Original Article

Comparison of anti-inflammatory effect and protein profile between the water extracts from Formosan sambar deer and red deer

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ABSTRACT

Velvet antler (VA), the unossified antler from members of the family Cervidae, has been used in traditional Chinese medicines and health foods for over 2000 years in enhancement of kidney function and treatment or prevention of cardiovascular, immunological and gynaecological disease. The aim of this study was to investigate the anti-inflammatory effect of velvet antler water extracts from Formosan sambar deer (*Rusa unicolor swinhoei*, SVAE) and red deer (*Cervus elaphus*, RVAE). Results indicated that both SVAE and RVAE significantly reduced the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) productions in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells at concentrations above 200 μg mL^{-1}. SVAE seems to demonstrate a better anti-inflammatory effect than that of RVAE *in vitro*. Both SVAE and RAVE also enhanced the anti-inflammatory cytokine IL-10 production in LPS-stimulated RAW 264.7 cells. The results of MTT assay indicated that SVAE and RVAE did not exhibit any cytotoxicity in LPS-stimulated RAW 264.7 cells. Two-dimensional (2D) gel electrophoresis analysis revealed that the levels of 6 specific proteins were different between these two velvet antlers samples. Furthermore, the storage period was the major factor affecting the anti-inflammatory activity of SAVE. In this study, we demonstrated the difference of anti-inflammatory effect and the protein profile between SVAE and RVAE. SVAE showed better anti-inflammatory potential than RVAE. In the future, the anti-inflammatory active components and their related mechanisms should be further investigated.

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1.   Introduction

Inflammatory response plays a crucial role in body's defense mechanisms that protect the host from invading pathogens and biochemicals [1]. However, dysregulation of inflammatory response caused many disorders such as atherosclerosis, Alzheimer's disease, ischaemic heart and brain diseases, cancer, Grohn's disease, colitis, obesity, metabolic syndrome, asthma, and type 1 & 2 diabetes [2,3]. Using anti-inflammatory drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) and pro-inflammatory cytokine inhibitor that ameliorating inflammatory response is one of the most common treatment for inflammatory-related disease [4]. Unfortunately, NSAIDs has side effects, including gastrointestinal tract injury, renal damage and heart problems, high blood pressure, swelling, and rashes. Due to the side effects of current NSAIDs, researchers have focused on finding the new source of less toxic anti-inflammatory agents.

Recently, natural products from animals and plants seem to be a possible source for developing the potential pharmaceutical agent and food supplement. Velvet antler (VA) is one of the most famous animal-divided medicine material, which is the unossified tissue that isolated from deer or elk (members of family Cervidae). The Chinese medical classics, *Shen Nong Ben Cao Jing* and *Compendium of Materia Medica,* recorded that VA possessed beneficial effect on kidney function, body strengthening, and anti-aging. In addition, VA has been used as a traditional Chinese medicine for enhancement of kidney function and treatment or prevention of cardiovascular, immunological and gynaecological disease for centuries in East Asia. Many previous reports and clinical observations have convincingly demonstrated that VA and its extracts can alleviate the symptoms of rheumatoid arthritis, osteoporosis and osteoarthritis, promote dermal cell proliferation and angiogenesis, and treat heart failure [5–10]. Although VA and its extracts have showed various beneficial effects *in vitro* and *in vivo*, no previous studies have distinguished the difference of anti-inflammatory effect among VA from different species or different extraction methods.

Sui et al. [7] reported that various components including mineral elements, amino acids, proteins and peptides, saccharides, lipids and polyamines are the major substrates that contributed the bio-activities to VA and VA extracts. Among these components, amino acid, polypeptides, and proteins are the most abundant components in VA, and also have been reported with excellent bio-activities. Fox example, the polypeptides from VA possess anti-osteoporosis effects in both of the *in vitro* osteoarthritic rabbit chondrocytes model and the *in vivo* retinoic acid-induced osteoporotic rat model [11,12]. In addition, the polypeptides from VA also exhibited anti-heart failure, anti-fatigue, and wound healing effects [13–15].

In Taiwan, there is an indigenous subspecies of deer, which inhabit at low to middle elevation forest, named Formosan sambar deer (*Rusa unicolor swinhoei*). The farming deer industry began in the 1960's in Taiwan, thus the use of products from Formosan sambar deer have attracted many attentions, especially in VA and its pharmaceutical components [5,6]. However, there is another popular VA source from red deer (*Cervus elaphus*). No previous study evaluated the differences between Taiwan indigenous deer species and other species in the bio-activities and the components. The influence of storage conditions on its anti-inflammatory effect also remains unclearly. Therefore, the aim of this study was to assess the anti-inflammatory effects of different VA extracts through RAW 264.7 cell model, and to identify the major components of each sample using 2D SDS-PAGE electrophoresis (2DE) and LCMS^2. The effect of storage conditions on the anti-inflammatory effect of the VA extracts was also evaluated. The final aim of this study was to develop a potential anti-inflammatory agent from VA extracts.

2.   Material and methods

2.1.   Chemicals and reagents

Lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trichloroacetic acid (TCA), and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMEM (Dulbecco's Modified Eagle Medium) medium, fetal bovine serum (FBS), and other cell culture reagents were obtained from Corning (Tewksbury, MA, USA). Urea, Dithiothreitol (DTT), and triton X-100 were purchased from Merck (Darmstadt, Germany). All of the other chemicals and solvents used in this research were analytical grade.

2.2.   Cell culture

RAW 264.7 cell line was purchased from Bioresource Collection and Research Center (BCRC, Taiwan). Cells were cultured in DMEM containing 10% FBS and 1% antibiotic antimycotic in a 37 °C humidified incubator containing 5% CO_2. Cell subculture was prepared by scraping 2 to 3 times per week.

2.3.   Morphology and chemical composition analysis of velvet antler

The morphology of velvet antler from Formosan sambar deer and red deer were observed using microscopy (SG-EX30, SAGE Vision, Taiwan). Chemical composition analysis, including moisture, ash, crude protein, and crude fat contents were analyzed according to Chinese National Standards (CNS) 5033, 5034, 5035, and 5036, respectively.

2.4.   Preparation and extraction of velvet antler

The 70–75 days' VA samples of Formosan sambar deer were obtained from Kaohsiung Animal Propagation Station, Taiwan Live Stock Research Institute (Pintong, Taiwan). The 60–65 days' VA samples of red deer were purchased from Feng Ying Deer Ranch (Tainan, Taiwan). After harvested and sliced, the VA samples were stored in a −80 °C freezer until analyzed. The frozen VA samples were lyophilized by a freeze dryer (Kingmech Co. Ltd., Taipei, Taiwan) and then ground into a fine powder (VA powder) by a pulverizing machine. The VA power extracted by soaking with cold water (50 g L^{-1}) at 4 °C for 24 h, then collected and freeze-dried the supernatant to obtain the VA water extract. For the storage test, the lyophilized slides

were stored in various environment (−20, 4, and 25 °C) for 2, 4, and 6 months, respectively. After that, the VA samples were extracted by cold water following abovementioned procedure. In this study, all VA extracts were dissolved in DMEM and diluted to the indicated concentrations.

2.5. Cytotoxicity assays

The cytotoxic assays were performed following the method described by Zhai et al. [16]. Briefly, seeding cell onto 24-well plates at densities of 2×10^5 cells/well for 24 h. After 24 h, the test samples (500, 200, 100, and 50 μg mL^{-1} in DMEM medium) were added to a 24-well plate followed by another 24 h of incubation. Additionally, medium alone was used as the control in this assay. After incubation, the supernatant was removed, and cells were washed twice by $1 \times$ PBS. Then, 200 μL of MTT solution (1 mg mL^{-1}) was added to each well and incubated in 37 °C for 4 h. The cell viabilities were calculated by measuring the absorbance at 570 nm using an ELISA reader (Labsystems Multiskan MS, Helsinki, Finland).

2.6. Pro-inflammatory cytokine production assays

Cytokine detection was performed as previously described by Zhai et al. [16]. RAW 264.7 cells were seeded onto 24-well plate at a density of 1×10^6 cells/well for 24 h. After 24 h, the cells were pretreated with various concentrations of VA extract for 1 h, and were added LPS (1 μg mL^{-1} in PBS) to each well for 4 h or 24 h to induce tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production, respectively. At the indicated time point, the medium in each well was collected by centrifugation at 1500 rpm for 5 min, and then the resulting supernatant was stored at −80 °C freezer until analyzed. All Cytokine productions were measured using commercial kits (PeproTech, Rocky Hill, NJ, USA; R & D Systems, McKinley Place NE, MN, USA) according to manufacturer's instructions.

2.7. 2D electrophoresis analysis and protein identification of velvet antler

The assay was performed as previously reported with slight modification [17]. In brief, 100 mg of velvet antler powder was soaked in 0.5 mL ddH$_2$O, then precipitated with equal volume of TCA at ice bath for 20 min and then centrifuged (4 °C, 14,000 rpm, 15 min). After centrifugation, discarded supernatant and harvested the precipitated proteins. The proteins were washed by ice cold acetone for 3 times and removed the organic solvent. The protein samples were then dissolved in sample buffer (9 M urea, 16 mM DTT, 0.5% Triton X-100) for further quantification and analyzation using the Bradford microassay (Bio-Rad Laboratories, Hercules, CA, USA) and 2DE, respectively. For 2DE, the samples were separated by isoelectric focusing at ranges of pH 4.0 to pH 7.0 in the first dimension. The second dimension, SDS-PAGE gels, were run in 12.5% (w/v) acrylamide gels using Hoefer SE 600 Ruby System (GE Healthcare Biosciences, Uppsala, Sweden). After electrophoresis, the gel was stained using commasie blue dye (Amresco, Solon, Ohio, USA). The protein spots were quantitated by ImageMaster 2D Platinum 7.0 software (GE Healthcare, Little Chalfont, UK). The protein identification

process was entrusted to Mithra Biotechnology Inc. (Taipei, Taiwan). In brief, the excised gel spot was first de-stained, and then reduced with 10 mM DTT at 56 °C for 45 min, followed by cysteine-blocking with 55 mM iodoacetamide (IAM, Sigma, St. Louis, MO, USA) at 25 °C for 30 min. The samples were digested with sequencing-grade modified porcine trypsin (Promega, Madison, WI, USA) at 37 °C for 16 h. The peptides were then extracted from gel, and stored at −80 °C until analyze. The dried peptide mixtures were reconstituted in high performance liquid chromatography (HPLC) buffer A (0.1% formic acid) and loaded onto a reverse-phase column (Zorbax 300SB−C18, 0.3 × 5 mm; Agilent Technologies, Wilmington, DE, USA). The desalted peptides were then separated on a homemade column (HydroRP 2.5 μm, 75 μm I.D. x 20 cm with a 15 μm tip) using a multi-step gradient of HPLC buffer B (99.9% acetonitrile/0.1% formic acid) for 45 min with a flow rate of 0.25 μl/min. The LC apparatus was coupled with a 2D linear ion trap mass spectrometer (Orbitrap Elite ETD; Thermo Fisher, San Jose, CA, USA) operated using Xcalibur 2.2 software (Thermo Fisher, San Jose, CA, USA). The full-scan Mass Spectrometer (MS) was performed in the Orbitrap over a range of 400 to 2000 Da and a resolution of 60,000 at m/z 400. Internal calibration was performed using the ion signal of $[Si(CH_3)_2O]_6H^+$ at m/z 536.165,365 as lock mass. The 20 data-dependent MS/MS scan events were followed by one MS scan for the 20 most abundant precursor ions in the preview MS scan. The m/z values selected for MS/MS were dynamically excluded for 60 s with a relative mass window of 15 ppm. The electrospray voltage was set to 2.0 kV, and the temperature of the capillary was set to 200 °C. MS and MS/MS automatic gain control was set to 1000 ms (full scan) and 200 ms (MS/MS), or 3×10^6 ions (full scan) and 3×10^3 ions (MS/MS) for maximum accumulated time or ions, respectively.

2.8. Statistical analysis

All results are expressed as the mean ± SD (n = 3). Statistical analysis was performed with Tukey's range test or Student t-test using the SPSS program (version 10.0). The results with P < 0.05 were considered to be statistically significant.

3. Results

3.1. Morphology and chemical compositions of fresh velvet antler from Formosan sambar deer and red deer

As shown in Fig. 1, the morphology of fresh velvet antler slides from two deer were different. Before freeze dried, the VA from red deer (RVA) showed a dark red appearance, while the VA from Formosan sambar deer (SVA) showed a crimson red appearance (Fig. 1A and B). After lyophilization, RVA remained dark red appearance, but SVA faded to white color (Fig. 1C and D). The lyophilization sample was then milled as powders (Fig. 1E and F) for further analyzation. The results of chemical composition analysis of SVA and RVA were listed in Table 1. The SVA sample had higher ash and crude fat contents, but lower crude protein and moisture content than the RVA analog.
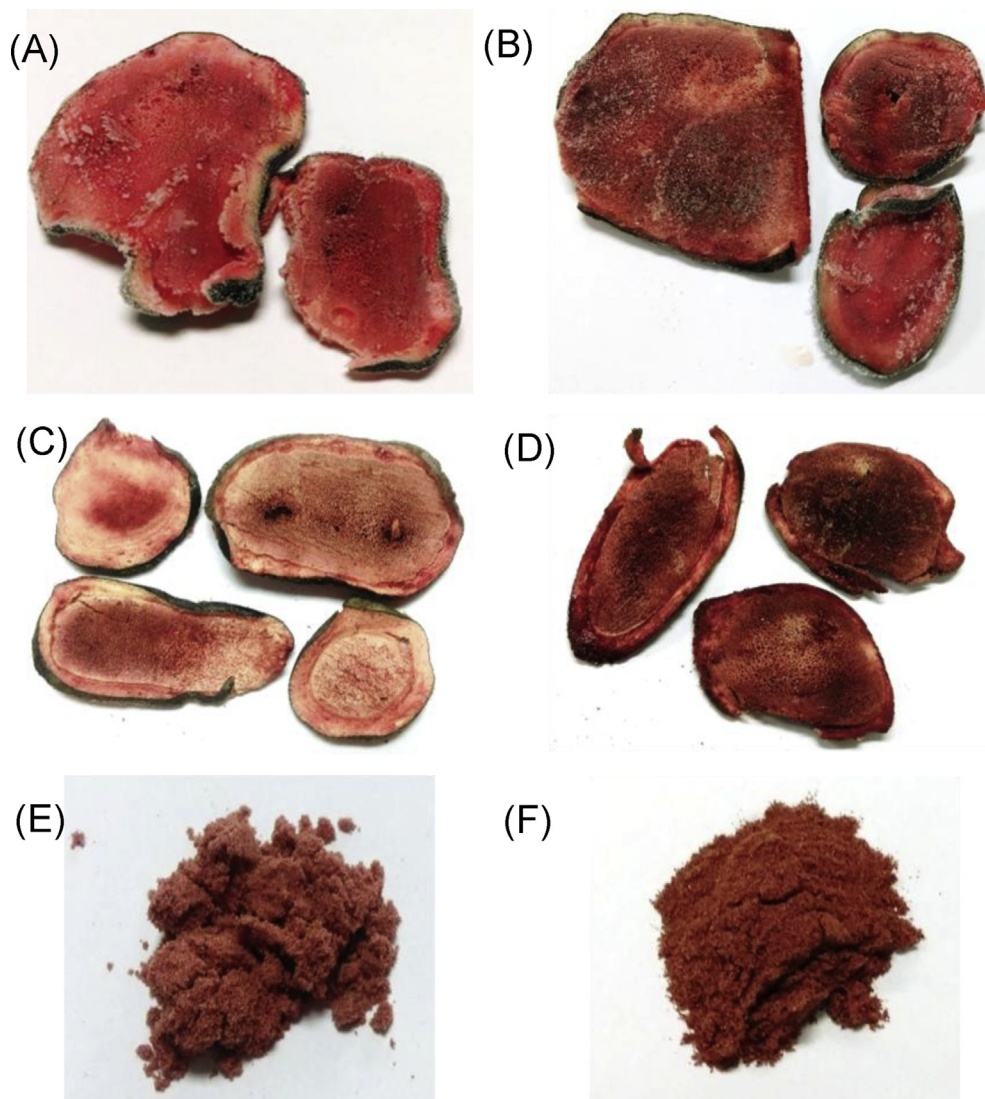
Fig. 1 — The appearances of fresh velvet antler slides from Formosan sambar deer (A) and red deer (B); lyophilized velvet antler slides from Formosan sambar deer (C) and red deer (D); milled powder of velvet antler from Formosan sambar deer (E) and red deer (F).

3.2.   Anti-inflammatory activity of velvet antler extracts from Formosan sambar deer and red deer

To compare the anti-inflammatory effect of the different VA extracts, we determined the pro-inflammatory cytokine production using ELISA assay. The TNF-α production in LPS-

Table 1 — Chemical composition of fresh velvet antler from Formosan sambar deer and red deer.

Species	Crude protein (%)	Ash (%)	Moisture (%)	Crude fat (%)
Formosan sambar deer	18.3 ± 0.2	17.6 ± 0.7	62.8 ± 1.1	1.5 ± 0.2
Red deer	$20.5 \pm 0.5^*$	$11.0 \pm 0.8^*$	$67.2 \pm 0.6^*$	$0.9 \pm 0.1^*$

Values are Mean ± SD (n = 3). * significantly different (P < 0.05) as compared to Formosan sambar deer.

stimulated RAW 264.7 cells was 335.0 pg mL^{-1} (Fig. 2A). After treatment with 200 and 500 µg mL^{-1} of SVA and RVA extracts (SVAE and RVAE), the TNF-α production of LPS-stimulated RAW 264.7 cells were significantly down-regulated. The SVAE demonstrated stronger inhibitory effect (93.4 pg mL^{-1}) than that of RVAE (155.8 pg mL^{-1}) at 500 µg mL^{-1} treatment on TNF-α production.

For IL-6 production in LPS-stimulated RAW 264.7 cells (Fig. 2B), the SAVE samples showed a stronger inhibiting ability at the levels of 200 and 500 µg mL^{-1} when compared with the LPS group. Only RVAE with 500 µg mL^{-1} significantly inhibited IL-6 production. SVAE also showed a better inhibitory effect than that of RAVE at the concentration of 200 µg mL^{-1}.

To further confirm the anti-inflammatory effect of SVAE and RVAE, we also determined the production of anti-inflammatory cytokine IL-10 on RAW 264.7 cells (Fig. 2C). It was shown that LPS-stimulated RAW 264.7 cells co-treated with SVAE enhanced the level of IL-10 production.
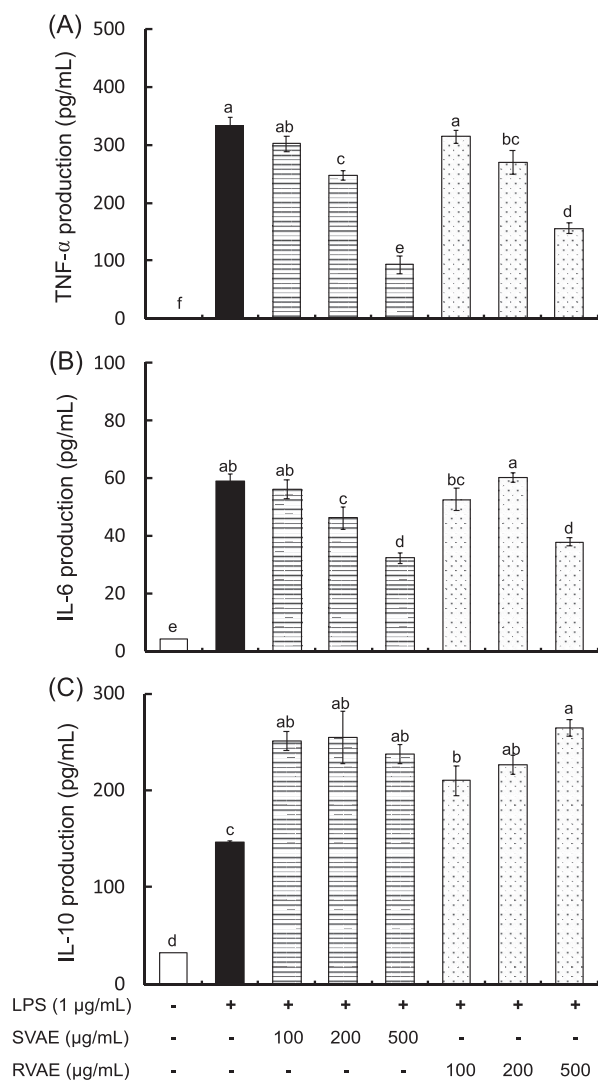
Fig. 2 – Effects of velvet antler extracts from Formosan sambar deer (SVAE) and red deer (RVAE) on cytokines including (A)TNF-α; (B) IL-6, and (C) IL-10 production in LPS-stimulated RAW 264.7 cells. Bars with different capital letters indicate significant differences at P < 0.05.
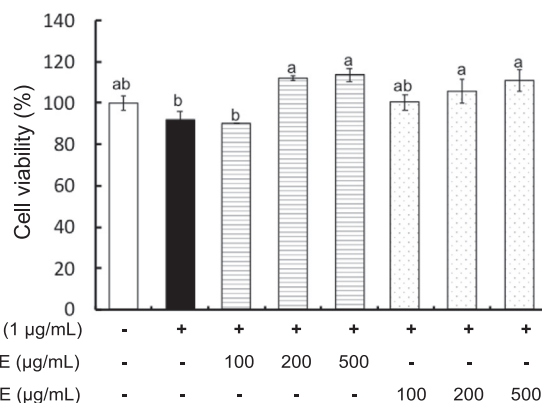

Fig. 3 – Effects of SVAE and RVAE on cell viability of LPS-stimulated RAW 264.7. Cell viabilities were measured using the MTT method after treated with various concentrations of SVAE and RVAE. Values represented as means ± S.E.M. (n = 3). Bars with different capital letters indicate significant differences at P < 0.05.

3.4. Comparative analysis of proteomes of velvet antler extracts from Formosan sambar deer and red deer by 2D SDS-PAGE

To further distinguish the difference of bioactive protein components between SVAE and RVAE, a 2D SDS-PAGE electrophoresis (2DE) was used to visualize the proteins profile of these two VAE. The analysis of the protein profile indicated that approximately 50 small molecular weight protein (<65 kDa) spots existed in each gel (Fig. 4). Among these spots, 6 spots demonstrated significant differences in the protein expression between SVAE and RVAE, and 2 specific spots only appeared in one sample and absented in another group. The spots were picked and then identified by LC/MS/MS (Table 2). The fold change of cellular retinoic acid-binding protein 1 (CRABP 1, spot 1 and 9), apolipoprotein A-I (APOA-I, spot 2,3, and 10, 11), collagen alpha-1(I) chain (spot 4 and 12), serum albumin (spot 5 and 13), and vimentin (spot 6 and 14) in SVAE comparing with RVAE were 1.95, 1.98, 2.89, 1.40, 0.92, and 4.25, respectively. The 2 specific proteins identified from SVAE were hemoglobin subunit beta-3 (spot 7), and creatine kinase B-type (spot 8). The percentage of these two proteins in total protein contents were 1.39, and 2.38%, respectively. Peroxiredoxin-2 (spot 15) was the specific proteins that found in RVAE, the content of peroxiredoxin-2 in total protein contents was 5.20%.

3.5. Effects of storage conditions on anti-inflammatory activity of velvet antler extracts from Formosan sambar deer

Since SVAE demonstrated better anti-inflammatory effects in LPS-stimulated RAW 264.7 cells *in vitro*, we further investigated the anti-inflammatory activities of SVAE under various storage conditions including different storage temperatures (−20, 4, and 25 °C) and periods (0, 2, 4, and 6 months) using ELISA assay. The results indicated that (Table 3), under the same storage temperature, the inhibitory effects of SVAE on

3.3. Cytotoxicity of velvet antler extracts from Formosan sambar deer and red deer

To ensure that the inhibitory effect of SVAE and RVAE on LPS-stimulated RAW 264.7 cells was not due to their cytotoxic effects, the cytotoxicity of various concentrations of SVAE and RVAE were also assessed using MTT assay in this study. As shown in Fig. 3, after 24 h treatment, the cell viabilities of the treatment groups were higher than 90%, indicating that SVAE and RVAE had no significant cytotoxic effects on RAW 264.7 cells. Thus, we can exclude the possibility of cytotoxic effect that affected the anti-inflammatory activities of SVAE and RVAE on RAW 264.7 cells.
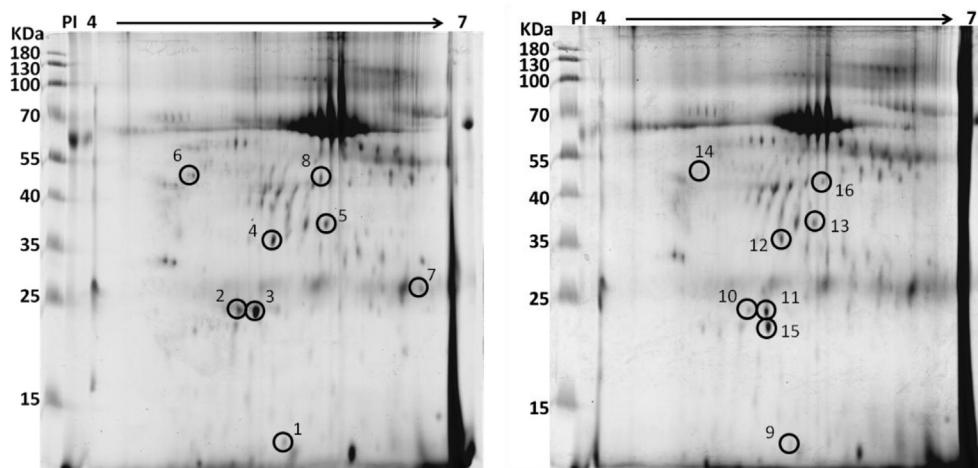
Fig. 4 — Protein profile of (A) SVAE and (B) RVAE. The major protein components of SVAE and RVAE were determined using two-dimensional electrophoresis, the first dimension: IEF with pH 4–7; second dimension: 12.5% SDS-PAGE.

Table 2 — Identification of specific protein components in SVAE and RVAE.

Spot no.	Protein identification	M.W.[a]	pI	%Vol in SVAE[b]	%Vol in RVAE
Sharing spots					
1, 9	Cellular retinoic acid-binding protein I	15.753	5.30	1.607	0.822
2, 10	Apolipoprotein A-I	30.258	5.61	4.889	2.469
3, 11	Apolipoprotein A-I	30.258	5.71	12.348	4.276
4, 12	Collagen alpha-1 chain	139.88	5.60	4.618	3.295
5, 13	Serum albumin	70.556	5.52	2.361	2.563
6, 14	Vimentin	53.752	5.06	1.322	0.311
Specific spots					
7	Hemoglobin subunit beta-3	15.871	6.64	1.392	—
8	Creatine kinase B-type	42.977	5.47	2.383	—
15	Peroxiredoxin-2	22.217	5.37	—	5.197

[a] M.W.: molecular weight (kDa).
[b] %Vol: % of total protein content.

Table 3 — Effects of storage conditions on anti-inflammatory activity of velvet antler extracts from Formosan sambar deer and red deer.

Storage conditions		Pro-inflammatory production (pg/mL)	
°C	Period (Month)	IL-6	
		200 μg/mL SVAE	500 μg/mL SVAE
−20	2	20.4 ± 1.8^d	22.1 ± 0.2^d
	4	20.9 ± 0.4^d	17.9 ± 0.3^d
	6	34.1 ± 0.0^a	19.82 ± 0.8^d
4	2	20.4 ± 0.8^d	18.9 ± 0.0^d
	4	28.0 ± 0.0^{ab}	22.1 ± 0.3^d
	6	31.8 ± 0.4^{ab}	21.9 ± 0.2^d
25	2	22.1 ± 0.1^d	17.1 ± 0.1^d
	4	22.9 ± 0.9^{cd}	24.7 ± 0.1^{bcd}
	6	30.5 ± 0.6^{abc}	21.2 ± 1.7^d
Interaction effect (P value)			
Temperature		0.0863	0.2969
Period		<0.0001	0.0146

Values (Mean ± SD, n = 3) with different letters are significant differences at P < 0.05.

the IL-6 production decreased as their storage period extended at the levels of 200 μg mL^{-1}, the statistical analysis also showed a significant effect of storage period on the anti-inflammatory effect of SVAE (P < 0.05). However, the storage temperatures did not influence the inhibitory effects of SVAE even during different storage periods (P > 0.05).

4.   Discussion

Although velvet antler has been used in many countries for very long time, only few studies focused on the pharmaceutical effects of Formosan sambar deer. The present study evaluated that the difference of appearance, anti-inflammatory effect and protein profile between velvet antlers extract from Formosan sambar deer and red deer. The appearance and composition differences between slides of SVA and RVA might be partly due to the harvested method and growing period of the velvet antlers. Cheng et al. [18] indicated that the hardness of VA sections increased, and that the color of these samples tended to become reddish-yellow due to the Ca content in the base section without

ossifying. Jeon et al. [19] also indicated that growth periods of antlers affected the chemical compositions of VA. The velvet antler from Formosan sambar deer was harvested at 70—75 days with removing the blood for other usage, resulting in the pale color appearance, while RVA was harvested at 60—65 days without removing blood, and the color of RVA was reddish.

We further investigated the anti-inflammatory effects of both samples. Results showed that both SVAE and RVAE reduced the TNF-α and IL-6 and increased IL-10 in LPS-stimulated RAW 264.7 macrophages. LPS, the Gram-negative bacteria product, could initiate inflammatory response through activating Toll-like receptor 4 (TLR-4) pathway, and then trigger transcription of nuclear factor κB (NF-κB), leading to massive release of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 [20]. IL-10 can induce the soluble inhibitors such as sTNFR and IL-1Ra secretion for mitigating the inflammatory actions of IL-1 and TNF cytokines [21,22]. Inhibiting production of TNF-αand IL-6 and stimulating IL-10 production indicated that both SVAE and RVAE possess anti-inflammatory activity. Extracts of velvet antler have shown to have anti-inflammatory effect including anti-allergic effect on ovalbumin-sensitized mouse model [6] and prevention of the development of collagen-induced arthritis [23] with reduction in the expression of inflammatory cytokines.

It is worth noting that SVAE, the VA extracts from Taiwan indigenous deer species, seems to demonstrate a better anti-inflammatory activity as compared with the RVAE *in vitro*. Velvet antlers are rich in amino acids, polypeptides and proteins, which are considered as the most prominent bioactive components. Thus, we investigated the bioactive components of SVAE and RVAE using 2DE. Among the protein components identified by LC-MS, the level of APOA-I in SVAE showed a 2—4 time higher than RAVE. APOA-I, the principal protein fraction of high-density lipoprotein (HDL), possessed anti-inflammatory ability on human monocytes, macrophages, adipocytes, and endothelial [24,25] with regulating the activation of human monocytes by inhibiting expression of CD11b through ATP-binding cassette transporter A1 (ABCA1) pathway [24]. Additionally, several anti-inflammatory peptides from simulated gastrointestinal digest (pepsin-pancreatin hydrolysate) of velvet antler protein components were identified, including VH (Val—His), LAN (Leu-Ala-Asn), AL (Ala—Leu), and IA (Ile-Ala) [26]. Other peptide, nVAP32 (3.2 kDa) isolated from velvet antler of *Cervus nippon* Temminck, also exhibited immunomodulatory potential in an *in vitro* mice cell model [27]. However, we did not identify these peptides might due to a lack of *in vitro* digestion process and the differences in extraction methods and deer species.

Finally, we evaluated the effects of different storage conditions on anti-inflammatory effect, and found that the storage period was the main influence factor in the storage test ($P < 0.05$), suggesting that some bioactive components of SVAE might be degraded by increasing storage time. The major bioactive components in water extract of velvet antlers are amino acids, polypeptides, proteins and polysaccharides. According to previous study, the results also revealed that the quality changes occurring in different food protein and their hydrolysates during storage, and the resulting changes in the structure and texture [28].

5. Conclusion

In this study, we demonstrated that SVAE possesses better anti-inflammatory effects than RVAE. The protein profile indicated higher APOA-I level in SAVE supports the finding. To the best of our knowledge, no previous study compares the effects of SVAE and RVAE on the anti-inflammatory activity, specific active components, and evaluating the influences of storage conditions on anti-inflammatory activity of velvet antler extracts from Formosan sambar deer. The future research will focus on the exactly mechanism and *in vivo* anti-inflammatory effect of SVAE.

Author contributions

C.-Y. Kuo, M.-J. Chen, and Y.-T. Cheng designed the experiments. C.-Y. Kuo, Y.-T. Cheng, and C.-C. Yu performed the experiments. Y.-T. Cheng and S.-T. Ho analyzed data. C.-Y. Kuo and M.-J. Chen supported reagents and kits. S.-T. Ho, Y.-T. Cheng, and M.-J. Chen wrote the manuscript. All of authors read and approved the final manuscript.

Conflict of interests

The authors have no conflict of interests.

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