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Effect of plants containing secondary metabolites on ruminal fermentation and methanogenesis *in vitro*

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ABSTRACT

The effect of *kalmegh* (*Andrographis paniculata*), neem (*Azadirachta indica*), bamboo (*Bambusa balcooa*), *kesuti* (*Eclipta alba*), *kanchan* (*Alternantherd sessilis)* leaves and *akanda* (*Calotrips gigantea*) bark were tested at 3 different levels of replacement (0, 30 and 60 mg per 200 mg of substrate, consisting of paddy straw and concentrate mixture in 1:1 ratio) for *in vitro* methane production, ciliate protozoal population, enzyme profile and rumen fermentation characteristics. The gas as well as methane production (ml/g DM) was significantly lower due to inclusion of *Andrographis paniculata*, *Azadirachta indica and Calotrips gigantean* in the incubation medium. Holotrich and spirotrich protozoal number also reduced due to inclusion of *Andrographis paniculata*, *Azadirachta indica and Calotrips gigantean. Azadirachta indica*, *Alternantherd sessilis* and *Eclipta alba* had no influence on the enzyme activity of carboxymethyl cellulase, xylanase and β-glucosidase. However, specific activity of carboxymethyl cellulase and xylanase reduced significantly due to addition of *Andrographis paniculata* and *Calotrips gigantea* in the incubation medium. The results indicated that plant tested in the present experiment (leaves of *Andrographis paniculata* and *Azadirachta indica and* bark of *Calotrips gigantea*) reduces ruminal methanogenesis without much adverse effect in rumen fermentation.

Key words: Ciliate protozoa, Methane, Plant secondary metabolites, Rumen enzyme activities, Rumen metabolites

Modification of rumen microbial fermentation to decrease ruminal methane and ammonia nitrogen production by using feed additives is a useful strategy to improve production efficiency of ruminant animals (Alexander *et al*. 2008). Recently, there has been an increase interest to use natural products containing plant secondary compounds instead of chemical feed additives to modify rumen fermentation for improving feed utilization and productive performances of ruminant animals. The plant containing high amount of saponins such as *Sapindus saponaria, Yucca schidigear, Enterolobium ciclocarpum and Sessbania sesban* have a potential to suppress rumen protozoal populations, increase bacterial and fungal populations, propionate production, microbial yield, microbial protein synthesis and decreased ruminal methanogenesis and ammonia production which leads to better productivity of ruminant animals (Hess *et al.* 2003, Santoso *et al.* 2004, Patra and Saxena 2009). Similarly, condensed tannin containing plants reduced dietary protein degradation and methanogenesis in the rumen and increased microbial protein synthesis (Puchala *et al.* 2005, Patra and Saxena 2010). However, effectiveness of plant to manipulate rumen fermentation varied depending upon the source and type of secondary metabolites present in these plants. The present experiment was conducted to evaluate the plants containing secondary metabolites for their anti-protozoal and anti-methanogenic activities to manipulate the rumen fermentation.

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MATERIALS AND METHODS

Leaves of *kalmegh* (*Andrographis paniculata*), neem (*Azadirachta indica*), bamboo (*Bambusa balcooa*), *kesuti* (*Eclipta alba*), *kanchan* (*Alternantherd sessilis)* and bark of *akanda* (*Calotrips gigantea*) were dried at 50°C for 72 h and grinded to pass through 1 mm sieve. These ground plant materials were tested for their effect on ruminal fermentation characteristics and methanogenesis by *in vitro* gas production test.

In vitro *gas production test:* For *in vitro* gas production test, 200±5 mg of substrate comprising of air dried milled (<1.0 mm) paddy straw (OM, 89.8%; CP, 2.2%; NDF, 61.3%; and ADF, 55.9%) and concentrate mixture (crushed maize grain 30%; groundnut cake 22%; wheat bran 45%; mineral mixture 2% and common salt 1% with 88.9% OM, 16.5%

CP, 41.3% NDF and 37.1% ADF) in 1:1 ratio was weighed and taken in the 100 ml glass syringes. In the syringes parts of the substrate was replaced (w/w) with 30 and 60 mg of different tested/selected ground plant material. Rumen liquor was collected by using stomach tube just before morning feeding from cattle fed on a diet containing paddy straw and concentrate mixture in 1:1 ratio, which was same as the substrate used for the *in vitro* gas production test. The inoculum/incubation medium was prepared by mixing rumen liquor with buffer in the ratio of 1:2 (Menke and Steingass 1988). 30 ml of incubation medium was dispensed anaerobically in each syringe. Syringes were incubated and total gas as well as methane was estimated as described by Patra *et al.* (2006a)

Chemical analysis of substrate and statistical analysis: The substrate was analysed for dry matter (DM) by drying at 100 °C for 24 h, organic matter (OM) by ashing at 550°C for 4 h and crude protein (CP) by Kjeldahl technique (AOAC 1995). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were estimated as per Van Soest *et al*. (1991). Total volatile fatty acids (TVFA) estimation of incubation medium was carried out as per Barnett and Reid (1957) and fractionation of VFA as well as estimation of the enzyme activity and *in vitro* true dry matter degradability were done as described by Patra *et al.* (2006a). Microbial biomass was estimated according to Blumel *et al.* (1997). Staining and counting of rumen protozoa was done as described by Veira *et al.* (1983). Ammonia nitrogen concentration in the incubation medium was estimated as per the method of Weatherburn (1967).

The data generated in the experiment were analyzed using Generalized Linear model ANOVA procedures of SPSS (1996). The differences between means were compared using Duncan's multiple range tests (Duncan 1955).

RESULT AND DISCUSSION

Effect on gas and methane production: The results of the effect of different plant leaves and bark on ruminal gas and methane production *in vitro* are presented in Table 1. As compared to control, *in vitro* gas production was reduced ($P<0.05$) due to inclusion of dried ground leaves of *Andrographis paniculata* and A*zadirachta indica* and bark of *Calotrips gigantean*. The reduction in gas production by inclusion of ground *Andrographis paniculata, Azadirachta indica* leaves and *Caoltrips gigantean bark* might be due to high concentration of tannic acid, phenolic compounds and saponins present in them, respectively. Earlier it was reported that tannic acid (Makkar *et al.* 1995, Patra *et al.* 2006b, Kumar *et al*. 2011), phenolic compounds and saponins (Goel *et al.* 2008) reduce gas production significantly under *in vitro* gas production test. The lowest methane production was observed in the treatment where substrate was incubated with *Andrographis paniculata* leaves followed by *Azadirachta indica* leaves and *Calotrips gigantean* bark. There was about 6.0 ml/g DM reduction in methane production when *Andrographis paniculata* leaves added in higher doses (60 mg/30 ml incubation medium) in comparison to control. This reduction in methane production might be due to the presence of different anti-microbial compounds present in these three plants (Calsamiglia *et al.* 2007). The report from literatures indicated that phenolic acids such as p-coumaric acid, ferulic acid, cinnamic acid have found to decrease methane production (Asiegbu *et al.* 1995, Goel *et al.* 2008).

Effect on rumen ciliate protozoal population: The protozoal number was decreased due to addition of *Andrographis paniculata* and *Azadirachta indica* leaves and *Caoltrips gigantea* bark in the incubation medium (Table 2). Inclusion of *Andrographis paniculata* at 30 mg level in the

Table 1. Effect of different plant leaves and bark on ruminal gas and methane production (ml/g DM) *in vitro*.

Attributes	Additives (mg)	Additives						Standard error
		Andrographis paniculata	*Azadirachta indica*	*Bambusa balcooa*	*Eclipta alba*	*Alternantherd sessilis*	*Caoltrips gigantea*	
Ruminal gas								2.79
	00	167.9^{x}	167.9^{x}	167.9	167.9	167.9	167.9^{x}	
	30	148.8^{y}	158.2^{y}	166.7	166.5	169.4	159.2^{y}	
	60	140.4^{z}	152.8^{y}	168.9	164.7	162.7	158.8^{y}	
	Mean	152.4^{c}	159.6^{b}	167.8^{a}	166.4^{a}	166.7^{a}	161.9^{b}	
Methane								0.37
	00	35.1^{x}	35.1^{x}	35.1	35.1	35.1	35.1^{x}	
	30	32.1^{y}	33.5^{xy}	33.5	34.6	34.9	32.9^{xy}	
	60	29.1^{y}	32.2^{y}	34.4	34.8	34.1	32.2^{y}	
	Mean	32.1^{b}	33.6^{ab}	34.3^{ab}	34.5^{a}	34.7^{a}	33.4^{ab}	

^{xyz}Mean with different superscripts in a column and ^{abc}in a row among levels and treatments, respectively differ significantly ($P<0.05$).

Table 2. Effect of different plant leaves and bark on rumen ciliate protozoal population (× 10^3 ml)

Attributes	Additives (mg)	Additives						Standard error
		Andrographis paniculata	*Azadirachta indica*	*Bambusa balcooa*	*Eclipta alba*	*Alternanthera sessilis*	*Calotrips gigantea*	
Holotrich protozoa								0.03
	00	1.73^{x}	1.73^{x}	1.73	1.73	1.73	1.73^{x}	
	30	0.41^{y}	1.31^{y}	1.85	1.69	1.91	1.11^{y}	
	60	0.17^{y}	0.74^{z}	1.79	1.67	1.83	0.68^{z}	
	Mean	0.77^{c}	1.26^{b}	1.79^{a}	1.70^{a}	1.82^{a}	1.17^{b}	
Spirotrich protozoa								0.58
	00	43.67^{x}	43.67^{x}	43.67	43.67	43.67	43.67^{x}	
	30	41.73^{y}	42.20^{y}	44.08	43.06	44.22	41.81^{y}	
	60	40.14^{z}	40.99^{z}	43.96	42.65	43.98	41.05^{y}	
	Mean	41.85^{b}	42.29^{b}	43.90^{a}	43.13^{ab}	43.96^{a}	42.18^{b}	
Total rumen protozoa								1.64
	00	45.40^{x}	45.40^{x}	45.40	45.40	45.40	45.40^{x}	
	30	42.14^{y}	43.51^{xy}	45.93	44.75	46.13	42.92^{xy}	
	60	40.31^{y}	41.73^{y}	45.75	44.32	45.81	41.73^{y}	
	Mean	42.62^{b}	43.55^{ab}	45.69^{a}	44.82^{a}	45.78^{a}	43.35^{a}	

^{xyz} Mean with different superscripts in a column and ^{abc} in a row among levels and treatments, respectively differ significantly (P<0.05).

incubation medium resulted in a significant reduction of total protozoal number where as *Azadirechta indica* and *Calotrips gigantean* reduced prozoal count only at 60 mg level. Other tested leaves (*Bambusa balcooa*, *Eclipta alba*, *Alternanthera sessilis*) had no influence on total rumen protozoal counts. The inhibitory effect of *Calotrips gigantea* bark on rumen protozoa could be due to saponin content (Kamra *et al*. 2006)

Effect on volatile fatty acids and ammonia nitrogen production: The effect of different plant leaves and bark on total volatile fatty acids (TVFA), molar proportion and concentration of acetate, propionate, butyrate and ammonia nitrogen concentration are presented in Table 3. Concentration of TVFA was significantly lower due to inclusion of *Andrographis paniculata, Azadirachta indica and Calotrips gigantean* in the incubating media compared to control. The ratio of acetate to propionate was not affected by the addition of additives in the incubation medium. On the contrary, Hess *et al.* (2003) reported that reduced rumen protozoal number was associated with increase in propionate % and decrease in A:P ratio. However, according to Jouany *et al*. (1988) changes in the VFA pattern due to reduction in protozoa population is not always consistent because nature of diet also plays an important role in VFA pattern. Ammonia nitrogen concentration was significantly lower due to addition of *Andrographis paniculata* and *Azadirachta indica* leaves and *Calotrips gigantean* bark in comparison to control and this is might be due to lower rumen ciliate protozoal population resulting in more utilization of ruminal ammonia by rumen bacteria.

Effect on ruminal enzyme activities: The effect of plant additives on rumen enzyme activity are presented in Table 4. The activity of carboxymethyl cellulase, xylanase and β glucosidase enzyme was decreased (P<0.05) due to addition of *Andrographis paniculata* leaves and *Calotrips gigantean* bark in higher doses (60 mg/30 ml incubation media). This might be due to antiprotozoal activity of *Andrographis paniculata* leaves and *Calotrips gigantean* bark (Agarwal *et al*. 1991, Patra *et al.* 2006a). Further, *Calotrips gigantean* bark contain saponin which may suppressed the activity of carboxy-methyl cellulase and xylanase enzyme. A decrease in carboxymethyl cellulase and xylanase enzyme activity by addition of yucca and quillaja saponins have been observed by Hristov *et al*. (2003).

Effect on ruminal dry-matter degradability and microbial biomass production: In vitro dry matter degradability and *in vitro* organic matter degradability was suppressed (P<0.05) due to addition of all tested plant additives (Table 5). The suppression in degradability varied only between 1–3% in comparison to control. It seems that these extracts had some secondary metabolite which might be detrimental to one or the important rumen microbes. A depression in feed degradability due to addition of dried plant powder of bamboo *Bambusa balcooa*, *Eclipta alba*, *Alternantherd sessilis* leaves could be due to phenolic compounds such as tannins, gallic acids, ellagic acids and tannic acid. Tannins have been implicated for their inhibitory effect on feed digestion, microbial population and enzyme activity in many experiments (Hristov *et al*. 2003, Patra *et al*. 2006b, Alexander *et al*. 2008, Kumar *et al.* 2011). Reduction in IVDMD and IVOMD due to addition of *Andrographis*

Table 3. Effect of different plant leaves and bark on concentration of fatty acids and ammonia nitrogen (NH_3-N) *in vitro*

Attributes	Additives (mg)	Additives						Standard error
		Andrographis paniculata	*Azadirachta indica*	*Bambusa balcooa*	*Eclipta alba*	*Alternanthera sessilis*	*Calotrips gigantea*	
TVFAs (MEq/dl)								0.21
	00	3.5^{x}	3.5^{x}	3.5	3.5	3.5	3.5^{x}	
	30	3.3^{x}	3.4^{x}	3.1	3.3	3.1	3.2^{x}	
	60	2.6^{y}	2.8^{y}	3.1	3.1	3.0	2.9^{y}	
	Mean	3.1	3.2	3.2	3.3	3.2	3.2	
Acetate (% of TVFA)								0.31
	00	67.3	67.3	67.3	67.3	67.3	67.3	
	30	67.7	67.2	67.8	67.0	67.7	67.5	
	60	67.4	67.5	67.1	67.2	67.6	67.2	
	Mean	67.5	67.3	67.4	67.2	67.5	67.3	
Propionate (% of TVFA)							0.11	
	00	22.6	22.6	22.6	22.6	22.6	22.6	
	30	22.7	22.1	21.8	22.4	21.4	22.3	
	60	22.2	22.5	22.2	22.2	21.7	22.1	
	Mean	22.5	22.4	22.2	22.4	21.9	22.3	
Butyrate (% of TVFA)								0.28
	00	10.1	10.1	10.1	10.1	10.1	10.1	
	30	9.6	10.7	10.4	10.6	10.9	10.2	
	60	10.4	10.0	10.7	10.6	10.7	10.7	
	Mean	10.0	10.3	10.4	10.4	10.6	10.3	
A:P ratio								0.08
	00	2.98	2.98	2.98	2.98	2.98	2.98	
	30	2.98	3.04	3.11	2.99	3.16	3.03	
	60	3.04	3.00	3.02	3.03	3.12	3.04	
	Mean	3.00	3.01	3.04	3.00	3.09	3.02	
NH_3-N (mg/dl)							0.83	
	00	19.6^{x}	19.6^{x}	19.6	19.6	19.6	19.6^{x}	
	30	17.7^{y}	17.9^{y}	18.9	19.8	19.3	18.2^{x}	
	60	16.2^{z}	17.1^{y}	19.3	19.4	19.1	17.1^{y}	
	Mean	17.8^{b}	18.2^{ab}	19.3^{a}	19.6^{a}	19.3^{a}	18.1^{b}	

^{xyz} Mean with different superscripts in a column and ^{abc} in a row among levels and treatments, respectively differ significantly (P<0.05).

Table 4. Effect of different plant leaves and bark on ruminal enzyme activites (IU/dl/h) of carboxymethyl cellulase, xylanase and β-glucosidase *in vitro*

Attributes	Additives (mg)	Additives						Standard error
		Andrographis paniculata	*Azadirachta indica*	*Bambusa balcooa*	*Eclipta alba*	*Alternanthera sessilis*	*Calotrips gigantea*	
Carboxymethyl cellulose								0.92
	00	13.7^{x}	13.7	13.7	13.7	13.7	13.7^{x}	
	30	11.8^{xy}	12.5	13.2	12.1	13.2	11.5^{xy}	
	60	10.1^{y}	12.1	12.4	13.6	12.7	10.6^{y}	
	Mean	11.8^{b}	12.8^{a}	13.1^{a}	13.2^{a}	13.2^{a}	11.9^{b}	
Xylanase								2.09
	00	32.5^{x}	32.5	32.5	32.5	32.5	32.5^{x}	
	30	28.9^{y}	30.6	31.3	33.9	31.5	29.7^{y}	
	60	25.9^{z}	28.5	32.7	32.5	32.1	26.2^{z}	
	Mean	29.1^{b}	30.5^{a}	32.1^{a}	32.9^{a}	32.1^{a}	29.4^{b}	
b-glucosidase							0.68	
	00	7.9^{x}	7.9	7.9	7.9	7.9		7.9^{x}
	30	6.3^{y}	6.8	7.3	7.7	7.3	7.1^{y}	
	60	5.9^{y}	6.2	7.1	8.1	7.8	6.7^{y}	
	Mean	6.7	6.9	7.4	7.9	7.7	7.2	

^{xyz} Mean with different superscripts in a column and ^{abc} in a row among levels and treatments, respectively differ significantly (P<0.05).

Table 5. Effect of different plant leaves and bark on dry matter degradability and microbial biomass production *in vitro*.

Attributes	Additives (mg)	Additives						Standard error
		Andrographis paniculata	*Azadirachta indica*	*Bambusa balcooa*	*Eclipta alba*	*Alternanthera sessilis*	*Calotrips gigantea*	
Dry matter degradibility (%)								0.39
	00	64.2^{x}	64.2^{x}	64.2^{x}	64.2^{x}	64.2^{x}	64.2^{x}	
	30	63.3^{y}	63.5^{x}	63.6^{x}	63.5^{xy}	63.7^{x}	62.7^{y}	
	60	61.3^{z}	61.9^{y}	62.2^{y}	62.9^{y}	62.5^{y}	62.1^{y}	
	Mean	62.9	63.2	63.3	63.5	63.1	62.8	
Organic matter degradibility (%)								0.41
	00	65.8 ^{x}	65.8 ^{x}	65.8 ^{x}	65.8 ^{x}	65.8 ^{x}	65.8 ^{x}	
	30	64.2 ^{y}	64.7 ^{y}	64.9 ^{y}	64.7 ^{y}	64.5 ^{y}	63.9 ^{y}	
	60	62.7 ^{z}	63.1 ^{z}	64.1 ^{y}	63.9 ^{y}	64.3 ^{y}	62.5 ^{z}	
	Mean	64.2	64.5	64.9	64.8	64.9	64.1	
Total microbial biomass production (mg)								0.37
	00	34.2 ^{x}	34.2 ^{x}	34.2	34.2	34.2	34.2 ^{x}	
	30	36.8 ^{xy}	33.5 ^{xy}	34.7	33.9	34.5	33.6 ^{xy}	
	60	37.1 ^{y}	32.7 ^{y}	35.3	33.5	34.9	33.1 ^{y}	
	Mean	33.2^{b}	33.4^{b}	34.7^{a}	33.9^{ab}	34.5^{a}	33.6^{b}	

^{xyz} Mean with different superscripts in a column and ^{abc} in a row among levels and treatments, respectively differ significantly (P<0.05).

paniculata and *Azadirachta indica* leaves powder in incubation medium might be due to different bitter principles which significantly reduced rumen protozoal numbers. A depression in feed degradability (IVDMD and IVOMD) due to addition of dried *Calotrips gigantean* bark could be due to presence of saponin. Lila *et al.* (2003) also observed that sarsaponin reduced IVDMD of hay plus concentrate after 24 h of incubation.

Our results indicated that the plant secondary metabolites appear to have a potential to manipulate rumen fermentation favorably. Dried powder of *Andrographis paniculata*, *Azadirachta indica leaves and Calotrips gigantean* bark have the potential to reduce ruminal methanogenesis and rumen protozoal population *in vitro*. Therefore, further studies are needed to explore chemical nature of the active principles responsible for the anti-methanogenic activity and testing of these plants products in *in vivo* experiments for their effect on ruminal fermentation and nutrient utilization by the ruminant animals.

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