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Research Article

EFFECT OF ADDITION OF TEA SEED (*CAMELLIA SINENSIS* VAR. *KUNTE*) AND TEA SEED SAPONIN EXTRACT WITH DIFFERENT CONCENTRATE TO ROUGHAGE RATIO ON *IN VITRO* RUMEN FERMENTATION

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ABSTRACT

This study was conducted to assess the beneficial effect of tea seed (*Camellia sinensis* var. *Kunte*) and tea seed saponin on *in vitro* rumen fermentation and to evaluate its potential as feed additive in ruminants. Different ratios of roughage to concentrate mixture i.e. 70:30 (Substrate-I), 60:40 (Substrate-II) and 50:50 (Substrate-III) were used in triplicate to study the effect of tea seed and tea seed saponin on *in vitro* rumen fermentation. Tea seed saponin was added in a concentration ranging from 0.2 to 0.6% of substrate incubated and tea seed was added with equivalent dose of tea saponin from 0.2 to 0.6 % of substrate incubated. Protozoa number, ammonia N, methane production, true dry matter and organic matter digestibility linearly decreased ($P<0.01$) with increasing level of tea saponin and tea seed in all the three diets. Total gas production increased significantly ($P<0.01$) with increasing levels of both tea saponin and tea seed. The total gas production (24 hrs) was increased by 16, 20 and 17% with tea saponin and 14, 18 and 15% with tea seed for substrate I, II and III, respectively. The ammonia-N was decreased by 45, 19 and 22% with tea saponin and 21, 10 and 11% with tea seed for substrate I, II and III, respectively. Methane production was decreased by 39, 24 and 25% with tea saponin and 17, 7 and 14% with tea seed for substrate I, II and III, respectively at 0.6% level of saponins. Better results were obtained from crude saponin mixture as compare to tea seed which was added as a raw source of saponin in all three substrates. It is suggested that tea saponin and tea seed could modify the rumen fermentation pattern by inhibiting methane and ammonia release, thus beneficial for improving the growth and nutrient utilization in ruminants.

Keywords: Ammonia-N, Gas production, *In vitro*, Methane, Tea saponin, Tea seed

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Plants produce a variety of plant secondary metabolites (PSM) such as saponins, tannins, organic acids, essential oils etc. These compounds have been shown to selectively modulate the rumen fermentation pattern by modulating ruminal microbial ecosystem resulting in an improvement in efficiency of nutrient utilization and growth (Wallace, 2002; Kamra *et al*., 2006 and Rochfort *et al.*, 2008). The possible use of natural plant products as a productivity enhancer provide cheap, safe, sustainable and more consumer acceptable. Global warming and climate change has become a global issue in last few decades. One of the important green house gas is methane and its amount in the atmosphere has increased from 315 ppm in 1958 to 417 ppm in 2022 (Duan *et al.*, 2023)). Methane produced during the process of ruminal fermentation represents a loss of 2-15% of gross energy intake (Johnson and Johnson, 1995). Therefore, reducing methaneemmisision from animals has implications for global warming as well as efficient animal production. Saponins are high molecular weight glycosides found in different plant parts. They can be used as natural feed additives mostly to reduce methane emission and improve nutrient utilization in ruminants. Saponins have nutraceutical, insecticidal, antimicrobial and several other properties. Tea seeds are rich source of saponins and they are commercially used in China and Japan (Zhan, 1999). India and china are the leading producer of tea in the world. Tea plants are vegetatively propagated, seeds are not used for the propagation of this plant. Many Chinese workers have shown that supplementation of tea saponins in ruminants improves the growth performance and nutrient utilization. Both Yucca and Quillaja saponins have been used as feed additives (Cheeke, 2000), but little information is available about the use of tea seed saponins of Indian origin as ruminant feed additives. Saponins from different sources shown to have antiprotozoal properties (Wallace *et al.*, 1994 and Newbold *et al.*,1997). Hu *et al.* (2005a) and Guo *et al.* (2008) reported decrease in protozoal count *in vitro* on addition of tea saponins to the basal substrate. *In vitro* studies have shown that addition of tea saponin to the basal substrate decreases ruminal ammonia nitrogen (Hu *et al.*, 2005a; Zhou *et al.*, 2011). Wang *et al.* (2000) and Hu *et al.* (2005b) reported decrease in methane production with the addition of Yucca and tea saponin, respectively. The feed additives should only contribute to profitable livestock farming and superior quality animal products, but also be in line with the steadily increasing food safety and agricultural regulations. Phytogenic feed additives like tea seed saponins have the great potential to be used in animal

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feeding and to get maximum proformance from the animals.

MATERIALS AND METHODS

Saponin extraction

Tea seeds (*Camellia sinensis* var. *Kunte*) used for this study were purchased from the Department of Tea Husbandry, CSK HPKV, Palampur (H.P.). Extraction of tea seed saponin was done as per the method of Joshi *et al.* (2012). Dried and powered tea seeds were defatted with hexane in a percolator at room temperature. After concentrated under reduced pressure, the percolate was suspended in 70% methanol. This extract was concentrated and dried into powder form containing tea saponins. The powder was chromatographed on Diaion HP-20 eluting with H2O-MeOH-CHCl3 to get H2O, MeOH and CHCl3 fractions. Methanolic fraction was separated on silica gel column to get eight fractions. On DM basis, saponin yield was 14.11-16.59 % (15.35% avg.). Detection and purity estimation of saponin was performed on a HPLC equipped with evaporative light scattering detector (ELSD), quaternary gradient pump and Lichrosphere C-18 column. The mobile phase consisted of acetonitrile:water (75:25) (isocratic elution) with the flow rate 1.0 ml/min. Purity of the crude saponins mixture was found to be 73.6 %. By HPLC four types of saponins were detected in crude saponins mixture named as S1, S2, S3 and S4. This mixed saponin extracted from tea seed was used for both in vitro as well as in vivo studies.

Chemical composition of *in vitro* medium

A) Macro-mineral solution: Consisting of Na_2HPO_4 anhydrous (5.7g), KH_2PO_4 anhydrous (6.2g) and $MgSO_4.7H_2O$ (0.60g). All these constituents were dissolved in 1000 ml of distilled water.

B) Micro-mineral solution: Consisting of $CaCl_2$. $2H_2O$ (13.2g), $MnCl_2$. $4H_2O$ (10.0g), $COCl_2$. $6H_2O$ (1.0g) and $FeCl_3$. $6H_2O$ (8g). All these constituents were dissolved in 1000 ml of distilled water.

C) Rumen buffer solution: This solution consist of NH_4HCO_3 (4.0g) and $NaHCO_3$ (35g). All these constituents were dissolved in 1000 ml of distilled water.

D) Resazurine solution: 0.10% w/v

Incubation Medium

Solution-I: Distilled water (365ml), Micro-mineral solution (0.1ml), Rumen buffer solution (183ml), Macro-mineral solution (183ml), Resazurin solution (0.95ml). Prepared on previous day of incubation and stored at 39º C. **Solution-II:** 1N NaOH (1.6ml), Na_2S. $7H_2SO_4$ (220 mg) anddistilled water (37ml). Solution II were prepared on the day of incubation. Solution III: Rumen liquor 330 ml (collected from the fistulated cattle fed with roughage: concentrate at 70:30 ratios). All three solutions were mixed to prepare incubation medium.

Equipment and technique

Accurately weighed 200 mg substrate consisting of roughage and concentrate in three different ratios such as 70:30 (Substrate-I), 60:40 (Substrate-II) and 50:50 (Substrate-III) was taken in graduated 100 ml capacity syringe with the help of spatula without touching walls of syringe. The pistons of the syringes were lubricated with petroleum jelly. The in vitro medium was bubbled with CO_2 for a few minutes to maintain anaerobic environment and then 50.0 ml of reducing agent was added. Rumen liquor collected from the rumen of fistulated male cattle before morning feeding was strained with muslin cloth and incubated under CO_2 at 39º C. When in vitro medium turned colourless, 330 ml of rumen liquor was added. Then 30.0 ml of in vitro medium was dispensed in 100 ml syringes. Different substrates (I, II, III) along with blanks were incubated inawater bath maintained at 39º C. Syringes were shaken at an interval of every 30 minutes for first 2 hours from the start of incubation and thereafter at every 2 hours up to 10 hours of incubation. The gas production was recorded at 0, 1, 2, 3, 6, 12 and 24 h of incubation. At the end of incubation period (24 h) methane was estimated by GLC by sampling the gas from the silicon tube of syringes and contents were further processed for estimation of ammonia N and protozoal counts. For the determination of DM and OM digestibility, the sample weight was increased to 400 mg to increase the residue so as to reduce analytical error inherent to gravimetric determinations.

Experimental design and substrate composition

In vitro gas production was estimated as described by Menke *et al.* (1979). Three different ratios of roughage to concentrate mixture (70:30, 60:40 and 50:50) were used in triplicate to study the effect of tea seed and tea seed saponin on *in vitro* rumen fermentation. Concentrate mixture was prepared by mixing properly 34% ground maize grains 28% soyabean meal, 35% wheat bran, 2% mineral mixture and 1% common salt (NaCl). Precaution was taken so that mineral mixture and salt were properly distributed in feed. Oat fodder was used as roughage. Tea seed and tea seed saponin extract were added in a dose equivalent to 0.2 to 0.6 % of the basal substrate.

Measurement of *in vitro* fermentation parameters

Methane was estimated in a gas liquid chromatography (Nucon 5765, Nucon Engineers, New Delhi) equipped with flame ionization detector and glass column packed with chromosorb - 101. Column was made up of stainless

steel and packed with porapak-q (length 6'; o.d. 1.8"; i.d. 2 mm; mesh range 80-100). The temperature of injection port was 150º C; column 60º C; detector 130º C. Injection volume was 100 µl. The flow rate of carrier gas (nitrogen) was 40 ml/min; air 300 ml/min and hydrogen 30 ml/min; From the head space of each syringe 100 µl gas was collected by puncturing the silicon tube and injected in gas chromatography for the estimation of methane (Hamilton Company, Nevada, SA). The standard gas used for methane estimation composed of 50% methane and 50% carbon ioxide (SPANCAN Calibration gas, Spantech, Surrey, England). The peak was identified by comparison of above standard and peak area obtained was used to calculate methane concentration in the gas sample. The volume of methane produced (ml) was estimatedby multiplying the gas produced in ml by the concentration of methane in the given sample. The methane produced from the substrate after 24 h incubation was calculated by correcting the corresponding blank values.

Ammonia nitrogen was estimated using micro Kjeldahl distillation apparatus. To 5 ml of rumen fluid sample and 2 ml of 1 N NaOH was added and steam distilled using micro Kjeldahl distillation apparatus; and the NH_3 evolved was collected in boric acid and titrated against 0.01N H_2SO_4. The numbers of protozoa were counted in a known volume of medium and the total number was calculated as per the procedure of Kamra *et al.* (1991). For counting protozoa in the contents of syringe, 0.5 ml content pipetted with a wide orifice pipette was mixed with 1 ml methyl green formal saline solution (composition: 0.06 g methyl green, 0.85 g sodium chloride, 10 ml formaldehyde solution and 90 ml distilled water) and allowed to stand overnight at room temperature. Counting was done in 20 microscopic fields in a hemocytometer counting chamber at a magnification of 100X. True digestibility (TD) was estimated as per the method outlined by Goering and Van Soest (1970) with modifications. For the determination of DM and OM digestibility, the sample weight was increased to 400 mg to increase the residue so as to reduce analytical error inherent to gravimetric determinations. After 24 hours of incubation, the contents of the syringe were directly transferred to a 500 ml spoutless beaker. The syringe was washed with 40 ml double strength neutral detergent solution (NDS). And washings added to the beaker. The contents in the beaker were refluxed for 1 hour, filtered under vacuum through pre-weighed Gooch crucibles. NDF content of the residue was determined. Ash content of the residue was also determined.

Statistical Analysis

Data generated from this study was analyzed through SAS 9.2 software.

RESULTS AND DISCUSSION

Chemical composition of the basal substrates used in *in vitro* studies and tea seed saponin yield: The chemical composition of basal substrates-I, II and III are given in Table 1. The substrate-III was lower in NDF (55.35%) and ADF (26.32%) and high in CP (17.09%) while substrate-I contained low level of CP (15.12%) and high level of NDF (58.43%) and ADF (30.91%). The CP (16.10%), NDF (56.89 %) and ADF (28.62%) content were intermediate of above two in the substrate-II. The OM content of all the substrates used was more or less similar (88.68, 89.28 and 89.87% respectively for substrates-I, II and III).

Tea plant contains triterpenoid saponins. The saponin content of tea seed was found to be in between 14.11-16.59% (15.35% avg.). Kohata *et al.* (2004) reported that in the seeds of Camellia sinensis the content of saponins amounts up to 10% of the dry weight. Crude saponin powder obtained was light brown in colour, easily soluble in water. Jadhav *et al.* (2016) reported that tea seaponin content in tea seed was 6.5 to 25.1%. This variation in content of saponin in tea seed may be due to seasonal variation, climatic conditions, stage of maturity of seed and geographical location of area.

Effect of addition of tea seed and tea seed saponin on total gas (24 hr) production: Volume of gas produced in different treatments is given in Table 2. Gas production values were comparatively more in high concentrate substrate. Gas production was significantly increased with increasing level of saponin and tea seed in all three substrates. For substrate 1 values were ranging from 156.66 to 186.66 ml/gram for 0.2% to 0.6% tea saponin, respectively and 172.73 to 180.66 ml/gram for tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, respectively, whereas that for control group (0% tea saponin) was 156.66 ml/gram. For substrate 2 values were ranging from 160.33 to 200.16 ml/gram for 0.2 to 0.6% tea saponin, respectively and 173.83 ml/gram to 196.66 ml/gram for tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, whereas that for control group was 160.33 ml/gram. For substrate 3 values were ranging from 181.66 ml/gram to 216.66 ml/gram for 0.2 to 0.6% tea saponin, respectively and for tea seed it varied from 182.00 (equivalent to 0.2% tea saponin) to 211.66 ml/gram (equivalent to 0.6% tea saponin), whereas that for control group was 181.66 ml/gram. Gas production was significantly higher in substrate 3 as compared to substrate 2 in which it was higher than substrate 1.

24-hour gas production increased with increasing levels of in tea saponin and tea seed in all three substrates.

Table 1. Chemical composition of the basal substrates used in *in vitro* studies

Attributes	Roughage to concentrate ratio		
	70:30 (Substrate-I)	60:40 (Substrate-II)	50:50 (Substrate-III)
Proximate composition			
Organic matter	88.68	89.28	89.87
Crude protein	15.12	16.10	17.09
Ether extract	2.06	2.02	2.06
Total ash	11.32	10.72	10.13
Cell wall constituents			
NDF	58.43	56.89	55.35
ADF	30.91	28.62	26.32
Cellulose	26.46	24.28	22.10
Hemicellulose	27.52	28.27	29.03
ADL	4.45	4.33	4.22
Minerals			
Calcium	0.92	0.98	1.04
Phosphorus	0.36	0.39	0.44

The total gas production (24 hrs) was increased by 16, 20, and 17% with tea saponin and 14, 18, and 15% with tea seed for substrate-I, II and III, respectively at 0.6% level of saponins. For 24 h gas production results are in agreement with Jadhav *et al.* 2016) and Hu *et al.* (2005a). Jadhav et al. (2016) reported that with the increasing levels of tea saponins (from 0.3 to 1 % of substrate incubated) the total gas (ml/200mg substrate) increased significantly. The total gas production was increased by 22, 21.7 23% at 1% tea saponin for 30:70, 50:50 and 70:30 roughages to concentrate ratio, respectively.

Hu *et al.* (2005a) who reported, inclusion of 2, 4 and 6 mg TSS increased the 24 h gas production to 48.5, 49.5 and 50.2 ml/200 g of substrate, respectively while the GP at 8 mg level kept little change from 47.9 ml in the control using Menke and Steingass (1988) technique. Hu *et al.*

Table 2. Effect of addition of tea seed and tea seed saponin on 24 hr gas production (ml/g) in *in vitro* gas production test

S.No.		Roughage: Conc. ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea saponin	0.0	$156.66^{Be} \pm 1.66$	$160.33^{Be} \pm 0.33$	$181.66^{Ae} \pm 1.66$
2.	0.2	$156.66^{Be} \pm 3.00$	$163.33^{Be} \pm 1.66$	$195.83^{Ad} \pm 0.83$
3.	0.3	$170.83^{Bd} \pm 0.83$	$171.66^{Bd} \pm 1.68$	$200.00^{Adc} \pm 2.88$
4.	0.4	$175.50^{Cc} \pm 0.50$	$185.16^{Bc} \pm 1.66$	$202.50^{Ac} \pm 2.05$
5.	0.5	$180.16^{Cb} \pm 0.16$	$192.83^{Bb} \pm 1.48$	$209.66^{Ab} \pm 0.33$
6.	0.6	$186.66^{Ca} \pm 1.66$	$200.16^{Ba} \pm 1.60$	$216.66^{Aa} \pm 1.58$
7. Tea seed	0.2	$172.73^{Bdc} \pm 0.89$	$173.83^{Bd} \pm 1.96$	$182.00^{Ae} \pm 2.00$
8.	0.3	$175.66^{Cc} \pm 0.66$	$184.00^{Bc} \pm 1.00$	$196.16^{Ad} \pm 0.60$
9.	0.4	$180.33^{Cb} \pm 0.34$	$185.33^{Bc} \pm 0.33$	$202.16^{Ac} \pm 1.48$
10.	0.5	$180.66^{Cb} \pm 0.66$	$190.83^{Bb} \pm 0.83$	$203.16^{Ac} \pm 3.16$
11.	0.6	$180.66^{Cb} \pm 0.67$	$196.66^{Ba} \pm 1.66$	$211.66^{Aab} \pm 1.68$
SEM		1.66	2.25	1.92
P value		<0.001	<0.001	<0.001

Table 3. Effect of addition of tea seed and tea seed saponin on *in vitro* methane production (ml/litre) in *in vitro* gas production test

S.No.		Roughage: Conc. ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea saponin	0.0	$49.01^{a} \pm 0.16$	$47.01^{a} \pm 1.43$	$48.25^{a} \pm 0.31$
2.	0.2	$40.83^{Be} \pm 0.16$	$40.67^{Bbcd} \pm 0.84$	$44.33^{Ab} \pm 0.54$
3.	0.3	$35.88^{f} \pm 0.19$	$39.36^{edc} \pm 1.42$	$37.96^{e} \pm 1.10$
4.	0.4	$32.79^{Bg} \pm 0.74$	$36.93^{Aed} \pm 0.93$	$37.63^{Ae} \pm 0.20$
5.	0.5	$32.69^{Bg} \pm 0.85$	$38.29^{Aed} \pm 1.39$	$37.59^{Ae} \pm 0.37$
6.	0.6	$30.00^{Bh} \pm 0.58$	$35.33^{Ae} \pm 0.60$	$36.45^{Ae} \pm 0.17$
7. Tea seed	0.2	$44.33^{ABb} \pm 0.16$	$46.05^{Aa} \pm 0.74$	$42.10^{Bcd} \pm 1.10$
8.	0.3	$43.25^{Bcb} \pm 0.14$	$45.91^{Aa} \pm 0.46$	$43.80^{Bcb} \pm 0.57$
9.	0.4	$42.90^{cbd} \pm 0.18$	$45.13^{ab} \pm 2.88$	$42.95^{cbd} \pm 0.15$
10.	0.5	$41.50^{ced} \pm 1.50$	$44.84^{ab} \pm 2.46$	$42.38^{cd} \pm 0.21$
11.	0.6	$41.16^{ed} \pm 0.16$	$43.97^{abc} \pm 2.04$	$41.93^{d} \pm 0.06$
SEM		0.99	0.80	0.62
P value		<0.001	<0.001	<0.001

Table 4. Effect of addition of tea seed and tea seed saponin on protozoa count (*104/ml) in *in vitro* gas production test

S.No.		Roughage: Conc. ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea saponin	0.0	$2.45^{Aa} \pm 0.02$	$2.28^{Ba} \pm 0.01$	$2.22^{Ba} \pm 0.01$
2.	0.2	$2.26^{Abc} \pm 0.04$	$2.20^{ABb} \pm 0.01$	$2.13^{Bbc} \pm 0.02$
3.	0.3	$1.96^{Bf} \pm 0.05$	$2.10^{Ac} \pm 0.03$	$1.98^{Bed} \pm 0.01$
4.	0.4	$1.79^{Cg} \pm 0.01$	$1.98^{Ae} \pm 0.01$	$1.85^{Bf} \pm 0.01$
5.	0.5	$1.75^{Bg} \pm 0.02$	$1.96^{Ae} \pm 0.02$	$1.81^{Bf} \pm 0.02$
6.	0.6	$1.54^{Bh} \pm 0.02$	$1.72^{Af} \pm 0.01$	$1.61^{Bg} \pm 0.01$
7. Tea seed	0.2	$2.43^{Aa} \pm 0.01$	$2.20^{Bb} \pm 0.02$	$2.17^{abB} \pm 0.02$
8.	0.3	$2.34^{Ab} \pm 0.03$	$2.20^{Bb} \pm 0.02$	$2.11^{Cc} \pm 0.01$
9.	0.4	$2.23^{Acd} \pm 0.02$	$2.11^{Bc} \pm 0.01$	$2.02^{Cd} \pm 0.01$
10.	0.5	$2.16^{Aed} \pm 0.01$	$2.04^{Bd} \pm 0.01$	$2.00^{Bed} \pm 0.01$
11.	0.6	$2.12^{Ae} \pm 0.01$	$2.05^{Ad} \pm 0.02$	$1.95^{Be} \pm 0.02$
SEM		0.04	0.03	0.02
P value		<0.001	<0.001	0.003

Table 5. Effect of addition of tea seed and tea seed saponin on ammonia N (mmole/L) in *in vitro* gas production test

S.No.		Roughage: Conc. ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea saponin	0.0	$13.62^{a} \pm 0.07$	$12.86^{a} \pm 0.09$	$13.12^{a} \pm 0.49$
2.	0.2	$12.40^{ab} \pm 0.44$	$12.85^{a} \pm 0.14$	$12.96^{ab} \pm 0.08$
3.	0.3	$10.44^{Bdc} \pm 0.44$	$12.31^{Aa} \pm 0.16$	$12.24^{Abc} \pm 0.11$
4.	0.4	$9.29^{Bd} \pm 0.76$	$11.66^{Ab} \pm 0.09$	$11.62^{Ac} \pm 0.07$
5.	0.5	$9.27^{Cd} \pm 0.08$	$11.49^{Ab} \pm 0.09$	$10.83^{Bd} \pm 0.18$
6.	0.6	$7.43^{Be} \pm 0.81$	$10.49^{Ac} \pm 0.16$	$10.26^{Ad} \pm$d 0.42
7. Tea seed	0.2	$11.46^{Bbc} \pm 0.14$	$12.97^{Aa} \pm 0.04$	$13.00^{Aab} \pm 0.05$
8.	0.3	$11.13^{Bbc} \pm 0.30$	$12.80^{Aa} \pm 0.16$	$12.74^{Aab} \pm 0.48$
9.	0.4	$11.11^{Cbc} \pm 0.31$	$12.78^{Aa} \pm 0.10$	$11.82^{Bc} \pm 0.05$
10.	0.5	$10.76^{Cc} \pm 0.19$	$12.65^{Aa} \pm 0.30$	$11.76^{Bc} \pm 0.01$
11.	0.6	$10.76^{Bc} \pm 0.19$	$11.67^{Ab} \pm 0.47$	$11.74^{ABc} \pm 0.01$
SEM		0.29	0.11	0.16
P value		<0.001	<0.001	<0.001

(2006) reported increased 24 h gas production using the Reading Pressure Techniques (RPT). Increased gas production may be due to higher bacterial and fungal population due to antiprotozoal action of saponins.

Effect of addition of tea seed and tea seed saponin on Methane production: Methane production in different treatments is given in Table 3. Methane production was comparatively more in high concentrate substrate. Methane production was significantly decreased with increasing level of saponins. For substrate 1, values were ranging from 40.83 to 30.00ml/L for 0.2 to 0.6% tea saponin, respectively and 44.33 to 41.16ml/Lfor tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, respectively, whereas that for control group (0% TSS) was 49.01 ml/L. For substrate 2 values were ranging from 40.67 to 35.33ml/L for 0.2 to 0.6 % tea saponin and 46.05 to 43.97ml/L for tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, whereas that for control group was 47.01 ml/L. For substrate 3 values were ranging from 44.33 to 36.45 ml/L (0.2 to 0.6 % TSS) for tea saponin and for tea seed it varied from 42.10 to 41.93 ml/L (equivalent to 0.2 to 0.6% tea saponin), whereas that for control group was 48.25 ml/L.

Methane produced during enteric fermentation leads to a loss of feed energy for the animals, and increased environmentalproblems through greenhouse effect. Addition of tea saponin in the diet significantly decreased the in vitro methane production, but with tea seeds best results were obtained in 70:30 and 50:50 roughage to concentrate ratio. Methane production was decreased by

Table 6. Effect of addition of tea seed and tea seed saponin on true dry matter digestibility (%) in *in vitro* gas production test

S.No.		Roughage: Concentrate ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea saponin	0.0	$78.73^{a} \pm 0.37$	$78.75^{a} \pm 0.38$	$78.33^{a} \pm 0.33$
2.	0.2	$78.66^{Aa} \pm 0.33$	$78.40^{Ba} \pm 0.30$	$77.55^{Bb} \pm 0.29$
3.	0.3	$78.65^{a} \pm 0.32$	$78.13^{a} \pm 0.70$	$77.36^{cb} \pm 0.25$
4.	0.4	$77.75^{b} \pm 0.25$	$78.04^{a} \pm 0.42$	$77.41^{cb} \pm 0.30$
5.	0.5	$74.26^{c} \pm 0.26$	$74.85^{b} \pm 0.30$	$75.00^{d} \pm 0.05$
6.	0.6	$74.70^{c} \pm 0.15$	$74.80^{b} \pm 0.11$	$74.83^{d} \pm 0.16$
7. Tea Seed	0.2	$77.66^{cb} \pm 0.33$	$78.47^{a} \pm 0.66$	$76.93^{bc} \pm 0.06$
8.	0.3	$78.11^{Aab} \pm 0.11$	$78.20^{Aa} \pm 0.11$	$76.92^{Bbc} \pm 0.07$
9.	0.4	$78.09^{Aab} \pm 0.04$	$78.18^{Aa} \pm 0.18$	$76.92^{Bbc} \pm 0.10$
10.	0.5	$78.06^{Aab} \pm 0.04$	$78.06^{Aa} \pm 0.03$	$76.90^{Bbc} \pm 0.09$
11.	0.6	$77.93^{Aab} \pm 0.06$	$78.04^{Aa} \pm 0.24$	$76.88^{Bc} \pm 0.08$
SEM		0.26	0.25	0.18
P value		<0.001	0.001	<0.001

Table 7. Effect of addition of tea seed and tea seed saponin on true organic matter digestibility (%) in *in vitro* gas production test

S.No.		Roughage: Concentrate ratio		
	Percentage	70:30 (Sb-1)	60:40 (Sb-2)	50:50 (Sb-3)
1. Tea Saponin	0.0	$80.54^{ABa} \pm 0.32$	$81.06^{Aa} \pm 0.16$	$80.18^{Ba} \pm 0.05$
2.	0.2	$80.51^{Aa} \pm 0.16$	$80.88^{Aa} \pm 0.01$	$79.23^{Bb} \pm 0.29$
3.	0.3	$80.47^{Aa} \pm 0.03$	$80.21^{Aab} \pm 0.13$	$78.96^{Bb} \pm 0.16$
4.	0.4	$78.76^{cd} \pm 0.28$	$79.33^{bc} \pm 0.66$	$78.91^{b} \pm 0.07$
5.	0.5	$78.59^{cd} \pm 0.016$	$77.66^{c} \pm 0.33$	$76.13^{c} \pm 0.18$
6.	0.6	$77.44^{Ad} \pm 0.01$	$77.16^{Ac} \pm 0.03$	$75.86^{Bc} \pm 0.52$
7. Tea Seed	0.2	$79.16^{Bbc} \pm 0.26$	$80.55^{Aa} \pm 0.23$	$78.87^{Bb} \pm 0.06$
8.	0.3	$79.66^{ABb} \pm 0.11$	$80.28^{Aab} \pm 0.42$	$78.85^{Bb} \pm 0.04$
9.	0.4	$79.66^{Bb} \pm 0.23$	$80.26^{Aab} \pm 0.17$	$78.84^{Cb} \pm 0.08$
10.	0.5	$79.62^{Bb} \pm 0.99$	$80.14^{Aab} \pm 0.14$	$78.79^{Cb} \pm 0.02$
11.	0.6	$79.51^{Ab} \pm 0.08$	$80.13^{Aab} \pm 0.03$	$78.78^{Bb} \pm 0.07$
SEM		0.13	0.14	0.16
P value		<0.001	0.003	<0.001

39, 24 and 25% with tea saponin and 17, 7 and 14% with tea seed for substrate I, II and III, respectively at 0.6% level of saponins. Many workers also reported reduction *in vitro* methane production due to addition of saponin or saponin extracts. Results are in agreement with Jadhav (2016), Hu *et al.* (2005b) and Wang *et al.* (2000). Jadhav (2016) reported that with the increasing levels of tea saponins (0.3% to 1%), the methane production decreased significantly. The methane production was decreased by 27%, 30% and 40% at 0.8% level of tea saponin for three different roughages to concentrate ratio (30:70, 50:50 and 70:30). Hu *et al.* (2005b) reported inclusion of 0.2 and 0.4 mg/ml tea saponin decreased methane production by 13.3 and 14.3%, respectively, in the faunated rumen fluid. Wang et al. (2000) observed that the methane production was 15% lower in Yucca saponin-fed group compared to the control. This reduced methane production may be either due to direct inhibition of methanogenic microbes or due to reduction of protozoal population. Protozoa produce H+ ions in their metabolic pathways, which are used by archea to produce methane. Zhou et al. (2011) in a study with defaunated and faunated sheep observed that tea saponin appeared to reduce methane production by inhibiting protozoa and presumable lowering methanogenic activity of protozoal associated methanogens.

Effect of addition of tea seed and tea seed saponin on Protozoal population and Ammonia nitrogen: Protozoal number in different treatments is given in Table 4.

Protozoal number was significantly decreased with increasing level of saponins. For substrate-1 values were ranging from 2.26 to 1.54 for 0.2 to 0.6 % TSS, respectively and 2.43 to 2.12 for tea seed with equivalent dose of 0.2 to 0.6% tea saponin, respectively, whereas that for control group (0% TSS) was 2.45*104/ml. For substrate 2 values were ranging from 2.20 to 1.72 for 0.2 to 0.6% TSS, respectively and 2.20 to 2.05 for tea seed with equivalent dose of 0.2 to 0.6% tea saponin, respectively whereas that for control group was 2.28*104/ml. For substrate 3 values were ranging from 2.13 to 1.61 (0.2% to 0.6 % TSS) for tea saponin and for tea seed it varied from 2.17 to 1.95 (equivalent to 0.2 to 0.6% tea saponin), whereas that for control group was 2.22*104/ml.

The protozoal count was reduced with increasing levels of tea saponin and tea seed. Protozoal count was reduced by 38, 25 and 28% with tea saponin and 14, 11 and 13% with tea seed for substrate I, II and III, respectively at 0.6% level of saponins. Results are in agreement with Jadhav (2016), Hu *et al.* (2005a) and Guo *et al.* (2008). Jadhav (2016) reported that with the increasing levels of tea saponins (from 0.3 to 1%), the protozoal count (*104/ml) decreased significantly from 1.88, 1.85 and 2.30*104/ml to 0.93, 0.95 and 0.89*104/ml, respectively for three different roughage to concentrate ratio (30: 70, 50: 50 and 70: 30). The protozoal count was decreased by 55, 58 and 60% at 1% level of tea saponin for three different roughages to concentrate ratio (30:70, 50:50 and 70:30). Hu *et al.* (2005a) reported that after 24-h incubations, protozoal counts were reduced by 19, 25, 45 and 79% when the tea saponin was added at 10, 20, 30 and 40 g/kg substrate, respectively. Guo *et al.* (2008) found that at 0.4 mg tea saponin/ml medium containing rumen fluid or 53 g tea saponin/kg substrate, decreased protozoa count substantially. Wallace *et al.* (2002) reported that Saponins form complexes with sterols in protozoal cell membrane and the membrane become impaired and eventually disintegrate, leads to death of protozoal cell. This leads to reduced protozoal population.

Concentration of ammonia-N in different treatments is given in Table 5. Ammonia-N concentration was significantly decreased with increasing level of saponin and tea seed. For substrate-1 values were ranging from 12.40 (0.2% TSS) to 7.43 mmole/L (0.6% TSS) for tea saponin and 11.46 to 10.76 mmole/L for tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, respectively, whereas that for control group (0% TSS) was 13.62 mmole/L. For substrate-2 values were ranging from 12.85 (0.2% TSS) to 10.49 mmole/L (0.6% TSS) for tea saponin and 12.97 to 11.67 mmole/L for tea seed containing equivalent dose of 0.2 to 0.6% tea saponin, whereas that for control group was 12.86 mmole/L. For substrate 3 values were ranging from 12.96 (0.2% TSS) to 10.26 mmole/L (0.6 % TSS) for tea saponin and for tea seed it varied from 13.00 to 11.74 mmole/L (equivalent to 0.2 to 0.6% tea saponin), whereas that for control group was 13.12 mmole/L.

Addition of tea seed saponins, decreased the ammonia N concentration in all the three substrates, but with tea seed, best results are obtained in 50:50 and 70:30 roughage concentrate ratio. The ammonia-N was decreased by 45, 19 and 22 % with tea saponin and 21, 10, and 11% with tea seed for substrate I, II and III, respectively at 0.6% level of saponins. Results are in agreement with Jadhav (2016), Hu *et al.* (2005a) and Hu *et al.* (2006). Jadhav (2016) reported that with the increasing levels of tea saponins (from 0.3 to 1%), the ammonia nitrogen decreased significantly from 7.9, 7.40 and 7.05 mg to 5.55, 5.20 and 4.8mg, respectively for three different roughages to concentrate ratio (30:70, 50:50 and 70:30). The ammonia nitrogen was reduced by 34, 36 and 34 % at 1% tea saponin level for three different roughages to concentrate ratio (30:70, 50:50 and 70:30). Hu (2005a) reported decreased ammonia-N concentration from 15 mmol/L to 14.39, 12.77, 12.31 and 11.41 when tea saponins were added 2, 4, 6 and 8 mg to the basal substrate. Hu *et al.* (2006) reported decreased ammonia-N concentration from11.6 mmol/L to 10.4, 8.9 and 8.4 when tea saponins were added 0.2, 0.4 and 0.8 mg/ml. Reduced ammonia nitrogen may be due to reduced protozoal population, which are responsible for degradation of bacterial proteins and thereby increasing rumen ammonia levels.

Effect of addition of tea seed and tea seed saponin on digestibility: For all substrates with addition of tea saponintrue dry matter as well as true organic matter digestibility was decreased significantly. With the addition of tea seed digestibility was not affected (Table 6 and 7). For all substrates with addition of TSS true dry matter as well as true organic matter digestibility was decreased significantly. With the addition of tea seed digestibility decreased in substrate 1 and 2, with increasing levels of tea seed. Jadhav (2016) reported that with the addition of increasing concentrations of tea saponin to the substrate, the dry matter and organic matter digestibility decreased significantly as compare to control in an *in vitro* study. Addition of saponins in the basal substrate from Sapindus saponaria (Hess *et al.*, 2003), Yucca and Quillaja (Holtshausen *et al.*, 2009) plants reduced the digestibility of fibre components *in vitro*. It is suggested that tea saponin and tea seed could modify the rumen fermentation pattern by inhibiting methane and ammonia release, thus beneficial for improving the growth and nutrient utilization in ruminants

CONCLUSION

The primary effect of saponin is to modify the composition of rumen microbial populations resulting in the modification of rumen fermentation. The mechanisms and effects of saponins on rumen fermentation depend on the type and level of saponin, composition of diets, microbial populations affected etc. In this study the tea seed saponins extract shown to have antiprotozoal effect, reduce methane and ammonia release in vitro. Teasaponin and tea seed could modify the rumen fermentation pattern and beneficial for improving the growth and nutrient utilization in ruminants. Although best results were obtained from tea saponin extract as compare totea seed, but futures studies in different concentration of tea saponins and somewhat higher doses of tea seed are needed to explore the possibility of tea seed saponin and tea seed as a feed additive in ruminants.

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