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Research Article

Extraction and Preformulation Study of Deer Antler Velvet Extract: Physical Characterization of Aqueous and Ethanol Extract

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ABSTRACT

Objective: The aim of the research was to extract the deer antler velvet from Kalimantan Indonesia and to study physical characteristics between 70% ethanol and aqueous extract. Materials and methods: Ethanol extracts was extracted from deer antler velvet using maceration and modified maceration method. Ethanol extracts were compared to aqueous extract which produced using maceration technique. The extract profiles were determined by screening test and physicochemical properties as preformulation study were characterized using Thin Layer Chromatography (TLC), Nuclear Magnetic Resonance (NMR), X-ray diffractometer, differential thermal analysis (DTA), solubility test, BCA protein content, and molecular weight using SDS PAGE assay. Results: Extracts were successfully prepared and determined. Physicochemical properties of 70% ethanol extract and aqueous extract resulted different characteristics in melting point, solubility, crystallinity and protein content. Both ethanol extract and aqueous deer antler velvet extract contained group compounds of terpenoids and steroids and contains high amount of proteins at molecular weight of 17 to 43 kDa. In terms of crystallinity, ethanol and aqueous extracts had different crystal lattices. Conclusion: The aqueous extract of deer antler velvet was then recommended for further in vitro drug formulation and characterization. The molecular weight of majority protein inside aqueous deer antler velvet extract was 17 kDa and this datas will be useful for further drug formulation.

Keywords: deer antler velvet extract, preformulation study, diffraction, thermal analysis, protein.

INTRODUCTION

Development of medicines derived from animals has been still very limited. Indonesia has Deer Antler as one of Indonesia's natural medicinal agents which has mineral content, amino acids such as aspartic acid, glutamic acid, glycine and arginine of 32.5-37.2% of total acids, polypeptides, proteins, polysaccharides, fatty acids and phospholipid which has been proven as a bioactive component, and may has the potential to provide some pharmacological effects. Deer antlers velvet extracts have been claimed to have numerous medical benefits. In China, it was claimed to replenish the vital essence, strengthened bones, promoted virility, nourished the blood, and promoted both male and female sexual functions[1].

Although the science behind these claims remained lacking, deer antler velvet-related products showed potential positive effects on modern ailments such as those associated with aging, infection, and immune dysfunction. However, the mechanisms of actions and the bioactive compounds responsible were mostly not clear[2,3].

Compounds of active chemical agents of deer antler velvet revealed that there are local differences in chemical composition which usually contents of proteins and lipids decrease downward from the tip to the base, while those of ash, calcium, and collagen increased[4]. Normally, the market values of antlers are downgraded with increasing degree of calcification. However, which parts of deer antler velvet are suitable for preventing and managing disease had not been clarified. In addition, extraction process of animal source or plants produced different yield of production. Some extraction methods have been widely used such as maceration or reflux technique[5]. Therefore in this study, we extracted and evaluated the physical characteristics of deer antler velvet from Kalimantan Indonesia as preformulation study to be completed information for further drug formulation.

MATERIAL AND METHODS

Material

Deer antler velvets were obtained from Kalimantan as

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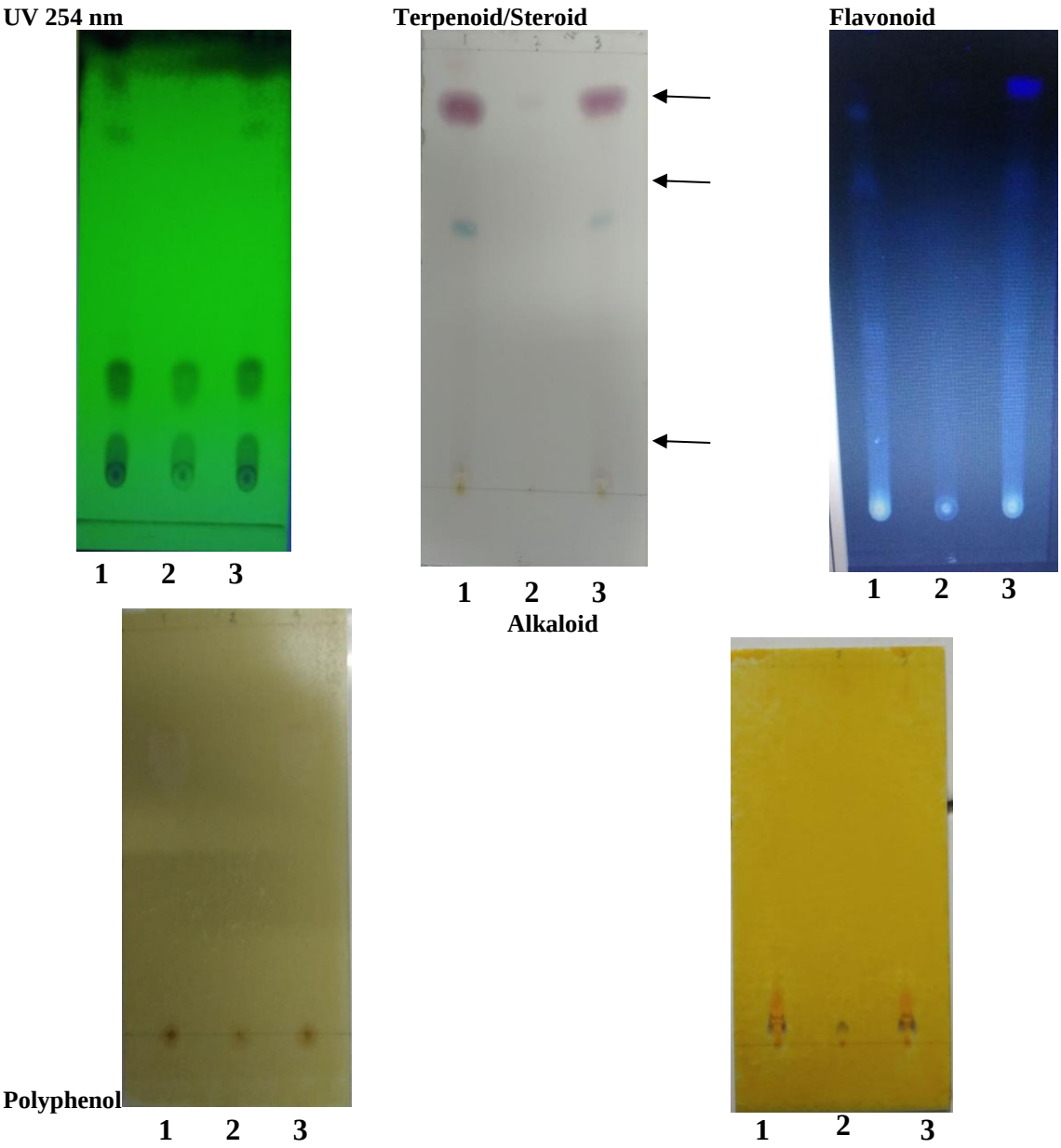


Figure 1: Profile Identification of Flavonoid, Terpenoids, Steroid, Polyphenol and Alkaloid Extracts (1) ethanol extract using macerated method (2) aqueous extract (3) ethanol extract using reflux/modified macerated method.

collaboration between Airlangga University and UPTD-Kalimantan Indonesia. Deer antler velvet was mashed to form powder and powder was extracted using maceration and reflux method. The 70% ethanol extract produced by maceration and reflux method were used then for this study compared with aqueous extract by modified maceration method.

Extraction Method

Conventional Maceration Method

This method was conducted only for extraction with 70% ethanol. A total of 991 grams of deer antler powder was added by 70% ethanol (2.4 Litre) and was soaked for 24 hours. The filtrate was then strained using a Buchner funnel. The residue was extracted again with 70% ethanol. The extraction process was repeated three times. The filtrate extracted was collected; the solvent was evaporated with a rotary evaporator followed by drying in an oven at 40 °C until extract was obtained.

Modified Maceration/Reflux Method

This method was conducted for gaining 70% ethanol extract and aqueous extract. Aquadest of 500 mL was heated at a hot plate temperature of 70 °C. 100 grams of deer antler velvet powder was added into the extractor and warm distilled water was added. This mixture was then soaked for about 5 minutes. The filtrate and residue was then separated. The obtained filtrate was heated at 70 °C for 5 minutes and was used again for extraction. This process was repeated six times. The extraction cycle used was 6 x 10 minutes. The extraction results were then dried by freeze drying. For drying the ethanol extract, it was firstly carried out with a rotary evaporator to evaporate ethanol, then continued after drying, the water was left with freeze drying.

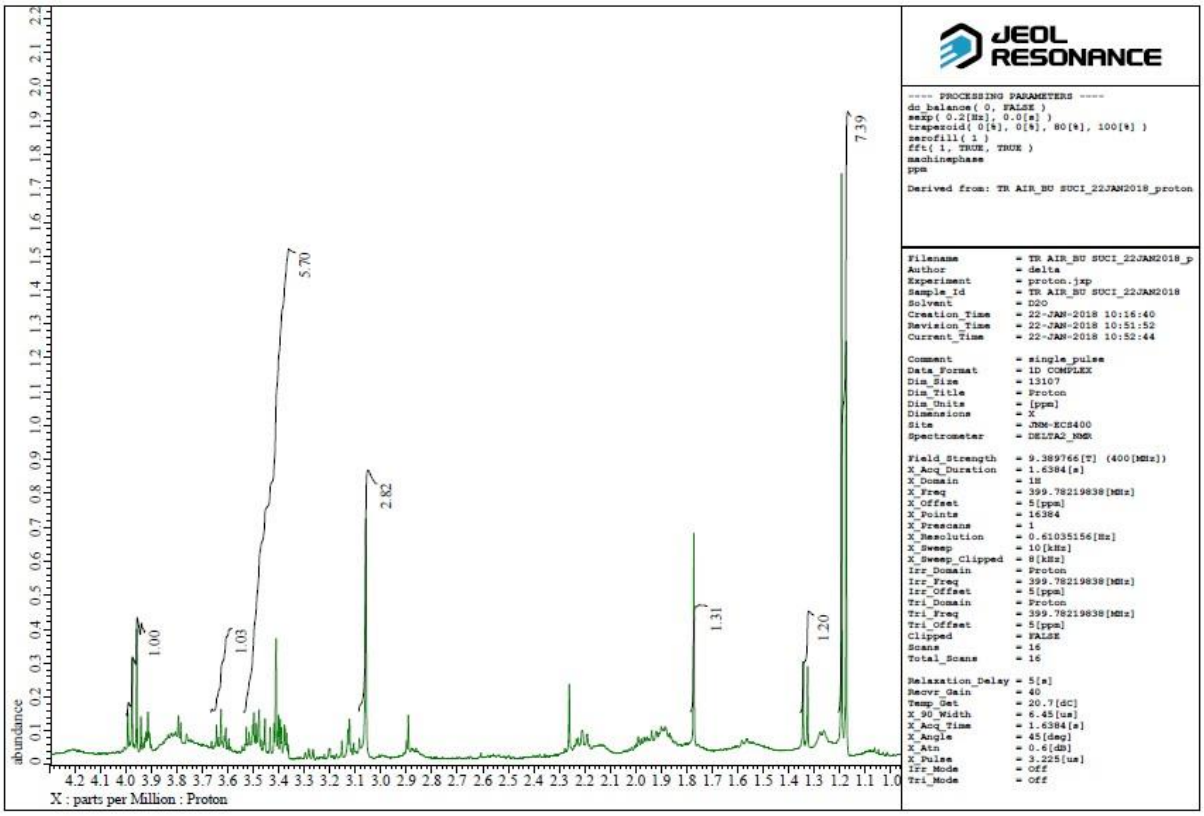


Figure 2: ^{1}H NMR from aqueous extract.

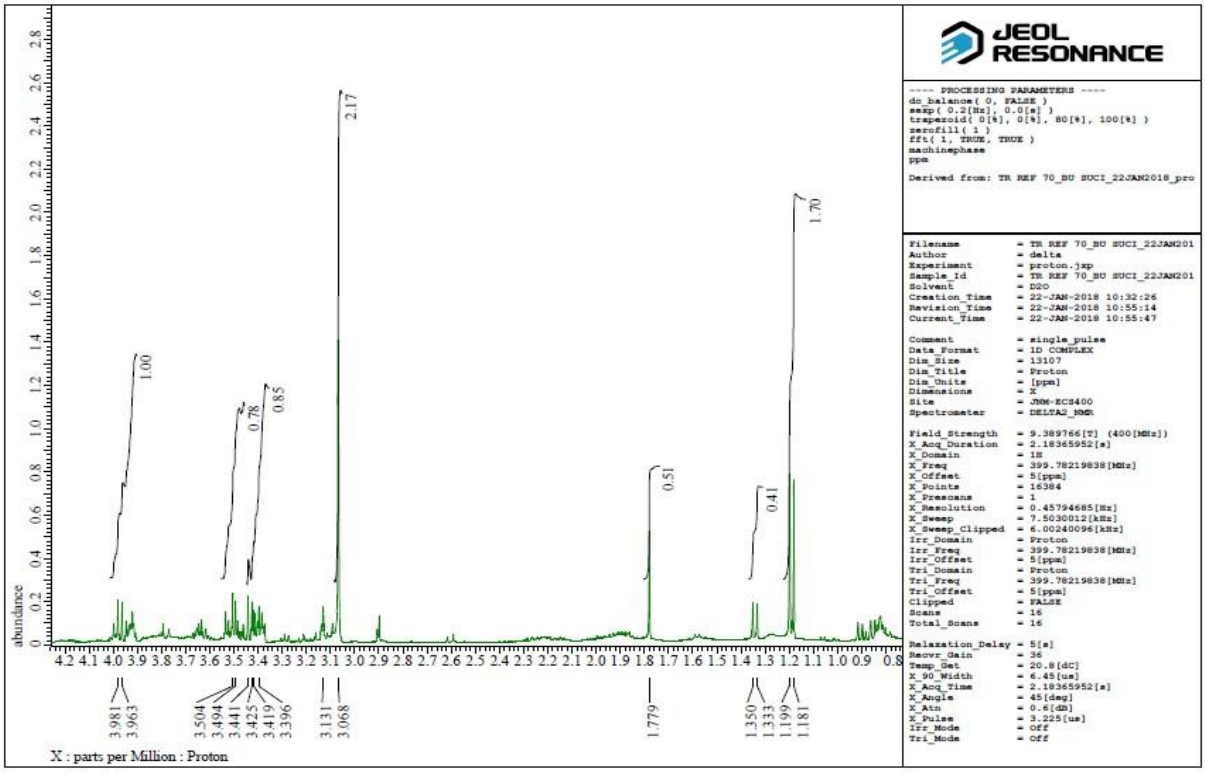


Figure 3: ^{1}H NMR from ethanol extract using modified maceration method

Screening of compound groups of deer antler velvet extract profiles

Screening the profile of the compounds group was carried out by thin layer chromatography (TLC) and was visualized using UV and reagent for staining.

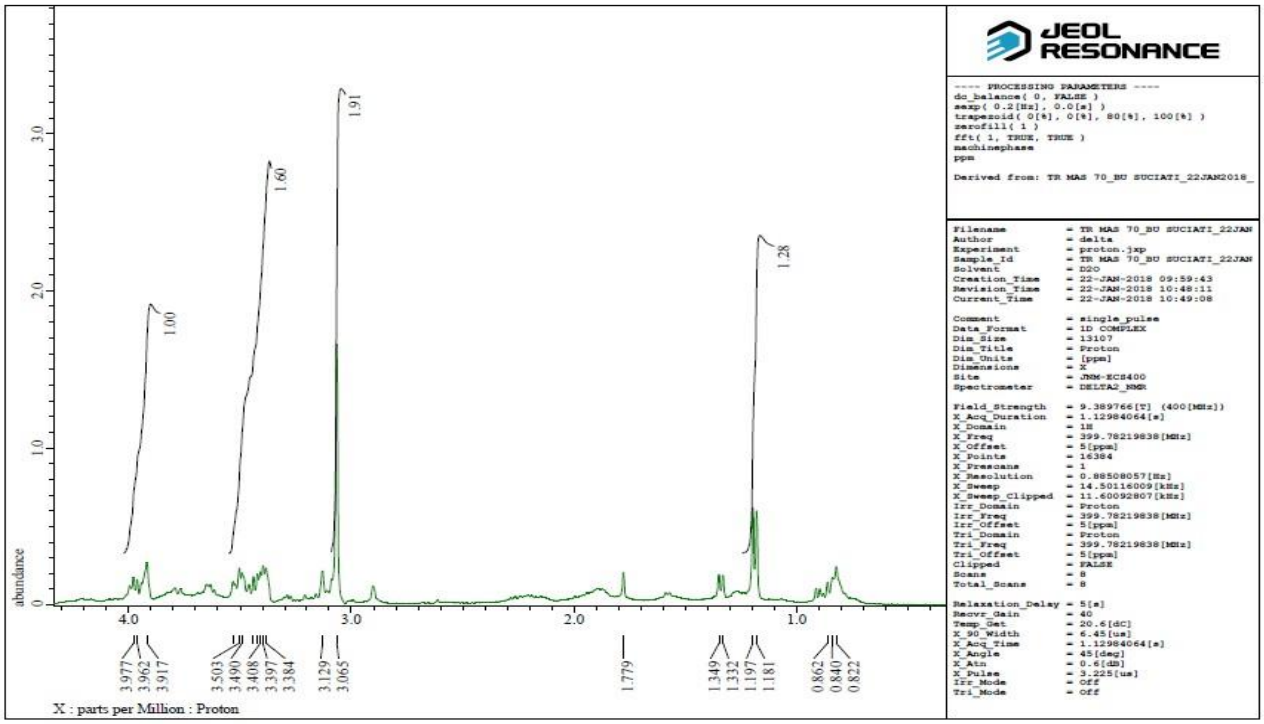
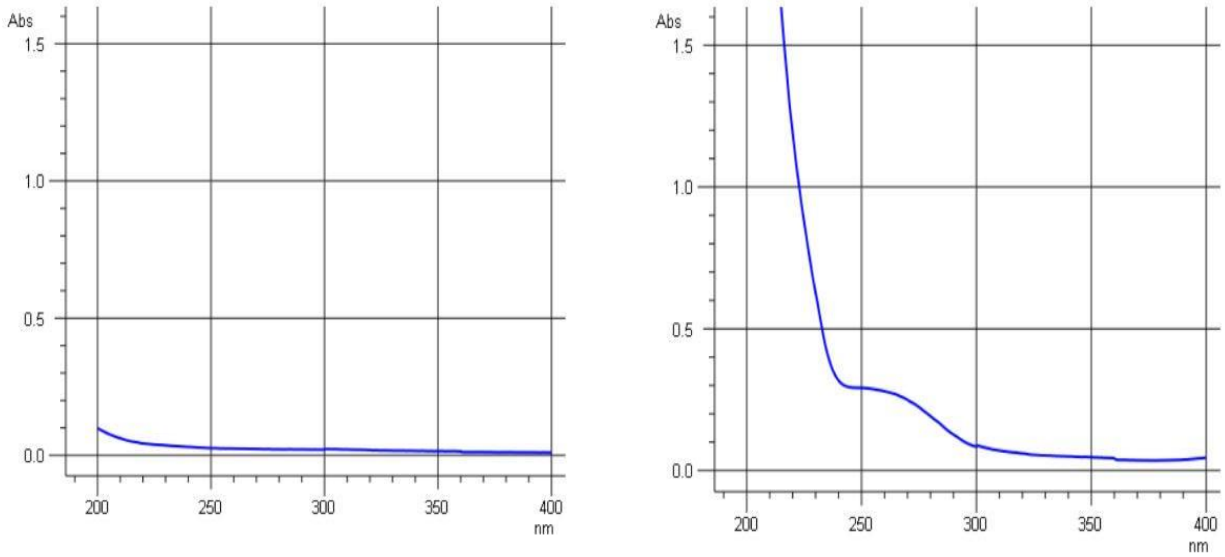


Figure 4. ^{1}H NMR from ethanol extract using maceration method.



a b

Figure 5: The solubility profile of aqueous extract at concentration of 10 ppm (A) and solubility profile of aqueous extract of deer antler at 100 times dilution (B).

Identification of terpenoids/steroids

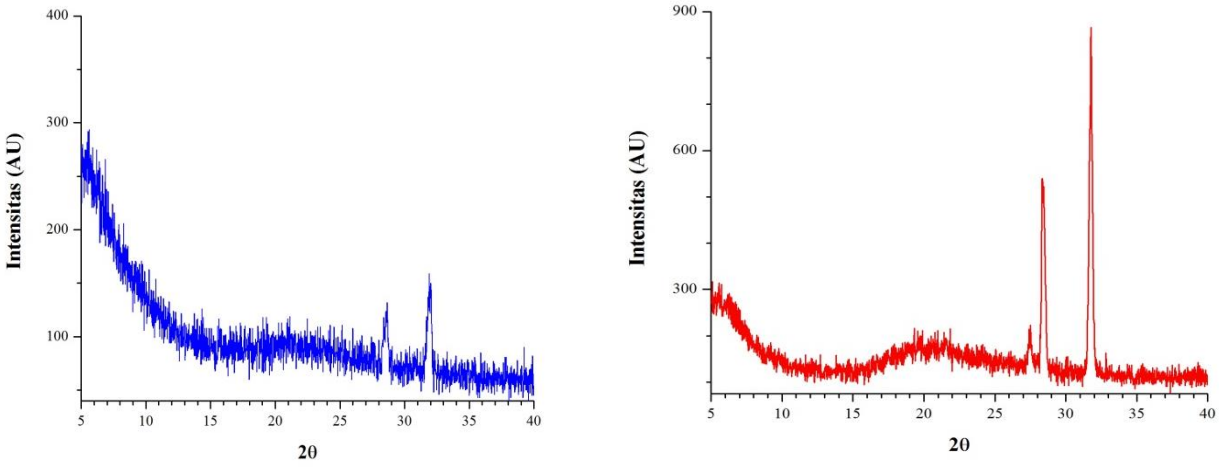
This thin layer chromatography (TLC) test used Stationary phase of Kiesel gel GF 254 and mobile phase of mixture of Dicloroethane: Ethyl acetate: methanol (8: 1: 1). Stain reagent used Anisaldehyde sulfuric acid. The presence of terpenoids/steroids was indicated by the occurrence of purple or blue red.

Identification of Flavonoids

This TLC test used stationary phase of Kiesel gel GF 254, mobile phase of mixture of Dicloroethane: Ethyl acetate: methanol (8: 1: 1). Stain reagent used Borate citrate. The presence of flavonoids was indicated by the occurrence of intensive yellow after the TLC plate was heated and was visualized with UV 366 nm.

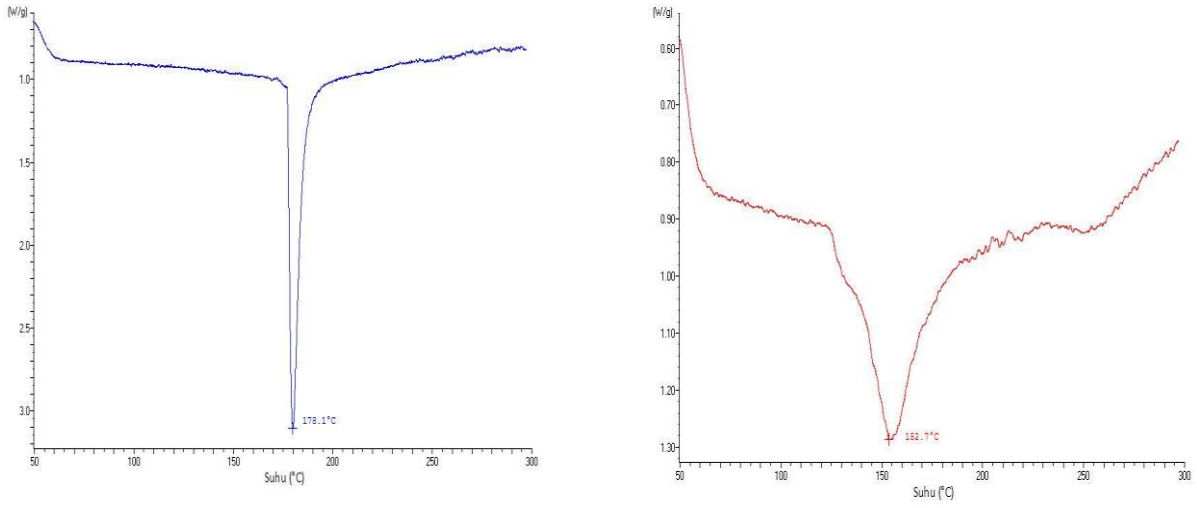
Identification of Polyphenols

This TLC test used stationary phase of Kiesel gel GF 254, mobile phase of mixture of Dicloroethane: Ethyl acetate: methanol (8: 1: 1). Stain reagent used FeCl3. The presence of flavonoids was indicated by the occurrence of black stains.



A B

Figure 6: Diffractograms of aqueous extract (A) and 70% ethanol extract (B) of deer antler velvet.



A B

Figure 7: Thermogram of aqueous extract (A) and 70% ethanol extract (B) of deer antler velvet

Identification of Alkaloids

This TLC test used stationary phase of Kiesel gel GF 254, mobile phase of mixture of Dicloroethane: Ethyl acetate: methanol (8: 1: 1). Stain reagent used Dragendorf. The presence of flavonoids was indicated by the occurrence of orange stains.

NMR Identification

The sample was dissolved in D2O solvent. Measurement of 1H NMR samples was carried out on JEOL 400 MHz instrument with 128 scan counts, reference solvent at δH at 4.79 ppm.

Characterization with Powder X-ray Diffraction (PXRD)

PXRD analysis was conducted at room temperature (25 °C) using diffractometer (Philips X`Pert, PABalytical, Almelo Almelo, Netherlands). Measurement was then performed under certain conditions, involving Cu metal target, Kαfilter, voltage 40 kV, and 40 mA electrical current. Analysis was performed in the range of 5-50°. The powder X-ray diffractometer was used to obtain the diffraction pattern of the sample with the following settings: CuKα radiation source (1.54 Å), at 40 kV and 30 mA, and the sample read at a speed of 0.017 º/sec in the range 2θ 5-40º. The diffractogram obtained was analyzed using Origin Pro7 software.

Characterization with Differential Thermal Analyzer (DTA)

Thermal analysis of the samples was performed by DTA (Mettler Toledo FP85 TA Cell, Polaris Parkway Columbus, USA) with a calibrated temperature using indium. A volume of 3.0-7.0 mg of the samples was put on a covered aluminium pan. DTA was then performed in the temperature range from 50 to 300°C with a heating rate of 10°C per minute. Thermal phenomena that occur are observed through the thermogram profile produced.

Determination of saturated solubility

The extract powder was added to 5 mL of the solvent and was confirmed to be saturated (the extract could not dissolve again and indicated by the presence of precipitate). The extract solution was then filtered using filter paper, then the absorbance was read on UV-Vis spectrophotometry with 100 times dilution. The absorbance that was read was compared to the absorbance of the extract which was known to be at 10 ppm.

BCA (Bicinchoninic Acid) protein assay

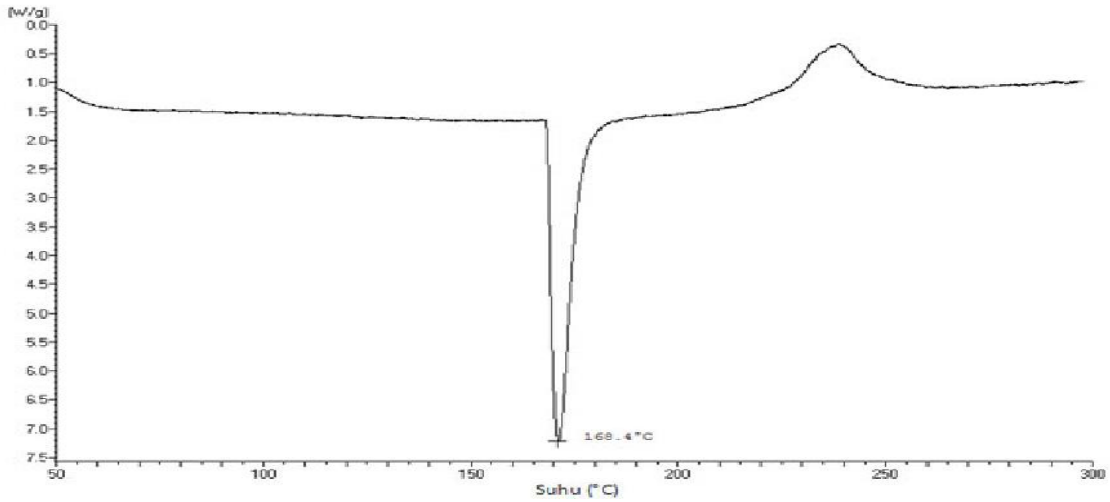


Figure 8: Thermogram of Chondroitin sulphate standard.

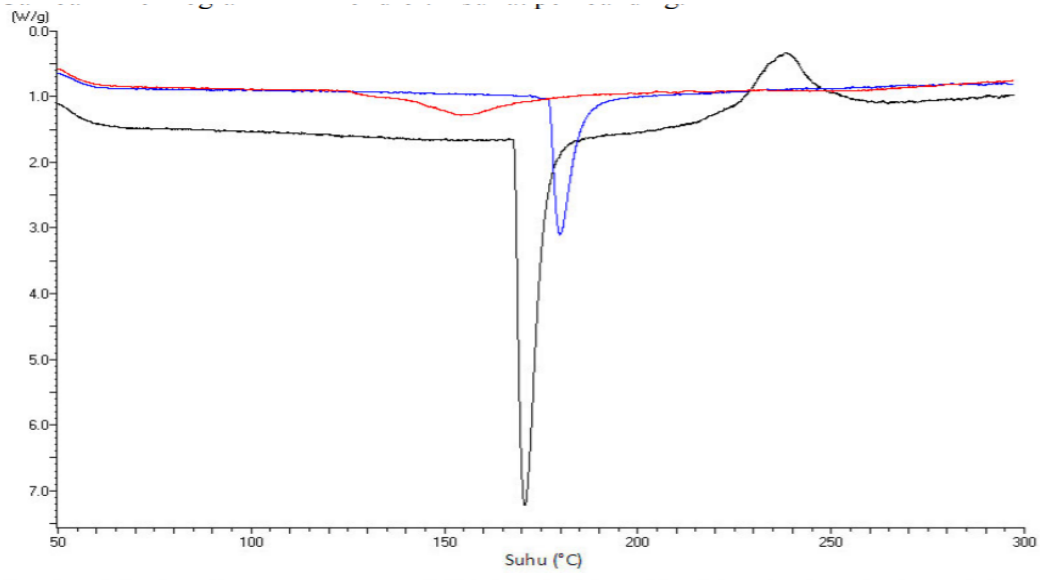


Figure 9: Thermogram of aqueous extracts of deer antlers (blue), ethanol extract of deer antlers (red) compared with chondroitin sulphate standard (black).

This assay was done to determine the presence of a protein in a sample. Albumin standard solution (BSA) is made with several concentrations such as table 3.1. Also prepared WR (working reagent) by mixing 50 parts of Reagent A with 1 part reagent B (50: 1, Reagent A: B). A total of 25 µl of standard (AI) and samples (100, 50 and 10 µg/ml of 70% maceration ethanol extract, modified 70% ethanol extract and deer horn water extract) were incubated with 200 µl WR for 30 minutes at 96 well plates, 37ºC. Then the absorbance is measured at 562 nm using an ELISA reader.

Identification of protein molecular weight

The molecular weight of the protein contained in the test sample can be determined using a protein assay kit (Bio-Rad) by comparing the results with the BSA standard. A total of 10 mg/ml of 70% ethanol extract maceration (M), modified 70% ethanol extract (Et) and water extract (A) from deer antlers were suspended with a buffer. Then it was heated in a water bath, transferred to the sample tube in an SDS-Phage vessel and flushed for 1 hour. The tape obtained was compared with the standard band to determine the magnitude of the molecular weight of the test sample.

RESULTS AND DISCUSSION

Extraction of deer antler velvet

Yield percentage of ethanol extracts by maceration method was 3.5%, reflux/modified maceration method was 1.4% and aqueous velvet extract was 0.8%. Considering the high yield of conventional maceration method for ethanol extract, it was recommended to use the maceration method for further production.

Screening of groups compound of deer antler velvet extract profiles

Figure 1 showed screening result by TLC in terms of compounds screening of terpenoids, flavonoid, polyphenol and alkaloid of ethanol and aqueous extracts.

Based on the TLC results, ethanol extract of deer antlers velvet contained terpenoids or steroids, while the identification of flavonoid and polyphenol compounds

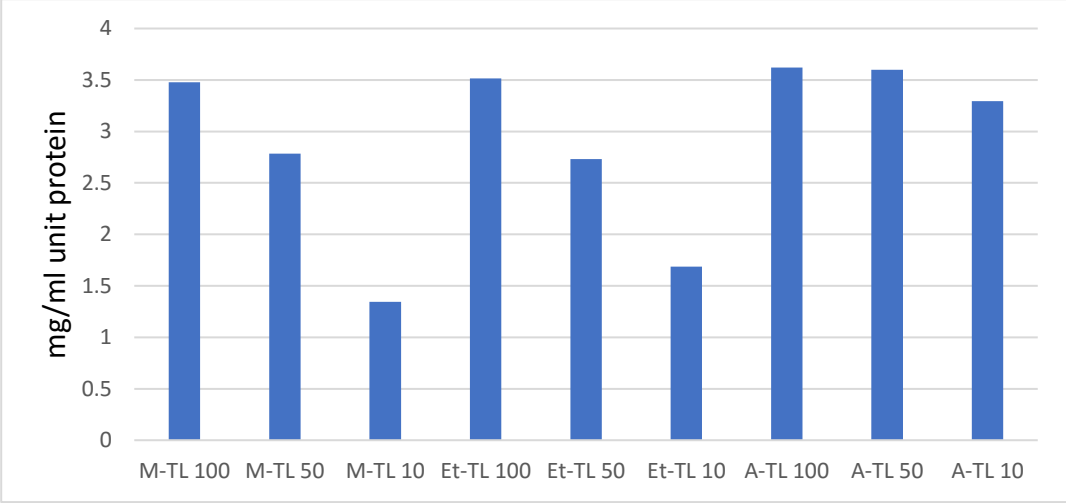


Figure 10: Protein content of deer antler velvet extracts of ethanol extract using maceration method (Et), ethanol extract using modified maceration method (M), and aqueous extract (A).

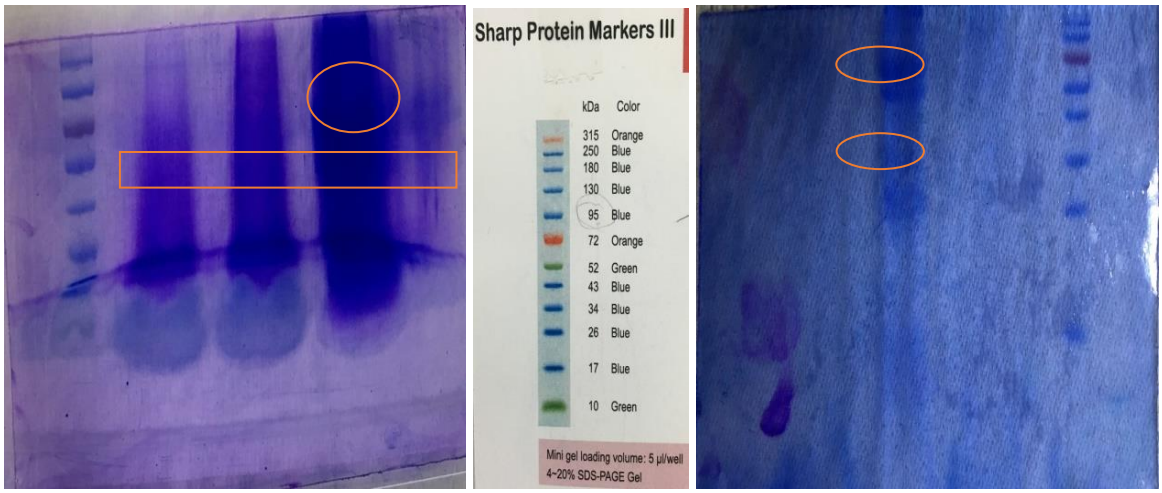


Figure 11: SDS-PAGE assay of protein content inside deer antlers.

showed negative results. The presence of terpenoids / steroids and alkaloids were further confirmed by using Nuclear Magnetic Resonance (NMR). For identification of alkaloids with Dragendorf stain showed that there were orange stains, but it cannot be ascertained that in deer antlers there were alkaloids because some compounds such as proteins can caused false positives, therefore it was necessary to rearrange test of firstly doing TLC of extract from samples which processed by the acid base method using Dragendorf with acid-base to confirm.

The 70% ethanol extract made by conventional maceration and modification methods showed the same TLC profiles. Both ethanol and aqueous extract contained terpenoids and steroid compounds.

Identification of Compounds using Nuclear Magnetic Resonance (NMR)

^{1}H NMR all samples of both aqueous extract and ethanol extract showed signals on chemical shifts of 1.0 - 4.0 ppm which confirmed the presence of terpenoids compounds. Comparison with ^{1}H NMR data from chondroitin sulphate standard showed no certainty of the presence of these compounds in the sample. This was because the peak that was formed was too broad and there were several signals on the overlapping samples.

Measurement of Compounds using Thin Layer Chromatography-Densitometry (TLC-Densitometry)

Based on quantitative measurement of chondroitin sulphate content and glucosamine content inside the deer antler velvet extract which was predicted as the main compounds in the deer antler extract, it was found using TLC-densitometry that glucosamine content in aqueous extract was 1.45% and about 1.0% in the ethanol extracts, whereas the chondroitin sulphate content had not confirmed yet in NMR as well as in densitometry (unpublished data).

Solubility test

Solubility test of deer antler extract was tested in aqueous solvent and was read the absorbance using a UV-Vis spectrophotometer. Determination of saturation solubility of aqueous extract in water cannot be determined because the extract solution with a level of 10 ppm did not show any absorption, and the test solution with 100 times dilution also did not provide maximum peaks in the wavelength range 200-400 nm (Figure 5).

The 10 ppm concentration of aqueous extract did not provide absorption in the wavelength range of 200-400 nm. However, this aqueous extract was soluble in water because it required a sufficient amount of sample to saturate 5 mL of water and was needed to be diluted a hundred times to produce a good absorbance value. The results of UV-Vis absorbance however were not obtained at maximum wavelength. Therefore it caused the solubility of deer antler velvet extract cannot be determined.

Analysis using X-Ray Diffraction

The results of examination of powder X-ray diffraction patterns of deer antler extract both extracted with water and 70% ethanol showed successive diffraction peaks at 2 theta 28,62; 32.05 and 27.53; 28,32; 31.77°. The diffraction intensity of deer aqueous extract was very low, even lower than the noise that appeared at the initial angles (Figure 6). When compared with the diffraction intensity of 70% crystalline ethanol extract of deer, it can be seen that crystallinity profile of aqueous extract was seem to be more amorphous. From the value of 2 theta, both extracts had peaks at different angles, indicated that the aqueous extract and 70% ethanol extract had different crystal lattices. It has been demonstrated that the formation of stable crystals can improve several properties of active agents such as flow properties and stability[6].

Thermal Analysis using DTA

Thermal analysis was conducted at a temperature range of 50-350°C with a heating rate of 10°C/min to evaluate both extracts compared to the main compound of deer antler velvet extract which were glucosamine and chondroitin sulphate.

DTA of extracts

Thermal analysis graph using the DTA showed that aqueous extract and ethanol extract of deer antlers each gave a single endothermic peak, ie, 178.1 and 152.7 °C (Figure 7). Aqueous deer antler velvet extract produced sharp endothermic peaks which were generally indicated by pure single components (slightly impurities). Meanwhile, 70% ethanol extract produced a wide peak which indicated that the extract was in the form of a mixture of components/polymorphs. Both extracts provided different melting points, and were in harmony with the results of X-ray diffraction patterns, this indicated that the results of extracting deer antlers with different solvents produced different components.

DTA of extracts and chondroitin sulphate

The melting points of extracts of both water and ethanol extract had a melting point which was almost the same as compared to the standard melting point of chondroitin sulfate (Figure 8 and 9). Many applications of different thermal analysis techniques were used in pharmaceutical technology and characterization of dry extracts from medicinal plants[7,8].

BCA protein test

Figure 10 showed results of evaluated samples consisting of 100, 50 and 10 µg/ml of 70% maceration ethanol extract, modified 70% ethanol extract and aqueous extract of deer antler velvet showed that in deer velvet contained a number of proteins. Protein content was mostly found in aqueous extracts.

Identification of protein molecular weight

Protein content of the aqueous deer antler velvet extract from BCA assay findings was then further determined for their molecular weight of extract using SDS-PAGE analysis. Based on Figure 11, this showed that the molecular weight in aqueous extract at a concentration of 10 mg/ml was 17-43 kDa, while all extracts of deer antler velvets at higher concentration of 50 mg/ml were detected to have a confirmed molecular weight of 17 kDa. This confirmed of the molecular weight of major protein inside the extract of deer antler velvet. Chondroitin sulphate chains as major compounds in the deer antler velvet theoretically have moleular weight of 10-25 kDa[9].

From protein findings, this information could be useful to further investigate protein activity for example in treatment for heart diseases, including ischemic heart disease, heart failure, and arrhythmia[10,11].

CONCLUSION

Extracts of deer antler velvet from Kalimantan Indonesia were successfully prepared and determined. Characterization of ethanol and aqueous deer antler velvet extract showed different characteristics in compounds content, melting point, solubility, crystallinity and protein content. Yield percentage of ethanol extracts by maceration method was 3.5%, reflux/modified maceration method was 1.4% and aqueous velvet extract was 0.8%. Both ethanol and aqueous extract contained terpenoids and steroid compounds. In terms of crystallinity, ethanol and aqueous extracts had different crystal lattices. Both ethanol extract and aqueous deer antler velvet extract contained high amount of proteins at molecular weight of 17 to 43 kDa. The aqueous extract of deer antler velvet was then recommended for further in vitro drug formulation and characterization.

ACKNOWLEDGEMENTS

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