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RESEARCH ARTICLE

Ruminal fermentation and methane production in vitro, milk production, nutrient utilization, blood profile, and immune responses of lactating goats fed polyphenolic and saponin-rich plant extracts

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Abstract

This study was conducted to evaluate the effect of a composite plant extract (CPE) rich in polyphenolics and saponins from seeds of *Dolichos biflorus* (horse gram), root of *Asparagus racemosus* (shatavari), bark of *Amoora rohituka* (rohitaka), and peel of *Punica granatum* (pomegranate) on ruminal fermentation and methanogenesis in vitro, milk production, nutrient digestibility, immune response, and blood profiles in lactating Beetal goats fed CPE at 20 g/kg diet. Dose effect of CPE was assessed using different doses (0, 10, 20, 30, and 40 g/kg substrate) to find out an optimum dose for the in vivo study. The in vivo experiment lasted 70 days including a 10-day adaptation period. In the in vitro study, dry matter (DM) and fiber degradability increased linearly ($P < 0.05$) and methane production and ammonia concentration decreased linearly ($P < 0.05$) with increasing doses of CPE. Concentrations of total VFA and proportion of propionate increased ($P < 0.001$) linearly, whereas proportion of acetate and acetate to propionate ratio decreased with a linear effect. Dietary CPE increased milk yield ($P = 0.017$) and concentrations of protein and lactose ($P = 0.045$) by CPE, but concentrations of fat and solid not fat in milk were not affected ($P > 0.10$). Somatic cell counts in milk reduced ($P = 0.045$) in the CPE-fed goats. Apparent digestibility of DM ($P = 0.037$) increased significantly and NDF ($P = 0.066$) tended to increase due to supplementation of CPE. Blood glucose ($P = 0.028$) and albumin ($P = 0.007$) concentrations increased, while other liver-marker metabolites and enzyme activities and superoxide dismutase activity were not altered in goats due to feeding of CPE. Concentrations of total amino acids ($P = 0.010$), total essential amino acids ($P = 0.012$), and total ketogenic amino acids ($P < 0.001$) were greater in the CPE-fed goats than the control goats. Cell-mediated immune response improved due to CPE feeding. This study suggests that the CPE rich in both phenolics and saponins could improve ruminal fermentation, milk production, and nutrient utilization in lactating goats with better health status while decreasing methane emission.

Keywords Plant extract · Methanogenesis · Milk production · Immune response · Nutrient utilization · Lactating goat

Introduction

Ruminant production systems contribute to enhancing nutritional food security and economic uplift of millions of rural and periurban smallholders in low- and medium-income countries of the world (Herrero et al. 2013). Ruminants in traditional production systems convert inedible fiber-rich feed resources (agricultural wastes and underutilized and non-arable grasslands) to high-quality meat and milk, which reduce the competition of natural resource use for foods, feeds, or fuel production (Bateki et al. 2019). They are capable of converting low-quality fiber-rich or protein feeds to high-quality food products due to the presence of a complex ruminal microbiome with dynamic, redundant, and plasticity in nature, which mediates the fermentation of feeds (Patra

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2020; Mizrahi et al. 2021). However, the ruminal microbial communities are also responsible for various undesirable processes, for example, production of methane, excessive degradation of protein, and biohydrogenation of unsaturated fatty acids (Belanche et al. 2021). These undesirable processes not only cause a loss of dietary energy and protein utilization inefficiency, but also aggravate the environmental problems due to methane emission and urea-ammonia-nitrous oxide emission—two most potent greenhouse gases and reduction of food product quality. Moreover, the microbial conversions of fiber feeds to useful metabolites are often not efficient. Therefore, ruminant nutritionists have attempted for a long time to overcome these constraints by modulating ruminal fermentation using various chemical, microbial feed additives. The recent focus has centered on the use of plant secondary metabolites to improve ruminal fermentation, ruminant production, and health while minimizing the environmental burdens (Patra and Saxena 2009; Singh et al. 2021; Singla et al. 2021).

Around 150 to 200 thousand bioactive plant metabolites have been identified in different broad classes such as phenolics, alkaloids, terpenoids, saponins, and glucosinolates (Berdy 2005; Patra 2012). Plant secondary metabolites exhibit numerous beneficial biological and pharmacological properties, commonly, antimicrobial, antioxidant, immunomodulating activities besides many specific pharmacological actions such as antidiabetic, anticancer, antihypertensive, and antilipidemic actions. Due to their beneficial antimicrobial actions, they have been exploited to modulate ruminal fermentation (Patra and Saxena 2009) and owing to the antioxidant and immune stimulating activities, they have been used to improve health and productivity of ruminants (Olagaray and Bradford 2019). Plant polyphenolics and saponins have most widely studied in ruminant nutrition. Saponins have specific effects on ruminal protozoa that are responsible for protozoa-linked methanogenesis and turnover of microbial proteins in the rumen (Patra and Saxena 2009; Tan et al. 2020). Polyphenolic compounds can reduce protein degradation and methanogenesis by forming tannin-protein complex and directly inhibiting methanogens and protein degraders in the rumen (Patra et al. 2012a; Puchala et al. 2012; Vasta et al. 2019). These compounds exert detrimental responses on ruminal fermentation and nutrient utilization at high doses, whereas lower doses may not be highly effective to reduce methane production. Therefore, it has been suggested that plant compounds with complementary actions on the ruminal functions may be effective to address these issues (Patra and Yu 2015b). The extract from bark of *Amoora rohituka* (rohida) and peel of *Punica granatum* (pomegranate) contain high concentrations of polyphenolic compounds including flavonoids, whereas the extracts from seeds of *Dolichos biflorus* (kulthi) and root of *Asparagus racemosus* (shatavari) are rich in saponins along with phenolic compounds (Singh et al. 2018; Hundal et al. 2019; Hundal et al. 2020b). These extracts up to 30 g/kg substrate individually decreased methanogenesis, but reduced fiber degradability in vitro (Singh et al. 2018; Hundal et al. 2019; Hundal et al. 2020a). We hypothesized that using these extracts as a mixture could avoid adverse effects on feed degradability while exerting additive and broader antimethanogenic effects due to the complementary action of saponins and polyphenolic compounds. Therefore, this study was conducted to investigate the effect of a composite plant extract (CPE) rich in polyphenolics and saponins from seeds of *Dolichos biflorus*, root of *Asparagus racemosus* (shatavari), bark of *Amoora rohituka* (rohitaka), and peel of *Punica granatum* (pomegranate) on ruminal fermentation and methanogenesis *in vitro*, milk production, nutrient digestibility, immune response, and blood profiles in lactating Beetal goats.

Materials and methods

The study was conducted at the Directorate of Livestock Farm (30°54′07″ N, 75°48′20″ E), Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India, in 2021 after necessary ethical approval for animal experiment (CPCSEA/2021/83-1 dated 06.01.2021).

Selection of optimum dose of the composite dry plant extract

There are four different plant extracts, namely, bark of *Amoora rohituka* (rohitaka or rohida), peel of *Punica granatum* (pomegranate), seeds of *Dolichos biflorus* (horse gram or kulthi), and root of *Asparagus racemosus* (shatavari), which were procured from Konark Herbals and Health Care, Mumbai, India. The extracts were kept in room temperature. The extracts of *A. rohituka* and peel of *P. granatum* were rich in polyphenolic compounds, whereas the extract of *D. biflorus* and *A. racemosus* contained high concentrations of saponins as well as phenolic compounds. These plant extracts were mixed in an equal ratio to prepare the phenolics and saponins rich composite plant extract (CPE).

In vitro gas production technique (Menke and Steingass 1988) was used to determine a proper dose for dietary inclusion of CPE in lactating dairy goats. Two different in vitro experiments were conducted by using glass syringes (Haberle Labortechnik, Germany).

In vitro ruminal fermentation characteristics (experiment 1)

For the first experiment to determine substrate degradability and volatile fatty acid profile, 375 mg substrate (moisture free) was weighed carefully in graduated glass

syringes. In this in vitro study, the greater amount of substrate was used to obtain higher accuracy in substrate degradability and fermentation profile. The CPE was added with the substrate as a top-dressing at five different dose levels (0, 10, 20, 30, and 40 mg/kg substrate DM) in triplicates for each dose in each run. The substrate was prepared by mixing pearl millet and concentrate mixture in the ratio of 60:40 on dry matter (DM) basis (Table 1). Concentrate mixture and pearl millet were dried for 72 h (at 60°C) in a hot air oven (NSW-143, Narang Scientific Works, New Delhi, India), ground in a hammer mill (1 mm sieve), and used for in vitro evaluation. Three adult dairy goats of Beetal breed (49.7 ± 3.88 kg) maintained on a total mixed ration (TMR) with ingredient and nutrient composition similar to the substrate used for the in vitro study were used as a source of inocula (ruminal fluid) for in vitro evaluation. Rumen contents were withdrawn using a pedal suction apparatus (Model: 7B, Ishneel Health Care Pvt. Ltd. Mumbai, India) through oro-ruminal passage before morning feeding and watering. The rumen contents were immediately transferred to preheated (39°C) insulated flasks maintained under anaerobic conditions and transported to the laboratory. The ruminal contents were filtered through double layered-muslin cloth before pooled together with equal volumes and used to prepare buffered ruminal inoculum by mixing buffer and ruminal fluid (2:1) as per procedure described by Menke and Steingass (1988). The fermentation medium was continuously maintained at 39°C under a stream of CO_2. The substrate was incubated with 30 mL of fermentation medium for 24 h in a water bath (maintained at 39°C) and swirled hourly over a period of 24 h during incubation. The syringes without substrate (blank) and with reference standard (200 mg berseem hay) in triplicates were also incubated for 24 h. The incubations with triplicate syringes for each dose were repeated thrice with a total replicates of 9 per dose of CPE.

The gas from syringes were recorded and released if the gas volume exceeded 70 mL after 8 h. After the stipulation period (24 h), gas production in the syringes was recorded and net gas production (NGP) was calculated (gas production in treatment syringe minus gas volume in blank syringe). Samples of fermented fluid were taken from incubated syringes and preserved for volatile fatty acid (VFA) and ammonia nitrogen analysis (AOAC International 2007) at −20°C till further analysis. For determination of in vitro true organic matter (IVTDMD), in vitro true organic matter (IVTOMD), and neutral detergent fiber degradability (IVNDFD), about 20 mL neutral detergent solution was used to transfer the contents of syringes to spoutless beakers. The content was refluxed for 1 h on a hot plate (NSW-255, Narang Scientific Works, New Delhi, India), subsequently filtered through pre-weighed sintered crucibles (grade 1), and IVTDMD and IVTOMD of substrate were calculated (Robertson and Van Soest 1981). The microbial biomass production (IVTDOM, mg/g – net gas production, ml/g × 2.2) was calculated as per Grings et al. (2005). The metabolizable energy content (ME) of the substrate was calculated from gas production and dietary composition using the equation developed by Menke et al. (1979): ME (MJ/kg DM) = 1.54 + 0.145 × net gas production (ml/200 mg DM) + 4.12 × CP (g/g DM) + 6.5 × CP (g/gDM) × CP (g/g) + 20.6 × ether extract (g/g DM).

For VFA determination, 0.2 mL of metaphosphoric acid (25%) was added per milliliter of fermented ruminal fluid of in vitro syringes and the content was allowed to stand for 2 h. Then, samples were centrifuged at 4000 rpm for 7 min and supernatant was collected to determine VFA concentrations by using gas chromatograph (Netchrom 9100, Netel (India) Ltd., Mumbai, India). The machine was equipped with a capillary column (Porapak-Q; 2 m in length and 3.18 mm outer diameter) and flame ionization detector (Cottyn and Boucque 1968). The temperature of injection port, column,

Table 1 Ingredient and chemical composition (g/kg dry matter) of total mixed ration (TMR) and ingredient concentrate mixture fed lactating goats

Ingredient composition		Chemical composition	
Ingredient	Composition	Item	Composition
Total mixed ration		Total mixed ration	
Bajra green	600	Organic matter	913.5
Concentrate	400	Crude protein	113.5
Concentrate mixture		Ether extract	36.0
Maize grains	300	Neutral detergent fibre	541
Soybean meal	200	Acid detergent fibre	309
Wheat bran	220	Acid detergent lignin	49.5
Deoiled rice bran	250	Cellulose	259.5
Mineral mixture[1]	20	Hemicellulose	232
Salt (sodium chloride)	10	Gross energy, MJ/kg	20.68

[1]Minerals content (g/kg mineral mixture): calcium, 160; phosphorus, 90; magnesium, 40; sulfur, 14; copper, 0.78; iron, 3; zinc, 3; manganese, 1; cobalt, 0.09; iodine, 0.2

and detector was set at 250, 175, and 270°C, respectively. The flow rate of carrier nitrogen gas through the column was 15 ml/min; the flow rates of hydrogen and zero air through flame ionization detector were 30 and 300 ml/min, respectively. Standard VFA mixture was prepared by mixing stock solutions of each standard VFA (acetic acid, propionic acid, and butyric acid). From VFA concentration, the fermentation efficiency (FE) was worked out (Orskov 1975) using following equation:

$$\text{FE}, \% = (0.622 \times \text{A} + 1.092 \times \text{P} + 1.56 \times \text{B}) \times 100/(\text{A} + \text{P} + 2\,\text{B})$$

where A, B, and P stand for the concentration of acetate, butyrate, and propionate in ruminal fluid samples.

In vitro methanogenesis (experiment 2)

For the second experiment to measure methane production, 200 mg of moisture free substrate (low amount of substrate was used to avoid gas release from the syringes for methane analysis) was incubated with five different levels (0 to 40 mg/kg of substrate DM) of CPE in graduated glass syringes in triplicates with three runs in the same way as in the first experiment (Menke and Steingass 1988). The gas samples from the headspace of each syringe were collected in an airtight syringe and injected into a gas chromatograph (Netchrom 9100, Netel (India) Ltd., Mumbai, India) equipped with a stainless steel packed column (30 m length and 0.53 mm internal diameter) with Porapak Q and a flame ionization detector. The gas standard composed of methane and carbon dioxide in an equal proportion (Sigma Gases and Services, New Delhi-110020, India) was used for calibration. The flow rates for nitrogen, hydrogen, and air were 30, 30, and 320 ml/min, respectively. Temperature of injector oven, column oven, and detector were 70, 50, and 100°C, respectively. The methane production expressed as mL/g substrate DM, mL/g in vitro true digested dry matter (IVTDDM), and mL/g in vitro true digested organic matter (IVTDOM) was calculated.

In vivo assessment of CPE at a selected dose

Goats, treatment, and management

The lactating Beetal goats ($n = 14$) were divided into two groups ($n = 7$) depending upon their initial milk yield (1.51 ± 0.5 kg/day) and days in milk (19.6 ± 1.57 day). The control group was offered a total mixed ration as per NRC (2007), whereas CPE was supplemented as top-dressing at 20 g/kg of DM intake along with the control TMR in the experiment group. The TMR was prepared by mixing green bajra and concentrate mixture in the ratio of 60:40 on DM basis in the same way as for the substrate (for in vitro assessment). The lactating goats were raised under similar management conditions. The experiment period consisted of a 10-day adaptation period followed by a 60-day observational period. Due to standard management conditions in the farm for the lactating goats, they were not allowed to keep in individual stalls. In this condition, control and test treatment replicates were applied to the animals by providing the CPE individually. All animals were tied at 8 am daily and the experimental group was offered the required quantity of CPE by mixing it with 100 g of concentrate mixture, whereas the control goats were fed 100 g of concentrate mixture (without CPE) daily in individual manger. After ensuring complete intake of the mixture, all animals were untied and offered rest of concentrate mixture and roughage as TMR at 9 am in two groups in the similar farm conditions. During the adaptation period, experimental animals were offered CPE at 5 g/kg DM intake for the first 2 days, at 10 g/kg DM intake for the next 3 days, and then at 20 g/kg DM intake for the last 5 days. All animals started consuming the entire quantity of the offered feeds on day 9 of the adaptation period. Animals had free access to drink water throughout the day except between 8.00 and 9.00 am daily. Milk output from individual animals was recorded for 2 consecutive days (morning-evening) at weekly intervals, whereas feed intake was calculated as the difference between daily feed offered and refusals in groups. The milk samples were collected fortnightly. The animals were weighed at fortnight intervals and the feeding schedule was revised accordingly.

Digestibility, blood collection, and immune response

On day 47 of the observational period, the fecal sampling directly from rectum was started from all animals daily (at 8 am and 4 pm) for 7 days. The samples from each goat were placed separately in a hot air oven (NSW-143, Narang Scientific Works, New Delhi, India) maintained at 60°C for 48 h for determination of DM content. For nitrogen content, about 10 g of feces from each collection was stored in plastic containers (containing 25 mL of 20% sulfuric acid solution) and mixed thoroughly. The fecal samples from each animal were pooled after oven drying, finely ground (1 mm sieve), and stored for further analysis. The digestibility of nutrients was calculated by using acid detergent lignin as an internal marker (Kanani et al. 2014).

On day 55 of the observation period, blood samples from all goats were collected from jugular vein into tubes containing heparin 4-h postprandial, centrifuged at 3500 rpm for 15 min within 30 min of collection, and stored at −20°C until analysis of select biochemical and free amino acid profile in plasma.

On day 56 of the trial, all goats were shaved on the neck region to assess the effect of supplementing CPE on cell-mediated immune (CMI) response (Agazzi et al. 2004; Singh et al. 2021). On day 57, 0.15 mg of a plant antigen phytohaemagglutinin-P (TC-226; Cat No. 9008-97-3; HiMedia Laboratories Pvt. Ltd. Mumbai, India) dissolved in 0.2 mL saline solution was injected intradermally at

two different sites with a distance of 4 cm at the cleaned neck regions. The increase in double skin thickness was measured by vernier calliper first after 12 h of injection and then at 24-h intervals up to 72 h.

Analytical techniques

Analysis of concentrate mixture, forage, orts, CPE and fecal samples

The concentrate mixture, forage, orts, CPE, and fecal samples were analyzed for DM, crude protein, ether extract, and total ash as per protocol prescribed by AOAC International (2007), whereas neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined following the procedures of Robertson and Van Soest (1981). The organic matter (OM) and cellulose and hemicellulose contents were calculated by the difference method. Using a bomb calorimeter (Toshniwal Bros, Delhi, India), the gross energy (GE) values of CPE and TMR were determined. The bioactive components in individual plant extracts and CPE were analyzed using the methods described by Makkar et al. (1993) for total phenolics, Porter et al. (1986) for condensed tannins, Balabaa et al. (1974) for flavonoids, Baccou et al. (1977) for saponins, Kumaran and Karakumaran (2007) for 1,1-diphenyl-2-picryl-hydrazyl (DPPH) activity, and Jagota and Dani (1982) for vitamin C.

Analysis of milk samples

After collection, milk samples were transported to the laboratory, stirred by using digital ultrasonic stirrer (BGS-11A, Biogenic Scientific, Meerut, India) and analyzed for fat (%), total protein (%), solid not fat (%), and lactose (%) by using Ultra Scan Milk Analyzer (BGS-11, Biogenic Scientific, Meerut, India). Fat and protein corrected milk (FPCM) was calculated as per Schau and Fet (2008) by using the following equation:

$$\text{FPCM (kg/d)} = \text{milk yield (kg/day)} \times \left(0.337 + 0.0116 \times \text{fat (g/kg)} + 0.006 \times \text{protein (g/kg)}\right).$$

The somatic cells in milk samples (cells/mL of milk) were counted by using a direct cell counter (De Laval, Tumba, Sweden).

Analysis of plasma metabolites

Blood plasma samples were analyzed by an Automatic Biochemical Analyzer (Global 240, BPC BioSed, Italy) for total protein, glucose, albumin, cholesterol, triglycerides, urea, creatinine, aspartate aminotransaminase, and alanine aminotransaminase using commercial Erba Manheim diagnostic kits. The superoxide dismutase (SOD; IU/L) was determined in plasma samples by using SOD ELISA kit (Cat No. E0003Bo; Bioassay Technology Laboratory, Shanghai Korain Biotech, Shanghai, China) and a microplate ELISA reader (BioTek Instruments, Vermont, USA).

Blood plasma amino acid profiling

Plasma samples (50 µL) were vortexed with 50 µL of sulfosalicylic acid (10%) in micro-centrifuge tubes for 1 min and centrifuged at 14000 rpm for 8 min. The supernatant was collected to analyze the derivatized free amino acids using AccQ•Tag^{TM} ultra reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) as per manufacturer's protocol. Briefly, 10 µL of supernatant, 70 µL of borate buffer, and 20 µL of AccQ•Tag^{TM} ultra reagent were vortexed at room temperature after mixing in an Eppendorf tube (Armenta et al. 2010). After 10 min incubation in a water bath (55°C), and at least 2 min at room temperature, this mixture was used for analysis of amino acids. The free amino acid contents were determined with Water's ACQUITY UPLC system (Waters India Pvt. Ltd.). The equipment was fitted with a column heater, a binary solvent manager, an autosampler, and a UV (TUV) detector. The Waters AccQ•Tagultra-column (2.1mm ×100 mm, 1.7 µm particles) was used as a separation column. The column temperature and mobile phase flow rate were maintained at 55°C and 0.7 µL/min, respectively. One microliter of prepared samples was injected into the Water's ACQUITY UPLC system individually. The protein hydrolysate amino acid (at 2.5 µmole/mL; except for cysteine at 1.5 µmole/mL) standards (Waters Corporation, Milford, USA.) were used and concentrations of individual amino acids in samples were determined by comparing its area with that of diluted standards (100 µL standard: 900 µL 0.1 N HCl). After analysis of individual amino acids, total essential amino acid (EAA; Thr + Phe + His + Val + Leu + Met + Ile + Arg + Lys), non-essential amino acid (NEAA; Glu + Asp +Gly+ Cys + Ser +Pro + Ala + Tyr), glucogenic amino acids (GAA; Val + Met + Glu + Gly + Thr + His + Arg+ Ser + Ala + Pro + Cys + Asp), ketogenic amino acid (KAA; Lys + Leu), and branched-chain amino acid (BCAA; Ile + Leu + Val) contents in plasma were calculated.

Statistical analysis

The data generated during in vitro evaluation experiment was subjected to analysis of variance (SPSS 2009) in a completely randomized design with the model: Yij= µ+ Li+ eij; where each observation (NGP, IVTOMD, IVNDFD, methane, VFA profile) was Yij; mean was µ; effect of ith level of CPE (0, 10, 20, 30, 40 g/kg diet) was Li; and residual error

was eij. The means were subjected to post-hoc comparison with Tukey's b test ($P<0.05$). The dose effect of CPE was analyzed by using linear and quadratic polynomial contrast. Immune response was analyzed in a repeated measure model with dose, time, and its interaction as main effects, time as the repeated measure, and goat as the subject using SAS (2001). The best covariance structure, i.e., type = arh(1), was used, which showed better model fit as indicated by the lowest Akaike information criteria. The slice option was used to find out the significant time points. Probability values at $P < 0.05$ were considered significant and $0.05 \leq P < 0.10$ as tendency to become significant.

Table 2 Chemical and phytochemical composition of composite plant extract (CPE) fed to lactating goats

Chemical composition		Phytochemical composition	
Item	g/kg DM	Item	Concentration
Total ash	12.5	Phenolic compounds (g/kg DM)	
Organic matter	987.5	Total phenolics	96.4
Crude protein	113.5	Non tannin phenolics	15.3
Ether extract	7.00	Total tannins	81.2
Neutral detergent fiber	5.00	Condensed tannins*	3.13
Acid detergent fiber	1.50	Antioxidants (g/kg DM)	
Acid detergent lignin	0.00	Vitamin C	11.2
Cellulose	1.50	Flavonoids	64.1
Hemicellulose	3.50	DPPH activity (%)	42.9
Gross energy, MJ/kg	15.7	Saponins (g/kg DM)	
		Aqueous saponin	41.0
		Methanolic saponin	27.9

*Leucocyanidin equivalent

DM, dry matter; *DPPH*, 1,1-diphenyl-2-picrylhydrazine

Results

Phytochemical composition

The CPE was rich in polyphenolic compounds with mainly flavonoids (Table 2). Condensed tannin concentration was low. Also, the extract contained saponins in high amounts. Moreover, the antioxidant activity of the CPE was high with DPPH activity of 43% and vitamin C content.

In vitro gas production and feed degradability

Gas production, IVTOMD, IVNDFD, and metabolizable energy content increased linearly ($P < 0.05$) with increasing doses of CPE (Table 3). Methane production expressed as mL/g DM, mL/g IVTDDM, and mL/g IVTDOM decreased linearly ($P < 0.05$) with increasing doses of CPE. Ruminal ammonia concentration decreased with a linear effect with increasing doses of CPE.

In vitro ruminal volatile fatty acids

Concentrations of total VFA, propionate, butyrate, and branched-chain VFA increased linearly ($P < 0.01$), whereas concentration of acetate tended to increase ($P = 0.052$) linearly with increasing doses of CPE (Table 4). Proportion of acetate decreased ($P < 0.001$) linearly, but proportion of

Table 3 Effect of supplementation of composite plant extract (CPE) on in vitro total gas production, substrate degradability, partitioning factor, and methane production (in vitro experiment)

Variable	Dose of composite plant extract, g/kg					SEM	*P*-value	Contrast *P*-value	
	Control	10	20	30	40			Linear	Quadratic
NGP, mL/g at 24 h	147^{a}	149^{ab}	151^{b}	152^{b}	152^{b}	0.81	<0.001	<0.001	0.074
IVTOMD, g/kg	670^{a}	677^{ab}	694^{bc}	696^{bc}	706^{c}	5.13	<0.001	<0.001	0.619
IVNDFD, g/kg	435^{a}	446^{ab}	472^{bc}	480^{bc}	497^{c}	8.86	<0.001	<0.001	0.807
MBP, mg/g	347^{a}	349^{ab}	362^{ab}	361^{ab}	371^{b}	5.17	0.021	0.001	0.887
ME, MJ/kg	7.73^{a}	7.81^{b}	7.86^{bc}	7.90^{c}	7.90^{c}	0.013	<0.001	<0.001	0.010
In vitro methane production									
CH_4, mL/g DM	48.1^{b}	47.0^{b}	44.6^{ab}	43.1^{a}	41.9^{a}	0.88	0.003	<0.001	0.851
CH_4, mL/g IVTDDM	65.2^{c}	62.2^{bc}	58.6^{ab}	56.2^{ab}	53.1^{a}	1.46	0.001	<0.001	0.857
CH_4, mL/g IVTDOM	67.9^{c}	64.2^{bc}	60.3^{ab}	57.7^{ab}	54.3^{a}	1.65	0.001	0.001	0.761
Ammonia-N, mg/L	177^{d}	172^{cd}	163^{bc}	155^{ab}	149^{a}	2.56	0.001	0.001	0.850

NGP, net gas production; *IVNDFD*, in vitro neutral detergent fiber degradability; *IVTOMD*, in vitro true organic matter degradability; *PF*, partitioning factor; the ratio of true substrate degradability to gas production; *MBP*, microbial biomass production; *ME*, metabolizable energy; *DM*, dry matter; *CH_4*, methane; *IVTDDM*, in vitro true digested dry matter; *IVTDOM*, in vitro true digested organic matter; *SEM*, pooled standard error

^{a,b,c,d}Means with different superscripts in a row differ significantly ($P < 0.05$)

propionate increased ($P < 0.001$) linearly, which resulted in decreased ($P < 0.001$) acetate to propionate ratio with a linear effect. The CPE exerted quadratic effect ($P < 0.001$) on the proportion of branched-chain VFA with highest proportion at 20 g CPE/kg feed. Butyrate proportion was not altered by CPE. Supplementation of CPE improved ($P < 0.001$) fermentation efficiency with a linear effect.

Milk production and composition

Dietary CPE increased milk yield ($P = 0.017$) and FPCM ($P = 0.025$) production in goats (Table 5). Concentrations of fat and solid not fat in milk were not affected ($P > 0.10$), but concentrations of protein and lactose were increased ($P = 0.045$) by CPE. Yields of milk fat ($P = 0.082$) and milk solid not fat ($P = 0.061$) tended to increase, whereas yields of milk protein ($P = 0.039$) and milk lactose ($P = 0.049$) increased due to feeding of CPE. Somatic cell counts in milk reduced ($P = 0.045$) in the CPE-fed goats.

Nutrient digestibility

Apparent digestibility of DM ($P = 0.037$) increased significantly and apparent digestibility of organic matter ($P = 0.058$) and NDF ($P = 0.066$) tended to increase due to supplementation of CPE (Table 5). Digestibility of other nutrients was similar in both groups.

Table 5 Effect of supplementation of composite plant extract (CPE) to a total mixed ration on feed intake, milk yield, and composition and digestibility of nutrients in lactating goats

Variable	Control	CPE	SEM	*P*-value
DMI, kg/day	1.59	1.45	-	-
GE intake, MJ/day	32.9	29.8	-	-
Milk production				
Initial milk yield, kg/day	1.52	1.51	0.214	0.994
Milk yield, kg/day	1.40	1.63	0.069	0.017
FPCM, kg/day	1.71	2.01	0.060	0.025
Milk component and yield				
Fat, g/kg	44.5	45.9	0.97	0.302
SNF, g/kg	87.6	89.1	0.71	0.140
Protein, g/kg	33.6	34.7	0.28	0.045
Lactose, g/kg	45.1	46.4	0.43	0.045
Fat, kg/day	0.63	0.72	0.050	0.082
SNF, kg/day	1.15	1.45	0.084	0.061
Protein, kg/day	0.47	0.57	0.033	0.039
Lactose, kg/day	0.63	0.75	0.044	0.049
SCC ×1000 cells/ml	443	289	36.5	0.045
Digestibility of nutrients, g/kg				
Dry matter	691	727	10.9	0.037
Organic matter	604	624	6.77	0.058
Crude protein	609	631	11.0	0.182
Ether extract	659	658	29.4	0.983
Neutral detergent fiber	510	531	7.30	0.066
Acid detergent fiber	481	499	7.97	0.134
Cellulose	573	594	9.49	0.134

DMI, dry matter intake; *FPCM*, fat and protein corrected milk; *SNF*, solid not fat; *SCC*, somatic cell counts; *SEM*, pooled standard error or mean

Table 4 Effect of supplementation of composite plant extract (CPE) in vitro ruminal volatile fatty acid concentration and proportion

Variable	Dose of composite plant extract, g/kg					SEM	*P*-value	Contrast *P*-value	
	Control	10	20	30	40			Linear	Quadratic
VFA production, mM									
Total VFA	71.7^{a}	73.1^{a}	78.0^{ab}	83.8^{b}	83.9^{b}	1.94	<0.001	<0.001	0.810
Acetate	48.7	48.3	49.2	52.5	51.5	1.50	0.235	0.052	0.853
Propionate	11.1^{a}	12.3^{a}	14.2^{b}	16.6^{c}	17.5^{c}	0.49	<0.001	<0.001	0.816
Butyrate	6.66^{a}	6.90^{ab}	8.02^{abc}	8.40^{c}	8.20^{bc}	0.46	0.036	0.004	0.371
BCVFA	5.28^{a}	5.64^{ab}	6.36^{b}	6.46^{b}	6.70^{b}	0.28	0.006	<0.001	0.372
A:P	4.40^{d}	3.96^{c}	3.41^{b}	3.20^{ab}	2.94^{a}	0.134	<0.001	<0.001	0.178
Molar proportion of VFA, %									
Acetate	67.9^{b}	66.1^{b}	63.0^{a}	62.6^{a}	61.2^{a}	0.84	<0.001	<0.001	0.273
Propionate	15.5^{a}	16.7^{ab}	18.5^{bc}	19.7^{cd}	20.9^{d}	0.55	<0.001	<0.001	0.845
Butyrate	9.22	9.42	10.3	9.95	9.80	0.526	0.619	0.319	0.329
BCVFA	7.32	7.71	8.17	7.69	7.98	0.312	0.381	0.196	<0.001
FE, %	72.2^{a}	72.9^{a}	73.9^{b}	74.4^{b}	74.9^{b}	0.26	<0.001	<0.001	0.496

TVFA, volatile fatty acids; *A:P*, acetate to propionate ratio; *BCVFA*, total branched-chain volatile fatty acids (isovalerate and isobutyrate); *FE*, fermentation efficiency; *FE*, fermentation efficiency; *SEM*, pooled standard error or mean

[a,b,c]Means with different superscripts in a row differ significantly ($P < 0.05$)

Blood biochemical markers

Blood glucose ($P = 0.028$) and albumin ($P = 0.007$) concentrations were increased by CPE (Table 6). Concentration of creatinine tended to decrease ($P = 0.075$) in the CPE-supplemented group compared to the control group. Concentrations of other blood metabolites, different liver-marker enzyme activities, and superoxide dismutase activity were not altered in goats due to feeding of CPE.

Plasma free amino acid profile

Among the individual amino acids, the concentration of free serine ($P = 0.002$), lysine ($P < 0.001$), and leucine ($P = 0.038$) elevated and concentration of aspartic acid tended to increase ($P = 0.089$) in goats fed CPE-containing diet (Table 7). Concentrations of total amino acids ($P = 0.010$), total essential amino acids ($P = 0.012$), total ketogenic amino acids ($P < 0.001$), total glucogenic amino acids ($P = 0.096$), and total branched-chain amino acids ($P = 0.072$) were greater or tended to be greater in the CPE-fed goats than the control goats.

Immune response

Cell-mediated immunity in goats was affected ($P = 0.046$) by time and treatment interaction (Fig. 1). The skin thickness tended ($P = 0.095$) to be greater at 12 h and was significantly greater ($P = 0.039$) at 72 h after phytohemagglutinin-P injection. The skin thickness after 24 h and 48 h of injection was similar ($P > 0.10$) in both groups.

Table 6 Effect of supplementation of composite plant extract (CPE) to the total mixed ration on the blood profile of lactating goats

Variable	Control	CPE	SEM	*P*-value
Glucose, mg/L	764	801	10.5	0.028
Total protein, g/L	79.1	78.2	3.08	0.834
Albumin (A), g/L	33.1	36.5	0.76	0.007
Globulin (G), g/L	46.0	41.6	2.88	0.305
A:G	0.73	0.93	0.082	0.105
Cholesterol, mg/L	642.6	648.4	37.4	0.914
Triglycerides, mg/L	336.5	323.2	13.7	0.506
Urea, mg/L	205.9	208.3	11.8	0.887
Creatinine, mg/L	8.84	6.59	0.82	0.075
AST, IU/L	85.7	81.9	5.76	0.655
ALT, IU/L	14.2	14.3	1.34	0.970
SOD, IU/L	357	289	28.0	0.166

AST, aspartate aminotransferase; *ALT*, alanine aminotransferase; *SOD*, superoxide dismutase; *SEM*, pooled standard error or mean

Table 7 Effect of inclusion of composite plant extracts (CPE) to the total mixed ration on the plasma free amino acid concentration (μM) in lactating goats

Variable	Control	CPE	SEM	*P*-value
Individual amino acids				
Histidine	394	412	7.80	0.130
Serine	67.1	82.6	2.73	0.002
Arginine	264	250	5.80	0.111
Glycine	485	508	13.6	0.257
Aspartic acid	19.1	22.8	1.41	0.089
Glutamic acid	148	139	4.46	0.179
Threonine	43.8	48.6	8.46	0.348
Alanine	153	148	5.10	0.465
Proline	88.0	86.0	3.46	0.684
Cysteine	9.96	12.0	0.92	0.141
Lysine	55.9	68.9	2.44	<0.001
Tyrosine	55.9	62.0	2.51	0.114
Methionine	18.2	19.4	0.84	0.325
Valine	318	330	8.72	0.336
Isoleucine	116	114	3.31	0.668
Leucine	145	155	3.01	0.038
Phenylalanine	37.8	36.4	1.99	0.621
Group of amino acids				
∑TAA	2415	2495	18.7	0.010
∑EAA	1388	1434	11.1	0.012
∑NEAA	1027	1060	14.0	0.115
∑KAA	196	224	3.23	<0.001
∑GAA	2009	2059	19.5	0.096
∑BCAA	579	599	7.26	0.072

EAA, essential amino acid; *NEAA*, non-essential amino acid; *TAA*, total amino acid; *KAA*, ketogenic amino acid; *GAA*, glucogenic amino acid; *BCAA*, branched-chain amino acid; *SEM*, pooled standard error of mean

EAA: Thr + Val + Met + Ile + Leu + Phe + His + Lys + Arg

NEAA: Ser + Glu + Asp + Pro + Gly + Ala + Cys + Tyr

TAA: EAA + NEAA

GAA:Thr + Val + Met + His + Arg + Ser + Glu + Gly + Ala + Pro + Asp + Cys

KAA:Leu + Lys

BCAA: Val + Ile + Leu

Discussion

Amoora rohituka belongs to *Meliaceae* family, which contains different types of plant metabolites such as flavonoids, terpenoids, glycosides, and anthraquinones (Singh et al. 2020; Kumara et al. 2014). *A. rohituka* is one of important Indian medicinal plants, which is commonly used in several herbal medicinal formulations (Kumara et al. 2014; Singh et al. 2020). In Ayurveda, the seeds of *Dolichos biflorus* are used in the treatment of different types of diseases and disorders and contain alkaloids, flavonoids, saponins, and

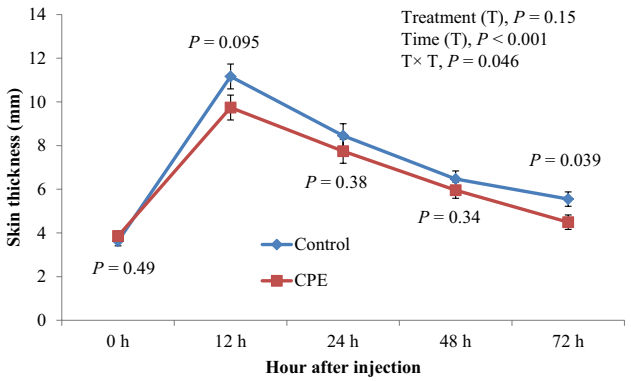

Fig. 1 Effect of supplementation of composite plant extract (CPE) on the on cell mediated immune response (skin thickness in mm) in lactating goats

tannins (Alok et al. 2014; Mathew et al. 2014). The therapeutic applications of *Asparagus racemosus* are reported in traditional systems of medicine and Indian and British Pharmacopoeias (Singh and Geetanjali 2016). Many bioactive phytochemicals mostly saponins, phenolics including flavonoids and tannins, and glycosides have been identified from the root of this plant which display various pharmacological activities (Kaur and Mondal 2014; Singh and Geetanjali 2016). The extract of *A. racemosus* is reported to contain total phenolic and flavonoid contents at 12.9 to 365 mg/g and 0.80 to 15.9 g/g dry weight, respectively, with high antioxidants and superoxide radical activities (Kaur and Mondal 2014; Behera 2018). Many steroidal saponins, i.e., shatavarin I (or asparoside B), immunoside and schidigerasaponin D5 (or asparanin A), shatavarin IV (or asparinin B), and shatavarins V–X, have been isolated from the roots of *Asparagus racemosus (*Hayes et al. 2008*)*. The peel of *Punica granatum* contains several polyphenolic compounds including flavonoids and their derivatives, tannins, and phenolic acids with many biological and pharmacological activities (Singh et al. 2018b).

The in vitro study was performed to determine a dose of the CPE that may exert beneficial responses on ruminal fermentation and reduce methane production. Gas production, IVTOMD, and IVNDFD increased due to supplementation of CPE linearly. The CPE dose of 20 g/kg DM showed significantly higher values compared with the control and even the CPE at 40 g/kg DM did not decrease ruminal fermentation efficiency. Increased degradability and gas production was attributed to the presence of both phenolics and saponins in CPE. In the present in vivo study also, DM digestibility increased and NDF digestibility tended to increase in goats fed CPE at 20 g/kg diet compared with the control goats. In this context, saponin-rich plant extract at 20 g/kg diet fed to goats did not increase nutrient digestibility (Hundal et al. 2020b), suggesting the only one plant extract at this dose did not stimulate ruminal microbiota. Saponins that have strong anti-protozoal property increased DM degradability in forage-based diets (Patra and Yu 2015a) and abundances of total bacteria and some fiber degrading bacteria in the rumen probably due to reduction of protozoa-associated bacterial engulfment and direct stimulation of select bacterial population (Patra et al. 2012b; Patra and Yu 2015b). The fermentation and microbial responses of saponins were dose- and type-dependent with lower doses showing stimulating effects, but higher doses exhibiting inhibitory effects (Patra et al. 2012b). Low doses of saponins were found to directly stimulate the growth of *Ruminococcus flavefaciens*, *Fibrobacter succinogenes*, and *Prevotella* genus (Patra et al. 2012b). Phenolics with antioxidant properties may also stimulate the strictly anaerobic microbiota by reducing the oxidative stress (Cattani et al. 2012). Growth of specific groups of microorganisms such as *Lactobacillus*, *Bifidobacteria*, and *Akkermansia* in the gut may be enhanced by polyphenolic compounds and their metabolites (Zhou et al. 2020). In a pure culture study, some phenolic acids stimulated the growth rate of ruminal bacteria in species and dose-specific manners (Borneman et al. 1986). High concentrations of polyphenolics and saponins are always toxic ruminal bacteria (Borneman et al. 1986; Patra and Saxena 2011; Patra et al. 2012a, 2012b2012b). In the present study, the doses of CPE up to 40 g/kg diet did not reduce IVTOMD, IVNDFD, and gas production probably due to the use of four extracts containing different types of saponins and polyphenolics at low concentrations.

In the present study, increased IVTOMD was associated with increased concentrations of total SCFA and all individual SCFA, but propionate and butyrate concentrations increased to greater extents. However, proportion of propionate elevated and acetate proportion decreased. This result signifies that this CPE improved all types of fermentative activities of the microbiota although there were preferential influences on the microbial communities particularly on the propionate producers. Saponins present in the CPE may increase propionate production (Patra et al. 2012b). Defaunating effect of saponins might be accountable to increased propionate production (Agarwal et al. 2006; Patra et al. 2012b). The increase in molar proportion of propionate that is energetically more efficient than acetate and butyrate is responsible for improved fermentation efficiency and this is expected to increase animal productivity. The CPE also decreased ammonia concentration linearly. Excessive ammonia production in the rumen arises due to degradation of dietary protein, which is absorbed from the rumen and excreted through urine as urea (Patra 2015) causing lower efficiency of protein utilization and environmental pollution (Agle et al. 2010; Patra et al. 2020). Lowered ammonia concentrations in the rumen occur due to formation of protein-phenolic compound complex,

inhibition of predominant protein degrading or ammonia producing microbiota, use of ammonia for synthesis of microbial protein, and inhibition of protozoa-associated ammonia production (Patra and Saxena 2009, 2011; Lagrange and Villalba 2019). In the present study, decreased ammonia concentration is unlikely contributed from decreased deamination from amino acids, because proportion of branched-chain fatty acids that are mainly formed from deamination of branched-chain amino acids by ruminal microbes (Apajalahti et al. 2019) did not decrease, rather it increased at 20 g/kg of CPE. Therefore, reduced ammonia concentration may be caused by increased microbial protein synthesis using amino acids or ammonia for their growth. Increased estimated microbial biomass was noted in the present in vitro experiment. The presence of saponins in the CPE may also decrease protozoal growth with the resultant of reduced bacterial protein turnover and ammonia production (Patra and Saxena 2009).

Furthermore, this CPE lowered methane production in vitro, which was probably attributed to predominantly increased propionate production. Propionate acts as a hydrogen sink redirecting hydrogen from methanogenesis to propionigenesis. Polyphenols, flavonoids, tannins, and saponins also decrease methane production directly inhibiting the methanogens, protozoa-related methanogens (Patra and Saxena 2010; Patra et al. 2017). Methane production was also lowered by CPE despite increased IVNDFD, suggesting that methane inhibition was not associated with decreased fiber digestibility by ruminal microbiota. Polyphenolic extracts or feeds from different plant sources also reduced methane production (Sinz et al. 2019; Huang et al. 2021).

Polyphenolics including flavonoids in diets can boost immunity, antioxidant balance, and productivity in ruminants (Oh et al. 2017; Olagaray and Bradford 2019). The effect of CPE was investigated for apparent toxic effects and health benefits. Blood lipid (concentrations of cholesterol and triglyceride) and liver enzyme (alanine aminotransferase and aspartate aminotransferase) markers were not altered suggesting that CPE had no effect on lipid metabolism and liver toxicity. Dietary CPE increased glucose concentration in blood, which was, as noted in the in vitro study, likely due to greater feed degradability and increased concentrations of propionate, a precursor of glucose formation. Blood albumin concentration increased in the CPE group, which indicated better protein nutrition and liver function in goats (Jordan and Swanson 1979). Furthermore, free plasma amino acid concentrations were greater in goats fed CPE than those in control goats, which may be ascribed due to greater microbial protein synthesis. Microbial protein usually has better protein quality compared to average dietary proteins fed to ruminants. Creatinine (a waste product of muscle protein breakdown) levels in blood, a marker of kidney function, tended to be lower due to CPE feeding, suggesting that CPE improved kidney function or protein nutrition in goats fed CPE. Increased protein intake reduced plasma creatinine concentration from 84 µmol/L for a high protein diet (140 g/kg) to 125 µmol/L for a low protein diet (120 g/kg) in goats (Valtonen et al. 1982).

Cell-mediated immunity in goats improved to a certain extent due to feeding of CPE. The impairments of immune cell integrity in a delayed-type hypersensitivity reaction due to oxidative stress may decrease immune responses (Latshaw 1991). This feed additive was rich in phenolic compounds including flavonoids, which have shown to boost endogenous antioxidant systems along with catalase, glutathione peroxidase, and superoxide dismutase and by upregulating genes and transcription factors related to antioxidant status (Oliveira et al. 2010; Oh et al. 2017). Polyphenol-rich extracts can improve lymphocyte functions, mitogen-induced lymphocyte cytokine releases, and balancing between oxidants and antioxidants in immune cells, leading to enhanced immune response (Oliveira et al. 2010; Ma et al. 2021). In the present study, superoxide dismutase activity in plasma was not affected by CPE, which might be attributed to the degradation in the rumen and intestine, use of a low level, and high health status (i.e., low oxidative stress) of goats. Similar to the present study, improved humoral and cell-mediated immunity was also reported due to feeding of pomegranate extract (15 and 30 mg gallic acid equivalent/kg body weight) to calves (Oliveira et al. 2010). Polyphenolic rich extract increased cell-mediated delayed type hypersensitivity reactions in buffaloes (Singh et al. 2022)

Finally, feeding CPE to lactating goats increased milk production, concentration of lactose and protein, and their yield, which was combined effects of increased feed digestibility, plasma free amino acid concentrations, ruminal propionate concentration, and blood glucose concentration in these goats. Similar to the present study, pomegranate peel extract, a component of CPE, enhanced milk production and efficiency in lactating cows fed 800 mL extract (11 g/kg feed DM), but not at a high (1200 mL/day) or a low (400 mL/day) dose (Abarghuei et al. 2013). In this study, propionate concentrations in the rumen and glucose concentration in blood were increased by CPE, which might increase lactose content in milk (Fisher and Elliot 1996). Pomegranate peel extract increased protein concentration in milk (Jami et al. 2012). High antioxidant properties of the CPE may reduce SCC in milk due to alleviation of oxidative stress in udders. A low level of SCC in raw milk indicates better udder health and hygienic milk quality because high SCC can arise due to inflammation of udders (Suriyasathaporn et al. 2006). Lipid peroxidation and malondialdehyde concentration may enhance oxidative stress, which may increase SCC in milk (Suriyasathaporn et al. 2006). A diet with condensed tannins was also reported to alleviate oxidative stress and decrease SCC in raw milk (Liu et al. 2013). Also, feeding *Emblica*

officinalis (rich in phenolic compounds and antioxidants) residue to lactating buffaloes reduced SCC in milk (Singla et al. 2021). Collectively, this study demonstrated that CPE rich in polyphenols and saponins reduced methanogenesis and improved feed degradability and fermentation in vitro and improved milk production, nutrient utilization, and health status of lactating goats. However, ruminal methane production and microbial communities affected by CPE feeding in goats are required to be investigated to better understand the findings.

Conclusions

In vitro study clearly showed that the use of CPE up to the dose of 40 g/kg diet increased dry matter and fiber degradability, whereas it decreased methane production and ammonia concentration. Also, concentrations of total VFA and proportion of propionate increased, whereas proportion of acetate and acetate to propionate ratio decreased linearly. In lactating goats fed CPE at 20 g/kg diet, milk yield and concentrations of protein and lactose increased and somatic cell counts in milk reduced. Moreover, apparent digestibility of DM and fiber improved due to supplementation of CPE. Blood biochemical profile either improved or not affected, while immune response was better in CPE-fed goats than the control goats. Therefore, this study demonstrates that the CPE rich in phenolics and saponins has potential to improve milk production and nutrient utilization in lactating goats with better health status while decreasing environmental burdens.

Author contribution JSH and SS conceived and designed research. SS, JSH, and MS conducted experiments. SS performed laboratory analysis. JSH and AKP analyzed the data. AKP and JSH wrote the manuscript. All authors read and approved the manuscript.

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Data availability Most of the data are presented in the tables. The data in the figure can be available from the corresponding author after a reasonable request.

Declarations

Ethics approval The study was conducted after getting the necessary approval from the Institutional Animal Ethics Committee of the University (CPCSEA/2021/83-1 dated 06.01.2021) working under CPCSEA, New Delhi, India.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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