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 10⁷ to 10⁹ 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 of feed additives is the most direct and permissible means of lowering CH₄ emissions from ruminants in most systems. Because it is well established that feeding grain-based diets reduces enteric CH₄ (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. Ishitlyak et al. (2010) [9] also reported an improvement in the in vitro dry matter and organic matter digestibility after addition of Trigonella foenugracrum 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 guajaba, Terminalia chebula [5], horsetail and sage [14], Patra et al. (2006) [7] found that addition of extracts of A. concina, E. officinalis and T. berclica resulted in a significantly (P<0.05) higher production of gas per gram dry matter as compared to control. Herbal feed additives Oicium sanctum, Curcuma longa, Emblica officinalis and Clerodendrum phlomidium 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 plants 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 phlomidium (Arni). All five plants were mix. with each other. Ten different combinations of herbal treatments were divided like T1 Control, T2 Tulsi: Haldi, T3 Haldi: Amla, T4 Amla: Neem, T5 Neem: Arni, T6 Arni: Tulsi, T7 Tulsi: Amla, T8 Tulsi: Neem, T9 Haldi: Neem, T10 Haldi: Arni and T11 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 Chungi 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 CO2 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 °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 connector 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-H2SO4), stand overnight and centrifuged at 5000 rpm for 15 minutes. The supernatant was used for VFA fractionation in Ami 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×height*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 T5 to 62.09 in T1 and T8. However, the difference was statistically similar. The data showed that combined effect of two herbs 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 T5 to 130.08 in T10. Methane production (mg/g of substrate DM) was significantly (P<0.05) reduced in T4, T5 than control T1; Table 2 (see supplementary material).

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 T2, T3, T6, T9, T10 and T11 as compared to control T1. TCA-perceptible nitrogen (mg/dl) was statistically (P<0.01) higher in T2, T3, T4 and T6 than control T1. Total nitrogen (mg/dl) was increased (P<0.01) in T2 and T4 than control T1 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 T4 to 9.36 in T2. 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 ecosystem, 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 T2 (Tulsi: Haldi), T3 (Haldi: Amla), T4 (Amla: Neem) and T5 (Neem: Arni). NH3-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 T2 (Tulsi: Haldi) and T11 (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 significantly increased in T2 (Tulsi: Haldi), T3 (Haldi: Amla), T4 (Amla: Neem), T5 (Neem: Arni). NH3-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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Reference:
[1] Kamtra DN Current Science India. 2005 89: 124
[2] AI Hasin A et al. Environ Sci Technol. 2010 44: 400 [PMID: 20039753]
Table 1: Locally available herbal plants and its parts used in the study.

| S. No. | Common name | Botanical 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 |
|--------|-------|-------|-------|-------|------|------|------|----------------|-----------|--------|
| T1     | 13.87 | 2.82  | 73.45 | 90.14 | 9.86 | 53.88| 28.40| 25.48          | 21.57     | 5.56   |
| T2     | 13.65 | 2.82  | 73.59 | 90.06 | 9.94 | 53.68| 28.75| 25.93          | 21.75     | 5.91   |
| T3     | 13.68 | 2.77  | 73.47 | 90.19 | 9.80 | 53.42| 28.52| 25.90          | 21.45     | 5.75   |
| T4     | 13.61 | 2.80  | 73.67 | 90.08 | 9.92 | 53.53| 28.50| 25.03          | 21.16     | 5.78   |
| T5     | 13.68 | 2.81  | 73.64 | 90.13 | 9.87 | 53.60| 28.52| 25.08          | 21.29     | 5.96   |
| T6     | 13.69 | 2.79  | 74.54 | 90.02 | 9.98 | 53.10| 28.47| 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. T1=Control** substrate without herbs, Treatments=T1 to T6.

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) | NH3-N (mg/dl) |
|-----------|------|-----------------|-------------|-------------------|--------------|
| T1 (Control) | 6.69 | 63.89           | 32.70        | 31.20             | 24.94        |
| T2 (Tulsi; Haldi) | 6.50 | 82.88           | 26.88        | 56.00             | 17.64        |
| T3 (Haldi; Amla) | 6.47 | 70.48           | 24.40        | 46.08             | 20.44        |
| T4 (Amla; Neem) | 6.49 | 84.00           | 25.20        | 58.80             | 20.28        |
| T5 (Neem; Arni) | 6.47 | 70.32           | 23.68        | 46.64             | 19.88        |
| T6 (Arni; Tulsi) | 6.51 | 62.24           | 25.52        | 36.72             | 22.12        |
| T7 (Tulsi; Amla) | 6.49 | 59.52           | 21.92        | 37.60             | 15.80        |
| T8 (Amla; Neem) | 6.46 | 59.28           | 21.00        | 38.28             | 16.24        |
| T9 (Haldi; Neem) | 6.47 | 59.52           | 19.04        | 40.48             | 16.80        |
| T10 (Haldi; Arni) | 6.47 | 61.04           | 23.52        | 37.52             | 19.04        |
| T11 (Amla; Arni) | 6.44 | 64.96           | 21.56        | 43.40             | 17.36        |
| 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) | CH4 (mg)/g substrate DM | CH4 (mg)/g digested DM |
|--------------------|---------|---------------------|-------------------------|------------------------|
| T1 (Control)       | 57.55   | 122.35              | 51.41                   | 88.48                  |
| T2 (Tulsi; Haldi)  | 61.56   | 124.64              | 51.97                   | 84.80                  |
| T3 (Haldi; Amla)   | 62.01   | 119.00              | 46.75                   | 75.35                  |
| T4 (Amla; Neem)    | 59.47   | 117.24              | 35.51                   | 59.80                  |
| T5 (Neem; Arni)    | 55.71   | 125.96              | 36.93                   | 66.83                  |
| T6 (Arni; Tulsi)   | 58.26   | 114.56              | 47.63                   | 82.19                  |
| T7 (Tulsi; Amla)   | 62.09   | 114.32              | 45.72                   | 73.88                  |
| T8 (Amla; Neem)    | 62.09   | 115.04              | 44.76                   | 72.23                  |
| T9 (Haldi; Neem)   | 61.35   | 119.72              | 46.75                   | 76.36                  |
| T10 (Haldi; Arni)  | 60.73   | 130.08              | 53.01                   | 87.20                  |
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 |
|-----------------|----------------|-------------|----------------|--------------|-----------|
| T₁ (Control)    | 8.82           | 67.26       | 21.53          | 11.21        | 3.13      |
| T₂ (Tulsi: Haldi)| 9.36           | 69.63       | 23.12          | 8.38         | 3.03      |
| T₃ (Haldi: Amla)| 9.14           | 72.66       | 19.96          | 7.45         | 3.66      |
| T₄ (Amla: Neem) | 8.02           | 70.11       | 20.19          | 10.90        | 3.47      |
| T₅ (Neem: Arni) | 8.42           | 68.19       | 19.72          | 12.08        | 3.49      |
| T₆ (Arni:Tulsi) | 8.98           | 66.55       | 19.68          | 13.77        | 3.45      |
| T₇ (Tulsi: Amla)| 8.48           | 71.66       | 18.41          | 9.11         | 3.95      |
| T₈ (Tulsi: Neem)| 8.90           | 67.53       | 21.55          | 10.92        | 3.24      |
| T₉ (Haldi: Neem)| 8.80           | 71.62       | 19.09          | 9.29         | 3.76      |
| T₁₀ (Haldi:Arni)| 8.40           | 71.10       | 18.21          | 10.69        | 4.24      |
| T₁₁ (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     |