EFFECT OF WATER EXTRACT OF PLANTS CONTAINING TANNIN ON IN VITRO METHAGONESIS AND FERMENTATION CHARACTERISTICS OF THE GRASS *Pennisetum purpureophoides*

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ABSTRAK

Penelitian ini dilaksanakan untuk mengevaluasi pengaruh perbedaan ekstrak tanaman yang mengandung tanin terhadap produksi CH_4, karakteristik fermentasi dan degradasi nutrien secara *in vitro*. Enam jenis daun tanaman yaitu *Gliricidia sepium*, *Acacia mangium*, *Leucaena leucocephala*, *Desmodium intortum*, *Camellia sinensis*, *Calliandra calothyrsus* dan biji *Areca catechu* diekstraksi menggunakan pelarut air. Perlakuan percobaan terdiri atas *P. purpureophoides* (300±5 mg) diinkubasi tunggal atau ditambah 1,2 mL ekstrak tanaman. Degradasi NDF diukur menggunakan prosedur Tilley and Terry tahap pertama. Hasil penelitian menunjukkan bahwa konsentrasi tanin total pada ekstrak tanaman bervariasi antara 34-95 g/kg BK, terendah pada *D. intortum* dan tertinggi pada *A. mangium.* Produksi CH_4 pada inkubasi 48 jam signifikan lebih rendah (P<0,001) dengan penambahan ekstrak *A. mangium, L. leucocephala, A. catechu, C. sinensis* dan *C. calothyrsus* dibandingkan kontrol. Tanin total mempunyai hubungan yang erat dengan produksi CH_4 (*r*=-0,79). Terdapat korelasi yang kuat antara produksi CH_4 dan konsentrasi NDF (*r*=0,61). Disimpulkan bahwa ekstrak tanaman *A. mangium, L. leucocephala, A. catechu, C. sinensis* dan *C. calothyrsus* menggunakan air berpotensi sebagai manipulator untuk menurunkan produksi CH_4 pada ternak ruminansia.

Kata kunci: in vitro, metana, ruminansia, tanin, ekstrak

ABSTRACT

This experiment was conducted to evaluate the effect of extract of plants containing tannin on *in vitro* CH_4 production, fermentation characteristics and nutrient degradability. Six of plant leaves *i.e. Gliricidia sepium, Acacia mangium, Leucaena leucocephala, Desmodium intortum, Camellia sinensis, Calliandra calothyrsus* and seed of *Areca catechu* were extracted by using water. Experimental treatments consisted of *P. purpureophoides* (300±5 mg) incubated alone or added with 1.2 mL of plant extracts. The *in vitro* neutral detergent fibre (NDF) degradability was determined using the first stage technique of Tilley and Terry. The results showed that total tannin concentration of plant extract ranged from 34 to 95 g/kg DM, and was lowest in *D. intortum* and highest in *A. mangium*. Methane production was significantly (P<0.001) lower with addition of *A. mangium, L. leucocephala, A. catechu, C. sinensis and C. calothyrsus* extracts compared to control. Total tannin had a close relationship with CH_4 production (*r*=-0.79). There was strong correlation between CH_4 production and NDF degradability (*r*=0.61). It was concluded that water extracts of *A. mangium, L. leucocephala, A. catechu, C. sinensis* and *C. calothyrsus* have potential to be used as rumen manipulator in order to reduce CH_4 production in ruminants.

Keywords: in vitro, methane, ruminant, tannin, extract

INTRODUCTION

Methane (CH_4) is produced as a result of anaerobic fermentation of the soluble and structural carbohydrates by methanogens in the rumen of ruminant animals, which is released into the environment by eructation. The CH_4 emissions from ruminant animals range from 2 to 12% of the gross energy intake (Johnson and Johnson, 1995). Besides, it has been estimated that the world's population of ruminants produce about 15% of total atmospheric CH_4 emissions

(Moss, 1993). This means that CH_4 production from ruminant is not only represents a substantial loss in efficiency of animal production, but also contributes significantly to global warming as the greenhouse gas.

Recently, there is an increasing interest in research activities to evaluate the potential of secondary plant compound as feed additives instead of chemical compounds *i.e*. ionophores and antibiotics as manipulators of rumen fermentation including decrease CH_4 production. As previously stated by Russell and Rychlik (2001), there has been an increased perception that antibiotics and chemical compounds should not be routinely used as feed additives.

Tannin is a phenolic plant secondary compound and is widely distributed through plant kingdom especially legume and browse. Makkar *et al*. (1995) noted that secondary compound tannin is prevalent in many tropical fodder plants. Effect of tannin from some plants such *Acacia mangium, Biophytum petersianum* and *Psidium guajava* has been demonstrated as supplement to the tropical grass *Pennisetum purpureum* (Hariadi and Santoso, 2010). Plant extract containing tannin have been shown to decrease CH_4 production (Śliwiński *et al*., 2002; Sirohi *et al*., 2009) and ruminal NDF digestibility (McSweeney *et al*., 2001; Oliveira *et al*., 2007). Previous study with tropical plants, Jayanegara *et al*. (2011) concluded that total tannin had close relationship with CH_4/digestible OM (r=-0.74). However, use of water as a solvent is more applicable and safe for animals than chemical solvents *i.e.* methanol, ethanol or acetone. The objective of this study was to evaluate the effect of water extracts from plant containing tannin on *in vitro* CH_4 production, fermentation characteristics, and nutrient degradability.

MATERIALS AND METHODS

Samples and Extract Preparations

King grass (*P. purpureophoides*) was planted in a 6 m^2 plot without fertilizer at the Animal Research Station of State University of Papua in Manokwari. Grass was harvested after 50 days, chopped to 5 cm in length and oven-dried at 60°C for 48 h.

The leaves of *G. sepium, A. mangium, L. leucocephala, C. sinensis, D. intortum* and *C. calothyrsus* and seed of *A. catechu* were collected from the Manokwari Regency. The collected samples were then pooled and oven-dried at 60°C for 48 h. A commercial leaf of *C. sinensis* was used in this experiment. Samples of grass, leaves and seed of plants were ground to pass a 1 mm sieve in a Wiley mill.

Plants extract were prepared in water following a modified method of Patra *et al*. (2006). About 5 g of finely ground plants material were weighed into 100 ml beaker glass and added 50 ml of water. Plant materials were boiled for 10 min on a hotplate. The beakers were stoppered and incubated at 39°C on a shaker for 24 h and filtered through 2 layers of cheesecloth. The filtrates were stored at 4°C for further use.

Experimental Design and Treatments

The experiment was arranged in a completely randomized design with eight treatments and three replications. Experimental treatments consisted of *P. purpureophoides* incubated alone as control or added with plant extract at level of 1.2 mL/300 mg of substrate.

In vitro CH_4 Production and Nutrient Degradability Measurements

The *in vitro* gas production method was essentially according to Menke and Steingass (1988) as previously demonstrated by Hariadi and Santoso (2010). Oven-dried samples of about 300±5 mg were weighed into 100 mL glass syringes with pistons lubricated with Vaseline. Rumen liquor was obtained from two ruminally fistulated Holstein Frisian cross-bred cows fed elephant grass twice a day at maintenance level. About 30 mL of rumen liquor-buffer mixtures (1 : 2, v/v) was added into each syringe and then incubated in a water bath at 39°C for 48 h. The volume of gas released from each syringe was recorded before incubation (0 h) and 2, 4, 6, 12, 24 and 48 h of incubation.

Ten milliliter of gas was collected at 24 and 48 h of incubation from the silicon tube at the syringe tip using Terumo syringe and pooled to vacutainer tube for CH_4 analysis. Methane was determined by injection 100 mL of gas into a chromatograph gas. The volume of CH_4 production was calculated by using formula: CH_4 production (ml) = total gas produced (ml) × % CH_4 in the sample.

At the end of the incubation period, about 10 ml of syringe contents were sampled. The pH of medium incubation was recorded using a digital pH meter. Subsequently, 0.2 mL of sub-

samples were pipetted into 1.5 mL micro centrifuge tube containing 1 ml of 25 g/100 mL (w/v) metaphosphoric acid and centrifuged at 9000 x *g* for 10 min for volatile fatty acids (VFA) determination. Further on 2 mL of sub-samples were added to 2 mL of 20 g/l (w/v) NaCl for NH_3-N (Chaney and Marbach, 1962).

The *in vitro* organic matter (OM) and NDF degradability was determined using the first-stage technique as proposed by Tilley and Terry (1963) as previously demonstrated by Hariadi and Santoso (2010).

Laboratory Analyses

Dried samples were used to determine DM, ash and crude protein (CP) according to procedure of AOAC (1990). Total tannin and condensed tannin contents were assayed using Folin-Ciocalteu and butanol-HCl methods respectively (Makkar, 2003). Concentrations of NDF and acid detergent fibre (ADF) were determined following Van Soest *et al.* (1991).

Statistical Analysis

Data was analyzed using the procedure GLM of SAS (version 6.12, SAS institute, Cary, NC, USA). Duncan's multiple range test was used to separate treatment means. Correlation analysis was done to establish the relationship between variables.

RESULTS AND DISCUSSION

Results

Chemical Composition of Plants

The chemical composition of experimental plants is presented in Table 1. The NDF and ADF content in plants containing tannin ranging from 269 to 596 and 115 to 381 g/kg DM, respectively. The ranking order of plant samples on the basis their total tannin content was *A. mangium* > *C. calothyrsus* < *L. leucocephala* < *A. catechu* > *C. sinensis* > *G. sepium* > *D. intortum.* However, on the basis of their condensed tannin concentration the plant could be ranked as *A. mangium* > *A. catechu* > *L. leucocephala* > *G. sepium* > *C.calothyrsus* > *C.sinensis* > *D. intortum.*

***In vitro* Gas and CH_4 Productions**

Effect of plant extract containing tannin on gas and CH_4 production are given in Table 2. Gas production was significantly (P<0.001) different among treatments at 6, 24 and 48 h of incubation. Addition of plant extracts in grass substrate decreased (P<0.001) gas production at 48 h of incubation compared to control. Methane production in substrate with addition of *A. mangium*, *L. leucocephala*, *A. catechu*, *C. sinensis* and *C. calothyrsus* extracts was significantly (P<0.001) lower as compared to control. The CH_4 produced at 48 h of incubation averaged 12.3% of total gas at 48 h of incubation.

Table 1. Chemical Composition (g/kg DM) of *P. purpureophoides* Incubated as Substrate, and Leaves or Seed Plant Containing Tannin

	OM	CP	NDF	ADF	Hemi-celullose	TT	CT
P. purpureophoides	953	148	751	404	347	5	N.D.
G. sepium	916	219	466	315	261	45	25
A. mangium	934	241	477	216	261	95	36
L. leucocephala	920	344	269	115	154	81	28
A. catecú	975	102	496	135	361	77	35
D. intortum	881	207	403	209	194	34	5
C. sinensis	936	197	424	269	155	69	20
C. calothyrsus	944	188	596	381	215	82	21

DM = dry matter; OM = organic matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; TT = total tannin; CT = condensed tannin; ND = not detected

Table 2. Gas and CH_4 Productions of *in vitro* Incubation of *P. purpureophoides* Alone or with Addition of Plant Extracts

	Total gas (ml/300 mg of DM)			CH_4 (ml/300 mg of DM)	CH_4/total gas 48 h (ml/l)
	6 h	24 h	48 h		
P. purpureophoides	12.6^{a}	54.1^{a}	71.2^{a}	11.0^{a}	154^{A}
G. sepium	9.3^{c}	43.6^{cde}	64.0^{cd}	8.4^{ab}	131^{AB}
A. mangium	7.3^{cd}	37.6^{ef}	61.3^{e}	6.2^{b}	101^{B}
L. leucocephala	10.3^{b}	50.9^{ab}	66.3^{de}	7.6^{b}	114^{B}
A. catecú	6.9^{d}	33.3^{f}	69.5^{e}	7.6^{b}	129^{AB}
D. intortum	12.1^{a}	49.6^{abc}	68.3^{b}	8.3^{ab}	121^{B}
C. sinensis	6.8^{d}	40.3^{de}	60.5^{e}	7.0^{b}	115^{B}
C. calothyrsus	11.1^{ab}	45.3^{bcd}	64.8^{c}	7.5^{b}	115^{B}
SEM	0.52	1.54	0.67	0.56	9.15
P	<0.001	<0.001	<0.001	<0.001	0.03

Means in the same column followed by different letters are different ($^{A-B}P<0.05$; $^{a-f}P<0.01$)

Fermentation Characteristics

The pH value, concentrations of NH_3-N and VFA in the medium incubation are present in Table 3. The pH value in medium incubation was not significantly (P>0.05) different among treatments. Concentration of NH_3-N significantly (P<0.05) increased in grass substrate with addition of extract of *L. leucocephala* as compared to control. Addition of extract of *G. sepium*, *A. mangium*, *A. catechu*, *C. sinensis* and *C. calothyrsus* in grass substrate decreased (P<0.001) total VFA concentration. Extracts of *G. sepium*, *A. catechu* and *C. sinensis* increased (P<0.001) proportion of acetate compared to control, whereas *A. mangium* and *C. sinensis* extracts reduced (P<0.001) proportion of propionate. Acetate:propionate ratio was highest in *A. mangium* and lowest in *A. catechu* extracts.

***In vitro* Nutrient Degradability**

In vitro OM and NDF degradability of grass substrate with addition of tannin extracts is presented Table 4. Addition of *L. leucochepala, C. sinensis* and *C. calothyrsus* extracts to grass substrate decreased (P<0.001) IVOMD, whereas IVNDFD was decreased (P<0.001) by addition of *A. mangium, L. leucocephala, A.catechu, D. intortum, C. sinensis* and *C. calothyrsus* extracts as compared to control.

Relationships between Tannin Content and Fermentation Variables

The correlations between total tannin, condensed tannin and fermentation variables are in Table 5. There was a negative correlation between total tannin and the gas production at 6, 24 and 48 h of incubation and CH_4 production, indicating that as total tannin concentration increased, the gas and CH_4 production decreased. Total tannin concentration had closer correlation with CH_4 and CH_4/total gas production than condensed tannin concentration. There was negative correlation between total tannin content and both IVOMD and IVNDFD.

Discussion

The total tannin content in *A. mangium* was comparable to that value reported by Jayanegara *et al*. (2011), but condensed tannin content was lower than value of 42 g/kg DM as reported by Jayanegara *et al*.(2011) Concentration of total tannin in *D. intortum* was lower than in *Desmodium intortum* (Getachew *et al*., 2000). Concentration of condensed tannin in *L.*

Table 3. The pH, Concentrations of NH_3-N and VFA in the Medium Incubation After 48 h of *in vitro* Incubation of *P. purpureophoides* Alone or with Addition of Plant Extracts

	pH	NH_3-N (mg/100 ml)	Total VFA (mmol/l)	Acetate (C2) (mol/ 100 mol)	Propionate (C3) (mol/ 100 mol)	Butyrate (mol/100 mol)	C2/C3
P. purpureophoides	6.53	19.5^{BC}	139.2^{a}	71.0^{d}	15.9^{ab}	13.1^{a}	4.5^{cd}
G. sepium	6.53	22.2^{C}	111.5^{b}	75.8^{abc}	14.2^{bc}	10.0^{abc}	5.4^{ab}
A. mangium	6.54	21.2^{ABC}	88.5^{d}	76.7^{ab}	13.2^{c}	10.1^{abc}	5.8^{a}
L. leucocephala	6.51	25.4^{A}	137.9^{a}	75.0^{abc}	15.6^{ab}	9.3^{bc}	4.8^{bcd}
A. catecú	6.50	17.2^{BC}	87.9^{d}	73.8^{c}	17.0^{a}	9.2^{bc}	4.3^{d}
D. intortum	6.48	22.6^{AB}	128.9^{a}	76.0^{abc}	17.0^{a}	7.1^{c}	4.5^{cd}
C. sinensis	6.50	19.4^{ABC}	97.7^{cd}	74.3^{c}	14.4^{c}	11.3^{ab}	5.2^{abc}
C. calothyrsus	6.53	19.3^{ABC}	103.3^{bc}	77.2^{a}	15.7^{ab}	7.1^{c}	4.9^{bcd}
SEM	0.01	1.54	2.69	0.63	0.53	0.73	0.17
P	0.11	0.04	<0.001	<0.001	<0.001	<0.001	<0.001

Means in the same column followed by different letters are different ($^{A-C}P<0.05$; $^{a-d}P<0.01$)

Table 4. The IVOMD and IVNDFD after 48 h of Incubation of *P. purpureophoides* Alone or with Addition of Plant Extracts

	IVOMD (g/kg)	IVNDFD (g/kg)
P. purpureophoides	606^{a}	336^{a}
G. sepium	588^{abc}	309^{a}
A. mangium	594^{ab}	222^{bc}
L. leucocephala	562^{c}	260^{b}
A. catechu	589^{abc}	189^{c}
D. intortum	575^{abc}	207^{c}
C. sinensis	560^{c}	187^{c}
C. calothyrsus	484^{d}	180^{c}
SEM	0.74	10.1
P	<0.001	<0.001

Means in the same column followed by different letters are different ($^{A-B}P<0.05$; $^{a-d}P<0.01$)

leucocephala and *C. calothyrsus* was lower than values of 76 and 240 g/kg DM, respectively (Tiemann *et al*., 2008). Different of plant nutrients content in this study as compared to previous study could be due to difference in location of sample source and plants maturity.

Higher gas production with addition of plant extract could be due to the presence of higher of soluble sugar from these extract. In previous studies of Śliwiński *et al.* (2002); Patra *et al.* (2010), increasing gas production by plant extracts that contain phenol or saponin caused by increasing soluble sugar from these plant extract.

No differences in pH values obtained in the present study, consistent with previous studies of Śliwiński *et al*. (2002); Oliviera *et al.*(2007); Animut *et al.* (2008) who found that H was not changed by addition of tannin in both sheep and cattle rumens.

Tannin has beneficial effect on protection on dietary protein in the rumen and subsequently enhancement of amino acid absorption and utilization by the ruminant animal (Waghorn *et al*., 1994). McSweeney *et al*. (2001) revealed that tannin has ability to bind protein by forming hydrogen bonds between the phenolic sub-units of

Table 5. Coefficient of Correlation (*r*) Between Total Tannin, Condensed Tannin Contents and Gas Production, Fermentation Characteristics, *in vitro* Nutrients Degradability

Variables	Total Tannin	Condensed Tannin
Gas 6 h	-0.63***	0.70***
Gas 24 h	-0.61**	0.60**
Gas 48 h	-0.72**	0.67**
CH_4	-0.79***	-0.37ns
CH_4/total gas	-0.66***	-0.12ns
Total VFA	-0.65***	-0.53*
pH	0.10ns	0.41ns
C2	0.56**	-0.15ns
C3	-0.30ns	-0.35ns
C4	-0.32ns	0.43*
C2/C3	0.41*	0.33**
NH_3-N	-0.04ns	-0.17ns
IVOMD	-0.40*	0.21ns
IVNDFD	-0.21ns	0.17ns

ns: not significant (P>0.05); * (P<0.05); ** (P<0.01); ***(P<0.001)

the polymer and the carbonyl groups of peptides of the protein. *In vivo* study, Min *et al*. (2005) suggested that the action of condensed tannin in forages markedly reduced both growth and population of proteolytic bacterial. However, the extent of positive or negative effects of tannins may vary depending on the type and level of tannins in plants and their biological activity (Getachew *et al*., 2000). Relatively higher concentration ammonia-N in the medium incubation with addition of extract of *G. sepium*, *A. mangium*, *L. leucochepala*, *A. catechu* and *D. intortum* could be due to higher crude protein content in those plants or tannin concentration is lower than minimum concentration needed to produce maximum inhibition of proteolysis.

The inhibition of methanogenesis has long been considered from nutritional aspects, and more recently from the perspectives on greenhouse gas emissions. In the previous studies by Śliwiński *et al*. (2002); Patra *et al*. (2006); Patra *et al*. (2010); Rodríguez *et al*. (2011) who reported that CH_4 production reduced by addition of plant extract containing tannin. In contrast, Beauchemin *et al*. (2007) described that supplementing a forage-based diet with quebracho tannin extract failed to reduce CH_4 production from growing cattle. The same result has been reported by Oliviera *et al*. (2007) that feeding sorghum silage containing high or low concentrations of tannin did not affect nutrient digestibility and CH_4 production in cattle. However, effect of tannin on methanogenesis depends on the source, type and dose of tannins (Patra *et al*., 2006). In this study, relative to control, addition 1.2 ml extract of *A. mangium*, *L. leucocephala*, *A. catechu*, *C. sinensis* or *C. calothyrsus* decreased CH_4 production by 43.6, 30.9, 30.9, 36.3 and 31.8%, respectively. Decreased CH_4 production in this study, however might be related to decreasing of fibre degradation which is shown by high correlation coefficient value between CH_4 production and NDF degradability (r =0.61; P<0.01) (result not shown). This result was supported by previous study of Estermann *et al*. (2002) that there was a strong relationship between CH_4 production and digestible NDF for cows and calves. Bhatta *et al*. (2009) concluded that tannins suppress methanogenesis by reducing methanogenic populations in the rumen either directly or by reducing the protozoal population, thereby reducing methanogens symbiotically associated with the protozoal population. Tavendale *et al*. (2005) revealed that condensed tannin reduce CH_4 production could be due to indirect effect by reduced hydrogen production as result of reduced feed degradability, and by direct inhibitory effect on methanogens. A high correlation (r=-0.79, P<0.001) between total tannin concentration and CH_4 production in this study (Table 5) was agreed with previous studies of Jayanegara *et al*. (2009, 2011); Hariadi and Santoso (2010) that found r values -0.60, -0.66 and -0.76, respectively. A similar observation was found by Bhatta *et al*. (2009) that total tannin had close correlation with CH_4 output (r=-0.97). In our study, IVNDFD in substrate with addition of *L. leucocephala, A. catechu, D. intortum, C. sinensis* or *C. calothyrsus* extracts was significantly lower than control. This finding agrees with previous study of Bhatta *et al*. (2009) who found that tannin significantly suppressed bacteria population, through a direct effect (Koike and Kobayashi, 2009) and by reducing nutrient availability

(Sallam *et al*., 2010). In addition, tannin could reduce fibre digestion by complexing with lignocellulose and preventing microbial digestion or by directly inhibiting cellulolytic microorganism or both (McSweeney *et al*., 2001). In our results, IVOMD decreased ranged from 7.3 to 20.1% when addition of *L. leucocephala, C. sinensis* or *C. calothyrsus* extract to *P. purpurophoides* substrate. Similar result has been reported by Patra *et al*. (2006) that addition of extract of plants containing tannin reduced IVDMD and IVOMD by about 7% in comparison to control. Hess *et al*. (2003) also reported that tannins from *C. calothyrsus* may cause significant shifts in rumen microbial populations, especially a reduced total number of cellulolytic bacteria, which could have contributed to lower OM degradation.

CONCLUSION

Plant extracts from *A. mangium*, *L. leucocephala*, *A. catechu*, *C. sinensis* and *C. calothyrsus* have potential to be used as rumen manipulator in order to reduce CH_4 production in ruminants. Among plant extracts, *A. mangium* had the strongest effect on *in vitro* CH_4 production. The mode of action tannin on methanogenesis might be partially attributed to reduce fibre degradability.

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