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Evaluation of Local Tropical Plants by *In vitro* Rumen Fermentation and Their Effects on Fermentation End-Products

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Abstract: Seventeen local feed resources were collected and analyzed for chemical composition and were incubated in *in vitro* fermentation using gas technique. Based on the condensed tannins (CT) values, these feed resources could be divided into three groups: high (11.4-16.8%), medium (2.1-4.6%) and low (0.7-1.7%). Mangosteen peel had the highest and Pak Kayaeng had the lowest in terms of CT value. All samples were added with rumen fluid mixed with artificial saliva and incubated in *in vitro* gas fermentation for 48 h during which gas production and volatile fatty acids (VFAs) were measured. There were significant differences ($P< 0.001$) in gas production during 48 h incubation. Gas production was lower in the group having the highest level of CT, from 32.2 to 181.9 ml/gDM. There were significant differences ($P<0.05$) in total VFAs (from 48 to 88 mmol/L^{1}), individual VFA production and acetate to propionate ratio (from 1.6 to 4.6), however the values were respectively variable. Propionate production tended to be higher in the group with higher CT. The correlation coefficients (*r*) were relatively low between gas production after 48 h incubation with total VFAs (0.39), acetate (0.16) and butyrate (0.05) production. Furthermore, negative correlations were obtained between gas and propionate production (-0.20).

Key words: Local feed resources, *in vitro* gas technique, condensed tannins, saponins

Introduction

Feed resources for ruminant production in the tropics are becoming increasingly important because of rising costs and limited supplies. This is especially critical during the time of feed shortage in the dry season and also during the hectic season of cultivation where labor is limited and paddy fields are being cropped (Wanapat, 1986; Wanapat and Devendra, 1992). Local feed resources such as cassava (root/hay/silage), corn stovers, kapok meal, baby corn, cow-pea, cotton seed meal, leuceana leaves, sweet potatoes, sugarcane, sesbania (seed/leaves), mulberry leaves, moringa seed, sapindus fruit etc, are potential as ruminant feeds to improve and increase the efficiency of the production system. However, some of these feeds contain secondary plant compounds namely condensed tannins, saponins, gossypol, mimosine, and trypsin inhibitor, which may diminish the effects of these feedstuffs with respect to feed quality and animal production. Therefore, the objective of this study was to investigate condensed tannins (CT) and/or crude saponins (CS) concentrations in local plants and their relationship with the fermentation end-products by using the *in vitro* gas technique.

Materials and Methods

Experimental design and feed samples: A Completely randomized design (CRD) with seventeen treatments and three replicates per treatment was used. Seventeen kinds of local plants were used as substrates in this experiment. All samples were collected at their mature physiological stages, Samples were dried in a 60 ^{o}C forced air oven then ground to pass a 2 mm sieve and used for chemical analysis and *in vitro* gas production. Local plant materials investigated were as follows:

1. Fresh banana *(Musa sapientum),* Fruit
2. Indian mulberry *(Morinda citrifolia*), Fruit
3. Bitter cucumber *(Mormormdica charantia)*, Fruit
4. Siam neem tree *(Azadirachta indica)*, Leaf
5. Sugar apple *(Annona squamosa)*, Leaf
6. Guava *(Psidium guajava)*, Leaf
7. Mangosteen *(Garcinia mangostana)*, Peel of fruit
8. Sesbania *(Sesbania grandoflora*), Leaf
9. Cassava hay *(Manihot esculenta* Crantz)
10. Banana *(Musa sapientum),* Flower
11. Mulbery *(Morus indica)*, Leaf
12. Pak Kayaeng; Paddy rice weed (*Limnophila aromatica*), Leaf
13. Bai Yanang, Leaf (*Tiliacora triandra*)
14. Coral, Leaf (*Erythrina variegate)*
15. Star gooseberry, Leaf (*Phyllanthus acidus)*
16. Banana *(Musa sapientum),*Leaf
17. Rice straw (*Oryza saliva*)

***In vitro* gas production:** The method used for *in vitro* fermentation was based on the technique described by

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Menke *et al.* (1979) with some modifications. Two hundred milligrams of feed samples were weighed into 60 ml plastic syringes with pistons lubricated with vaseline. Buffered mineral solution was prepared and placed on a magnetic stirrer at 39°C under continuous flushing with CO_2. Rumen fluid was collected before the morning feeding from two ruminally fistulated steers fed on rice straw as roughage. Rumen fluid was taken from the middle part of the rumen by using a 60-ml hand syringe and transferred into two pre-warmed thermos flasks, combined, filtered through three layers of cheesecloth and flushed with CO_2. About 30 ml of buffered rumen fluid was taken into syringes containing the feeds. After closing the three way clips, the syringes were gently swirled and tubes opened to remove gas by pushing the piston upwards to achieve complete removal. The clip was closed, this initial volume recorded, and syringes placed in an incubator at 39°C. Gas production rates were recorded at 2, 4, 6, 12, 18, 24 and 48 h of incubation and each syringe was gently swirled after reading. At 2, 4, 6 and 48 h of incubation, the fluid samples were drawn into plastic bottles where 3 ml of H_2SO_4 solution (1M) were added to 30 ml of sample. The mixture was centrifuged at 16,000 x *g* for 15 minutes and the supernatant stored at –20°C prior to VFA analysis using High performance liquid chromatography (HPLC; Model Water 600; UV detector, Millipore Crop.), according to the method of Samuel *et al.* (1997). Rate and extent of gas production were determined for each substrate by fitting gas production data to the non-linear equation $Y = b\ (1\text{-}e^{-ct})$ (Orskov and McDonald, 1979), where Y is the volume of gas production at time t, b the potential gas production(ml g^{-1}DM), and *c* the fraction rate of gas production (h^{-1}).

Chemical analysis: Substrates were analyzed for DM, Ash, CP, P, K, Ca, Mg and S using the procedure of AOAC (1990), and NDF and ADF according to Goering and Van Soest (1970). Condensed tannins (CT) were estimated by the Vanillin-HCl method modified by (Wanapat and Poungchompu, 2001) and saponins were measured by using methanol extraction (Kwon *et al.*, 2003).

Statistical analysis: The means of each parameter measured in the digestibility studies, nutrient intake and rumen microorganisms were analyzed by the analysis of variance (ANOVA) techniques using the General Linear Model (GLM) procedures of the Statistical Analysis System Institute (1998). Mean separations with a significant F (P<0.05) for treatment were statistically compared using Duncan's New Multiple Range Test (DMRT) (Steel and Torrie, 1980).

Results and Discussion

Chemical composition of feeds: The chemical composition of the feedstuffs are shown in Table 1. The CP content ranged from 1.3 % in bitter cucumber to 28.0% in sesbania leaf. More than half the samples had CP, NDF and ADF contents ranging from 10 to 20%, 27.4 to 85.6% and 11.3 to 53.8%, respectively. P, K, Ca, Mg and S contents ranged from 0.03 to 0.48%, 0.85 to 6.29%, 0.06 to 2.56%, 0.04 to 0.51% and 0.05 to 0.43%, respectively. Feed resources could be divided into three groups: high, medium and low, depending on CT concentration, ranging from 11.4 to 16.8%, 2.1 to 4.6% and 0.7 to 1.7%, respectively. Mangosteen peel had the highest values of CT and crude saponins (CS).

***In vitro* gas production:** There was a significant difference (P<0.001) in gas production among substrates (Table 2). Gas production was lower in groups with had higher levels of CT, ranging from 32.2 to 181.9 ml g^{-1} DM. There was a wide range in potential gas production (b), while rates of gas production ranged from 0.001 h^{-1} in rice straw to 0.083 in Indian mulberry fruit (Table 2). *In vitro* gas production values at 48 h from this experiment were lower than in previous studies (Menke *et al.*, 1979; Liu *et al.*, 2002; Getachew and Makkar, 2002). Nevertheless, this result could be due to differences in chemical compositions of the feeds used, especially CP and NDF contents.

Volatile fatty acids (VFA) production: The results of VFA production are shown in Tables 3 for 48 h of incubation. There were significant differences (P<0.05) in total (from 48 to 88 mmol/L^{1}) and individual VFA production and acetate to propionate ratio (from 1.6 to 4.6) among substrates. However, propionate production was slightly higher in the group higher CT. Based on this study, the VFAs especially proportion of acetate, propionate and butyrate were different among substrates. The proportion of propionate production was slightly higher and C2/C3 ratio was lower in the group whit higher CT. This result could have been due to differences in the chemical composition of the feeds, particularly CT content. The effect of CT on TVFAs and molar proportions of individual VFA production could have been resulted from reduced protozoal and increased bacterial populations, since acetate and butyrate are the major fermentation end-products of protozoa (Jouany, 1994) and agrees with reports by Getachew and Makkar (2002).

Relationship between gas and VFA production: The relationships between gas and TVFs and individual VFA production are shown in Table 4. The correlation coefficients (r) were relatively low between gas production after 48 h incubation and with total VFA (0.39), acetate (0.16) and butyrate (0.05) production. Moreover, negative correlation was obtained between gas and propionate production (-0.20). Overall, there were no

Table 1: Chemical composition of local feed resources used in *in vitro* gas production incubated in rumen fluid mixed with artificial saliva

Substrate	DM	CP	Ash	NDF	ADF	P	K	Ca	Mg	S	CT[1]	CS[2]
	%	%DM										
Mangosteen peel	93.0	18.3	2.8	56.8	51.3	0.03	1.23	0.16	0.04	0.05	16.8	10.0
Guava leaf	91.0	10.1	6.4	54.0	29.1	0.12	1.67	0.95	0.25	0.10	15.8	2.8
Siam neem leaf	89.9	14.9	6.3	52.1	30.0	0.12	1.43	1.47	0.40	0.14	11.4	2.8
Sesbania leaf	89.5	28.0	10.3	27.4	14.6	0.30	2.93	1.51	0.46	0.22	4.6	2.0
Sugar apple leaf	90.1	18.6	7.7	49.9	23.1	0.14	1.80	1.99	0.23	0.12	3.8	n.d
Star gooseberry leaf	89.3	17.4	7.4	54.1	28.9	0.17	1.81	1.34	0.35	0.25	3.4	n.d
Coral leaf	87.2	19.2	11.9	50.5	31.1	0.24	0.96	2.56	0.38	0.20	2.6	1.8
Bai yanang	90.5	16.4	7.1	62.3	37.1	0.16	1.57	0.94	0.21	0.20	2.3	1.3
Cassava hay	88.9	21.7	9.9	54.0	31.2	0.17	0.85	1.33	0.51	0.16	2.2	1.7
Bitter cucumber	85.8	1.3	8.8	50.9	30.1	0.48	3.29	0.20	0.26	0.12	2.1	4.1
Banana leaf	89.4	13.8	10.1	78.2	35.6	0.28	3.41	0.27	0.25	0.23	1.7	1.3
Mulberry leaf	87.1	15.2	10.8	56.6	18.6	0.21	1.36	1.82	0.27	0.12	1.6	2.3
Pak kayaeng	87.6	14.9	16.7	63.1	53.8	0.16	2.04	1.34	0.37	0.43	0.7	1.3
Fresh banana fruit	83.4	2.3	2.9	45.4	11.3	0.07	1.30	0.06	0.10	0.06	c.d	1.9
Indian mulberry fruit	88.8	7.1	6.3	49.8	39.9	0.14	2.24	0.44	0.16	0.23	c.d	3.1
Banana flower	89.2	12.4	14.1	68.5	52.8	0.39	6.29	0.21	0.39	0.17	c.d	n.d
Rice straw	90.6	3.0	13.5	85.6	53.2	0.10	2.04	0.47	0.17	0.13	c.d	n.d

[1]CT = Condensed tannins; [2]CS = Crude saponins; n.d = not detectable.

Table 2: *In vitro* gas production of feed samples during hours of incubation

Substrates	Gas production (ml/g DM)							Gas production constants[a]	
hour	2	4	6	12	18	24	48	b	c
Bitter cucumber	16.2^{b}	30.7^{b}	43.7^{b}	75.4^{b}	98.3^{ab}	115.0^{a}	146.9^{b}	159.2^{ab}	0.053^{c}
Banana flower	5.3^{gh}	10.4^{fg}	15.2^{g}	28.1^{ij}	39.2^{hi}	48.8^{gh}	75.0^{hi}	105.9^{fghi}	0.025^{e}
Banana fruit	8.1^{ef}	16.2^{e}	24.3^{e}	48.1^{ef}	71.5^{d}	94.5^{cd}	181.9^{a}	166.2^{a}	0.003^{f}
Banana leaf	4.0^{hi}	8.0^{gh}	11.8^{gh}	22.8^{ikl}	33.1^{i}	42.7^{h}	75.5^{hi}	137.3^{bcde}	0.010^{f}
Bai yanang	12.7^{cd}	23.8^{cd}	33.4^{cd}	55.5^{d}	70.2^{d}	79.8^{e}	95.2^{ef}	98.8^{ghi}	0.069^{b}
Cassava hay	11.3^{d}	21.0^{d}	29.6^{d}	49.0^{e}	61.7^{e}	70.0^{f}	83.0^{fgh}	86.1^{ij}	0.070^{b}
Coral leaf	8.4^{ef}	16.3^{e}	23.6^{ef}	42.2^{fg}	56.9^{ef}	68.6^{f}	95.5^{ef}	113.0^{efgh}	0.039^{d}
Guava leaf	4.5^{hi}	8.3^{gh}	11.7^{gh}	19.2^{kl}	24.2^{ij}	27.3^{i}	32.2^{j}	33.4^{k}	0.072^{b}
Indian mulbery fruit	18.5^{a}	34.0^{a}	47.2^{ab}	75.8^{b}	93.1^{bc}	103.6^{b}	118.1^{d}	120.7^{defg}	0.083^{a}
Mulberry leaf	6.9^{fg}	13.3^{ef}	19.5^{f}	36.3^{gh}	50.8^{fg}	63.2^{f}	97.7^{e}	139.7^{bcd}	0.025^{e}
Mangosteen peel	3.4^{i}	6.5^{h}	9.3^{h}	16.4^{l}	21.8^{j}	25.9^{i}	34.6^{j}	39.2^{k}	0.045^{cd}
Star gooseberry leaf	7.3^{ef}	13.8^{e}	19.8^{f}	34.2^{hi}	44.8^{gh}	52.6^{g}	67.8^{i}	74.1^{j}	0.052^{c}
Pak kayaeng	13.7^{c}	26.1^{e}	37.3^{c}	65.1^{c}	85.8^{c}	101.2^{bc}	132.6^{c}	147.0^{abc}	0.049^{c}
Sugar apple leaf	11.2^{d}	21.5^{d}	30.8^{d}	54.2^{de}	72.1^{d}	85.6^{de}	114.5^{d}	129.7^{cdef}	0.045^{cd}
Siam neem leaf	8.6^{e}	16.4^{e}	23.4^{ef}	40.7^{g}	53.5^{f}	63.0^{f}	82.2^{gh}	91.0^{hij}	0.050^{c}
Sesbania leaf	19.2^{a}	35.8^{a}	50.3^{a}	83.2^{a}	104.7^{a}	118.8^{a}	140.6^{bc}	145.5^{abc}	0.071^{b}
Rice straw	4.1^{hi}	8.1^{gh}	12.2^{gh}	24.3^{ik}	36.3^{i}	48.2^{gh}	95.1^{efg}	167.2^{a}	0.001^{b}
SEM	0.5	1.0	1.4	2.2	2.7	3.1	4.5	8.5	0.003

Means on the same a column with different letters are significantly different ($p<0.05$).

[a]b : potential gas production (ml/g DM); *c* : fractional rate of gas production (h^{-1}).

correlations between gas production volume and rumen fermentation parameters among this tropical feed resources. The relatively weak correlation obtained between 48 h gas production and TVFs (r = 0.39) and individual VFA production were lower than previous study with browse species (Getachew and Makkar, 2002) and industrial by-product feeds (r = 0.76). This finding could be due to proportionally higher condensed tannins and/or saponins in feeds and available carbohydrates. Generally, gas is produced mainly when substrate is fermented to acetate and butyrate, while propionate was found to be relatively higher in all feeds, especially at 48h. When substrate were fermented to yields propionate, gas being produced only from buffering of the acid from an extra carbon atom in propionate would otherwise have appeared as CO_2 (Wolin, 1960). Therefore, relatively lower gas production is associated with propionate production as shown by the negative correlation in this study (r = -0.20). One of the most challenging problems associated with using gas production methods is that the amount of gas produced varies with different molar proportions of VFAs as obtained in this study.

Effects of condensed tannins on gas and VFA production: Lower production of gas and changes in proportion of VFA productions were observed in substrates with higher CT content and were consistent with the finding by Getachew and Makkar (2002). Pell and Schofield (1993) reported high correlation between

Table 3: Total (mmol/L) and individual (mol/dl) volatile fatty acid production and acetate to propionate ratio (A : P) in buffered rumen fluid after 48 h incubation of 200 mg of feed samples

Substrates	Total	Acetate	Propionate	Butyrate	A : P ratio
Bitter cucumber	88.0^{a}	57.6^{gh}	34.6^{a}	7.7^{cdef}	1.6^{fg}
Banana flower	66.2^{efg}	65.8^{cde}	23.2^{de}	10.9^{b}	2.8^{bcde}
Banana fruit	74.6^{bcd}	57.9^{gh}	26.6^{bcd}	15.4^{a}	2.1^{defg}
Banana leaf	72.8^{cde}	58.6^{fgh}	34.1^{a}	7.2^{def}	1.7^{fg}
Bai yanang	68.0^{def}	73.1^{ab}	19.9^{ef}	6.8^{ef}	3.7^{ab}
Cassava hay	49.0^{i}	65.4^{cde}	25.3^{cde}	9.2^{bcde}	3.2^{bc}
Coral leaf	56.7^{h}	66.7^{bcd}	23.4^{de}	9.7^{bcd}	2.8^{bcde}
Guava leaf	59.5^{gh}	52.1^{h}	31.3^{ab}	16.4^{a}	1.6^{g}
Indian mulbery fruit	67.8^{def}	60.9^{defg}	30.9^{abc}	8.1^{cdef}	1.9^{fg}
Mulberry leaf	62.6^{fgh}	67.6^{bcd}	24.1^{de}	8.2^{cdef}	2.8^{efg}
Mangosteen peel	80.6^{b}	59.9^{efg}	33.8^{a}	6.1^{f}	1.7^{fg}
Star gooseberry leaf	55.8^{hi}	64.7^{cdef}	26.7^{bcd}	8.5^{bcdef}	2.4^{cdefg}
Pak kayaeng	74.9^{bcd}	75.3^{a}	16.2^{f}	8.4^{bcdef}	4.6^{a}
Sugar apple leaf	66.2^{efg}	65.1^{cdef}	24.6^{de}	10.1^{bc}	2.6^{cdef}
Siam neem leaf	48.5^{i}	67.5^{bcd}	23.2^{de}	9.1^{bcde}	2.9^{bcde}
Sesbania leaf	79.3^{bc}	69.0^{abc}	22.7^{de}	8.1^{cdef}	3.0^{bcd}
Rice straw	66.5^{efg}	66.6^{bcde}	26.5^{bcd}	6.8^{ef}	2.5^{cdefg}
SEM	2.5	2.3	2.1	0.9	0.3

Means in the same column with different letters are significantly different ($p<0.05$).

Table 4: Correlation (*r*) between gas production (ml/g DM) and total volatile fatty acids(TVFs)(mmol/L), individual VFA production (mol/dl) for feed samples

Item	Gas	P <
TVFs		
h 2	0.12	0.363
4	0.18	0.187
6	0.38	0.004
48	0.39	0.003
Acetate		
h 2	-0.07	0.618
4	0.08	0.561
6	-0.10	0.462
48	0.16	0.259
Propionate		
h 2	-0.01	0.901
4	0.00	0.989
6	0.12	0.386
48	-0.20	0.139
Butyrate		
h 2	0.17	0.222
4	-0.12	0.374
6	-0.00	0.983
48	0.05	0.715

gas production and NDF disappearance (r = 0.99) or gas production and DM disappearance (r = 0.95) (Prasard *et al.*, 1994) Higher concentration of tannins in the diet was associated with reduction in organic matter digestibility (Waghorn and Shelton, 1997). Therefore, substrates especially, Mangosteen peel and Guava leaves, which higher CT contents, resulted in low gas production in this study. Barry *et al.* (1986) suggested a level of CT in diet of 30-40 g/kg DM for efficient utilization by ruminants. Moreover, a relatively weak correlation between CT and percent increase in gas production (r = 0.23) were observed by Wood and Plumb (1995), Abdulrazak *et al.* (2000) due to the variation in structural and biological activity of tannins. Changing the pattern of fermentation towards higher molar proportion of propionate could have been due to the changing of rumen microbial population and fermentation process.

Conclusion and recommendations: Based on this experiment, it can be concluded that local tropical feed resources contained variable contents of condensed tannins and crude saponins. However, these resources also contained other nutrients especially the macromineral and were potentially used for ruminants. In vitro gas production had relatively low correlation with volatile fatty acid production particularly that of propionate. Evaluation of tropical feed resources containing secondary plant compounds should not solely depend on in vitro gas fermentation but subsequent in vivo trials should be conducted.

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