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Extracted Saponin from *Sapindus rarak* and *Hibiscus* sp. as an Additive in Cassava Leaf Silage: Effects on Chemical Composition, Rumen Fermentation and Microbial Population

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Abstract | This study aimed to evaluate the addition of saponin extracts from lerak fruit (*Sapindus rarak*) or hibiscus leaf (*Hibiscus* sp.) on chemical composition, *in vitro* rumen fermentation and microbial population of cassava leaf silage. Lerak and hibiscus extracts were added to cassava leaf by following a factorial design 2 × 3, in which the first factor was saponin source (*Sapindus rarak* or *Hibiscus* sp.) and the second factor was addition level (0, 2 or 4% of cassava leaf dry matter). Cassava leaf was then ensiled in a lab-scale silo (1 L capacity) for 30 d under room temperature. The silage was subjected to further chemical composition determination and *in vitro* rumen fermentation analysis, including rumen microbial population by using real-time PCR. Results showed thatthe chemical composition of cassava leaf silage added with various levels of lerak and hibiscus extracts showed significant differences (P<0.05) for a number of variables such as crude protein, ether extract and saponin. The addition of 4% lerak extract on cassava leaf increased significantly gas production after 24 and 48 h, and organic matter digestibility than that of control. The addition of lerak extract at 2% or 4% hibiscus extract in cassava silage significantly decreased (P<0.05) ruminal ammonia concentration. However, the addition of lerak or hibiscus extract to cassava leaf silage did not alter rumen microbial population. It is concluded that the addition of lerak extract at a level 4% to cassava leaf silage increases gas production, organic matter digestibility, and decrease ruminal ammonia concentration.

Keywords | Lerak, Hibiscus, Cassava, Silage, Rumen

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INTRODUCTION

Methane is among the main sources of green house gases in the atmosphere, precisely the second after carbon dioxide. Its global warming potential is more than 25 times greater than carbon dioxide (Tian et al., 2016). Livestock is a major source of methane emission through enteric fermentation, and it may account for 25% of the total methane emission (Hristov, 2003). Besides its effect on global warming, methane emission from ruminants also results in the loss of feed energy that would otherwise be used to support productivity. Enteric methane emission from ruminants represents approximately 5–9 % of dietary gross energy loss (Jeyanathan, 2014).

Several approaches have been studied to mitigate the enteric methane emission from ruminants by using natural bioactive compounds such as tannin, saponin, and essen-

tial oil (Cottle et al., 2011). The effects of plant secondary metabolites on methane emission have been reported previously (Kumar et al., 2014). These natural compounds secondary are preferred (Kondo et al., 2014) since many countries have prohibited the use of antibioticsas feed additives. Plants of tropical origin generally contain high concentration of natural compounds including saponin. Fruit of lerak (*Sapindus rarak*) and leaf of *Hibiscus* sp. have been known to contain a considerable amount of saponin. Saponin consists of fat-soluble nucleus with one part of the bond iseither steroid or triterpenoid (Cheok et al., 2014). The structure possesses by saponin has membranolytic activity and also has anti-bacterial, anti-tumor, anti-inflammatory properties in animals (Wojciechowski et al., 2016), and reduces methane emission (Rira et al., 2015). However, to our knowledge, there is no study so far attempted to compare between saponin extracts from both sources, i.e., lerak fruit (*Sapindus rarak*) and hibiscus leaf (*Hibiscus* sp.) regarding their effects on silage quality of cassava and rumen fermentation profiles.

This study aimed to evaluate the addition of saponin extracts from lerak fruit (*Sapindus rarak*) or hibiscus leaf (*Hibiscus* sp.) on chemical composition, *in vitro* rumen fermentation and microbial population of cassava leaf silage.

MATERIALS AND METHODS

Sample Preparation

Cassava leaves were collected from field research station of Faculty of Agriculture, Universiti Putra Malaysia, whereas *Sapindus rarak* fruit and *Hibiscus* sp. leaves were obtained from Bogor Agricultural University, Indonesia. Extraction of saponin was according to Wina et al. (2005); the extract was subsequently lyophilized to obtain a powdered form. The extracts were added to cassava leaf by following a factorial design 2 × 3, in which the first factor was saponin source (*Sapindus rarak* or *Hibiscus* sp.) and the second factor was addition level (0, 2 or 4% of cassava leaf dry matter). Cassava leaves were then ensiled in a lab-scale silo (1 L capacity) for 30 d under room temperature. The silage was subjected to further chemical composition determination and *in vitro* rumen fermentation analysis.

Determination of Chemical Composition

Silage samples were subjected to analysis of crude protein (CP), ether extract (EE) and crude fiber (CF) according to the standard procedures of AOAC (AOAC, 1990). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Van Soest et al. (1994). Measure of total saponin was done in accordance with the method of Hiai and Nakajima (1976) and calibrated against diosgenin standard (Sigma-Aldrich D1634, Sigma Aldrich Chemie GmbH, Steinheim, Germany). A pH meter was used to determine pH value of the silage.

In vitro Rumen Fermentation Procedure

Dried and ground forms of cassava leaf samples were incubated *in vitro* with buffered rumen fluid according to the procedure of Menke and Steingass (1988). The rumen fluid was obtained from two fistulated Brahman-cross cattle prior to morning feed.It was filtered and mixed with buffer solution in a ratio of rumen fluid: buffer 1:4 v/v. The incubation was performed in four replicates by employing a randomized complete block design. Total gas generated was recorded at 0, 3, 6, 9, 12, 24, 30, 36, and 48 h.The kinetic parameters of *in vitro* cumulative gas produced were estimated as per Ørskov and McDonald (1979). Rumen fluid pH was determined by a pH meter while the ammonia nitrogen (NH_3-N) concentration was measured according to Parsons et al. (1984). Volatile fatty acids (VFA) were determined by employing a gas chromatograph (Hewlett Packard 6890 GC system) according to the procedure of Cottyn and Boucque (1968). The amount of methane produced was estimated by employing the stoichiometrical equation of Moss et al. (2000).

Microbial Population Analysis using Real Time PCR

The rumen fluid sample was extracted using a tool kit extractor (Qiagen Inc., Valencia, USA). The target of rumen microbial population, primer sequences of DNA microbial, annealing temperature and references in this study were provided in Table 1. The real time PCR was performed with Bio Rad CFX96 real-time PCR system (Bio Rad, USA) using an optical grade plate. An amount of 25 µl Quanti Fast® SYBR® Green PCR kit (Qiagen Inc., Valencia, USA), which included 12.5 µl of 2x SYBR Green Master Mix, 1 µl of 10 µM forward primer, 2 µl of DNA sample and 8.5 µl nuclease-free water for each reaction were analyzed in duplicate. To prevent contamination for each sample, a number of plate control was established in the real-time PCR amplification to rule. The real-time cycle condition had been set up with annealing temperature of 94°C for 5 min for initial denaturation, then 40 cycles at 94°C for 20 s. The total annealing for total bacteria, methanogen, total protozoa, *Fibrobacter succinogenes, Ruminococcus albus*, and *Ruminococcus flavefaciens*was performed at 60°C and extension at 72°C to 20 s (Navidshad et al., 2012).

Statistical Analysis

The obtained data were subjected to analysis of variance (Steel and Torrie, 1993). When a parameter exhibited a significance at $P<0.05$ among the experimental treatments, Duncan's multiple range test was performed to compare the different treatments. The statistical analysis was determined by using SPSS statistical software version 22.0.

Table 1: Sequence of primers used for targeting total bacteria, total protozoa, *Fibrobacter succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens* and methanogen

Targeted microbe	Sequence 5'- 3'	Annealing Temperature (°C)	Reference
Total bacteria	F-CGGCAACGAGCGCAACCC R-CCATTGTAGCACGTGTGTAGCC	55	Koike and Kobayashi (2001)
Methanogen	F-CCGGAGATGGAACCTGAGAC R-CGGTCTTTGCCCAGCTCTTATTC	55	Zhou *et al.* (2009)
Total protozoa	F-CTTGCCCTCYAATCGTWCT R-GCTTTCGWTGGTAGTGTATT	55	Sylvester *et al.* (2004)
F. succinogenes	F-GTTCGGAATTACTGGGCGTAAA R-CGCCTGCCCTGAACTATC	55	Lane (1991)
R.albus	F-CCCTAAAAGCAGTCTTAGTTCG R-CCTCCTTGCGGTTAGAACA	55	Koike and Kobayashi (2001)
R. flavefaciens	F-TCTGGAAACGGATGGTA R-CCTCCTTGCGTTAGAACA	60	Koike and Kobayashi (2001)

Table 2: Chemical composition of cassava leaf silage added with saponin extract (% dry matter)

Parameter	Saponin source	Level (% dry matter)			SEM	*P*-value
		0	2	4		
CP	Lerak	31.87^{a}	27.81^{a}	32.87^{a}	0.874	<0.001
	Hibiscus	32.41^{a}	28.84^{a}	38.41^{b}		
EE	Lerak	3.33^{a}	5.76^{c}	5.38^{bc}	0.237	0.019
	Hibiscus	4.10^{ab}	5.04^{bc}	5.68^{bc}		
CF	Lerak	14.32	16.61	15.49	0.573	0.363
	Hibiscus	15.68	12.37	15.68		
NDF	Lerak	46.69	48.09	40.37	2.120	0.326
	Hibiscus	49.23	54.67	39.40		
ADF	Lerak	29.84	30.78	25.49	1.145	0.541
	Hibiscus	32.07	26.34	27.56		
pH	Lerak	4.00^{a}	4.48^{b}	4.92^{c}	0.081	0.002
	Hibiscus	4.47^{a}	4.56^{b}	4.51^{b}		
Saponin	Lerak	0.24^{a}	3.10^{b}	6.01^{c}	0.468	<0.001
	Hibiscus	0.17^{a}	0.17^{a}	2.98^{b}		

Different superscripts within the same parameter are significantly different at P<0.05.
CP: crude protein, EE: ether extract,CF: crude fiber, NDF: neutral detergent fiber, ADF: acid detergent fiber, SEM:standard error mean.

Table 3: The effect of saponin extract addition on *in vitro* gas production and digestibility of cassava leaf silage

Parameter	Saponin source	Level (% dry matter)			SEM	*P*-value
		0	2	4		
Gas 24 h (ml)	Lerak	24.53^{b}	21.19^{a}	27.01^{c}	0.589	0.002
	Hibiscus	24.63^{b}	22.32^{a}	26.86^{b}		
Gas 48 (ml)	Lerak	29.60^{b}	23.72^{a}	32.93^{c}	0.722	0.002
	Hibiscus	29.20^{b}	29.16^{b}	29.62^{b}		
b (ml)	Lerak	34.17^{b}	28.32^{a}	32.83^{b}	0.589	0.056
	Hibiscus	32.30^{b}	32.54^{b}	34.24^{b}		
c (/ml)	Lerak	0.07	0.07	0.07	0.722	0.354
	Hibiscus	0.06	0.06	0.85		

IVOMD (%)	Lerak	54.45[b]	49.76[a]	59.21[c]	0.603	<0.001
	Hibiscus	54.17[b]	50.49[a]	55.81[b]		

Different superscripts within the same parameter are significantly different at P<0.05.

b: Potential gas production, c: Gas production rate constant, IVOMD:*in vitro* organic matter digestibility, SEM:standard error mean.

Table 4: The effect of saponin extract addition on ruminal volatile fatty acid profile of cassava leaf silage

Parameter	Saponin source	Level (% dry matter)			SEM	*P*-value
		0	2	4		
TVFA	Lerak	53.84	52.59	56.71	2.098	0.798
(mM)	Hibiscus	56.74	49.69	54.00		
C_2 (%)	Lerak	67.88[a]	69.70[c]	66.86[a]	0.264	<0.001
	Hibiscus	68.32[b]	70.16[c]	69.02[b]		
C_3 (%)	Lerak	18.13[c]	17.43[b]	17.62[b]	0.141	0.035
	Hibiscus	18.16[c]	17.15[a]	17.30[a]		
$IsoC_4$ (%)	Lerak	1.46[b]	1.36[a]	1.53[b]	0.024	0.022
	Hibiscus	1.46[b]	1.29[a]	1.53[b]		
C_4 (%)	Lerak	8.65[a]	8.10[a]	9.86[b]	0.172	0.007
	Hibiscus	8.27[a]	8.17[a]	8.17[a]		
$IsoC_5$ (%)	Lerak	2.64[b]	2.33[a]	2.81[b]	0.062	0.012
	Hibiscus	2.65[b]	2.19[a]	2.72[b]		
C_5 (%)	Lerak	1.21[b]	1.06[b]	1.30[c]	0.031	0.017
	Hibiscus	1.12[b]	1.02[a]	1.24[b]		

Different superscripts within the same parameter are significantly different at P<0.05.

TVFA: total volatile fatty acid, C_2: acetate, C_3: propionate,$IsoC_4$: isobutyrate, C_4: butyrate, $IsoC_5$: isovalerate, C_5:valerate, SEM:standard error mean.

Table 5: The effect of saponin extract addition on ruminal pH, ammonia, and methane formation of cassava leaf silage

Parameter	Saponin source	Level (% dry matter)			SEM	*P*-value
		0	2	4		
pH	Lerak	7.3[a]	7.5[b]	7.5[b]	0.043	0.007
	Hibiscus	7.4[a]	7.5[b]	7.5[b]		
NH_3 (mM)	Lerak	17.3[b]	17.8[b]	14.2[a]	0.369	0.007
	Hibiscus	17.3[b]	15.3[a]	16.9[b]		
CH_4	Lerak	22.65[a]	22.98[b]	22.64[a]	0.069	0.002
(%TVFA)	Hibiscus	22.66[a]	23.07[b]	22.90[b]		

Different superscripts within the same parameter are significantly different at P<0.05.

NH_3: ammonia, CH_4: methane, TVFA: total volatile fatty acid, SEM: standard error mean.

Table 6: The effect of saponin extract addition on ruminalmicrobial population of cassava leaf silage (log cell/ml)

Parameter	Saponin source	Level (% dry matter)			SEM	*P*-value
		0	2	4		
Total bacteria	Lerak	10.6	10.5	10.6	0.042	0.699
	Hibiscus	10.4	10.4	10.5		
R. flavefaciens	Lerak	4.62	4.89	4.91	0.070	0.437
	Hibiscus	4.52	4.78	4.76		
R. albus	Lerak	6.47	6.39	6.30	0.193	0.844
	Hibiscus	6.36	5.76	6.34		
F. succinogenes	Lerak	6.69	6.47	6.81	0.121	0.659

	Hibiscus	6.72	6.78	6.27		
Total protozoa	Lerak	5.02	5.75	6.03	0.166	0.141
	Hibiscus	5.07	5.02	5.34		
Methanogen	Lerak	6.70	6.61	6.65	0.037	0.630
	Hibiscus	6.56	6.48	6.56		

Different superscripts within the same parameter are significantly different at P<0.05.
SEM: standard error mean.

RESULTS AND DISCUSSION

The chemical composition of cassava leaf silage added with various levels of lerak and hibiscus extracts showed significant differences (P<0.05) for a number of variables such as CP, EE and saponin (Table 2). The nutrient contents of the experimental silages were quite diverse due to different saponin level addition. All silages in this study had higher CP content than the minimum 6-7% as required for effective rumen function (Milford & Haydock, 1965). The increase in CP content of cassava leaf silage after adding lerak extract was expected because the lerak extract contained a small amount of protein and fat. Higher level of saponin in the cassava leaf silage added with lerak extract was expected due to the high content of saponin present in lerak. This is in agreement with Wina et al. (2005) who observed that lerak fruit extract contained 48-87% saponin. This also indicates that saponin isrelatively resistant to degradation during the ensiling process.

The addition of 4% lerak extract on cassava leaf significantly increased (P<0.05) gas production after 24 and 48 h as compared to the control (Table 3). The addition of 4% lerak extract also, siginificantly increased the organic matter digestibility of cassava leaf silage than that of control. Total gas production in all treatments increased with the increasing incubation time. Gas production will continue to increase as long as the microbial substrate is still available. The gas production during *in vitro* rumen incubation is a product of microbial metabolism in degrading feed, and in addition, it isalso as a result of the buffering effect of artificial saliva (buffer solution) when VFA is produced (Getachew et al., 1998). The increase in gas production after the addition of lerak extract is possiblesince some saponin is cleaved to aglycon and glycon (sugar) component, and then the sugar component is metabolized by the microbes to generate gas (Patra et al., 2010). Gas production is positively correlated with organic matter digestibility because both parameters reflect the level of feed degradation in the rumen (Jayanegara et al., 2016).

Addition of lerak extract did not increase propionate proportion of cassava leaf silage (Table 4). This was in contrast to some other related studies which an increase in propionate proportion from the total SCFA production with increasing levels of the saponins (Jayanegara et al., 2014), that saponin generally increases propionate in the rumen. Propionate is related to methane emission since both products require H_2 for their synthesis in the rumen system, and hence compete for similar substrate. Methane was also did not decrease by addition of lerak or hibiscus extract (Table 5). Methane is produced in the rumen by methanogen which possesses enzyme system to use H_2 and combined with CO_2 to form methane (Morgavi et al., 2011). Saponin addition has been known to increase proportion of propionate and its respective ratio to total VFA in the rumen particularly when saponin is given in high concentration (Goel et al., 2008). The CO_2, CH_4, and volatile fatty acids (VFA) are the final products of rumen fermentation, and the VFA is a major energy source for ruminant (Banik et al., 2013).

The addition of lerak extract at a level 4% or 2% hibiscus extract in cassava silage significantly decreased (P<0.05) ruminal ammonia concentration. Ammonia concentration produced from all treatments ranged between 14.2 and 17.8 mM and these values are considered to be satisfactory for rumen microbial growth. McDonald et al. (2002) stated that the optimum ammonia concentration to support microbial protein synthesis in rumen fluid varies widely, ranging from 6 to 21 mM. A common observation about saponin is its typical effect to decrease NH_3 concentration in the rumen (Goel et al., 2008). Supporting the current finding, Lila et al. (2005) observed that the administration of saponin reduced NH_3 concentration and accompanied with an increase of total VFA and propionate concentration. Hu et al. (2005) also observed a decrease of 27% NH_3 proportion and an increase proportion of propionate by giving saponin from tea at 8 mg/200 g of feed under *in vitro* rumen fermentation.

Addition of lerak or hibiscus extract to cassava leaf silage in the present study did not alter microbial population in the rumen *in vitro*, i.e. total bacteria, *R. flavefaciens*, *R. Albus*, *F. succinogenes*, total protozoa and methanogen (Table 6). This is in contrast to some other findings that saponin or plant extract rich in saponin generally has a defaunation activity against protozoa and affects the H_2 pathway so that it could not be used by methanogen (Johnson & Johnson, 1995). Saponins has the capability to bind the sterol component found in the protozoa cell membrane and may cause cell lysis (Wina et al., 2005). Supplemen-

tation of *Sesbania sesban* leaves that rich in saponin was able to increase the flow of protein from rumen by pressing the existing protozoa (Newbold et al., 1997). Methane production in the rumen is very dependent on the interaction level among methanogens and rumen protozoa, as well as the methane production level per methanogen cell (Machmuller et al., 2003). Methane can also be influenced by saponin as a result of a decrease in the rate of methanogenesis with decreasing methane-producing gene activity without changing the total population of methanogen (Hess et al., 2003).

CONCLUSION

Addition of lerak extract at a level 4% to cassava leaf silage can increase gas production, organic matter digestibility, and decrease ruminal ammonia concentration.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Pristian Yuliana conducted research, analyzed the data and wrote the manuscript. Erika B. Laconi supervised the experiment, Anuraga Jayanegara supervised the experiment, analyzed the data and revisedthe manuscript, Suminar S. Achmadi revised the manuscript. Anjas A. Samsudin supervised the analysis of rumen microbial population using Real Time PCR and revised the manuscript.

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