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The Effect of Nutmeg Leaves Tannin (*Myristica fragrans Houtt*) as Protein Protecting Agents on *In Vitro* Nutrient Digestibility

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ABSTRACT

This experiment was aimed to study the effect of nutmeg leaf tannin addition on in vitro nutrient digestibility. Treatments in this experiment consisted of: P0 (control without tannin), P1 (feed + 2% tannin) and P2 (feed + 4% tannin). Feed for fermentation substrate consisted of *Pennisetum purpureum* and soybean meal with ratio 60:40. Fermentation was carried out using Tilley and Terry two stages in vitro technique for 48 hours. Variables measured were the digestibility of dry matter, organic matter, and crude protein in the rumen as well as the total digestive tract digestibility based on in vitro technique. The data obtained were analyzed by One Way ANOVA, and followed by the Duncan Multiple Range Test (DMRT). The results showed that rumen dry matter digestibility was lower (P<0.05) in P1 and P2 (59.03±3.24 and 57.19±1.32) compared to P0 (70.77±1.05), but did not show a significant difference (P>0.05) in the total dry matter digestibility of P0, P1, and P2 (74.88±5.28, 67.70±3.21, and 64.83±4.96). Organic matter digestibility in the rumen was also lower (P<0.05) in P1 and P2 (55.55±6.29 and 55.76±6.88) compared to P0 (75.39±0.91), but did not show significant difference (P>0.05) in total organic matter digestibility from P0, P1, and P2 (64.69±6.44, 64.33±6.34, and 61.20±5.11). The digestibility of crude protein in the rumen at P1 and P2 (45.48±5.12 and 38.47±3.44) was also significantly lower (P<0.05) compared to P0 (60.93±9.72), whereas total digestibility did not show any significant difference (P>0.05). Addition of tannin leaf nutmeg 2% optimally reduced rumen dry matter and crude protein digestibility without causing excessive negative impact on results of *in vitro* digestibility, so it can be used as a protective agent protein feed.

Keywords: *In vitro* digestibility, Nutmeg leaves, Protein protection, Tannin

Introduction

Feed is one of the main components in livestock which functions as the source of nutrients that directly affects livestock growth and productivity. One of the essential nutrients for livestock is protein. Livestock feeding with high protein content generally intended to provide amino acids that can increase their productivity. However, some factors should be considered while feeding it to ruminants, including appropriate levels or amounts, as well as fermentability and degradation resistance in the rumen (Puastuti, 2005; Stern *et al*., 2006). It is related to the digestion process of feed ingredients in ruminants that are different from other livestock.

Rumen microbes have a crucial role in the fermentation process of feed ingredients in the rumen by producing enzymes that can degrade feed. Feed ingredients degradation, including protein and carbohydrates, in the rumen, will produce fermentation products such as volatile fatty acids (VFA), ammonia (NH3), and methane gas (CH_4), also CO_2. Protein in the ruminant digestive tract system is degraded and deaminated by rumen microbes into ammonia (NH_3), which will be used by microbes to form microbial proteins, so that the utilization of protein by livestock becomes less efficient, especially when fed with high-quality protein. There is a way to modify feed so that the efficiency of protein utilization in the digestive tract will increase. One of feed modifications is carrying out several agents that act as protein protection, including using formaldehyde, tannins, and saponins (Makkar, 2005; Ani *et al*., 2015).

Tannin is a phenolic compound that can bind with other macromolecules, such as proteins and polysaccharides, including fibers so that it can protect feed ingredients from degradation by rumen microbes. There are two types of tannins,

namely hydrolyzed tannins and condensed tannins (Makkar, 1998). Hydrolyzed tannins are way more effective in reducing methane compared to condensed tannins (Jayanegara *et al*., 2009; Rira *et al*., 2019). Condensed tannins can bind to proteins and are stable at rumen neutral pH conditions. The bond between condensed tannins and these proteins are released at acidic pH as in the post rumen digestive organs, abomasum and intestines, so that the protein can be used as a source of amino acids directly by animals (Sasongko *et al*., 2010).

The application of tannin needs to be adequately regulated to avoid excessive adverse effects to the ruminants. The binding of tannins with feed ingredients can cause a lower availability of nutrients to meet the needs of microbes growth so that it will affect the digestibility of feed ingredients in the rumen. Decreased digestibility of feed nutrients, including proteins and carbohydrates, causes a drop in fermentation products. Sugoro (2004) stated that tannins can reduce digestion, thereby reducing the concentration of ammonia, VFA, and gas production. Widyobroto *et al*. (2007) stated that the availability of NH_3 precursor influences microbial protein synthesis as well as the availability of energy from the degradation process of feed ingredients.

One of the plants that produce high amounts of tannin compounds is nutmeg; therefore, nutmeg leaves becomes a potential source to tannin compounds that can protect soybean meal protein. Though soybean meal is classified as a high-quality protein source, it degrades in the rumen easily. Soybean meal contains high levels of crude protein and contains complete essential amino acids. The protein content in soybean meal can reach up to 41.7% (Sajati *et al*., 2012).

Several previous studies regarding the use of tannins from various plant sources for in vitro protein protection can reduce rumen fermentation products and feed nutrients digestibility. Some of them were including protein protection with tannin tea extract, sengon leaves, gambir leaves pulp, guava leaves, and jackfruit leaves which can reduce the fermentation and nutrients digestibility as well as increase the levels undegraded protein and total protein production (Subrata *et al*., 2005; Anas *et al*., 2015; Ramaiyulis *et al*., 2019; Rosita, 2015; Sasongko, 2010).

This study aimed to determine the effect of the addition of nutmeg leaves as a source of tannin to the digestibility of dry matter, organic matter, and crude protein in the rumen and total digestive tract, which were incubated using Tilley and Terry method of two-stage in vitro for 96 hours.

Materials and Methods

Nutmeg leaves sample preparation

As much as 500 g samples of young nutmeg leaves from female trees were taken from Ngobo Afdeling Gebugan PTPN IX Bergas, Semarang. The nutmeg leaves were then directly dried in an oven at 55^0C for 3x24 hours. Samples obtained were then ground and filtered with a size of 1 mm filters, then the chemical compositions were analyzed by the proximate method (AOAC, 2005) as well as the content of phenols and tannins (Makkar, 2003).

Tilley and Terry (1963) *in vitro* digestibility technique

Tilley and Terry's *in vitro* digestibility technique consists of two stages, namely stage one describing the fermentation stage in the rumen and stage two describing the stages of fermentation in the post rumen digestive tract. In vitro stage one reflects the data on nutrient digestibility in the rumen in the first incubation period. In vitro stage two represents the second incubation period in which total digestibility data obtained consist of the rumen as well as post rumen nutrient digestion (abomasum and intestines). Each stage passed through 48 hours incubation time. Rumen fluid was obtained from a fistulated Bali cows in the morning before being fed. The rumen fluid was filtered with gauze before it was mixed with artificial saliva. Rumen fluid and artificial saliva solution are predetermined with the ratio of 1: 4. As much as 25 ml of rumen and medium were taken and put into 20 small test tubes and 50 ml in 20 large test tubes filled with feed mixture. The composition of the feed used can be seen in Table 1. CO_2 gas always flowed into the tube to create an anaerobic atmosphere. The tube was then incubated in a water bath at 39^oC and occupied 48 hours in each stages; moreover, 8 hours shake was needed.

The first stage potrays the fermentation stage in the rumen and lasts for 48 hours. Then, a total of 10 large test tubes and ten small tubes were harvested and filtered, so that the filtrate and the undigested substrate were separated, using a crucible given a glass wool filter with known weight and digestibility as a way to analyze the nutrients digestibility. The remaining substrate filtered from a small tube were then processed to obtain dry matter and organic matter digestibility data, while the results of a filtered large tube of crude were analyzed to determine the crude protein digestibility.

The remaining ten large and ten small test tubes that had not yet harvested resumed to the second stage incubation. By adding 3 ml of 20% HCl and 1 ml of pepsin 5%, this creates an acidic atmosphere as in abomasum. The tubes were

Table 1. Chemical composition and proportion of feed ingredients used in in vitro fermentation

Chemical composition of feed ingredients	*Pennisetum purpureum*	Soybean mean	Nutmeg leaves
Dry matter (%)	89.89	92.30	91.64
Organic matter (%)	84.05	92.80	93.29
Crude protein (%)	13.05	41.30	8.98
Tannin (%)	-	-	13.40
Proportion used:			
P0	60	40	-
P1	60	40	2
P2	60	40	4

P0: control treatment without tannin addition, P1: feed mixture + 2% nutmeg leaves tannin, P2: feed mixture + 4% nutmeg tannin.

harvested after the second incubation and then filtered to separate the filtrate and the undigested substrate using *glass wool inside the crucible*, which was known for its weight to examine the nutrient digestibility. The remaining substrate filtered from a small tube was then analyzed to obtain the digestibility of dry matter and organic matter, while the results of filtering a large tube were analyzed to determine the crude protein digestibility. Therefore, it can portray whether the tannin bonds with the protein in the post rumen digestive tract or not.

Observed variable

The observed variables were rumen and total nutrient digestibility. Nutrient digestibility included dry matter digestibility (DMD), organic matter digestibility (OMD), and crude protein digestibility (CPD).

Data analysis

The experimental design used in this study was Completely Randomized Design (CRD) of one-way analysis. The treatments in this study were P0 (control feed mixture without tannin addition), P1 (feed mixture + 2% nutmeg leaves tannin), P2 (feed mixture + 4% nutmeg leaves tannin), each treatment were carried out with three replications. Feed mixtures used included *Pennisetum purpureum* and soybean meal with a ratio of 60: 40. Data obtained from this study were analyzed using a one-way analysis of variance (*One Way Anova*) using SPSS. *Duncan's Multiple Range* Test (DMRT) was used as further tests to measure specific differences between pairs of means (Steel and Torie, 1993).

Result and Discussion

Dry matter digestibility and organic matter digestibility

Nutmeg leaves contain 13.40% total tannins, 10.29% condensed tannins, and 3.11% hydrolyzed tannins. Table 2 shows the effect of adding nutmeg leaves as a source of tannin on nutrient digestibility in vitro fermentation. These included rumen and total digestibility of dry matter (DMD), organic matter (OMD), and crude protein (CPD).

The addition of nutmeg leaves as a source of tannin showed a significant reduction of dry matter digestibility ($P<0.05$) in the rumen along with the higher tannin level but did not show any significant difference ($P>0.05$) in total dry matter in vitro digestibility. Decreased digestibility of dry matter in the rumen is related to decreased digestibility of nutrients due to the binding of tannins with organic compounds in feed ingredients, so that rumen microbes become inadequate to degrade the nutrients while in the rumen neutral pH condition. Sasongko *et al*. (2010) stated that condensed tannins can form bonds that are stable at rumen neutral pH, however, it is easy to be broken down at acidic pH in the post rumen digestive tract.

The digestibility of organic matter in the rumen decreased significantly ($P<0.05$) along with the addition of nutmeg leaves tannin. Total organic matter digestibility did not show any significant difference ($P>0.05$). It proven that the bonds between tannins and organic compounds are difficult to degrade by rumen microbes. However, the process in post rumen digestive organs can break down the bonds; therefore, the total digestibility did not show any significant difference from the control treatment. The tannin from the nutmeg leaves can form bonds with organic compounds, including protein and other organic compounds. Jayanegara *et al*. (2009) stated that tannins can interact with proteins as well as fiber and other components of feed ingredients such as vitamins and minerals.

Significant decreased in digestibility of dry matter and organic matter also occurred in the addition of Lamtoro leaves and Calliandra leaves as a source of condensed tannins in fish waste silage with the best level of addition is as much as 2% condensed tannins in Lamtoro leaves and 1.5% condensed tannins in Calliandra leaves (Rimbawanto *et al.*, 2015). A treatment of adding oak leaves (*Quercus libani*) in the feed also showed a significant decrease in the digestibility of dry matter and organic matter (Abarghuei *et al*., 2010).

Crude protein digestibility

The results showed a significant decrease in crude protein in vitro rumen digestibility ($P<0.05$) along with the addition of nutmeg leaves tannin levels; however, the total digestibility was not significantly different ($P>0.05$). The addition of nutmeg leaves tannin as much as 2, and 4% significantly reduced protein digestibility ($P<0.05$) in the rumen. The addition of condensed tannin content as much as 4% had a beneficial effect; however, each tannin has a different biological activity that should be considered (Makkar, 2003).

Table 2. Dry matter digestibility (%), organic matter digestibility (%), and crude protein digestibility (%) in the rumen and total digestive tract in vitro in feed fermentation with the addition of nutmeg leaves tannin

Parameter	Feed treatments		
	P0	P1	P2
Rumen digestion			
DMD (%)	70.77±1.05[a]	59.03±3.24[b]	57.19±1.32[b]
OMD (%)	75.39±0.91[a]	55.55±6.29[b]	55.76±6.88[b]
CPD (%)	60.93±9.72[a]	45.48±5.12[b]	38.47±3.44[b]
Total digestion			
DMD (%)	74.88±5.28	67.70±3.21	64.83±4.96
OMD (%)	64.69±6.44	64.33±6.34	61.20±5.11
CPD (%)	62.96±5.12	52.12±6.82	47.42±6.21

P0: control treatment without tannin addition, P1: feed mixture + 2% nutmeg leaves tannin, P2: feed mixture + 4% nutmeg tannin.
[a, b] Different superscripts on the same row show significant differences (P<0.05); DMD: dry matter digestibility, OMD: organic matter digestibility, CPD: crude protein digestibility.

The results relate to the ability of nutmeg leaves tannin to bind to the protein in the feed, especially soybean meal in the rumen, and the post rumen digestive organs will break down the bond. Value of pH and affinity of tannins towards the macromolecules influence the bonds-breaking between tannins and proteins. Conditions close to the electrostatic pH of the protein trigger the formation of complex bonds between tannins and proteins, where the tannin hydroxyl group will bind to the protein carbonyl group. This binding is not only influenced by pH, but the presence of inorganic ions such as Ca, Mg, Na, and K will also accelerate its formation. Another factor that also affects binding is the structure of the flavanol subunit in tannins (Perez-Maldonado *et al.*, 1995).

The bonds formed between tannins and proteins are stable and difficult to degrade by microbes in the rumen, causing a decrease in protein digestibility in the rumen. Protein protected by tannins can be digested in the post rumen digestive organs. This was indicated by the absence of a significant difference (P>0.05) between the control treatment and the addition of nutmeg leaves tannin to total digestibility. Broderick *et al*. (1991) stated that under normal rumen pH conditions, the protein remains bound to tannins, but at low pH as in the abomasum, the protein will be released.

Decreased protein in vitro digestibility in the rumen with the addition of tannin also occurs in the addition of guava leaves by 0, 2, 4, and 6% where it reduced the digestibility of crude protein, with digestibility values are 70.49, 68.66, 66.61, and 61.49% (Rosita, 2015). Rochman *et al*. (2012) reported that the used tea grounds tannin was able to protect castor bean meal in vitro digestibility compared to control treatment without used tea grounds. The level of tannin addition were 0.25, 0.5, and 0.75% then significantly increased the total rumen undegraded protein (RUP).

Conclusions

The addition of nutmeg leaves tannin by 2 and 4% can reduce the digestibility of dry matter, organic matter and crude protein in the rumen, but did not show any significant difference in dry matter, organic matter, and crude protein in the total digestibility. The optimal level of nutmeg leaves tannin addition to reduce the digestibility of crude protein in the rumen without causing excessive negative impact on total in vitro nutrient digestibility was 2%, so that it can be used as protein protective agent in feed. Further research needs to be done to determine the effect of using nutmeg leaves as a source of tannin on livestock productivity in a way of in vivo, which observes the feed conversion and body weight gain.

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