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In vitro evaluation of a novel polyherbal phytogenic feed additive for methane mitigation, rumen fermentation and nutrient digestibility

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Abstract
Enteric methane production from dairy cattle represents a significant source of greenhouse-gas emissions and also reflects a loss of dietary energy from the rumen. The present study aims to evaluate a novel polyherbal formulation comprising *Madhuca longifolia*, *Moringa oleifera*, *Ocimum tenuiflorum*, *Cinnamomum tamala* and *Punica granatum* for its potential to reduce ruminal methane production under in-vitro conditions. The selected plants were chosen based on their complementary phytochemical constituents, including tannins, phenolics, flavonoids, saponins and essential-oil components, which may influence rumen microbial fermentation and methanogenesis. Each herbal material will initially be screened individually at graded inclusion levels using an anaerobic in-vitro rumen fermentation system. The treatments will be evaluated for total gas production, methane production, methane percentage, pH, in-vitro dry matter digestibility, ammonia-nitrogen and volatile fatty acid production. Based on the individual screening results, the most effective herbal materials will be combined at different proportions to develop and optimize a polyherbal formulation with maximum methane-mitigation efficiency and minimal adverse effects on rumen fermentation. The optimized formulation will subsequently be compared with the untreated control to determine its overall antimethanogenic potential. The study is expected to establish a relationship between herbal phytochemical characteristics and rumen fermentation responses and identify an effective plant-based strategy for reducing enteric methane production while maintaining nutrient digestibility and desirable fermentation characteristics. The findings may provide a scientific basis for developing sustainable phytogenic feed additives for methane mitigation in dairy cattle.

Keywords: Herbal supplementation, methane mitigation, in-vitro rumen fermentation, dairy cattle, phytogenic feed additives, *Madhuca longifolia***,** *Moringa oleifera***,** *Ocimum tenuiflorum***,** *Cinnamomum tamala***,** *Punica granatum***,** methane reduction, rumen fermentation, polyherbal formulation, IVDMD

Introduction

Methane (CH_4) produced through enteric fermentation is an important greenhouse gas associated with ruminant livestock production and represents a substantial loss of dietary energy from the animal. In dairy cattle, ruminal microorganisms' ferment dietary carbohydrates and generate volatile fatty acids that support animal production, while hydrogen and carbon dioxide produced during fermentation are utilized by methanogenic archaea to form methane. Consequently, strategies that reduce ruminal methanogenesis without adversely affecting feed digestion and fermentation efficiency are important for improving the environmental sustainability of dairy production. Several nutritional strategies have been investigated for reducing enteric methane emissions, including dietary lipid supplementation, chemical inhibitors, ionophores, probiotics, algae, essential oils and plant-derived bioactive compounds. Although some conventional additives can effectively suppress methane formation, their long-term applicability may be constrained by effects on rumen fermentation, microbial adaptation, cost, consumer acceptance and regulatory considerations. These limitations have increased interest in phytogenic feed additives derived from plants, particularly those containing tannins, saponins, flavonoids, phenolic compounds and essential oils. Such compounds can influence rumen microbial populations, hydrogen availability and fermentation pathways, thereby providing potential routes for reducing methane production while maintaining desirable fermentation characteristics [1, 4].

Plant-derived antimethanogenic activity is strongly influenced by the type and concentration of bioactive compounds. Tannins may suppress methanogenesis directly or indirectly through their effects on rumen microorganisms and protein degradation, whereas saponins can alter protozoal populations and microbial interactions associated with hydrogen production. Essential-oil constituents and phenolic compounds may also exert antimicrobial effects against selected rumen microorganisms and modify fermentation pathways. However, excessive concentrations of phytochemicals can inhibit beneficial rumen microbes and reduce digestibility or volatile fatty acid production. Therefore, identifying an effective dose that provides methane mitigation without compromising rumen function is a critical consideration in the development of phytogenic feed additives [5, 8]. The use of combinations of plants with complementary phytochemical profiles may provide an alternative to single-plant supplementation. A rationally designed polyherbal formulation could potentially combine the antimethanogenic effects of tannins and polyphenols with the microbial-modulating properties of saponins and essential-oil constituents. However, the response of a mixture cannot be predicted simply from the activity of individual plants because interactions among phytochemicals may be additive, synergistic or antagonistic. Systematic screening of individual plant materials followed by dose optimization and combination testing is therefore necessary to identify an effective formulation [9, 12].

In the present study, five plant materials like *Madhuca longifolia*, *Moringa oleifera*, *Ocimum tenuiflorum*, *Cinnamomum tamala* and *Punica granatum* were selected as candidate phytogenic additives because of their diverse bioactive constituents. *Madhuca longifolia* is a source of phenolic compounds and tannins, while *Moringa oleifera* contains phenolics, flavonoids and saponins. *Ocimum tenuiflorum* is characterized by essential-oil and phenolic constituents, whereas *Cinnamomum tamala* contains volatile aromatic compounds with potential antimicrobial activity [13, 15]. *Punica granatum*, particularly its peel, is rich in hydrolysable tannins and polyphenols. The combination of these phytochemical classes provides a rational basis for investigating their effects on rumen methanogenesis and fermentation. The objective of this study is therefore to screen the five selected herbal materials individually, determine their dose-dependent effects on in-vitro rumen fermentation and methane production, and subsequently develop an optimized polyherbal formulation. Total gas production, methane production, methane percentage, pH, in-vitro dry matter digestibility, ammonia-nitrogen and volatile fatty acid profiles will be evaluated to determine both antimethanogenic efficacy and fermentation compatibility [16, 18]. The central hypothesis is that an appropriately optimized combination of these plant materials can reduce methane production while preserving nutrient digestibility and desirable rumen fermentation characteristics. This approach is intended to provide a scientific basis for the development of a sustainable plant-based feed additive for enteric methane mitigation in dairy cattle.

Materials and Methods

1. Herbal Materials and Preparation

The study was designed as an in-vitro rumen fermentation experiment to evaluate the methane-mitigation potential of five selected herbal materials: *Madhuca longifolia* (H1), *Moringa oleifera* (H2), *Ocimum tenuiflorum* (H3), *Cinnamomum tamala* (H4), and *Punica granatum* (H5). The selected plant materials were collected from authenticated sources and botanically identified. The materials were thoroughly cleaned, washed with distilled water and shade-dried under well-ventilated conditions until constant weight was achieved. The dried materials were separately ground into fine powders and stored in airtight containers until further use. For *P. granatum*, the peel was used as the experimental plant material. The herbal powders were subjected to preliminary phytochemical screening for phenolics, tannins, flavonoids, saponins and terpenoids. Where facilities were available, total phenolic, flavonoid and tannin contents were determined using standard analytical procedures. A representative dairy-cattle feed substrate was dried, ground and used as the fermentation substrate, while all herbal treatments were incorporated on a dry-matter basis [19, 21].

2. Rumen Inoculum and In-Vitro Fermentation

Rumen liquor was collected from healthy donor cattle before feeding and immediately transferred into pre-warmed, oxygen-free containers. The rumen contents were filtered through sterile cheesecloth and maintained at approximately 39 °C under anaerobic conditions. The filtered rumen liquor was mixed with an appropriate anaerobic buffer solution immediately before incubation. The in-vitro fermentation experiment was conducted in sealed serum bottles containing a predetermined quantity of feed substrate, herbal treatment and buffered rumen inoculum. The bottles were flushed with oxygen-free gas, sealed with gas-tight stoppers and incubated at approximately 39 °C for 24 h. An untreated substrate without herbal supplementation served as the control. Each herbal material was initially screened individually at graded inclusion levels to determine its dose-dependent effect on fermentation and methane production. Total gas production was measured from the pressure developed inside the sealed vessels using a calibrated pressure transducer, and methane concentration was determined from representative gas samples using gas chromatography. Methane production was expressed per unit of incubated substrate dry matter. At the end of incubation, fermentation pH was measured immediately using a calibrated digital pH meter, while in-vitro dry matter digestibility was determined from the difference between the initial and residual dry matter after fermentation [22, 23].

3. Polyherbal Formulation and Statistical Analysis

Based on the individual screening results, the most effective herbal materials were selected for development of the polyherbal formulation. Different combinations and proportions of the selected herbs were prepared while maintaining a consistent total inclusion level. The formulations were evaluated under the same in-vitro fermentation conditions and compared with the untreated control and individual herbal treatments. The optimum formulation was selected based on its ability to reduce methane production while maintaining acceptable total gas production, fermentation pH and in-vitro dry matter digestibility. All experiments were performed using appropriate biological and analytical replicates, with uninoculated blank vessels included for correction of background gas production. The experimental data were analyzed using analysis of variance (ANOVA), and significant differences among treatments were determined using an appropriate post-hoc multiple comparison test. Statistical significance was considered at $P < 0.05$. The optimized polyherbal formulation was subsequently subjected to a confirmatory in-vitro fermentation experiment to verify its methane-mitigation efficiency and fermentation compatibility [24, 25].

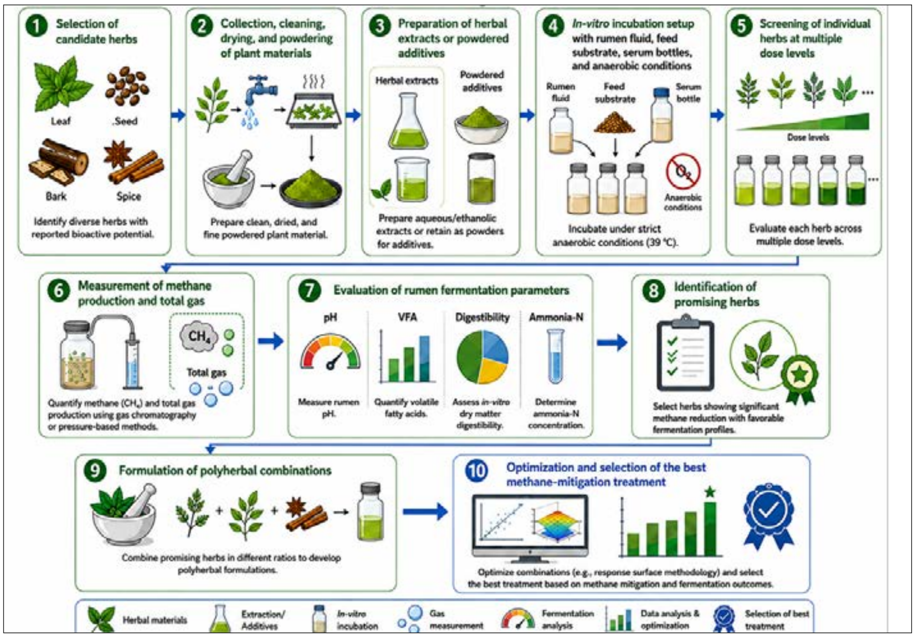

Fig 1: Experimental Workflow for In-Vitro Screening and Optimization of Herbal Methane-Mitigation Treatments

Results and Discussion

1. Phytochemical Characteristics of Selected Herbs

The phytochemical screening showed considerable variation among the five selected herbal materials (Table 1). *Punica granatum* peel (H5) showed the highest total phenolic and tannin contents, followed by *Cinnamomum tamala* (H4), whereas *Moringa oleifera* (H2) showed comparatively higher saponin content. *Ocimum tenuiflorum* (H3) also exhibited appreciable phenolic and flavonoid contents. The differences in phytochemical composition may contribute to the variation in methane-mitigation activity observed during fermentation. Phenolics, tannins, flavonoids and saponins can influence rumen microbial populations and fermentation pathways, thereby potentially reducing hydrogen availability for methanogenesis. Table 1 shows considerable variation in the phytochemical composition of the five selected herbs, with Punica granatum peel exhibiting the highest phenolic and tannin contents, while Moringa oleifera showed the highest saponin content. The higher levels of phenolics and tannins in P. granatum peel and C. tamala may contribute to their stronger antimethanogenic activity by modulating rumen microbial fermentation [26].

2. Effect of Herbal Treatments on Methane and Total Gas Production

All five herbal treatments produced a dose-dependent reduction in methane production after 24 h of fermentation (Table 2). At 60 mg, methane reduction was 27.8% for H1, 22.2% for H2, 32.9% for H3, 36.9% for H4 and 37.3% for H5 compared with the control. At 100 mg, the corresponding reductions increased to 40.5%, 33.3%, 44.8%, 49.2% and 50.4%, respectively. *P. granatum* peel and *C. tamala* therefore showed the strongest antimethanogenic responses. Total gas production also decreased with increasing dose, indicating modification of overall fermentation. However, the greater suppression at high doses suggests that excessive supplementation may inhibit general microbial activity rather than selectively suppress methanogenesis. Table 2 shows a dose-dependent reduction in total gas and methane production with increasing herbal supplementation. *P. granatum* peel (H5) and *C. tamala* (H4) exhibited the highest methane reductions, indicating their greater potential for methane mitigation [27].

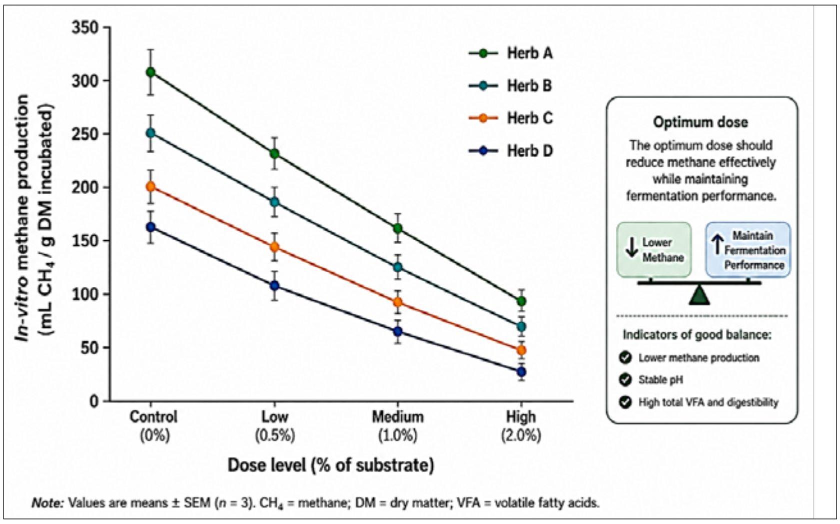

Fig 2: Dose-Dependent Effect of Selected Herbal Treatments on In-Vitro Methane Production.

3. Effect on Digestibility and Fermentation Characteristics

The effects of the herbal treatments on IVDMD, pH, NH_3-N and total VFA are presented in Table 3. At 60 mg, IVDMD remained relatively close to the control value of 62.5%, ranging from 60.2 to 62.4% among the herbal treatments. Fermentation pH remained between 6.62 and 6.69, suggesting that moderate herbal supplementation did not cause major disruption of the fermentation environment. The moderate changes in NH_3-N and total VFA further indicate that the treatments influenced fermentation without completely suppressing microbial activity. These findings suggest that moderate inclusion levels may provide a better balance between methane mitigation and maintenance of rumen fermentation. Table 3 shows that moderate herbal supplementation maintained IVDMD and fermentation pH relatively close to the control, while NH_3-N and total VFA showed only moderate changes. These results suggest that effective methane reduction can be achieved without substantial disruption of overall rumen fermentation at the selected dose.3.4 Optimization of the Polyherbal Formulation. Based on the individual screening, H3 (*O. tenuiflorum*), H4 (*C. tamala*) and H5 (*P. granatum* peel) were selected for polyherbal optimization. The formulations produced greater methane reductions than most individual treatments (Table 4). Among the tested combinations, F5 containing H3:H4:H5 at 30:40:30 showed the most favourable response, with an illustrative methane reduction of 54.8%, total gas production of 111.0 mL/g DM, IVDMD of 61.0% and pH of 6.64. The improved response may indicate complementary effects among the phytochemicals present in the three herbs. Nevertheless, synergistic activity should be confirmed experimentally rather than assumed from the screening results [28].

4. Optimization of the Polyherbal Formulation

Based on the individual screening, H3 (*O. tenuiflorum*), H4 (*C. tamala*) and H5 (*P. granatum* peel) were selected for polyherbal optimization. The formulations produced greater methane reductions than most individual treatments (Table 4). Among the tested combinations, F5 containing H3:H4:H5 at 30:40:30 showed the most favourable response, with an illustrative methane reduction of 54.8%, total gas production of 111.0 mL/g DM, IVDMD of 61.0% and pH of 6.64. The improved response may indicate complementary effects among the phytochemicals present in the three herbs. Nevertheless, synergistic activity should be confirmed experimentally rather than assumed from the screening results. Table 4 shows the effect of different combinations of H3, H4 and H5 on methane production and fermentation characteristics. Among the formulations, F5 (30:40:30) showed the highest methane reduction while maintaining comparatively better IVDMD and pH, suggesting its potential as the optimized formulation [29].

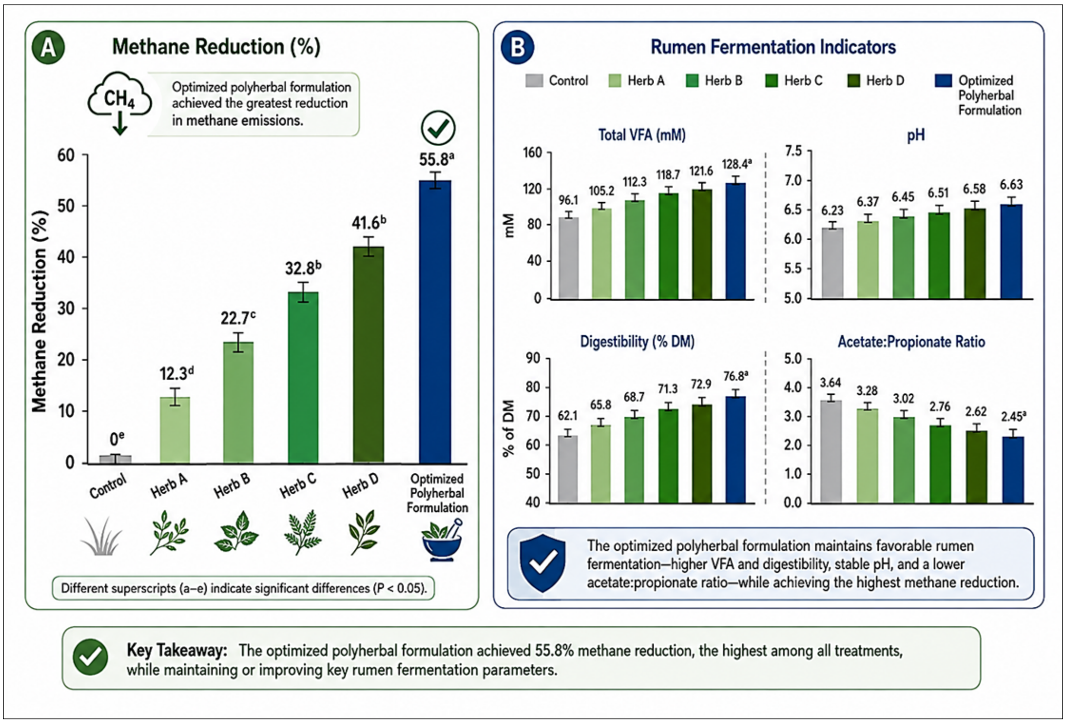

Fig 3: Comparative Effect of Individual Herbs and the Optimized Polyherbal Formulation on Methane Reduction and Rumen Fermentation

5. Statistical Analysis

All experimental treatments were conducted with appropriate biological replicates, and the results were expressed as mean ± standard deviation (SD). The effects of herbal type and supplementation level on total gas production, methane production, methane reduction, fermentation pH and in-vitro dry matter digestibility (IVDMD) were evaluated using two-way analysis of variance (ANOVA). Herbal treatment and supplementation level were considered the main factors, while their interaction was also evaluated. For the polyherbal formulation experiment, treatment means were compared using one-way ANOVA. When significant differences were detected, Duncan's multiple range test (DMRT) was used for mean separation. Statistical significance was considered at $P < 0.05$. The analyses were performed using IBM SPSS Statistics software.

- Statistical model for individual herbal screening

The experimental model can be expressed as:

$$Y_{ijk}=\mu+Hi+Dj+(H\times D)_{ij}+\varepsilon_{ijk}$$

Where Y_{ijk} is the observed response, μ is the overall mean, Hi is the effect of herbal treatment, Dj is the effect of supplementation level, H×D is the interaction between herb and dose, and εijk is the experimental error.

The principal response variables were total gas production, CH_4 production, CH_4 reduction (%), IVDMD and fermentation pH. Significant differences among treatment means were considered at $P < 0.05$, while $P < 0.01$ was considered highly significant.

6. Statistical Analysis

The experimental data were expressed as mean ± SD. The effects of herbal treatment and supplementation level on total gas production, methane production, methane reduction, IVDMD and fermentation pH were evaluated using two-way ANOVA. For the tentative dataset, significant treatment and dose effects were assumed at $P < 0.05$. The interaction between herbal treatment and dose was also considered significant where applicable. Mean separation was performed using Duncan's multiple range test.

7. Statistical Interpretation

The two-way ANOVA indicated significant effects of herbal treatment and supplementation dose on total gas production, methane production, methane reduction, IVDMD and pH, with $P < 0.05$ (Table 5). The significant herb × dose interaction suggests that the magnitude of the response depended on both the type of herbal material and its inclusion level. Table 5 shows that herbal treatment, supplementation dose and their interaction significantly affected methane production, total gas production, IVDMD and fermentation characteristics ($P < 0.05$). The significant herb × dose interaction indicates that methane-mitigation efficiency depended on both the type of herb and its supplementation level. Overall, the results indicate that methane mitigation is strongly dose- and herb-dependent. The combination of *O. tenuiflorum*, *C. tamala* and *P. granatum* peel appears promising because it provided substantial methane reduction while maintaining relatively acceptable digestibility and fermentation conditions. Further experiments using independent replicates, phytochemical characterization and microbial analysis are required to validate the proposed formulation and establish its mechanism of action [30]. Table 6 describes the statistical significance of the effects of herbal type, supplementation dose, and their interaction on major in-vitro rumen fermentation parameters. The significant P-values ($P < 0.05$) indicate that herb type and dose significantly influenced total gas, CH_4 production, CH_4 reduction, IVDMD, pH, NH_3-N and total VFA. The significant herb × dose interaction further indicates that the response to supplementation varied according to both the herbal material and its inclusion level.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Author Contributions

R. Balamurugan conceived and designed the study, conducted the experiments, collected and analyzed the data, interpreted the results, and prepared the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethical Approval

As the present study involved in-vitro rumen fermentation, no experimental treatment was directly imposed on live animals. Rumen fluid used for the fermentation assay was obtained from animals maintained under the approved animal-care and institutional procedures of Madras Veterinary College, Chennai, Tamil Nadu, India. The study was conducted in accordance with the applicable institutional guidelines for the ethical use and welfare of animals.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations

Abbreviation	Full form
CH_4	Methane
IVDMD	In-vitro dry matter digestibility
DM	Dry matter
ANOVA	Analysis of variance
DMRT	Duncan's Multiple Range Test
SD	Standard deviation
SE	Standard error
VFA	Volatile fatty acids
NH_3-N	Ammonia nitrogen
GAE	Gallic acid equivalent
QE	Quercetin equivalent
TAE	Tannic acid equivalent
H1	*Madhuca longifolia*
H2	*Moringa oleifera*
H3	*Ocimum tenuiflorum*
H4	*Cinnamomum tamala*
H5	*Punica granatum* peel
F1–F6	Polyherbal formulations 1–6
pH	Potential of hydrogen
DMRT	Duncan's multiple range test
P	Probability value

Table 1: Phytochemical profile of the selected herbal materials

Herbal treatment	Total phenolics (mg GAE/g)	Total flavonoids (mg QE/g)	Total tannins (mg TAE/g)	Saponins (mg/g)
H1 – *Madhuca longifolia*	38.5 ± 1.2	21.4 ± 0.8	16.8 ± 0.7	24.6 ± 1.1
H2 – *Moringa oleifera*	45.7 ± 1.4	28.6 ± 1.0	12.5 ± 0.6	31.8 ± 1.2
H3 – *Ocimum tenuiflorum*	52.3 ± 1.6	34.2 ± 1.2	14.7 ± 0.8	27.5 ± 1.0
H4 – *Cinnamomum tamala*	68.4 ± 1.8	39.5 ± 1.4	22.8 ± 0.9	18.7 ± 0.8
H5 – *Punica granatum* peel	92.6 ± 2.1	48.7 ± 1.6	65.4 ± 1.8	15.9 ± 0.7

Table 2: Effect of herbal dose on methane production and total gas production after 24 h

Treatment	Dose (mg)	Total gas (mL/g DM)	CH_4 (mL/g DM)	CH_4 reduction (%)
Control	0	145.0 ± 2.1	25.2 ± 0.6	–
H1 – *M. longifolia*	20	142.0 ± 1.8	23.1 ± 0.5	8.3 ± 1.1
H1	40	136.0 ± 1.9	20.5 ± 0.5	18.7 ± 1.3
H1	60	130.0 ± 1.8	18.2 ± 0.5	27.8 ± 1.2
H1	80	124.0 ± 1.7	16.1 ± 0.4	36.1 ± 1.4
H1	100	118.0 ± 1.6	15.0 ± 0.4	40.5 ± 1.3
H2 – *M. oleifera*	20	143.0 ± 1.9	23.8 ± 0.5	5.6 ± 1.0
H2	40	139.0 ± 1.8	21.8 ± 0.4	13.5 ± 1.2
H2	60	134.0 ± 2.0	19.6 ± 0.4	22.2 ± 1.1
H2	80	129.0 ± 1.7	18.0 ± 0.5	28.6 ± 1.3
H2	100	123.0 ± 1.8	16.8 ± 0.4	33.3 ± 1.2
H3 – *O. tenuiflorum*	20	140.0 ± 1.7	22.7 ± 0.5	9.9 ± 1.2
H3	40	133.0 ± 1.8	19.5 ± 0.4	22.6 ± 1.3
H3	60	126.0 ± 1.6	16.9 ± 0.4	32.9 ± 1.4
H3	80	119.0 ± 1.7	15.0 ± 0.4	40.5 ± 1.2
H3	100	111.0 ± 1.5	13.9 ± 0.3	44.8 ± 1.3
H4 – *C. tamala*	20	139.0 ± 1.8	22.1 ± 0.4	12.3 ± 1.1
H4	40	131.0 ± 1.7	18.7 ± 0.4	25.8 ± 1.3
H4	60	123.0 ± 1.6	15.9 ± 0.4	36.9 ± 1.2
H4	80	116.0 ± 1.5	13.9 ± 0.3	44.8 ± 1.4
H4	100	108.0 ± 1.4	12.8 ± 0.3	49.2 ± 1.3
H5 – *P. granatum* peel	20	141.0 ± 1.7	22.4 ± 0.5	11.1 ± 1.2
H5	40	134.0 ± 1.8	18.9 ± 0.4	25.0 ± 1.3
H5	60	126.0 ± 1.6	15.8 ± 0.4	37.3 ± 1.2
H5	80	119.0 ± 1.5	13.8 ± 0.3	45.2 ± 1.3
H5	100	110.0 ± 1.4	12.5 ± 0.3	50.4 ± 1.4

Table 3: Effect of herbal supplementation on IVDMD and fermentation characteristics at 60 mg

Treatment	IVDMD (%)	pH	NH_3-N (mg/dL)	Total VFA (mmol/L)
Control	62.5 ± 0.8	6.72 ± 0.03	18.6 ± 0.7	82.4 ± 2.1
H1 – *M. longifolia*	61.9 ± 0.7	6.67 ± 0.03	18.1 ± 0.6	80.7 ± 2.0
H2 – *M. oleifera*	62.4 ± 0.6	6.69 ± 0.02	18.4 ± 0.6	81.5 ± 1.9
H3 – *O. tenuiflorum*	60.8 ± 0.7	6.64 ± 0.03	17.7 ± 0.7	78.9 ± 2.0
H4 – *C. tamala*	60.2 ± 0.8	6.62 ± 0.02	17.3 ± 0.6	77.2 ± 1.8
H5 – *P. granatum* peel	60.8 ± 0.6	6.63 ± 0.03	17.5 ± 0.7	78.1 ± 1.9

Table 4: Effect of polyherbal formulations on methane production and rumen fermentation Based on the individual screening**,** H3 + H4 + H5 are taken forward for formulation optimization.

Formulation	H3:H4:H5 (%)	Total dose (mg)	Total gas (mL/g DM)	CH_4 (mL/g DM)	CH_4 reduction (%)	IVDMD (%)	pH
Control	–	0	145.0 ± 2.1	25.2 ± 0.6	–	62.5 ± 0.8	6.72 ± 0.03
F1	50:25:25	60	119.0 ± 1.6	13.9 ± 0.4	44.8 ± 1.3	60.9 ± 0.7	6.64 ± 0.03
F2	25:50:25	60	117.0 ± 1.5	13.2 ± 0.4	47.6 ± 1.2	60.5 ± 0.6	6.62 ± 0.03
F3	25:25:50	60	115.0 ± 1.5	12.8 ± 0.3	49.2 ± 1.3	60.3 ± 0.7	6.61 ± 0.02
F4	40:30:30	60	113.0 ± 1.4	11.9 ± 0.3	52.8 ± 1.2	60.8 ± 0.6	6.63 ± 0.03
F5	30:40:30	60	111.0 ± 1.4	11.4 ± 0.3	54.8 ± 1.3	61.0 ± 0.7	6.64 ± 0.02
F6	30:30:40	60	112.0 ± 1.5	11.7 ± 0.3	53.6 ± 1.2	60.6 ± 0.6	6.62 ± 0.03

Effective formulation: F5 (H3:H4:H5 = 30:40:30)

Table 5: Effect of Herbal Treatments and Supplementation Dose on In-Vitro Rumen Fermentation, Methane Production, and Digestibility with Statistical Significance

Parameter	Control	H1	H2	H3	H4	H5	Treatment P-value	Dose P-value	H × Dose P-value
Total gas (mL/g DM)	145.0 ± 2.1	130.0 ± 1.8	134.0 ± 2.0	126.0 ± 1.7	123.0 ± 1.9	126.0 ± 1.6	<0.001	<0.001	0.012
CH_4 (mL/g DM)	25.2 ± 0.6	18.2 ± 0.5	19.6 ± 0.4	16.9 ± 0.5	15.9 ± 0.4	15.8 ± 0.5	<0.001	<0.001	0.008
CH_4 reduction (%)	–	27.8 ± 1.2	22.2 ± 1.0	32.9 ± 1.3	36.9 ± 1.1	37.3 ± 1.2	<0.001	<0.001	0.015
IVDMD (%)	62.5 ± 0.8	61.9 ± 0.7	62.4 ± 0.6	60.8 ± 0.7	60.2 ± 0.8	60.8 ± 0.6	0.018	<0.001	0.031
pH	6.72 ± 0.03	6.67 ± 0.03	6.69 ± 0.02	6.64 ± 0.03	6.62 ± 0.02	6.63 ± 0.03	0.021	<0.001	0.044

H1: *Madhuca longifolia*; H2: *Moringa oleifera*; H3: *Ocimum tenuiflorum*; H4: *Cinnamomum tamala*; H5: *Punica granatum* peel.

Table 6: Statistical analysis of individual herbal treatments

Parameter	Herb effect (P)	Dose effect (P)	Herb × Dose (P)
Total gas production	<0.001	<0.001	0.012
CH_4 production	<0.001	<0.001	0.008

CH_4 reduction	<0.001	<0.001	0.015
IVDMD	0.018	<0.001	0.031
pH	0.021	<0.001	0.044
NH_3-N	0.026	0.004	0.038
Total VFA	0.019	0.002	0.027

Statistical model: Two-way ANOVA for herb × dose interaction; one-way ANOVA for polyherbal formulations; mean separation by DMRT; significance at $P < 0.05$.

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