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Synergistic effects of bioactive plant extract mixtures on methane reduction and rumen fermentation of eragrostis curvula hay in vitro

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Building on prior research indicating the methane-mitigating potential of specific medicinal plants for ruminant animals. This study aimed to investigate the associative effects of combining six medicinal plant extracts known for their methane-reducing properties: *Aloe vera* (AV), *Carica papaya* (CP), *Azadirachta indica* (AZ), *Tithonia diversifolia* (TD), *Jatropha curcas* (JA), and *Moringa oleifera* (MO). Methanolic extracts of the plants were combined in equal proportions into two-way mixtures and applied at a concentration of 50 mg/L to 400 mg Eragrostis curvula hay, followed by a 48-hour in vitro incubation. Phytochemical profiling of individual plant extracts was performed using LC-MS and HPLC methods. Evaluated parameters included methane (CH_4) production, total gas production (TGP), in vitro organic matter digestibility (IVOMD), ammonia nitrogen, and volatile fatty acids (VFA). Phytochemical profiling revealed diverse bioactive compounds such as flavonoids, saponins, anthraquinones, phenols, alkaloids, and terpenoids in all extracts, with AZ showing the highest phenolic content. The mixtures significantly reduced CH4 production by over 50%, individual plant extracts generally showed greater improvements in IVOMD compared to mixtures. Moreover, the mixture displayed positive associative effects on various parameters, including TVFA, CH_4/IVOMD, CH_4/TGP, and CH_4/TVFA production. Two-way mixture containing AV or CP (AV + CP, AV + JA) notably increased propionic acid concentration, differentiating them from single plant extracts, monensin, and control treatments. The study highlights that specific combinations of these medicinal plant extracts can significantly reduce methane emissions while positively modulating rumen fermentation parameters, indicating their potential as natural additives for sustainable livestock production.

Keywords Methane emission, Medicinal plants, Phytochemicals, Antibiotics alternatives

Ruminants' unique ability to convert dietary fibre into milk, meat, and wool has made them an important component of agro ecosystem. Over the recent years, they have been intensively managed to increase animal performance and production efficiency. The use of antibiotic growth promoters and rumen modulators enabled individual animals to decrease energy loss through eructation, increase bowel movement, suppress actions and activities of protozoans in the rumen, and reduce rumen methanogens[1] which converts surplus H_2 in the rumen to CH_4. Methane is a greenhouse gas that is 23 times more potent as a greenhouse gas than CO_2[2].

However, several countries have put restrictions on the use of antibiotic growth promoters in livestock feeding[3], This is because traces of antibiotics that might be present in the products of livestock raised with antibiotic growth promoters may be associated to resistance to these antibiotics when used in human medicine. This necessitates the search for alternative products preferably from natural sources. Medicinal plants have been used for different purposes, and in traditional medicine, some plants have been identified for the treatment of different diseases[4]. Scientific research on these plants has revealed the presence of some secondary plant metabolites responsible for their activities and ability to cure certain conditions. These secondary metabolites were developed over time in plants as defensive mechanisms to ensure their survival against grazing, drought, and endemic or new disease conditions[5]. In ruminant nutrition, different plant secondary metabolites have been

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assessed and researched to determine potential in terms of reducing CH_4, combating diseases, and improving digestibility without adversely affecting animal health and performance[6–8].

Aloe vera (AV), *Azadirachta indica* (AZ), *Carica papaya* (CP), *Jatropha curcas* (JA), *Moringa oleifera* (MO), and *Tithonia diversifolia* (TD) were specifically selected due to their previously demonstrated effectiveness in mitigating methane emissions in ruminants' feeds[6]. AV and AZ are particularly rich in bioactive anthraquinones, flavonoids, and phenolic compounds exhibiting antimicrobial, antiparasitic, and anti-methanogenic properties[9–11]. CP and JA extracts contain significant concentrations of saponins, cardiac glycosides, alkaloids, and fatty acids known to inhibit rumen methanogens, decrease protozoal activity, and thereby reduce methane production[12,13]. MO is recognised for its abundant flavonoids, tannins, and saponins, which positively modulate rumen microbial ecology, enhancing fibre digestion and reducing methane emissions[14,15]. While TD is valued for its diverse phenolic and terpenoid constituents that influence microbial populations and promote rumen fermentation efficiency[16,17].

Several of these plants are known to contain more than one type of secondary plant metabolite at various concentration levels. There is a need to analyse the phytochemicals present in these medicinal plants, which may be responsible for their antimicrobial, anti-protozoan, and anti-methanogenic properties. Furthermore, since there is a variety of secondary compounds found in different plants, mixtures of these selected medicinal plants might have synergistic effects at mitigating the activities of methanogens, combating protozoans in the rumen, or working to break down cell walls present in roughages. This study, therefore, profiled the metabolomics of the methanol extract of AV, AZ, CP, JA, MO, and TD plant extracts. We hypothesised that two-way combinations of these medicinal plant extracts would exhibit associative effects on CH_4 production, organic matter digestibility, and concentrations of volatile fatty acids compared to their individual effects.

Materials and methods

Approval for use of animal was obtained from the Animal Ethics Committee, University of Pretoria (ECO-03014), all methods were carried out in accordance with relevant guidelines and regulations and reported in accordance with ARRIVE guidelines.

Collection, preparation, and plant extracts metabolites

Fresh 4 kg samples of AV, AZ, TD, CP, JA, and MO were each harvested from at least 10 individual trees between March and July during the growing season at the University of Ibadan campus. The samples were washed and then freeze-dried for 5 days. The dried samples were milled through a 1 mm sieve and extracted by dissolving 200 g dried plant materials in a flask containing 2000 mL of pure methanol. The mixture was placed in a shaker at 20 °C for 96 h. The contents of the flask were then filtered through a 150 µm aperture. Excess methanol in the filtrate was removed under a vacuum using a Rotorvapor Hie-VAP (Hiedolph, Germany) at 35 °C to eliminate moisture. The resulting extract was then transferred to a freeze dryer for 2 days to complete drying. All extracts were obtained in powder form, except for AV, which yielded a gel-like substance. The crude extracts were stored at 4 °C for future use.

Phytochemical profiling of the methanolic extracts of AV, AZ, CP, JA, MO, and TD was conducted using high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS). HPLC analysis was performed using a Thermo Scientific Finnigan Surveyor system equipped with a PDA Plus detector (220–340 nm), using a Gemini 5 µm C6-Phenyl 110 Å column (250 × 4.60 mm) and a linear gradient elution system. For LC-MS, approximately 100 mg of each plant extract was reconstituted in 2 mL of 50% acetonitrile-water solution, vortexed, sonicated, centrifuged, diluted tenfold with 50% methanol containing 0.1% formic acid, and analysed using a Waters Acquity UPLC coupled with a Waters Synapt G2 instrument. Compounds were identified and quantified by comparison with authenticated standards (Sigma-Aldrich Ltd, TCI AMERICA, Apin Chemicals Ltd, ACROS Chemicals). Total phenolic content was quantified using the Folin–Ciocalteu method[18]. Phytochemical classification was based on Harborne[19], covering key secondary metabolites such as flavonoids, tannins, saponins, alkaloids, and anthraquinones.

Experimental design

The extracts of AV, AZ, TD, CP, JA, and MO were reconstituted by dissolving 50 mg of each extract in 1000 mL of distilled water. Equal proportions of each plant extract solution were combined to create two-way mixture, and 4 mL of these mixture were administered as different treatments to 400 mg of *E. curvula* hay in the following sequence: AZ + AV, AV + JA, AV + CP, AV + MO, AV + TD, AZ + JA, AZ + CP, AZ + MO, AZ + TD, JA + CP, JA + MO, JA + TD, CP + MO, CP + TD, and MO + TD. Monensin sodium was used as a positive control at a recommended dose of 15 mg/kg of feed DM, 15 mg was dissolved in 1000 mL distilled water. A negative control group received 4 mL of distilled water as a placebo. Individual plant extracts were also included in the treatments to assess the disparity between expected and observed values, using the Chi-Square test. In total, 23 different treatments were tested, comprising 2 control groups, six single plant extracts, and 15 distinct mixtures, with each receiving 4 mL of various plant extracts, monensin, or water.

In vitro gas and methane production

Two ruminally fistulated South African Merino sheep, housed at the experimental farm, Innovation Africa campus, University of Pretoria, served as rumen fluid donors for the duration of the experiment. They were provided with unlimited access to alfalfa hay as their sole diet. Ruminal content was collected via the cannula from the rumen. Approximately 1000 mL of fluid was obtained from each sheep after the rumen content was carefully strained through four layers of cheesecloth and collected in pre-heated thermos flasks. The collected rumen fluid was swiftly transported to the laboratory within 10 min of collection. Upon arrival, it was placed in a water bath set at 39 °C, where it was thoroughly mixed and continuously flushed with CO_2. This process aimed

to maintain an anaerobic environment and minimize oxygen contamination. A buffer mineral solution was prepared in accordance with the procedure outlined by Menke and Steingass[20]. The substitution of $MgSO_4.7H_2O$ with $MgCl_2.6H_2O$ in the buffer media was undertaken to lower SO_4 in the media[21]. Reducing solution L-cysteine and $Na_2S_9.H_2O$ were added as previously reported[6]. The solution was bathed at 39 °C.

In vitro incubation

In vitro ruminal incubations were conducted in 100 mL serum bottles. Each treatment, including the controls and plant extract combinations, was tested in three (3) replicates per experimental run, arranged in a randomised complete block design. The entire experiment comprised five independent runs. Before incubation, 400 mg of *E. curvula* hay was weighed into each 150 mL serum bottle. Subsequently, 4 mL of pre-prepared single and two-way mixture consisting of AV, CP, AZ, MO, TD, and JA; were added to the bottles, along with 25 mL of prepared media and 15 mL of rumen fluid. After adding rumen fluid, the bottles were purged with CO_2 gas and immediately sealed with rubber stoppers. They were then crimp-sealed and transferred to an incubator set at 39 °C with an oscillatory motion of 120 rpm. A modified needle syringe tap, capable of opening and closing, was inserted into each vial. These taps were opened for 5 s to release any accumulated gas and establish a common starting point for all the vials. The replicates within a single run were used to calculate average values, and the runs themselves were treated as blocks. Additionally, three blanks were consistently included in each run.

Chemical analysis, total gas, methane, volatile fatty acids, and in vitro organic matter digestibility

The chemical analysis of the test feed sample, *E. curvula* was carried out as follows: Nitrogen was analysed with a N-Analyser (Leco TruMac N determinator Leco Corporation, St. Joseph, USA). Ether extract content was analysed for using a Tecator Soxtec (HT6) system. Neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were all analysed using ANKOM200/220 fibre analyser and ANKOM daisy incubator (ANKOM Technology, Fairport, NY) as described previously[22]. The NDF assay employed the ANKOM filter bag technique, with the inclusion of heat-stable alpha-amylase and sodium sulphite. Additionally, the determination of ADF was conducted using the ANKOM filter bag technique. Both NDF and ADF values were expressed exclusive of residual ash. AOAC[18] method was used to analyse dry matter (ID 934.01), and ash (ID 942.05).

Gas production was measured at various time points (3, 6, 12, 24, and 48 h) during incubation. This was done using a pressure transducer (PX4200-015GI; Omega Engineering Inc.) attached to a digital data logger (Tracker 220 series indicators; Omega Engineering Inc., Laval, QC, Canada)[23]. The transducer, fitted with a modified tip, was securely placed over the syringe tap already attached to the vials. The syringe tap was opened to release the gas accumulated in the vials, and the digital data tracker recorded the gas pressure in psi units. Gas pressure readings at each time interval were cumulatively summed to obtain the total gas production. Methane gas production was measured in all replicated bottles at the same time intervals. Methane concentration in each sample was analysed using gas chromatography equipped with a flame ionization detector (FID) and a solenoid column packed with silica gel (8610 C Gas Chromatograph (GC) BTU Gas Analyser GC System; SRI Instruments GmbH, Bad Honnef, Germany). Gas samples were injected using the pull-and-push method into the GC, which had been previously calibrated with standard CH_4. Blanks were also analysed and used to correct for CH_4 produced by the inoculum. The fermentation process was terminated at 48 h by placing all the bottles on ice in a cold room. Subsequently, they were centrifuged at 4500 g to obtain 5 mL supernatant samples for the determination of acetic, propionic, butyrate, isobutyric, isovaleric, and valeric acids[24]. Finally, a two-stage in vitro organic matter digestibility was conducted[25], which involved modifying the procedure by incubating the residue from fermentation vials with an HCL-pepsin solution for 48 h.

Gas pressure was converted to volume using Boyle's law relationship as reported previously[23]:

$$Gas\ volume\ (mL) = \frac{Vh}{Pa} \times \mathrm{Pt.}$$

Where Vh is the volume of head space in the incubating vials (mL); Pa is the atmospheric pressure (psi); Pt is the reading from the pressure transducer attached to a data tracker (psi).

Methane concentration captured from GC in ppm was converted to mL by the following formula.

$$Methane\ (mls) = Total\ gas\ produced\ (mls)\ \times\ \%\ methane\ concentration\ of\ a\ gas\ sample$$

Statistical analysis

All data were analysed using one-way analysis of variance (ANOVA) with SAS software (version 9.4, SAS Institute Inc., Cary, USA). Treatment means were compared using Tukey's post hoc test, and significance was declared at $p < 0.05$. Before conducting ANOVA, the data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. Principal component analysis (PCA) was performed using XLSTAT software, and associative effects were determined using a Chi-Square test comparing observed and expected values calculated from single extract effects.

$$Associative\ effect\ = (Observed\ value - Expected\ value)\ \times\ 100/Expected\ value$$

Chi Square was calculated using the formula below, and significance was tested by comparing Chi Square value with critical value on the chi square distribution table.

$$Chi\ Square = \sum (Observed\ value - Expected\ value)^2 / Expected\ value$$

The expected value for each cocktail was calculated from the 2 single plant extracts that forms the cocktail as follows.

$$The\ expected\ value\ = \sum Observed\ value\ \left(\frac{plant\ extract1}{2} + \frac{plant\ extract\ 2}{2} \right)$$

Results

Phytochemical analyses

The results of plant extract preparation and the quantification of the total phenolic content are summarized in Table 1. The yield of plant extracts, expressed as a percentage, ranged from 7.4 to 20%. Among the tested plant extracts, AV had the highest yield at 13%. The total phenolic content in the extracts varied among the plant species. Plant extracts displayed varying levels of total phenolic content. Phytochemical analysis of the plant extracts revealed a diverse array of compounds, as shown in Table 2. The major families identified are flavonoids, alkaloids, coumarin, anthraquinones, and terpenoids in all the plant extracts. All the families listed have diverse functions and activities, they have a common role as plants defence compounds against herbivores.

Phytochemical compounds' relationship with parameters measured

The Principal Component Analysis (PCA) conducted on extracts of AV, AZ, TD, CP, JA, and MO, individually and in combination, revealed the presence of two prominent principal components (PCs) that collectively accounted for 55% of the total variation, as visually represented in Fig. 1. Specific phytochemical compounds such as Aloin A and B, Wistin, and Marmesin galactoside, identified within AV extracts, exhibited discernible associations, positive or negative, with fermentation parameters such as acetic acid levels and in vitro organic matter digestibility. Furthermore, our analysis highlights that phytochemical constituents present in AZ extracts contribute to variations in propionic acid concentrations observed across diverse treatment conditions. While most mixture and individual extracts showed similar pattern in terms of all parameters tested, extracts of AZ, TD and many of their combinations have different association patterns as shown in Fig. 1. This figure also underscores the interplay between botanical extracts, their phytochemical composition, and the resulting fermentation parameters, shedding light on the potential impact of these natural compounds on the fermentation process.

Figure 2 shows the PCA encompassing all parameters measured within the scope of this study, spanning the relative concentrations of phytochemicals inherent to both individual extracts and their various combinations. The analysis unveils positive correlations between identified phytochemicals, namely gypsogenin, rutin, lonicerin, xanthohumol, and terretonin, and a range of treatment groups, including AZ + AV, AZ + CP, AZ + TD, AZ + MO, AZ + JA, and AZ. Measured parameters associated are propionic acid, acetic acid and CH_4 production. The interrelationships between various treatment combinations, including AV + JA, AV + TD, AZ + MO, JA + CP, JA + MO, JA + TD, CP + MO, MO + TD, and all individual extracts except AZ, have been meticulously scrutinized with respect to their correlations with PC2. It is noteworthy that PC2 exhibits perceptible associations, both positive and negative, with these treatment groups. Furthermore, PC2 demonstrates a positive alignment with key parameters such as CH_4 production and IVOMD. It also provides a comprehensive depiction of the interplay, highlighting the positive correlations observed between PC2 and specific phytochemicals, including but not limited to marmesin galactoside, aloin, wistin, lamoxirene, tanzawaic acid, and others. These correlations show the role of these phytochemicals in contributing to the observed reductions in CH_4 emissions and potential enhancements in IVOMD.

Plants	Extract weight (mg)	Yield (%)	Total phenols (mg/g) equivalent Quebracho or Tannic acid
T. diversifolia	370	7.4	Quebracho: 72 mg/g
			Tannic acid: 52 mg/g
J. curcas	370	7.4	Quebracho: 88 mg/g
			Tannic acid: 65 mg/g
M. oleifera	610	12	Quebracho: 105 mg/g
			Tannic acid: 78 mg/g
A. vera	990	20	Quebracho: 54 mg/g
			Tannic acid: 38 mg/g
C. papaya	650	13	Quebracho: 97 mg/g
			Tannic acid: 73 mg/g
A. indica	660	13	Quebracho: 248 mg/g
			Tannic acid:193 mg/g

Table 1. HPLC analysis showing total yields and phenols equivalent when 5 g of different medicinal plants were extracted with methanol.

A. vera			C. papaya		
RT (min)	RC*	Phytochemical compounds	RT (min)	RC*	Phytochemical compounds
3.52	1.28	Lonicerin	8.5	3.61	Cyclopentaneoctanoic acid
4.03	6.42	Isosalipurposide	8.95	22.52	Alpha-dimorphecolic acid
4.4	0.95	Marmesin galactoside	9.58	2.52	18-Hydroxy-9-octadecenoic acid
4.59	8.74	Aloin A	10.75	5.13	Crepenynic acid
4.74	63.23	Aloin B	11.34	1.82	Linoleic acid
4.89	4.06	Wistin	11.91	38.91	Palmitic acid
4.99	0.44	Epifisetinidol	***A. indica***		
5.73	12.32	protoaphin aglucone	RT (min)	RC*	Phytochemical compounds
6.13	2.56	9,12,13-TriHOME	2.39	2.83	alpha-bradyrhizose
J. curcas			3.83	6.79	Rutin
RT (min)	RC *	Phytochemical compounds	3.96	1.84	Quercetin 3-galactoside
6.13	13.79	9,12,13-TriHOME	4.09	1.92	Lonicerin
7.17	6.28	Ovalimethoxy I	4.84	1.29	Lariciresinol
9.07	30.35	Alpha-dimorphecolic acid	4.86	3.4	Gaultherin B
9.6	3.35	18-Hydroxy-9-octadecenoic acid	4.92	0.37	Glaucolide M
9.9	10.83	Alpha-dimorphecolic acid	4.98	1.6	Sethukarailin
10.03	4.09	16-oxohexadecanoic acid	5.09	1.93	Terretonin
11.86	19.26	20-Hydroxyeicosanoic acid	5.13	5.75	Quercetin
11.88	1.33	Fasciculic acid B	5.25	2.02	Terretonin
11.92	5.9	Palmitic acid	5.69	1.36	Taiwanschirin D
12.01	1.37	Rotiorinol A	5.7	3.01	Kaempferol
T. diversifolia			5.77	1.59	Dracunculifoside F
RT (min)	RC*	Phytochemical compounds	5.9	7.06	Interiotherin B
5.06	11.66	Neurolenin C	6	8.42	Xylogranatin Q
5.79	15.84	Lobetyol	6.1	5.12	Terretonin A
5.81	8.37	Corchorifatty acid F	6.41	0.56	Khivorin
6.13	7.81	9,12,13-TriHOME	6.74	1.23	Orthosiphol X
6.33	2.41	Lamoxirene	7.16	2.02	6-Desmethylmonacolin J
6.33	2.12	Dehydrooreadone	7.63	0.58	Desmethylxanthohumol
6.47	3.64	Neurolenin D	8.77	2.62	Xanthohumol
7.16	5.87	Tanzawaic acid H	8.9	6.36	Fukanemarin B
9.06	16	Alpha-dimorphecolic acid	9.07	3.21	Alpha-dimorphecolic acid
9.24	4.11	Cyclopentaneoctanoic acid	9.2	1.61	Fukanemarin B
10.89	1.84	11-Hydroxyhexadecanoic acid	9.81	1.04	Gypsogenin
11.85	12.38	20-Hydroxyeicosanoic acid	9.89	1.53	Alpha-dimorphecolic acid
11.93	4.56	Lesquerolic acid	10.22	4.96	Hippuristerone H
12.18	1.92	Lesquerolic acid	10.47	1.23	Abyssinone V
M. oleifera			10.89	1.26	11-Hydroxyhexadecanoic acid
RT (min)	RC*	Phytochemical compounds	11.86	14.34	20-Hydroxyeicosanoic acid
5.81	28.8	Corchorifatty acid F			
6.13	6.47	9,12,13-TriHOME			
6.76	9.6	Corchorifatty acid D			
8.49	2.82	Cyclopentaneoctanoic acid			
9.06	21.18	Alpha-dimorphecolic acid			
10.74	3.72	Crepenynic acid			
11.35	1.31	Linoleic acid			
11.9	19.19	Palmitic acid			

Table 2. Metabolomics profiling of *Aloe vera*, *Azadirachta indica*, *carica papaya*, *Jatropha curcas*, *tithonia diversifolia*, and *Moringa Oleifera* using LC-MS method. *RC Relative concentration of each phytochemical

In vitro gas and methane production, digestibility, and volatile fatty acids

The substrate used in this study, *E. curvula* hay, was of low quality. It had a crude protein content of 5.12%, 9.1% ash, 1.3% ether extract, 75.5% neutral detergent fibre, 44.5% acid detergent fibre, and 8.1% acid detergent lignin. Figure 3 presents the data for total gas production (TGP), methane production, organic matter digestibility (IVOMD), and total volatile fatty acids (TVFA) across all treatments, while Table 3 showed the calculated ratios

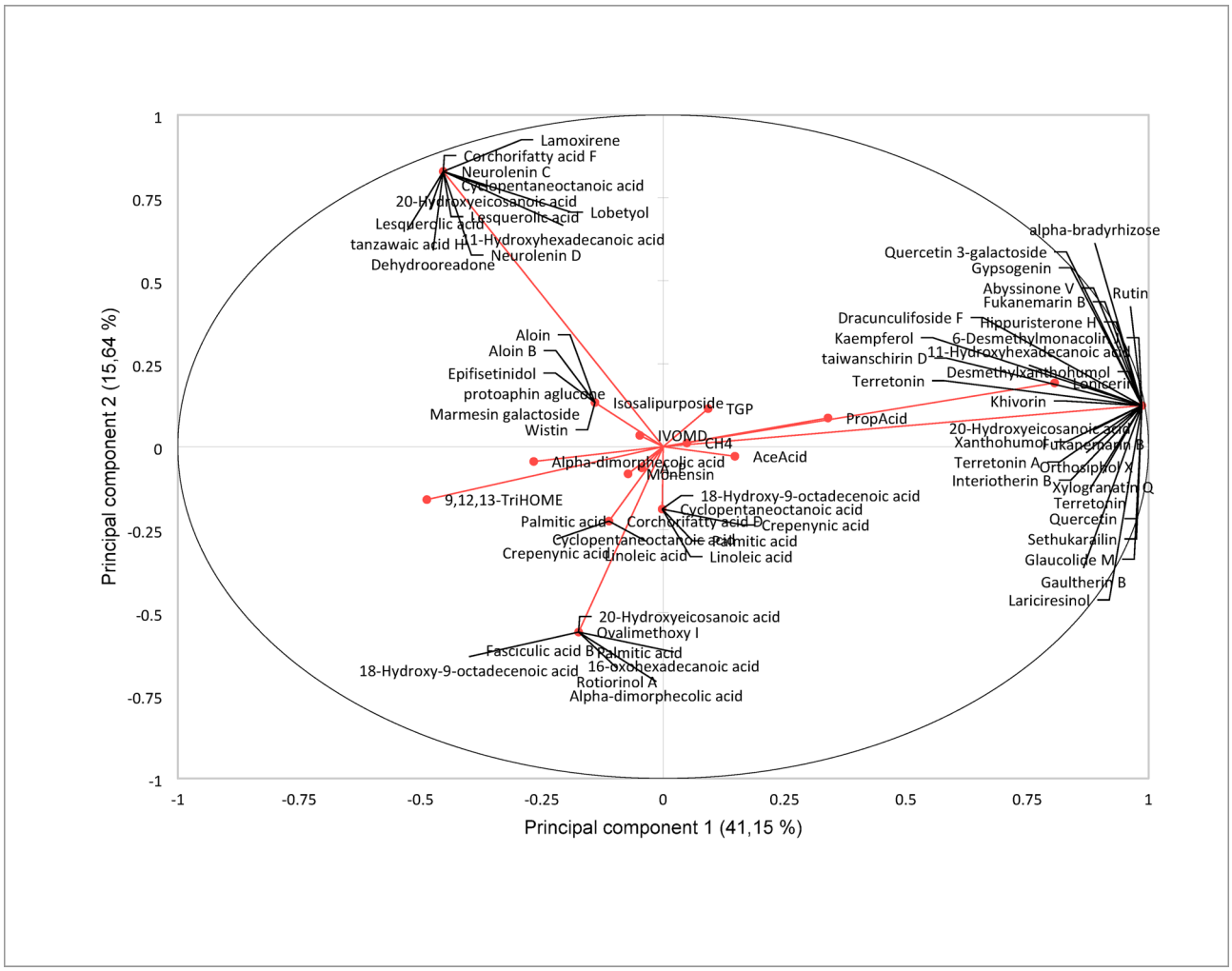

Fig. 1. Biplots showing the relationships between the phytochemical extracts and in vitro fermentation parameters of Eragrostis curvula hay. TGP Total gas produced, IVOMD In vitro organic matter digestibility.

of TGP, CH4, IVOMD and TVFA. Cumulative gas production after 48 h indicated that the combinations AV + JA and AZ + AV reduced ($p < 0.05$) TGP compared to the control (Fig. 3A). Other combinations and individual plant extracts did not differ significantly ($P > 0.05$) from the control or monensin. Leaf extracts of AV, AZ, TD, CP, JA, and MO decreased CH_4 production ($p < 0.05$) relative to the control ($p < 0.05$), with AZ and CP showing least and greatest reductions, at 57% and 71%, respectively (Fig. 3B). In general, the two-way mixture also led to significantly reduced CH_4 production compared to the control ($p < 0.05$). Reductions between 50% and 60% were recorded for mixture such as AV + CP, AZ + CP, AZ + MO, AZ + TD, JA + TD, while reductions between 60% and 74% were observed for AZ + AV, AV + JA, AV + MO, JA + CP, CP + MO, and MO + TD. Although the monensin group showed a numerical reduction in CH_4 compared to the control, this difference was not significant ($p > 0.05$). Among treatments, CP, and JA individual extracts, as well as the mixture AV + JA and CP + MO significantly reduced CH_4 compared to both the control and monensin ($p < 0.05$), indicating synergistic effects. IVOMD results are presented in Fig. 3C. Among the individual plant extracts, CP, JA, and MO significantly increased IVOMD compared to the control ($P < 0.05$), indicating improved fibre degradation potential. In contrast, the AV + CP and AV + MO mixtures showed significantly lower IVOMD than both their respective individual components and the control ($P < 0.05$), suggesting antagonistic interactions. Other mixtures, including JA + TD and CP + MO, produced IVOMD values comparable to the control ($P > 0.05$). Monensin treatment also did not significantly affect IVOMD relative to the control ($P > 0.05$).

Figure 3D showed the concentrations of TVFA while Table 4 displays the molar proportions of VFAs, the acetic-to-propionic acid ratio, and NH_3-N concentrations. The control group exhibited the highest ($p < 0.05$) NH_3-N concentrations across treatments, followed by the monensin group, which had values comparable to those of individual plant extracts and plant extract mixture. NH_3-N values for individual plant extracts and all mixtures were significantly lower than the control ($P < 0.05$) but did not differ significantly from one another ($P > 0.05$). The AV + CP, AV + MO, and JA + MO mixtures showed significantly lower acetic acid concentrations than both the control and monensin groups ($P < 0.05$; Table 4), indicating a shift in fermentation pathways towards more reduced VFA profiles. Apart from JA + MO, JA + TD, and MO + TD, all mixture had higher

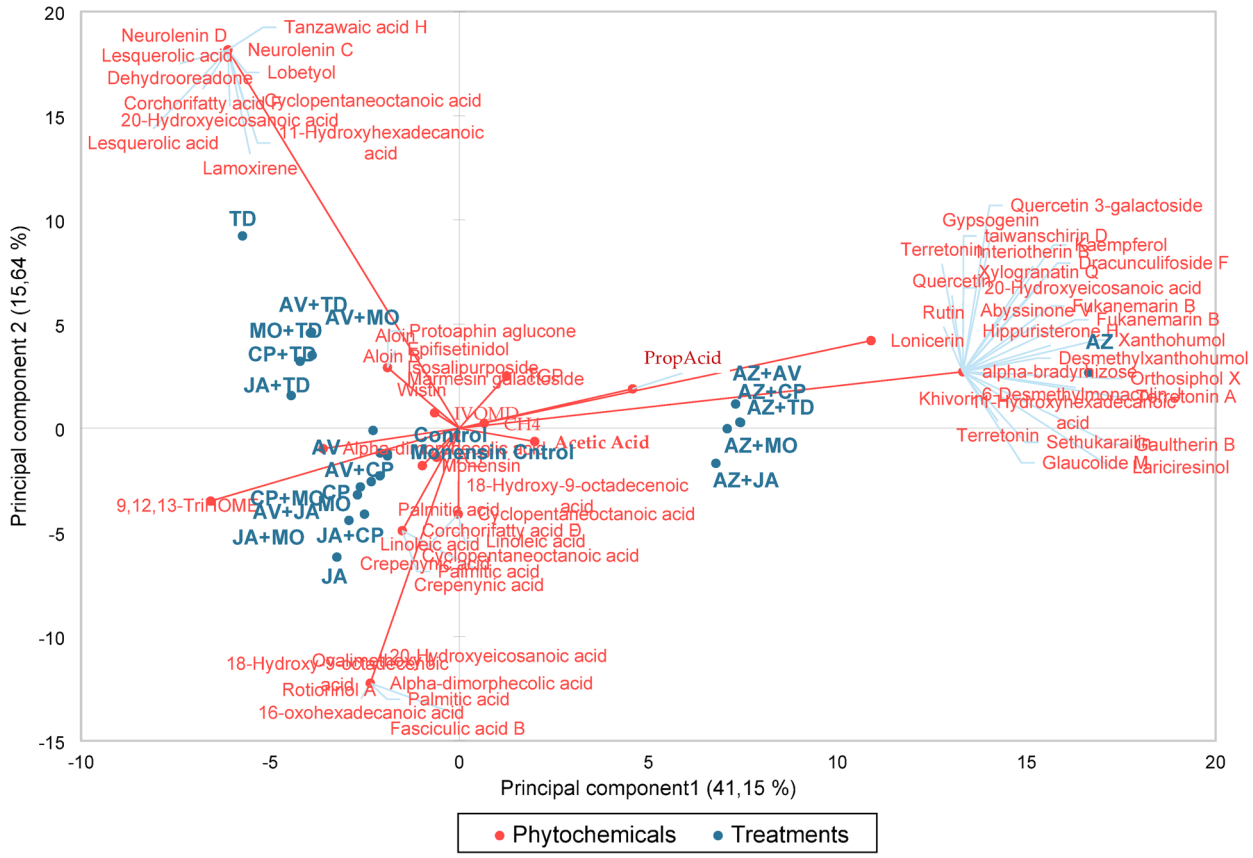

Fig. 2. Principal component analysis showing the relationship within the treatments, in vitro gas and digestibility parameters, and phytochemicals present in different plant extracts. AV *Aloe vera*, CP *Carica papaya*, AZ *Azadirachta indica*, JA *Jatropha curcas*, MO *Moringa oleifera*, TD *Tithonia diversifolia*.

($p < 0.05$) propionic acid concentrations compared to the control. In contrast, individual plant extracts did not increase propionic acid concentration compared to the control or monensin. The AV plant extract had lower butyric acid values compared to the control or monensin.

Mixture of AV + MO, JA + MO, JA + TD, and CP + TD exhibited higher iso-butyric acid values compared to the control. On the other hand, mixture of AZ + TD and CP + MO resulted in lower valeric acid values compared to the control. Conversely, the JA + MO and MO + TD mixture led to higher iso-valeric acid values compared to the control. The lowest acetate-to-propionate ratio was observed for the AV + MO and JA + MO mixture. Mixture of AV + MO, CP + TD, MO + TD, JA + MO, as well as individual plant extracts AV, JA, MO, and TD reduced total volatile fatty acid production compared to the control. The volume of CH_4 per unit of TVFA was reduced ($p < 0.05$) by AZ + AV and AV + JA mixture compared to the control. However, there was no difference ($p > 0.05$) between the control, plant extracts, and monensin in terms of TGP per unit of TVFA.

Associative effect of different plant extracts

Mixture of AZ + AV, AV + JA, AV + CP, AV + MO, and AZ + JA had negative associative effects on IVOMD (Table 5), with significantly lower values than their individual components ($p < 0.05$). All other single plant extracts and plant extract mixture had higher or equal IVOMD compared to the control and the monensin group. Total gas production per unit IVOMD was generally lower in the single plant extracts compared to the control and monensin group, with AV + MO having the highest value among the mixture. CH_4 per unit of total gas produced (CH_4/TGP) was significantly lower ($P < 0.05$) for AV, AZ, CP, JA, MO, and TD, as well as for mixtures such as AV + JA, AV + MO, JA + CP, CP + MO, and MO + TD, compared to the control. Monensin did not significantly reduce CH_4/TGP compared to the control ($P > 0.05$). Similarly, CH_4 per unit of IVOMD was significantly higher in the control (63.8%) and monensin (57.1%) treatments than in all plant extract treatments ($P < 0.05$). Mixtures including AZ + AV, AV + JA, AV + TD, AZ + MO, AZ + TD, JA + CP, CP + MO, and MO + TD, along with all individual extracts, significantly reduced CH_4/IVOMD values compared to the control ($P < 0.05$).

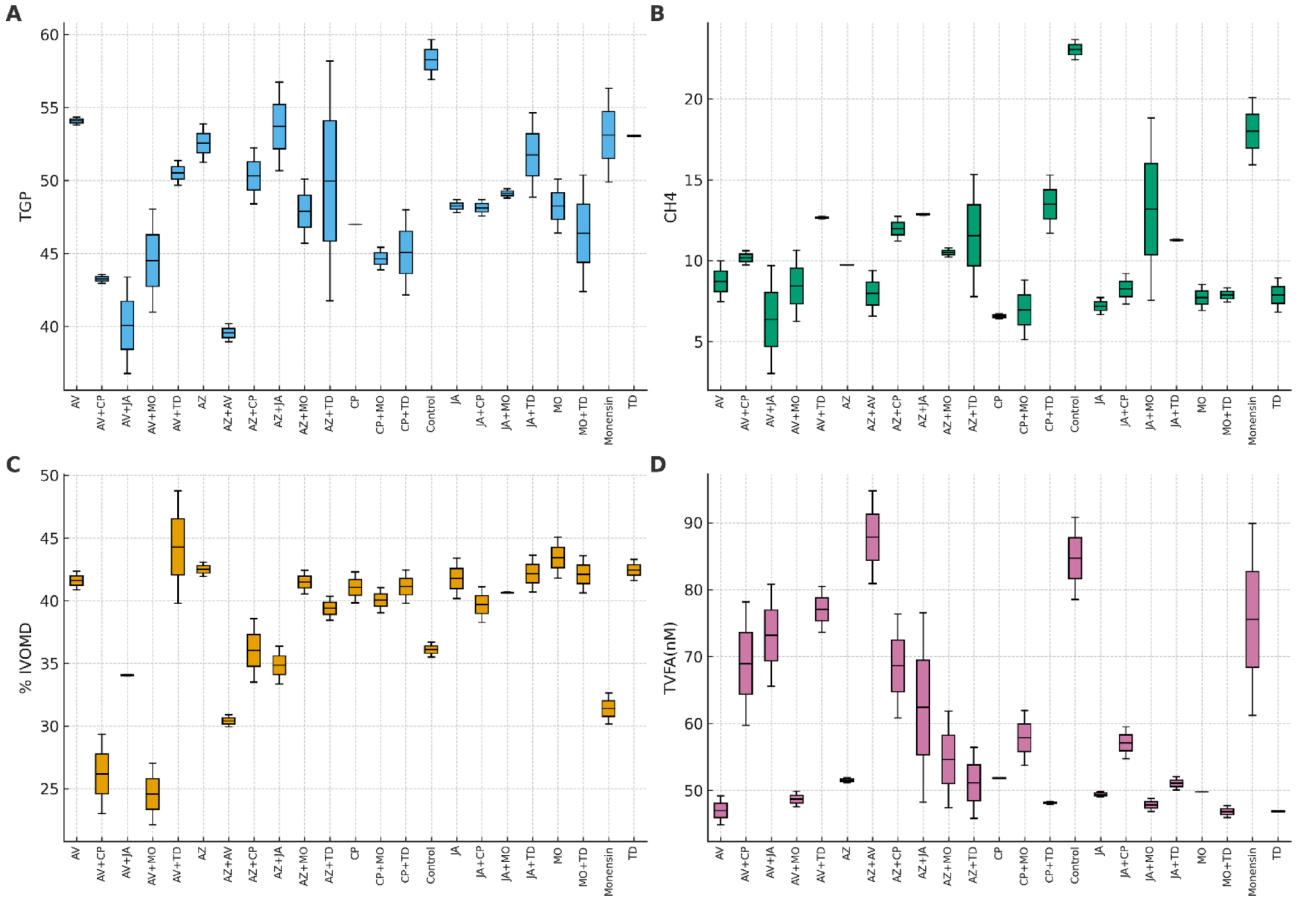

Fig. 3. Boxplots of (**A**) total gas production (TGP), (**B**) methane (CH_4) production, (**C**) in vitro organic matter digestibility (IVOMD), and (**D**) total volatile fatty acid concentration (TVFA) in *Eragrostis curvula* hay treated with individual or plant extract mixtures. AV *Aloe vera*, CP *Carica papaya*, AZ *Azadirachta indica*, JA *Jatropha curcas*, MO *Moringa oleifera*, TD *Tithonia diversifolia*.

However, only the AV + JA and CP + MO mixtures performed significantly better than monensin in reducing CH_4 per unit of IVOMD ($P < 0.05$), suggesting these combinations offered superior methane mitigation efficiency.

Discussion

The low-quality substrate allows for the evaluation of the effectiveness of individual plant extracts and extract combinations on fermentation parameters. The negative control group consistently maintained higher TGP levels throughout the experiment, likely indicating no treatment effect. It is noteworthy that antibiotic growth promoters and plant extracts have been previously reported to reduce gas production[6]. Monensin reduced CH_4 production by reducing total gas production (TGP). Therefore, it can be hypothesized that the plant extracts' activity is similar to that of antibiotic growth promoters. Antibiotic growth promoters, such as monensin, operate by inhibiting both total gas production and CH_4. They achieve this effect through the presence of polyether ionophores, which are broad-spectrum antibiotics with significant anti-parasitic properties and effective against archaea. These ionophores work by blocking the intracellular transport of Golgi apparatus proteins in selected cells[26] and catalyses the exchange of Na^+ for H^+ across cellular membranes thereby reducing the actions of protozoans and methanogens. This reduction of CH_4 by medicinal plants agrees with other studies[27,28]. There is evidence that the antimicrobial properties present in these plants might have significantly influenced methanogenesis (Table 2). Rutin, quercetin, lonicerin, and other flavonoids were identified in extracts of AZ. They are effective antimicrobials, improve feed palatability[29], reduce stress, promote a balanced gut microbiome, and assist in the absorption and utilization of essential nutrients[30] in the digestive system leading to increased nutrient availability for growth and metabolism. This can explain the higher propionic acid concentration recorded for AZ and its mixture. The inclusion of these compounds in animal feed can also lead to a higher absorption rate of volatile fatty acids in the rumen thereby leading to higher feed efficiency and performance of the animal.

Methane was reduced by all the plant extracts and their respective mixture. The lack of a significant difference between the single plant extracts and the mixture suggests that there was no associative effect in terms of CH_4 volume reduction when combining these plant extracts. Notably, reductions exceeding 70% in CH_4 production observed in CP, CP + MO, and AV + JA treatments are likely attributable to the antimicrobial properties and

Treatments	TGP/IVOMD (%)	TGP/TVFA	CH_4/TGP (%)	CH_4/IVOMD (%)	CH_4/TVFA
Control	161ab	0.69a-c	39.5a	63.8a	27.3a
Monensin	169ab	0.72a-c	33.7ab	57.1ab	24.2ab
AZ + AV	130ab	0.45c	20.1ab	26.1bc	9.00b
AV + JA	117b	0.54bc	15.2b	18.6c	8.30b
AV + CP	167ab	0.63a-c	23.5ab	39.6a-c	14.9ab
AV + MO	184a	0.92a-c	18.8b	35.6a-c	17.4ab
AV + TD	115b	0.65a-c	25.1ab	28.8bc	16.5ab
AZ + JA	154ab	0.89a-c	24.2ab	36.9a-c	21.7ab
AZ + CP	140ab	0.74a-c	23.9ab	33.2a-c	17.5ab
AZ + MO	115b	0.89a-c	21.9ab	25.3bc	19.6ab
AZ + TD	127ab	1.00ab	22.4ab	29.5bc	23.6ab
JA + CP	121ab	0.84a-c	17.2b	20.9bc	14.4ab
JA + MO	120ab	1.03ab	26.7ab	32.4a-c	15.8ab
JA + TD	122ab	1.02ab	21.8ab	26.7a-c	22.1ab
CP + MO	111b	0.77a-c	15.5b	17.2c	11.8ab
CP + TD	109b	0.94a-c	30.3ab	32.9a-c	27.9a
MO + TD	110b	0.99ab	17.2b	18.7c	16.8ab
AV	129ab	1.15a	16.1b	20.9c	18.7ab
AZ	123ab	1.02ab	18.5b	22.8c	18.4ab
CP	114b	0.91a-c	13.9b	15.9c	12.7ab
JA	115b	0.97a-c	14.8b	17.2c	14.5ab
MO	111b	0.97a-c	15.9b	17.8c	15.5ab
TD	125ab	1.13a	14.8b	18.5c	16.8ab
SEM	16.3	0.12	5.10	7.9	4.31

Table 3. Total gas produced (TGP), methane (CH_4), in vitro organic matter digestibility (IVOMD), and total volatile fatty acids ratios of 400 Mg *E. curvula* hay sprayed with individual and combination of different plant extracts. Means with different letters across the column are significantly different ($p < 0.05$). AV *Aloe vera*, CP *Carica papaya*, AZ *Azadirachta indica*, JA *Jatropha curcas*, MO *Moringa oleifera*, TD *Tithonia diversifolia*.

phytochemicals present in these individual plant extracts. Previous phytochemical screening[31] indicated the presence of saponins, cardiac glycoside, and alkaloids in the green leaf of CP which is similar to those obtained in this study. Cardiac glycosides, which function similarly to polyether ionophores as discussed earlier, may also be partly responsible for the reduction observed in CH_4 production[13]. The lower CH_4 values achieved without negatively impacting TGP indicate a reduction in energy loss by the animal. Previous studies have reported the effectiveness of plant extracts in modulating rumen fermentation parameters without adversely affecting ruminal fermentation[32–35].

In addition to methane mitigation, notable effects were observed on nutrient digestibility and fermentation characteristics. The significantly higher IVOMD values in CP, MO, and JA suggest these extracts may enhance fibre degradation and energy release from low-quality roughages[36,37]. In contrast, mixtures such as AV + CP and AV + MO showed negative associative effects on IVOMD, indicating that certain plant extract combinations can impair digestibility, possibly due to antagonistic interactions among their secondary metabolites[38]. The observed shifts in VFA profiles, especially the reduction in acetic acid and increased propionic acid in most mixtures, are indicative of a fermentation pattern that favours glucogenic energy production, which has been associated with reduced methane formation and improved animal performance[39,40]. Furthermore, the decline in NH_3-N concentrations across treatments may reflect decreased proteolysis or improved microbial nitrogen assimilation[41], supporting the potential of these extracts to enhance nitrogen utilisation efficiency in ruminants.

The observed reductions in IVOMD for mixtures such as AV + CP and AV + MO, despite both components having individually favourable effects, suggest possible antagonistic interactions. These antagonisms may arise from overlapping or competing phytochemical actions, such as saponins interfering with the emulsifying effect of fatty acids, or tannins complexing with proteins or enzymes, thereby impairing microbial activity[38,42]. For example, the anthraquinones in AV, such as aloin A and B, are known to influence gut motility and digestive secretions[43], but when combined with other lipid-rich or bioactive extracts, they may disrupt microbial balance or fibre breakdown pathways. In addition, long-chain fatty acids like linoleic acid and alpha-dimorphecolic acid, present in JA, MO, CP, and TD, can modulate rumen fermentation depending on their concentrations and interactions[44]. Such interactions could explain the reduced IVOMD observed in certain combinations. Therefore, when developing plant extract blends, it is critical to consider potential phytochemical interference or inhibition among compounds, rather than assuming additive or synergistic effects.

In the present study, the relationship between plant extracts and their impact on CH_4 production in relation to TGP and IVOMD yielded favourable results for the plant extracts compared to the control group. However, there

Treatments	NH_3-*N* (mg/100mL)	Acetic (mM)	Propionic (mM)	Butyric (mM)	Iso-butyric (mM)	Valeric (mM)	Iso-valeric (mM)	Acetic/ Propionic
Control	5.12a	39.1ab	20.5 g	12.9a-d	7.69f-h	11.4a-c	8.32 cd	1.89a
Monensin	5.08b	37.5ab	22.0d-g	12.1a-d	7.88d-h	11.7ab	8.64 cd	1.72ab
AZ + AV	4.40 m	42.5a	23.5a-d	10.8de	6.74 h	10.1c-e	6.08e	1.80ab
AV + JA	4.51i	37.0b	22.9a-f	13.7ab	7.45gh	10.4b-e	8.43 cd	1.62a-c
AV + CP	4.69e	33.8 cd	23.2a-e	14.1a	8.10c-h	10.5a-e	10.0a-d	1.46a-c
AV + MO	4.21p	29.6d	24.0a	14.2a	9.69a-e	11.6a	10.3a-c	1.23c
AV + TD	4.49ij	38.4ab	22.6a-f	12.2a-d	7.76e-h	11.0a-e	7.79de	1.70ab
AZ + JA	4.79c	34.4b-d	22.6a-f	12.9a-d	8.89b-g	11.1a-d	9.87a-d	1.53a-c
AZ + CP	4.54 h	36.6bc	23.9ab	12.4a-d	7.47gh	10.5a-e	8.89b-d	1.53a-c
AZ + M0	4.14q	34.7bc	23.5a-d	13.0a-c	8.99b-g	10.0c-e	9.57a-d	1.48a-c
AZ + TD	4.42 L	34.8bc	23.9ab	12.2a-d	8.98b-g	9.61e	10.3a-c	1.45a-c
JA + CP	4.47j	35.1bc	23.8a-c	12.4a-d	8.85b-g	10.5a-e	9.08a-d	1.47a-c
JA + MO	4.44k	31.5 cd	22.3b-g	12.8a-d	10.7ab	11.5a-c	11.1ab	1.41bc
JA + TD	4.43kl	35.9bc	22.0c-g	11.5c-e	9.89a-c	10.2b-e	10.1a-c	1.63a-c
CP + MO	4.18p	38.1ab	22.3a-f	11.5c-e	8.67b-g	9.91de	9.38a-d	1.71ab
CP + TD	4.64f	34.2b-d	22.3a-f	12.6a-d	9.80a-d	10.3b-e	10.5a-c	1.53a-c
MO + TD	4.57 h	34.0b-d	22.1c-g	11.7b-e	9.49a-f	11.2a-d	11.2a	1.54a-c
AV	4.79c	36.1bc	21.7e-g	9.99e	11.3a	11.1a-d	9.77a-d	1.65a-c
AZ	4.75d	36.9b	22.0c-g	11.7b-e	9.08b-g	9.81de	10.3a-c	1.67a-c
CP	4.50 h	37.6ab	21.4 fg	11.7b-e	9.58a-f	9.87de	9.64a-d	1.75ab
JA	4.33n	37.2b	21.3 fg	11.4c-e	9.66a-e	10.2b-e	10.3a-d	1.76ab
MO	4.54gh	37.9ab	22.2b-g	11.2c-e	8.9b-g	10.1c-e	9.58a-d	1.70ab
TD	4.23o	37.9ab	21.7e-g	11.0c-e	9.18b-g	10.0c-e	10.0a-d	1.74ab
SEM	0.02	7.06	0.79	1.09	0.99	0.58	1.32	0.05

Table 4. Ammonia-Nitrogen (NH_3-N) and molar proportions of volatile fatty acids expressed as the percentage of total volatile fatty acids of *E. curvula* hay sprayed with individual and combination of different plant extracts. Means with different letters across each column are significantly different ($P \leq 0.05$). AV *Aloe vera*, CP *Carica papaya*, AZ *Azadirachta indica*, JA *Jatropha curcas*, MO *Moringa oleifera*, TD *Tithonia diversifolia*. TGP Total gas production, CH_4 Methane SEM Standard error of mean.

Treatment	TGP	CH_4	IVOMD	TVFA	Propionic	Acetic	Acetic/Propionic
AZ + AV	− 25.81	− 12.11	− 27.65	78.04*	7.43	16.55	8.58
AV + JA	− 21.64	− 21.74	− 18.27	52.25*	6.58	1.07	− 4.83
AV + CP	− 14.39	33.39	− 36.69	39.94*	7.89	− 8.22	− 14.58
AV + MO	− 13.07	3.45	− 42.32	− 1.38	9.51	− 19.66	− 26.58
AV + TD	− 5.68	52.64	5.34	64.04*	4.19	4.16	− 0.02
AZ + JA	6.46	52.24	− 17.31	23.43*	4.52	− 6.55	− 9.82
AZ + CP	1.15	46.93	− 13.78	32.71*	10.14	− 1.64	− 10.85
AZ + MO	− 4.75	20.83	− 3.46	7.83	6.14	− 6.99	− 12.36
AZ + TD	− 5.55	29.22	− 7.23	3.98	9.36	− 6.85	− 14.87
JA + CP	1.07	21.02	− 4.14	12.8	11.6	− 6.01	− 15.77
JA + MO	1.88	85.14*	− 4.46	− 3.54	2.45	− 15.72	− 17.78
JA + TD	2.13	51.45	0.15	6.01	2.71	− 4.04	− 6.12
CP + MO	− 6.17	− 0.35	− 5.22	13.87	2.32	0.9	− 1.17
CP + TD	− 9.9	90.32*	− 1.51	− 2.51	3.64	− 9.26	− 12.48
MO + TD	− 8.54	1.76	− 1.99	− 3.12	0.54	− 10.16	− 10.62

Table 5. Associative effect (Chi square value) of plant extract mixture on total gas produced (TGP), methane (CH_4), organic matter digestibility (IVOMD) and volatile fatty acids (VFA) of 400 Mg *E. curvula* hay. *Indicates significance $p < 0.05$. AV *Aloe vera*, CP *Carica papaya*, AZ *Azadirachta indica*, JA *Jatropha curcas*, MO *Moringa oleifera*, TD *Tithonia diversifolia*. TGP Total gas produced, CH_4 Methane, IVOMD In vitro organic matter digestibility.

was no evidence of a favourable synergistic effect from the mixture in this regard. The positive associative effect observed in the molar proportions of propionic acid and the reduction of total volatile fatty acids in both mixture and single extracts could be attributed to the enhanced antimicrobial effect on ruminal microorganisms[45] which causes reduction in total VFA and subsequent increased concentration of propionic acid. A previous study[46] reported that at higher concentration of inclusion of extracts *Humulus lupulus*, increased propionic acid and decreased C2/C3 ratio. A decrease in CH_4 production by more than 40% when substrate was treated with plant extracts was reported[32] and associated with reductions in ciliate population by more than 60% and incresed diversity of fibrolytic bacteria. The test feed ingredient has a lot of fibre and cellulose which is the main source of fibre fragments and would have resulted in the production of high volume of acetic acids through gradual degradation during fermentation.

Two mechanisms could explain the higher proportion of propionic acid observed in the two-way plant extract mixture treatments. One possible scenario is that they phytochemicals in the extracts altered rumen microbial activity, leading to changes in fibre degradation dynamics and potentially modifying substrate passage rates[38,42]. The second scenario could be the suppressive effect of the antimicrobial and antiprotozoal properties of the plant extracts on methanogens[47], which, in turn, reduced the usage rate of H_2 in the rumen, as previously discussed. Several reports have shown that polyhalogens, such as chloroform, alter the fermentation pattern in the rumen by decreasing the production of acetic acid and increasing the production of propionic acid. Ammonia N concentration, often regarded as waste[48], was reduced by plant extracts, probably due to a reduction in CP degradability in the rumen[49]. MO and other plants used in this study have been reported to have activity against rumen protozoans; however, most of the mixture had no associative effect on other fermentation parameters. The CH_4 per unit TGP, IVOMD, TVFA, and TGP per unit IVOMD and TVFA all showed that single plant extracts and their mixture performed the same or better than the control and monensin groups.

Overall, individual extracts such as CP, MO, and JA showed strong potential to improve digestibility and reduce methane emissions, with CP and MO in particular enhancing IVOMD while reducing CH_4 per unit of TGP and IVOMD[36]. Among the mixtures, AV + JA and CP + MO consistently outperformed both the control and monensin in methane mitigation efficiency while maintaining favourable fermentation characteristics[6]. Although some combinations like AV + CP and AV + MO exhibited antagonistic effects on digestibility likely due to conflicting phytochemical actions[38,42], while others such as AZ + AV and AV + JA demonstrated synergistic reductions in CH_4 per unit of TVFA. These findings support the strategic use of plant extract combinations based on known phytochemical interactions and confirm their potential as sustainable alternatives to ionophores for improving rumen fermentation and reducing methane output in ruminants[39].

Conclusions

Plant extracts of CP, JA, MO were the most effective at improving IVOMD and reducing CH_4 emissions, outperforming both the control and monensin. Mixtures such as AV + JA and CP + MO showed strong synergistic effects, significantly lowering CH_4 per unit of IVOMD and CH_4 per unit of TVFA, while maintaining favourable fermentation profiles. However, some combinations like AV + CP and AV + MO exhibited antagonistic effects on digestibility, suggesting that phytochemical compatibility should be carefully considered when designing mixtures. The consistent involvement of AV and JA in synergistic combinations suggests they may play key roles in modulating microbial fermentation. These findings support the potential of targeted plant extract blends as natural alternatives to ionophores in methane mitigation. Future studies should explore the bioactivity and optimal ratios of key phytochemical compounds in AV, JA, and CP, with a view to standardising formulations for practical application in ruminant feeding systems.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

AMA and AH conceived and designed the research. AMA conducted the experiment. HA, ZA, SYL contributed to further laboratory analysis. AH and EVMK supervised the study. AMA analysed the data and wrote the manuscript. All authors read, edited and approved the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Author contribution statement

AMA and AH conceived and designed the research. AMA conducted the experiment. HA, ZA, SYL contributed to further laboratory analysis. AH and EVMK supervised the study. AMA analysed the data and wrote the manuscript. All authors read, edited and approved the manuscript.

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