Indian Journal of Animal Sciences **88** (4): 443–452, April 2018/Article

Assessment of potential of some tannins and saponins containing herbs on digestibility of nutrients, fermentation kinetics and enteric methane production under different feeding systems: An *in vitro* study

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Received: 8 September 2017; Accepted: 13 December 2017

ABSTRACT

The study was undertaken to assess the effect of some tannin and saponin containing herbs, viz. *Anarchal*, *Amla*, *Baheda* and *Rohitaka* at four levels (0–3% of substrate) on digestibility of nutrients and enteric methane (CH_4) production under different feeding systems (roughage to concentrate (R:C) ratios; 80:20, 75:25, 70:30 and 65:35 on DM basis) *in vitro* in 4×4×4 factorial design. Irrespective of level of herbs and R:C ratios, the fermentation parameters and CH_4 mitigation potential varied significantly among different herbs. Digestibility of OM, TVFA and A:P ratio for *Anarchal* whereas NGP, ME availability, VFA efficiency and depression in CH_4 production as % of DM at $t_{1/2}$ for *Rohitaka* were observed to be highest. Irrespective of type of herbs and R:C ratios, the net gas production (NGP), digestibility of NDF, ME availability and TVFA increased linearly with increase in the level of supplementation of herbs and was significantly higher at 2 and 3% level; however, 2%, level had an edge over 3% level w.r.t. partitioning factor, VFA efficiency and A:P ratio. At $t_{1/2}$, CH_4 production as percent of NGP was observed to be lowest at 2 and 3% level. Irrespective of type of herb and their level, NGP, digestibility, ME availability, and TVFAs increased linearly with increase in concentrate in TMRs. At $t_{1/2}$, CH_4 production as percent of NGP was lowest at 65:35 in comparison to others. It was concluded that best results on digestibility of nutrients, VFA production, ME availability and in reducing CH_4 production was given by *Anarchal* and *Rohitaka* supplemented @ 2% of TMR with R:C ratio of 65:35 on DM basis.

Key words: Gas production, Herbs, *In-vitro* digestibility, Male buffaloes, Methane, Rumen fermentation, Saponins, Tannins

Agriculture contributes 10–12% towards global anthropogenic greenhouse gases (GHG) emissions, excluding land use change (Todd *et al.* 2011), particularly livestock is increasingly recognized as a potential victim of it (Casssandro *et al.* 2013). Livestock is assumed to be responsible for the largest part at nearly 80% of total agricultural GHG emissions. This is particularly due to methane (CH_4) emissions from enteric fermentation and manure handling (Mihina *et al.* 2012). Methane is the major GHG produced from enteric fermentation during the normal digestive process of ruminants (Moss *et al.* 2000). It is important to note that production of greenhouse gases from animals and their impact on climate changes are a major concern worldwide (Martin *et al.* 2010).

There are many rumen methane mitigation strategies developed by the different researchers such as probiotics, acetogens, bacteriocins, organic acids and herbal plant extracts. However, the natural products with high concentration of secondary metabolites appear to be good candidates for utilization as alternate feed additives (Teferedegne 2000) and seem to have a potential for rumen manipulation to reduce methane emission (Kamra *et al.* 2006). So, there is an increasing interest in exploiting natural plant extracts containing secondary metabolites such as tannins and saponins as feed additives to manipulate enteric fermentation and possibly reduce methane emission from livestock. But the effects of tannins and saponins on methanogenesis and rumen function are variable and depend upon source, type and level of tannin (Mueller-Harvey 2006, Patra *et al.* 2011) or saponin (Wina *et al.* 2005a). Even these compounds from different plants exhibit variable effects at the same concentration as evidenced by difference in magnitudes of net gas production (NGP) and digestibility (Makkar 2003, Guglielmelli *et al.* 2011). Therefore, the study was taken up to investigate the effect of different tannin and saponin containing herbs (*Anarchal*, *Amla*, *Baheda* and *Rohitaka*) at different levels (0%, 1%, 2%, 3%) on TMR with variable roughage to concentrate ratios on

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digestibility of nutrients, fermentation kinetics and methane mitigation potential in *in-vitro*.

MATERIALS AND METHODS

Four herbs, i.e. *Emblica officinalis* (Amla or Indian gooseberry), *Terminalia bellirica* (Baheda), *Punica granatum* (Anarchal or pomegranate) and *Tecomella undulate* (Rohitaka) were procured from Konark Herbals, Mumbai, Maharashtra and screened for bioactive components. Herbs (100 mg) in duplicate were extracted with 7.5 ml of distilled water. The contents were centrifuged at 3000 g for 10 min. The extraction was repeated again and the extracts were pooled. The aqueous extract obtained was used for the estimation of phenolics (Makkar *et al.* 1993), condensed tannins (Porter *et al.* 1986), flavonoids (Balabaa *et al.* 1974), saponins (Baccou *et al.* 1977) and 2, 2-diphenyl-1-picrylhydrazyl (Kumaran and Karakumaran 2007). Simultaneously, to 1 ml of aqueous extract, 1 ml of 20% TCA was added. The contents were kept overnight at 4°C, centrifuged and the supernatants used for the estimation of vitamin-C (Jagota and Dani 1982). The herbs were extracted with 80% methanol following the same procedure as mentioned above. The saponins were estimated from the menthanolic extract as well (Baccou *et al.* 1977).

The TMRs were formulated with roughage to concentrate ratio of 80:20, 75:25, 70:30 and 65:35 on DM basis. The roughage portion was made up of wheat straw and maize green fodder in 70:30 ratio, while the conventional concentrate was made up of maize 15, wheat 15, de-oiled mustard cake 15, mustard cake 10, soybean meal 10, rice bran 15, de-oiled rice bran 16, urea 1, salt 1 and mineral mixture 2% each. Therefore, in present study, the effect of four herbs at four levels (0–3% of substrate) on digestibility of nutrients, fermentation kinetics and enteric methane (CH_4) production under different feeding systems (roughage to concentrate (R:C) ratios; 80:20, 75:25, 70:30 and 65:35 on DM basis) *in vitro* in 4×4×4 factorial design was assessed.

Rumen fistulated male buffalo fed as ICAR (2013) was used as a donor for rumen liquor. The rumen contents were collected before feeding at 9:00 AM in a thermos flask flushed with CO_2 and maintained at 39°C. The rumen contents were blended for 2–3 min in a blender and strained through four-layers of muslin cloth. The solution, containing 960 ml distilled water, 0.16 ml micro-mineral solution, 660 ml bicarbonate buffer, 30 ml macro-mineral solution and 1.6 ml resazurine (0.1%) were mixed in a Woulff flask (3 litres capacity) with magnetic stirrer in a water bath at 39°C. The mixture was continuously flushed with CO_2. Then strained rumen liquor (SRL) was added to the buffer media in the ratio of 1:2. Different herbs were added to 100 ml calibrated glass syringes (Haberle Labortechnik, Germany) containing 375 mg complete feed (as percent over 375 mg) with buffered rumen fluid. Syringes in triplicate were incubated in a water bath at 39°C and swirled every 60 min over a 24 h incubation period. If the volume of gas in the syringe exceeded 70 ml after 8 h, the volume was recorded and the gas was expelled (Menke *et al.* 1979, Menke and Steingass 1988). After 24 h, the volume of gas produced in each syringe was recorded and the contents of syringes were transferred to spout-less beaker, boiled with neutral detergent solution for assessing the true OM and NDF digestibility. For determining *in vitro* true digestibility, the content of syringes was transferred to spoutless beaker by repeated washing with 20 ml neutral detergent solution. The flask content were refluxed for 1 h and filtered through pre weighed sintered crucibles (grade GI). The dry matter content of the residue was weighed and *in vitro* true digestibility of feeds was calculated (Van Soest and Robertson 1988) by using equation:

TOMD (%) = (Initial OM of feed taken for incubation-OM residue)/ Initial OM of feed taken for incubation ×100. The amount of gas produced was used to calculate the ME value the substrate by using the equation

ME (MJ/kg DM) = 1.24 + 0.146 G (ml/200 mg DM) + 0.007 CP + 0.0244 EE (Menke *et al.* 1979)

where G, Net gas production (ml/200mg DM); CP, Crude protein (g/g DM) and EE, Ether extract (g/g). Hydrogen recovery (%) was estimated as (4M+2P+2B)/(2A+P+4B) × 100, the ratio of hydrogen consumed *via* CH4/VFA was estimated as 4M/(2P+2B), where acetate (A), propionate (P), butyrate (B) and methane (M) production was expressed in mmol (Demeyer 1991). The fermentation efficiency was calculated on the basis of the equation worked out by Orskov (1975) and modified by Baran and Zitnan (2002)

i.e. FE = (0.622a + 1.092p + 1.56b) 100/ (a + p + 2b)

where, a, p and b express the concentrations (µmol) of acetic, propionic and butyric acid, respectively in the total concentration of VFAs produced. VFAs utilization index was calculated as per Orskov (1975) from equation

$$A + 2B + V/P+V$$

where A, P, B and V express the concentrations (µmol) of acetic, propionic, butyric and valeric acids respectively. The partitioning factor (PF) defined as the ratio of substrate truly degraded *in vitro* (mg) to the volume of gas (ml) produced by it (France *et al.* 1993).

To determine the rate of degradation (t½) of the substrate, the samples were incubated for 96 h, with recording of the gas volumes after 2, 4, 6, 8, 10, 12, 24, 36, 48, 72 and 96 h. The data was subjected to graph-pad prism programme to determine Y min (minimum amount of gas production), Y max (maximum potential of gas production), t½ and k (rate of gas production). The metabolizable energy (ME) was calculated using the equation suggested by Menke *et al.* (1979).

After the initial 96 h gas run, t½ was calculated and a second incubation with the diet as substrate was conducted to obtain degradability measures at substrate-specific times (i.e. t½ for each supplement). Collection and handling of ruminal fluid was same as that described for the 96 h incubations. The incubations were terminated at t½ and the volume of gas was recorded.

For methane estimation, 200 mg of substrate was incubated for 24 h with buffered rumen liquor and respective

tannin solution in duplicate. After the stipulated period, total gas production was measured. For methane estimation, representative gas was sampled from the headspace of syringe in an airtight syringe and injected into Netchrom 9100 gas chromatograph equipped with flame ionization detector (FID) and stainless steel column packed with Porapak-Q. The gas flow rates for hydrogen, nitrogen and air were 30, 15 and 300 ml/min, respectively. Temperature of injector oven, column oven and detector were 70, 50 and 100°C, respectively. A 50/50 mixture of CH_4 and CO_2 (Spancan; Spantech Products Ltd., England) was used as a standard. Methane was also estimated by using the equation based on VFA proportions (Wolin 1960).

After 24 h of incubation, 5 ml aliquot from each syringe was mixed with 1 ml of 25% meta-phosphoric and kept for 1 h at ambient temperature and centrifuged at 5500 rpm for 10 min. The supernatant was collected and stored at -20°C until analyzed. The VFAs were estimated using Netchrom 9100 gas chromatograph equipped with glass column and flame ionization detector (Cottyn and Boucque 1968). Temperature of injection port, column and detector was set at 250, 175 and 270°C, respectively. The flow rate of carrier gas (N) through the column was 15 ml/min; the flow rate of H_2 and air through FID was 30 and 300 ml/min, respectively. Sample (2 ml) was injected through the injection port using a Hamilton syringe. Individual VFA's of the samples were identified on the basis of their retention time and concentration (mmol) and calculated by comparing the retention time as well as the peak area of standards after deducting the corresponding blank values.

The finely ground samples of the substrate were analyzed for DM, CP, EE and total ash (AOAC 2000) and NDF (Robertson and Van Soest 1991). For ammonia estimation, 5 ml of supernatant was mixed with 1 N NaOH and steam distillated and the NH_3 evolved was collected in boric acid solution containing mixed indicator and titrated against 0.01N H_2SO_4 (AOAC 2000).The data were analyzed by using SPSS (2012) version 20.0 and the means were tested for the significant difference by using Tukey's b test.

RESULTS AND DISCUSSION

The chemical composition of different TMRs revealed that with increase in level of concentrate, the OM, EE and CP content increased whereas that of cellulose, neutral and acid detergent fibre (NDF and ADF) and acid detergent lignin (ADL) decreased (Table 1). Screening of herbs for bioactive components (Table 2) revealed that total phenols and true tannins were highest (P<0.001) in *Anarchal* followed by that in *Baheda*, *Amla and Rohitaka* whereas *Amla* was rich in total flavonoids and *Anarchal* in vitamin C in comparison to the other herbs. Extraction of herbs with water and methanol revealed that water and methanol soluble saponin content was highest (P<0.001) in *Baheda*, followed by that in *Anarchal*, *Amla* and *Rohitaka*.

Effect of different herbs on rumen fermentation and methane mitigation potential, irrespective of R:C ratio and level of supplementation of herb: The results (Table 3) revealed that irrespective of the level of supplementation and R:C ratio (P<0.001), NGP varied from from 142.74 (*Baheda*) to 153.02 ml/24h/g DM (*Rohitaka*). NGP from substrate was highest (P<0.001) when it was supplemented with *Rohitaka* whereas comparable between *Anarchal* and *Amla* supplemented group.The digestibility of nutrients (OM and NDF) varied significantly (P<0.001) amongst the tannin and saponin containing herbs being evaluated. Supplementation of diet with the *Baheda* (40.67%) showed higher (P<0.001) NDF digestibility, closely followed by *Anarchal* (40.22%). The digestibility of OM was significantly higher in *Anarchal* (58.82%) followed by

Table 1. Chemical composition of TMRs used for *in vitro* studies, (% DM basis)

Parameter	Roughage: Concentrate ratio			
	80:20	75:25	70:30	65:35
Total ash	12.22	11.22	10.95	10.78
Organic matter	87.78	88.78	89.05	89.22
Crude protein	14.65	17.9	18.55	21.45
Ether extract	2.05	2.25	2.85	3.35
Neutral detergent fibre	69.2	64.9	60.7	57.4
Acid detergent fibre	37.6	32.9	30.3	26.1
Acid detergent lignin	8.10	7.55	6.60	5.60
Hemicellulose	31.6	32.0	30.4	31.3
Cellulose	29.5	25.35	23.7	20.5

Table 2. Active components in herbal feed additives (% DM basis)

Parameter	Herbs				PSE	P-value
	Anarchal	*Amla*	*Baheda*	*Rohitaka*		
Tannins						
Total phenolics**	15.74^{c}	14.77^{c}	13.51^{b}	4.15^{a}	0.110	0.000
Non tannin phenols*	2.51^{b}	1.94^{a}	2.27^{ab}	2.10^{ab}	0.041	0.032
True tannins**	13.23^{c}	12.83^{c}	11.24^{b}	2.10^{a}	0.132	0.000
CT, Leucocyanidin**	0.08^{a}	0.03^{a}	0.25^{a}	0.75^{b}	0.014	0.000
Antioxidants						
Vitamin C**	0.61^{a}	3.69^{c}	7.30^{d}	1.30^{b}	0.185	0.000
Flavonoids**	5.66^{a}	8.75^{c}	7.54^{b}	9.20^{c}	0.218	0.000
DPPH AA%**	459.93^{b}	453.77^{b}	449.06^{b}	396.75^{a}	2.248	0.002
DPPH mg%**	2.30^{b}	2.27^{b}	2.24^{b}	2.00^{a}	0.011	0.002
Saponins						
Aq. Saponin**	2.59^{b}	1.93^{a}	4.65^{d}	4.05^{c}	0.049	0.000
Meth sapon**	2.44^{b}	1.48^{a}	3.31^{c}	1.65^{a}	0.028	0.000

CT, Condensed tannins; DPPH, 2, 2-diphenyl-1-picrylhydrazyl. Figures with different superscripts in a row differ significantly [**P<0.01, *P<0.05]

Table 3. Effect of supplementation of herbs (% DMB) on *in-vitro* gas production, digestibility of nutrients and ME availability

Parameter	Herbs used					Levels of herbs (%)					Roughage to Concentrate ratio				
	Anarchal	*Amla*	*Baheda*	*Rohitaka*	*PSE*	Control	1%	2%	3%	PSE	80:20	75:25	70:30	65:35	PSE
NGP (ml/ 24h/375mg)	55.09^{b}	56.32^{c}	53.53^{a}	57.38^{d}	0.002	55.08^{b}	54.11^{a}	56.18^{c}	57.06^{d}	0.002	52.98^{a}	55.08^{b}	56.83^{c}	57.42^{d}	0.002
NGP (ml/24h/g)	146.90^{b}	150.19^{c}	142.74^{a}	153.02^{d}	0.003	146.89^{b}	144.29^{a}	149.81^{c}	152.16^{d}	0.003	141.29^{a}	146.87^{b}	151.56^{c}	153.12^{d}	0.003
NDFD (%)	40.22^{bc}	39.68^{b}	40.67^{c}	38.24^{a}	0.274	37.85^{a}	39.37^{b}	39.47^{b}	39.12^{b}	0.274	38.16^{a}	38.15^{b}	38.89^{b}	39.37^{b}	0.274
TOMD (%)	58.82^{c}	54.66^{a}	55.14^{ab}	55.53^{b}	0.166	56.53^{b}	55.93^{a}	55.74^{a}	55.96^{a}	0.166	49.58^{a}	54.24^{b}	57.92^{c}	62.40^{d}	0.166
PF	2.48^{c}	2.41^{b}	2.53^{d}	2.36^{a}	0.007	2.47^{c}	2.51^{c}	2.43^{b}	2.37^{a}	0.007	2.56^{d}	2.46^{b}	2.40^{a}	2.37^{a}	0.007
ME (MJ/kg DM)	7.62^{a}	7.79^{b}	7.60^{a}	7.87^{b}	0.028	7.70^{b}	7.57^{a}	7.77^{bc}	7.84^{c}	0.028	7.17^{a}	7.64^{b}	7.89^{c}	8.18^{d}	0.028

NGP, Net gas production; D, Digestibility; NDF, Neutral detergent fibre; TOM, True organic matter; PF, Partitioning factor; ME, Metabolizable energy. Figures with different superscripts in a row differ significantly (P<0.01).

Table 4. Effect of supplementation of herbs (% DMB) on the *in-vitro* volatile fatty acid production (mM/dl)

Parameter	Herbs used					Levels of herbs (%)					Roughage to Concentrate ratio				
	Anarchal	*Amla*	*Baheda*	*Rohitaka*	PSE	Control	1%	2%	3%	PSE	80:20	75:25	70:30	65:35	PSE
TVFA	8.05^{d}	6.160^{a}	6.62^{b}	6.86^{c}	0.008	6.40^{a}	6.80^{b}	7.24^{c}	7.26^{c}	0.008	6.35^{a}	6.80^{b}	7.04^{c}	7.50^{d}	0.008
Acetate	5.44^{d}	4.080^{a}	4.39^{b}	4.53^{c}	0.005	4.20^{a}	4.53^{b}	4.84^{c}	4.88^{d}	0.005	4.26^{a}	4.52^{b}	4.68^{c}	4.97^{d}	0.005
Propionate	1.88^{d}	1.450^{a}	1.55^{b}	1.60^{c}	0.004	1.54^{a}	1.59^{b}	1.66^{c}	1.69^{d}	0.004	1.47^{a}	1.60^{b}	1.67^{c}	1.74^{d}	0.004
Isobutyrate	0.039^{d}	0.033^{a}	0.037^{b}	0.038^{c}	0.000	0.035^{a}	0.036^{a}	0.038^{b}	0.038^{b}	0.000	0.032^{a}	0.035^{b}	0.038^{c}	0.0410^{d}	0.000
Butyrate	0.587^{d}	0.493^{a}	0.528^{b}	0.571^{c}	0.001	0.515^{a}	0.532^{b}	0.573^{d}	0.559^{c}	0.001	0.51^{a}	0.54^{b}	0.55^{c}	0.585^{d}	0.001
Isovalerate	0.069^{b}	0.060^{a}	0.070^{b}	0.079^{c}	0.000	0.067^{a}	0.069^{b}	0.073^{c}	0.069^{b}	0.000	0.062^{a}	0.066^{b}	0.072^{c}	0.0787^{d}	0.000
Valerate	0.049^{b}	0.045^{a}	0.0487^{b}	0.0487^{b}	0.001	0.469^{a}	0.464^{a}	0.511^{b}	0.482^{b}	0.001	0.043^{a}	0.046^{b}	0.0487^{b}	0.055^{c}	0.001
A:P	2.89^{b}	2.82^{a}	2.83^{a}	2.83^{a}	0.007	2.77^{a}	2.88^{b}	2.94^{c}	2.88^{b}	0.007	2.89^{c}	2.82^{b}	2.81^{a}	2.82^{b}	0.007

TVFA, Total volatile fatty acids. Figures with different superscripts in a row differ significantly (P<0.01).

Table 5. Fermentation gases and hydrogen balance of TMRs

Parameter	Herbs used					Levels of herbs (%)					Roughage to Concentrate ratio				
	Anarchal	*Amla*	*Baheda*	*Rohitaka*	PSE	Control	1%	2%	3%	PSE	80:20	75:25	70:30	65:35	PSE
FCO_2 (mmoles)	50.53^{a}	51.03^{b}	50.96^{b}	51.25^{c}	0.023	30.79^{a}	31.37^{b}	31.57^{c}	31.66^{c}	0.001	31.78^{c}	31.48^{b}	31.31^{a}	31.31^{a}	0.037
FCH_4 (mmoles)	31.57^{b}	31.24^{a}	31.26^{a}	31.33^{a}	0.037	50.92^{a}	50.92^{a}	50.88^{a}	51.05^{b}	0.023	51.25	50.96	50.88	50.68	0.023
H- recovery	70.03^{a}	82.46^{d}	78.52^{c}	77.48^{b}	0.056	81.26^{c}	77.89^{b}	74.68^{a}	74.66^{a}	0.037	81.18^{d}	77.89^{b}	76.22^{c}	73.21^{a}	0.056
HC via CH_4/VFA	1.138^{a}	1.453^{d}	1.342^{c}	1.303^{b}	0.004	1.363^{c}	1.338^{b}	1.270^{a}	1.264^{a}	0.056	1.458^{d}	1.317^{c}	1.262^{b}	1.198^{a}	0.004
F efficiency	74.79^{a}	75.05^{b}	75.01^{b}	75.03^{b}	0.019	75.30^{d}	74.94^{c}	74.78^{a}	74.85^{b}	0.004	74.83^{a}	75.04^{c}	75.07^{c}	74.94^{b}	0.019
VFA efficiency	3.45^{ab}	3.43^{a}	3.43^{a}	3.47^{b}	0.008	3.33^{a}	3.45^{b}	3.53^{c}	3.47^{b}	0.008	3.51^{c}	3.42^{b}	3.39^{a}	3.45^{b}	0.008

F, Fermentative; H, Hydrogen; R, Recovery; C, Consumed; E, Efficiency; I, Index; CO_2, Carbon dioxide; CH_4, Methane; VFA, Volatile fatty acids. Figures with different superscripts in a row differ significantly (P<0.001).

Rohitaka (55.53%) and was observed lowest in *Amla* (54.66%).The partitioning factor (PF) varied from 2.36 (*Rohitaka*) to 2.53 (*Baheda*) on supplementation of herbs indicated significantly higher efficiency of producing microbial protein for *Baheda*. The availability of ME from complete diet was observed to vary from 7.62 (*Anarchal*) to 7.87 MJ/kg DM (*Rohitaka*) and was highest for *Amla* and *Rohitaka* supplemented group. The findings were in agreement with Hundal (2011), who reported varied NGP, digestibility of nutrients, PF and ME availability when wheat straw was supplemented with different tannin containing herbs, i.e. *Green tea, Kayphal, Babul chal* and sapon in containing herbs *Kulthi, Shatavary* or *Shikakai.* The difference in w.r.t. rumen parameters may be attributed to nature of plant secondary metabolites in different herbs.

VFA, CO_2 and methane are the universal end-product of anaerobic microbial fermentation of carbohydrates in the rumen (Camero and Franco 2001) and the values for total volatile fatty acid concentration (Table 4) obtained in this study varied (P<0.01) from 6.16 mM/dl (*Amla*) to 8.05 mM/dl (*Anarchal*). The acetate levels followed the same trend as that in TVFAs (maximum in *Anarchal* and minimum in *Amla*). The propionate levels varied (P<0.01) from 1.45 (substrate supplemented with *Amla*) to 1.88 mM/dl (substrate supplemented with *Anarchal*). The molar proportions of major volatile fatty acids were significantly different (P<0.001) amongst the diets supplemented with different tannin containing herbs. Acetate was significantly (P<0.001) higher in diet supplemented with *Anarchal* (67.54%) compared to other diets supplemented with *Amla* (66.23%), *Baheda* (66.23%) and *Rohitaka* (65.92%). Propionate was significantly higher (P<0.001) in diet supplemented with *Amla* (23.52%) closely followed by diet supplemented with *Baheda* (23.42%) and *Anar* (23.37%). Widiawati and Thalib (2009) reported that fermentation of high fibre content in a diet will result in higher proportion of acetate concentration and CO_2 released. The molar proportion of butyrate varied (P<0.05) from 7.28% (*Anarchal* supplemented diet) to 8.33% in diet supplemented with *Rohitaka*, irrespective of the level of supplementation and R:C ratio of TMR as substrate. The difference in molar proportion among different herbs may be due to the nature and type of plant secondary metabolites they contained. Researcher studied the effect of the pure tannins, viz. catechin hydrate, gallic acid, tannin acid and ellagic acid (Hundal *et al.* 2016) and that of pure saponins (Hundal *et al.* 2011) supplemented individually at varying levels of the substrate DM (wheat straw) by *in vitro* technique and found that irrespective of the level used, the TVFAs, acetate and propionate production varied significantly (P<0.01) among different types of tannins or saponins.

Methane gas is an important gas among gases produced by ruminants during fermentation which have negative correlation with feed energy and represents inefficient utilization of the feed (Varga and Kolver 1997). Metabolic hydrogen in the form of reduced protons can be used during the synthesis of volatile fatty acids (VFA) or incorporated into microbial organic matter. Acetate and butyrate promote methane production while propionate formation can be considered as a competitive pathway for hydrogen use in the rumen (Moss *et al.* 2000, Hegarty and Nolan 2007). Therefore, the proportions of acetate, butyrate and propionate determine the amounts of available H_2 in the rumen to be used by methanogens. By this relation, CH_4 emission was calculated stoichiometrically from the respective VFA. Herbs containing tannins and saponins were evaluated for their anti-methanogenic properties and the data (Table 5) revealed that supplementation of diet with *Anarchal* showed the higher (P<0.001) methane production in comparison to other herbs evaluated. The type of tannin and saponin present in herb could be the reason for different response. Fermentation efficiency based on VFA production is very useful for analyzing the effect of some feed additives on ruminal fluid fermentation via microbial metabolism modulation. The VFA efficiency varied between 3.43 (*Amla or Baheda*) to 3.47 (*Rohitaka*). The VFA efficiency of *Anarchal* closely followed *Rohitaka*, indicated the potential of *Rohitaka* and *Anarchal* as a feed supplement.

The fermentation kinetics presented in Table 6 indicated that y max (maximum potential of gas production) varied (P<0.01) amongst the herbs evaluated (maximum for *Baheda* and minimum for *Rohitaka*). However, the maximum rate of degradation (k), t½ (time taken for reaching half asymptote) were not affected by type of supplementation of herb.The effect of supplementation of herbs on methane production from fermentation of TMRs, irrespective of their level and R:C ratio is given in Table 7. The proportion of methane at $t_{1/2}$ of net gas produced was lowest (P<0.001) in the diet supplemented with *Baheda* and *Amla*. Methane production expressed in volume (methane production ml/100mg DM and ml/100mg IVDMD) also varied significantly (P<0.05) amongst the different herbs. Different sources of tannins and saponins have been shown to have different impacts on CH_4 production, likely due to tannin composition, type and concentration. Tannins reduce methane production from ruminants either by reducing fibre digestion, which in turn decreases H_2 production and/or by inhibition of the growth of methanogens (Tavendale *et al.* 2005).

The data clearly revealed that amongst the four herbs evaluated, *Rohitaka* due to more NGP, highest ME availability, better VFA profile, improved OM digestibility and lowest methane production was selected for *in vivo* evaluation. The potentail of *Anarchal* and *Amla* were comparable but former had an edge over *Amla* w.r.t. NDF and OM digestibility, PF, TVFA and A:P ratio.

Effect of level of supplementation of herbs on rumen fermentation and methane mitigation potential, irrespective of R:C ratio and type of supplementation of herb: The tannin and saponin containing herbs screened at four levels, viz. 0, 1, 2, 3% of substrate and it's the effect on rumen fermentation, irrespective of R:C ratio and type of supplementation of herbs is presented in Table 3. NGP was

Table 6. Effect of herbs (% DMB) on fermentation kinetics of TMR in *in-vitro*

Parameter	Herbs used					Levels of herbs (%)				Roughage to Concentrate ratio			
	Anarchal	*Amla*	*Baheda*	*Rohitaka*	PSE	Control	1%	2%	3%	PSE	80:20	75:25	70:30	65:35	PSE
Lag time (h)	-2.85^{ab}	-2.26^{ab}	-2.00^{b}	-3.20^{a}	0.114	-2.73^{a}	-2.67^{a}	-2.42^{a}	-2.49^{a}	0.136	-2.81^{a}	-2.76^{a}	-2.50^{a}	-2.25^{a}	0.134
Ymax (ml)	46.79^{b}	47.79^{bc}	49.29^{c}	42.08^{a}	0.277	44.11^{a}	44.33^{a}	46.78^{ab}	48.74^{b}	0.385	45.73^{a}	46.08^{a}	47.42^{a}	46.73^{a}	0.427
Rate of degradation, per hour	0.044^{a}	0.047^{a}	0.044^{a}	0.047^{a}	0.000	0.044^{a}	0.047^{a}	0.047^{a}	0.045^{a}	0.001	0.043^{a}	0.044^{ab}	0.046^{bc}	0.048^{c}	0.000
Ymin (ml)	23.05^{a}	21.36^{a}	21.33^{a}	22.98^{a}	0.245	21.91^{a}	21.43^{a}	21.54^{a}	22.84^{a}	0.288	23.59^{c}	22.75^{c}	21.66^{ab}	20.72^{a}	0.268
t½ (h)	15.98^{a}	14.81^{a}	14.79^{a}	15.93^{a}	0.170	14.88^{a}	14.86^{a}	14.93^{a}	15.83^{a}	0.200	16.35^{c}	15.77^{bc}	15.02^{ab}	14.36^{a}	0.186

Ymax, Maximum potential of gas production; Ymin, Minimum potential of gas production; t½, Time taken for reaching half asymptote. Figures with different superscripts in a row differ significantly (P<0.01).

Table 7. Effect of level of tannins (% DMB) on methane production at $t_{1/2}$

Parameter	Herbs used					Levels of herbs (%)					Roughage to Concentrate ratio				
	Anarchal	*Amla*	*Baheda*	*Rohitaka*	PSE	Control	1%	2%	3%	PSE	80:20	75:25	70:30	65:35	PSE
NGP (ml/g DM $t^{1/2}$)	75.57^{a}	78.15^{b}	79.09^{b}	78.23^{b}	0.462	77.54^{c}	76.10^{b}	74.35^{a}	82.04^{d}	0.462	68.73^{a}	74.23^{b}	80.48^{c}	86.60^{d}	0.462
CH_4 (% of NGP)	16.65^{c}	15.41^{b}	13.16^{a}	13.06^{a}	0.265	16.14^{c}	15.10^{b}	13.57^{a}	12.81^{a}	0.265	15.96^{d}	14.85^{c}	13.90^{b}	12.91^{a}	0.265
CH_4 (ml/100mg DM)	1.26^{c}	1.21^{b}	1.06^{a}	1.03^{a}	0.023	1.26^{c}	1.16^{b}	1.01^{a}	1.06^{a}	0.023	1.38^{d}	1.19^{c}	1.03^{b}	0.88^{a}	0.023
CH_4 (ml/100mg DMD)	2.09^{cd}	2.21^{d}	1.92^{bc}	1.70^{a}	0.014	2.21^{c}	2.00^{b}	1.76^{a}	1.84^{ab}	0.014	2.39^{c}	2.00^{b}	1.83^{b}	1.58^{a}	0.014

NGP, Net Gas Production; CH_4, Methane. Figures with different superscripts in a row difference significantly (P<0.01).

significantly (P<0.001) higher at 2 and 3% level of supplementation as compared to control and 1% level. Digestibility of NDF and organic matter was comparable at all three levels and higher (P<0.01) in comparison to control group. These findings were in line with Hundal (2011), who reported that supplementation of tannin containing herbs to wheat straw based diet improved NGP by 2.26% at 2% level and 1.27% at 3% level of supplementation in comparison to control group whereas saponin containing herb increased NGP by 4.88% (P>0.05) when supplemented @ 2% of the substrate. Partitioning factor (PF) varied from 2.37 to 2.51 and was significantly higher (P<0.001) at 1% level of supplementation, indicating more efficiency of microbial protein synthesis. PF was also significantly higher (P<0.001) at 2% level as compared to 3% level of herb supplementation. The availability of metabolizable energy (MJ/kg DM) followed the trend of NGP, varied from 7.57 (1% level) to 7.84 (3% level of supplementation) and was observed 7.70 in control (un-supplemented diet). The increase in level of supplementation of herbs resulted in increased availability of ME.

The VFAs was a major energy source for ruminants. The data on fermentation pattern revealed that total volatile fatty acids were higher (P<0.001) when the diet was supplemented at 2 and 3% levels, irrespective of the type of herb and the type of diet used. The levels of acetate and propionate also increased with the increased level of supplementation but level of butyrate was observed maximum at 2% (0.573). Ningrat *et al.* (2016) reported that supplementation of tannin source different levels showed increasing propionate production compared to control. Formation of higher acetate level may be due to the reason that the bacteria which produce acetate better evolve with doses and source herbs that were given. Hundal *et al.* (2016) studied the effect of the pure tannins, viz. catechin hydrate, gallic acid, tannin acid and ellagic acid supplemented individually at 1 to 5% levels of the substrate DM (wheat straw) by *in vitro* technique and found that irrespective of type of tannins, the TVFAs, acetate and propionate production in

tannin supplemented groups was lowest (P<0.01) at 1% level as compared to the control whereas irrespective of the level of tannins, the TVFAs, acetate and propionate production was higher (P<0.01), while A:P ratio was lowest (P<0.01) from catechin hydrate as compared to other tannin supplemented groups.

The fermentative methane emission calculated stoichiometrically from the respective VFA was observed to be lowest (P<0.05) when the diet was supplemented at 1% on DM basis, irrespective of type of herb and the diet used (Table 5). The level of supplementation of herbs significantly (P<0.001) influenced H recovery and fermentation efficiency, the later was lowest at 2% level whereas VFA efficiency was significantly higher (P<0.001) at same level of supplementation of herbs in the present study.

The fermentation kinetics presented in Table 6 indicated that y max (maximum potential of gas production) increased (P<0.01) linearly with the increase in level of supplementation, irrespective of type of herb and that of diet, which was also depicted by decreased lag phase. However, the maximum rate of degradation (k), t½ (time taken for reaching half asymptote) were not affected by the level of supplementation of herb. The effect of level of supplementation of tannin containing herbs was quite visible, the methane as percent of total NGP decreased linearly with increase in level of supplementation of tannin containing herbs (Table 7). Methane production expressed in volume (methane production ml/100mg DM and ml/100mg IVDMD) also decreased linearly (P<0.05) with increase in level of herbs. The decrease in fermentation and digestibility with increasing levels of tannin might be due to their continuous effect during fermentation and digestibility. Tan *et al.* (2011) and Gemedaand Hassen (2015) had reported similar effects with increasing tannin level supplementation on methane production. The absence of tannin had increased methane production that was depressed by tannins.

Analysis revealed that amongst the four level evaluated, herbs at 3% level of supplementation had better NGP, high NDF digestibility, more ME availability, improved VFA production and reduced methane production. However, fermentation parameters at 2 and 3% level were comparable; but level of supplementation of herbs beyond 2% had no additional benefit.

Effect of TMR varying in R:C ratio on rumen fermentation and methane mitigation potential, irrespective of type and level of supplementation of herb: The results revealed that NGP observed using roughage to concentrate ratios 80:20, 70:30, 75:25 and 65:35 was 141.29 ml/g, 146.87 ml/g, 151.56 ml/g and 153.12 ml/g, respectively (Table 3). With increase in level of concentrate in TMRs, the net gas production increased linearly (P<0.001). The digestibility of organic matter and availability of ME followed the trend of NGP and increased (P<0.001) with increase in level of concentrate in TMRs. However, the partitioning factor decreased (P<0.001) with increase in level of concentrate in TMRs. A high NGP indicates greater fermentation to support rapid rumen microbial growth. Total and individual VFA's were observed (Table 4) minimum at

Table 8. Correlation of active components in herbal feed additives with *in vitro* gas production and related parameters

Parameter	NGP (ml/24h/g)	NDFD (%)	TOMD (%)	PF	ME (MJ/kg DM)	TVFA (mM/dl)	A:P	CH_4 (%)	Rate of degradation
Type of herb used irrespective of roughage to concentrate ratio and level of supplementation of herbs									
True tannins	-0.629	0.962	0.269	0.671	-0.706	0.127	0.344	0.697	-0.526
CT, Leucocyanidin	0.502	-0.913	-0.227	-0.550	0.591	-0.069	-0.297	-0.745	0.395
Vitamin C	-0.679	0.481	-0.619	-0.591	-0.406	-0.605	-0.557	-0.494	-0.279
Flavonoids	0.612	-0.637	-0.863	-0.700	0.836	-0.823	-0.903	-0.646	0.867
DPPH	-0.644	0.964	0.318	0.694	-0.738	0.180	0.393	0.707	-0.568
Aq. Saponins	-0.332	-0.274	-0.235	0.261	-0.150	-0.069	-0.241	0.851	-0.289
Meth saponins	-0.940	0.569	0.198	0.925	-0.878	0.256	0.256	-0.190	-0.902
Level of herbs irrespective of roughage to concentrate ratio and type of supplementation of herbs									
True tannins	-0.827	-0.260	-0.984*	0.894	-0.760	-0.669	-0.259	0.813	0.846
CT, Leucocyanidin	0.903	0.243	0.955*	-0.952	0.849	0.704	0.287	-0.843	-0.830
Vitamin C	0.018	0.716	-0.442	0.089	-0.043	0.527	0.829	-0.335	0.709
Flavonoids	0.259	0.803	0.582	-0.328	0.101	0.716	0.651	0.713	-0.223
DPPH	-0.803	-0.304	-0.980	0.873	-0.728	-0.686	-0.290	0.823	0.824
Aq. Saponins	0.882	0.365	0.385	-0.812	0.866	0.789	0.594	-0.796	-0.245
Meth saponins	0.244	-0.044	-0.457	0.108	0.320	0.126	0.231	-0.035	0.399
Roughage to concentrate ratio irrespective of type and level of supplementation of herbs									
True tannins	0.727	-0.896	-0.863	0.723	-0.807	-0.877	-0.259	0.852	-0.907
CT, Leucocyanidin	0.748	0.943	0.870	-0.733	0.808	0.873	0.287	-0.862	0.929
Vitamin C	0.449	0.119	0.233	-0.438	0.312	0.186	0.829	-0.257	0.153
Flavonoids	0.773	0.537	0.794	-0.817	0.837	0.829	0.651	-0.786	0.692
DPPH	-0.746	-0.890	-0.878	0.746	-0.828	-0.895	-0.290	0.867	-0.912
Aq. Saponins	0.743	0.842	0.697	-0.692	0.664	0.643	0.594	-0.713	0.762
Meth saponins	0.024	0.076	-0.122	0.037	-0.129	-0.196	0.231	0.096	-0.057

Table 9. Interaction between herb, level and R:C ratio w.r.t. fermentation parameters

Parameter	Herb* Level	Level* R:C ratio	Herb* R:C ratio	Level* R:C ratio
NGP (ml/24h/375 mg)	**	**	**	**
NGP (ml/24h/g)	**	**	**	**
NDFD (%)	**	**	NS	NS
TOMD (%)	*	**	NS	NS
PF	**	**	**	**
ME (MJ/kg DM)	*	NS	*	NS
TVFA (mM/dl)	**	**	**	**
Acetate (mM/dl)	**	**	**	**
Propionate (mM/dl)	**	**	**	**
Isobutyrate (mM/dl)	**	**	**	**
Butyrate (mM/dl)	**	**	**	**
Isovalerate (mM/dl)	**	**	**	**
Valerate (mM/dl)	**	**	**	**
A:P	**	**	**	**
FCO_2 (mmoles)	**	**	**	**
FCH_4 (mmoles)	**	**	**	**
H-recovery	**	**	**	**
HC via CH_4/VFA	**	**	**	**
F efficiency	**	**	**	**
VFA efficiency	**	**	**	**
CH_4 (%)	**	**	**	**

R:C, Roughage to concentrate; NS, Non-significant. **P<0.01, *P<0.05.

lower level of concentrate used (80:20) and were maximum at higher level of concentrate (65:30). Thus, with increase in level of concentrate in TMRs, total and individual VFAs increased linearly (P<0.01). The levels of acetate, butyrate and propionate showed significant difference by change in R:C ratios in TMR. However, the ratio of acetate to propionate decreased with increase in concentrate level in TMRs and was reported maximum at 80:20 R:C ratio. Tannins might increase the propionate production as a result of rechanneling of hydrogen from methane to propionate and decrease the A:P ratio, which is nutritionally beneficial for ruminants.

The fermentation gases and hydrogen balance of TMRs, irrespective of type and level of herb used is given in Table 5. It is expected that increased proportion of concentrates in the diet reduces CH_4 production in ruminants by promoting propionate fermentation in the rumen. The fermentative methane, hydrogen recovery, hydrogen consumption via VFA, fermentation efficiency and VFA efficiency differed significantly amongst the diets prepared using different roughage to concentrate ratios but no such significant difference was observed in FCO_2. In *in vitro* studies, the recovery rate of metabolic H_2 varies between 78 and 96% (Demeyer 1991).

The fermentation kinetics indicated that with increase in level of concentrate in the diet, irrespective of type and level of supplementation of herbs, the lag period for fermentation of diet decreased (P<0.01) linearly as presented in Table 6. With increase in concentrate in diet, the cell soluble increased, which gets fermented at a faster rate, as indicated by rate of degradation (k), which increased linearly (P<0.01) with increase in concentrate in the diet. The y min (minimum potential of gas production) followed the reverse trend (P<0.01) and decreased linearly with increase in the concentrate in diet. However, the maximum, t½ (time taken for reaching half asymptote) decreased (P<0.01) with increase in level of concentrate in the diet, irrespective of type of herbs and their level of supplementation of herb.

The results revealed that methane as percent of total NGP increased linearly with increase in level of concentrate in TMR (Table 7). Methane production expressed in volume (methane production ml/100mg DM and ml/100mg IVDMD) also decreased linearly (P<0.05) with increase in concentrate proportion in TMR. The decrease in methane is due to linear increase in propionate and decrease in acetate production with increase in R:C ratio in TMR, which in turn enhanced hydrogen recovery as compared to high fibre TMR and less H_2 is available for methane formation. Several researches showed that the effectiveness of tannins on methanogenesis was different depending on the composition of diets. Feeding of high tannin (CT) forage *Lespedeza striata* to Boer wether goats at increasing levels (0, 33, 67 and 100% inclusion), decreased methane (P<0.05) emission quadratically (26.2, 17.6, 13.8 and 10.9 l/d) along with decreased (P<0.05) methanogen and protozoa population with stable microbial population (Animut *et al.* 2008). In a meta-analysis published by Jayanegara *et al.* (2012), it was conclusively demonstrated that a clear relationship exist between the concentration of CT in the ration and the reduction in the synthesis of enteric methane.

Roughage to concentrate ratio is a tool to manipulate ruminal microbial ecosystem to alter nutrient digestibility, fermentation efficiency, methane emission and nitrogen excretion. The types of diet are potential modifiers of ruminal fermentation and may offer a strategy to reduce protozoal and methanogen populations, thus improving the efficiency of feed utilization in the ruminants (Anantasook and Wanapat 2012).As far as the effect of TMR varying in R:C ratio on rumen fermentation and methane mitigation potential, irrespective of type and level of supplementation of herb is concerned, TMR with roughage to concentrate ratio 65:35 had better NGP, high NDF digestibility, more ME availability, improved VFA production and reduced methane production. Hence, TMR with roughage to concentrate ratio 65:35 was selected for *in vivo* studies.

Interactions between herb, level and R:C ratio w.r.t. fermentation parameters: Interactions between herb*level, herb*R:C ratio, level*R:C ratio and herb*level*R:C ratio w.r.t. fermentation parameters are given in Table 9. It was found that interactions between type of herb used with its level and type of herb used with R:C ratio influenced all fermentation parameters (P<0.01) except ME availability. On the other hand, interactions between level of herb used and R:C ratio didn't influence NDFD and TOMD, however it significantly influenced ME availability (P<0.05) in

addition to other fermentation parameters (P<0.01). Overall the interaction among type of herb used with its level and R:C ratio (herb*level*R:C ratio) influenced all fermentation parameters (P<0.01) except ME availability, NDFD and TOMD. The results collectively indicated that interaction between the factors and most of the fermentation parameters were highly significant.

Correlation of active components in herbal feed additives with fermentation parameters: Correlation of active components in herbal feed additives with fermentation parameters is given in Table 8. In general fermentation parameters were negatively or positively correlated with presence of different active components within herbs, i.e. tannins, saponins and antioxidants but the correlation was statistically non-significant.

It was concluded that best response with respect to digestibility of nutrients, methane production, VFA production and ME availability from TMRs with different roughage to concentrate ratios was given by *Anarchal* and *Rohitaka* supplemented @ 2% of TMR with R:C ratio of 65:35 on DM basis.

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