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Research Article

EFFECTS OF ESSENTIAL OILS RICH PLANT EXTRACTS AND THEIR BLENDS ON IN VITRO METHANOGENESIS AND FERMENTATION CHRACTERISTICS OF OAT HAY BASED SUBSTRATE

KRISHAN KUMAR[1-2], AVIJIT DEY[1]* and M.K. ROSE[2]
[1]Division of Animal Nutrition and Feed Technology, ICAR- Central Institute for Research on Buffaloes, Hisar, India
[2]Department of Veterinary Physiology and Biochemistry, College of Veterinary Science, LUVAS, Hisar, India

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ABSTRACT

The present study was conducted to assess the effects of essential oils extracts from Eucalyptus (*Eucalyptus citriodora*) leaves (ECL), Poplar (*Populus deltoids*) leaves (POL) and Clove (*Syzygium arometicum*) buds (CLB) and their blendson feed digestion, rumen fermentation pattern and methanogenesis by *in vitro* gas production technique. The essential oils extracted with petroleum were supplemented individually (at1ml per 30 ml of buffered rumen fluid) or in different combinations (Blend-1: Poplar, Eucalyptus and Clove extracts, 0.1 ml each; Blend- 2: Poplar and Eucalyptus extracts, 0.1 ml each; Blend- 3: Eucalyptus and Clove extracts, 0.1 ml each; Blend- 4: Poplar, Eucalyptus and Clove extracts, 0.25 ml each; Blend-5: Poplar and Eucalyptus extracts, 0.25 ml each; Blend- 6: Eucalyptus and Clove extracts, 0.25 ml each per 30 ml buffered rumen fluid) to oat hay based substrate to assess its effects. At the specific dose level, ECL and POL, significantly ($p<0.001$) reduced methane production without ($p>0.05$) affecting *in vitro* feed digestibility, while at the same dose, clove bud extract significantly ($p<0.001$) reduced both methane production and feed digestibility. The blends of these extracts modulate rumen fermentation parameters more distinctly at low dose levels than their individual inclusion, showing positive associative effect. The present experiment demonstrated significant effects of the BLEND- 4 and BLEND- 5 in maximum reduction in methane production without affecting feed digestibility. The study suggests *in vivo* experimentation with supplementation of these blends to find out their effects on methane emission and animal performance for adopting as phytogenic feed additive for ruminants.

Keywords: Blends, Essential oils, *In Vitro*, Methanogenesis, Rumen fermentation

According to Inter-governmental Panel on Climate Change (IPCC), the major greenhouse gases (GHG) are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) and are responsible for global warming and climate change. Methane is the second largest anthropogenic greenhouse gas, it contributes about 14% of total anthropogenic greenhouse gas production (Eggleston *et al.*, 2006). The production of enteric methane in ruminant animals can be reduced mainly by modulation of rumen ecosystem through dietary manipulation. Antibiotics and ionophores have been used as additives in diet to transform rumen fermentation from long back, but animal nutritionists have been searching alternate source of rumen modifiers because antibiotics have negative perceptions. Essential oils have powerful antimicrobial properties and the plant extracts containing essential oils could be used as a potential substitute to modify rumen fermentation as well as growth and feed efficiency in ruminants (Benchaar *et al.*, 2008).

Therefore, the present study was planned to assess the effect of some essential oil rich promising plant extracts or their combinations on methane production for development of potential feed additives for ruminants. Thus, an experiment was conducted to examine the effects of extracts from Poplar (*Populus deltoids*), Eucalyptus (*Eucalyptus citriodora*) leaves and clove buds (*Syzygium arometicum*) and their various blends on *in vitro* methanogenesis, volatile fatty acids production and fermentation characteristics of feed by *in vitro* gas production technique.

MATERIAL AND METHODS

The present study was carried out in the Rumen Microbiome Laboratory, Division ofAnimal Nutrition and Feed Technology, ICAR- Central Institute for Research on Buffaloes, Hisar, Haryana, India.

Collection of plant parts and preparation of extracts: Poplar and Eucalyptus leaves were collected form the ICAR- Central Institute for Research on Buffaloes, Hisar campus. Half of them were dried in hot air ovenat 50 °C and ground to pass through 1 mm sieve and extracts were prepared with petroleum ether and remaining were used for dry matter estimation in hot air oven at 100 °C. Clove bud was purchased from local market of Hisar and extract was prepared with the same solvent. For preparation of plant extracts from poplar, eucalyptus leaves and clove buds, 15 gm sample was extracted in petroleum ether by Soxhlet's apparatus for 72 hours (Tongnuanchan and Benjakul, 2014) and volume made to 100 ml with the solvent to get final concentration.

Collection on rumen liquor: Rumen liquor (RL) was collected from three rumen fistulated adult male Murrah

*Corresponding author: akshays482@gmail.com

buffaloes (Live Weight, 550 ± 40 kg) before feeding and watering at the animal farm. The sample was collected from different locations of rumen and at different depths, from each of fistulated buffalo in order to get a representative and homogenous sample. Equal volume of RL from each buffalo was pooled and strained through four layered muslin cloth into a pre-warmed thermos flask (1 L capacity) which was previously flushed with carbon dioxide to maintain anaerobic condition and immediately brought to laboratory for *in vitro* studies.

Weighing of feed sample and preparation of syringes: The sample of oats hay was ground to pass through 1.0 mm screen and about 200 mg of air-dried samples were weighed and transferred into the 100 ml calibrated syringes (Fortuna, Germany). Various plants extract at specific doses (1ml/30 ml buffered rumen fluid) individually and their blends [BLEND- 1, BLEND- 2, BLEND- 3, BLEND- 4, BLEND- 5, and BLEND- 6 @ 0.1 ml each (Poplar, Eucalyptus and Clove), 0.1 ml each (Poplar and Eucalyptus), 0.1 ml each (Eucalyptus, Clove), 0.25 ml each (Poplar, Eucalyptus and Clove), 0.25 ml each (Poplar and Eucalyptus), 0.25 ml each (Eucalyptus, Clove) /30ml of buffered rumen fluid, respectively] were added to the substrate through micropipette from the top of syringes. The blends were prepared with the hypothesis that combination of different plant extracts may act synergistically to modulate rumen fermentation. After adding extracts, the piston was greased with paraffin soft white LR (Hi Media; M.P. 39-56 °C) and pushed into the barrel of the syringe. Lubrication with paraffin soft makes syringe gas-water tight and prevents the piston from getting stuck in the barrel. Chemical analyses of feed sample and residue was done as per the methods of AOAC (2007) and fibre fractions according to Van Soest *et al.* (1991).

Preparation of buffer media: Buffer media was prepared as per the Menke and Steingass (1988).Carbon dioxide gas was passed continuously through the submerged tube in the flask during buffer media preparation. Strained RL at required quantity was mixed with the media to make final concentration of 2:1 (Buffer: RL) for dispensing to the syringes.

Estimation of total gas and ethane production: After 24 h of incubation, the total gas production was recorded by displacement of piston. Concentration of methane in head space gas, was measured by taking sample (200 µl) in 1000 µl graduated Hamilton gas tight syringe (Hamilton, Switzerland) using gas chromatograph (Nucon -5700, Nucon Engineers, New Delhi) fitted with flame ionization detector (FID) and a column (Porapak 'Q'). Total methane production was calculated by multiplying methane concentration in the head space gas with the total gas produced and expressed as per g DM incubated as well as per g DM digested.

Estimation of *in vitro* dry matter digestibility (IVDMD): For the determination of dry matter digestibility, the contents of each syringe were transferred into a beaker of refluxing apparatus. Neutral detergent solution (100 ml) was added and refluxed for 60 minutes, time started from the onset of refluxing. After complete refluxing, filtered through sintered crucible (G1) and washed thrice with hot water and twice with acetone under vacuum. The residue was dried at1000 ºC overnight and *in vitro* true dry matter digestibility was calculated.

Estimation of ammonia nitrogen (NH3-N): For estimation of NH3-N concentration, conway disc method was applied. One ml supernatant of each syringe was transferred in outer compartment of conway disc (Conway, 1962). One ml of saturated Na_2CO_3 was kept in outer compartment, just opposite to the sample and 1 ml of 2 % boric acid solution was added in inner compartment. Capped the disc, gently mixed the content of outer chamber by rotation and placed in incubator at- 39 °C for 3 hrs. After 3 hrs, discs were taken out and inner chamber content titrated against N/100 H_2SO_4 to calculate ammonia-N.

Individual volatile fatty acids (IVFAs): After 24 hr incubation, 1 ml of the supernatant of each syringe content was taken in a micro centrifuge tube containing 0.20 ml metaphosphoric acid (25% w/v). The mixture was allowed to stand for overnight at room temperature and centrifuged at 5000 g to get clear supernatant. The supernatant (1µL)was injected into gas chromatograph equipped with flame ionization detector (FID) and glass column packed with chromosorb- 101as described by Cottyn and Boucque (1968).

Statistical analysis: An average of three replicate syringes within a run, were the statistical unit. The data obtained were analyzed statistically with SPSS (version 17) software using one-way ANOVA (Snedecor and Cochran, 1994) followed by Duncan's Multiple Range tests. The data are expressed as significance level, $p \leq 0.05$.

RESULTS AND DISCUSSION

Plants rich in essential oils have well known antimicrobial activities against rumen microflora but their

efficiency varies with types and dose. In the present study, the essential oils extract from Eucalyptus leaves (ECL), Poplar leaves (POL) and Clove bud (CLB) were added individually at similar dose level (1ml/30 ml buffered rumen fluid), but the variations (Table 1) in modulating rumen fermentation and methane production of oats hay substrate (OM, 93.30%; CP, 7.80%; CF, 31.40%; EE, 2.07%; Ash, 6.70%; NDF, 58.40%; ADF, 43.70%) clearly demonstrated that the sources of essential oils have significant effect on rumen fermentation.

Reduction ($p<0.001$)in total gas and methane production (ml/g DM) in all the treatment groups i.e. ECL, POL and CLB as compared to control were evident. However, methane concentrations in head space gas and thereby, total methane production during fermentation were more pronounced by CLB followed by POL and ECL. Interestingly, the IVDMD of oats hay was reduced ($p<0.001$) with addition of CLB extract only. The decrease in total gas and methane productions by essential oils rich extracts possibly due to selective effect on specific ruminal microbial communities, especially methanogens (Benchaar and Greathead, 2011) with inhibition of some other microbes depending on the source and dose of essential oils (Benchaar *et al.*, 2008). Present study corroborates with the findings of Singh *et al.* (2019), which demonstrated the reduction ($p<0.05$) in methane production with pure essential oils of Eucalyptus leaves. The major compound of clove, eugenol has strong antimicrobial activity against Gram positive and negative bacteria. Gunal *et al.* (2017) reported that clove bud oil at 500 mg/L culture fluid significantly ($p<0.05$) reduced methane production as well as dry matter digestibility but at low dose (250 mg/L and 125 gm/L), there was only reduction in methane production as compared to control. The reduction in feed digestibility, in the present study (Table 1) by CLB could be due to the composition of essential oils present in the CLB extract and their specific activity to rumen microbes, which suppressed the fibrolytic microbes in the rumen microbial consortia.

Ammonia-N concentration was reduced in all the treatments (ECL, POL, CLB) in comparison to control, however, reduction was more pronounced in ECL and POL supplemented oat hay than CLB supplemented substrate. Therefore, ECL and POL treatment groups at similar dose level (1 ml extract/30 ml) were found to be better for reducing methane and ammonia concentration without affecting feed digestibility. The reduced ammonia-N concentration with addition of essential oils rich plant extracts could be due to lowered protein degradation (Wallace *et al.*, 2002), as essential oils affect the final stage of protein degradation, deamination of amino acids to ammonia probably due to inhibition of hyper-ammonia (HAB) producing bacteria (*Clostridium sticklandii, Peptostreptococcus anaerobius*) in rumen. Our study gets support from Merry Chanu (2018), who also observed reduced ammonia-N production with inclusion of essential oils rich plant extracts due to inhibition of hyper ammonia producing bacteria in the rumen.

The concentration of individual volatile fatty acids (Table 2) remained similar to control in ECL and POL extracts added groups, while significant ($p<0.05$) reduction in CLB treatment was evident. Castillejos *et al.* (2006) reported modulation of VFA profile without decreasing total VFA concentration in a study with eugenol in batch fermentation and thymol in continuous culture. However, the decrease in all the fatty acids with higher

Table 1

Effects of extracts from Eucalyptus leaves, Poplar leaves and Clove buds on *in vitro* rumen fermentation and methane production of oats (*Avena sativa*) hay

Attributes	CON	ECL	POL	CLB	SEM	P value
Gas, ml/g DM	118.39^{c}	96.56^{b}	80.77^{a}	103.9^{b}	7.45	<0.001
Gas, ml/g DMD	168.00^{c}	137.72^{b}	118.02^{a}	180.96^{d}	12.56	<0.001
Methane, ml/g DM	14.25^{c}	8.27^{b}	4.45^{a}	4.09^{a}	2.14	<0.001
Methane, ml/g DMD	20.22^{c}	11.81^{b}	6.63^{a}	6.88^{a}	2.90	<0.001
IVDMD, %	70.49^{b}	70.10^{b}	68.42^{b}	59.38^{a}	2.42	<0.001
Ammonia N, mg/dl	28.47^{c}	21.47^{a}	20.53^{a}	23.33^{b}	1.64	<0.001

CON- Control, ECL, POL and CLB are treatment groups containing 1 ml extracts from Eucalyptus leaves, Poplar leaves and Clove buds/30ml of buffered rumen fluid, respectively

Mean values bearing a, b, c, d superscripts in a row varies significantly ($p<0.001$)

DM = Dry matter, DMD = Digestibledry matter, IVDMD = *In vitro* dry matter digestibility

Table 2

Individual volatile fatty acids production on *in vitro* incubation of oats hay with extracts from Eucalyptus leaves, Poplar leaves and Clove buds

Attributes	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P
CON	3.83[b]	0.87[b]	0.33[b]	4.42
ECL	3.71[b]	0.87[b]	0.34[b]	4.23
POL	3.71[b]	0.88[b]	0.34[b]	4.48
CLB	2.89[a]	0.59[a]	0.23[a]	4.91
SEM	0.17	0.13	0.02	0.19
P Value	0.01	0.00	0.02	0.40

CON- Control, ECL, POL and CLB are treatment groups containing 1 ml extracts from Eucalyptus leaves, Poplar leaves and Clove buds /30ml of buffered rumen fluid, respectively

Mean values bearing a, b superscripts in a column varies significantly ($p<0.05$)

dose as compared to control suggested detrimental effects on rumen microbes (Busquet *et al.,* 2006). As the production of VFA represents the principal source of energy for ruminants, decreasing VFA production could yield adverse nutritional consequences if this same effect was expressed *in vivo*. The comparable VFA concentrations in ECL and POL with reduction in CLB could be due to their effects on feed digestibility (Table 1) due to modulation of rumen microbiome as supported by earlier studies(Busquet *et al.*, 2006; Dey *et al.*, 2017).

Experiment conducted to find out suitable blends of feed additives, which could decrease methane production with minimum effects on feed digestibility (Table 3), revealed that various combinations of essential oils rich plant extracts affect differently in modulating rumen fermentation pattern. The total gas production remained comparable with the control for all the blends except BLEND- 5, where a reduction ($p<0.001$) was evident. However, comparable IVDMD in BLEND-5 with control, suggest more production of microbial protein instead of diverting feed dry matter towards gas production (Blummel *et al.*, 1997). BLEND-3 and BLEND-6, which were combinations of ECL and CLB at two different dose levels (0.1 ml each, BLEND-3 and 0.25 ml each, BLEND-6) did not vary in gas production and IVDMD between them, however, both the blends differ ($p< 0.05$) in these parameters from other blends (Table 3). Reduction of digestibility without affecting gas production in these blends from others suggest diverting energy produced in fermentation process towards gas production with minimal microbial biomass production, as gas production and microbial protein synthesis are inversely proportional (Blummel *et al.*, 1997). The total methane produced was significantly ($p<0.001$) decreased in all the BLENDS groups as compared to control, however, more pronounced ($p<0.001$) effect was revealed in BLEND-4, where a combination of all three ECL, POL and CLB at their higher dose (0.25 ml each) were used. As the IVDMD remained comparable for BLEND-4 with control, the methane production per unit dry matter digested was significantly ($p<0.001$) lower, suggesting selective inhibition of methanogenesis without affecting feed digestion. Comparative evaluation of IVDMD between BLEND-4 and BLEND-6 revealed higher ($p<0.05$) feed digestibility in BLEND-4, which suggest possible stimulation of fibrolytic rumen microbes by addition of POL extract. Several studies with various blends of essential oils either *in vitro* or *in vivo* suggested reduced methane production and protein degradation by selectively inhibiting archaea and hyper ammonia producing bacteria (Newbold *et al.*, 2004).

The concentration of ammonia-N nitrogen was significantly ($p<0.001$) decreased in all the treatment groups except BLEND- 2 treatment group as compared to control. The reduction in ammonia-N was significantly ($p<0.001$) more in BLEND-4 and Blend-6 than the other blends. The addition (1µl/ml) of Eucalyptus oil (ECO) in *in vitro* fermentation media, reduced the ammonia-N concentration, however at lowest dose (0.5 µl/ ml), no effect on ammonia-N was evident (Singh *et al.*, 2019). These researchers also reported associative effects of blends of extracts on ruminal ammonia production, suggesting inhibitory effect on HAB bacteria. The present study corroborates with the earlier workers in synergistic effects of blends of essential oils and other bioactive compounds in modulating rumen fermentation and deamination process.

The concentrations of acetate, propionate and

Table 3

Variations in gas production, methane production and feed digestibility on incubation of oats hay with blends of extracts from Poplar leaves, Eucalyptus leaves and Clove buds

Attributes	Gas ml/g DM	Gas ml/g DMD	Methane ml/g DM	Methane ml/g DMD	Ammonia-N mg/dl	IVDMD %
CON	159.49^{b}	226.43^{b}	22.74^{d}	30.43^{d}	22.40^{c}	70.46^{b}
BLEND- 1	156.67^{b}	228.57^{b}	16.36^{b}	23.86^{b}	20.07^{b}	68.68^{b}
BLEND- 2	164.48^{b}	223.03^{b}	19.08^{c}	27.03^{c}	21.00^{bc}	70.57^{b}
BLEND- 3	161.77^{b}	227.68^{b}	17.61^{bc}	30.17^{cd}	20.07^{b}	58.29^{a}
BLEND- 4	158.24^{b}	228.31^{b}	11.66^{a}	16.82^{a}	17.27^{a}	69.31^{b}
BLEND- 5	130.33^{a}	187.96^{a}	16.97^{bc}	22.91^{b}	19.13^{b}	69.33^{b}
BLEND- 6	157.74^{b}	274.31^{c}	16.75^{bc}	29.14^{cd}	17.33^{a}	57.53^{a}
SEM	4.35	11.45	1.08	1.78	0.74	2.14
P value	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

CON- Control, BLEND- 1(Poplar, Eucalyptus and Clove extracts, 0.1 ml each) BLEND- 2 (Poplar and Eucalyptus extracts, 0.1 ml each) BLEND- 3 (Eucalyptus and Clove extracts, 0.1 ml each), BLEND- 4 (Poplar, Eucalyptus and Clove extracts, 0.25 ml each) BLEND- 5 (Poplar and Eucalyptus extracts, 0.25 ml each) and BLEND- 6 (Eucalyptus and Clove extracts, 0.25 ml each) are treatment groups. Mean values bearing a, b, c superscripts in a column varies significantly ($p<0.001$); DM = Dry matter, DMD = Digestibledry matter, IVDMD = *In vitro* dry matter digestibility.

Table 4

Changes in volatile fatty acids production on inclusion of blends of Poplar leaves, Eucalyptus leaves and Clove buds extractin fermentation media incubated with oats hay

Attributes	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P
CON	7.04^{a}	1.67^{ab}	0.67^{b}	4.21
BLEND- 1	6.81^{a}	1.65^{ab}	0.56^{a}	4.14
BLEND- 2	7.84^{b}	1.79^{b}	0.79^{d}	4.39
BLEND- 3	6.53^{a}	1.61^{a}	0.65^{b}	4.03
BLEND- 4	7.26^{ab}	1.69^{ab}	0.69^{b}	4.28
BLEND- 5	7.88^{b}	1.85^{c}	0.72^{cd}	4.27
BLEND- 6	6.83^{a}	1.72^{ab}	0.72^{bc}	4.00
SEM	0.22	0.05	0.03	0.09
P Value	0.007	0.005	<0.001	0.453

CON- Control, BLEND- 1(Poplar, Eucalyptus and Clove extracts, 0.1 ml each) BLEND- 2 (Poplar and Eucalyptus extracts, 0.1 ml each) BLEND- 3 (Eucalyptus and Clove extracts, 0.1 ml each), BLEND- 4 (Poplar, Eucalyptus and Clove extracts, 0.25 ml each) BLEND- 5 (Poplar and Eucalyptus extracts, 0.25 ml each) and BLEND- 6 (Eucalyptus and Clove extracts, 0.25 ml each) are treatment groups. Mean values bearing a, b, c, d superscripts in a column varies significantly

butyrate varied among the treatments (Table 4) owing to different combinations of source and dose levels. Busquet *et al.* (2006) reported that at high dose, Clove bud oil reduced the VFA concentration suggesting detrimental effect on fermentation processes. However, experiments conducted by Castillejos *et al.* (2007) on various blends of essential oils suggests inconsistent effects on VFA production.

CONCLUSION

The present study demonstrated dominance of the BLEND-4 (0.25 ml each extract from Eucalyptus leaves, Poplar leaves and Clove buds) and BLEND-5 (0.25 ml each extract from Poplar leaves and Eucalyptus leaves) over others in maximum reduction in methane production without affecting feed digestibility. Therefore, in vivo experimentation with supplementation of these blends need to be conducted to find out their effects on methane emission and animal performance, for adopting as phytogenic feed additive for ruminants.

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