Journal of
Advanced Veterinary Research
Volume 2 (2012) 120-126 **Original Research**

Estimation of Total Saponins and Evaluate Their Effect on in vitro Methanogenesis and Rumen Fermentation Pattern in Wheat Straw Based Diet

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(Recieved 26 November 2011/ Accepted 6 April 2012)

Abstract

The present experiment was carried out to estimate the total saponins and evaluate their effect on methanogenesis and rumen fermentation by in vitro gas production techniques. Three plant material, rough chaff tree seed (*Achyranthus aspara*, T_1), gokhru seed (*Tribulus terrestris*, T2) and Siris seed (*Albizia lebbeck*, T3) were selected for present study. The total saponins content in T_1, T_2 and T_3 were 45.75, 25.65 and 48.26% (w/w), respectively. Three levels of each saponins (3, 6 and 9% on DM basis) and wheat straw based (50R:50C) medium fiber diet (200±10 mg) were used for the evaluation of their effect on methanogenesis and rumen fermentation pattern. Results showed the maximum methane reduction (49.66% in term of mM/gDDM) and acetate propionate ration (35.08%) were found in T1 at 6 and 3% levels. Result show that propionate production (mM/ml) was increased; protozoa population decreased (75%) significantly on addition with T_3 at 6% level. No significant variation was found in dry matter digestibility in all cases. The present results demonstrate that total saponins extracted from different herbal plants are a promising rumen modifying agent. They have the potential to modulate the methane production, dry matter digestibility and microbial biomass synthesis.

*Keywords:*Saponins; In vitro gas production technique; methane; IVDMD; rumen fermentation

Introduction

The recent goal of ruminant microbiologists and nutritionists is to manipulate the ruminal microbial ecosystem to improve the feed conversion efficiency and reduce methane production. Most of the recent studies deal with the diversity analysis of rumen microbes especially methanogens (Chaudhary 2009; Chaudhary *et al*., 2009, 2012) but the studies that deal with the manipulation in the rumen microbial ecosystem for increasing digestion and improving other nutritional parameters are still limited. Increasing fiber digestion, propionate production, yield and efficiency of microbial protein synthesis and decreasing methanogenesis, extensive dietary protein degradation and bacterial recycling by protozoa in rumen are some of the well recognized ways to improve efficiency of nutrient utilization and ruminant productivity under roughage based feeding systems. Recent studies have shown that PSM at lower concentrations could be used to manipulate rumen fermentation and to improve animal productivity. Plant secondary metabolites present in different plant extract positively affected the ruminal methanogenesis (Sirohi *et al*., 2009; Goel *et al*., 2011). Although effort have also been made to select the potential inhibitors from chemical compounds available to check methanogensis by computational means to get better results (Sharma *et al*., 2011). Saponins, tannins and EO (Essential oil) are the group of secondary metabolites that have been widely investigated for their pro nutritional effects in ruminants. Among the all PSM, saponins attract the researchers due to their presence of large group of plants, their antimethanogenic activity and many beneficial effects on animal systems (Francis *et al*., 2002a). Saponins are surface-active glycosides with detergent, wetting, emulsifying, and foaming properties (Wang *et al*., 2005, Sarnthein *et al*., 2004; Mitra *et al*., 1997) and occurring widely in plants and some lower marine animals such as star fish and sea cucumber and bacteria (Riguera 1997; Yoshiki *et al*., 1998). They are abundant in many

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foods consumed by animals and man (Cheeke 1971). Methanolic extract of soapnut decreased *in vitro* methane production and increased molar proportion of propionate (Agarwal *et al*., 2004). Saponin rich aqueous extract of *Asparagus adscendens* (satavar) root and aqueous methanol of *Moringa oleifera* (drumstick) seed decreased protozoal number and methanogenesis and increased yield and efficiency of microbial protein synthesis (Alexander, 2005).

Materials and methods

Procedure of saponins extracts preparation

Three plant parts, rough chaff tree seed (*Achyranthus aspara*, T_1), gokhru seed (*Tribulus terrestris*, T_2) and Siris seed (*Albizia lebbeck*, T_3) were used for the preparation of crude saponins extracts. Seeds of T_1 and T_3 were collected from the campus of National Dairy Research Institute, Karnal while T_2 seeds were purchased from local market of Karnal. Seeds of these plants were dried at 70^0C and ground in mills to pass a 1 mm sieve. All plant parts were defatted with petroleum ether in a soxhlet apparatus.

Take 25 g fat free sample in 500 ml conical flask and add 250 ml absolute methanol (99.9%) in ration of dry weight of the sample to methanol as 1:10. Flask was tightly sealed and kept in a shaker at 25^0C and 120 rpm for 24 hour, followed by centrifuging the contents at 3500 rpm for 20 min. After centrifugation, methanol extract was filtered using Whatman filter paper No.1. The resulting methanolic extracts were evaporated to dryness under in vacuo condition using a rota-evaporator. After evaporation dried plant extract dissolve in minimum amount of distilled water (10 ml), transferred in to a separating funnel and extracted with equal volume of n-butanol (3 times). Again, solvent, n-butanol was evaporated under *in vacuo* condition not higher than 45^0C. Dissolved the dried saponins content in 5-10 ml of distilled water and transferred the solution in to a separate pre-weighed container and freeze dried.

Estimation of total saponins

Total saponins contents in different plant materials were estimated by prescribed colorimetric methods (Hiai *et al.*, 1976). Ten milligram of saponins extract was dissolve in 5 ml 80% aqueous methanol and 50 µl of solution was taken in different test tubes to which 0.25 ml of vanillin reagent (8%, w/v in 99.9% ethanol) was added. Test tubes were placed in ice-cold water bath and 2.5 ml of 72% (v/v) sulphuric acid was added slowly on the inner side of the wall. After mixing the content in each tube, these were left as such for 3 min. warmed the tubes to 60^0C for 10 min using a water bath and then cooled them in ice-cold water bath. Absorbance was measured at 544 nm using spectrophotometer against the reagent blank and prepared the standards curve. Quillaja saponin (Sigma-Aldrich) was used as a reference standard (Shiau *et al*., 2009) and the content of total saponins was expressed as Quillaja saponin equivalents (QS µg/mg extract).

Preparation of diet

To evaluate the effect of different saponins extract, diet was prepared by taking roughage concentrate ratio of 50:50. The roughage part composed of wheat straw and the concentrate part composed of maize (33%), GNC (21%), mustard cake (12%), wheat bran (20%), deoiled rice bran (11%), mineral mixture (2%) and salt (1%) respectively.

Treatments and experimental design

For the evaluation, stock solution (2mg/ml) of extracted saponins was prepared in distilled water and its different volumes were added in calibrated 100ml glass syringe containing 200±10 mg of milled (1mm) wheat straw based diet to have final concentrations of 3, 6 and 9% on DM basis. All the treatments were arranged in RBD with three replicates. Sets was also incubated devoid of substrate with and with out plant powder combinations which served as blanks for particular treatment and values were corrected for different parameters with these blanks.

In vitro gas production

The incubation medium was prepared by prescribed method (Menke and Steingass, 1988). Rumen liquor was collected from a fistulated male buffalo (Bubalus bubalis) maintained on a standard diet (60 parts roughage: 40 parts concentrate) before morning feeding into an insulated flask and

brought into the laboratory. The rumen liquor filtered through four layers of muslin cloth and then the required amount of filtered rumen liquor used as a source of inoculum. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. The 30 ml incubation medium was dispensed anaerobically in each syringe, closed the clamps and incubated at 39±0.5^{0}C for 24 h.

Total gas production and methane estimation

After 24 h incubation, total gas production was calculated by subtracting gas produced in blank syringe (containing no substrate, but only the inoculum and buffer) from total gas produced in the syringe containing substrate and inoculum and buffer. Methane content in fermentation gas was determined by gas chromatography (GC) using Nucon-5765 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q (length 6';o.d.1/8" i.d. 2 mm; mesh range 80-100). Temperatures were 40, 50 and 50^{0}C, in injector oven, column oven and detector, respectively and the flow rates of carrier gas (nitrogen), hydrogen and air were 30, 30 and 300 ml/min, respectively. For methane estimation, each gas sample (250µl) was manually injected using Hamilton airtight syringe. Methane content in sample was calculated by external calibration, using a certified gases mixture with 50% CH_4 and 50% CO_2 (Spantech calibration gas, Surrey, England). The volume of methane produced was calculated as follows:

Methane production (ml) = Total gas produced (ml) × % methane in the sample

Partitioning factor and Microbial biomass yield

The partitioning factor is calculated as the ratio of substrate truly degraded in vitro (mg) to the volume of gas (ml) produced by it. The microbial biomass (MBM) yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor (Blummel *et al*., 1997).

MBM (mg) = Substrate truly degraded - (gas volume × stoichiometrical factor)

Where the stoichiometrical factor used was 2.25.

Total volatile fatty acid (TVFA) estimation

TVFA concentration (mM/100ml) in the supernatant was estimated according to prescribed method (Barnet and Reid, 1957).

Individual volatile fatty acid (IVFA) estimation

Individual volatile fatty acid estimated by gas chromatograph according to the prescribed method (Erwin *et al*., 1961).

Estimation of ammonia nitrogen

The supernatant of each syringe including that of blank was used for NH_3-N estimation. Supernatant (5 ml) was mixed with 1 N NaOH (2 ml) and steam passed on this using KEL PLUS - N analyzer (Pelican, India) and the NH_3 evolved was collected in boric acid solution having mixed indicator and titrated against N/100 H_2SO_4.

Protozoa counting

The protozoa in fermentation fluid were counted by Haemocytometer as per the prescribed method (Dehority, 1984).

In vitro true DM degradability

In vitro true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per the prescribed method (Van soest *et al*., 1991).

Proximate analysis and Cell wall constituents

The proximate analysis (Organic matter, Crude protein, Ether extract, Total Ash) of substrate was carried out as per the methods of AOAC (1995). The Neutral detergent fibre of substrates were determined according to prescribed method (Van Soest *et al*., 1991) and other cell wall components like Acid detergent fiber (ADF) and Hemicellulose (HC) as per the method (Van Soest *et al*., 1991) .

Statistical analysis

Experimental data of different parameters were analyzed in randomized block design with three replicates for analysis of variance (Snedecor and Cochran, 1968).

Results

The physical and chemical composition of wheat

straws based medium fiber diet was presented in table 1. The OM, CP and NDF content of wheat straws based medium fiber diet were 878.4, 125.3 and 604.5 g/kg, respectively.

Table 1. Physical and chemical composition of wheat straw based diet used as substrate in in vitro incubation

Ingredient of diet							
Particulars	g/kg on DM basis						
Wheat straw	500						
Concentrate	500						
Ingredient of concentrate							
Particulars	g/kg on DM basis						
Maize	330						
Ground nut cake	210						
Mustard cake	120						
Wheat bran	200						
Deoiled rice bran	110						
Mineral mixture	20						
Salt	10						
Chemical constituents of diet (g/kg on DM basis)							
Diet	OM	CP	EE	NDF	ADF	HC	TA
R:C (50:50)	878.4	125.3	30.4	604.5	329.5	275.0	121.6

OM= Organic matter, CP= Crude protein, EE= Ether extract, NDF= Neutral detergent fiber, ADF= Acid detergent fiber, HC= Hemicelluloses, TA = Total ash

The total saponins content in *Achyranthus aspara* (T_1), *Tribulus terrestris* (T_2) and *Albizia lebbeck* (T_3) seeds were shown in table 2. T_3 contain highest total saponins content 48.26% (w/w) while T_1 and T_2 seeds have 45.75, 25.65% (w/w), respectively.

Effects of different doses of total saponins supplementation on in vitro rumen fermentation pattern and methane production of wheat straws based medium fiber diet were shown in table 3 and table 4, respectively.

Table 2. Total saponin contents in herbal plants parts (% w/w) as Quillaja saponin equivalents

Herbal Plants	Total saponins % (w/w)
Achyranthus aspara, (T_1)	45.70
Tribulus terrestris, (T_2)	25.65
Albizia lebbeck, (T_3)	48.26

Methane production was reduced in each treatment and every level of doses. The highest inhibition of CH_4 (mM/gm digestible dry matter) formation occurred with T_1 (49.66%) at 6% level. A good reduction percentage in methane production were also observed in T_2 (47.61%) and T_3 (48.99%) at 9 and 6% levels, respectively. Significant reduction in protozoa population observed except T_2 treatment. Maximum 75% reduction of protozoa population was found in T_3 at 6% level. Among the all treatment and doses, total volatile fatty acids concentration (mM/100ml) was maximum increased (9.55%) in T_1 at 3% level. T_3 at 3% level showed the maximum increase in Propionate concentration (13.39%), it was also show the maximum reduction in acetate (26.56%) and butyrate (30.35%) concentration (mM/100ml). Reduction in acetate propionate ratio was highest in T_1 (35.08%) at 3% level. At 6% level of T_2 and T_3 were also show the good reduction percentage in acetate propionate ratio i.e. 24 and 21.23% respectively.Supplementation of total saponins at differ-

Table 3. Supplementation effect of different saponins extracts on in vitro rumen fermentation pattern and methane inhibition on wheat straw based diet (50R:50C)

Treatment	Dose (%DM Basis)	IVDMD%	CH_4 (ml/gm DM)	CH_4 (mM/gm DM)	MBM (mg)	PF	Protozoa ($x10^4$/ml)
Control	00	64.65±0.70	37.57±1.52	1.47±0.06	40.06±1.70	3.26±0.05	2.00±.0.29
T_1	3	70.80±0.64	24.14±0.29	0.94±0.01	50.47±0.72	3.50±0.04	1.67±0.17
T_1	6	68.68±0.55	18.96±0.43	0.74±0.02	58.62±1.81	3.93±0.07	1.33±0.33
T_1	9	67.09±1.24	24.47±0.73	0.96±0.03	50.93±0.22	3.63±0.03	1.00±0.29
T_2	3	66.52±1.46	20.83±0.97	0.81±0.04	55.78±5.62	3.89±0.23	1.50±0.29
T_2	6	66.13±0.85	21.40±0.91	0.84±0.03	52.38±3.02	3.74±0.14	1.25±1.14
T_2	9	64.60±1.69	19.74±1.26	0.77±0.05	47.82±4.36	3.58±0.14	1.50±0.29
T_3	3	64.10±1.11	21.82±0.37	0.85±0.02	45.69±1.88	3.50±0.05	0.67±0.17
T_3	6	67.54±1.86	19.23±0.86	0.75±0.02	61.20±3.25	4.12±0.11	0.50±0.29
T_3	9	72.49±3.80	22.44±1.01	0.88±0.04	61.36±11.17	3.93±0.39	1.00±0.29

T_1 = Achyranthus aspara, T_2 = Tribulus terrestris, T_3 = Albizia lebbeck, IVDMD = In vitro dry matter digestibility, CH_4 = Methane, MBM = microbial biomass, PF = Partition factor

Table 4. Supplementation effect of different saponins extracts on in vitro IVFA and Acetate: propionate ratio on wheat straw based diet (50R:50C)

Treatment	Dose (% DM Basis)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P	TVFA (mM/100 ml)	NH_3-N (mg/100ml)
Control	00	10.46±0.41	3.21±0.03	1.68±0.02	3.25±0.09	7.85±0.08	21.75±0.10
T_1	3	7.68±0.07	3.64±0.06	1.17±0.04	2.11±0.07	8.60±0.10	18.20±0.16
T_1	6	7.88±0.48	3.62±0.14	1.14±0.07	2.18±0.04	8.07±0.09	18.39±0.09
T_1	9	7.87±0.25	3.56±0.12	1.19±0.06	2.20±0.07	7.95±0.10	18.48±0.16
T_2	3	8.43±0.17	3.15±0.13	1.18±0.01	2.67±0.11	7.72±0.02	18.85±0.41
T_2	6	8.57±0.09	3.47±0.03	1.37±0.01	2.47±0.07	8.13±0.06	18.67±0.25
T_2	9	8.78±0.35	3.04±0.08	1.38±0.08	2.89±0.06	7.72±0.06	19.60±0.16
T_3	3	10.14±0.14	3.37±0.06	1.50±0.03	3.01±0.08	8.08±0.02	18.01±0.25
T_3	6	9.20±0.12	3.59±0.12	1.35±0.05	2.56±0.07	8.05±0.03	18.39±0.34
T_3	9	9.29±0.25	3.22±0.08	1.33±0.11	2.88±0.02	7.93±0.11	17.55±0.28

T_1 = Achyranthus aspara, T_2 = Tribulus terrestris, T_3 = Albizia lebbeck, A:P = Acetate to propionate ratio, TVFA = Total volatile fatty acid, NH_3-N =Ammonia nitrogen

ent levels did not affect dry matter digestibility of wheat straw based medium fiber diet. The highest increase in dry matter digestibility was in T_3 (12%) at 9% level. The partition factor value were increased in all cases but the maximum raise was found in T3 (26.38%) at 6% level. Among the all treatment, Microbial biomass (mg) was significantly increased in T_3 (53.17%) at 9% level and it was also increased more then 50% in case of T_1 at 6% level.

Discussion

Ruminants produced methane by enteric fermentation, which is leads to a sever loss of feed energy. Methane is potent greenhouse gas; therefore, reducing methane production has significant economical and environmental benefits. Inclusion of T_3 at 6% level reduced the 24 h methane emission significantly. Inhibition of methanogenesis by T_3 was primarily due to their anti-protozoal activity. As some methanogens live in association with protozoa (New bold *et al*., 1995; Tokura *et al*., 1997), it was expected that reducing protozoa would also reduce methanogens, hence reducing methane production.

In present study, among the all treatment, T_3 showed the significantly reduced protozoa count (table 3). Saponins possibly bind with sterol of cell membrane of protozoa and change the permeability of cell membrane. Decreased protozoal counts with supplementation of saponins rich extract (Hristov *et al*., 1999b; Kamra *et al*., 2000) or saponin rich forages (Newbold *et al*., 1997, Teferedegne et al., 1999) or fruits (Thalib *et al*., 1998; Hess *et al*., 2003) have been reported. Increase in propionate (percent) and decrease in acetate (percent) and consequently decrease in acetate and propionate ratio by T3 could be due to the high percentage of saponins and its inhibitory effect on protozoa, which is in agreement with previous studies (Wang *et al*., 2000; Ye *et al*., 2001; Lila *et al*., 2003). Over all, the present results demonstrate that 6% T_3 is a promising level of supplementation than the other levels of T_3 and other saponins in 50:50 wheat straw based roughage concentrate diet. Total saponins of T_3 have the potential for use as a defaunating agent and should be tested to find out a suitable dose to get maximum inhibition in methane emission without adversely affecting feed degradability.

Acknowledgements

Authors would like to thank and acknowledge for the grant provided by NFBSRA, Ministry of Agriculture, ICAR, New Delhi-100012 to carry out this research work.

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