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Supplementing blends of plant secondary metabolites as phytobiotics for modulation of *in vitro* methanogenesis and rumen fermentation in buffalo

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ABSTRACT
Blends of various plant secondary metabolites (PSM) containing saponins, tannins and essential oils were examined for their effects on rumen environment, especially in vitro methane inhibition and other ruminal characteristics. Two different blends were prepared with three plant secondary metabolites (essential oil obtained from eucalyptus, saponins from fruits extracts of reetha and tannins from leaves extracts of bargad). In vitro fermentation examinations were carried out in 125mL serum bottle under anaerobic condition with two types of substrates, oats hay as well as mixed feed containing oats hay and concentrate mixture (60:40) and incubated in 39℃ for 24h. Experiment was conducted in completely randomized design (CRD) with a control group and incubated PSM groups i.e. Blend-1 and Blend-2. Methane concentration (%) in the head space gas was reduced linearly with the increasing concentration of plant secondary metabolites, irrespective of substrate. However, truly degradable dry matter was comparable in Oat hay feed supplement group only, as were compared with control. Propionate production was increased in treatments with plant secondary metabolites. It may be concluded that blends of plant secondary compounds could be used as phytogenic feed additives to reduce enteric methane production from ruminants. However, the dose of blends should be used judiciously depending on the composition of the diet to maximise methane reduction without affeting feed digestibility and rumen fermentation parameters.
Key words: *Plant secondary metabolites (PSM), Rumen environment, Methanogenesis*

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INTRODUCTION

India has largest livestock population in the world, consisting of 304.42 million bovine, 71.56 million sheep, 140.54 million goats, 0.61 million equines, 0.52 million camels and 468.88 million poultry (2), contributing considerably to the national economy and the livelihood of the people. Livestock population is expected to grow at the rate of 0.55% in the coming years and the population is likely to be around 781 million by 2050 (3). The livestock sector shares about 30% of the total agricultural gross domestic product and 4.4 % of the total gross domestic product of the nation. With incredible advantages and benefits of livestock rearing, this sector is also bound to harm the lower growth rate and environmental issues by producing greenhouse gases (GHG), which shifts dietary energy away from growth and production and ultimately lower the animal production and contributes to global warming. In India, ruminants are reared on lingo-cellulosic rich feed residue resulting in poor feed utilization and more methane emission. Indian livestock produces about 14.3 Tg/year methane by enteric fermentation (7). The amount of energy loss as methane within ruminant animals may ranges from 6-10% of gross energy intake, or 8-14% of digestible energy intake (1). Use of antibiotics are one of the measures for rumen modifier and animal health prospective, but their long term uses have catastrophic effects such as antibiotics resistance.

Thus, to minimize the dietary losses and promoting animal's health, hazardous impacts on environment and antibiotics resistance, some blends of plant bio-active compounds re-examined for their effects on *in vitro* methane production, volatile fatty acids production and feed fermentation in buffalo with the view to develop a phytognic feed additive for improvement of animal production with less impact on environment.

MATERIALS AND METHODS

Oat hay used as substrate for *in vitro* studies was collected from agricultural farm of the ICAR- Central Institute for Research on Buffaloes, Hisar at late bloom stage of the plant and subjected to drying in hot air oven at 100°C overnight. The hay was ground to 1.0mm particle size and used as substrate for *in vitro* studies. However, concentrate feed substrate was prepared by mixing maize grains (37%), groundnut cake (35%), deoiled rice bran (25%), mineral mixture (2%) and common salt (1%). Fruits of reetha (*Sapindus mukorossi*) were subjected to extract saponin by its aqueous extraction. Aqueous extract of reetha was prepared by adding 10g finely ground dried fruits in 100ml of distilled water and placed in thermo scientific incubator cum shaker at 39°C for 48 h. After incubation, the aliquot was filtered by whatman filter paper No-1 and stored at 4˚C till further studies. Bargad leaves (*Ficus bengalensis*) were subjected to extract tannins by its acetonic extraction. Acetonic extract was prepared by adding 10g finely ground dried leaves in 100ml of 70% aqueous acetone (acetone: water:: 70:30) and placed in thermo scientific incubator cum shaker at 39°C for 48 h. After incubation, the aliquot was filtered by whatman filter paper No-1 and stored at 4˚C till further investigations. However, purified eucalyptus essential oil (EO) was purchased from *Sigma-Aldrich* Ltd India and used for the studies

Rumen liquor was collected manually from the three fistulated adult male buffaloes maintained at ICAR-CIRB, Hisar Haryana- India buffalo farm, before feeding and watering. Rumen liquor was collected from all fistulated animals from different locations of rumen and at different depths and finally mixed to get a representative and homogenous sample. *In vitro* incubation media was prepared (300ml incubation media) by mixing 157.2ml distilled water, 68.40ml rumen buffer solution, 68.40ml macro-minerals solution, 0.30ml resazurine solution, 0.036ml micro-minerals solution, 0.186g. Cysteine HCl and 0.0372g. $Na_2S.H_2O$. The media was boiled under continuous bubbling with CO_2 till the blue color of the medium got vanished (5). Ruminal fermentation parameters such as, *in vitro* head space methane concentration (%) was estimated by gas chromatograph (Nucon Model-5700, New Delhi, India), installed with flame ionization detector (FID) and a column ((Porapak 'Q'). Gas sample (200µl) from the 1000µl graduated Hamilton gas tight micro liter syringe (Hamilton, Switzerland) was injected into the injection port of GC. Simultaneously, methane gas standard (Centurion Scientific, New Delhi- India) having 50.95% concentrations of methane and 49.05% of CO_2 was also injected for comparison. The temperature of injector, detector and oven of GC was maintained at 140˚, 200˚ and 70°C, respectively. *In vitro* true degradability of dry matter (TDDM) was estimated by refluxing fermentation residue for 1h with neutral detergent solution (8). Ammonia nitrogen (NH_3-N) concentration was estimated by Conway disc method (Conway, 1967). Fermentation fluid of 1.0ml from supernatant of serum bottle was transferred in outer compartment of Conway disc. And 1.0ml of saturated Na_2CO_3 was placed in outer compartment, just opposite to the rumen liquor, and 1.0ml of of 2% boric acid solution was kept in inner compartment. The discs were closed air tight and incubated at 39°C for 2-3 h and the boric acid was titrated with N/100 H_2SO_4. Individual VFA in the rumen fluid samples was determined using gas chromatograph (Nucon 5700) equipped with flame ionization detector and glass column packed with chromosorb –101. Injection port, column and detector temperature was set at 250˚C, 175˚C and 260˚C, respectively. A processed sample of 1.0µl was injected by means of 10µl Hamilton syringe (Hamilton company, Nevada, USA) and concentrations of individual VFA were measured against standard mixture of VFA.

Statistical analysis

Data analysis was done by using SPSS (version 16) one-way ANOVA for ruminal parameters followed by Tukey's post hoc test. Data are presented as means and considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Methane concentration (%) in the head space gas was reduced ($p< 0.005$) linearly with increasing concentrations of Blends (Table: 1, 2, 3 and Fig 1, 2), irrespective of substrate. However, truly degradable dry matter (TDDM) remained comparable ($p>0.05$) in both the treatment of blends, where Oats hay was used as substrate, as compared with control (Figure:1). The TDDM was reduced ($p<0.001$) by both the blends after 24h fermentation of mixed feed containing Oats hay and concentrate mixtutre. Ammonia nitrogen production followed drastic decrease ($p<0.001$) in mixed feed substrate group, suggesting inhibition of hyper-ammonia producing bacteria by blends of plant secondary metabolites containing saponins, tannins and essential oils. However, it did not differ ($p>0.05$), irrespective of blends in Oat hay

substrate group, where ammonia-N concentration was much lower, probably due to less protein degradation. Volatile fatty acids composition varied with significant ($p<0.001$) reduction of acetate production in mixed feed group. However, it remained comparable ($p>0.05$) in Oats hay substrate group. Enhancement of propionate production, thereby reduction ($p<0.001$) in acetate to propionate ratio was evident by both the blends incubating in Oats hay or mixed feed substrate groups. Impacts of blends of plant secondary metabolites on rumen environments were also screened by (4), they screened blends of lemon grass oil, garlic oil, clove oil, turmeric and cinnamon @ 0.0, 167.0 and 333.0 µl/lt of rumen fluid and reported 31.26, 29.0 and 27.06 ml/g DM methane production, respectively. However, TDDM was 56.77%, 55.24% and 42.47%, respectively. (6) examined blend of reetha fruits with mango kernel @ 4.0% and found significantly ($p<0.05$) reduced methane concentration with minimal affecting feed digestibility. Simultaneous impacts of plant secondary metabolites (PSM) on rumen fermentation characteristics are possibly due to their effects on rumen microbial population, enhancement of propionate production, declining ruminal degradability etc.

CONCLUSIONS

From the present study, it may be concluded that blends of plant secondary compounds could be used as phytogenic feed additives to reduce enteric methane production from ruminants. However, the dose of blends should be used judiciously depending on the composition of the diet to maximise methane reduction without affeting feed digestibility and rumen fermentation parameters.

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Table:1 Composition of blends of plant secondary metabolites (PSM) used in *in vitro* investigations

Attributes	Control	Blend-1	Blend-2
Eucalyptus oil	Nil	2µl	5µl
Aqueous extract of reetha fruits	Nil	0.125ml	0.225ml
Acetonic extract of bargad leaves	Nil	0.125ml	0.225ml

Table:2 Effects of blends of plant secondary metabolites (PSM) on *in vitro* methanogenesis and fermentation of Oat hay substrate

Attributes	Control	Blend-1	Blend-2	SEM	P-Value
TDDM (%)	61.48	59.46	59.39	1.29	0.492
Methane conc. (%)	7.87^{b}	5.46^{a}	5.26^{a}	0.75	<0.001
NH_3-N (mg/dl)	14.47	14.00	13.53	0.57	0.579
Acetate (mM/dl)	6.69	6.64	6.58	0.04	0.182
Propionate (mM/dl)	1.25^{a}	1.49^{b}	1.53^{b}	0.08	0.008
Butyrate (mM/dl)	0.44^{b}	0.39^{a}	0.38^{a}	0.02	0.001
Acetate: Propionate	4.24^{b}	3.57^{a}	3.42^{a}	0.24	0.002

$^{a, b}$ means with different superscrips within a row differ significantly

control, Blend-1 and Blend-2 are incubated PSM mentioned in Table:1 /40ml of rumen liquor, respectively.

Table:3 Effects of blends of plant secondary metabolites (PSM) on *in vitro* methanogenesis and fermentation of mixed feed substrate containing Oat hay and concentrate mixture (60:40)

Attributes	Control	Blend-1	Blend-2	SEM	P-Value
TDDM (%)	65.09^{b}	63.29^{a}	56.46^{a}	2.25	<0.001
Methane conc. (%)	6.56^{b}	6.30^{b}	4.60^{a}	0.54	<0.001
NH_3-N (mg/dl)	22.40^{c}	15.40^{b}	10.27^{a}	0.79	0.004
Acetate (mM/dl)	6.67^{b}	6.40^{a}	6.37^{a}	0.09	0.001
Propionate (mM/dl)	1.54^{a}	1.83^{b}	1.84^{b}	0.09	0.001
Butyrate (mM/dl)	0.98^{c}	0.82^{b}	0.71^{a}	0.07	<0.001
Acetate: Propionate	4.32^{b}	3.49^{a}	3.47^{a}	0.25	<0.001

$^{a, b, c}$ means with different superscrips within a row differ significantly

control, Blend-1 and Blend-2 are incubated PSM mentioned in Table:1 /40ml of rumen liquor, respectively.

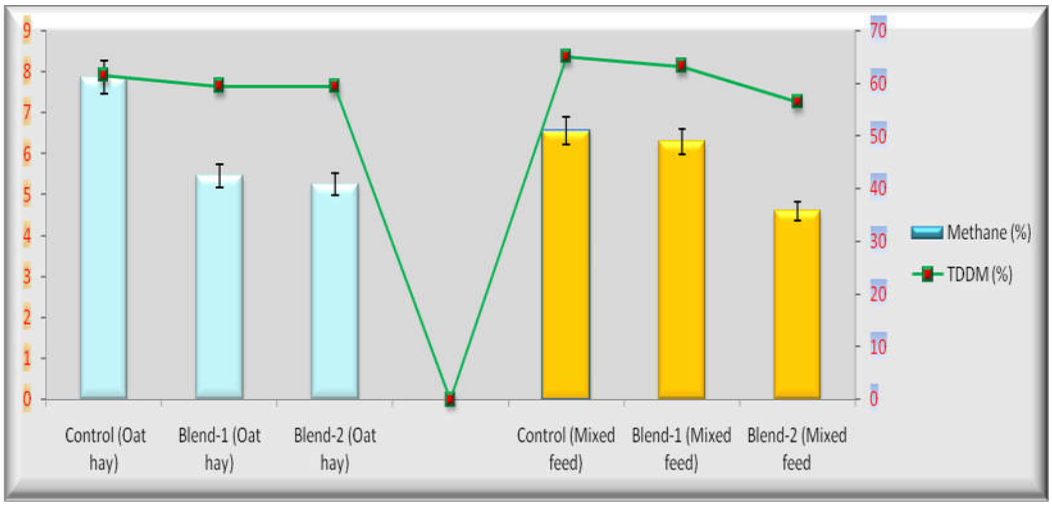

Fig.1: Truly degradable dry matter vs methane concentration *in vitro* on blends of plant secondary metabolites (PSM) with Oat hay and mixed feed as substrate

control, Blend-1 and Blend-2 are incubated PSM mentioned in Table:1 /40ml of rumen liquor, respectively.

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