N.D	N.D

N.D: Not detected; NS: Not significant. Different superscript letters within each row indicate significant differences ($P<0.05$).

indicates that pesticide residues are ubiquitous in Chinese herbal medicines[55,56]. The residual pesticides were quantified by the gas chromatography analysis. We quantified 464 residual pesticides (526 residual pesticide compounds) from raw, dried, and freeze-dried extracts. The results indicated that all of the pesticides were not detected except for one cypermethrin (0.044-0.098 mg/kg). According to the WHO, cypermethrin (isomers) is an insecticide among residual pesticide items, and the acceptance standard is reported to be 1.0 mg/kg. In Korea, as the PLS system has been implemented for all agricultural products since January 1, 2019, the uniform standard (0.01 mg/kg or less) is applied when pesticides without residual acceptance standards are detected.

Naturally, fed feeds are the primary source of heavy metals in deer products; although this issue requires greater attention during processing and production, it is frequently unavoidable[12]. The concentrations of toxic heavy metals (Cd, Pb, As, and Hg) in raw, dried, and freeze-dried velvet extract were determined by ICP-OES and are shown in Table 3. The findings showed that not all velvet samples contained Pb or Hg. On the other hand, Cd was found in both dried and freeze-dried antler extracts (0.1 mg/kg) and in raw (0.08 mg/kg) extract. The arsenic (As) levels were 0.33 mg/kg in raw extract and 0.14 mg/kg in freeze-dried rust extract. The management of farms, the preparation of feed, medicine, storage, transit, and other activities are probably the sources of heavy metal and pesticide pollution in antlers[12]. All velvet extracted samples, however, had As, Cd, Hg, and Pb levels below the allowable limits set by the ASEAN guidelines on limits of contaminants for traditional medicines and health supplements version 1.0 for oral use[7]. According to the acceptable standards for heavy metals such as raw drugs and the test method, the acceptable standards for arsenic for antlers and antlers were less than 3 mg/kg, and all three types of antlers were below the acceptable standards[57].

Cytoprotective effect of raw, dried, and freeze-dried antler extract on HT22, BV2, RAW264.7, and HaCaT cells

To evaluate the effects of velvet antler extracts (samples 1-3) on cytotoxicity of HT22 from mouse hippocampal cells, BV2 cells derived from mouse microglia cells, RAW264.7 cells derived from mouse macrophages, and HaCaT keratinocyte cells with various concentrations (125, 250, 500, 1000 μg/mL) for 24 h. In the results, we found that the HT22 cells showed the cytotoxicity effect at 500 and 1000 μg/mL with dried and freeze-dried extract treatments (Fig. 4A). However, BV2 cells showed the cytotoxic effect at 500 and 1000 μg/mL treated with freeze-dried extract (Fig. 4B). In RAW264.7 cells showed cytotoxicity with dried antler extract at 1000 μg/mL concentration, and freeze-dried extract confirmed cytotoxic effect at a concentration of 500 and 1000 μg/mL (Fig. 4C). However, deer antler protein fraction treatment with RAW264.7 macrophages for 24 hours significantly reduced inflammatory cytokines in dose dose-dependent manner[58]. While, HaCaT cells did not show cytotoxic effects with 125, 250, 500, or 1000 μg/mL concentrations (Fig. 4D).

Acute toxicity in ICR mice model

One of the broadest tests in toxicology is the repeated oral administration toxicity study, the results of which are used to forecast and ascertain the safe doses for humans after the level at which no adverse effects are observed[59,60]. In the sighting study, five male and female mice per group were used to assess the potential acute toxicity of raw, dried, and freeze-dried deer velvet antler extracts. According to mortality and clinical signs of toxicity over a 14-day period, the results showed that there was no acute toxicity at dose levels of 0, 500, 1000, and 2000 mg/kg. In addition, pathological analysis and gross necropsy results in mice given antler-extracted sample treatment did not show any abnormalities in the morphology of the stomach, lungs, liver, pancreas, kidneys, bladder, or spleen. Following their deer velvet dosage, none of the rats experienced weight loss (data not shown). In the primary investigation, animals did not exhibit any obvious behavioral abnormalities or symptoms of toxicity after receiving 2000 mg/kg of deer velvet orally or acutely. These macroscopic pathological results showed that all mice given extracts up to 2000 mg/kg body weight

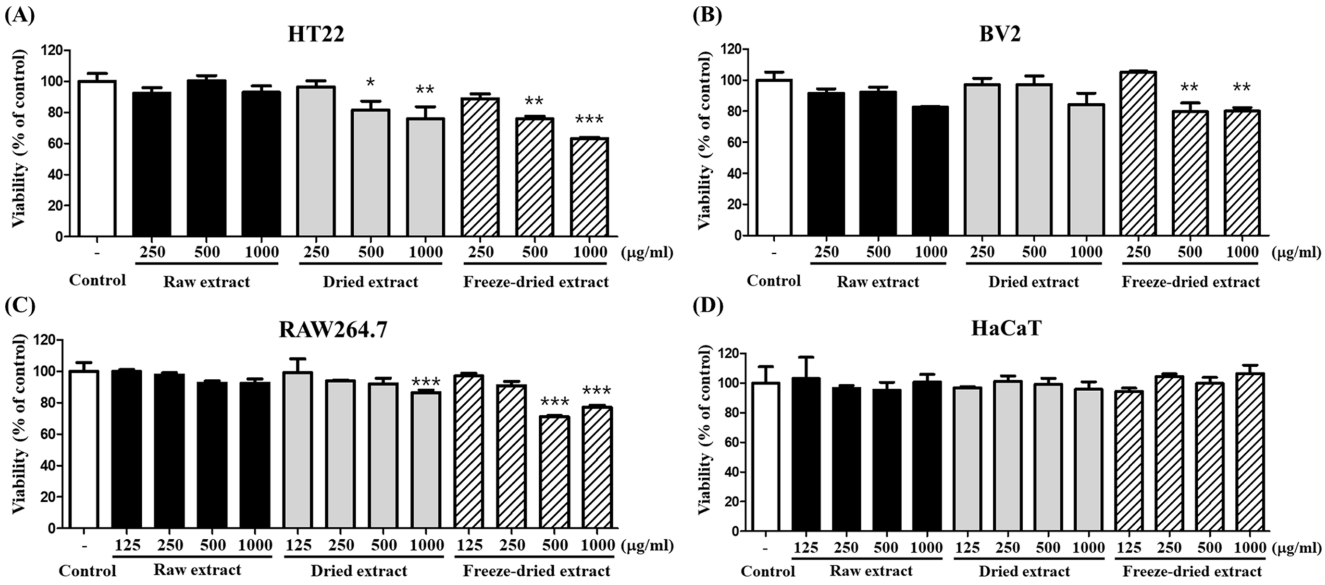

Fig. 4. Effect of velvet antler (raw, dried, and freeze-dried) extracts on cell viability in HT22, BV2, RAW264.7, and HaCaT cells. ***P<0.001, **P<0.01, *P<0.05 compared with control group.

Table 4. Evaluation of the acute toxicity over a period of 14 days in terms of mortality, clinical signs, and necropsy results.

Samples	Dose (mg/kg BW)	Groups	Mortality	Clinical signs	Necropsy findings (External and Internal)
Raw antler	0	Male/Female	0%	Normal	No gross findings
	500	Male/Female	0%	Normal	No gross findings
	1000	Male/Female	0%	Normal	No gross findings
	2000	Male/Female	0%	Normal	No gross findings
Dried antler	0	Male/Female	0%	Normal	No gross findings
	500	Male/Female	0%	Normal	No gross findings
	1000	Male/Female	0%	Normal	No gross findings
	2000	Male/Female	0%	Normal	No gross findings
Freeze-dried antlers	0	Male/Female	0%	Normal	No gross findings
	500	Male/Female	0%	Normal	No gross findings
	1000	Male/Female	0%	Normal	No gross findings
	2000	Male/Female	0%	Normal	No gross findings

B.W: Body weight

of raw, dried, and freeze-dried extracts did not exhibit any signs of acute toxicity as shown in Table 4. Therefore, the approximate lethal dose of rust extract in mice is considered to be more than 2,000 mg/kg B.W. for both males and females. According to Zhang et al.[1], rats treated with 2000 mg/kg of New Zealand deer velvet extracts for 14 days did not show any pathological signs or toxicity, and none of the rats experienced weight loss as a result of the antler extracts. Furthermore, no evidence of possible reproductive toxicity was found in the sperm analysis, fertility-related indices, or the estrus cycle[61]. This investigation concluded with confidence that consuming deer antlers does not result in any possible buildup of active constituents that could be harmful.

Microbiological analysis of deer velvet extracts

To have an overview of the pathogenic and non-pathogenic bacterial types, the bacterial flora in the extracts from plants and animals must be identified[62]. Therefore, the total bacteria and *E. Coli* bacterial count analysis results of three types of velvet extracts are shown in Table 5. The analysis results indicate total bacterial contents in the raw

Table 5. Total aerobic bacteria and *E. Coli* contents of deer antler extracts (log CFU/g)

Sample	Total bacteria	E.Coli
Raw extract	3.68±0.06[b]	N.D
Dried extract	3.91±0.01[a]	N.D
Freeze-dried extract	3.95±0.02[a]	N.D

N.D: Not detected

extract was 3.68 log CFU/g, the dried antler extract was 3.91 log CFU/g, and the highest bacterial contents were present in the freeze-dried antler extract (3.95 log CFU/g). However, *E. coli* bacterial contents were not detected in raw, dried, and freeze-dried velvet antler extracts. According to the Ministry of Food and Drug Safety's microbial standards for animal processed foods, *E. coli* groups are set as n=5, c=2, m=10, M=100 (limited to sterilized products), bacterial counts n=5, c=0, m=0/25g (limited to raw meat). In general, when the total number of bacteria per 1g (or 1 mL) of food reaches 5 to 6 log CFU/g, it is known that it shows decay in the early stages of decay, and at 7 log CFU/g, so it is no longer possible to consume it. As per the ASEAN guidelines on the limits of contaminants for traditional medicines and health supplements version 1.0 for oral use, extracts from animal origin are acceptable as long as they don't exceed 2×10^4 and 2×10^2 CFU/g, respectively, for total aerobic microbial count (TAMC) and total yeast and mold count (TYMC). Furthermore, the quantity of gram-negative bacteria that can withstand bile must not exceed 2×10^2 CFU/g[7].

In summary, the present study concluded that the deer velvet extracts contain various chemical compositions, and nine major metabolic compounds. All extracts had lower levels of pesticides, hazardous heavy metals, and microbiological contamination than the ASEAN guidelines' allowable limits. The findings of this investigation demonstrated that the administration of velvet antler extract at a concentration of up to 500 μg/mL had no cytotoxic effect on a variety of cells when tested *in vitro*. Moreover, this study showed that the deer velvet extracts with different doses (500 to 2000 mg/kg) for 14 days oral treatment in ICR mice exhibited no treatment-related toxicological evidence. These results show that extracts from velvet antlers that are less than 2000 mg/kg BW/day do not appear to have any negative effects. This gives assurance that consuming deer velvet won't potentially accumulate active ingredients to the point of toxicity. It stated that deer velvet extracts are among the animal medications that patients have preferred due to their minimal adverse effects, strong therapeutic efficacy, and medication safety, all of which have been progressively acknowledged and accepted by the global community.

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국문요약

녹용은 수많은 연구에서 면역력 강화를 포함한 영양 및 의학적 가치를 입증하였으며 전통적인 약으로 널리 사용되고 있다. 본 연구는 녹용 추출물 (sample 1: 생녹용 추출물, sample 2: 건녹용 추출물, sample 3: 동결 건조 추추물)의 일반성분과 우론산, 황산화 글리코사미노글리칸, 시알릭산, 콜라겐을 포함한 유효성분을 조사하고, 액체 크로마토그래피-quadrupole-time-of-light mass spectrometry (UPLC/QTOF-MS)를 사용하여 녹용 추출물의 화학 성분을 분석하는데 목적이 있다. 또한, HT22 해마 세포, BV2 미세아교세포, RAW264.7 대식세포 및 HaCaT 케라틴 세포를 사용하여 MTT 분석을 통해 녹용 추출물의 세포 독성 효과 평가와 암컷과 수컷 ICR 마우스에 녹용 추출물을 각각 (0, 500, 1000, 2000 mg/kg) 경구투여 하여 급성 독성평가를 실시하였다. 투여후에는 OECD 가이드라인에 따라 마우스의 일반독성, 생존율, 체중 변화, 사망률, 임상 징후 및 부검 결과를 관찰하였다. 결과적으로 녹용 추출물은 HaCaT 케라틴 세포에서 세포 독성 효과가 없었으며, 건녹용 추출물에서는 HT22 해마 세포에서 500 μg/mL, RAW264.7 대식세포의 경우 1000 μg/mL 에서, 동결건조추출물에서는 RAW264.7 세포와 BV2 미세아교세포의 경우 500 μg/mL 및 1000 μg/mL 농도에서 세포 독성을 가지고 있음을 보였다. 그러나 마우스를 이용한 급성 독성 평가에서는 녹용 추출물 시료를 처리한 모든 마우스에서 사망률, 임상 징후 및 부검 결과 특이사항이 없었으며 이는 LD50이 2000 mg/kg 이상으로 사료된다. 그러나 인간에 대한 안전성에 대한 충분한 증거를 확보하기 위해서는 동물과 사람에 대한 추가적인 연구가 필요하다.

Conflict of interests

The authors declare no potential conflict of interest.

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