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Effect of Cashew Nutshell Oil Supplementation as Phenol Source for Protein Protection on *In Vitro* Nutrient Digestibility

Rahma Fitriastuti*, Lies Mira Yusiati, Budi Prasetyo Widyobroto, Zaenal Bachruddin, and Chusnul Hanim

Faculty of Animal Science, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia

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* Corresponding author:
Telp. +62 82324257055
E-mail: rahma110589@gmail.com

ABSTRACT

The current study was aimed to evaluate the ability of phenol content in cashew nutshell liquid (CNSL) in binding protein and its effect on *in vitro* nutrient digestibility. This research divided into three steps, there were determination of total phenol and tannins content in CNSL, optimalization binding of CNSL tannin to *bovine serum albumin* with the determination of protein-percipitable phenolics compound and protein content using Lowry method, after that amount of optimalization supplementation of CNSL was used for measuring on *in vitro* nutrient digestibility by Tilley and Terry method. The results showed that phenol and tannin content of CNSL were 148.69 g/100 ml and 28.3 g/100 ml. One gram tannin in CNSL could be bound by 65.83 g protein. Optimum tannin were used in this study was 5.85 g tannin or equivalent to 163.91 mg of CNSL. Amount of CNSL were used in various levels, there are 0, 50, 100, 150 dan 200 mg. CNSL supplementation reduced dry matter, organic matter crude protein digestibility in rumen, and didn't reduce dry matter dan crude protein *in vitro* post rumen digestibility. Supplementation of CNSL increased feed efficiency on *in vitro*.

Keywords: Cashew nutshell liquid (CNSL), *In vitro* digestibility, Phenol, Tannin

Introduction

Cashew nutshell is an agricultural waste that has not been used by farmers. Cashew nutshell can be extracted into cashew nutshell liquid (CNSL) which contains phenolic compound (Trevisan *et al.,* 2006). Generally, phenolic compound consists of two types of tannin, namely tannin that is able to bind to and precipitate protein and non-tannin phenol that is able to bind to protein but unable to precipitate protein. Commonly, tannin contains more complex molecular weight and structure (Jayanegara *et al.,* 2011). There are many researches show that tannin is used as an agent in protecting feed protein due to its ability to bind to protein. The results of research (Beauchemin *et al*., 2007) show that adding tannin extract of *quebracho* plant as much as 1% dan 2% BK can decrease protein degradation in the rumen as much as 5% and 15% in the level of 1% and 2%. The existence of tannin content in the rumen is able to cause protein degradation into ammonia and is able to increase the efficiency of N use in the rumen. It is expected that decreased digestibility of dietary protein in the rumen is able to maintain the quality of high-protein feed sources to be more beneficial for cattle in the small intestine (Frutos *et al.,* 2004). This research is conducted to determine the binding of tannin in cashew nutshell liquid (CNSL) with optimal amount of protein and to increase the efficiency of the use of nutrients in the rumen without decreasing the digestibility of crude protein, dry matter, and organic matter in vitro.

Materials and Methods

This research was conducted in three steps, namely determining the total content of phenol, tannin, and non-tannin phenol in cashew nutshell liquid (CNSL); optimizing the binding of tannin in cashew nutshell to protein; and the effects of adding cashew nutshell liquid (CNSL) on the in vitro digestion of crude protein, dry matter, and organic matter. Material used in this research is cashew nutshell liquid (CNSL) as a source of phenol and tannin taken from Dompu, West Nusa Tenggara, while the source of protein used is Bovine Serum Albumin (BSA). Reagen used to determine the total of phenol and tannin content in cashew nutshell liquid (CNSL) is *folin-ciocialteu*, *sodium carbonate* 20%, *polyvinyl polypyrrolidone* (PVPP), and tannic acid as the standard. Reagan used to optimize the binding of tannin in cashew nutshell liquid (CNSL)to BSA protein is *acetate buffer* of pH 4.9, 1% of *sodium dodecyl sulfate*

(SDS) solution, *SDS-triethanolamine* (SDS-TEA), and *ferric chloride*.

Determining the total of phenol and tannin in cashew nutshell liquid (CNSL)

About 1,300 grams of cashew nutshell that has been cleaned was dried using oven at the temperature of 50ºC for 1 hour, then extracted using *hydraulic press* to obtain 250 ml of cashew nutshell liquid (CNSL).

Makkar (2000) method was used to determine the total of phenol and tannin contents. One milliliter of cashew nutshell liquid (CNSL) was added by 0.5 ml of *folin* and 2.5 ml of *sodium carbonate.* After then, it was conditioned into homogenous using vortex, incubated in a dark room for 40 minutes, and the results were read using spectrophotometer at a wavelength of 725 nm.

The content of non-tannin phenol was determined by adding 100 milligram of *polivinilpolipyroledone* (PVPP) then put into test tube and added 1 ml of aquades and 1 ml of extract. Cashew nutshell extract had a total phenol content of more than 10%, so it must be diluted and made into homogeneous by using vortex. The tube containing solution was incubated for 15 minutes in the refrigerator. The tube containing solution was homogenized and centrifuged at 3,000 grams for 10 minutes. Supernatant inserted into the test tube is used to determine non-tannin phenols as in the previous determination of phenol. The total tannin produced was obtained from total phenols minus non-tannin phenol.

Optimizing the binding of cashew nutshell liquid (CNSL) tannin to BSA protein

The second step is determining the binding of tannin and protein conducted using Makkar method (2000). Determination of tannin in the tannin-protein complex is done by using a solution that has been made into vortex and taken as much as 1 ml then put into a test tube, and then added by 3 ml SDS TEA (*Sodium Dodecyl Sulfate Triethanolamine*) and 1 ml of $FeCl_3$. The solution was made into vortex and incubated for 15 minutes then read by using spectrophotometer at a wavelength of 510 nm, while the supernatant was tested by using Lowry method using the BSA protein standard.

Digestibility of in vitro nutrient using Tilley and Terry method (1963)

According to the results of research in the second step, the level of cashew nutshell liquid (CNSL) added into digestibility of crude protein, dry matter, and organic matter was determined by *in vitro* method. This research was conducted for six times. The treatment was done by controlling without adding cashew nutshell liquid (CNSL) and by adding cashew nutshell liquid (CNSL) based on the optimization of the binding of cashew nutshell liquid (CNSL) to protein. *In vitro* nutrient digestibility in stage I was carried out for 48 hours. Rumen liquid was taken from two Bligon goats fed by adaptation to the proportion of forage: concentrate = 65 : 35 for one week. The fermentation medium was made of a mixture of rumen and saliva (McDougall) with a ratio of rumen: buffer = 1: 4. Rumen liquid and saliva were mixed and put into a water bath while flowing with CO_2 gas.

Medium was put into in vitro tube with the volume of 100 ml that had been filled by forage as a substrate. The substrate consists of king grass, peanut straw, pollard, and soybean, with the proportion of forage: concentrate = 65: 35 which contained 71.47% of total digestible nutrients (TDN), 42.75% of dry matter (BK), 13.96 % of crude protein (PK), 6.64% of ether extract (EE), and 28.27% of crude fiber (SK). Cashew seed oil was added to the soybean meal first then mixed with other feed ingredients.

About 500 mg of substrate was tested to know its crude protein digestibility and 250 mg of substrate was tested to know its dry matter and organic matter digestibility then covered with rubber plugs. After that, they were incubated at 39°C in a water bath for 48 hours and homogenized every 8 hours. Blanks were made for correction of digestibility but there was no addition of samples of feed ingredients. The digestibility test samples of dry matter and organic matter were taken from the solid phase by filtering using *Gooch crucible*, while the crude protein digestibility test was taken from the solid phase and filtered using filter paper then the crude protein is tested.

In vitro digestibility method *(*Tilley and Terry 1963) on step II was conducted for 72 hours to test crude protein, dry matter, and organic matter digestibility by adding HCl and pepsin to reduce pH into 3.5 as in abomasum. This was conducted to test the strength of tannin and protein bond whether it can be released at that level of pH, then the samples were incubated at 39°C in a water bath for 48 hours and homogenized every 8 hours. Blanks were made for correction of digestibility but there was no addition of samples of feed ingredients. The digestibility test samples of dry matter and organic matter were taken from solid phase by filtering using *Gooch crucible*, while the crude protein digestibility test was taken from the solid phase and filtered using filter paper then tested by proximate analysis of crude protein.

Data analysis

Data of the addition of cashew nutshell liquid to nutrient digestibility was analyzed by using variance analysis in a completely randomized design. Differences in mean values between treatments were further tested using *Duncan's new Multiple Range Test* (DMRT).

Result and Discussion

The total of phenol and tannin content in cashew nutshell liquid (CNSL)

The total of phenol and tannin content in cashew nutshell liquid (CNSL) is 148.69 g/100 ml and 28.3 g/100 ml. These results indicate that cashew seed liquid (CNSL) is rich in phenol. Cashew seed liquid (CNSL) is one of the sources of long chain phenol that is bound to C saturated and unsaturated fatty acids (*anacardic acid, cardanol, cardol*). Cashew seed liquid (CNSL) contains *meta alkylphenol* with various unsaturated benzene rings (Santos *et al*., 2011). Trevisan *et al*. (2006) state that the total of *alkylphenol* (*anacardic acid, cardol and cardanol*) in cashew nutshell liquid is 71.67 g/100 ml. The phenol content of plants depends on genetics, varieties, extraction methods, and the environment (Fratianni *et al*., 2007).

The binding of cashew seed shell liquid (CNSL) tannin to protein

The measurement of the amount of protein that can be bound by tannin is done by measuring the remaining BSA that is not bound by tannin using Lowry method. Based on the results of the measurement, the amount of protein bound by tannins can be calculated. The results of proteins bound by tannins can be seen in Table 1.

Table 1 shows that the ability of 1 gram of tannin in cashew nutshell liquid (CNSL) can bind to 65.83 grams of protein. This result is used to determine the level of addition of cashew nutshell liquid (CNSL) *in vitro*. A research done by Sasongko *et al.* (2010) shows that 1 gram of tannin in jackfruit leaf can bind 23.149 grams of protein.

Interaction between tannin and protein occurs through three types of bonds, namely hydrogen bond in tannin hydroxyl group, hydrophobic bond between aromatic structures of tannin and hydrophobic parts of protein, and covalent bond through oxidative polymerization reactions induced by heat, ultraviolet radiation, and enzyme action of polyphenol oxidase. (Silanikove *et al.,* 2001).

Polyphenol is able to form complex with protein by changing their structure or function, or both (Ozdal *et al.,* 2013). The difference in the binding ability to protein by phenolic compound depends on the type and chemical structure of phenolic compound and amino acids in the protein (Santos-Buelga and Freitas, 2009).

The more oil added, the more the amount of bound protein. However, at the level of 0.03 ml of extract solution, cashew nutshell liquid is unable to bind to protein. Adding too much phenol (tannin) source will cause tannin to be unable to bind to protein or to be already too saturated. This is in line with Michaelis-Menten equation that states that the reaction speed depends on substrate concentration. However, at high substrate concentration, the reaction speed is not affected by the concentration, and the reaction speed is already maximum (Nelson and Cox, 2004).

Tannin used to protect the high-protein feed ingredients in this study was soybean with PK of 38.54%, and the optimum amount of tannin used was 5.85 gram, that is equivalent to 163.91 mg of cashew nutshell liquid (CNSL). The basis of this addition is used for further research, with level below the optimum and above the optimum amount. The levels of addition of cashew seed shell liquid (CNSL) are 50, 100, 150 and 200 mg.

Digestibility of crude protein

The effects of adding cashew nutshell liquid (CNSL) on the *in vitro* digestibility of crude protein is presented in Table 2. Significant reduction in protein degradation in the rumen is caused by tannin effects. Tannin's affinity on protein is very big, and the rumen pH ranging from 4 to 7 is a suitable medium to form tannin-protein complex. Phenolic compound will interact with protein both reversibly and irreversibly. Phenolic group is a good hydrogen donor that will form hydrogen bond with carboxyl group of protein. This will affect protein structure and protein digestibility (Ozdal *et al.*, 2013). Tannin will bind to the feed protein, so it will reduce the degradation of protein in the rumen, and protein digestibility in the rumen is low. Meanwhile, in abomasum, tannin and protein bond will be released, so the digestibility of post-rumen protein increases (Silanikove *et al.*, 2001). Tannin binds to proteins with hydrogen bond that is sensitive to pH

Table 1. Protein that is bound to phenolic compounds (tannin) in cashew nutshell liquid (CNSL)

Tube	Extract Solution (ml)	The amount of bound protein		
		mg	mg/ml of liquid	g/g tannin
1	0.01	0.094	0.094	3.32
2	0.02	1.863	0.931	65.83
3	0.03	1.367	0.456	48.30

Table 2. The digestibility of crude protein from ration fermentation in the rumen and post-rumen by adding cashew seed shell liquid (CNSL)

Parameters	Levels of addition of cashew seed shell liquid (CNSL) (mg/kg BK)				
	0	50	100	150	200
KcPK rumen	61.94±5.13^{a}	57.66±5.07^{ab}	51.21±4.54^{c}	53.74±3.61^{bc}	50.09±5.08^{c}
KcPKpost- rumen	21.89±3.43^{b}	23.20±6.44^{b}	30.66±5.50^{a}	28.08±5.71^{ab}	30.25±4.41^{a}
Kc PK total^{ns}	86.67±3.29	85.01±4.63	87.02±5.93	86.95±5.72	82.48±3.63

^{ns}: No significant difference (P>0.05)

^{abc}: Different superscripts on the same line show significant differences (P<0.05).

changes. Tannin is able to bind to protein because of the hydrogen bond in OH group in tannin and its receptor group, namely the bond between NH and OH. Besides, the ionic bond between the anion group in tannin and cation in protein causes protein, that has amino acid composition with more than one NH group, will be able to bind more strongly to tannin (El-Waziry *et al*., 2005).

This study shows an increase in digestibility of post-rumen crude protein at the level of adding cashew nutshell liquid (CNSL) compared to the control group. This is because tannin and protein bond is released on post-rumen pH (pH less than 3), and the post-rumen digestibility of protein increases (Frutos *et al.*, 2004). The formation of protein and tannin complex is also influenced by pH of protein isoelectric point. Tannin is only able to bind to certain protein at certain level of pH (Naumann *et al.*, 2017).

Levels of 100 and 200 mg/kg of BK indicate a greater increase in post-rumen digestibility when compared to the levels of 150 mg/kg of BK. Adding cashew nutshell liquid (CNSL) did not show any significant difference in the digestibility of crude protein. Tannin content in cashew nutshell liquid binds to protein in the rumen, so there is a decrease in protein degradation in the rumen, and an increase in the post-rumen digestibility of crude protein. This is because tannin and protein bond is released on post-rumen bond, and the post-rumen digestibility of proteins increases (Frutos *et al.*, 2004). This is in line with a research by Coutinho *et al.* (2014) stating that adding cashew nutshell liquid (CNSL) up to 0.036% does not affect the crude protein digestibility. Shinkai *et al*. (2012) state that adding cashew nutshell liquid (CNSL) increases up to 5.7% of digestibility of crude protein compared to the control group.

Digestibility of dry matter and organic matter

The effects of adding cashew nutshell liquid (CNSL) on the in vitro digestibility of dry matter and organic matter is presented in Table 3 and 4. In this research, adding cashew nutshell liquid (CNSL) decrease the rumen digestibility of dry matter. It is caused by an addition of cashew nutshell liquid (CNSL) that can inhibit Gram-positive microorganisms in the rumen (Watanabe *et al*., 2010), and tannin content in cashew nutshell liquid (CNSL) can reduce the protein degradation in the rumen (Frutos *et al*., 2004).

Digestibility of dry matter in the post-rumen increases significantly, in proportion to the increase in the addition level of cashew nutshell liquid (CNSL), along with the increased level of cashew nutshell liquid (CNSL) added to 100 mg/kg of BK, but decreased at the addition level of 150 and 200 mg/kg of BK. This is because tannin and protein bond is released at acidic pH (pots-rumen). Meanwhile, there is a decrease at the level of 150 and 200 mg/kg of BK possibly due to some tannin bonds with other saturated nutrients that make them unable to release and disrupt the degradation of nutrients or dry matter. Protein can be degraded by digestive enzymes in the abomasum, increasing the digestibility of post-dried ingredients, organic matter, and crude protein.

The effect of adding plant metabolites in the rumen is a decrease in protein and soluble carbohydrate degradation, and inhibition of amino acid degradation through a selective mechanism in the rumen liquid microorganisms, especially bacteria (Hart *et al*., 2008).

In this research, the rumen digestibility of organic matter decreases, along with the level of addition of cashew nutshell liquid (CNSL). It is similar to the digestibility of dry matter in this study. Tannin prevents the attachment of microbes to the cell walls of feed ingredients that can reduce protein degradation. Tannin and protein complex makes the food containing carbohydrates and protein difficult to be degraded by rumen microbes (Frutos *et al*., 2004). Decreased digestibility of organic matter is caused by the inhibition of cashew nutshell liquid (CNSL) against Gram-positive bacteria (Watanabe *et al*., 2010).

Post-rumen digestibility of organic matter increases, along with the addition level of cashew nutshell liquid. This is because tannin and protein bond is released on the post-rumen pH, and post-rumen digestibility of protein increases (Frutos *et al.,* 2004). In this research, the total of digestibility

Table 3. Digestibility of dry matter from ration fermentation in the rumen and post-rumen by adding cashew nutshell liquid (CNSL)

Parameters	Levels of addition of cashew nutshell liquid (CNSL) (mg/kg BK)				
	0	50	100	150	200
KcBK rumen	70.45±2.81^{a}	66.01±2.65^{b}	59.08±3.98^{c}	55.07±2.12^{d}	51.15±2.94^{e}
KcBK post-rumen	3.58±1.63^{c}	5.70±2.52^{bc}	10.5±2.97^{ab}	12.89±8.73^{a}	16.5±5.82^{a}
KcBK total ^{ns}	74.03±2.37	71.71±3.24	69.58±4.70	67.96±8.47	67.65±6.78

^{ns}: No significant difference (P>0.05)

^{abc}: Different superscripts on the same line show significant differences (P<0.05)

Table 4. The rumen and post-rumen digestibility of organic matter from ration fermentation by adding cashew nutshell liquid (CNSL)

Parameters	Levels of addition of cashew nutshell liquid (CNSL) (mg/kg BK)				
	0	50	100	150	200
KcBO rumen	74.89±2.29^{a}	70.74±2.37^{a}	64.90±6.50^{b}	56.96±2.37^{c}	56.17±2.32^{c}
KcBO post-rumen	2.45±0.84^{c}	3.96±3.23^{ab}	4.55±1.77^{ab}	7.04±4.07^{a}	7.41±3.21^{a}
KcBO total	77.34±1.68^{a}	74.70±4.42^{ab}	69.45±7.99^{b}	63.99±3.12^{c}	63.57±2.58^{c}

^{ns}: No significant difference (P>0.05)

^{abc}: Different superscripts on the same line show significant differences (P<0.05).

of organic matter decreases. Shinkai *et al.* (2012) states that the decreased the rumen digestibility of dry matter and organic matter is caused by a decreased number of bacteria that plays a role as fiber-degrading bacteria (*B. fibrisolvens* and *Ruminococcus*).

Conclusions

Based on the results of research, it can be concluded that the total of phenol and tannin contents in cashew nutshell liquid (CNSL) respectively are 148.69 g/100 ml and 28.3 g/100 ml. In this case, 1 gram of tannin in cashew nutshell liquid (CNSL) is able to bind 65.83 grams of protein optimally, which is equal to 163.91 milligram of cashew nutshell liquid (CNSL) to protect high-protein food ingredients. Phenolic compound (tannin) of cashew nutshell liquid (CNSL) is able to bind to protein optimally in 150 mg/kg of BK, so it can reduce the rumen digestibility of dry matter, organic matter, and crude protein, but cannot reduce the post-rumen *in vitro* digestibility of dry matter and crude protein. Adding cashew nutshell liquid (CNSL) increases the efficiency of feed use on *in vitro* method.

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