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In vitro Nutrient Digestibility, Gas Production and Tannin Metabolites of *Acacia nilotica* Pods in Goats

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ABSTRACT : Six total mixed rations (TMR) containing 0, 4, 6, 8, 10, 12% tannin (TMR I-VI), using *Accacia nilotica* pods as a source of tannin, were used to study the effect of *Acacia* tannin on *in vitro* nutrient digestibility and gas production in goats. This study also investigated the degraded products of *Acacia nilotica* tannin in goat rumen liquor. Degraded products of tannins were identified using high performance liquid chromatography (HPLC) at different hours of incubation. *In vitro* digestibility of dry matter (IVDMD) and organic matter (IVOMD) were similar in TMR II, and I, but declined ($p<0.05$) thereafter to a stable pattern until the concentration of tannin was raised to 10%. *In vitro* crude protein digestibility (IVCPD) decreased ($p<0.05$) with increased levels of tannins in the total mixed rations. Crude protein digestibility was much more affected than digestibility of dry matter and organic matter. *In vitro* gas production (IVGP) was also reduced ($p<0.05$) with increased levels of tannins in the TMR during the first 24 h of incubation and tended to increase ($p>0.05$) during 24-48 h of incubation. Gallic acid, phloroglucinol, resorcinol and catechin were identified at different hours of incubation. Phloroglucinol and catechin were the major end products of tannin degradation while gallate and resorcinol were produced in traces. It is inferred that *in vitro* nutrient digestibility was reduced by metabolites of *Acacia nilotica* tannins and ruminal microbes of goat were capable of withstanding up to 4% tannin of *Acacia nilotica* pods in the TMR without affecting *in vitro* nutrient digestibility. (**Key Words :** *Acacia nilotica* Pods, *In vitro* Digestibility, Tannin Degradation Products, HPLC)

INTRODUCTION

Availability of conventional feed to sustain livestock feeding is a major constraint in developing countries. There is a deficit of about 18.9 metric tonnes (MT) of digestible crude protein and 2000 MT of total digestible nutrients as animal feeds in India (Jain et al., 1996). Hence, pressure on utilization of unconventional feed resources has been increasing to develop least cost rations (Rai and Shukla, 1977; Rai and Barman, 2004). The use of unconventional feed is limited due to the presence of one or other toxic factors, including tannins (Makkar et al., 1990b; Barman and Rai, 2006; Kondo et al., 2007). Tannins may negatively affect the utilization of nutrients in animals (McLeod, 1974; Mueller-Harvey et al., 1988; Hagerman et al., 1992). Tannin reduces feed intake by decreasing palatability of the ration because of its astringent effect on the oral cavity (Glick and Joslyn, 1970). Tannin affects digestibility either by binding the digestive enzymes or by binding feed nutrients (Barman and Rai, 2000). Tannin also lowers rumen turnover rate (Barman and Rai, unpublished report) as well as digestibility of nutrients which has a greater impact on reducing feed intake than decreased palatability (Waghorn et al., 1994). Degen et al. (1995) found negative N balance as well as reduction in digestibility of NDF, ADF and ADL when *Acacia saligna* leaves were fed to sheep and goats. However, recently it has been reported that a few species of ruminal bacteria, namely *Streptococcus caprinus* (predominant in goats), *Selenomonas ruminantium*, *Prevotella ruminicola*, *Butyrivibrio* sp., *Lactobacillus* sp. and *Enterobacteriacae* sp., can utilize both condensed and hydrolysable tannin as a sole source of energy (Pell et al., 2001). *Streptococcus caprinus* bacteria found in the goat rumen had the ability to tolerate up to 3% of hydrolysable or condensed tannins, but did not utilize them as an energy source (Brooker et al., 1994). Reduction in ruminal protozoa was also reported by Wang et al. (1994). Tannin reduces ruminal ammonia nitrogen and total volatile fatty acids (Salawu et al., 1999; Krebs et. al., 2007) and reduces bioavailability of several minerals (Ally and Kunjikutty, 2003).

Acacia nilotica pods are one of the highly nutritious (Ngwa et al., 2001; Barman and Rai, 2003c) unconventional

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Table 1. Ingredient composition (%, w/v) of the total mixed ration containing different levels of tannin[a]

Ingredients	TMR-I (0% Tannin)	TMR-II (4% Tannin)	TMR-III (6% Tannin)	TMR-IV (8% Tannin)	TMR-V (10% Tannin)	TMR-VI (12% Tannin)
Maize Hay	5.0	30.0	30.0	30.0	30.0	30.0
Wheat straw	25.0	-	-	-	-	-
A. nilotica pods[b]	-	22.0	32.0	43.0	54.0	65.0
Ground nut cake	15.0	8.0	8.0	8.0	7.0	5.0
Maize grain	25.0	6.0	8.0	10.0	9.0	-
Wheat bran	30.0	34.0	22.0	9.0	-	-
Total	100.0	100.0	100.0	100.0	100.0	100.0

[a]2% mineral mixture and 1% NaCl were also added.
[b]*Acacia* pods contained 94.83% OM, 13.15% CP, 14.94% CF, 1.44% EE, 63.0% NFE, 5.17% ash, 30.40% NDF, 25.18% ADF, 22.54% cellulose, 5.22% hemicellulose, 1.90% lignin, and 18.71% total tannin; 17.31 hydrolysable tannin and 1.4% condensed tannin, TMR = Total mixed ration

feeds. About 60×10^3 tonnes of *Acacia nilotica* pods are available annually in India (Punj, 1988). *Acacia nilotica* pods can be used as an energy source in a concentrate mixture for ruminants and improves the efficiency of energy utilization in cattle (Barman and Rai, 2005). They also contain all the essential amino acids in good proportions comparable to egg protein (Barman and Rai, 2006). Scanty of literature is available on the nutritional potential of *Acacia nilotica* pods and their tannin metabolites in ruminants. Hence, an attempt was made to assess *in vitro* nutrient digestibility, gas production and tannin metabolites with goat rumen fluid using different levels of *Acacia nilotica* pods in a total mixed ration (TMR) as substrate.

MATERIALS AND METHODS

Composition of total mixed ration (TMR)

Total mixed rations were formulated (%, w/v) using concentrate ingredients, namely *Acacia nilotica* pods, groundnut cake, maize grain, wheat bran, and roughage ingredients, namely maize stover and wheat straw. The concentrate to roughage ratio was kept at 70:30 (Table 1). In addition, to each TMR diet (100 g), a mineral mixture (2 g) and common salts (1 g) were added.

Experimental design

The chemical composition and tannin fractionation of TMRs were determined using standard procedures. Three replicates from each TMR were used for *in vitro* DM, OM and CP digestibility estimation together with triplicate blanks. TMR I was used as a control. *In vitro* gas production was measured at different time intervals to correlate with digestibility of dry matter and organic matter. Tannin metabolites in the rumen fluid were estimated using HPLC at 18, 30 and 42 h post-incubation.

Tannin estimation of TMR

The TMRs were dried at 55±1°C to constant weight, ground to pass through a sieve of 1 mm diameter and stored in plastic containers with lids until further analysis. Total phenol and tannins (tannic acid equivalent) were analyzed as per Makkar et al. (1993). Total tannins (tannic acid equivalent) were estimated from the difference between total phenol and non-tannin phenol obtained after precipitating with polyvinyl polypyrrolidone (P-6755, SIGMA Comp.). Concentrations of total phenol and non-tannin phenol were calculated from a tannic acid (T-0125, SIGMA Comp.) standard curve. Condensed tannin (leucocyanidin equivalent) was estimated by the butanol-HCl method of Porter et al. (1986). The concentration of condensed tannin was calculated (% DM) from the formula $A_{550nm}\times78.26\times$dilution factor/% DM. Hydrolysable tannin was calculated from the difference between total tannin phenol and condensed tannin.

In vitro nutrient digestibility and gas production

Selection, housing, management and feeding of experimental animals : Three male crossbred (Alpinex Beetal; Saanen×Beetal) goats were selected for experimental purposes and were maintained on a diet comprising 0.5 kg concentrate mixture (wheat grain 49%, wheat bran 30%, ground nut cake 18%, mineral mixture 2% and common salt 1%) per animal per day and green maize *ad libitum* as basal roughage. Rumen fluid was collected through a stomach tube, fitted with a perforated stainless steel probe at one end and a 100 ml plastic syringe at the other end, in the morning 3 h post-feeding. The first withdrawal was discarded to prevent possible contamination of saliva with rumen fluid. Rumen fluid was filtrated through a double layer of muslin cloth into pre-warmed thermos previously flushed with CO_2. After transferring to the laboratory, rumen fluid was flushed with CO_2 gas and then fractionated for *in vitro* studies.

In vitro nutrient digestibility was estimated as described by Tilley and Terry (1963). Dry TMR (500 mg, 1 mm mesh size) was taken in a 100 ml glass bottle and 40 ml McDougall's buffer (McDougall, 1948) was added and pre-warmed to 39±1°C. Then into each bottle 10ml strained rumen fluid was dispensed, keeping the buffer and rumen

Table 2. Tannin content (%) of the diet after incorporation of *Acacia* pods at different levels

Total mixed ration	Total phenol[a]	Non tannin phenol	Total tannin[a]	Hydrolysable tannin	Condensed tannin[b]
TMR II (4% Tannin)	5.38±0.00	1.26±0.11	4.12±0.01	3.81±0.12	0.31±0.00
TMR III (6% Tannin)	7.62±0.14	1.62±0.00	6.00±0.14	5.55±0.15	0.45±0.01
TMR IV (8% Tannin)	10.08±0.14	2.08±0.05	8.01±0.09	7.40±0.08	0.60±0.01
TMR V (10% Tannin)	12.81±0.11	2.78±0.01	10.03±0.11	9.28±0.12	0.76±0.1
TMR VI (12% Tannin)	14.98±0.02	2.86±0.00	12.12±0.04	11.21±0.03	0.91±0.01
Acacia pods	21.67±0.63	2.96±0.06	18.71±0.68	17.31±0.68	1.40±0.02

* For detailed composition of diet (%, w/v) ingredients see Table 1. [a] Tannic acid equivalent. [b] Leucocyanidin equivalent. TMR = Total mixed ration.

Table 3. Chemical composition (% DM) of the total mixed ration containing different levels of tannin

TMR	OM	CP	EE	CF	NFE	Ash	NDF	ADF	Cellulose	HC	Lignin
TMR I	91.64	16.64	4.18	23.08	47.75	8.36	62.45	34.06	22.25	28.39	10.70
(0% Tannin)	±0.10	±0.04	±0.45	±0.10	±0.41	±0.11	±0.60	±0.04	±0.13	±0.56	±0.18
TMR II	90.60	14.77	2.65	22.46	50.72	9.04	55.05	35.80	25.05	19.25	9.94
(4% Tannin)	±0.16	±0.44	±0.09	±0.17	±0.20	±0.65	±0.35	±0.26	±0.46	±0.09	±0.59
TMR III	89.25	14.76	2.98	16.36	55.15	10.75	53.41	35.14	25.30	18.27	9.35
(6% Tannin)	±0.06	±0.14	±0.10	±0.10	±0.08	±0.05	±0.15	±0.25	±0.17	±0.40	±0.31
TMR IV	91.03	14.73	2.83	19.44	54.03	8.97	53.55	31.06	22.19	22.48	8.27
(8% Tannin)	±0.06	±0.13	±0.10	±0.07	±0.05	±0.06	±1.60	±5.69	±5.31	±7.30	±0.29
TMR V	90.62	13.85	2.68	22.14	51.95	9.38	48.93	35.86	28.16	13.07	6.83
(10% Tannin)	±0.15	±0.35	±0.09	±0.05	±0.46	±0.15	±2.10	±2.65	±1.85	±0.55	±0.67
TMR VI	90.51	16.54	2.65	17.57	53.75	9.49	49.11	38.95	28.71	10.16	9.15
(12%Tannin)	±0.08	±0.20	±0.15	±0.07	±0.11	±0.08	±0.72	±1.59	±0.26	±0.87	±1.12

OM = Organic matter, CP = Crude protein, EE = Ether extract, CF = Crude fibre, NFE = Nitrogen free extractives.
NDF = Neutral detergent fibre, ADF = Acid detergent fibre, HC = Hemicellulose; TMR = Total mixed ration.
* For detail composition of ingredients in different TMRs see Table 1.

fluid ratio at 4:1. The bottles were sealed under a continuous supply of CO_2 gas and incubated at 39±1°C. After 48 h of incubation, 2 ml 5% pepsin (1:3,000, Hi-Media Laboratories Pvt. Ltd., Mumbai, India) solution prepared in 6 N HCl (2 ml contained 0.1 g) was injected into the bottle and the contents incubated for another 24 h. Then the contents were filtered through a sintered glass crucible for the estimation of IVDMD and IVOMD. Gas production was measured using a pressure transducer (Bailey and Mackey Ltd., UK) fitted with a 60cc plastic syringe and 3-way valve (Theodorou et al., 1994). Gas production was measured at 6, 12, 18, 24, 30, 36, 42 and 48 h post incubation and expressed as ml/g substrate and ml/h/g substrate. The period from 0 h to 24 h was considered as first stage kinetics (fast degradation) and 24 to 48 h as second stage kinetics (slow degradation) and expressed as two separate values. Gas production was measured to correlate gas production with digestibility of DM and OM. *In vitro* CP digestibility was estimated at the end of the incubation period by filtering the content of vials through Grade 1 filter paper (SONAR). The residues were digested for protein estimation (AOAC, 1990). Digestibility was calculated from the difference in the protein content of the sample before and after incubation.

Chemical composition and fibre fractionation of TMR

Chemical composition was estimated by the methods of AOAC (1990) and fibre fractionation was carried out by the method of Van Soest et al. (1991).

Determination of tannin degraded products using HPLC

A separate set of *in vitro* bottles containing 0.5 g dry samples from TMR I, II, III and IV containing 0, 4, 8 and 12% tannin were used to estimate tannin degradation products. Samples (2 ml) were removed from the *in vitro* bottles after 18, 30 and 42 h to determine the tannin degradation products. Rumen fluid was centrifuged at 19,000 g for 10 min. After filtration through a 0.45 μ Millipore membrane filter, supernatant was stored at +4°C. The (+) catechin, (-) epicatechin, gallic acid, phloroglucinol and resorcinol were used as standards. The sample was analysed using a reverse phase HPLC column (25 cm×4.6 mm packed with 5 μm particle supelcosil LC 8DB, Sigma Chemical Company, USA) on a CTO 10A SHIMADZU (Japan) liquid chromatograph fitted with two pumps (LC10 AU SHIMADZU). The peaks were detected at 280 nm

Table 4. *In vitro* digestibility of dry matter, organic matter and protein of different TMRs containing different tannin levels

Parameters	TMRs						SEM
	I (0% Tannin)	II (4% Tannin)	III (6% Tannin)	IV (8% Tannin)	V (10% Tannin)	VI (12% Tannin)	
IVDMD	67.04[a]±0.21	67.17[a]±0.28	61.15[b]±1.44	60.47[b]±0.96	61.04[b]±0.33	58.24[c]±0.51	0.44
IVOMD	66.76[a]±0.61	67.06[a]±0.55	59.63[b]±1.48	59.97[b]±1.52	60.33[b]±0.30	57.08[c]±0.35	0.63
IVCPD	84.93[a]±0.69	72.77[b]±0.96	70.05[c]±0.55	66.15[d]±1.83	63.19[e]±0.32	63.04[ef]±1.78	0.78
Gas production							
Total (ml)	87.50±1.80	84.83±1.01	85.33±2.41	88.33±1.43	85.67±1.45	81.50±3.18	4.32[NS]
0-24 h (ml)	55.17[a]±2.09	53.50[a]±2.29	49.67[b]±1.77	48.17[b]±0.60	43.67[c]±0.67	42.50±3.50	1.20
24-48 h (ml)	32.33[e]±0.67	31.33[f]±1.33	35.66[d]±0.67	40.16[b]±0.83	42.00[a]±1.00	39.00[c]±0.58	0.51
ml/g substrate	175.00±3.61	169.67±2.03	170.67±4.81	176.67±2.85	171.33±2.91	163.00±6.36	8.64[NS]
ml/h/g substrate	3.65±0.08	3.53±0.04	3.56±0.10	3.68±0.06	3.57±0.06	3.40±0.13	0.18[NS]

IVDMD = *In vitro* dry matter digestibility, IVOMD = *In vitro* organic matter digestibility.
IVCPD = *In vitro* crude protein digestibility, TMR = Total mixed ration, SEM = Standard error of mean.
[a, b, c, d, e, f] Different superscripts in a row differ significantly, ($p<0.05$).
* For detail composition of ingredients in different TMRs see Table 1.

Table 5. Correlation coefficient of different parameters with increasing level of tannins in the TMRs

Parameters	Tannin	IVDMD	IVOMD	IVCPD	IVGP (ml/g)	IVGP (ml/h/g)
Tannin	1					
IVDMD	-0.89*	1				
IVOMD	-0.88*	0.99*	1			
IVCPD	-0.96*	0.83*	0.82*	1		
IVGP (ml/g)	-0.55	0.32*	0.41*	0.44*	1	
IVGP (ml/h/g)	-0.55	0.30*	0.40*	0.45*	0.99*	1

IVDMD = *In vitro* dry matter digestibility, IVOMD = *In vitro* organic matter digestibility.
IVCPD = *In vitro* crude protein digestibility, IVGP= *In vitro* gas production. * $p<0.05$.

using a SPD-M10 AU SHIMADZU diode array detector. Glacial acetic acid and water (975:25 ml, v/v) was used as eluent A. Pure methanol (HPLC grade) was used as eluent B. The gradient was started with 100% eluent A and ended with 100% eluent B over a period of 58 min. Tannin metabolites in the samples were identified by comparing with the readings of the standard.

Statistical analysis of the data

The data were analyzed by one-way ANOVA as per Snedecor and Cochran (1989) using SYSTAT software. Correlations were estimated to find out the relationship of different levels of tannins with IVDMD, IVOMD, IVCPD and IVGP. Regression analysis was used to estimate the effect of different tannin levels on IVDMD, IVOMD, IVCPD and IVGP.

RESULTS

Tannin content and chemical composition of the TMR

Total mixed ration II, III, IV, V and VI contained total tannins ranging from 4.12 to 12.12% (Table 2). Percentage (on DM basis) of crude protein, crude fiber, nitrogen free extract and neutral detergent fiber of different TMRs ranged from 14.76±0.14 to 16.64±0.04; 16.36±0.10 to 23.08±0.10; 47.75±0.41 to 55.15±0.08; 48.93±2.10 to 62.45±0.60, respectively, in TMR I, II, III, IV, V and VI (Table 3).

***In vitro* digestibility and gas production**

Dry matter digestibility was decreased ($p<0.05$) in all samples compared to the control (TMR I), but the difference was only significant ($p<0.05$) between tannin levels 0% and 12% (Table 4). Likewise, organic matter digestibility showed a similar pattern. However, variation in crude protein digestibility was much more pronounced compared to IVDMD and IVOMD and values decreased significantly ($p<0.05$) with increasing level of tannin from 0% to 12% (Table 4).

In vitro total gas production was similar across all TMRs (Table 4). However, during the first 24 h of incubation there was a gradual reduction ($p<0.05$) in gas production, which was reflected in reduced *in vitro* digestibility of DM and OM. The gas production in the next 24 h (24-48 h), first decreased ($p<0.05$) and then gradually increased ($p<0.05$) up to a tannin level of 10% and then suddenly decreased (Table 4). Tannin concentration was negatively correlated with IVDMD, IVOMD, IVCPD and IVGP and IVDMD, IVOMD and IVCPD were positively correlated with IVGP (Table 5).

Tannin degradation products

Phloroglucinol, gallic acid, resorcinol and catechin were

Table 6. HPLC analysis of *in vitro* degradation products of tannins of TMR II, IV and VI after different incubation periods

Parameters	TMRs								
	II (4% Tannin)			IV (8% Tannin)			VI (12% Tannin)		
	18 h	30 h	42 h	18 h	30 h	42 h	18 h	30 h	42 h
Tannin degraded products (mg/g tannin)									
Phloroglucinol	50.54	75.99	-	86.25	297.79	18.75	63.34	298.40	213.07
Gallic acid	-	0.05	-	-	0.05	0.07	0.01	0.02	0.05
Resorcinol	-	-	-	-	-	7.10	-	-	1.09
Catechine	16.40	11.58	-	5.62	10.00	11.45	1.55	3.39	7.06

- Not detected. TMR = Total mixed ration.

identified as degradation products of *Acacia* tannins in goat rumen fluid. Phloroglucinol concentration increased over the first 30 h of incubation. Catechin concentration declined with time at the lowest tannin concentration (4%) while it increased with time at a higher level of tannin (12%). Gallic acid was only present in trace amounts (Table 6).

DISCUSSION

In vitro nutrient digestibility decreased with increased level of tannins ranging from 0 to 12%. There was no significant difference in DM and OM of TMRs containing 6, 8 and 10% tannin, which indicated that goats can utilize nutrients from TMRs containing 6% tannin equally as well as from TMRs containing 8 and 10% tannin. It became clear from this study that digestibility of DM, OM and CP was reduced with increased level of tannin. Crude protein digestibility was greatly affected compared to dry matter and organic matter. There was sudden drop of 12.16% in crude protein digestibility when tannin level was increased from 0 to 4%. However, digestibility thereafter did not show much variation when tannin concentration was increased from 6 to 12% in the TMRs. The pronounced reduction in protein digestibility in this case might be due to the high ratio of soluble to insoluble tannins in *Acacia nilotica* pods as a high ratio of soluble tannins reduces protein digestibility more (Hagerman et al., 1992; Smith and Brown, 2001). Similar results were also reported by Barman and Rai (2003a). Moreover, tannin of *Acacia* has high protein binding capacity (Alam et al., 2007). In contrast, it was reported (Martinez and Moyano, 2003) that enzymatic hydrolysis of the protein of casein, pea meal and soybean meal increased in the presence of tannic acid (1 to 5% w/w). However, tannins of *Acacia nilotica* pods differ from tannic acid as they contain epigallocatechin gallate (Ayoub, 1985; Barman and Rai, unpublished report). Decreased ($p<0.05$) digestibility of other nutrients was observed with increased level of tannin which was reflected through decreased ($p<0.05$) IVGP (ml/g) during the first 24 h (Table 4).

Since catechin but not epicatechin was produced as one of the degradation products of *Acacia nilotica* tannin, it is unambiguous that *Acacia nilotica* contained catechin gallate (Barman and Rai, unpublished report). Tanner et al. (1990) also reported the presence of catechin gallate in *Acacia nilotica* pods. Concentration of phloroglucinol was highest (18.75-298.40 mg/g tannin) followed by catechin (1.55-16.40 mg/g tannin) and gallic acid (0.01-0.07 mg/g tannin). Resorcinol was produced in traces (Table 6). Lower concentrations of gallic acid and catechin found in this study might be due to rapid degradation of these two products to either phloroglucinol or resorcinol or both by rumen microbes (Murdiati et al., 1992; Lowry et al., 1996; Arunachalam et al., 2003). Pyrogallol is produced as one of the metabolite of gallic acid, but in this study pyrogallol was also not detected. This might be due to rapid degradation of pyrogallol to phloroglucinol (Zhu et al., 1995; Tor et al., 1996). Several authors (Nelson et al., 1995; Odenyo and Osuji, 1998) found pyrogallol and gallic acid as metabolites of tannic acid. The uncommon nature of tannin of *Acacia nilotica* might follow a different degradation path in which either pyrogallol is not produced or it is rapidly degraded to subsequent metabolites.

The metabolites of *Acacia nilotica* tannin reduced the *in vitro* digestibility of dry matter, organic matter and crude protein. Degraded products of tannins from *Acacia nilotica* pods in rumen fluid of goats were phloroglucinol, gallic acid, resorcinol and catechin. Phloroglucinol was the major degradation product while gallate was produced in traces. Goats harbour the tannin degrading bacteria in the rumen microflora without pre-exposure to a tannin-containing diet. It is recommended that *Acacia nilotica* pods can be incorporated up to 22% in the TMRs of goats without affecting DM and OM digestibility. Further research is necessary to determine the metabolites of *Acacia* tannins responsible for reducing nutrient digestibility.

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