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EXTRACTION OF TANNINS AND SAPONINS FROM PLANT SOURCES AND THEIR EFFECTS ON *In vitro* METHANOGENESIS AND RUMEN FERMENTATION

P. Yuliana[1], E.B. Laconi[1], E. Wina[2] and A. Jayanegara[1]
[1]*Faculty of Animal Science, Bogor Agricultural University,*
Jl. Agatis, Darmaga Campus, Bogor 16680 - Indonesia
[2]*Indonesian Research Center for Animal Production, Ciawi, Bogor 16002 - Indonesia*
Corresponding E-mail: anu_jayanegara@yahoo.com

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ABSTRAK

Penelitian ini bertujuan untuk mengekstrak tanin dari daun mahoni (*Swietenia mahagony*) dan saponin dari buah lerak (*Sapindus rarak*) menggunakan sejumlah pelarut, serta mengamati efek penambahan ekstrak terhadap fermentasi rumen dan metanogenesis secara *in vitro*. Pelarut yang digunakan untuk ekstraksi adalah air, metanol, aseton dan kombinasinya. Ekstrak tanin dan saponin ditambahkan pada botol inkubasi yang mengandung rumput *Brachiaria humidicola* dan legum *Indigofera* sp. (1:1 w/w) dengan perlakuan sebagai berikut (dalam empat ulangan): R1: kontrol, R2: R1 + 0,5 mg/mL ekstrak tanin, R3: R1 + 1 mg/mL ekstrak tanin, R4: R1 + 0,5 mg/mL ekstrak saponin, R5: R1 + 1 mg/mL ekstrak saponin, R6: R1 + 0,5 mg/mL ekstrak tanin + 0.5 mg/mL ekstrak saponin, dan R7: R1 + 1 mg/mL ekstrak tanin + 1 mg/mL ekstrak saponin. Hasil menunjukkan bahwa 75% air + 25% metanol merupakan pelarut terbaik untuk mengekstrak tanin dari daun mahoni sedangkan 100% metanol adalah yang terbaik untuk mengekstrak saponin dari buah lerak. Produksi gas tertinggi dan penurunan gas metana terbaik didapatkan dari perlakuan R7. Sebagai kesimpulan, kombinasi ekstrak tanin dan saponin berpotensi untuk menurunkan emisi metana dari rumen.

Kata kunci: tanin, saponin, metana, rumen, in vitro

ABSTRACT

This study was aimed to extract tannins from *Swietenia mahagony* and saponins from *Sapindus rarak* by using different solvents, and to test their extracts on *in vitro* rumen fermentation and methanogenesis. Solvents used for extraction were water, methanol, acetone and their combinations. Tannin and saponin extracts were added into each incubation bottle containing *Brachiaria humidicola* grass and *Indigofera* sp. legume (1:1 w/w) according to the following treatments (in four replicates): R1: control substrate, R2: R1 + 0.5 mg/ml tannin extract, R3: R1 + 1 mg/mL tannin extract, R4: R1 + 0.5 mg/mL saponin extract, R5: R1 + 1 mg/mL saponin extract, R6: R1 + 0.5 mg/mL tannin extract + 0.5 mg/mL saponin extract, and R7: R1 + 1 mg/mL tannin extract + 1 mg/mL saponin extract. Results revealed that 75% water + 25% methanol was the best solvent to extract tannins from *S. mahagony* whereas 100% methanol was the best to extract saponins from *S. rarak*. The highest gas production and the lowest methane emission were obtained in R7. It can be concluded that combination of tannin and saponin extracts were potential in mitigating ruminal methane emissions.

Keywords: tannin, saponin, methane, rumen, in vitro

INTRODUCTION

Methane (CH_4) is the second largest contributor of greenhouse gases after CO_2 in the atmosphere. Despite the fact, the ability of methane to retain heat is 21 times bigger than that

of CO_2. Ruminant livestock in particular is one of the contributors to the anthropogenic methane gas accumulation which is about 28% of the total methane (Beauchemin *et al.*, 2008). In addition to its impact on global warming, methane emission from ruminants is also a form of energy loss that would otherwise be used to support productivity; the amount of energy loss from ruminants is approximately 8-14% from the digestible energy intake (Cottle *et al.*, 2011).

An approach for mitigating methane emission from ruminants is through feeding strategy. A number of attempts have been conducted and shown to be effective in decreasing the methane emission (Cottle *et al.*, 2011). Accordingly, natural compounds are preferred especially after the ban of using antibiotics as feed additives in many countries. Plants of tropical origin are generally high in plant secondary compounds such as polyphenols (tannins) and saponins. Plants containing tannins and saponins can be used for mitigating enteric methane emission (Jayanegara *et al.*, 2013; 2014). However, there were limited studies that attempted to extract the compounds from plant sources and to further examine their effects on methane emission. Moreover, although some studies have tested such effects of tannins or saponins individually, none of them have combined both tannins and saponins simultaneously. It would be of interest to investigate whether the compounds interact each other, either synergistically or antagonistically, in mitigating methane emission or no interaction at all (additive effect only).

This study was aimed to extract tannins from *Swietenia mahagony* and saponins from *Sapindus rarak* by using different solvents. Subsequently, both extracts were tested using *in vitro* rumen fermentation system to observe their effects of rumen fermentation and methanogenesis.

MATERIALS AND METHODS

Extraction Procedures

The leaves of *S. mahagony* (source of tannins) and the fruits of *S. rarak* (source of saponins) were oven-dried at 50 ^{o}C for 12 h, and then ground to pass a 0.5 mm sieve. The materials were subjected to various solvent extractions, i.e. 100% water (P1), 75% water + 25% methanol (P2), 50% water + 50% methanol (P3), 25% water + 75% methanol (P4), 100% methanol (P5), 75% water + 25% acetone (P6), 50% water + 50% acetone (P7), 25% water + 75% acetone (P8), and 100% acetone (P9).

For extraction and quantification of tannins in *S. mahagony*, 10 mL of each solvent was inserted into a test tube containing 500 mg of the sample, then put into a beaker glass that was filled with water. The tube was then placed in an ultrasonic water bath (Barnstead/Lab Line Aqua Wave 9377, E60H, Germany) and extracted for 20 min at room temperature. Each sample was centrifuged (Thermo Scientific IEC Centra CL2 Centrifuge, Fisher Scientific Pte Ltd, Singapore) at 4^{o}C for 10 min; this procedure was repeated twice and the supernatants were combined. Total phenols (TP) and total tannins (TT) were measured according to Makkar (2003) by employing Folin-Ciocalteu method. Polyvinyl polypyrrolidone (PVPP) was used to separate tannin phenols from non-tannin phenols. The absorbance was read by using a UV-Vis spectrophotometer (UV-Vis spectrophotometer, U-1800, 5930482, High Technology Corporation, Tokyo, Japan) with a wavelength of 724 nm.

For extraction and quantification of saponins in *S. rarak*, 10 mL of each solvent was inserted into a test tube containing 500 mg of the sample, then put into a beaker glass that was filled with water. The tube was then placed in an ultrasonic water bath (Barnstead/Lab Line Aqua Wave 9377, E60H, Germany) and extracted for 20 min at room temperature. Each sample was centrifuged (Thermo Scientific IEC Centra CL2 Centrifuge, Fisher Scientific Pte Ltd, Singapore) at 4^{o}C for 10 min; this procedure was repeated twice and the supernatants were combined. Analysis of total saponins was performed according to Hiai and Nakajima (1976) and calibrated against Diosgenin standard (Sigma-Aldrich D1634, Sigma Aldrich Chemie GmbH, Steinheim, Germany). The sample was added with 0.2 mL vanillin, 0.25 mL ethanol and 2.5 mL 72% H_2SO_4, and then vortexed. The sample was heated in a water bath (Watson Victor Ltd., Bw6t, Watson Victor Limited, New Zealand) at 60^{o}C for 10 min. After being cool, the absorbance was read in UV-Vis spectrophotometer (UV-Vis spectro-photometer, U-1800, 5930482, High Technology Corporation, Tokyo, Japan) with a wavelength of 544 nm.

The solvents resulted in the highest tannin and saponin concentrations were further used for the *in vitro* rumen fermentation experiment. The

organic solvent was removed by using a rotary evaporator (Buchi Rotavapor R-200, Germany) followed by freeze drying (lyophilization) for 24 h to obtain dry extracts of tannins and saponins.

***In Vitro* Rumen Fermentation Procedures**

Substrate used in this study was a mixture of *Brachiaria humidicola* grass and *Indigofera* sp. legume. Both materials were dried at 50 ^{o}C for 12 h, ground to pass a 1 mm sieve and homogeneously mixed in the same ratio (DM basis). Chemical composition of the basal substrate is presented in Table 1.

The *in vitro* incubation was conducted by the method of Theodorou and Brooks (1990). Briefly, one gram of substrate was added into each incubation bottle. Rumen fluid was taken from two fistulated Friesian-Holstein cows before morning feeding, and then brought to the laboratory to be filtered and mixed with buffer solution (rumen fluid:buffer = 1:4 v/v). The buffer solution was comprised of 385.6 mL bicarbonate, 193.6 mL macro minerals, 0.256 mL micro minerals, 0.975 mL resazurine, 36.8 mL reducing solution and 579 mL distilled water. Rumen-buffer solution was saturated with CO_2 during the mixing process. Similarly, the incubation bottles were saturated with CO_2 before closing in order to ensure the anaerobic condition. An amount of 100 mL rumen-buffer solution was added into each incubation unit. Tannin and saponin extracts were added into each incubation bottle according to the following treatments: R1: control substrate, R2: R1 + 0.5 mg/mL tannin extract, R3: R1 + 1 mg/mL tannin extract, R4: R1 + 0.5 mg/mL saponin extract, R5: R1 + 1 mg/mL saponin extract, R6: R1 + 0.5 mg/mL tannin extract + 0.5 mg/mL saponin extract, and R7: R1 + 1 mg/mL tannin extract + 1 mg/mL saponin extract. The incubation bottles were then immediately closed with rubber caps. The bottles were incubated in a water bath at 39^{o}C for 48 h.

Total gas production was recorded at 4, 6, 9, 12, 24, 30, 36, and 48 h after incubation by using a gas syringe (Sigma-Aldrich Z314382-1EA, Poulten and Graf GmbH, Wertheim, Germany) equipped with an injected needle (BD REF Precision Glide Needle TM 302 008, Singapore). Measurement of methane production was conducted based on Fievez *et al.* (2005). Total gas produced was flown into 5 N NaOH. Carbon dioxide, the main gas produced during *in vitro* rumen fermentation, was bound by NaOH and methane volume was read in another syringe connected to the system.

Table 1. Nutrients Composition in Feed as Substrate (in % Dry Matter)

Nutrients	*Brachiaria humidicola*	*Indigofera* sp.	BH:I
CP	7.15	25.60	16.38
EE	1.25	2.51	1.88
NDF	82.14	53.66	67.90
ADF	38.04	49.05	43.55
Lignin	3.82	19.49	11.66
GE	4366	3579	3973

BH:I, *Brachiaria humidicola*:*Indigofera* sp. (1:1 w/w); CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; GE: gross energy

Statistical Analysis

The experiment on extraction of tannins and saponins was based on a completely randomized design, and each treatment was performed in three replicates. The experiment on *in vitro* rumen fermentation employed a randomized complete block design with four replicates, and each replicate was represented by two incubation bottles. Different batches of rumen fluid (runs) served as the block. Data obtained from both experiments were analyzed by analysis of variance (ANOVA). When a parameter showed significantly different at $P<0.05$ for various experimental treatments, Duncan's multiple range test was conducted for comparison among treatment means. All of the statistical analyses were performed by using SPSS statistical software version 16.0.

RESULTS AND DISCUSSION

Extraction of Tannins and Saponins

Total phenol and total tannin contents of *S. mahagony* leaves extracted with various solvents are presented in Table 2. Total tannins were highest when extracted with a mixture of solvents, i.e. 25% water + 75% methanol. Total phenol contents were also high when extracted with the solvent mixture. Apparently the compounds could

not be extracted optimally by using a single solvent only like water, methanol or acetone. Chemical structure of tannins contains both polar (hydrophilic) and non-polar (hydrophobic) groups; hydroxyl groups are polar and the aromatic phenolic structures are non-polar (Mueller-Harvey, 2006). Therefore, for extraction of tannins, mixtures of polar and less polar solvents are required. Mixture of water and methanol represented polar and less polar solvents, respectively. However, Makkar (2003) recommended the use of 30% water + 70% acetone to extract tannins from various plants sources. Apparently generalization of a certain optimum solvent composition to extract tannins from different plant sources was uneasy since tannins are quite a diverse chemical structure (Mueller-Harvey, 2006) and, hence, their chemical properties may differ from one to another. For instance, Iqbal *et al.* (2012) found that the use of high polarity solvent, i.e. methanol improved the recovery of total phenols in the extract. Further, the author stated that the efficiency of different solvents for phenols extraction was (ordered from highest to lowest): methanol > water > ethanol > acetone > chloroform > hexane.

Total saponin contents of *S. rarak* fruits extracted with various solvents are presented in Table 3. The best solvent to extract saponins from the fruits was 100% methanol; the yield of total saponins of *S. rarak* fruits extracted using 100% methanol was higher by about two fold as compared to the other solvents ($P<0.05$). Although chemical structure of saponins contains both polar (glycone) and non-polar (sapogenin) groups (Wina *et al.*, 2005), it seems that extraction of saponins did not need mixture of solvents like tannins. Based on these results, therefore, the respective solvents were used to extract the contents of tannins or saponins from *S. mahagony* and *S. rarak* for the subsequent *in vitro* rumen fermentation experiment.

Methanogenesis and Rumen Fermentation

Total gas production by addition of tannin and/or saponin extracts to basal substrate is presented in Figure 1. Addition of tannins and/or saponin extracts had no significant effects on total gas production at various time point intervals. Gas produced by rumen microbes during incubation partly was a product of microbial metabolism to digest and ferment feed or substrate, and also as a result of the buffering effect of artificial saliva (buffer solution) when the volatile fatty acids were produced (Getachew *et al.*, 1998). Such

Table 2. Total Phenol (TP) and Total Tannin (TT) Contents of *Swietenia mahagony* Leaves Extracted by Various Solvents (n=3)

Treatment	Solvent	TP (% DM)	TT (% DM)
P1	100% water	26.6^{b}	19.0^{b}
P2	75% water + 25% methanol	30.4^{bc}	22.0^{bc}
P3	50% water + 50% methanol	31.7^{bcd}	18.2^{b}
P4	25% water + 75% methanol	41.6^{de}	29.9^{c}
P5	100% methanol	31.0^{bcd}	17.1^{b}
P6	75% water + 25% acetone	43.1^{e}	26.7^{bc}
P7	50% water + 50% acetone	39.0^{cde}	25.7^{bc}
P8	25% water + 75% acetone	39.9^{cde}	26.9^{bc}
P9	100% acetone	3.7^{a}	2.3^{a}
	SEM	0.92	1.76
	P-Value	<0.001	<0.001

Different superscripts in the same column are significantlydifferent at $P<0.05$; DM: dry matter; SEM: standard error of the mean

Table 3. Total Saponin Contents of *Sapindus rarak* Fruits Extracted by Various Solvents (n=3)

Treatment	Solvent	Total saponins (% DM)
P1	100% water	19.6[b]
P2	75% water + 25% methanol	22.6[b]
P3	50% water + 50% methanol	23.4[b]
P4	25% water + 75% methanol	22.5[b]
P5	100% methanol	44.1[c]
P6	75% water + 25% acetone	20.4[b]
P7	50% water + 50% acetone	18.8[b]
P8	25% water + 75% acetone	24.0[b]
P9	100% acetone	12.6[a]
	SEM	3.30
	P-Value	<0.001

Different superscripts in the same column are significantly at P<0.05; DM: dry matter; SEM: standard error of the mean

results may suggest that the addition of tannin (from *S. mahagony*) and/or saponin (from *S. rarak*) extracts at a level of 0.5 or 1.0 mg/mL did not impair rumen fermentation. This was confirmed by the other rumen fermentation parameters, i.e. dry matter digestibility (DMD), organic matter digestibility (OMD), bacteria and protozoa population, and ammonia concentration, which were not significantly different from control (Table 4). Kinetics of methane concentration (%) in gas total is shown in Figure 2. In general, all treatments increased methane concentration during early incubation and then began to decline after 8 h and stabilized after 24 h. Apparently methanogens were more active during early incubation and less active as the fermentation substrate was depleted. The addition of *S. mahagony* and *S. rarak* extracts either single (R2-R5) or in combination (R6-R7) tended to decrease methane concentration as compared to control treatment (R1) at 48 h. The best treatment in lowering methane was shown by R7 (combination of tannin and saponin extracts at 1 mg/mL) which decreased the methane concentration by approximately 17%.

Dry matter digestibility (DMD) and organic matter digestibility (OMD) did not differ significantly among all treatments as shown in Table 4. Digestibility of nutrients can determine the quality of the feed. The higher the digestibility of a feed, the higher the chance of nutrient in the feed to be utilized by livestock. This was in agreement with a study reported that saponin extracts or plant containing saponins did not affect digestibility (Hess *et al.*, 2003), but decrease methane production (Santoso *et al.*, 2004). The addition of tannin extracts (R2-R3) numerically decreased digestibility of substrate since tannins exert anti-microbial actions in the rumen (Patra and Saxena, 2009), which may show adverse effects on rumen fermentation and digestion of feeds. Tannin and saponin extracts addition did not alter $N\text{-}NH_3$ concentration as compared to the control treatment. The range of $N\text{-}NH_3$ in the present study was between 24.92 to 30.89 mM, which was slightly above the optimum concentration to support microbial protein synthesis according to McDonald *et al.* (2002), i.e. 6-21 mM.

The addition of tannin and saponin extracts either in single or in combination tended to decrease rumen protozoa population; the best treatment was shown by R7. The existence of protozoa in rumen often disrupts the bacteria ecosystem because they have predatory activities against the bacteria. In addition, the presence of protozoa affects methanogens in the rumen since parts of the methanogens are in symbiosis with protozoa. Saponins may reduce methane emissions through elimination of rumen protozoa population due to the capacity of saponins in binding the sterol present in protozoa cell membrane, causing the cell lysis (Wina *et al.*, 2005; Beauchemin *et al.*, 2008). On the other hand, tannins act as anti-methanogenic compounds that directly influence the methanogens and indirectly by decreasing feed digestibility (Jayanegara et al., 2009; Jayanegara and Palupi, 2010) and, hence, limiting hydrogen supply for methanogenesis. Tannins also inhibit the growth of cellulolytic and proteolytic bacteria (McSweeney *et al.*, 2001).

CONCLUSION

Extraction of tannins and saponins from plants sources was strongly influenced by the type and composition of the solvents used. The optimal solvent to extract tannins from *S. mahagony*

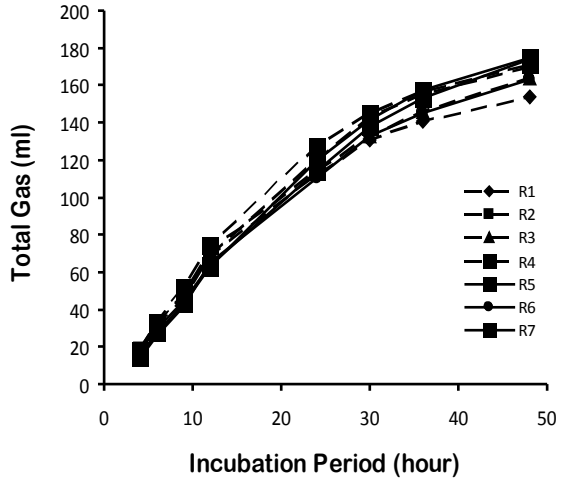

Figure 1. Total Gas Production of Added Tannin and Saponin Extracts in Subtrate when Incubated in Buffered-rumen Fluid. R1: control substrate, R2: R1 + 0.5 mg/mL tannin extract, R3: R1 + 1 mg/mL tannin extract, R4: R1 + 0.5 mg/mL saponin extract, R5: R1 + 1 mg/mL saponin extract, R6: R1 + 0.5 mg/mL tannin extract + 0.5 mg/mL saponin extract, and R7: R1 + 1 mg/mL tannin extract + 1 mg/mL saponin extract.

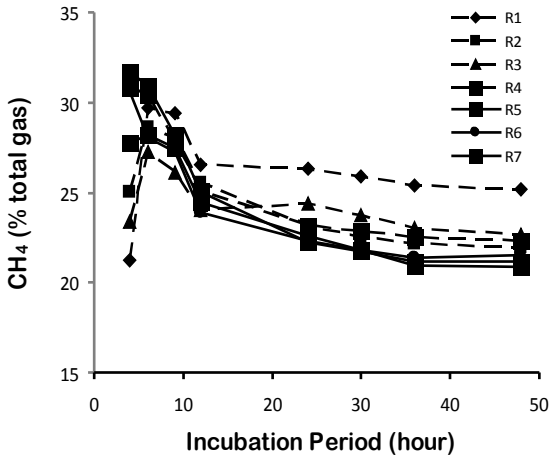

Figure 2. Methane Concentration of Added Tannin and Saponin Extracts in Subtrate when Incubated in Buffered-rumen Fluid. R1: control substrate, R2: R1 + 0.5 mg/mL tannin extract, R3: R1 + 1 mg/mL tannin extract, R4: R1 + 0.5 mg/mL saponin extract, R5: R1 + 1 mg/mL saponin extract, R6: R1 + 0.5 mg/mL tannin extract + 0.5 mg/mL saponin extract, and R7: R1 + 1 mg/mL tannin extract + 1 mg/mL saponin extract.

Table 4. Dry Matter Digestibility (DMD), Organic Matter Digestibility (OMD), Microbial Population and N-NH_3 Concentration of Added Tannin and Saponin Extracts in Subtrate when Incubated in Buffered-rumen Fluid at 48 h

Treatment	DMD (%)	OMD (%)	Bacteria (log cfu/ml)	Protozoa (log cell/ml)	N-NH_3 (mM)
R1	67.20	67.10	10.15	7.17	30.89
R2	64.02	63.79	9.92	7.08	26.30
R3	53.32	52.62	10.05	7.11	27.38
R4	59.96	59.39	9.98	7.15	25.93
R5	51.66	57.75	9.91	7.10	24.92
R6	64.06	64.79	9.92	7.15	25.22
R7	59.24	59.15	9.93	7.03	25.50
SEM	1.99	2.19	0.02	0.02	0.80
P-value	0.28	0.60	0.59	0.07	0.31

R1: control substrate, R2: R1 + 0.5 mg/mL tannin extract, R3: R1 + 1 mg/mL tannin extract, R4: R1 + 0.5 mg/mL saponin extract, R5: R1 + 1 mg/mL saponin extract, R6: R1 + 0.5 mg/mL tannin extract + 0.5 mg/mL saponin extract, and R7: R1 + 1 mg/mL tannin extract + 1 mg/mL saponin extract

leaves was 25% water + 75% methanol, while 100% methanol was optimal to extract saponins from *S. rarak* fruits. The addition of tannin and saponin extracts simultaneously in the basal substrate reduced methane emissions especially when added at 1 mg/mL. The treatment was able to reduce methane emission by 17% without reducing feed digestibility and ammonia concentration.

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