Short Communication

EFFECT OF MIXTURE OF AJWAIN AND SOAPNUT PLANT EXTRACTS ON *IN VITRO* RUMEN FERMENTATION, METHANE PRODUCTION AND TRUE DIGESTIBILITY OF DIET AT DIFFERENT ROUGHAGE AND CONCENTRATE RATIOS

M. Palanivel

Department of Animal Nutrition
Veterinary College and Research Institute
Tamil Nadu Veterinary and Animal Sciences University
Orathanadu – 614 625, Thanjavur District
Tamil Nadu, India

ABSTRACT

An in vitro study evaluated the anti-methanogenic potentiality of aqueous and alcoholic plant extract mixture of ajwainseed and soapnut berries under different roughage and concentrate ratio-based diets in a 3x3 factorial design. Dried and milled plant mixture was extracted (10 g/100 ml) in three solvents, viz., water (control), ethanol (95 %), and methanol (98 %). Substrate (200 mg) prepared by mixing wheat straw and concentrate mixture at the ratio of 30:70, 50:50 and 70:30 was taken in glass syringes (six per treatment) and incubation medium (30 ml) dispensed anaerobically. Aqueous, ethanol and methanol plant extract (0.5 ml) were taken in three dietary treatments of groups I, II, and III respectively. All the syringes were incubated at 39°C for a running duration of 24 hours and total gas production was calculated. Hundred ml of emitted gas was injected into gas chromatograph equipped with flame ionization detector for methane estimation. In vitro true digestibility of diet and ammonia nitrogen content of fermented medium were determined. Experimental data generated were analysed by adopting factorial ANOVA procedures. Results revealed that ethanol plant extract mixture had significantly (P<0.01) reduced the in vitro total gas and methane production by suppressing the true dietary digestibility of high roughage to low concentrate (70:30) based diet.

Key words: Digestibility of diet, *In vitro* gas production, Methane, Plant extracts.

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INTRODUCTION

Methane production during ruminal fermentation is considered as loss of energy of 2–12 % of the gross energy intake by

Professor, Corresponding author Email id: palanivelvet@yahoo.co.in

ruminants (Kobayashi, 2010), and this greenhouse gas is having 23 times more global warming potential than carbon di-oxide (Moss *et al.,* 2000). However, methane mitigation can be done by different feed additives including plant secondary metabolites possessing antimicrobial activity and also in modulating rumen microbial ecosystem to reduce methanogenesis has been conceptualized (Schultes, 1978). Recently, use of alcoholic plant extracts rich in phytochemicals has been of interest among nutritionists and rumen microbiologists to modify the rumen fermentation and to decrease methanogenesis. Germicidal, carminative and laxative properties of thymol in the ajwain (*Trachyspermum ammi*) seed (Bairwa *et al.,* 2012), and antiprotozoal activity of saponinsin the soapnut (*Sapindus mukorossi*) berry reported to have anti methanogenic activity (Kamra *et al.,* 2000; Hess *et al.,* 2003). In addition, types and dietary proportions of carbohydrates largely affect ruminal fermentation conditions, VFA profile and, concomitantly, methane production (Lascano and Cardenas, 2010). Therefore, the present experiment was designed to evaluate the mixture of soapnut berry and ajwain seed extracted in alcoholic solvents and compared with aquatic plant extract of the same (Control) on *in vitro* total gas, methane, ammonia gas production and nutrients utilization from three roughage: concentrateratio-based diets.

MATERIALS AND METHODS

In vitro gas production test

Comparative screening of plant extract mixture containing secondary metabolites on rumen fermentation and methanogenesis was done by *in vitro* gas production test as described by Menke and Steingass (1988) with three roughage and concentrate based diets as substrate in a 3x3 factorial experiment. A mixture (50:50) of ajwain (*Trachyspermum ammi)* seed and soapnut (*Sapindus mukorossi*) berry was dried at 60°C, milled to pass through 1 mm sieve and extracted in three solvents *viz*, water (Control), ethanol (95 %) and methanol (98 %) at the rate of 10 g/100 ml and the solvents were removed by drying at 60°C to obtain dry plant extracts. Three groups of substrate was prepared by mixing air dried, milled (<1.0 mm) wheat straw (contained 913 g OM, 34 g CP, 16 g EE, 827 g NDF and 532 g ADF per kg of dry matter) and concentrate mixture (containing wheat bran, 82 %; soybean meal, 15 %; mineral mixture, 2 % and salt, 1 %; contained CP - 18.8 % and TDN - 66.2 %) at the ratio of 30:70, 50:50 and 70:30. Substrate (200 mg) of each dietary group was taken in six numbers of glass syringes and incubation medium (30 ml) was dispensed anaerobically in all the syringes. 0.5 g of aqueous plant extract (control) was added in all the syringes of three dietary treatments of group I. 0.5 g of ethanol plant extract was added in all the syringes of three dietary treatments of group II. 0.5 g of methanol plant extract was added in all the syringes of three dietary treatments of group III. All the syringes were incubated at 39°C for running duration of 24 hours.

Estimation of total gas and methane production

After incubation period, total gas production was estimated by the displacement of piston during incubation. Total gas produced due to fermentation of substrate with plant extract mixture was calculated by subtracting

gas produced in blank syringe (containing no substrate with plant extract mixture, but only the incubation medium and buffer) from total gas produced in the syringe containing substrate with plant extract mixture and incubation medium. For methane estimation, 100 ml of gas was sampled from the head space of syringe in an airtight syringe and injected into Nucon-5765 gas chromatograph equipped with flame ionization detector and stainless-steel column packed with Porapak-Q. The gas flow rates for nitrogen, hydrogen and air were 30, 30 and 300 ml/min, respectively. Temperature of the injector oven, column oven and detector were 40°C, 50°C and 50°C respectively. A 50/50 mixture of methane and carbon di-oxide (Spancan; Spantech Products Ltd) was used as a standard.

In vitro true digestibility of diet

The IVTD of experimental diets was determined after termination of incubation. The contents of syringes were transferred quantitatively in to spoutless beakers by repeated washings with 100 ml of neutral detergent solution. The beaker contents were refluxed for 1 hour and filtered through pre-weighed gooch crucibles. NDF content of the residue in the crucible was weighed and *in vitro* true digestibility of diet was calculated as per Van Soest *et al.* (1991).

Ammonia nitrogen production

$$\text{*In vitro* true digestibility of diet} = \frac{\text{Initial DM content of diet - NDF residue in the crucible}}{\text{Initial DM content of diet}}$$

Ammonia nitrogen in the fermented medium was estimated as per the method described by Weatherburn (1967). To the 0.1 ml of fermented liquor from each syringe of three dietary treatment groups in test tube, 0.9 ml of distilled water was added, followed by addition of 5.0 ml of solution A and then immediately 5.0 ml of solution B and mix thoroughly. The tubes were incubated at 39°C for 15 minutes for colour development. Samples were then read spectrophotometrically at 625 nm against a reagent blank. In a similar way, standard samples (ammonia nitrogen concentration ranging from 0.5 to 10.0µg) were processed and a calibration curve was plotted. The ammonia nitrogen concentration of experimental samples was calculated by the standard curve.

Statistical analysis

Experimental data generated were analysed using SPSS computer package adopting standard statistical procedures (SPSS, 2010). Parameters were analyzed using 3x3 factorial ANOVA to compare the experimental groups.

RESULTS AND DISCUSSION

Total gas production

Alcoholic plant extracts had shown significantly ($P<0.01$) lower total gas production as compared to Control (Table 1), indicating that majority of plant secondary metabolites were alcohol soluble (Cowan, 1999; Kamra *et al.,* 2008). Similarly, diet containing high roughage to low concentrate (50:50 and 70:30) ratio significantly ($P<0.01$) reduced the total gas production as compared to that of low roughage to high concentrate (30:70) based diet. The inclusion of high concentrate proportion results in increased fermentation rate (Anantasook and Wanapat,

2012). *In vitro* gas production which reflects the apparent substrate degradability increased linearly in accordance with the proportion of concentrate in the substrate (Kang *et al.,* 2017).

Methane production

Methane production was significantly (P<0.01) reduced with ethanol plant extract among the treatment groups (Table 2). An experiment was conducted earlier with three saponin rich tropical fruits and found that *S. saponaria* was detrimental for methanogenesis. Unlike production of gas, ethanol plant extract was highly detrimental for protozoa followed by methanol and aquatic plant extracts when the protozoa numbers were counted microscopically (Hess *et al.,* 2003). The antiprotozoal effect of saponins is attributed to the binding of saponins to cholesterol in protozoal cell membrane, causing cell lysis (Cheeke, 2000). Similarly, ethanol extract of *S. mukorossi* was the most effective causing 95 % inhibition in methanogenesis accompanied with suppression in *in vitro* degradability of feed (Agarwal *et al.,* 2006).

Similarly, on dietary basis, methane production was significantly (P<0.01) reduced with high roughage to low concentrate-based diet (70:30). When methane production is evaluated with respect to the amount of dietary source intake, data shows that the percentage of ingested gross energy converted to methane drops when the amount of concentrate in the diet is reduced from 70 % to 30 % of DM, indicating a greater energetic efficiency of concentrate based diets (Pedreira *et al.,* 2013).

Digestibility of diet

Alcoholic plant extracts significantly (P<0.01) reduced the IVTD of diet as compared to control (Table 3). *In vitro* degradability of roughage was adversely affected by adding plant extract in the incubation medium and this was most pronounced with alcohol plant extracts (Kim *et al.,* 2013). Similarly, IVTD of diet was suppressed by addition of the plant extracts of *Eugenia jambolana* (jamun), *Aegle marmelos* (bel) and *Zyzipus jujuba* leaves, and the suppression was more with alcoholic extracts (Kumar *et al.,* 2011). Tannins content of plants was also correlated with depression of dietary degradability with plant extracts of *Terminalia chebula, Terminalia Belerica* and *Emblica officinalis* (Patra *et al.,* 2006).

The IVTD of diet was significantly (P<0.01) reduced with increasing levels of roughage in the diet. This is likely due to the difference in roughage to concentrate ratios. The decrease in degradability in roughage content can be due to more lignin content in roughage compared to concentrate. It is generally agreed that lignin content of forages is negatively correlated with extent of digestion (Moore and Jung, 2001). The lignin-suppressing effect is probably resulting from a reduction in attachment of ruminal microbes to feed particles and inhibition of microbial growth and microbial enzyme activity (McSweeney *et al.,* 2001).

Ammonia nitrogen production

No significant difference was observed in ammonia nitrogen production among the dietary treatment groups by inclusion of ajwain and soapnut plant extract mixture

Table 1. Effect of plant extract mixture on *in vitro* total gas production under different roughage and concentrate ratio-based diets

Total gas (ml/g DM)	Roughage: Concentrate ratio			Mean	P value			
	30:70	50:50	70:30		SEM	F	T	F*T
Aqueous plant extract (Group I)	150.00	143.52	125.00	**139.51^{A}**	5.72	P<0.01	0.001	0.398
Ethanol plant extract (Group II)	144.44	113.89	104.17	**120.83^{B}**				
Methanol plant extract (Group III)	135.18	133.33	103.70	**124.07^{B}**				
Mean	**143.21^{a}**	**130.25^{b}**	**110.96^{c}**	**128.14**				

^{AB}Mean values bearing different superscripts in a column differ significantly (P<0.01)
^{abc}Mean values bearing different superscripts in a row differ significantly (P<0.01)

Table 2. Effect of plant extract mixture on *in vitro* methane production under different roughage and concentrate ratio-based diets

Methane (ml/g DM)	Roughage: Concentrate ratio			Mean	P value			
	30:70	50:50	70:30		SEM	F	T	F*T
Aqueous plant extract (Group I)	22.50	22.67	20.87	**22.01^{A}**	1.57	P<0.01	0.026	0.56
Ethanol plant extract (Group II)	16.34	15.28	14.85	**15.49^{B}**				
Methanol plant extract (Group III)	21.66	17.85	18.60	**19.37^{A}**				
Mean	**20.17^{a}**	**18.60^{ab}**	**18.11^{b}**	**18.96**				

^{AB}Mean values bearing different superscripts in a column differ significantly (P<0.01)
^{ab}Mean values bearing different superscripts in a row differ significantly (P<0.01)

Table 3. Effect of plant extract mixture on *in vitro* true digestibility of diet under different roughage and concentrate ratio-based diets

IVTD (%)	Roughage: Concentrate ratio			Mean	P value			
	30:70	50:50	70:30		SEM	F	T	F*T
Aqueous plant extract (Group I)	64.11	55.20	41.66	**53.66**^{A}	4.65	P<0.01	0.015	0.81
Ethanol plant extract (Group II)	58.82	46.00	36.92	**47.25**^{B}				
Methanol plant extract (Group III)	54.81	45.91	42.80	**47.84**^{B}				
Mean	**59.25**^{a}	**49.04**^{b}	**40.46**^{c}	**49.58**				

^{AB}Mean values bearing different superscripts in a column differ significantly (P<0.01)
^{abc}Mean values bearing different superscripts in a row differ significantly (P<0.01)

Table 4. Effect of plant extract mixture on *in vitro* ammonia nitrogen production under different roughage and concentrate ratio-based diets

Ammonia nitrogen (mg/dl)	Roughage: Concentrate ratio			Mean	P value			
	30:70	50:50	70:30		SEM	F	T	F*T
Aqueous plant extract (Group I)	19.15	18.87	18.82	**18.95**	0.47	0.38	0.49	0.57
Ethanol plant extract (Group II)	19.36	18.77	19.64	**19.26**				
Methanol plant extract (Group III)	19.96	19.36	18.75	**19.36**				
Mean	**19.49**	**19.00**	**19.07**	**19.19**				

(Table 4). Likewise, no significant change in the concentration of rumen ammonia nitrogen by feeding various types of herbal additives including essential oils to dairy calves was observed (Akbarian-Tefaghi *et al.,* 2018). The levels of ammonia nitrogen ranged from 18.82 - 19.96 mg/dl which was similar to that reported earlier (15.0 -30.0 mg/dl) in an *in vivo* study (Wanapat and Pimpa, 1999) and in an *in vitro* study (Polyorach *et al.,* 2014).

CONCLUSION

Ethanol plant extract mixture of ajwain seed and soapnut berry significantly reduced the *in vitro* total gas and methane production by suppressing the true dietary digestibility of high roughage to low concentrate (70:30) based diet.

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