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Effect of combined herbal feed additives on methane, total gas production and rumen fermentation

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Abstract:
The present study was to evaluate effect of herbal feed additives on methane and total gas production during the rumen fermentation for environment and animal health concern. Different parts of the five medicinal plants were selected such as leaf and small stems of Ocimum sanctum (Tulsi), roots of Curcuma longa (Haldi), fruits of Emblica officinalis (Amla), leaves of Azadirachta indica (Neem) and leaves and small stem of Clerodendrum phlomidis (Arni) for our study. Addition of different herbal additive combinations did not influence IVDMD and total gas production however methane production (mg/g of substrate DM) was significantly ($P<0.05$) reduced in Amla: Neem and Neem: Arni combinations. Total nitrogen significantly ($P<0.01$) increased in the combinations of Tulsi: Haldi and Amla: Neem. TCA–ppt-N is significantly ($P<0.01$) increased in Tulsi: Haldi, Haldi: Amla, Amla: Neem and Neem: Arni however NH3-N (mg/dl) significantly decreased in all treatments. We conclude that the screening of plant combinations, Amla: Neem and Neem: Arni have potential to decrease methane production and our herbal feed supplements have no side-effects on the ruminant in small amount.

Keywords: Herbal feed additives, Methane, Rumen fermentation, Total gas, Medicinal plants

Background:
Methanogens are the methane producing bacteria accommodate the rumen liquor of animals in large numbers varying from 10^7 to 10^9 cells/ml. This large concentration of methanogens in the rumen liquor depends upon the feed resources provided to the animals as regular diet, especially the fiber content in the ration. Moreover, rumen fermentation is coordinated by supporting role of methanogens and making this a continuous process however it leads to a significant loss of gross energy consumed by the animals **[1, 2].** Manipulation in the basic ingredients of feed additives is the most direct and permissible means of lowering CH_4 emissions from ruminants in most systems. Because it is well established that feeding grain-based diets reduces enteric CH_4 (g/kg of DM) as compared with feeding forage-based diets **[3].**

Herbal plants are used in animal feeds as the growth promoters. They play a major role as antibacterial, antioxidant, anthelmintic and anticoccidial. Majority of medicinal plants do not have the residual effects. It has been shown that phytochemicals and plant secondary metabolites could increase

protein flow to the duodenum **[4].** The plants containing saponins have been found to suppress or eliminate protozoa from the rumen and reduce methane and ammonia production **[5].** Cheeke *et al*. (2000) **[6]** reported that plant secondary metabolites i.e., saponin- containing plants are reported to suppress or inhibit protozoa and certain bacteria in the rumen. Patra *et al.* (2006) **[7]** reported decrease in DM and OM digestibilities due to *Acacia concina* extract addition. Dey and Ghosh (1995) **[8]** reported that the dry matter consumption and digestibility of DM, CP, OM, EE, and NFE were higher (P<0.05) in kids supplemented with livol, an herbal preparation. Ishtiyak *et al.* (2010) **[9]** also reported an improvement in the *in vitro* dry matter and organic matter digestibility after addition of *Trigonella foenumgraecum* in ration. Earlier studies with different herbal additives reported different types of results. Kumar *et al.* (2009) **[10]** reported that addition of eucalyptus oil at different dose level reduced methane production and protozoa number under *in-vitro* system. Sirohi *et al.* (2009) **[11]** reported that acetone and methanol extract of *E. globules* and aquos extract of *S. mukorossi* and *E. globules* were the best inhibitor of methane production. Patra *et al.* (2010) **[12]** reported that ethanol and methanol extract of fennel, cloves and garlic had inhibitory effect on methane production. Similar finding of methane reduction, with *Acacia angustissima*, *Sesbania sesban* **[13]**, *Sapindus* spp., *Populus tremuloides*, *Syzygium zromaticum*, *Psidium guayaba*, *Terminalia chebula* **[5]**, horsetail and sage **[14].** Patra *et al.* (2006) **[7]** found that addition of extracts of *A. concinna*, *E. officinalis* and *T. belerica* resulted in a significantly (P<0.05) higher production of gas per gram dry matter as compared to control. Herbal feed additives *Ocimum sanctum, Curcuma longa, Emblica officinalis* and *Clerodendrum phlomidis* did not show any adverse effect on blood haematology in weaned Barari kid **[15].** It was observed that feeding Neem leaf powder improved growth of broilers **[16].** Yang *et al.* (2009) **[17]** suggested that supplementation with Neem oil inhibited bacterial activity, which could be beneficial in treating acute acidosis in feedlot cattle fed high-grain diets.

Therefore, because of beneficial effect of herbal plants, our present study was to evaluate effects of feed additive on methane production, IVDMD, and gas production. According to ruminant grazing behavior selected plants were used in their crude form in substrate feed. Plant combination sometimes enhances the activity of other plants and sometime shows antagonistic effect. Herbal plants individually mixed at the percentage of 0.5% with the Substrate feed and further their effects were analyzed.

Methodology:

Selection of plants

Based on the available literatures on the beneficial effects of herbal pants on the ruminant and availability of such plants in northern Indo-Gangetic plain of India following herbal plants were selected for the evaluation under *in-vitro* fermentation system. Selected five locally available medicinal plants taken parts are mentioned in **Table 1 (see supplementary material)**. First, the different parts of the five medicinal plants were selected such as leaf and small stems of *Ocimum sanctum* (Tulsi), roots of *Curcuma longa* (Haldi), fruits of *Emblica officinalis* (Amla), leaves of *Azadirachta indica* (Neem) and leaves and small stem of *Clerodendrum phlomidis* (Arni). All five plants were mix. with each other. Ten different combinations of herbal treatments were divided like T_1 Control, T_2 Tulsi: Haldi, T_3 Haldi: Amla, T_4 Amla: Neem, T_5 Neem: Arni, T_6 Arni: Tulsi, T_7 Tulsi: Amla, T_8 Tulsi: Neem, T_9 Haldi: Neem, T_{10} Haldi: Arni and T_{11} Amla: Arni. After estimating the Dry Mass (DM) content, the samples were grinded with Wiley mill in the laboratory. Furthermore, the samples were preserved in the polythene bags.

Preparation of the substrate feed

The substrate feed for all combinations was prepared using concentrate mixture (40%), gram straw (40%) and cowpea hay (20%). Concentrate mixture was prepared using Barley 37%, Linseed cake 30%, Gram Chuni 15%, Wheat bran 15%, Mineral mixture 2% and Common salt 1% Add Vitamins, minerals in feed supplements for *in-vitro* study.

Estimation of chemical composition

Concentrate mixture, gram straw and cowpea and all substrates were analyzed for proximate analysis of OM, CP, Total carbohydrate, EE and Total Ash **[18].** Representative samples of herbal samples were analyzed for cell wall components (NDF, ADF, Hemi cellulose, Cellulose and Lignin) in accordance with Goering and Van Soest method (1970) **[19].**

Collection of Rumen Liquor

Rumen liquor was taken from the kids maintained under uniform feeding system on (5-6 hour grazing, Gram straw, Concentrate mixture and Green fodder). Rumen liquor was collected from the donor bucks by the stomach tube from all parts of the rumen into a clean thermo flask. The rumen liquor was taken to ensure the maintenance of optimum temperature, while collecting and handling of rumen liquor.

In-vitro techniques

In each *in-vitro* bottle, 0.5g (DM) of same substrates was added. In each bottle, 40 ml McDougall's buffer and 10 ml of SRL collected from donor animals of respective groups were added. Each bottle was infused with CO_2 before sealing with aluminium cap and rubber cork. Further, the *in-vitro* bottles were incubated for 48h at 39°C±0.5°C.

Analysis of DMD, Total gas and Methane

After 48h of incubation, the contents of the flask were filtered through Grade-1 crucible. The DM was estimated according to AOAC (1984) **[18]** in the samples of substrates as well as the residues. Total gas production was observed in the *in vitro* bottles, which were incubated at 39±0.5 ^{0}C. Total gas production was measured by using siphon system at 48h of incubation. The siphon system was prepared with the help of two 50 ml burettes with connecter PVC tubes. The total gas produced in the bottle was measured with the help of 50 ml of syringe and tri-way valve. Water (in the burette) displaced by the pressure of gas was kept at initial level by sucking with the help of syringe. Total gas collected in the syringe was measured with the help of its graduation. The gas sample was taken from each bottle with the help of gas tight syringe (3ml capacity) and analyzed for methane using Gas Chromatograph (Amil Nucon 5700).

Rumen fermentation

The pH of rumen fluid was determined within 10 min. of aspiration using digital pH meter (PCS Tester 35, Eutech

Instruments Pte Ltd. Singapore), thereafter samples were strained through four layers of muslin cloth and stored at -20°C for further fluid (SRL) was used to determine total volatile fatty acids (TVFA). The Micro kjeldahl procedure **[18]** was followed for ammonia-N, and TCA-perceptible-N determinations in the SRF. Fractionations of VFA in rumen fluid were separated by GC according. In brief, a 5 ml SRL was mixed with 1.0 ml of 20% meta-phosphoric acid (w/v in 5N-H_2SO_4), stand over-night and centrifuged at 5000 rpm for 15 minutes. The supernatant was used for VFA fractionation in Amil Nucon Gas chromatography, series-5700 fitted with glass columns (chromosorb 101). The standard ratio 60:25:15 was used to Acetate: Propionate: Butyrate by GC and Area of peak for acetate, propionate and butyrate was calculated as '1/2×hight×width' and were compared with the area of the peaks of standard and presented as percentage of total VFA concentration. With the help of data station in the GC machine the area of each peak was calculated in terms of *mv* for each corresponding peak.

Statistical analysis

Data pertaining to the *in-vitro* studies were statistically analyzed using randomized block design (RBD) with one-way ANOVA. Computerized SPSS 7.5 statistical package was used for the analysis.

Results:

Chemical composition of substrates measuring

Chemical composition of different substrates is presented in **Table 1 (see supplementary material).** Herbs analyze during current investigation reflect full conformity in proximate composition and hold nutritional worthiness commensurate to traditional feed.

In-vitro DMD

Data on IVDMD using double combination of herbal plants were presented in **Table 2 (see supplementary material)**. IVDMD ranged from 55.71 in T_5 to 62.09 in T_7 and T_8. However, the difference was statistically similar. The data showed that combined effect of two herbals had no specific effect on IVDMD under *in-vitro* system using goat rumen liquor.

Total gas and methane production

Total gas (ml) production/g of DM ranged from 114.32 in T_7 to 130.08 in T_{10}. Methane production (mg/g of substrate DM) was significantly ($P<0.05$) reduced in T_4, T_5 than control T_1 **Table 2 (see supplementary material).**

Effect of herbals on pH

Observation regarding to pH concentration of rumen liquor were given in **Table 3 (see supplementary material)**. *In-vitro* pH values found statistically similar. The values ranged from 6.44 in T_{11} to 6.69 in T_1 (control).

Effect of herbal additives on ammonia-N and other nitrogen fractions

Data on rumen fermentation pattern were presented in **Table 4 (see supplementary material).** Ammonia nitrogen (mg/dl) was statistically ($P<0.05$) reduced in treatments T_2, T_7, T_8, T_9, T_{10} and T_{11} as compared to control T_1. TCA-precipitable nitrogen (mg/dl) was statistically ($P<0.01$) higher in T_2, T_3, T_4 and T_5 than control T_1. Total nitrogen (mg/dl) was increased ($P<0.01$) in T_2 and T_4 than control T_1 and other treatments. NPN (mg/dl) concentration in the incubation medium were significantly ($P<0.01$) reduced in all treatments than control. Studies with different herbal plants revealed variable types of results on ammonia-N concentration.

Total VFA concentration and its fractionation

Total VFA concentration (mmol/dl) was similar among all treatments; it range between 8.02 in T_4, to 9.36 in T_2. Acetate, propionate and butyrate concentration were also unaffected due to such supplementation **Table 5 (see supplementary material).**

Discussion:

The addition of different herbal additives did not influence IVDMD in present study. However, Sirohi *et al.* (2009) **[11]** reported 15% increased in IVDMD due to supplementation of *Aloe-barbadanis* extract. Methane production (*in vitro*) was reduced by combination of Amla: Arni and Neem: Arni when supplemented @ 0.5% in the substrate. All double combinations of herbal plants had no adverse effect on pH of incubation medium under *in-vitro* system. pH were within normal range (6.5-7.0) showing no adverse effect of plants addition on rumen environment. However, different herbal components (crude or extract) may have variable effect on the pH in rumen eco-system, which is evident in the present study as well as earlier studies. Total nitrogen increased in treatment Tulsi: Haldi and Amla: Neem. TCA–ppt-N increased in T_2 (Tulsi: Haldi), T_3 (Haldi: Amla), T_4 (Amla: Neem) and T_5 (Neem: Arni). NH_3-N significantly decreased in all treatments **[7]** showed that adding water extracts of Neem seeds decreased total ruminal volatile fatty acid (VFA) concentrations, the ratio of acetate to propionate and ruminal feed digestibility. However, such negative effect was not seen in all combinations of herbal plants. TVFA and propionate production marginally increased in T_2 (Tulsi: Haldi) and T_{11} (Amla: Arni) because of improvement of propionate A/P ratio, decrease slightly.

Conclusion:

Our research work concludes that the screening of plant combinations, Amla: Neem and Neem: Arni have potential to decrease methane production. IVDMD and total gas shows that these herbal feed supplements have no side-effects on the ruminant in trace amount. Different herbal combinations increase the total nitrogen significantly however TCA–ppt-N is significantaly increased in T_2 (Tulsi: Haldi), T_3 (Haldi: Amla), T_4 (Amla: Neem), T_5 (Neem: Arni). NH_3-N (mg/dl) significantly decreased in all treatments. Because of natural origin of herbal plans, they are easily available and able to reduce the cost of designing effective and balanced diet for the ruminant. Our work will help the animal biologist to design potential herbal feeds for the ruminant.

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Supplementary material:

Table 1: Locally available herbal plants and its parts used in the study.

S. No.	Common name	Botenical name	Part of use
1	Tulsi	*Ocimum sanctum*	Leaves and small stem
2	Haldi	*Curcuma longa*	Root
3	Amla	*Emblica officinalis*	Fruit
4	Neem	*Azadirachta indica*	Leaves
5	Arni	*Clerodendrum phlomidis*	Leaves and small stem

Table 2: Chemical composition of substrates* incorporated with different herbal plants used for *in-vitro* estimation (% on DM basis).

Ration	CP	EE	TCHO	OM	Ash	NDF	ADF	Hemi Cellulose	Cellulose	Lignin
T_1 Control**	13.87	2.82	73.45	90.14	9.86	53.88	28.40	25.48	21.57	5.56
T_2 (Tulsi)	13.65	2.82	73.59	90.06	9.94	53.68	28.75	25.93	21.75	5.91
T_3 (Haldi)	13.68	2.77	73.47	90.19	9.80	53.42	28.52	25.90	21.45	5.75
T_4 (Amla)	13.61	2.80	73.67	90.08	9.92	53.53	28.50	25.03	21.16	5.78
T_5 (Neem)	13.68	2.81	73.64	90.13	9.87	53.60	28.52	25.08	21.29	5.96
T_6 (Arni)	13.69	2.79	74.54	90.02	9.98	53.10	28.57	25.53	21.42	5.97

Substrate* = 99.5% of (Concentrate mixture 40% + Gram straw 40% + Cowpea fodder 20%) + 0.5% of each herbal plant. T_1= Control** substrate without herbs, Treatments=T_1 to T_6.

Table 3: Effect of incorporation of double combination plants @ 0.05% in the substrate feed on *in-vitro* N-metabolism.

Treatment	pH	Total-N (mg/dl)	NPN (mg/dl)	TCA–ppt-N(mg/dl)	NH_3-N (mg/dl)
T_1 (Control)	6.69	63.89^{a}	32.70^{d}	31.20^{a}	24.94^{c}
T_2 (Tulsi: Haldi)	6.50	82.88^{b}	26.88^{c}	56.00^{cd}	17.64^{ab}
T_3 (Haldi: Amla)	6.47	70.48^{a}	24.40^{abc}	46.08^{bcd}	20.44^{abc}
T_4 (Amla: Neem)	6.49	84.00^{b}	25.20^{bc}	58.80^{d}	20.28^{abc}
T_5 (Neem: Arni)	6.47	70.32^{a}	23.68^{abc}	46.64^{bcd}	19.88^{abc}
T_6 (Arni: Tulsi)	6.51	62.24^{a}	25.52^{bc}	36.72^{ab}	22.12^{bc}
T_7 (Tulsi: Amla)	6.49	59.52^{a}	21.92^{abc}	37.60^{ab}	15.80^{a}
T_8 (Tulsi: Neem)	6.46	59.28^{a}	21.00^{ab}	38.28^{ab}	16.24^{a}
T_9 (Haldi: Neem)	6.47	59.52^{a}	19.04^{a}	40.48^{ab}	16.80^{ab}
T_{10}(Haldi:Arni)	6.47	61.04^{a}	23.52^{abc}	37.52^{ab}	19.04^{ab}
T_{11}(Amla: Arni)	6.44	64.96^{a}	21.56^{abc}	43.40^{abc}	17.36^{ab}
SEM	6.49	67.10	24.12	42.97	19.14
P-value	0.965	0.000	0.000	0.000	0.017

Means in the same row with the different superscripts (a, b, c and d) are significantly different

Table 4: Effect of two plant combinations on *in vitro* gas and methane production.

Treatment	IVDMD %	Total gas (ml)/g DM	CH_4 (mg)/ g substrate DM	CH_4 (mg)/g digested DM
T_1 (Control)	57.55	122.35	51.41^{b}	88.48
T_2 (Tulsi: Haldi)	61.56	124.64	51.97^{b}	84.80
T_3 (Haldi: Amla)	62.01	119.00	46.75^{ab}	75.35
T_4 (Amla: Neem)	59.47	117.24	35.51^{a}	59.80
T_5 (Neem: Arni)	55.71	125.96	36.93^{a}	66.83
T_6 (Arni: Tulsi)	58.26	114.56	47.63^{ab}	82.19
T_7 (Tulsi: Amla)	62.09	114.32	45.72^{ab}	73.88
T_8 (Tulsi: Neem)	62.09	115.04	44.76^{ab}	72.23
T_9 (Haldi: Neem)	61.35	119.72	46.75^{ab}	76.36
T_{10} (Haldi:Arni)	60.73	130.08	53.01^{b}	87.20

T_{11} (Amla: Arni)	60.49	116.80	52.44[b]	87.73
SEM	60.11	119.97	46.62	77.71
P-value	0.109	0.388	0.020	0.105

Means in the same row with the different superscripts (a and b) are significantly different (P<0.05)

Table 5: Production pattern of *in-vitro* volatile fatty acid as influenced by the addition of two plant combinations on substrate feed.

Treatment	TVFA (mmol/dl)	Acetate %	Propionate %	Butyrate %	A/P ratio
T_1 (Control)	8.82	67.26	21.53	11.21	3.13
T_2 (Tulsi: Haldi)	9.36	69.63	23.12	8.38	3.03
T_3 (Haldi: Amla)	9.14	72.66	19.96	7.45	3.66
T_4 (Amla: Neem)	8.02	70.11	20.19	10.90	3.47
T_5 (Neem: Arni)	8.42	68.19	19.72	12.08	3.49
T_6 (Arni:Tulsi)	8.98	66.55	19.68	13.77	3.45
T_7 (Tulsi: Amla)	8.48	71.66	18.41	9.11	3.95
T_8 (Tulsi: Neem)	8.90	67.53	21.55	10.92	3.24
T_9 (Haldi: Neem)	8.80	71.62	19.09	9.29	3.76
T_{10} (Haldi:Arni)	8.40	71.10	18.21	10.69	4.24
T_{11} (Amla:Arni)	9.14	64.75	24.39	10.58	2.94
SEM	8.76	69.18	20.53	10.39	3.48
P-value	0.783	0.320	0.138	0.223	0.150