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Research Article

Bioactive leaves extracts from Eucalyptus and poplar in modulating rumen fermentation for reducing enteric methane emission in buffalo (*Bubalus bubalis*)

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Abstract

Exploration of feed additives from plant origin to modulate rumen fermentation for improvement in feed utilization and reduction in enteric methane emissions from ruminant animals is not only the focus of animal nutritionists, but also the present day need to dispose of worldwide anti-microbial resistance. The present study examined the effects of Eucalyptus and Poplar leaves extracts on *in vitro* rumen fermentation, methanogenesis and feed digestibility in buffalo (*Bubalus bubalis*). The green and matured leaves of Eucalyptus (*Eucalyptus citriodora*) and Poplar (*Populus deltoides*) were collected from the trees of the CIRB campus. Half of the leaves were dried in an electric oven at 45 to 50°C for 24 h and ground to pass through a 1 mm sieve. Other portions of fresh leaves were crushed into small pieces and kept for extraction afresh. Both fresh and dried leaves were extracted in Soxhlet's apparatus with petroleum ether solvent. The extracts at graded dose levels (0.00, 0.5 and 2.0 mL) were added with 200 mg of oats hay substrate in 100 mL calibrated glass syringe and incubated with buffered rumen fluid (30 mL) at 39°C for 24 h following standard *in vitro* gas production protocol (IVGP). The study revealed enhanced ($p<0.05$) gas production (mL/g DM), feed digestibility, and volatile fatty acids with reduced ($p<0.05$) methane production at supplementation of either fresh or dried leaves of Eucalyptus extract at lower dose (0.5 mL) level. However, higher dose level (2.0 mL) impeded ($p<0.05$) all the rumen fermentation parameters. Poplar leaves extracts were ineffective ($p>0.05$) in methane reduction, feed digestibility, volatile fatty acids and ammonia production at a lower level of supplementation (0.5 mL). However, a higher dose (2.0 mL) also reduced ($p<0.05$) methane production and feed digestibility. Thus, the study demonstrated the potential of Eucalyptus leaves in reducing enteric methanogenesis and promising to be used as feed additive to improve animal performance and reduce environmental pollution through enteric methane mitigation.

Keywords: Buffalo, Eucalyptus leaves, Methane mitigation, Plant bioactives, Poplar leaves

Highlights

- Potential of bioactive plant extracts as enteric methane mitigating agents were examined *in vitro*.
- Eucalyptus leaves extract was found to reduce methane production and enhance feed digestibility. Poplar leaves extract at low dose (0.5 mL/ 30 mL) was ineffective in methane reduction.
- Thus, Eucalyptus leaves are promising to be used as an anti-methanogenic feed additive.

INTRODUCTION

As per IPCC (2019), enteric methane from ruminants is responsible for about half (46%) of the total methane emissions from agriculture, forestry and other land use. Indian livestock contributed about 15.1% of global enteric methane emissions, out of which cattle (49.1%) and buffalo (42.8%) are the major source (Patra, 2014). Ruminal methanogenesis also results in a loss of feed energy by 2-12 per cent (Moss *et al*., 2000; Benchaar *et al*., 2001; Beauchemin and McGinn, 2005; Ding *et al*., 2010). Numerous antibiotics have been used earlier to improve rumen fermentation by enhancing some of the final product (propionate) and reducing the overall methane production (Stanier and Davies, 1981). However, the European Union has prohibited the use of antibiotics as a feed additive since January 2006 because of the possibility that they may remain in animal products (milk and meat) and have negative effects on human health (Russell and Houlihan, 2003). Therefore, to enhance animal production systems, new alternatives are needed but they should be secure and affordable.

The plant bioactive compounds have the ability to modulate rumen fermentation and have antimicrobial activity; both these properties of plant bioactive compounds may be utilized for reduction in methane production. When the combination of hydrolyzable tannins and condensed tannins was incubated with a basal diet, there was a reduction in total gas and methane

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production with decreased archaeal and protozoal populations as the concentration of tannin increased (Bhatta *et al*., 2009). Plant secondary metabolites (essential oils, tannins, saponins), either in pure form or in extracts, were reported to reduce enteric methane production. However, feed digestibility is also reduced in a dose-dependent manner (Kamra *et al*., 2012; Dey *et al*., 2017; Singh *et al*., 2023).

Poplar (*Populus* sp.) trees are abundantly available in the northern part of India, and the leaves are rich in polyphenolic compounds, terpenoids, flavones, flavanones, and several other essential oils compounds (Rubert-Nason *et al*., 2013; Kis *et al*., 2020). Several volatile essential oil components, including 1,8-cineole, limonene, and alpha terpineol, as well as non-volatile phenolic compounds, including quercetin, epicatechin, and catechin, are found in eucalyptus leaves (Vuong *et al*., 2015). The phytochemicals in these tree leaves may modulate rumen fermentation in a way that prevents methanogenesis. Therefore, the present study was planned to examine the effects of extracts from fresh and dried leaves of Eucalyptus and Poplar on *in-vitro* rumen fermentation, dry matter digestibility and methanogenesis with the aim to develop a phytogenic feed additive mixture to reduce enteric methane production from ruminants.

MATERIALS AND METHODS

The experiment was conducted in the Animal Nutrition and Feed Technology Division of the ICAR-Central Institute for Research on Buffaloes, Hisar, India (29.1203 N, 75.8069 E). The approval (IAEC protocol no. 2/2016) of the Institute Animal Ethics Committee (IAEC) was taken for the care of the fistulated animals and collection of rumen fluid for experimentation.

Collection of tree leaves and preparation of extracts: From the campus of the institute, green and matured leaves of eucalyptus (*Eucalyptus citriodora*) and poplar (*Populus deltoides*) were collected. Half of the leaves were dried in an electric oven at 45 to 50°C for 24 h and ground to pass through a 1 mm sieve. Other portions of fresh leaves were crushed into small pieces and kept for extraction afresh. Petroleum ether as a solvent was used to prepare extract from both the leaves separately. A 15 g sample of either dry or fresh leaves was taken in a thimble of Soxhlet's apparatus and extracted with petroleum ether for 72 h. These extracts were then kept in a refrigerator at 4°C until use.

Collection of rumen liquor: Three mature male Murrah buffaloes with fistulated rumen were used for the collection of rumen liquor. A maintenance feed made up of 40% concentrate and 60% roughage was given to all fistulated animals.

Weighing of feed sample and greasing of syringes for *in vitro* fermentation study: Oats hay (*Avena sativa*) sample (1.0 mm particle size) was used as a substrate for *in vitro* studies. Without letting the sample stick on its side, the precisely weighed 200 mg air-dried sample was placed at the bottom of the 100 mL calibrated glass syringe (Fortuna®, Poulten & Graf Ltd., D 97877 Wertheim, Germany). From the top of the syringes, graded (0, 0.5 and 2 mL) concentrations of plant extracts were added to the substrate. Then, the piston, lubricated with paraffin soft white LR (Hi Media; M.P. 39-56°C), was pushed into the barrel of the syringe. Thus, the syringes become gas-water tight, and the piston is prevented from being fixed in the barrel by lubrication with paraffin soft.

Preparation of fermentation media and *in vitro* incubation: The buffer media (Menke, 1988) was prepared by constantly passing carbon dioxide gas through the flask's submerged tube. After the medium had been brought to a boil for a short time to release any dissolved oxygen, reducing agents (cysteine- HCl and $Na_2S.H_2O$) were added. After that, the medium's temperature was lowered to 39°C by keeping the flask in the incubator. To create a final concentration of 2:1 (Buffer : Rumen liquor), strained rumen liquor was mixed with the media in the desired quantity before being dispensed into the syringes. This buffered rumen fluid (30 mL) was dispensed into each syringe under anaerobic conditions and kept in an incubator at 39°C for 24 h with intermittent shaking at every 2 h interval.

Estimation of total gas and methane production: The total gas generation was measured by piston displacement after a 24 h incubation period. Methane concentration in head space gas was evaluated using a gas chromatograph (Nucon-5700, Nucon Engineers, New Delhi) equipped with a flame ionization detector (FID) and a column (Porapak 'Q').

Estimation of *in-vitro* dry matter digestibility (IVDMD): After 24 h of incubation, each syringe contents were put into a refluxing equipment beaker to determine the digestibility of dry matter. 100 mL of neutral detergent solution was added, and the 60-minute refluxing was performed. Following thorough refluxing, the material was filtered through a sintered gooch crucible (G1) and washed four times in hot water and twice in acetone while under vacuum. Dried the residue at 100°C overnight and calculated for *in vitro* dry matter digestibility (IVDMD).

Estimation of ammonia nitrogen (NH_3-N): Conway disc method (Conway, 1957) was used for the estimation of NH_3-N. Each syringe's supernatant of one millilitre was transferred to the Conway disc's outer compartment. Just across from the sample, 1 mL of saturated Na_2CO_3 was kept in the outer compartment, and 1 mL of a 2 per cent boric acid solution was introduced to the inner compartment. Placed the disc in the incubator at 39°C for three hours while gently rotating the outer chamber's contents. Discs were removed after three hours, and the inner chamber fluid was titrated against N/100 H_2SO_4.

Analysis of volatile fatty acids (VFAs): One millilitre of each syringe's supernatant was removed after a 24-hour incubation period, and it was placed in a microcentrifuge tube with 0.20 millilitres of metaphosphoric acid (25 per cent, w/v). The mixture was centrifuged at 5,000 g to obtain clear supernatant after being left to remain at room temperature for 8 h. The supernatant (1 ìL) was injected into gas chromatograph (NUCON 5700) equipped with flame ionization detector (FID) and glass column packed with chromosorb-101 as described by Cottyn and Boucque (1968). The column pressure for hydrogen and zero air were 20 PSI and 10 PSI, respectively. Temperature of the column oven, injector and detector were 170°C, 240°C and 250°C, respectively.

Statistical analysis: The data obtained from the experiments were analyzed for the various parameters as a completely randomized design using a general linear model procedure of SPSS (2008) with a fixed effect of treatment. The significance of difference between the treatment means was determined by one-way ANOVA, as per Snedecor and Cochran (1994). The differences between means were established using Tukey's HSD test, with significance declared if $p \leq 0.05$.

RESULTS

The study was conducted in four separate *in vitro* experiments. In the 1st experiment, the effects of Eucalyptus fresh leaves extract at graded dose levels [@ control (0.0 mL), T_1 (0.5 mL) and T_2 (2.0 mL)/30 mL BRF] were examined for *in-vitro* rumen fermentation and methane reduction potential (Table 1). The results demonstrated a significant ($p<0.001$) increase in total gas in T_1; however, it reduced ($p<0.001$) gas production in T_2 in comparison to the control. Likewise, gas mL/g DM and gas mL/g DMD were significantly ($p<0.001$) increased in T_1 and reduced ($p<0.001$) in T_2. There was a

Table 1. Effects of Eucalyptus leaves extract on *in vitro* rumen fermentation and methane production

Attributes	Control		T 1		T 2		SEM		P value	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Total Gas, mL	27.67^b	27.67^b	34.00^c	33.67^c	16.67^a	14.0^a	3.71	5.08	<0.001	<0.001
Gas, mL/g DM	141.62^b	141.62^b	177.89^c	177.29^c	88.21^a	74.34^a	22.65	26.29	<0.001	<0.001
Gas, mL/g DMD	200.12^b	200.12^b	222.55^c	221.92^c	171.57^a	154.32^a	13.45	17.67	0.052	<0.001
Methane Conc., %	9.69^c	9.69^c	7.80^b	8.24^b	2.14^a	1.89^a	1.99	2.01	<0.001	<0.001
Total Methane, mL	2.59^c	2.59^c	2.03^b	2.07^b	0.35^a	0.27^a	0.66	0.70	<0.001	<0.001
Methane, mL/g DM	13.77^c	13.77^b	11.88^b	14.60^b	1.89^a	1.41^a	3.50	3.75	<0.001	<0.001
Methane, mL/g DMD	19.47^c	19.47^b	15.29^b	18.27^b	3.67^a	2.92^a	4.37	4.70	<0.001	<0.001
IVDMD, %	70.45^b	70.45^b	79.57^c	79.09^c	51.20^a	48.70^a	7.26	7.48	<0.001	<0.001
Ammonia N, mg/dL	15.40^b	15.40^b	15.87^b	15.93^b	12.60^a	11.90^a	0.91	1.13	<0.001	<0.001

Control, T_1 and T_2 are treatment groups @ 0.0, 0.5, and 2.0 mL of Eucalyptus dry/fresh leaves extracts/30 mL BRF, respectively. Mean values bearing a, b, c superscripts either for fresh/dry in a row vary significantly ($p<0.05$). DM = Dry matter, DMD = Digested dry matter, IVDMD = *In-vitro* dry matter digestibility

Table 2. Effects of Eucalyptus leaves extract on *in vitro* volatile fatty acids production

Attributes		Acetate (mM/100 mL)	Propionate (mM/100 mL)	Butyrate (mM/100 mL)	A:P
Control	Fresh	2.91[a]	0.67[b]	0.27[a]	4.38[b]
	Dry	2.92[b]	0.67[b]	0.27[a]	4.38[b]
T1	Fresh	3.00[b]	0.71[b]	0.43[b]	4.23[a]
	Dry	2.83[b]	0.97[c]	0.42[b]	2.92[a]
T 2	Fresh	2.27[a]	0.45[a]	0.23[a]	5.09[c]
	Dry	2.34[a]	0.50[a]	0.18[a]	4.68[b]
SEM	Fresh	0.23	0.07	0.06	0.29
	Dry	0.17	0.12	0.06	0.58
P Value	Fresh	0.020	0.001	0.010	0.046
	Dry	0.004	0.001	0.001	0.022

Control, T_1 and T_2 are treatment groups @ 0.0, 0.5, 2.0 mL eucalyptus fresh/dry leaves extract/30 mL BRF, respectively. Mean values bearing a, b, c superscripts either for fresh/dry in a column vary significantly ($p<0.05$). A:P = Acetate to propionate ratio

significant ($p<0.001$) reduction in methane concentration, methane mL/g DM and methane mL/g DMD in all treatment groups in comparison to the control. IVDMD was increased ($p<0.001$) in T_1 and reduced ($p<0.001$) in T_2, in comparison to non-supplemented control. Ammonia nitrogen (NH_3-N) concentration remained similar ($p>0.05$) in T_1 but reduced ($p<0.001$) in T_2 in comparison to the control. Thus, the first treatment group (T_1) was found to be better for methane inhibition with increasing DM digestibility.

Analysis of volatile fatty acids (VFAs) demonstrated a significant ($p<0.01$) increase in acetate, propionate and butyrate concentration with a decrease ($p<0.05$) in the A/P ratio in T_1 in comparison to the control. However, propionate production was reduced ($p<0.05$) in T_2 without affecting acetate and butyrate production (Table 2).

In the 2nd experiment, the effect of Eucalyptus dry leaves extracts at graded levels [@ control (0.0 mL), T_1 (0.5 mL) and T_2 (2.0 mL)/30 Ml BRF] on *in-vitro* rumen fermentation parameters were described (Table 1). Similar to the 1st experiment, there was a significant ($p<0.001$) increase in total gas in T_1, but it was reduced ($p<0.001$) in T_2, in comparison to control. Likewise, gas mL/g DM and gas mL/g DMD were significantly ($p<0.001$) increased in T_1 and reduced ($p<0.001$) in T_2 group, in comparison to control. There was a significant ($p<0.001$) reduction in methane concentration, methane mL/g DM and methane mL/g DMD in all treatments in comparison to the control group. *In-vitro* dry matter digestibility was significantly ($p<0.001$) higher in T_1, however, it was reduced ($p<0.001$) in T_2, in comparison to the control. Ammonia nitrogen (NH_3-N) concentration remained similar ($p>0.05$) in T_1 but significantly reduced ($p<0.001$) in T_2 in comparison to the control. Thus, the first treatment group (T_1) was found to be better for methane inhibition with increasing DM digestibility.

The *in vitro* volatile fatty acid production was examined, and the results showed that propionate concentration was significantly ($p<0.01$) increased, although acetate and butyrate remained similar ($p>0.05$) with a decrease ($p<0.05$) in A/P ratio in T_1 in comparison to control. However, all the VFA production was decreased in T_2 (Table 2).

In 3rd experiment, a trial was conducted to find out the results of Poplar fresh leaves extract on *in vitro* rumen fermentation and methane production of oats hay substrate (Table 3) and found that there was a significant ($p<0.001$) reduction in total gas in T_2 treatment without any effect in T_1 as compared to control. However, the concentration of head space methane was significantly ($p<0.001$) decreased in both the treatment groups as compared to the control group. The total methane, methane, mL/g DM and methane, mL/g DMD remained similar ($p>0.05$) in the T_1 treatment group as compared to the control, but all parameters were significantly decreased ($p<0.05$) in the T_2 treatment group. *In-vitro* dry matter digestibility was significantly ($p<0.001$) reduced in T_2 as compared to control but not altered in the T_1 treatment group. The concentration of ammonia nitrogen was similar ($p>0.05$) in the control and T_1 treatment groups but significantly ($p<0.05$) reduced in the T_2 treatment group. Thus, the first treatment, T1, i.e. 0.5 mL Poplar fresh leaves extract/30 mL of BRF, was unable to reduce ($p>0.05$) methane production, whereas T_2 reduced methane production at the cost of impeding feed digestibility.

The data in Table 4 showed the effect of Poplar fresh leaves extract on *in vitro* individual volatile fatty acid production of oats hay substrate. There was no significant ($p>0.05$) variation in T_1 and control groups regarding the individual volatile fatty acids, but these were significantly reduced ($p<0.05$) in the T_2 group (Table 4).

Another experiment (Experiment 4) was conducted to study the effect of dosing Poplar dry leaves extracts in *in-vitro* rumen fermentation and methane production of oats hay (Table 3) and found that there was a significant ($p<0.001$) reduction in total gas in T_2 group without variation ($p>0.05$) in T_1 group as compared to control. Similar results were found when total gas was

Table 3. Effects of Poplar leaves extract on *in vitro* rumen fermentation and methane production

Attributes	Control		T 1		T 2		SEM		P value	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
Total Gas, mL	25.67^{b}	25.67^{b}	25.00^{b}	24.67^{b}	14.33^{a}	15.67^{a}	3.21	2.80	<0.001	<0.001
Gas, mL/g DM	136.93^{b}	136.93^{b}	135.31^{b}	130.58^{b}	78.80^{a}	82.64^{a}	18.08	15.07	<0.001	<0.001
Gas, mL/g DMD	199.31^{b}	196.31^{b}	195.18^{b}	196.28^{b}	146.27^{a}	176.87^{a}	14.53	6.24	<0.001	0.007
Methane Conc., %	9.36^{c}	9.36^{c}	8.08^{b}	8.15^{b}	2.46^{a}	3.16^{a}	1.86	1.55	<0.001	<0.001
Total Methane, mL	2.57^{b}	2.57^{b}	2.57^{b}	2.34^{b}	0.45^{a}	0.62^{a}	1.61	1.61	<0.001	<0.001
Methane, mL/g DM	13.51^{b}	13.5^{b}	13.65^{b}	12.38^{b}	2.36^{a}	3.28^{a}	3.24	2.38	<0.001	<0.001
Methane, mL/g DMD	19.27^{b}	19.27^{b}	20.29^{b}	17.84^{b}	4.44^{a}	7.02^{a}	4.45	3.40	<0.001	<0.001
IVDMD, %	70.86^{b}	70.86^{b}	69.00^{b}	70.22^{b}	53.66^{a}	47.28^{a}	5.01	6.78	<0.001	<0.001
Ammonia N, mg/dL	16.80^{b}	16.80^{b}	16.80^{b}	16.33^{b}	14.00^{a}	15.40^{a}	2.42	0.42	0.027	0.027

Control, T_1 and T_2 are treatment groups @ 0.0, 0.5, 2.0 mL of poplar fresh/dry leaves extracts/30mL BRF, respectively. Mean values bearing a, b, c superscripts in a row either for fresh/dry vary significantly ($p<0.05$). DM = Dry matter, DMD = Digested dry matter, IVDMD = *In-vitro* dry matter digestibility

expressed in terms of mL/g DM or mL/g DMD. The concentration of methane was significantly ($p<0.001$) decreased in both the treatments as compared to the control. However, the total methane, methane mL/g DM and methane mL/g DMD remained similar ($p>0.05$) in the T_1 group as compared to the control, but all parameters were significantly ($p<0.001$) decreased in the T_2 group. *In-vitro* dry matter digestibility was significantly ($p<0.001$) reduced in T_2 as compared to the control group but not altered ($p<0.001$) in T_1 treatment. The concentration of ammonia nitrogen remained similar in the control and T_1 treatment groups but significantly ($p<0.05$) reduced in the T_2 treatment group. Thus, similar to fresh leaves extract, again the first treatment group (T_1) was non effective for methane inhibition without affecting DM digestibility.

The data in Table 4 showed the effect of Poplar dry leaves extract on *in vitro* individual volatile fatty acid production of oats hay. The individual volatile fatty acid production was comparable ($p>0.05$) in both the control and T_1 group. However, there was a significant reduction ($p<0.05$) in acetate and propionate production in the group in comparison to the control and T_1.

Table 4. Effects of Poplar leaves extract on *in vitro* volatile fatty acids production

Attributes		Acetate (mM/100mL)	Propionate (mM/100mL)	Butyrate (mM/100mL)	A:P
Control	Fresh	2.76^{b}	0.55^{b}	0.04 1	5.01^{ab}
	Dry	2.76^{b}	0.55^{b}	0.04	5.00^{ab}
T1	Fresh	2.60^{b}	0.64^{b}	0.05	4.17^{a}
	Dry	2. 65^{b}	0.60^{b}	0.05	4.64^{a}
T2	Fresh	1.98^{a}	0.36^{a}	0.05	5.56^{b}
	Dry	2.22^{a}	0.41^{a}	0.05	05.38^{b}
SEM	Fresh	2.16	0.08	0.004	0.432
	Dry	0.16	0.05	0.003	0.23
P Value	Fresh	0.001	0.014	0.971	0.042
	Dry	0.003	<0.001	0.822	0.053

Control, T_1 and T_2 are treatment groups @ 0.0, 0.5, 2.0 mL Poplar leaves (Fresh/dry) extract /30 mL of incubation media, respectively. Mean values bearing a, b superscripts in a column either for fresh/ dry vary significantly ($p<0.05$).

DISCUSSION

Essential oils (EO) produced by different plant species can vary in chemical structures as well as bioactive activities (Burt, 2004). In the present study, the increased gas production and IVDMD with concomitant reduction of methane production at a lower

dose (0.5 mL/30 mL buffered rumen fluid) of eucalyptus leaves, irrespective of nature of leaves (dry or fresh), could be due to specific activity of bioactive compounds, where the compounds present in the extracts might have shown their effects against some of the rumen microbes (Dhanasekaran *et al*., 2020) which stimulated the fibrolytic activities and reduced the methanogenic archaeal population. But at a higher dose (2.0 mL) level, the bioactive compounds might be detrimental for rumen microbes in general because of their natural antimicrobial properties (Benchaar *et al*., 2008), which ultimately reduced the feed digestibility and gas production.

Sallam *et al*. (2009) also reported reduced methane production and gas production by dosing (25-150 µL/75mL incubation media) pure eucalyptus oil *in vitro* without affecting digestibility. However, Beauchemin and MacGinn (2006) reported reduced digestibility of dry matter as well as fibre at higher dose levels. Our results corroborate with the findings of others with improvement of feed digestibility by stimulating microbial activity (Calsamiglia *et al*., 2007) and gas production at lower dose and reduction, at higher dose as the effects of essential oils are dose dependent (Benchaar *et al*., 2008; Singh *et al*., 2023). Reduction in ammonia nitrogen concentration at higher dose level could be due to inhibition of hyper-ammonia producing (HAP) bacteria (Wallace *et al*., 2002; Patra and Saxena, 2009), but at lower dose level, it might not be effective in inhibiting those bacteria. Our study gets the support of Sallam *et al*. (2010), who also reported a reduction in ammonia nitrogen concentration in fresh and dry leaves of Eucalyptus as compared to alfalfa hay. The increased concentration of acetate, propionate and butyrate with decreased acetate to propionate ratio at low dose in the present study, are in agreement with those of Castillejos *et al*. (2006) who 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 dose as compared to control suggested detrimental effects on rumen microbes (Evans and Martin, 2000; 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*.

Poplar leaves have a bioactive essential oil constituent named isoprene (Muller *et al*., 2015). Studies conducted on poplar leaves extract @ 0.5 mL and 2.0 mL/30 mL buffered rumen fluid to assess the effects on *in-vitro* rumen fermentation and methanogenesis and result revealed that at low dose the concentration of methane was reduced by 13% while the total gas, ammonia nitrogen and IVDMD remained comparable. But at the high dose level, total gas, methane concentration, IVDMD and ammonia nitrogen were significantly reduced by 39%, 66%, 33% and 8.33%, respectively, as compared to control. An experiment conducted by Patra *et al*. (2006) reported a reduction of 20.6% in methane production when 10 mg poplar leaves were added in 30 mL incubation media. However, Kamra *et al*. (2008) observed a reduction in methane production by 8.49% and 85.86%, respectively by *in vitro* dosing (0.5 mL/30 mL BRF) of ethanolic and methanolic extracts of poplar leaves. These activities of methane inhibition might be due to the antibiotic principle of poplar leaves against methanogens (Muller *et al*., 2015). The non-effectiveness of total methane production at a low dose of poplar leaves extract in the present study might be due to the shortfall of the required concentration to inhibit methanogenic archaeal population. The comparable concentrations of acetate, propionate and butyrate to the control group in the lower dose support the result of Patra and Yu (2012), where the volatile fatty acid concentration was not affected by the supplementation of essential oils. The concentration of acetate, propionate, and butyrate was significantly lower than that of the control at the high dose. Roy *et al*. (2015) revealed that essential oils are likely to be harmful for rumen microbial fermentation when used at high dose to decrease in the VFA concentration, and reduction in concentration of VFA is responsible for decreased production in ruminants.

The extracts of Eucalyptus leaves, either in fresh or dry conditions, revealed reduced methanogenesis without impeding feed digestibility at a low level (0.5 mL/30 mL buffered rumen fluid) of supplementation. An enhanced feed digestibility and propionic acid production was also evidenced with the inclusion of Eucalyptus leaves extract. Thus, Eucalyptus leaves have the potential to be used as feed additive to improve animal performance and reduce environmental pollution. However, more studies on animal feeding experimentation are warranted before using it in practical ration formulation.

Conflict of interest: The authors declare no conflict of interest in the study.

Author's contributions: DA, KK, RMK: Conceptualization; KK: Data curation, validation, writing- original draft; KK, DA: Methodology and investigation; DA, LPC: Review and editing.

Data availability statement: All the data used in this study are included in the manuscript. The corresponding author is willing to provide the raw data upon reasonable request.

Ethical approval: Ethical clearance was obtained from the Institutional Animal Ethics Committee (IAEC) before commencing the experiment, and humane animal handling procedures were followed throughout the experiment.

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