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Effect of herbal plants oil addition in total mixed diets on anti-methanogenic activity, rumen fermentation and gas production kinetics *in vitro*

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ABSTRACT

The aim of the current study was to evaluate the efficacy of herbal plant oils on methane reduction and as rumen fermentation modulators in different diets by using in vitro gas production technique. Garlic (Allium sativum, GO), Eucalyptus (Eucalyptus globules, EuO) and Neem (Azadirachta indica, NO) oils were tested for their inhibitory action on methane production on HFD, MFD and LFD. Methane was done by Gas Chromatography and it has been shown that supplementation of all three oils, GO reduces the maximum (55.06%) methane emission in LFD as compared to that of control. Digestibility of dry matter also decreased due to GO supplementation, which may affect the production of volatile acid production.

Key Words: Essential oils, *In vitro* gas production technique, Anti-methanogenic activity, Gas kinetics.

INTRODUCTION

Methane is a potent green house gas and methane emission from livestock is one of the major significant contributors towards the accumulation of this gas in the environment which contributes to global warming. Scientists are working continuously on the strategies that will help in the mitigation of methane from the ruminant livestock either by dietary manipulations or by using molecular techniques. It has been estimated that the world wide production of methane from ruminants is around 86 million tonnes and 18 million tonnes from manure [1]. There are several strategies proposed by different workers to reduce methane production in ruminants like manipulation in diet, use of ionophores, antibiotics, probiotics, elimination of rumen protozoa etc [2, 3, 4, 5, 6]. The use of antibiotics as feed additives in beef cattle and dairy cows has been banned

in the European Union since January 2006 (Regulation 1831/2003/EC) due to the risk of antibiotics residues in animal products (e.g. milk and meat) and its subsequent effects on human health. For this reason, attention has recently shifted towards use of natural antimicrobials or bioactive PSM as a safe means of ruminal fermentation modulators. These secondary metabolites are difficult to classify because their metabolic pathways of synthesis and their properties and mechanisms of action are often overlapped, and differences are difficult to ascertain. However, they can generally be structured into 3 groups: saponins, tannins, and essential oils. Many researcher observed that plant secondary metabolites at low concentration inhibit the methanogenesis and favorably modulate the rumen fermentations [7, 8, 9].

Essential oils are volatile aromatic compounds extracted from whole plants and responsible for the odor and color of plants and spices. These are composed of more than 100 individual components [6]. Major components can constitute up to 95% of the essential oil, whereas other components are present only as traces [10]. Most essential oils are classified as Generally Recognized as Safe (GRAS), and have been approved for food and beverage consumption by the US Food and Drug Administration (www.cfsan.fda.gov). Essential oil has antimicrobial antibacterial, antifungal and antioxidant properties. Therefore, recently it has been great interest among nutritionist and rumen microbiologist to exploit essential oil as natural feed additives to improve rumen fermentation, inhibition of methanogenesis and efficiency of feed utilization.

The major components of garlic oil are organosulfur compounds such as allicin, diallyl sulfite. Presence of these compounds garlic oil shows the antimicrobial activity [11, 12]. Eucalyptus oil is variable mixtures of principally terpenoids, mainly monoterpenes (C10) and sesquiterpenes (C15). Eucalyptol (1, 8-cineole) is the main active ingredient of the eucalyptus oil [13]. The antimicrobial activity of EuO has been attributed to a number of terpenoid and phenolic compounds [14, 15, 16].

Neem oil comprises mainly triglycerides and large amounts of triterpenoid compounds, which are responsible for the bitter taste. Neem oil also contains steroids (campesterol, beta-sitosterol, sigmasterol) and a plethora of triterpenoids of which azadirachtin is the most well known and studied. The objective of the present study was to evaluate the effects of different herbal plant oil on methane inhibition and ruminal fermentation patterns.

MATERIALS AND METHODS

Plant material

The oils of Garlic (*Allium sativum,* GO), Eucalyptus (*Eucalyptus globules,* EuO) and Neem (*Azadirachta indica,* NO) were purchased from local drugs supplier of Karnal district, Haryana, India. These oils were manufactured by FAME DRUGS, India.

Preparation of Diets

To evaluate the effect of different herbal oils three diets were prepared by taking different roughage and concentrate ratio i.e. high fiber diet (HFD, 60R:40C), medium fiber diet (MFD, 50R:50C) and low fiber diet (LFD, 40R:60C) and milled to pass through 1 mm sieve and used as substrate. 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

2% (DM basis) of each treatment were added to the different wheat straw based HFD, MFD and LFD diets. All the treatment combinations were arranged in 4 x 3 factorial designs with three replicates. Set was also incubated devoid of substrate with and with out oils which served as blanks for particular treatment and values were corrected for different parameters with these blanks.

Preparation of Inoculums and *In Vitro* Gas Production

Rumen liquor was collected after manual mixing of rumen contents from a fistulated mature male buffalo (*Bubalus bubalis*) maintained on a standard diet (60 parts roughage: 40 parts concentrate) before morning feeding into a pre-warmed 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. The incubation medium was prepared as per previously described method [17]. Treatments was added in 100 ml glass syringe containing 200±10 mg of milled (1mm) three type wheat straw based diets. The 30 ml incubation medium was dispensed anaerobically in each syringe. Plungers of syringes applied with petroleum jelly for smooth movement and stop any leakage. Syringes were closed using clamps and were incubated at 39 ± 0.5^0 C for 24 h.

Estimation of Methane production by Gas Chromatography

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 50^0C, 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 peak of methane gas was identified on the basis of retention time of standard methane gas and the response factor obtained was used to calculate methane percentage in the gas sample. The methane produced from substrate during 24 hour incubation was compared for the blank values. The volume of methane produced was calculated as follows:

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

Total volatile fatty acid (TVFA) estimation

TVFA concentration (mM/100 ml) in the supernatant was estimated according to prescribed method [18].

Estimation of individual volatile fatty acids (IVFA)

Individual volatile fatty acid estimated by gas chromatograph according to the prescribed method [16, 19].

Partitioning factor and microbial biomass yield

The PF is calculated as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it. Substrate provides important information about partitioning of fermentation products. The MBM yield was calculated by using the degradability of substrate and gas volume and stoichiometrical factor [20].

Microbial mass = Substrate truly degraded - (gas volume × stoichiometrical factor)

Where the stoichiometrical factor used was 2.25.

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 (12 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

For protozoal count one milliliter of the fermentation fluid was diluted with 1 ml of formalin (18.5% formaldehyde) and 3-4 drops of brilliant green and then incubated for 24 hours at room temperature. The stained protozoa were diluted (if needed) and counted by haemocytometer as per the prescribed method [21].

***In vitro* true DM degradability**

To estimate true DM degradability of feed sample of each syringe containing residues after incubation was estimated as per the prescribed method [22].

Proximate analyses and Cell wall constituents

The proximate analysis of substrate was carried out as per the methods of AOAC [23]. The cell wall constituents of substrates were determined according to described method [24].

Gas production kinetics

The total gas production kinetics was carried out in different treatment combinations incubated as per procedure mentioned above for different intervals i.e. 0, 1, 2, 3, 6 9, 12, 24, 36, 48, 60, 72 and 96 h. The potential gas production and rate of gas production was calculated by fitting the modified equation [25].

Regression model = Orskow without lag
Equation,

$$F=b*(1-\exp(-c*x))$$

Statistical analysis

Experimental data of different parameters were analyzed in randomized block design with three replicates for analysis of variance [26].

RESULTS AND DISCUSSION

The physical and chemical composition of all diets is shown in Table 1. Results of different essential oils on *in vitro* rumen fermentation and methanogenesis are presented in Table 2 and table 3.

Table 1: Physical and chemical composition of wheat straw based diets used as substrate in *in vitro* incubation

Ingredient of diets							
Diets				**g/kg on DM basis**			
				Wheat straw		**Concentrate**	
HFD				600		400	
MFD				500		500	
LFD				400		600	
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 diets (g/kg on DM basis)							
Diets	**OM**	**CP**	**EE**	**NDF**	**ADF**	**HC**	**TA**
HFD (60R:40C)	867.6	108.6	23.4	623.1	372.0	251.1	132.4
MFD (50R:50C)	878.4	125.3	30.4	604.5	329.5	275.0	121.6
LFD (40R:60C)	875.6	142.7	34.8	538.7	298.7	240.0	124.4

OM= Organic Matter, CP= Crude Protein, EE= Ether Extract, NDF= Natural Detergent Fiber, ADF= Acid Detergent Fiber, HC= Hemicelluloses, TA = Total Ash

Table 2: Supplementation effect of different herbal oils on *in vitro* methane inhibition and IVDMD on wheat straw based diets

Diets	**Treatments**	**Parameters**					
		pH	**IVDMD%**	**PF**	**MBM (mg)**	**CH_4 mM/gDM**	**Protozoa x10^5/ml**
HFD	**Control**	7.02	62.1	4.71	62.00	2.66	1.30
	GO	7.01	46.0	2.86	17.70	2.06	0.41
	EuO	7.13	49.0	2.61	11.45	3.19	0.41
	NO	7.11	56.3	4.10	53.75	2.75	0.75
MFD	**Control**	7.02	64.5	3.26	35.30	3.16	1.75
	GO	7.01	52.8	3.02	24.20	1.60	0.83
	EuO	7.01	47.0	3.36	38.75	3.11	0,83
	NO	7.04	60.0	3.56	42.00	2.91	1.33
LFD	**Control**	7.03	64.0	3.57	46.70	3.56	1.50
	GO	6.98	54.3	3.15	30.50	1.60	0.58
	EuO	7.02	49.1	3.20	33.00	3.82	0.58
	NO	7.05	55.8	3.22	35.65	3.43	1.58
SEM	**Diet**	0.013	N.S	0.056	NS	NS	0.09
	Treatment	0.016	3.0	0.065	2.14	0.18	0.10
	D*T	N.S	N.S	0.11	3.71	N.S	0.18

GO = Garlic oil, EuO = Eucalyptus oil, NO = Neem oil, IVDMD= In vitro Dry Matter Digestibility, MBM= Microbial Biomass, PF=Partition Factor, SEM= Standard Error of Means

Effect of essential oil on pH was significant for all three diets. Maximum 0.11 unit variation was found. Maximum pH was found in EuO (7.13) in HFD and minimum was in GO (6.98) in LFD. In current experiment, IVDMD was significantly affected by essential oils. The digestibility of dry matter was decreased significantly (25.92%) due to addition of GO, in case of HFD, while, EuO reduced 27.13% and 23.28%, in case of MFD and LFD, respectively. A reduction in

methane production (ml/gDM) was seen in all the case except EuO treatment. Maximum methane reduction was observed in GO treatments. Results showed the GO reduced methane production 22.56, 49.36 and 55.06% on the addition of HFD, MFD and LFD, respectively. These results were similar to previous work [27].

Table 3: Supplementation effect of different herbal oils on *in vitro* Rumen fermentation pattern on wheat straw based diets

Diets	Treatments	Parameters					
		TVFA (mM/100ml)	Acetate (mM/100ml)	Propionate (mM/100ml)	Butyrate (mM/100ml)	A:P Ratio	NH_3-N (mg/100ml)
HFD	Control	5.45	4.06	1.12	0.26	3.62	20.16
	GO	4.91	3.55	1.04	0.32	3.42	18.66
	EuO	5.48	4.11	1.09	0.28	3.79	18.10
	NO	5.93	4.53	1.39	0.30	3.26	16.61
MFD	Control	7.00	5.25	1.40	0.34	3.71	18.85
	GO	6.83	4.79	1.54	0.50	3.10	16.24
	EuO	6.96	5.17	1.43	0.35	3.97	17.36
	NO	7.08	5.43	1.37	0.27	3.67	17.82
LFD	Control	7.01	5.21	1.59	0.36	3.26	17.26
	GO	6.65	4.65	1.50	0.50	3.09	21.46
	EuO	5.83	4.36	1.18	0.29	3.69	24.25
	NO	7.03	5.42	1.54	0.37	3.36	24.26
SEM	Diet	0.13	0.11	0.037	0.014	N.S.	1.11
	Treatment	0.16	0.13	0.042	0.016	0.10	N.S
	D*T	N.S	N.S	0.072	0.028	N.S.	N.S

TVFA= Total Volatile Fatty Acids, A/P= Acetate to Propionate Ration, NH_3-N= Ammonia Nitrogen

Table 4: Supplementation effect of different herbal oils on gas kinetics on wheat straw based diets

Diet	Treatments	b	c	R^2
HFD	Control	36.27	0.08	0.992
	GO	36.75	0.08	0.995
	EuO	36.23	0.07	0.990
	NO	29.38	0.07	0.999
MFD	Control	31.96	0.07	0.998
	GO	37.02	0.07	0.997
	EuO	35.93	0.10	0.992
	NO	36.22	0.09	0.996
LFD	Control	37.64	0.11	0.994
	GO	34.47	0.08	0.996
	EuO	40.68	0.10	0.993
	NO	34.09	0.09	0.998

b = Potential gas production (ml), c = Gas Production Rate Constant (ml/h), R^2 = Regression Coefficient

In present experiment, essential oils was significantly affected the PF and MBM (mg). NO reduced the PF and MBM (mg) in HFD and LFD, where as in MFD both are increase. On the supplementation of NO, the minimum reduction in PF and MBM (mg) was 12.95% and 13.31%; 9.80% and 23.66% in HFD and LFD, respectively, while increase 9.20% and 18.98% in MFD. A little variation in TVFA concentration was observed in all type of diets. Similar results were observed in earlier studies [28, 29, 30]. The TVFA concentration increased with NO in all the three diets. The maximum increase in TVFA concentration in HFD was 8.81%. The acetate concentration (mM) was increased with NO in all the three diet while, it was decreased with GO and EuO. The highest increasing (11.57%) and reduction (12.56%) in acetate concentration were observed in NO and GO, in HFD, respectively. Maximum increasing of propionate concentrate was seen with NO (24.11%) in HFD, (10%) with GO in MDF, while in case of LFD, reduction of propionate concentrate was observed in all treatments and highest reduction (25.79%) was

seen in EuO. A significant effect of these oils on butyrate concentration was seen in present study. Slightly reduction of A/P ration was observed in all cases except EuO, where 4.60, 7.01 and 13.19%, increasing of A/P rations was found in HFD, MFD and LFD, respectively. A non significant ($P \leq 0.05$) effect of these oils was observed in NH_3-N concentration. In the presence of NO, concentration of ammonia nitrogen increase (40.56%) in LFD, while 17.61% decreased in case of HFD.

The number of protozoa is significantly affected in both the control and treated diets which are closely associated with methanogens. Among the all treatment, GO showed the maximum reduction i.e. 68.46, 52.57 and 58.22% in HFD, MFD and LFD, respectively. Garlic oil might be reducing the methane emission by inhibiting protozoa population directly. Most of the studies till date give us knowledge about the essential oils which will inhibit protozoa population and hence decreasing the methane gas production [31].

Result of effect on oils in gas kinetics in HFD, MFD and LFD presented in table 4. It was observed from the results that potential gas production (b) was increased due to the addition of oils, and the increase was highest (15.83%) with GO in MFD, while it was slightly affected by oils in case of HFD and LFD. The gas production rate constant (c) also unaffected by tested oils in comparison to control in high, medium and low fiber diets.

CONCLUSION

On comparing the effects of GO, EuO and NO on three diets, it was seen that supplementation of GO significantly reduced the methane production. Potential gas production (b) also increased with GO. However detailed study about dosage, active components of essential oils and mechanism of their inhibitory action on methanogenesis is required.

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