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The Comparison of Tannin Fractions in *Calliandra calothyrsus* Leaves Utilizing Butanol-HCl_4 and Protein-Precipitation Methods

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ABSTRACT. Butanol-HCl_4 (Bu-HCl_4) and Protein-Precipitation (P-P) are methods that proposed to determine the content of free tannins, protein-bound tannins, and fiber-bound tannins. This study aims to determine the feasibility of tannin measurements by both methods and validation of Analytical Methods (VMA) evaluates specific parameters like accuracy, precision, specificity, and linearity through lab testing. The material used was twenty *C. calothyrsus* leaves which collected randomly from different trees. The samples were measured by both methods in triplicates for qantification and validation. The obtained data were analyzed by descriptive and t-test to compare both methods. The analysis of VMA shows that the P-P method yields higher accuracy compared to the Bu-HCl method in percentage recovery rates. The quantification of free tannins, protein-bound tannin, tannin fiber, and total tannin by the Bu-HCl_4 method versus the P-P method showed significantly higher values ($p<0.01$) for the Bu-HCl_4 method: free tannins (13.44±0.54 vs. 7.11±0.16), protein-bound tannin (2.80±0.44 vs. 1.52±0.20), tannin fiber (5.20±0.45 vs. 1.00±0.21), and total tannin (21.50±0.88 vs. 9.59±0.36). The methode validation result of Bu-HCl_4 and P-P methods in the accuracy was different, but the linearity test showed a good response. In conclusion, the tannin quantivication by the P-P and Bu-HCl_4 can be applied for *C. calothyrsus* analysis. The total tannin content was higher if quantified by the Bu-HCl_4 method compared to P-P. The accuracy of the P-P found higher than the Bu-HCl_4 method.

Keywords: Butanol-HCl_4, leaves *C. calothyrsus*, protein-precipitation methods, tannin.

INTRODUCTION

Small ruminants often survive on low-quality fiber alone, which limits production with limited feed intake and digestibility. The first limiting nutrient for the utilization of low-quality forages is protein. Fed with legumes is a solution because they are rich in protein, making them suitable as low-quality fiber supplements. This feed source is generally neglected in ruminant feed systems, mainly due to a lack of knowledge about various aspects of its potential use. Kurniawati et al., (2016) stated that the reduction of polyphenols content, particularly tannin can be used as animal feed. It is caused by tannins that normally contained in legumes can inhibit the growth of fiber degrading bacteria, in the digestive tract of ruminants. This was proven in the study by Ifani et al. (2021) that administering tannins at a level of 4.5% in ruminant feed will decrease digestibility in the rumen.

Calliandra calothyrsus is a leguminosae that can provide seasonal leaf feed with high nutritional value, even on acid infertile soils. Ifani et al., (2022) stated that *C. calothyrsus* is a nutrient-rich legume with a crude protein content of 23.45%, crude fat of 4.14%, and crude fiber of 19.58%. Another study shows that *C. calothyrsus* has a higher protein content in Novia et al., (2015) that the protein content of *C. calothyrsus* is about 31.35%. However, besides from having good nutrient content, *C. calothyrsus* has a weakness, which is contain quite high quantity of total tannins. Rimbawanto et al., (2015) stated that the total tannin content in *C. calothyrsus* was about 11.84%. Tannins are molecules which complexly bind to proteins, causing the lower digestibility of feed proteins and inhibits the digestive enzymes ability. Tannins are secondary metabolite compounds with a molecular weight of more than 400, containing phenolic compounds that are difficult to separate and hard to crystallize, which precipitate protein from its solution (Safitri et al., 2023).

Condensed tannins and hydrolysable tannins are two subgroups of tannins. Tannins that are easily hydrolyzed are polymers of gallic and ellagic acid that are esterified with a sugar molecule, while condensed tannins are polymers of flavonoid compounds with carbon-carbon bonds in the form of catechin and gallocatechin (Patra and Saxena, 2010). Considering on tannin concentration and structure, the kind of plants, animal species and physiological state, and nutrition, both groups above have either negative or positive effects (Piluzza et al., 2013). Condensed tannins in some feed plants are

beneficial because they can bind proteins, thus protecting feed protein from excessive degradation processes in the rumen, whereas hydrolysable tannins in excessive amounts have the potential to poison livestock (Ifani et al., 2021).

The protein digestion process in ruminants are depending on the separation of the tannin-protein and tannin-fiber complexes, because these complex bonds cannot completely release one another, this is related to the different structures of tannins, proteins, and fibers (Mueller and Harvey, 2006). These metabolite compounds can be qualified by quantitative and qualitative methods (Ratnaningtyas et al., 2022). Based on the problem above, it is necessary to quantify the total tannins in *C. calothyrsus* in order to determine the optimum amount of *C. calothyrsus* that can be fed to livestock. Plants containing condensed tannins can provide an alternative to the scarcity of animal feed resources in tropical countries, due to the low availability of good forages (Pratama et al., 2019).

The methods commonly used to measure tannin levels in plants are the Butanol-HCl_4 (Bu-HCl_4) and the Protein-Precipitation (P-P) method. These methods are oftenly used in the qantification of tannin content because of ease and inexpensive. However, a comparison of tannin measurements using both methods is still unavailable. Therefore, it is necessary to deliver this research to determine which method is more accurate and recommended. This research, can answer the lack of information regarding the measurement of tannins and can maximize the utilization of *C. calothyrsus* as a small ruminant animal feed and also for tannin measurement in other plants.

EXPERIMENTASL SECTION

Material

The material used was twenty *C. calothyrsus* leaves were collected randomly from different trees by cutting each stalk individually near the stem. The leaves were originated from various random area in Banyumas, Indonesia.

Reagents preparation

a. Two mg/mL standard protein solution (BSA, bovine serum albumin, Sigma, Fraction V) was made in 0.2 M buffer acetate solution pH 5.0.
b. SDS–TEA solution (I) would be used in the Bu-HCl_4 method by dissolving Sodium Dodecyl Sulphate (SDS) (0.5% w/v) (Merck) and β-mercaptoethanol (1% w/v) in 10 mM trichloride (Merck). The pH was then adjusted to 8 by adding HCl_4 (Merck).
c. SDS – TEA solution (II) was made by dissolving 1% (w/v) SDS (Sigma) and 5% (w/v) triethanol amine (Merck) in distilled water. This solution is then used for the P-P method.
d. Tannins standard solution was made from 1g/L concentrated tannins which were isolated from *C. calothyrsus* and purified through sephadex LH 20. Thereafter it was mixed thoroughly either with distilled water or SDS-TEA solution (I). The concentration of tannin standards was set to 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml. The standard solution which was mixed in water would be used to determine free tannin (FT) using Bu-HCl and P-P methods. The standard solution in SDS solution (II) would be used to determine tannins bond to protein (TP) and fibre (TF) using Bu-HCl_4 methods.

Sample Preparation

The collected leaves were weighed and freeze dried for three days. The dried leaves then ground through a 1 mm sieve and weighed to determine its' dry matter. The samples were quantified for its tannins by both methods in triplicates. The obtained data were analyzed by descriptive and t-tests to compare the two methods.

Validation Of Analytical Methods (VAM)

The results of the validity test using the VAM parameters were accuracy, precision, and linearity obtained from the Bu-HCl4 method and P-P method. The indicator of how closely the analyses results match the actual analyte reported in percent recovery is called accuracy (Harmono, 2020). The range of recovery percentages used as acceptance criteria for accuracy percent recovery is 98% - 102% (Mulyati et al., 2011). Precision is a parameter to assess the degree of closeness between individual test results and their measurements through the dispersion of individual results from the average (Budari et al., 2014). Linearity tests are performed to assess the correlation between specific variables and if they may be used to predict other variables in the relationship (Widana and Muliani, 2020). The data was analyzed descriptively including the mean, median, standard deviation, initial limit, and lower limit of each parameter.

Measurement of tannin content using butanol – HCl_4 (Bu-HCl_4) method

The measurement with the Bu-$HCl_{,4}$ method involves extracting tannins using a 70% acetone solution containing 0.1% ascorbic acid, followed by diethyl ether extraction after the acetone has evaporated. The rest of the procedure was the same as that of Terril et al. (1992).

Extraction

Extraction is performed by adding 20 mL of 70% acetone containing 0.1% ascorbic acid to 500 mg of dried *C. calothyrsus*. was mixed thoroughly and stirred for 150 minutes. Thereafter the mixture was centrifuged for 10 minutes at 3000 rpm. The solution was separated into supernatant and residue. The supernatant was used to determine free tannins and the residue was used to determine complex tannins (tannins bound to protein and fiber).

Determination Free Tannins

Diethyl ether was added to the supernatant of extracted *C. calothyrsus* leaves and stirred. This

solution would then separate into 2 layers. The acetone and diethyl ether phase on the top was discarded. The bottom layer was the water phase which contained condensed tannins. The water phase was evaporated at 40°C to remove the remaining solvents and the volume was left at around 10 ml. This was kept frozen until further analysis. Thereafter 1 mg of the sample was added to 6 ml Butanol – HCl_4 solution (95% butane-1–ol: 5% HCl_4 36%) in a flask that was filled with marbles. The flask was kept in a water bath at 95 °C for 45 minutes. Thereafter it was cooled. The absorption was read at a wavelength of 550 nm. This procedure was also done both for the tannin standards which were dissolved in water and for a blanc sample (the butanol – HCl_4 solution).

Determination Protein – tannins complex

The determination of the Protein-Tannin complex was performed by drying 100 mg of residue from extracted C. calothyrsus leaves at 60°C for 12 hours. Then, 15 ml of SDS – TEA (I) was added to a flask, and the mixture was thoroughly mixed. Next, the mixture was placed in a water bath set at 95°C for 45 minutes. After the heating process, the flask was cooled to room temperature and centrifuged at 3000 rpm for 12 minutes. 1 ml of the supernatant was taken and added to 6 ml of butanol – 36% HCl_4 in a flask containing marbles, then the flask was returned to a water bath at 95°C for another 45 minutes. After cooling, the absorption was measured at a wavelength of 550 nm. This procedure was also done both for the tannin standards which were dissolved in SDS – TEA solution (I) and a blanc sample.

Determination Complex of fiber – tannins

The determination of the fiber-tannin complex was performed by adding 30 ml of butanol-HCl_4 solution and 3 ml of SDS-TEA solution (I) to the residue from the determination of the protein-tannin complexes. The flask was then boiled for 75 minutes. After boiling, the contents were cooled and centrifuged at 3000 rpm for 10 minutes. Subsequently, the absorption was read at a wavelength of 550 nm. This procedure was also carried out for the tannin standards, which were dissolved in SDS-TEA solution (I), and for the blank sample (the butanol-HCl_4 solution).

Measurement of tannin content using protein precipitation (P-P) method

The measurement of tannin content was conducted using the protein precipitation (P-P) method, with modifications to the tannin extraction procedure allowing for the assessment of free, protein-bound, and fiber-bound tannins. Precipitation with bovine serum albumin (BSA) and the colorimetric reaction with $FeCl_3$ were carried out following the method described by Hagerman and Butler (1978).

Extraction

Extraction is carried out by adding 20 ml of 70% acetone containing 0.1% ascorbic acid to 500 mg of dried C. calothyrsus leaves, thoroughly mixing the mixture. Stirred it for 150 minutes before centrifuging at 3000 rpm for 10 minutes. Separated the solution into supernatant and residue. Used the supernatant to determine free tannins and the residue to determine complex tannins (tannins bound to protein and fibre).

Determination Free tannins

The supernatant of extracted *C. calothyrsus* leaves was collected and diethyl ether was added. The solution was separated into 2 layers. The acetone and diethyl ether phase on the top was discarded. The water phase at the bottom contains condensed tannins. Next, evaporate at a temperature of 40 degrees Celsius to remove the remaining solvents until the volume is reduced to 10 ml, then freeze it until further analysis. Add 1.0 ml of the standard protein solution to 1 ml of the extracted solution in a flask. Mix thoroughly and allow to stand for 15 minutes. Then centrifuge at 3000 rpm for 10 minutes. The supernatant was discarded. The residue was added to 4 ml SDS – TEA solution (II) and 1 ml $FeCl_3$ solution and mixed thoroughly. The absorption was read at a wavelength of 510 nm, 30 minutes after the addition of $FeCl_3$ solution. This procedure was also done for the blank sample (without the addition of standard protein solution).

Determination Protein – tannin complex

The determination of the Protein-Tannin complex was conducted by adding 3 ml of room-temperature diluted phenol and 5 ml of distilled water to 100 mg of dried residue from extracted C. calothyrsus leaves. The mixture was thoroughly mixed and then incubated in a water bath at 90 - 95°C for 120 minutes. After incubation, it was centrifuged at 3000 rpm for 10 minutes. The solution separated into two layers, with the top layer being washed with ether. The ether was then evaporated by blowing air over the solution. The residue was washed with diethyl ether to remove any remaining phenol. This cleaned residue was utilized for determining the fiber-bound tannin fraction. To proceed, 1.0 ml of standard protein solution was added to 1 ml of the solution from the top layer in a flask. After thorough mixing, the solution was left to stand for 15 minutes before being centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, and the residue was mixed with 4 ml of SDS – TEA solution (II) and 1 ml of FeCl3 solution. Following thorough mixing, the absorption was measured 30 minutes post the addition of the $FeCl_3$ solution, at a wavelength of 510 nm.

Determination Complex of fibre – tannins

Add 10 ml of 0.1 N HCl solution to the residue from the determination of the protein-tannin complex. The mixture was boiled for 4 hours and centrifuged. The solution was separated into 2 layers (liquid and solid). Then 1.0 ml standard protein solution was added to 1 ml of the liquid in a flask. The mixture was mixed thoroughly and kept standing for 15 minutes before centrifuging at 3000 rpm for 10 minutes. The supernatant was discarded. The residue was added to 4 ml SDS – TEA solution (II) and 1 ml $FeCl_3$ solution and mixed thoroughly. The absorption was read within 30 minutes after the addition of $FeCl_3$ solution at a wavelength of 510 nm.

RESULTS AND DISCUSSIONS

Validation Of Analytical Methods (VMA)

Accuracy test

The Validation of Analytical Method (VMA) is a technique for determining if specific parameters match the requirements for their use based on laboratory testing (Harmita, 2004; Amorim et al., 2008). Accuracy, precision, specificity, detection limit, quantitation limit, range, and linearity were among the parameters in VMA. **Table 1** presents the findings of the VMA analysis. The percent recovery between the Bu-HCl_4 and P-P methods was 101.05±11.00 vs.104.48±11.10 showed in **Table 1**. The P-P method provides a higher accuracy value than the Bu-HCl_4 method. The percentage recovery which was different and does not fall into the range of % recovery requirements indicates that the accuracy of the Bu-HCl_4 method had not been well received but the figure is close to the required range.

The National Standard Agency (2008) used SNI ISO/IEC 17025 to establish the recovery percentage; the required range for accuracy recovery is 85–115%. The percentage of recovery when referring to SNI shows that the use of Bu-HCl_4 has complied with Indonesia's accuracy requirements. The usage of the Bu-HCl_4 method outside of Indonesia is thought to use various accuracy standards, or it may employ a predetermined accuracy correction factor, in order to trust the test findings obtained using that approach. Based on this statement, the results of the accuracy test in this study with a percentage difference of 3.44±0.01% could be used as a correction factor for the accuracy of the Bu-HCl_4 method to obtain an accuracy level equivalent to the P-P method.

Precision test

The difference in relative standard deviation between P-P and Bu-HCl_4 (0.49 vs. 0.47%) (Shown in **Table 2**) It may be determined that both procedures have adequate repetition because they both satisfied the precision test's minimum relative standard deviation (RSD) standards (2% RSD). According to Sugihartini et al. (2014), precision with an RSD accuracy requirement of 2% can be seen from the relative standard deviation. According to Fatimah et al. (2018), an analytical method could be seen tested to be more exact the smaller the relative standard deviation value that was achieved. According to this assertion, the Bu-HCl_4 approach had a higher repeatability rate than the P-P method, but both have achieved the required accuracy. The precision test findings revealed no discernible difference ($P>0.05$), and both met the requirements for good precision. When employing the Bu-HCl_4 and P-P methods to detect tannin, accurate testing reveals that the findings for each sample tested to show a constant tannin concentration.

Linearity Test

The linear regression used to was $Y = 0.9676x + 8.4715$ showed that the outcomes were in line with Y's forecast. Since the Bu-HCl_4 method's reaction to the standard rate evaluated was linear, the coefficient of determination (R^2) was 0.99. The resultant linear response was displayed as indicated in **Figure 1**.

Table 1. VMA (Accuracy)

The concentration of tannin standards (mg/ml)	Result		%Recovery Accuracy	
	Bu-HCl_4	P-P	Bu-HCl_4	P-P
0.2	202.33±9.29	233.67±23.35		
0.4	398.67±11.93	395.00±5.00		
0.6	615.33±12.58	615.33±17.01	101,05±11.00	104.49±11.10
0.8	813.00±15.52	809.33±7.02		
1.0	1027.33±5.69	1028.00±3.00		

Notes: Tannins standard solution was made from 1g/L concentrated tannins which was isolated from *C. calothyrsus* purified through Sephadex LH 20.

Table 2. VMA (Precision)

method of measuring tannins	Average	Relative Standard Deviation (%)
Bu-HCl_4	611.33 ± 302.38	0.49
P-P	616.67 ± 294.33	0.47

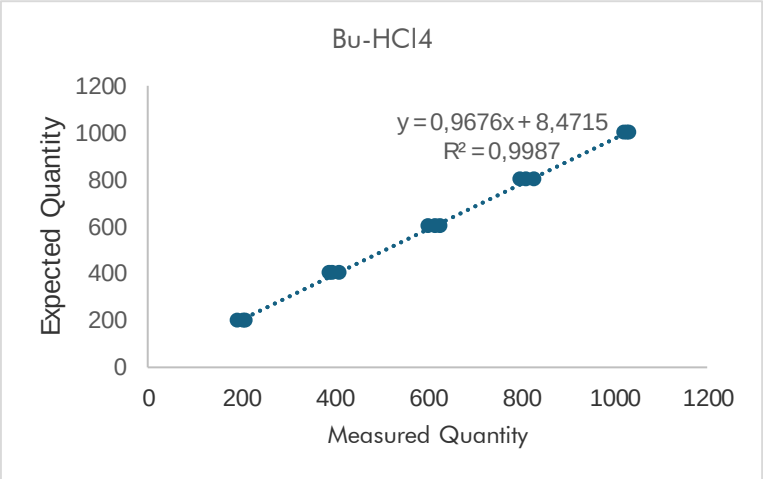

Figure 1. VMA (Linearity) Bu-HCl_4

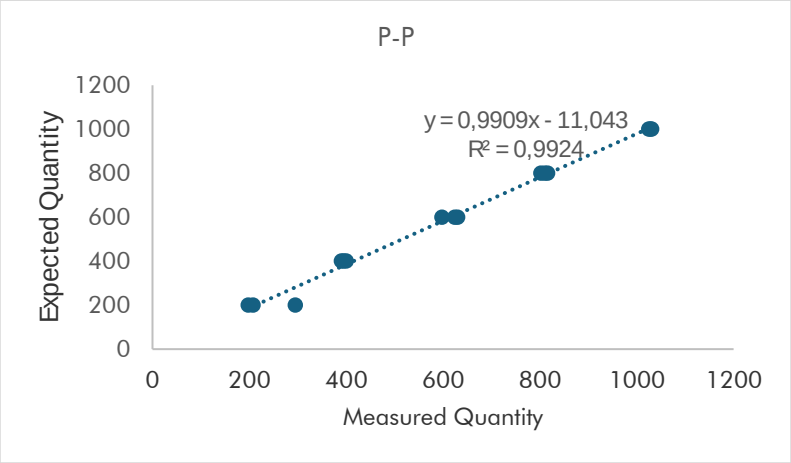

Figure 2. VMA (Linearity) P-P

The P-P method's linearity test was performed using the linear regression formula Y = 0.9909X - 11.043, suggesting that the obtained results were consistent with Y's prediction. The P-P method's reaction to the tested standard level was linear, as indicated by the correlation coefficient's r was 0.99 and R^2 0.99. The resultant linear response was displayed as indicated in **Figure 2**.

The linearity curve from each figure displays a very good linear line, indicating that the Bu-HCl_4 and P-P tannin content test has a good quality to be used. This is consistent with the findings of Fatimah et al. (2018), which state that different analytical approaches will be more accurate, as reflected by higher linearity values. A method's accuracy is ensured by strong linearity, according to Mulyati (2011). The outcomes of the analytical method's linearity test for the validation parameter corroborate this. The Bu-HCl_4 and P-P procedures were used to assess the level of validity of the tannin content test.

Tannin Content

The result of the recovery test of protein-precipitation showed that tannin isolate (TI) was very stable when heated in phenol, however, it was not stable when heated in 0.1 N HCl. This was proven by their percentage of recovery, 101 %, and 41 %, respectively. On the contrary, TI and xylan (tannin bound with fibre) were very stable when heated in 0.1 N HCl, (percentage recovery 109 %) but not stable when heated in phenol (percentage recovery 63 %). The detailed result of the recovery test of the method (P-P) is shown in **Table 3**.

The modified protein precipitation method (P-P) was then compared to Butanol-HCl_4 (Bu-HCl_4). The result showed that not only free tannin but also bound tannin in *C. calothyrsus* were higher when measured by Bu-HCl_4 compared to P-P method. The mean of free and bound tannin contents in *C. calothyrsus* leaves measured by P-P and Bu-HCl are shown in **Table 3**.

Based on the study's findings, *C. calothyrsus* had a total tannin content of 21.50±0.88% using the Bu-HCl_4 method and 9.59±0.36% using the P-P method. The T test was used to assess the test results for the two approaches, yielding the data analysis findings displayed in **Table 4**.

Table 3. Recovery test of the protein-precipitation method of *C. calothyrsus*

Tannin isolate/TI (3 mg/mL)	Treatment	Measured tannin (mg)	Percentage recovery (%)
TI	Heated in phenol	3.04	101
TI and BSA	Heated in phenol	2.30	63
TI	Heated in 0.1 N HCl	1.20	41
TI and xylan	Heated in 0.1 N HCl	3.28	109

Table 4. Free and bound tannin contents (% Dry Matter) in *C. calothyrsus* measured by P-P and Bu-HCl_4 methods

Methods of analysis	Tannins level (%)			
	FT[1]	TP[2]	TF[3]	Total
Bu-HCl_4	13.44±0.54[a]	2.80±0.44[a]	5.20±0.45[a]	21.50±0.88[a]
P-P[5]	7.11±0.16[b]	1.52±0.20[b]	1.00±0.21[b]	9.59±0.36[b]

FT[1] (Free Tannins); TP[2] (Tannins bound to protein); TF[3] (Tannins bound to fibre); Bu-HCl[4] (Butanol-HCl_4); P-P[5] (Protein-Precipitation). The different superscripts (a and b) in the same column showed a very significant differences ($P<0.01$).

The extraction procedure was extended to extract not only protein-free tannin in the legume, but also the protein-bound tannin and fibre-bound tannin. By extracting the protein-bound tannin in *C. calothyrsus* using phenol it is suggested by Markom et al., (2007) that phenol could dissolve the protein so that the binding of tannin-protein was broken. Previously, the temperature and extraction time was tested, so that 90 - 100º C and two hours were the optimum conditions for extraction.

Table 4 showed that there was no degradation of tannin when free tannin in *C. calothyrsus* was heated in phenol (the recovery of tannin reached 100 %). However, when protein (BSA)-tannin complex was heated in the phenol, the recovery decreased to 63%. So, it can be concluded that tannin itself could not be destroyed in heated phenol and some protein-tannin complex could not be degraded by heated phenol.

Table 4 also showed that there was a large degradation of tannin when free tannin in *C. calothyrsus* was heated at 0.1 N HCl (the recovery of tannin was only 41 %). However, when fibre (xylan)-tannin was heated in 0.1 N HCl, the percentage of tannin recovery was complete. This means that tannin bound to fibre is more stable when heated in acid than when tannin alone is treated in acid.

Table 4 showed that the Bu-HCl_4 method gave much higher values of free and bound or tannin content of *C. calothyrsus* leaves compared to the P-P method. The principle of the reaction of each method may explain the different values. The Bu-HCl_4 method is based on the formation of cyanidin from the depolymerisation of the tannin molecule (Shay et al., 2017), so it depends on the number of monomers that are released from tannin polymer to react with H^+ ions to form cyanidin (pink color). Therefore position or places for binding the protein in the polymer tannin would be limited whereas the formation of cyanidin would be much more abundant. The P-P method is based on the ability of tannin to bind with protein; therefore, it depends on the quantity of binding positions in the polymer of the tannin.

Most precipitated tannins ware protein, free or extractable tannins. This can be observed by the highly free tannin quantity. Actually, with the Bu-HCl_4 method, Jackson et al., (1996) found the similar result which found that 70-95% of tannins were in free or extractable forms, except from the *Flemingia* and *Gliciridae* forages. Therefore, the significant effect of tannins to the animals due to the amount of extractable tannin in the forage.

The implication is attention should be paid in interpreting or in comparing tannin values from various laboratories, because the differentcies of methods or standards used. Perez-Maldonado (1994) suggested that the Bu-HCl_4 method is not suggested to determine tannins in biological samples such as urine, rumen content, etc. The finding ware contradictive with Palmer (1997), which reported that free or extractable tannin content of 26 *C. calothyrsus* sampels measured by the P-P method had a higher correlation between freeze-dried and oven-dried (0.87**) compared to those of the Bu-HCl_4 method (0.83**).

However, the corelation between free tannins content and dry matter digestibility of fresh forage, the P-P method showed haiger corelation than the Bu-HCl_4 method (-0.80 vs -0.53). This indicates that tannin content values measured by the P-P method is more describing the nutritive values or the digestibility of *C. calothyrsus* (forage) than measured by the Bu-HCl_4 method.

The findings above emphasize comparison between both methode could not be performed. It is because of the principle of both method are different, which made the discrepancy results of these analyses. Therefore, reports on tannin content measurement should be clearly mantion in what kind of method and how the analyses were carried out. However, the

protein precipitation method was more suitable to quantify tannin content when considering the biological value of *C. calothyrsus.* It caused by the method was able to precipitate all kind of protein in the leaves.

CONCLUSIONS

The tannin analysis using the protein precipitation method (P-P) and Bu-HCl_4 could be applied for *C. calothyrsus* analysis. The total tannin content in the *C. calothyrsus* was higher when measured by Bu-HCl_4 method compared to P-P method. The accuracy level of the P-P method was higher than the Bu-HCl_4 method in testing tannin on *C. calothyrsus*.

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