Effect of (*Leucaena leucocephala*) Leaves as Tannin Source on Rumen Microbial Enzyme Activities and *In Vitro* Gas Production Kinetics

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**Abstract.** *Leucaena leucocephala* has become one legume most commonly used in ruminant feeding practices and which contains CT with potential to reduce CH₄ emissions, improve feed protein utilization. This study was aimed to determine the effect of *L. leucocephala* leaves as a tannin source on rumen amylase, CMC-ase, β-glucosidase, protease activity and kinetic of gas production. The study began with tannin protein-binding capacity evaluation. The levels of *L. leucocephala* leaves which used were 0%, 10%, and 25%. Feed fermentation was conducted using Menke and Steingass gas production technique for 48 hours. Kinetic of gas production was analyzed using the Fit Curve programme. Data obtained were analyzed using one way ANOVA, and continued by DMRT. The results of this study showed that the tannin protein-binding capacity was 1.2680 mg BSA/mg DM of *L. leucocephala* leaves. Amilase, CMC-ase, β-glucosidase, and protease activity decreased significantly (P<0.01) at 10% and 25% *L. leucocephala*. Gas production from soluble fractions (a) was not affected by the treatment. Gas production from the potentially degraded fraction (b) decreased significantly (P<0.05) at 10% and 25% *L. leucocephala*. The potential extent of gas production (a+b) decreased significantly (P<0.05) at 25% *L. leucocephala*. The gas production rate (c) increased significantly (P<0.05) at 25% *L. leucocephala*. Inclusion up to 25% of *L. leucocephala* leaves in the diet reduce rumen hydrolytic enzymes activity, the soluble and the potentially degraded fraction (a+b) and gas.

1. **Introduction**

Livestock is significant contributors to potential global warming through the emissions of Green House Gasses (GHGs) such as CO₂ and methane (CH₄). The increasing demand for livestock products contributes to accumulating greenhouse gases in the atmosphere. It is considered to make efforts to create environmentally friendly livestock by reducing methane gas production in ruminants [1]. Tannins are plant secondary metabolite compounds that can be used to reduce methane gas production by ruminants. Tannin compounds in plants have anti-methanogenic activity so that it can reduce methane production in the rumen [2]. Tannins can form complexes with methanogenic bacterial cell membranes that interfere with the transport of nutrients into cells and inhibit cell growth [3].

Besides being able to be used to reduce methane gas production, tannin can also be used as a protective agent for feed protein from microbial degradation in the rumen, so that the host animal can utilize feed protein optimally. Tannins are used as protein protection agents because they have a high
affinity to form complexes with feed proteins, so microbes in the rumen cannot degrade these proteins [4]. The tannin-protein complex can be formed in the rumen at pH 6-7, then the protein will be released from the complex in the abomasum when the pH is less than 3.5 so that the protein can be utilized by host animals [5]. Giving tannins at 1% levels reduced the digestibility of crude protein in the rumen, about 29.73% [6].

Rumen fermentation involve enzyme of rumen microbial. Enzymes are a functional form of protein so that tannins can form complexes with enzymes in the rumen [4,7]. Each tannin has a specific biological activity because it has a different structure from one to another so that the protein binding capacity of tannins is also different from each other [8,2].

Changes in enzyme activity caused by tannins can affect the in vitro gas production kinetics. Previous research stated that giving of 2% and 3% tannic acid reduced gas production from the potentially degraded fractions and gas production rate in in vitro fermentation [9]. Other studies stated that tannins from gambier extract with a level of 2% reduced gas production from soluble fractions and potentially degraded [10].

*L. leucocephala* leaves are a forage that contains high tannin compounds. *L. leucocephala* leaves have a total tannin content of 6.57% [11]. *L. leucocephala* leaves have high palatability for ruminants, functional adaptation, and lives well in tropical climates [12]. *L. leucocephala* is a legume plant that has been spread in various regions in Indonesia so that it is easily obtained and used as animal feed [13]. Based on this description, it is necessary to examine the effect of adding *L. leucocephala* leaves as a source of tannin in several different levels to determine its effect on enzyme activity in the rumen and gas production kinetics.

2. Materials and methods

2.1. Samples collection and preparation

Forages were collected from forages field laboratory of forages and pasture Universitas Gadjah Mada. Samples were dried at 55°C for 48 hours and ground to pass a 0.5 mm screen for chemical composition and tannis essay. Samples were proximate analyzed [14]. Forage samples were analyzed for the total tannins [15] and their condensed [16] tannins content.

2.2. Tannins extraction and protein-binding capacity

Dried *L. leucocephala* leaves (0.2 g) were extracted in 10 ml acetone 70% for 20 min with Bandelin Sonoplus ultrasound homogenizer at room temperature. The extract were centrifuged (3000 g, 10 min, 4°C). Supernatant were used for tannin protein-binding capacity assay. 0.05; 0.10; 0.15; 0.20; 0.25; 0.30 ml L. leucocephala leaves extract diluted with 0.95; 0.90; 0.85; 0.80; 0.75; 0.70 ml methanol (50%). The solution was then added with 2 ml of Bovine Serum Albumin (BSA) solution, so that the total volume of the mixture was 3 ml. The mixture was incubated for overnight in the chiller (4°C) and then centrifuged (3000 g, 10 min). Supernatants are separated from the precipitate slowly.

The precipitate was added with 1.5 ml of 1% Sodium Dodecyl Sulphate (SDS) solution and mixed with 3 ml of SDS-triethanolamine, after that the solution was added with 1 ml of ferric chloride reagent. The mixture was incubated 15 min, then the absorbance were measured with spectrophotometer at 510 nm. The absorbance were converted to tannic acid equivalent, using a standard curve.

2.3. In vitro gas production kinetics

Fat Tailed Sheep were fed with elephant grass, pollard and soybean meal (forages: concentrate, 70:30) for feed adaptation. Rumen fluid is taken by slaughtering sheep. In vitro fermentation were conducted by Menke and Steingass method [17] for 48 hours. Syringe is filled with substrate, which was feed material (elephant grass, pollard, and soybean meal) and tannin source forage with different levels (0%, 10%, and 25% *L. leucocephala* equivalent to total tannins of 0%, 0.98%, and 2.45%). Gas production from the fermentation was measured at 1, 2, 4, 6, 8, 12, 24, 36, dan 48 hours. Gas production kinetics
was measured using fitcurve programme by [18]. At the end of incubations, rumen liquid was centrifuged (3000 g, 10 min) to separate feed substrate from the rumen fluid. Supernatant was further centrifuged (10,000 g, 10 min) to separate microbial cells and supernatants that contain enzymes. Amylase, CMC-ase, β-glucosidase [19] and protease [20] activity were measured.

2.4. Data analysis
Data of fermentation were analyzed using one-way analysis of variance with SPSS 16 for Microsoft Windows and statistically significant differences between means were determined by Duncan’s Multiple Range Test (DMRT) when the effects of treatment (P<0.05) were detected.

3. Results and discussion

3.1. Tannin Protein-binding capacity
The results of the L. leucocephala leaves tannin protein-binding capacity can be observed in Table 1 and Figure 1.

| Volume of L. leucocephala leaves extract (µL) | Tannin-protein binding capacity (mg BSA /mg DM of L. leucocephala leaves) |
|---------------------------------------------|-------------------------------------------------------------------------|
| 50                                         | 1.2680<sup>d</sup>                                                      |
| 100                                        | 0.8693<sup>c</sup>                                                      |
| 150                                        | 0.7817<sup>b</sup>                                                      |
| 200                                        | 0.6130<sup>a</sup>                                                      |
| 250                                        | 0.6497<sup>a</sup>                                                      |
| 300                                        | 0.8400<sup>c</sup>                                                      |

Means within the same row with different superscript letters differ significantly (P<0.01)

![Figure 1. L. leucocephala leaves tannin protein-binding capacity](image)

The most optimal protein-binding capacity occurs in the addition of 50 µl extracts, that was 1,2680 mg BSA / mg DM L. leucocephala leaves. Previous studies reported that L. leucocephala tannin protein-binding capacity is 1,181 mg BSA / mg DM of L. leucocephala leaves [21].

3.2. Effect of L. leucocephala leaves on rumen hydrolytic enzymes activity
The effect of L. leucocephala leaves substitution on rumen hydrolytic enzymes activity can be observed in Table 2 and Figure 2.
Table 2. Effect of *L. leucocephala* Leaves on Rumen Hydrolytic Enzymes Activity

| Enzyme Activity | 0% (TT = 0%; CT = 0%) | 10% (TT = 0.98%; CT = 0.08%) | 25% (TT = 2.45%; CT = 0.20%) | Sig. |
|-----------------|----------------------|-------------------------------|-------------------------------|------|
| Amilase (U/g)   | 13.23±0.052b         | 12.92±0.065a                  | 12.80±0.040a                  | 0.003|
| CMC-ase (U/g)   | 8.46±0.020c          | 6.80±0.057b                   | 3.44±0.074a                   | 0.000|
| β-Glukosidase (U/g) | 159.72±0.753c | 102.07±0.457b                 | 65.62±1.171a                  | 0.000|
| Protease (U/g)  | 24.80±0.202c         | 20.87±0.572b                  | 7.44±0.002a                   | 0.000|

TT = Total tannin, CT = Condensed Tannin

Means within the same row with different superscript letters differ significantly (P<0.01)

Figure 2. Effect of *L. leucocephala* Leaves on Rumen Hydrolytic Enzymes Activity

3.2.1. Effect of *L. leucocephala* leaves on amylase activity

Based on the data in Table 2, the use of 10% and 25% *L. leucocephala* leaves significantly reduced amylase activity (P<0.01) about 2.03% and 3.38% compared from control, but increasing levels of *L. leucocephala* substitution from 10% to 25% did not cause significant differences. The previous study reported that giving tannins from cranberry, pomegranate, grapes, and cocoa extracts with a level of 1% reduced amylase activity about 15%, 8%, 3%, and 1% [22]. Other study reported that giving tannins from *Castanea sativa* and *Caesalpinia spinosa* extracts with a level of 4% reduced amylase activity in the rumen about 33.78% and 10.94% [23].

Decreased amylase activity might be caused by decreasing amylolytic microbes population. The previous study reported that 0.06% condensed tannins from *Onobrychis vicifolia* leaves extracts decreased the population of *Ruminobacter amylophilus* WP225 about 28.88% compared from control [24]. Other study reported that giving tannins from *Samanea saman* pod extract at 6% levels reduced amylolytic activity in the rumen [25].

3.2.2. Effect of *L. leucocephala* leaves on cMC-ase activity

Based on the data in Table 2, the use of 10% and 25% *L. leucocephala* leaves could significantly reduce CMC-ase activity (P<0.01). The use of 10% and 25% *L. leucocephala* reduced CMC-ase activity about 12.5% and 62.5% compared from control. The previous study reported that giving 0.5% condensed tannins from *Acacia nilotica* leaves extract decreased CMCase activity 62.58% [26]. Other studies reported that giving 5% tannins from quebracho extract reduced CMCase activity in the rumen about 8.3% [27], while giving 1.67% tannins from *Terminalia chebula* extract and *Emblica officinalis* reduced CMC-ase activity in buffalo’s rumen about 21.48% and 26.86% [28].

Tannins can disturb cellulolytic bacteria that are responsible for CMCase synthesis. The tannin content in feed can reduce fibre degradation because it decreases the number of cellulolytic bacteria or forms a complex between tannin and cellulose [29]. Previous studies reported that giving 6% tannins from *Samanea saman* pods extracts reduced cellulolytic activity up to 61.82% [25]. Other study reported that giving more than 0.1% condensed tannins from a mixture of quebracho and chestnut extracts reduced the population of *Fibrobacter succinogenes* in the rumen up to 95% [30].
3.2.3. Effect of *L. leucocephala* leaves on β-glucosidase activity
Based on the data in Table 2, the use of 10% and 25% of *L. leucocephala* leaves significantly reduced the β-glucosidase activity (P<0.01). The use of 10% and 25% *L. leucocephala* reduced β-glucosidase activity about 36.25% dan 58.75% compared from control. The previous study reported that giving 0.06% condensed tannins reduced about 95% to 98% the activity of β-glucosidase produced by fungi in the rumen [31]. Other study reported that giving 0.02% condensed tannins reduced β-glucosidase activity in the rumen about 77.78% while giving condensed tannins at 0.04% levels can stop the β-glucosidase activity in the rumen [32].

The decreased β-glucosidase activity can be caused by decreased microbes population that produce β-glucosidase. The previous study reported that giving condensed tannins from *Onobrychis vicifolia* leaf extracts at 0.01% levels inhibited *Butyrivibro fibrisolvens* growth about 24.41% [24], while giving tannin from *Styzolobium aterrorium* leaves extract at 3.8% levels reduced the population of *Fibrobacter succinogenes* in the rumen up to 73% [33].

3.2.4. Effect of *L. leucocephala* leaves on protease activity
Based on the data in Table 2, the use of 10% and 25% of *L. leucocephala* leaves significantly reduced protease activity (P<0.01). The use of 10% and 25% *L. leucocephala* reduced protease activity about 15.85% and 70.16% compared from control. The previous study reported that giving condensed tannins from *Acacia nilotica* leaves extracts at 0.5% levels reduced protease activity in the rumen about 59.65% [34]. Other study reported that giving 0.005%, 0.01%, and 0.02% of tannic acid inhibited the protease activity produced by *Pectobacterium chrysanthemi* about 58.34%, 65.42%, and 72.46% compared from control [35].

Decreased protease activity can be caused by decreased proteolytic microbes population that produce protease. Previous study reported that condensed tannins from *Lotus corniculatus* leaves extracts at 15% levels decreased proteolysis activity of *Prevotella ruminicola* 23, *Prevotella ruminicola* C21a, and *Clostridium proteoclasticum* B316 about 81.63%, 45.19%, and 85.27%, while giving condensed tannins from *Lotus corniculatus* extracts at 0.04% levels inhibited *Prevotella ruminicola* and *Clostridium proteoclasticum* growth about 55.56% and 45.45% [36]. Other study reported that giving condensed tannins from rain tree pod meal at 0.5% levels reduced protein digestibility, NH3 concentration, and population of proteolytic bacteria in the rumen about of 9.3%, 25.98%, and 17.95% [37].

3.3. Effect of *L. leucocephala* leaves on rumen gas production kinetics
The effect of *L. leucocephala* leaves on rumen gas production kinetics can be observed in Table 3 and Figure 3.

**Table 3.** The effect of *L. leucocephala* leaves on rumen gas production kinetics

| Fraction | Level of *L. leucocephala* | Sig. |
|----------|----------------------------|------|
|          | 0% (TT=0%; CT=0%) | 10% (TT=0.98%; CT=0.08%) | 25% (TT=2.45%; CT=0.20%) |
| a (ml)   | 0.560 ± 0.289          | 0.456 ± 0.136          | 0.813 ± 0.163          | 0.497 |
| b (ml)   | 58.198 ± 0.555<sup>b</sup> | 50.787 ± 3.253<sup>#</sup> | 46.074 ± 1.598<sup>a</sup> | 0.019 |
| a+b (ml) | 58.758 ± 0.757<sup>b</sup> | 51.243 ± 3.364<sup>ab</sup> | 46.887 ± 1.748<sup>a</sup> | 0.025 |
| c (ml/jam) | 0.052 ± 0.002<sup>a</sup> | 0.054 ± 0.004<sup>a</sup> | 0.083 ± 0.09<sup>b</sup> | 0.017 |

TT = Total tannin, CT = Condensed Tannin
<sup>a,b</sup> Means within the same row with different superscript letters differ significantly (P<0.05)
3.3.1. Effect of \textit{L. leucocephala} leaves on gas production from feed soluble fraction (a)
Based on the data in Table 3., the gas production produced by the soluble fraction (a) did not affect by the use of 10% and 25% \textit{L. leucocephala} (P>0.05). Degradation of soluble fractions involves several enzymes such as amylase and protease. Although the results of this study showed that \textit{L. leucocephala} leaves reduced amylase and protease activity, but this has not significantly affected the gas production produced by the soluble fraction (a). Previous study also reported that tannin-containing forages substitution like \textit{Quercus cercis}, \textit{Quercus libari}, and \textit{Quercus coccifera} did not affect the production of gas produced by the soluble fraction (a) [38].

3.3.2. Effect of \textit{L. leucocephala} leaves gas production from feed potentially degraded fraction (b)
Based on the data in Table 3., the use of 10% and 25% \textit{L. leucocephala} significantly reduced (P<0.05) gas production produced by the degraded potential fraction (b) about 12.73% and 20.83% compared from control. Increasing the level of \textit{L. leucocephala} from 10% to 25% does not cause a significant difference (P> 0.05) to the value of b. Degradation of potentially degraded fractions involves several enzymes such as CMC-ase and \(\beta\)-glucosidase. The reduced activity of CMC-ase and \(\beta\)-glucosidase enzymes in the 10% and 25% \textit{L. leucocephala} also decreased the value of b. Previous study also reported that tannins from gambier extracts at 2% levels reduced gas production from potentially degraded fractions (b) about 9.39% [10]. Other study reported that giving 3% tannins from the leaves and fruits of oak tree reduced gas production from potentially degraded fractions (b) about 17.09% and 27.77% [39].

The results showed that tannins could inhibit the activity of microbes that are responsible for degrading potentially degraded fractions in feed. Previous study reported that tannins extracted from \textit{Acacia nilotica} leaves can inhibit the activity of rumen fibrolytic enzymes such as xylanase, endocellulase, and exocellulase about 64.46%, 62.58%, and 2.6% compared from control [34]. Other study reported that 2% and 4% of condensed tannins extracted from quebracho reduces digestibility of crude fiber in goats about 1.79% and 6.22% compared from control [40].

3.3.3. Effect of \textit{L. leucocephala} leaves on gas production from soluble and potentially degraded fraction (a+b)
Based on the data in Table 3., the use of 10% \textit{L. leucocephala} did not cause a significant decrease (P<0.05) on the amount of gas production produced by the soluble fraction and the potential degraded fraction (a + b), but substitution of 25% \textit{L. leucocephala} caused a significant decrease (P <0.05). This shows that although the production of gas produced from the soluble fraction (a) did not significantly decrease, the total gas production (a+b) decreased significantly in 25% \textit{L. leucocephala} substitution. The same results obtained by previous study which stated that giving tannin from gambir extract at 2% levels reduced gas production from fractions a and b by 7.27% [10].
The results showed that tannins disturb the activity of microbes that produced hydrolytic enzymes. Decreased hydrolytic enzymes production may caused by tannin’s ability to protect feed protein. Previous study reported that tannins can be used as protective agents for feed protein in the rumen, tannins will form complexes with proteins so that degradation of proteins in the rumen decreases. Decreased protein degradation can reduce NH$_3$ concentration which acts as a source of N for microbial growth [41]. Other study reported that 1,5% and 3% of tannins from quebracho extract could reduce the NH$_3$ rumen concentration of dairy cows about 4.98% and 5.65% from control in in vitro fermentation [42].

3.3.4. Effect of L. leucocephala leaves on feed degradation rate (c)

Based on the data in Table 3. the use of 10% L. leucocephala did not affected significantly (P>0.05) to feed degradation rate (c), but 25% L. leucocephala caused a significant increase (P<0.05) about 36.88% compared from control. Increasing the level of L. leucocephala substitution from 10% to 25% also caused a significant increase (P<0.05) in the rate of feed degradation (c). Previous study reported that Acacia sieberriana woodii leaves that containing 9,6% tannin increased the rate of feed degradation about 15.05% compared from control [43].

The results showed that the higher levels of L. leucocephala leaves caused a higher feed degradation rate. This is likely to occur because tannin in L. leucocephala leaves is more likely to bind to the substrate than to bind to the enzyme, so enzyme activity decreases, but the rate of degradation is faster because of the small amount of substrate. Low substrate concentrations caused lower enzyme activity, but the reaction rate is getting faster [44].

4. Conclusion

The conclusion of this study is the use of 10% and 25% L. leucocephala reduced rumen hydrolytic enzymes activity and gas production kinetics.

Acknowledgment

The authors acknowledge to PDUPT program from Minister of Research, Technology and Higher Education of the Republic of Indonesia for financial support.

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