Journal of Animal and Feed Sciences, 28, 2019, 110–119 https://doi.org/10.22358/jafs/109237/2019
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

Effect of *Punica granatum* and *Tecomella undulata* supplementation on nutrient utilization, enteric methane emission and growth performance of Murrah male buffaloes

J.S. Hundal[1,3], I. Singh[1], M. Wadhwa[1], C. Singh[2], C. Uppal[1] and G. Kaur[1]

Guru Angad Dev Veterinary and Animal Sciences University,
[1] Department of Animal Nutrition, [2] Department of Veterinary Physiology and Biochemistry, Ludhiana, Punjab, India-141004

KEY WORDS: buffaloes, growth performance, herb extracts, methane, nutrient utilization

Received: 1 August 2018
Revised: 12 December 2018
Accepted: 21 May 2019

[3] Corresponding author:
e-mail: drjshundal@gmail.com

ABSTRACT. To assess the effect of herbal extracts on nutrient utilization, enteric methane emission and growth performance, 15 growing Murrah male buffaloes were divided into 3 groups and offered control diet (total mixed ration; roughage:concentrate ratio 65:35), control diet supplemented with *Punica granatum* or *Tecomella undulata* extract at 2% of dry matter intake (DMI) for 90 days. The supplementation of herbal extracts did not alter DMI and nutrient digestibility, however the tendency ($P = 0.058$) to higher nitrogen retention was observed in groups fed diets supplemented with *P. granatum* (72.9 g/day) and *T. undulata* extract (70.6 g/day) in comparison to control (64.3 g/day) group. No negative impact was seen as indicated by blood profile, which was within the physiological range. Supplementation of *P. granatum* or *T. undulata* extracts resulted in decrease in methane emission (g/kg average daily gain (ADG)) by 46 and 42%, respectively in comparison to control diet. The impact of decrease in methane emission and higher gross energy (GE) intake was visible in the daily body weight gain/day: the animals fed *P. granatum* and *T. undulata* extracts gained ($P < 0.001$) 28 and 21% more weight, respectively, in comparison to animals fed control diet. Therefore, the study established that the supplementation of diet with herbal extracts can help in ameliorating enteric methane production, thereby improving the growth performance in ruminants.

Introduction

Indian livestock produces 15% of total global enteric methane production with bovines accounted for 85% of total emission followed by other livestock species (Patra, 2014). Emissions of greenhouse gases from livestock-related anthropogenic activities trigger research towards methane mitigation in ruminants. Moreover, ruminal methane production is connected with the loss of carbon and consequently the loss of energy (Bell et al., 2011); the efforts have been made to inhibit methanogenesis and to re-channel hydrogen to improve feed conversion ratio. Existing methane mitigation strategies for ruminants comprise additions of ionophores, fats, defaunating agents, organic acids, antibiotics and anti-methanogens, but most of these feed additives have certain side effects on rumen ecosystem or animal health when long-term used. Therefore, alternative feed additives are needed. Plants consisting of plant secondary metabolites (PSMs) appear to have great potential to reduce enteric methane emission, to

control environmental pollution and to improve productive performance in animals (Teferedegne, 2000) by altering rumen fermentation, nutrient utilization or enhancing supply of polyunsaturated fatty acids to the blood for productive purposes (Szczechowiak et al., 2018; Yanza et al., 2018). Most researchers exploited pure PSMs (saponins, tannins, essential oils and flavonoids) or herbs rich in PSMs as feed additives in *in vitro* studies (Bakshi et al., 2004; Cieslak et al., 2012; Singh et al., 2018) and a few assessed its potential to alter enteric methane production *in vivo* (Qiao et al., 2013; Wanapat et al., 2013; Hundal et al., 2016). However, the effect of using herbs rich in tannins, saponins and flavonoids varies and depend on concentration, level and source of active compound.

Out of 199.8 mln buffalo population in the world, 97.04% (193.8 mln) and 57.8% (115.5 mln) are located in Asia and India, respectively, so it is necessary to find locally available herbal feed additives to tackle enteric methane emission. Moreover, potential of many herbs rich in PSMs as methane mitigator still needs to be exploited in India. *Punica granatum* (a native from the Himalayas in India and Iran) is a high value fruit rich in phenolic compounds, flavonoids, proanthocyanidin and antioxidant properties (Dhir and Shekhawat, 2012). Its peel, covering around 60% of fruit, is a rich source of ellagitannins, flavonoids and proanthocyanidin (Mirdehghan and Rahemi, 2007). *Tecomella undulata,* a deciduous shrub has tremendous medicinal and therapeutic potential (Rohilla and Garg, 2014), which is attributed to the presence of secondary metabolites (phytosterols, glycosides, tannins, phenolic compounds, flavonoids, saponins) in its heartwood, bark and leaf (Dhir and Shekhawat, 2012).

Therefore, due to the presence of secondary metabolites (tannins, saponins and flavonoids), the effect of supplementing *P. granatum* and *T. undulata* dry extracts on methane mitigation and productive performance, especially in *Bubalus bubalis* under Indian conditions was planned. Moreover, on the basis of our previous *in vitro* study (Singh et al., 2018), it can be hypothesized that dietary inclusion of *P. granatum* and *T. undulata* extracts may modulate nutrient digestibility, enteric methanogenesis and consequently body weight gain *in vivo*.

Material and methods

The study was conducted at Guru Angad Dev Veterinary and Animal Science University, Ludhiana-141 004, Punjab (India). All experimental protocols were approved (GADVASU/2015/IAEC/31/03) and compliant with the guidelines established by Institutional Animal Ethics Committee constituted (IAEC) under CPCSEA, New Delhi.

Herb extracts analysis

Dry herbal extracts of *P. granatum* fruit peel and *T. undulata* bark were procured from Konark Herbals, Mumbai, Maharashtra (India) and screened in triplicate for phenolics (Makkar et al., 1993), flavonoids (Balbaa et al., 1974) and saponins (Baccou et al., 1977).

Animals and feeding

Fifteen growing male buffaloes (average age 16.5 ± 0.13 months and average body weight 286.7 ± 6.75 kg) were assigned by completely randomized design to three groups of five animals each. The animals were offered control diet (total mixed ration, TMR) or control diet supplemented with *P. granatum* (pomegranate) or *T. undulata* (rohitaka) dry extracts at 2% of dry matter (DM) intake (DMI) for 90 days. Animals were fed TMR (ICAR-NIANP, 2013) with roughage:concentrate ratio of 65:35 and within roughage, the proportion of green and wheat straw was 30:70. The ingredients and chemical composition of TMR are presented in Table 1. The animals were housed in a concrete shed and were stall fed individually at 9:00 daily. The animals were weighed for 3 consecutive days every fortnight on digital electronic weighing balance (Swift, New Delhi, India) and the feeding schedule was revised accordingly. The animals were watered twice a day and were taken out in the yard for 1-h exercise daily.

Table 1. Chemical composition of total mixed ration (TMR), g/kg dry matter (DM)

Indices	Concentrate mixture[1]	Green fodder[1]	Wheat straw[1]	TMR[2,3]
Total ash	80	88	62	74
Organic matter	920	912	938	927
Crude protein	248	147	395	133
Ether extract	44	16	9	22.6
Cellulose	73	360	389	273
Neutral detergent fibre	363	702	856	654
Acid detergent fibre	151	436	562	394
Hemicellulose	212	266	291	259
Acid detergent lignin	40	59	79	62
Gross energy[1], MJ/kg DM	–	–	–	16

[1] analysed values; [2] calculated values; [3] TMR was prepared by mixing concentrate mixture (35%), green (19.5%) and wheat straw (45.5%)

Digestion-cum-metabolism trial

Before the end of growth experiment (90 days), a 7-day digestion-cum-metabolism trial was conducted in specially designed metabolic cages where urine and faeces were automatically collected in separate containers. The faeces, urine and orts (if any) were weighed daily at 9.00 before feeding. On the last day of the trial, blood samples were taken from each animal (4 h post feeding) by puncturing jugular vein.

Analysis

The dried and finely grounded feed, herb extracts, orts and faecal samples were analysed (AOAC International, 2007) for DM (method no. 934.01), total ash (method no. 942.05), ether extract (method no. 973.18) and nitrogen (method no. 976.05) concentration. Subtracting ash content from DM concentration is organic matter. The cellulose content was determined by Crampton and Maynard (1938) method and cell wall constituents were estimated by Robertson and Van Soest (1981) method. The pooled urine samples were analysed for nitrogen (AOAC International, 2007; method no. 976.05). After collection of blood samples, the serum was separated and stored at 0 °C until analysed for different biochemical constituents (total protein, albumin, globulin, cholesterol, triglycerides, glucose, blood urea nitrogen (BUN), urea, uric acid, serum glutamic oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT)) with Semi-automatic Analyzer (Model-Erbachem 5×, Transasia, Mumbai, India) by using Erba's diagnostic kits. The gross energy (GE) content of TMR and herb extracts were estimated by using Bomb calorimeter (Toshniwal Bros, Delhi, India). The energy content of methane was taken as 55.8 KJ/g (Brouwer, 1965).

Quantification of methane emission by SF_6 tracer technique

The enteric methane (CH_4) production in the rumen of growing male buffaloes was quantified by using SF_6 (sulphur hexafluoride) tracer technique (Johnson et al., 1994). The methane was estimated from day 45 to 74 during the experimental period and in total 30 observations (min. 5 and max. 7) from each animal were taken into account to calculate average methane emission value. The gas chromatograph (Ultima-2100, Netel (India) Ltd., Navi Mumbai, India) fitted with electron capture detector (ECD) and flame ionization detector (FID) was used to analyse SF_6 and CH_4 in breath samples of animals. Methane emission rate was calculated as the product of the permeation tube emission rate and the ratio of CH_4 to SF_6 concentration in the sample. The gas chromatograph was fitted with a 3.3 m molecular sieve 5 A column with inner diameter 0.32 mm for SF_6 and a 1.5 m Porapak Q stainless steel column with inner diameter 2 mm for CH_4. The injector, detector and oven temperature was maintained at 40, 220 and 50 °C for SF_6 and 40, 100 and 52 °C for CH_4 estimation, respectively. Nitrogen was used as a carrier gas, with flow rate of 30 ml/min both for SF_6 and CH_4 estimations. Prepared standards were used to standardize the gas chromatograph for SF_6 (39.2 ppt and 101.7 ppt) and CH_4 (10.4 ppm and 101.9 ppm, Scott-Marrin Inc., Riverside, CA, USA). Background methane [$(CH_4)b$] was subtracted from methane concentration in the canister [$(CH_4)y$]. The concentration of CH_4 was then calculated by using the equation of Johnson et al. (1994):

$$Q_{CH4} = Q_{SF6} \times (CH_4)y - (CH_4)b / (SF_6)$$

where: Q_{CH4} – CH_4 emission rate (g/min), Q_{SF6} – known release rate of SF_6 from permeation tube (g/min), $(CH_4)b$ – background CH_4, $(CH_4)y$ – CH_4 in sample in canister (μg/m^3), SF_6 – SF_6 concentration of collected sample in canister (μg/m^3).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SPSS (2012) software version 20.0 (IBM Corporation, Armonk, NY, USA) with the model:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij},$$

where: Y_{ij} – each observation (methane production, average daily gain etc.), μ – total mean, T_i – effect of i^{th} treatment (i = control, *P. granatum* extract and *T. undulata* extract) and ε_{ij} – residual error.

The means were tested for the significant difference ($P < 0.05$) by using Tukey's post hoc comparison test.

Results

Chemical composition of herbal extracts

The chemical analysis of herbal extracts (Table 2) revealed that crude protein and ether extract contents in *P. granatum* extract were 67.6 and 48 g/kg DM and in *T. undulata* extract were 18.5 and 8 g/kg DM, respectively. *T. undulata* extract had significantly ($P < 0.001$) lower organic matter, crude protein, ether extract and neutral detergent fibre contents as compared to *P. granatum*. However, higher ($P < 0.001$)

Table 2. Chemical composition and active components in herbal extracts, g/kg dry matter (DM) of herbal extract

Indices	*Punica granatum*	*Tecomella undulata*	PSE	*P*-value
Chemical composition				
total ash	1.5[a]	15.2[b]	0.036	0.000
organic matter	998.5[b]	984.8[a]	0.29	0.000
crude protein	67.6[b]	18.5[a]	1.01	0.000
ether extract	48.0[b]	8.0[a]	0.87	0.000
neutral detergent fibre	6.7[b]	4.0[a]	0.06	0.001
acid detergent fibre	1.5[a]	2.5[b]	0.022	0.000
gross energy[1], MJ/kg DM	16.07[b]	15.35[a]	0.18	0.027
Active components				
total phenolics	164.0[b]	41.5[a]	0.11	0.000
saponins	49.2[a]	55.3[b]	0.03	0.000
flavonoids	56.6[a]	92.0[b]	0.22	0.000

PSE – pooled standard error; [1] estimated value; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$ and $P < 0.001$

concentration of flavonoids (1.6 times) and saponins (1.13 times) were observed in *T. undulata* in comparison to *P. granatum* extract, while *P. granatum* extract had significantly higher ($P < 0.001$) amount of total phenolics (3.95 times) than *T. undulata* extract.

Nutrient digestibility and nitrogen balance

The supplementation of diet with neither *P. granatum* nor *T. undulata* herbal extracts affect the DMI and digestibility of nutrients (Table 3). The nitrogen intake and excretion through urine remained comparable ($P < 0.18$) among control and herbal extracts supplemented groups. Digestible nitrogen did not differ between groups; however the tendency ($P = 0.058$) to higher nitrogen retention was observed in groups fed diets supplemented with *P. granatum* and *T. undulata* (13.6 and 10%, respectively) in comparison to control group.

Table 3. Effect of supplementing herbal extracts on dry matter (DM) intake (DMI), digestibility of nutrients and nitrogen retention in male buffaloes

Indices	Diet supplemented with			PSE	*P*-value
	no supplement	*Punica granatum*	*Tecomella undulata*		
DMI, kg/day	7.49	7.90	7.82	0.09	0.559
Digestibility of nutrients, %					
DM	64.0	64.3	65. 7	0.56	0.086
organic matter	68.5	70.7	69.7	1.13	0.125
neutral detergent fibre	61.1	62.8	63.2	0.80	0.800
acid detergent fibre	51.3	51.6	51.6	1.01	0.307
crude protein	69.3	73.1	72.2	0.81	0.062
Nitrogen balance, g/day					
nitrogen intake	155.7	156.4	154.8	1.88	0.942
faecal nitrogen	47.9	42.1	43.0	2.25	0.189
urinary nitrogen	43.6	41.4	41.3	0.83	0.062
total excreted nitrogen	91.5	83.5	84.3	2.53	0.083
digestible nitrogen	107.8	114.3	111.8	2.42	0.203
retained nitrogen	64.2	72.9	70. 6	2.34	0.058

PSE – pooled standard error

Table 4. Effect of supplementing herbal extracts on the blood profile of male buffaloes

Indices	Diet supplemented with			PSE	*P*-value
	no supplement	*Punica granatum*	*Tecomella undulata*		
Total protein, g/dl	7.96	8.18	8.23	0.14	0.461
Albumin, g/dl	3.06	3.13	3.11	0.05	0.641
Globulin, g/dl	4.89	5.06	5.11	0.15	0.835
Albumin:Globulin ratio	0.64	0.62	0.62	0.02	0.890
Cholesterol, mg/dl	65.6	61.9	60.6	2.34	0.420
Triglycerides, mg/dl	21.5	21.9	22.4	1.38	0.810
Glucose, mg/dl	69.3	71.2	69.2	2.56	0.761
BUN, mg/dl	43.9	40.2	38.2	1.42	0.139
Urea, mg/dl	20.7	17.9	18.9	0.67	0.139
Uric acid, mg/dl	0.58	0.57	0.59	0.03	0.770
SGOT, IU/l	80.9	84.2	81.9	2.93	0.629
SGPT, IU/l	41.9	41.7	41.7	2.05	0.959

PSE – pooled standard error; BUN – blood urea nitrogen; SGOT – serum glutamic oxaloacetic transaminase; SGPT – serum glutamate pyruvate transaminase

Blood biochemical parameters

Blood profile analysis revealed that total protein, albumin, globulin, cholesterol and blood urea nitrogen levels in all animals were within normal range and showed no significant ($P > 0.05$) variation due to dietary supplementation of herbal extracts (Table 4). Blood cholesterol, SGOT and SGPT were also comparable in groups fed diets supplemented with *P. granatum* and *T. undulata* in comparison to control group.

Methane emission

The effect of supplementation of herbal extract on enteric CH_4 emission (g/kg DMI) was quite significant (Table 5) and was reduced ($P < 0.05$) by 25.3% with supplementation of diet with *P. granatum* extract and by 27.7% when diet was supplemented with *T. undulata* extract (at 2% of DMI) in comparison to the animals fed control diet. Supplementation of diet with *P. granatum* extract or *T. undulata* extract resulted in decrease in CH_4 emission (g/kg average daily gain (ADG)) by 46 and 42% in comparison to the control diet. Loss of energy through CH_4 was also lower in supplemented groups (8.16 (*P. granatum*) and 8.08 MJ/day (*T. undulata*)) in comparison to control animals (11.5 MJ/day).

Table 5. Effect of supplementing herbal extracts on methane emission in male buffaloes

Parameter	Diet supplemented with			PSE	*P*-value
	no supplement	*Punica granatum*	*Tecomella undulata*		
CH_4, g/day	206.1[b]	146.2[a]	144.8[a]	2.42	0.000
CH_4, g/day/kg DMI	24.5[b]	18.3[a]	17.7[a]	0.28	0.000
CH_4, g/day/kg DOMI	40.2[b]	28.6[a]	28.6[a]	0.37	0.000
Loss of energy through CH_4, MJ/day	11.5[b]	8.16[a]	8.08[a]	0.13	0.000
GE intake, MJ/day	121.0[a]	127.5[b]	126.2[b]	0.91	0.012
CH_4 energy loss (% of GE), MJ/day	9.5[b]	6.4[a]	6.4[a]	0.08	0.000
CH_4, g/kg ADG	428.1[b]	231.2[a]	248.2[a]	8.827	0.000

PSE – pooled standard error; CH_4 – methane; DMI – dry matter intake; DOMI – digestible organic matter intake; GE – gross energy; ADG – average daily gain; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$ and $P < 0.001$

Performance of animals

The impact of CH_4 emission decrease and higher GE intake was visible in gains (BWG/day). The animals fed *P. granatum* and *T. undulata* extracts gained ($P < 0.05$) 28 and 21% more weight, respectively, in comparison to animals fed control diet (Table 6). However, the gain on basis of GE intake (gain, g/day/MJ of GE intake) was 25 and 19% higher in *P. granatum* and *T. undulata* extracts supplemented animals, respectively.

Table 6. Effect of supplementing herbal extracts on growth performance of male buffaloes (n = 4 due to removal of outliers)

Parameter, kg	Diet supplemented with			PSE	*P*-value
	no supplement	*Punica granatum*	*Tecomella undulata*		
Initial body weight	285	287	288	6.75	0.985
Final body weight	330[a]	344[b]	342[b]	3.37	0.048
Total $gain_{90days}$	45[a]	57[b]	54[b]	0.983	0.000
Gain/day, g	498[a]	638[b]	601[ab]	11.0	0.000
Gain, g/day/MJ of GE intake	4.00[a]	5.00[b]	4.78[b]	0.090	0.000

PSE – pooled standard error; GE – gross energy; [ab] – means with different superscripts within a row are significantly different at $P < 0.05$ and $P < 0.001$

Discussion

Tannins, saponins and flavonoids, the major phytobiotics present in *P. granatum* and *T. undulata* extracts, are biologically active (Dhir and Shekhawat, 2012). In the studies on pure PSMs, researchers used single metabolite like tannins, saponins or flavonoids to modulate rumen microbial metabolism, to reduce methanogenesis and to improve productive performance in animals. Moreover, the usage of pure compounds have some practical problems and is difficult to use at the farm level. However, herbal extracts used in our study contained combinations of PSMs, which may have synergistic effect on mitigating enteric CH_4 emission and thereby improve the productive performance of animals. Interaction of different PSMs present in the same herb within rumen with microbial consortium or with each other might yield different results than expected or reported earlier.

Active components in herbal extracts. The presence of PSMs (tannins, saponins and flavonoids) in *P. granatum* and *T. undulata* extracts (Dhir and Shekhawat, 2012) enables these herbal extracts to manipulate rumen and perhaps to mitigate enteric CH_4 emission without adverse affection on the digestibility of nutrients. Recently, natural plant products (Ersahince and Kara, 2017; Hundal et al., 2019), which are often not expensive and environmentally safe, have been introduced in CH_4 mitigation strategies. These compounds are not only able to suppress the CH_4 production but also possess broad range of favourable effects on animal health.

Nutrient digestibility and nitrogen balance. DMI depends on the inhibitory or stimulatory effects of feed additives on organoleptic properties of feeds, however supplementation of *P. granatum* and *T. undulata* extracts at 2% of DMI did not influence organoleptic properties and consequently palatability of feeds, hence no change in DMI among animals of control and supplemented groups were reported. Our study confirmed the fact that the supplementation of PSM does not affect DMI (Samal et al., 2016).

Oliveira et al. (2010) found that *P. granatum* extract feeding (at 5–10 g/day equivalent to gallic acid intake of 0.8–1.6 g/day) to young calves for the first 70 days of life decreased intake of grains and whole tract digestibility of fat and crude protein, likely because of its high tannin content. Jami et al. (2012) noted a significant increase in the digestibility of DM, crude protein and neutral detergent fibre in cows fed 4% *P. granatum*-peel extract supplement. Dschaak et al. (2011) observed that the digestibility of DM, organic matter, crude protein and acid detergent fibre remained unchanged after the addition of quebracho condensed tannin extract at 3% of DM. In another study, supplementation of plant flavonoids at 300 mg/kg DMI did not influence feed consumption among Holstein Friesian crossbred heifers fed high concentrate diet (Balcells et al., 2012). Similarly, in the present study, supplementation of *P. granatum* and *T. undulata* extracts improved neither the digestibility of nutrients

nor retention of nitrogen but the tendency to higher digestibility and nitrogen retention was observed in comparison to control group. This variability of results of secondary metabolites supplementation on nutrients digestibility may be attributed to the concentration of active compound, chemical structure and source of the used plants (Abarghuei et al., 2010). According to Van Soest (1994) there is a positive correlation between voluntary intake and *in vivo* digestibility. In the present study, DMI was not influenced by inclusion of herbal extracts into diets, which was probably due to no differences in nutrient digestibility. The effects of secondary metabolites on feed intake in ruminants have been inconsistent among studies indicating either no change (Balcells et al., 2012), increase (Jami et al., 2012) or decrease (Abarghuei et al., 2011) in feed intake.

Abarghuei et al. (2013) also reported that supplementation of *P. granatum* peel extract (400–800 ml/day) did not affect nitrogen balance in dairy cows, which is due to lack of any effect on protein digestibility. Earlier Vaithiyanathan et al. (2007) found that adding secondary metabolite (69 g tannins/kg DM) had no effect on nitrogen balance in sheep. Tendency to improvement in nitrogen retention (8.9–13.3%) in the present study may be due to the combined effect of tannins, saponins or flavonoids present in herb extracts on utilization of feed, which in turn altered protein degradation in rumen and resulted in higher availability of amino acids at intestinal level (Barry and McNabb, 1999).

Blood biochemical parameters. The lack of differences in blood profile after supplementation of herbal extracts was consistent with lack of effect on DMI and digestibility of nutrients. Observations of Yang et al. (2010) revealed no effects on glucose and triglyceride concentrations in blood of growing cows supplemented with 400, 800 and 1600 mg eugenol per day. Comparable blood cholesterol, SGOT and SGPT between experimental and control group, may suggest that liver function was not altered by supplementation of herbal extracts. Blood glucose concentration remained comparable among animals of all groups. Blood total protein concentration is an indicator of the long-term protein status of dairy cows (Topps and Thompson, 1984). However, no changes in the total protein content of blood were observed in the present study indicating normal protein status. Similarly, Nasri and Ben Salem (2012) reported that oral administration of *Agave americana* extracts (containing 120, 240 and 360 mg saponins/kg DM) or *Quillaja saponaria* (containing 120 mg saponins/kg DM) to Barbarine female lamb had no effect on blood total protein concentration. The findings of present study are also in line with the observations of Dey and De (2014), who reported that dietary inclusion of tannins (1.5%) did not influence the most of the blood biochemical parameters in lactating cows.

Methane emission. Methane emission by ruminants is not only associated with environment pollution but also represents loss of carbon during ruminal fermentation and 1 g of CH_4 emission is related to a loss of 55.85 KJ of GE (Brouwer, 1965). The decrease in CH_4 emission in animals fed *P. granatum* and *T. undulata* extracts in comparison to those fed control diet supported the results of our previous *in vitro* study, in which depression in methanogenesis was reported due to supplementation of *P. granatum* and *T. undulata* extracts at 2% of the substrate (Singh et al., 2018). Similarly, depression in methanogenesis without compromising DM digestibility was observed by Cieslak et al. (2012) on supplementation of 140 g *Vaccinium vitis idaea* extract equivalent to 2 g tannins/kg DM in Polish Holstein Friesian dairy cows. Jayanegara et al. (2012) proposed two mechanisms of tannins action on CH_4 production: 1. by altering rumen microbial (protozoa and methanogens) growth and 2. by reducing fibre digestion, which leads to impaired hydrogen production. In the present study, 25–27.7% decrease ($P < 0.001$) in CH_4 emission was achievable though supplementation of herbal extracts at 2% of DMI in Murrah male buffaloes and the findings are in close agreement with that of Moreira et al. (2013) who reported 25.7% reduction in CH_4 emission after dietary supplementation of 40 g/kg DM of condensed tannins from *Leucaena leucocephala* in the ration of sheep. In our study, the reduction in ruminal CH_4 emission was not related to the decrease in digestibility. Although rumen microbiota diversity was not assessed in our study but reduced methanogenesis might be connected with direct toxic effect of tannins on protozoal and methanogens population as observed by other researchers (Cieslak et al., 2012).

The other possible explanation for decreased methanogenesis was the presence of saponins and flavonoids in used herbal extracts. *P. granatum* and *T. undulata* extracts contained 24.4 and 16.5 g of saponins per kg of DM, respectively. Similarly to tannins, saponins reduce protozoal population, which in turn leads to decrease in interspecies H_2 transfer to the methanogenic archea associated with protozoa, resulting in lower methane emission in the ruminants (Jayanegara et al., 2012). Flavonoids are benzo-1-pyrone derivatives with anti-inflammatory, antioxidant

and antimicrobial properties (Harborne and Williams, 2000) and potential of flavonoid rich plant extracts (*P. granatum*, *Betula scmidtii*, *Ginkgo biloba*, *Camellia japonica* and *Cudrania tricuspidata*) towards reduction in methane production by 39.6 to 48.8% without adversely affecting rumen fermentation characteristics at 5% of substrate *in vitro* (Kim et al., 2015) exhibited its possibility to act as bioactive regulator in ruminants. Ehsan et al. (2013) also reported reduction in CH_4 production with significant suppression in total protozoa and methanogen population along with compromised substrate degradability when used pure form of flavonoids naringin and quercetin at 4.5% of the substrate. However supplementation of flavonoids at 300 mg/kg DMI protected ruminal acidosis by increasing number of lactate consuming microbes (*Megasphaera elsdenii*) and raised molar proportion of propionate as well as lowered acetate:propionate ratio in the rumen of crossbred Holstein Friesian heifers but ADG or feed conversion ratio remained unaffected among control and test groups (Balcells et al., 2012). As propionate formation and CH_4 production are competitive (Cieslak et al., 2012), the reduction in methanogenesis is obvious with flavonoid supplementation. In the present study, herb extracts were also rich in flavonoids, as *P. granatum* and *T. undulata* extracts provided flavonoids at the tune of 1130 and 1840 mg/kg DMI to the respective groups. Change in protozoal and methanogen populations (Baker, 1999) or enhanced propionate formation (Cieslak et al., 2012) after dietary inclusion of flavonoids can be attributed to the significant decrease in enteric CH_4 emission in this study. Lower rumen protozoal population could enhance microbial protein synthesis which promotes production in ruminants by increasing supply of amino acids for absorption in intestine (Williams and Coleman, 1992).

Performance of animals. Productive performance of animals is the prime focus in animal nutrition studies in addition to control greenhouse gases while supplementing herbal feed additives in ruminant ration. Enteric methane emission represents loss of energy which should otherwise be used in the process of muscle and milk production in ruminants (Åby et al., 2013). After dietary inclusion of *P. granatum* or *T. undulata* extracts, CH_4 emissions (g/kg DMI, g/kg DOM intake and g/kg ADG) were reduced in comparison to control group. The CH_4 energy loss as % of GE intake was 6.4% for herb extract supplemented groups whereas 9.5% in the control group. Supplementing herb extracts into diet could provide an additional 800–820 KJ of energy/animal/day by considering 55.85 KJ of GE per g of CH_4 (Brouwer, 1965). Further, slightly higher DMI and herb supplementation itself resulted in higher energy intake by 6400 and 5100 KJ/animal/day in *P. granatum* and *T. undulata* extracts fed groups, respectively. The energy gained due to decrease in CH_4 emission and higher DMI on supplementation of herb extracts resulted in better performance of animals.

Improved average daily weight gain in *P. granatum* extract supplemented group in comparison to *T. undulata* fed group was attributed to their higher energy balance. Considerable antibacterial, immunomodulatory, antiatherosclerotic and antioxidant capacities demonstrated potential of *P. granatum* peel to enhance weight gain and health benefits without any adverse effects in ruminants and may yield animal products with higher antioxidants properties (Shabtay et al., 2008). Dietary supplementation of Karadi lambs with 1% *P. granatum* peels promoted an increase in feed intake, nutrient digestibility and weight gain due to its low to moderate tannin content whereas depression in digestibility and ADG was observed at 4% inclusion level of *P. granatum* peels (Sadq et al., 2016). Tannins are toxic at higher levels and lead to inactivation of rumen microbial enzymes with compromised digestibility (Barry and McNabb, 1999; Majewska et al., 2017) whereas at low and moderate level (20–45 mg/g DM) improved production efficiency in ruminants without affecting DMI, hence resulted in increase in weight gain and milk yield (Aerts et al., 1999). The dietary inclusion of 5% green tea by-product (containing condensed tannins (CT) 12.5 g/kg DM) with 0.5% bio-char improved average daily weight gain by 20.4% in beef cattle without affecting feed intake and nutrient digestibility (Khoa et al., 2018). Similarly, Dey and De (2014) reported significantly higher milk yield when supplementing diet with 1.5% CT through *Ficus bengalensis* leaves without affecting DMI and digestibility of nutrients among crossbred cows. Addition of saponins through *Saponaria officinalis* root at a dose of 440 g/day or 660 g/day in dairy cattle positively alter volatile fatty acid production which resulted in improved energy balance and consequently milk yield in experimental groups (Szczechowiak et al., 2016). In addition, dietary inclusion of flavonoids could positively affect ruminal fermentation, which in turn leads to higher milk yield in dairy cows (Theodorou et al., 1994). Broudiscou et al. (2002) observed that the flavonoid containing leaves (*Achillea millefolium, Arnica chamissonis* and *Lavandula angustifolia*) extracts at 15 g/kg of dietary DM had variable impact on efficiency of microbial

protein synthesis in continuous culture system and this variation in results may correspond to the type and concentration of the flavonoids present in different plant extracts. The difference in performance of animals in control and experimental groups and even within *P. granatum* and *T. undulata* extracts supplementing groups could be due to the presence of different plant secondary metabolites, their concentration and interaction within the system. So, the obtained results confirmed our hypothesis regarding potential PSMs towards CH_4 mitigation and productive performance of animals but without compromising digestibility.

Conclusions

The present study revealed that dietary inclusion of *Punica granatum* and *Tecomella undulata* extracts at 2% of dry matter intake of total mixed ration (roughage:concentrate ratio 65:35) resulted in methane mitigation *in vivo* by 25 to 27.7%. The energy gained from decrease in methane emission, slightly higher gross energy intake after supplementation of herbal extracts resulted in increased body weight gain. Therefore, the study established that the supplementation of diet with herbal extracts can help in ameliorating enteric methane production, thereby improving the growth performance in ruminants.

Funding

The study was conducted under the project 'Estimation of methane emission under different feeding systems and development of mitigation strategies' (Indian Council of Agricultural Research, New Delhi).

Acknowledgement

The authors would like to thank dr M.P.S. Bakshi, Former Head, Department of Animal Nutrition, GADVASU, Ludhiana, Punjab, India-141004 for technical guidance, and Mrs. Arvinder Kaur Beri, Head, Department of English, Sant Hira Das Kanya Mahavidyala (Women College) Kalesanghian, Punjab for his valuable time to eliminate grammatical errors from this manuscript.

References

Abarghuei M.J., Rouzbehan Y., Alipour D., 2010. The influence of the grape pomace on the ruminal parameters of sheep. Livest. Sci. 132, 73–79, https://doi.org/10.1016/j.livsci.2010.05.002

Abarghuei M.J., Rouzbehan Y., Alipour D., 2011. Effect of oak (*Quercus libani* Oliv.) leave tannin on ruminal fermentation of sheep. J. Agric. Sci. Technol. 13, 1021–1032

Abarghuei M.J., Rouzbehan Y., Salem A.Z.M., Zamiri M.J., 2013. Nutrient digestion, ruminal fermentation and performance of dairy cows fed pomegranate peel extract. Livest. Sci. 157, 452–461, https://doi.org/10.1016/j.livsci.2013.09.007

Åby B.A., Aass L., Sehested E., Vangen O., 2013. Effect of incorporating greenhouse gas emission costs into economic values of traits for intensive and extensive beef cattle breeds. Livest. Sci. 158, 1–11, https://doi.org/10.1016/j.livsci.2013.09.009

Aerts R.J., Barry T.N., McNabb W.C., 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agric. Ecosyt. Environ. 75, 1–12, https://doi.org/10.1016/S0167-8809(99)00062-6

AOAC International, 2007. Official Methods of Analysis of AOAC International. 18th Edition. 2nd Revision. Gaithersburg, MD (USA)

Baccou J.C., Lambert F., Sanvaire Y., 1977. Spectrophotometric method for the determination of total steroidal sapogenin. Analyst 102, 458–466, https://doi.org/10.1039/AN9770200458

Baker S.K., 1999. Rumen methanogens and inhibition of methanogens. Austral. J. Agric. Res. 50, 1293–1298, https://doi.org/10.1071/AR99005

Bakshi M.P.S., Rani N., Wadhwa M., Kaushal S.K., 2004. Impact of herbal feed additives on the degradability of feedstuffs in vitro. Indian J. Anim. Nutr. 21, 249–253

Balbaa S.I., Zaki A.Y., El-Shamy A.M., 1974. Total flavonoids and rutin content of the different organs of *Sophora japonica* L. J. Assoc. Off. Anal. Chem. 57, 752–755

Balcells J., Aris A., Serrano A., Seradj A.R., Crespo J., Devant M., 2012. Effects of an extract of plant flavonoids (Bioflavex) on rumen fermentation and performance in heifers fed high-concentrate diet. J. Anim. Sci. 90, 4975–4984, https://doi.org/10.2527/jas.2011-4955

Barry T.N., McNabb W.C., 1999. The implications of considered tannins on the nutritive value and temperature forage fed to ruminants. Br. J. Nutr. 81, 263–272, https://doi.org/10.1017/S0007114599000501

Bell M.J., Wall E., Simm G., Russel G., 2011. Effects of genetic line and feeding system on methane from dairy system. Anim. Feed Sci. Technol. 166–167, 699–707, https://doi.org/10.1016/j.anifeedsci.2011.04.049

Broudiscou L.-P., Papon Y., Broudiscou A.F., 2002. Effects of dry plant extracts on feed degradation and the production of rumen microbial biomass in a dual outflow fermenter. Anim. Feed. Sci. Technol. 101, 183–189, https://doi.org/10.1016/S0377-8401(02)00221-3

Brouwer E., 1965. Report of sub-committee on constants and factors. In: K.L. Blaxter (Editor). EAAP Publication No. 11. Energy Metabolism. Proceedings of the 3rd EAAP Symposium. Troon (Scotland). Academic Press Inc. London (UK), pp. 441–443

Cieslak A., Zmora P., Pers-Kamczyc E., Szumacher-Strabel M., 2012. Effects of tannin source (*Vaccinium vitis idaea* L.) on rumen microbial fermentation *in vivo*. Anim. Feed Sci. Technol. 176, 102–106, https://doi.org/10.1016/j.anifeedsci.2012.07.012

Crampton E.W., Maynard L.A., 1938. The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutr. 15, 383–395, https://doi.org/10.1093/jn/15.4.383

Dey A., De P.S., 2014. Influence of condensed tannins from *Ficus bengalensis* leaves on feed utilization, milk production and antioxidant status of crossbred cows. Asian-Australas. J. Anim. Sci. 27, 342–348, https://doi.org/10.5713/ajas.2013.13295

Dhir R., Shekhawat G.S., 2012. Critical review on *Tecomella undulata*: a medicinally potent endangered plant species of Indian Thar Desert. Int. J. Curr. Res. 6, 36–44

Dschaak C.M., Williams C.M., Holt M.S., Eun J.-S., Young A.J., Min B.R., 2011. Effects of supplementing condensed tannin extract on intake, digestion, ruminal fermentation, and milk production of lactating dairy cows. J. Dairy Sci. 94, 2508–2519, https://doi.org/10.3168/jds.2010-3818

Ehsan O., Norhani A., Armin O., 2013. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. Biomed. Res. Int. 2013, 349129, https://doi.org/10.1155/2013/349129

Ersahince A.C., Kara K., 2017. Nutrient composition and *in vitro* digestion parameters of Jerusalem artichoke (*Helianthus tuberosus* L.) herbage at different maturity stages in horse and ruminant. J. Anim. Feed Sci. 26, 213–225, https://doi.org/10.22358/jafs/76477/2017

Harborne J.B., Willams C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504, https://doi.org/10.1016/S0031-9422(00)00235-1

Hundal J.S., Wadhwa M., Bakshi M.P.S., 2016. Methane mitigation potential of tannins and their impact on digestibility of nutrients. Anim. Nutr. Feed Technol. 16, 505–513, https://doi.org/10.5958/0974-181X.2016.00047.0

Hundal J.S., Wadhwa M., Bakshi M.P.S., 2019. Herbal feed additives containing essential oil: 1. Impact on the nutritional worth of complete feed *in vitro.* Trop. Anim. Health Prod. https://doi.org/10.1007/s11250-019-01887-1

ICAR-NIANP (Indian Council of Agricultural Research- National Institute of Animal Nutrition and Physiology), 2013. Nutrient Requirements of Animals – Cattle and Buffalo. ICAR-NIANP, New Delhi (India)

Jami E., Shabtay A., Nikbachat M., Yosef E., Miron J., Mizrahi I., 2012. Effects of adding a concentrated pomegranate-residue extract to the ration of lactating cows on in vivo digestibility and profile of rumen bacterial population. J. Dairy Sci. 95, 5996–6005, http://doi.org/10.3168/jds.2012-5537

Jayanegara A., Leiber F., Kreuzer M., 2012. Meta-analysis of the relationship between dietary tannin level and methane formation in ruminants from *in vivo* and *in vitro* experiments. J. Anim. Physiol. Anim. Nutr. 96, 365–375, https://doi.org/10.1111/j.1439-0396.2011.01172.x

Johnson K., Huyler M., Westberg H., Lamb B., Zimmerman P., 1994. Measurement of methane emissions from ruminant livestock using a sulphur hexafluoride tracer technique. Environ. Sci. Technol. 28, 359–362, https://doi.org/10.1021/es00051a025

Khoa M.A., Quang N.H., Thang T.V., Phung T.V., Kien T.T., 2018. Effect of tannin in green tea by-product in combination with bio-char supplemented into basal beef cattle diet on nutrient digestibility, methane production and animal performance. Open J. Anim. Sci. 8, 206–214, https://doi.org/10.4236/ojas.2018.83015

Kim E.T., Guan L.L., Lee S.J., Lee S.M., Lee S.S., Lee I.D., Lee S.K., Lee S.S., 2015. Effects of flavonoid rich plant extracts on *in vitro* ruminal methanogenesis microbial populations and fermentation characteristics. Asian-Australas. J. Anim. Sci. 28, 530–537, https://doi.org/10.5713/ajas.14.0692

Majewska M.P., Pajak J.J., Skomiał J., Miltko R., Kowalik B., 2017. The effect of lingonberry leaves and oak cortex addition to sheep diets on pancreatic enzymes activity. J. Anim. Feed Sci. 26, 354–358, https://doi.org/10.22358/jafs/79748/2017

Makkar H.P.S., Blümmel M., Borowy N.K., Becker K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161–165, https://doi.org/10.1002/jsfa.2740610205

Mirdehghan S.H., Rahemi M., 2007. Seasonal changes of mineral nutrients and phenolics in pomegranate (*Punica granatum* L.) fruit. Sci. Hort. 111, 120–127, https://doi.org/10.1016/j.scienta.2006.10.001

Moreira G.D., Lima P.M.T., Borges B.O., Primavesi O., Longo C., McManus C., Abdalla A., Louvandini H., 2013. Tropical tanniniferous legumes used as an option to mitigate sheep enteric methane emission. Trop. Anim. Health Prod. 45, 879–882, https://doi.org/10.1007/s11250-012-0284-0

Nasri S., Ben Salem H., 2012. Effect of oral administration of *Agave Americana* or *Quillaja saponaria* extracts on digestion and growth of Barbarine female lamb. Livest. Sci. 147, 59–65, https://doi.org/10.1016/j.livsci.2012.04.001

Oliveira R.A., Narciso C.D., Bisinotto R.S., Perdomo M.C., Ballou M.A., Dreher M., Santos J.E.P., 2010. Effects of feeding polyphenols from pomegranate extract on health, growth, nutrient digestion and immunocompetence of calves. J. Dairy Sci. 93, 4280–4291, https://doi.org/10.3168/jds.2010-3314

Patra A.K., 2014. Trends projected estimates of GHG emissions from Indian livestock in comparisons with GHG emissions from world and developing countries. Asian-Australas. J. Anim. Sci. 27, 592–599, https://doi.org/10.5713/ajas.2013.13342

Qiao G., Shao T., Yang X., Zhu X., Li J., Lu Y., 2013. Effects of supplemental Chinese herbs on growth performance, blood antioxidant function and immunity status in Holstein dairy heifers fed high fibre diet. Italian J. Anim. Sci. 12, e20, https://doi.org/10.4081/ijas.2013.e20

Robertson J.B., Van Soest P.J., 1981. The detergent system of analysis and its application to human foods. In: W.P.T. James, O. Theander (Editors). The Analysis of Dietary Fiber in Food. Marcel Dekker Inc. New York, NY (USA), pp. 123–158

Rohilla R., Garg M., 2014. Phytochemistry and pharmacology of *Tecomella undulata*. Int. J. Green Pharm. 8, 1–6, http://dx.doi.org/10.4103/0973-8258.126811

Sadq S.M., Ramzi D.O.M., Hamasalim H.J., Ahmed K.A., 2016. Growth performance and digestibility in Karadi lambs receiving different levels of pomegranate peels. Open J. Anim. Sci. 6, 16–23. https://doi.org/10.4236/ojas.2016.61003

Samal L., Chaudhary L.C., Agarwal N., Kamra D.N., 2016. Effects of plants containing secondary metabolites as feed additives on rumen metabolites and methanogen diversity of buffaloes. Anim. Prod. Sci. 56, 472–481, https://doi.org/10.1071/AN15596

Shabtay A., Eitam H., Tadmor Y. et al., 2008. Nutritive and antioxidant potential of fresh and stored pomegranate industrial byproduct as a novel beef cattle feed. J. Agric. Food Chem. 56, 10063–10070, https://doi.org/10.1021/jf8016095

Singh I., Hundal J.S., Wadhwa M., Lamba J.S., 2018. 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. Indian J. Anim. Sci. 88, 443–452, https://doi.org/10.13140/RG.2.2.33520.35843

Szczechowiak J., Szkudelska K., Szumacher-Strabel M., Sadkowski S., Gwozdz K., El-Sherbiny M., Kozłowska M., Rodriguez V., Cieslak A., 2018. Blood hormones, metabolic parameters and fatty acid proportion in dairy fed condensed tannins and oils blend. Ann. Anim. Sci. 18, 155–166, https://doi.org/10.1515/aoas-2017-0039

Szczechowiak J., Szumacher-Strabel M., El-Sherbiny M., Bryszak M., Stochmal A., Cieslak A., 2016. Effect of dietary supplementation with *Saponaria officinalis* root on rumen and milk fatty acids proportion in dairy cattle. Anim. Sci. Rep. Pap. 34, 221–232

Teferedegne B., 2000. New perspective on the use of tropical plants to improve ruminant nutrition. Proc. Nutr. Soc. 59, 209–214, https://doi.org/10.1017/S0029665100000239

Theodorou M.K., Willams B.A., Dhanoa M.S., McAllan A.B., France J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Technol. 48, 185–197, https://doi.org/10.1016/0377-8401(94)90171-6

Topps J.H., Thompson J.K., 1984. Blood Characteristics and the Nutrition of Ruminants. Ministry of Agriculture, Fisheries and Food. Reference Book 260. London (UK), p. 33

Vaithiyanathan S., Raghavendra Bhatta, Mishra A.S., Prasad R., Verma D.L., Singh N.P., 2007. Effect of feeding graded levels of *Prosopis cinerarialeaves* on rumen ciliate protozoa, nitrogen balance and microbial protein supply in lambs and kids. Anim. Feed Sci. Technol. 133, 177–191, https://doi.org/10.1016/j.anifeedsci.2006.04.003

Van Soest P.J., 1994. Nutritional Ecology of the Ruminant. Cornell University Press. Ithaca, NY (USA)

Wanapat M., Kang S., Khejornsart P., Wanapat S., 2013. Effects of plant herb combination supplementation on rumen fermentation and nutrient digestibility in beef cattle. Asian-Australas. J. Anim. Sci. 26, 1127–1136, https://doi.org/10.5713/ajas.2013.13013

Williams A.G., Coleman G.S., 1992. The Rumen Protozoa. Springer-Verlag New York, Inc. New York, NY (USA), pp. 441, https://doi.org/10.1007/978-1-4612-2776-2

Yang W.Z., Ametaj B.N., Benchaar C., He M.L., Beauchemin K.A., 2010. Cinnamaldehyde in feedlot cattle diets: Intake, growth performance, carcass characteristics, and blood metabolites. J. Anim. Sci. 88, 1082–1092, https://doi.org/10.2527/jas.2008-1608

Yanza Y.R., Szumacher-Strabel M., Bryszak M., Gao M., Kolodziejski P., Stochmal A., Slusarczyk S., Patra A.K., Cieslak A., 2018. *Coleus amboinicus* (Lour) leaves as a modulator of ruminal methanogenesis and biohydrogenation in vitro. J. Anim. Sci. 96, 4868–4881, https://doi.org/10.1093/jas/sky321