DETERMINATION OF PROTEIN CONTENT IN SELECTED PLANTS

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Determination of Protein Content in Selected Plants** by Nurin Binti Che Azmi, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 55204 – Research Project.

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DEDICATIONS

To my family and numerous friends, I dedicate my dissertation. A special sense of thanks to my devoted parents, who always provide me numerous words of support and unconditional love. My very beloved brothers Aiman and Faezal, who have never left my side.

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BSA	Bovine serum albumin
BCA	Bicinchoninic Acid

ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement on the course DVT 55204 – Research Project

Plant proteins have always been one of the top protein sources consumed by farm animals throughout the years. Determination of protein content in selected plant extract can help us in providing information regarding the amount of protein available in each plant. This is a pilot study in determination of protein content in selected plant hexane extract. The selected plants used in this study are *Alpinia galangal***,** *Curcuma* spp. and *Musa* spp. leaves. Bradford method for protein quantification was used to measure the level of protein. Based on the result obtained, *Curcuma* spp. has the highest concentration of protein at 19, 000 ng/mL. Then, followed by *A. galangal* at 17, 000 ng/mL and *Musa* spp. leaves has the lowest value at – 20, 000 ng/L. These plants protein content can help to provide us information regarding suitability of these plants as substitutes for other plant protein in animal's feed.

Keywords: Protein Content, Hexane Extract, Plants

ABSTRAK

Abstrak daripada kertas penyelidikan dikemukakan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan untuk memenuhi sebahagian daripada keperluan kursus DVT 55204 – Projek Penyelidikan.

Protein tumbuhan sering menjadi salah satu sumber protein utama yang digunakan dalam makanan haiwan ternakan sepanjang tahun. Penentuan kandungan protein dalam ekstrak tumbuhan terpilih dapat membantu kita dalam memberikan maklumat tentang jumlah protein yang terdapat dalam setiap tumbuhan. Ini adalah kajian rintis dalam penentuan kandungan protein dalam ekstrak heksena tumbuhan terpilih. Tumbuhan terpilih yang digunakan dalam kajian ini ialah Alpinia galangal, Curcuma spp. dan Musa spp. daun. Berdasarkan keputusan yang diperolehi, Curcuma spp. mempunyai kandungan protein tertinggi pada 19, 000 ng/mL. Kemudian, diikuti oleh lengkuas Alpinia pada 17, 000 ng/mL dan Musa spp. daun mempunyai nilai terendah iaitu – 20, 000 ng/mL. Keputusan ini diperolehi menggunakan kaedah Bradford untuk kuantifikasikan protein. Dengan mengetahui kandungan protein tumbuhan-tumbuhan ini, ia boleh membantu memberikan kita maklumat mengenai kesesuaian tumbuhan ini sebagai pengganti protein tumbuhan lain dalam makanan haiwan.

Kata kunci: Penentuan Protein, Ekstrak Heksena, Ekstrak Tumbuhan

1.0 INTRODUCTION

Plant proteins have always been incorporated into farm animal's feed to meet their daily protein requirement. However, not many plants can be used as a good source of protein due to their low protein level. Currently in the feed mill industry, corn and soy have always been the most common main source of plant protein. Despite that, the amount of feed protein supplies, sources, and alternatives aside from corn and soy will undoubtedly need to be increased in response to the significant rise in the demand for animal products globally. Thus, if we're able to diversify the feed protein source for the animal, there will be considerable potential for improving food security and supply by better protein feed provision to the livestock.

1.1 Research problem

Proteins are part of the important macronutrients for the growth and physiologic demand of farm animals. There are many various sources of protein for animal feeds, and there are several potentials for substitution and further diversification. Currently, within the animal feed mill industry, plant proteins sourced from soy and corn are very saturated and high in demand. Dependent on these two types of plant protein sources alone are not economical and can be vulnerable when there's scarcity as most corn and soy in Malaysia are imported. Therefore, alternatives for other plant protein sources are necessary. Despite that being said, before much of the potential can be utilized in practice, further study on alternate sources is necessary.

1.2 Research questions

1.2.1 What is the protein level of the selected plant with hexane extract?

1.3 Research hypothesis

1.3.1 Each plant hexane extract will have its own protein quantity value.

1.4 Objectives

1.4.1 To determine protein level in selected plant extract by using hexane as the solvent.

2.0 LITERATURE REVIEW

2.1 Importance of Diversifying the Protein Source of Farm Animal's Feed.

For their overall health and welfare, farm animals rely on the type and quality of the protein they consume. In addition to serving as a source of energy, protein in the diet is necessary for the activity of enzymes as well as the transport of biochemicals across cellular membranes (Wu, 2014).

Despite the importance of protein as part of the farm animal's diet, another sprawling issue has been circulating throughout the year which is in addition to the fact that human population growth, particularly in developing nations, is a significant boost of demand for animal products. At the same time, feed providers also have to deal with rising safety concerns such as incidents involving chemical contamination of feeds (such as dioxin) and the usage of genetically modified crops like soy and maize. A lot of focus is being placed on the sources of feed protein and its compatibility, quality, and safety for future supply due to the significant and growing demand for animal protein. Sources of protein for animal feeds are many and varied, with considerable opportunities for further diversification and substitutions. More research is required on alternative sources before many of the opportunities can be exploited in practice (Food and Agriculture Organization, 2016).

Numerous methods need to be developed in order to diversify the protein source for animal's feed such as the usage of crop by-products. For example, crop leftovers and other fibrous materials that are given to ruminants may support adequate production levels with the right management.

2.1.1 Common Plant Protein in Animal's Feed.

It's indeed obvious that when the demand for animal products develops significantly, feed protein supply, sources, and alternatives must be continuously evaluated. Investments in research and development into a number of highly potential new sources appear to be strongly justified. There will undoubtedly need to be significant increases in feed production, necessitating a vibrant, prosperous, and sophisticated animal feed market.

According to the Food and Agriculture Organization (2016) the most significant and preferred source of high quality vegetable protein for the production of animal feed has to be soybean. When combined with maize meal in the formulation of feed, soybean meal, a by-product of oil extraction, has a high crude protein level of 44 to 50% and a balanced amino acid composition. The next source of plant protein traditionally used in animal feed is beans. Legumes have a positive effect on farms and feed producers because they can provide grain with high amounts of crude protein but often low levels of lysine and amino acids containing sulphur.

2.1.2 *Musa* spp. leaves as cattle's feed

High-quality conventional feed supplies like Napier grass, pallets, and wheat straw are relatively expensive to buy and are also less common in Malaysia, which is a problem for small-scale and rural farms (Dikshit and Birthal, 2010). Also, one of Malaysia's most common agricultural wastes is banana leaves. However, it must first be pre-treated before it may be fed to ruminants as feed. Despite this issue, there needs to be a greater selection of alternative cattle feed sources available (Amata, 2014). As a result, generating alternative feed supplies from ensiled banana peels or leaves that provide nutritional value requirements like glucose and protein is significant, particularly for addressing feed resource demand. These alternatives can also reduce

the strain on small-scale and rural farmers in providing adequate nutrition for their farm animals, especially cattle.

2.2.2 *Curcuma* spp. as part of farm animal's feed

The popular medical herb turmeric, sometimes referred to as the "golden spice," is made from the rhizomes of the *Curcuma longa* Linnaeus plant. It's more common to get information regarding turmeric being part of farm animal's feed additives instead of it being one the source of protein in the farm animal's feed. This is because turmeric is about 69.4% of it is made up of carbs, 5.1% of it is fat, 6.3% of it is protein, 3.5% of it is mineral, and 13.1% of it is moisture (Chattopadhyay et al, 2004).

2.2.3 *Alpinia galangal* in farm animal's feed

A. galangal or also known as galangal in English and lengkuas in Malay. It is commonly grown in Asian nations like China, India, Indonesia, Malaysia, Thailand, and Indonesia (Chitra et al., 2004; Bermawie et al., 2012). Many studies have been done for incorporation of galangal in animal's feed with many positive outcomes. For example, when broiler chicks are fed galangal at a rate of 0.25 %, their physiological status and performance are improved as evidenced by higher rates of body weight gain and feed utilisation. The blood biochemical markers of the chicks also showed an improvement, indicating enhanced antioxidative condition (Elghalid et al., 2021).

2.2 Protein Determination and Quantification

Standardized analytical techniques are essential for estimating the protein content of foods. Several methods exist that are used in different food industries to quantify the protein content in foods. The most frequently used methods for measuring protein content in foods include the Kjeldahl method, Dumas method, direct measurement methods using UV-spectroscopy and refractive index measurement (Hayes, 2020).

UV spectrophotometric methods, including the Biuret, Bradford and Lowry methods are easy to use, not costly and can quantify small amounts of protein. However, they can give false positive protein readings depending on the sample preparation method used and solubility of the test sample (Hayes, 2020).

2.2.1 UV Spectrophotometric method in protein quantification

There are few advantages and disadvantages of UV spectroscopy methods in protein quantification. Firstly, for the advantages of UV spectroscopy, it is simple and does not require any assay agents. It is also not costly and can quantify small amounts of protein. As for the disadvantages of UV spectroscopy, it is highly error prone due to other compounds that absorb at the selected absorbance wavelength (Maehre et al., 2018)

2.2.2 Bradford method in protein quantification

The concentration of total protein in a sample is determined using the Bradford protein assay. The assay's basic premise is that when protein molecules attach to coomassie dye in an acidic environment, the colour changes from brown to blue. The protein-dye complex is formed when the basic amino acid residues arginine, lysine, and histidine are present in sufficient amounts (He, 2011).

2.2.3 Kjeldahl method in protein quantification

Codex Alimentarius accepts the Kjeldahl method as the benchmark for measuring milk protein (FAO, 2017). Protein is quantified using the Kjeldahl method in three steps: digestion, distillation, and titration. To conduct it, strong sulfuric acid, heat, potassium sulphate, and a catalyst like selenium are used to break down organic material. Any nitrogen in the sample is converted to ammonium sulphate during this procedure. Next, sodium hydroxide is then added to the digestate to neutralise it. This process turns the

ammonium sulphate into ammonia, which will then be collected in a receiving flask with more boric acid to create ammonium borate. Then, to determine the sample's total nitrogen concentration, the remaining boric acid is subsequently titrated with a standard acid using an appropriate end-point indicator. The measured nitrogen content must be converted to the crude protein content using a specified conversion factor after the total nitrogen has been determined (Goulding et al., 2020).

2.2.4 Lowry method in protein quantification

Protein concentrations may be measured using the Lowry protein assay. Copper is used in the Lowry protein test, where it forms an alkaline bond with the peptide bonds in proteins. This produces a monovalent copper ion that reacts with the Folin reagent to produce a blue material when reduced. A spectrophotometer may be used to quantify this blue hue and calculate the amount of blue present in the sample. As a result, the protein concentration can be determined. In almost all situations involving protein mixes or crude extracts, Lowry protein estimates are a perfectly adequate substitute for an exact absolute measurement (Dawson & Heatlie, 1984).

3.0 MATERIALS AND METHODS

3.1 Sample collection

Alpinia galangal and *Curcuma* spp. were purchased from the wet market located in Pasar Siti Khadijah, Kota Bharu, Kelantan. As for the *Musa* spp. leaves, it was collected by a backyard farmer that sells bananas in Kota Bharu. The plants were kept in a secured plastic bag to prevent it from any form of contamination.

3.2 Sample preparation for plant extraction

A. galangal and *Curcuma* spp. were peeled and cut. As for the *Musa* spp. the leaves are cut into smaller pieces. These plants were weighed at 3 kg, then were air dried at a constant room temperature. Then, plant materials were freeze dried. Afterwards, the plants were grounded into powder form using a mechanical grinder. Then, the powder form of the plant was kept in a sterile beaker.

3.3 Maceration Technique for Plant Extraction

The powdered form of *A. galangal* was dissolved in hexane. Then, it was filtered using Whattman's Filter Paper into a beaker. The maceration was done 3 times as the residue on the Whattman's filter paper was filtered again using hexane. Then, the hexane in the beaker was put in the rotary evaporator to produce hexane extract. Same steps were done for *Curcuma* spp. and *Musa* spp. leaves.

3.4 Preparation of plant sample for protein determination

The obtained crude extract is weighed at 0.05 g and was pipette into a micro centrifuge tube. Then, sterile water was added to diluted the crude extract. The tube was then also vortexed for 10 minutes to ensure the crude extract has homogenized with the sterile water.

3.4.1 Protein Content Determination

3.4.1.1 Preparation of Bovine Serum Albumin (BSA) Standard Curve

Bovine Serum Albumin (BSA) Solution with initial concentration at 2 mg/ml was diluted into 500 µg/ml, 400 µg/ml, 300 µg/ml, 200 µg/ml, 100 µg/ml. The BSA solution was diluted with sterile water. The calculation used to diluted the BSA solution was by using the formula:-

$$M_1V_1 = M_2V_2$$

In a dark room, 50 µl of Bradford reagent was then added to each of the diluted 1000 µl BSA samples in the cuvette. In total there are six cuvettes of diluted BSA solution with concentration of 500 µg/ml, 400 µg/ml, 300 µg/ml, 200 µg/ml, 100 µg/ml and 0 mg/ml using a micropipette. Then, it was incubated for 10 minutes in a dark room as the Bradford Reagent is light sensitive.

The same procedure was also done for *A. galangal, Curcuma* spp. and *Musa* spp. leaves hexane extract in which the 50 µl of Bradford reagent was added into the 1000 µl plant sample and was also incubated in the dark room for 10 minutes.

After incubation, using the spectrophotometer at 595 nm, the blank cuvette was inserted first to get the standard absorbance. Then, followed by 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml and 500 µg/ml of the diluted BSA sample. A standard curve was prepared. Then, the plant protein of *Curcuma* spp., *A. galangal* and *Musa* spp. leaves hexane extract sample was also measured.

4.0 RESULTS

Table 4.1 showed absorbance obtained for the standard curve prepared for bovine serum albumin (BSA). As the BSA concentration increased, the absorbance also increased.

Table 4.1: Absorbance for different concentration of BSA at 595 nm

BSA Concentration (µg/ml)	Absorbance at 595 nm
0	0.000 A
100	0.096 A
200	0.382 A
300	0.498 A
400	0.587 A
500	0.714 A

From the absorbance obtained, a standard curve was plotted (Figure 4.1). The standard curve was used to determine protein content in the samples by using the equation from the graph.

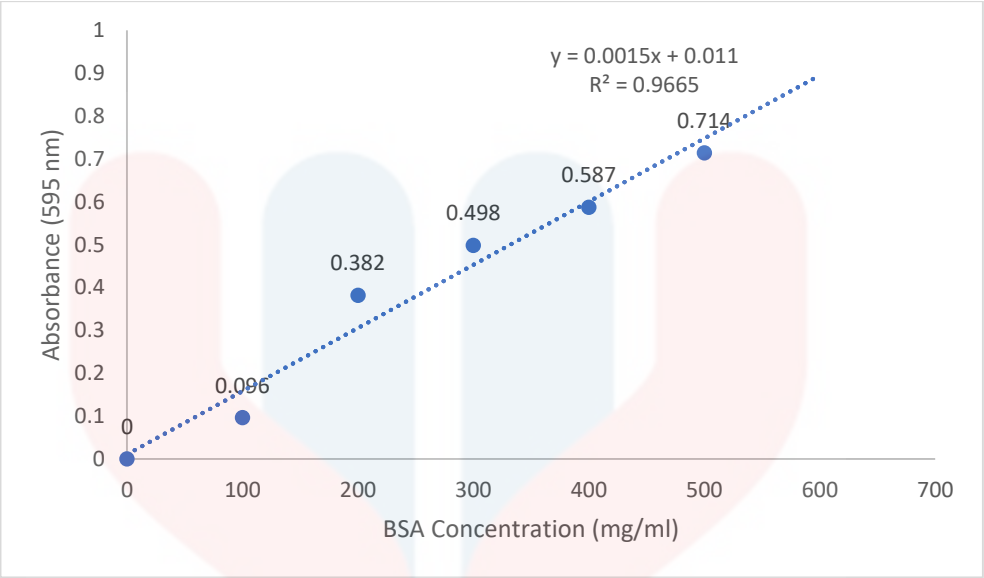

Figure 4.1: Graph showing the relationship between the Bovine Serum Albumin (BSA) Absorbance (595 nm) and the Bovine Serum Albumin (BSA) concentration (mg/ml).

Table 4.2 showed concentration of protein in hexane crude extract of *Alpinia galangal*, *Musa* spp. leaves and *Curcuma* spp.. From the results, Kunyit (*Curcuma* spp.) showed the highest protein concentration with 17, 000 ng/ml while Banana's (*Musa* spp.) leaves showed the lowest with -20, 000 ng/ml.

Table 4.2: Concentration of protein in hexane crude extract of *A. galangal*, *Curcuma* spp. *and Musa* spp. leaves.

Plant Hexane Extract Sample	First Absorbance (595 nm)	Second Absorbance (595 nm)	Average Absorbance (595 nm)	Concentration of protein (ng/mL)
A. galangal	0.008 A	0.065 A	0.0365 A	17, 000
Curcuma spp.	0.015 A	0.064 A	0.0395 A	19, 000
Musa spp. leaves	-0.055 A	0.017 A	-0.019 A	-20, 000

5.0 DISCUSSION

Based on the result obtained, *Curcuma* spp. has the highest concentration of protein at 19, 000 ng/mL. Then followed by *Alpinia galangal* at 17, 000 ng/mL and *Musa* spp. leaves has the lowest value at – 20, 000 ng/mL. This result was obtained using the Bradford method for protein quantification.

The positive and negative values of the absorbance obtained are highly influenced by the absorbance of the reference used in the blank measurement, in this case the 0 mg/ml of BSA solution. In this case, the negative value of the absorbance is due to absorbance of the reference used in the blank measurement in that given frequency is greater than the absorbance of the *Musa* spp. leaves. The detection limit for the Bradford assay is approximately 3000-5000 Daltons. If the protein is smaller than this, we might need to consider other methods in protein quantification such as Bicinchoninic Acid (BCA) Assay which is a copper-based colorimetric assay for total protein quantification (Otieno et al., 2016).

Aside from that, the reason why there's high and low value of the sample can be due to the protein content in the sample itself. This is because the method in sample preparation itself can influence the protein content in the sample. By right, the fresh plant sample should be processed immediately after being harvested but in this case the plant sample are market bought, the information regarding the storage and harvest condition prior is unavailable. Moreover, the proper storage of the fresh plant sample should be at 2 °C if the extraction is going to be done in 5 to 10 days or at -20 °C until extraction. This is because proteins are heat labile, especially in the case of *Musa* spp. leaves, it is better to have its protein content measured when it's in young and actively

growing state and not when the leaves are old and have prolonged exposure to heat or sun.

A good protein assay depends on its specificity, sensitivity, accuracy, rapidity and is easy to perform. Aside from the Bradford method that is being used in this study, there are other various methods available in protein quantification such as Kjeldahl method and Lowry method. A single sample will definitely give out different results when different methods in protein quantification and reagents are used. The ultimate or gold standard method don't really exist in protein quantification, however, the choice for proper method would depend on the characteristic of the protein, the purity of the extracts and the required accuracy with desired speed in protein quantification of the sample itself (Boyer, 1986).

Bradford was chosen in this case for protein quantification due to numerous advantages such as required to use only a single reagent which is the Coomassie dye (Bradford reagent), required a short time period due to the rapidity of the reaction, the stability of the protein-dye complex, high reproducibility and low interferences (Pedrol & Tamayo, 2001). The assay is based on the finding that protein binding induces Coomassie Brilliant Blue G-250's absorbance maximum in an acidic solution to shift from 465 nm to 595 nm. The anionic form of the dye is stabilized by both hydrophobic and ionic interactions, which results in a distinct color change (Bradford,1976). Histidine, lysine, tyrosine, tryptophan, and phenylalanine residues are less likely to be affected by the dye reagent's reaction than are arginine residues. Evidently, the assay is less accurate for proteins that are basic or acidic.

Kjeldahl method is another method in protein quantification and it's a very common method done in the dairy industry to assess the crude protein content in milk. The total

nitrogen content of organic and organic substances is measured using the Kjeldahl method, and the crude protein content is estimated using a specific conversion factor (Varelis, 2016). The Kjeldahl method has its own limitation in which it is not suitable to do in samples that contain nitrogen from the Azo and Nitro groups or when the nitrogen is in the form of rings such as quinoline and pyridine. In this case, if the plant sample hexane extract contains quinoline alkaloids or pyridine alkaloids which are a very common nitrogen containing compounds in plants, thus it can be undetected and give inaccurate results.

Another common method in protein quantification would be the Folin-Ciocalteu method or also known as Lowry method. Lowry method has its own disadvantages such as the measured protein cannot be recovered from the assay and many common substances and reagents within this method can interfere with the protein determination. Moreover, the Folin reagent is not stable and after addition it is reactive for a short period of time. Lowry method is much more time consuming compared to Bradfors or Bicinchoninic Acid (BCA) Assay as it has more steps and reagent used. Despite that, both the Bradford and Lowry methods are colorimetric assays; the former relies on the interaction of protein with an alkaline copper tartrate solution and the Folin reagent; the latter relies on the association of specific amino acid residues, arginine, lysine, and histidine. It is recommended considering the target protein's composition and buffers when deciding on a method for protein measurement (Lu et al., 2010).

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, *Curcuma spp.* has the highest concentration of protein at 19, 000 ng/mL. Then followed by *Alpinia galangal* at 17, 000 ng/mL and *Musa spp.* leaves at – 20, 000 ng/mL. Each of this plant extract protein quantification value obtained was by using Bradford method.

Recommendation for this study would be, since the plant sampling method prior to plant extraction and the methods used in this study influence the result and outcome of the protein quantification value, it is suggested to do the plant sampling method in a manner that will not affect the protein quantity of the plant. Also, in the case of plant such as *Musa spp*. having below than standard absorbance value, it may require to conduct Bicinchoninic Acid (BCA) Assay instead for further accurate protein determination instead of Bradford method. Also, another recommendation in this study would be, using of different solvent such as hexane and ethanol during the maceration process in which the result of the protein analysis will be compared.

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