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Balancing Risks and Benefits: Anti-Nutrient Content versus Methane-Suppressing Potential of Medicinal Plants in Maize Stover-Based Ruminant Diets

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ABSTRACT

In Ethiopia, greenhouse gas emissions from the agricultural sector are rising rapidly, with enteric fermentation and manure deposited on pastures contributing the majority of emissions. A study was carried out to determine the anti-nutrient profile and methane-suppressing potential of extracts derived from widely used Ethiopian medicinal plants, using maize stover as the basal substrate in an in vitro rumen simulation system.The total phenolic, flavonoid, tannin, and essential oil contents of the plant extracts were quantified using standard analytical methods. Leaf extracts from Acacia nilotica, Azadirachta indica, three accessions of Cymbopogon citratus (C. citratus-I, C. citratus java, and C. citratus upper Awash), Leucaena leucocephala, Moringa stenopetala, three accessions of Rosmarinus officinalis (R. officinalis I, II, and III), and Thymus schimperi; seeds of three coriander varieties (Coriandrum sativum Batu, Tulu, and Waltai); and roots of Echinops kebericho were tested at different inclusion levels. Their effects on total gas production, in vitro dry matter digestibility, and methane yield from maize stover were evaluated following established in vitro protocols. Results revealed that Acacia nilotica leaf extract exhibited the highest ($P < 0.001$) concentrations of total phenols and total tannins. All Cymbopogon citratus accessions contained significantly higher ($P < 0.001$) levels of flavonoids compared to the other species. Rosmarinus officinalis II showed the greatest ($P < 0.001$) essential oil content among all tested plants and rosemary accessions. At an inclusion level of 50 mg/kg dry matter (DM), Cymbopogon citratus java and Thymus schimperi extracts achieved the most pronounced methane suppression, reducing methane production by 22.5 % and 16.7 %, respectively, relative to the unsupplemented control ($P < 0.001$). A significant ($P < 0.001$) plant species × dose interaction was observed at this inclusion rate. Importantly, these reductions in methane were not accompanied by any adverse effect on substrate digestibility. It is concluded that supplementing maize stover with 50 mg/kg DM of Cymbopogon citratus java or Thymus schimperi extract offers a promising strategy for lowering enteric methane emissions in ruminants fed low-quality roughages. Additional research is recommended to assess the stability of these extracts during storage and to validate their efficacy and safety through in vivo feeding trials.

Keywords: Medicinal plant anti-methanogen, Crop residue, Ethiopia, Methane-Suppressing

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Introduction

Global greenhouse gas (GHG) emissions from agriculture are rising sharply, with crop and livestock production contributing approximately 5 billion metric tons of CO_2-equivalent annually to the atmosphere [1]. In Ethiopia, agricultural GHG emissions increased dramatically from 47,984 to 102,933 Gg CO_2eq year^{-1} (a 114.5 % rise) between 1993 and 2017, of which enteric fermentation accounted for 51.3 % and manure deposited on pastures for 36 % [2]. Within the country's production systems, ruminants reared under mixed crop-livestock systems are the primary source of enteric methane [3]. Enteric methane production represents an energy loss of 2–20 % of gross energy intake in ruminants [4] and, as a potent greenhouse gas, contributes significantly to global warming and associated environmental damage [5].

In Ethiopia, maize (Zea mays) stover constitutes a major basal feed resource in mixed crop-livestock systems, with roughly 56 % of the produced stover biomass being utilized as livestock feed [6]. However, maize stover is typically high in structural carbohydrates, exhibits low voluntary intake, slow ruminal digestibility, and limited protein and energy content [7]. Local in vitro studies have shown that methane production from maize stover is notably high, ranging from 18 to 32 % of total gas produced, compared to other roughage sources [8, 9]. Consequently, there is an urgent need to identify effective strategies to reduce ruminal methane emissions from fibrous feeds, thereby improving both environmental sustainability and livestock productivity.

Plant secondary metabolites are often classified as anti-nutritional factors because, at high levels, they can depress feed intake and nutrient utilization. Nevertheless, when supplied at optimal doses, extracts from medicinal plants can favorably modulate ruminal fermentation [10], enhance microbial protein synthesis [11], and inhibit methanogenesis [10, 12]. Phytochemical composition varies considerably among plant species and even cultivars; thus, detailed characterization of phenolic compounds and their effects on fermentation is essential for each candidate plant to fully exploit its potential in ruminant nutrition [13]. There is growing interest in identifying natural feed additives capable of improving the efficiency of dietary energy utilization while simultaneously reducing rumen methane production. Ethiopia possesses a rich diversity of indigenous and introduced medicinal plants that are traditionally used for treating human and animal ailments [14, 15] and as sources of food, feed, beverages, and spices [16]. Despite their widespread traditional application, limited scientific data exist regarding their anti-nutritional constituents, methanogenesis-suppressing capacity, and appropriate inclusion levels as feed additives.

Therefore, the present study was designed to quantify the anti-nutrient composition of selected Ethiopian medicinal plants, assess their in vitro anti-methanogenic potential when added to maize stover-based substrates, and determine the optimum inclusion rates of their extracts as natural rumen-modifying feed additives.

Materials and Methods

Selection and collection of experimental plants

A total of fifteen plant species were selected for the experiment, guided by earlier investigations into Ethiopia’s medicinal flora [14, 16, 17]. The plant materials were obtained from the southeastern region of the country, positioned around 8°54′N and 38°28′–39°27′E. This area spans elevations between 1715 and 4377 meters above sea level. Annual rainfall in the collection zones ranges from 760 to 1500 mm, while temperatures typically vary from 14 °C to 28 °C.

For each species, roughly 500 g of fresh plant material was harvested. Leaf samples were taken from Acacia nilotica (AN), Azadirachta indica (AZ), three types of Cymbopogon citratus (CC-UA, CC-Java, and CC-I), Moringa stenopetala (MS), Leucaena leucocephala (LL), and three forms of Rosmarinus officinalis (RO-I, RO-II, and RO-III). Thyme schimperi (TS) leaves were also collected. Additionally, seeds of three Coriandrum sativum varieties (Batu, Tulu, and Waltai) and roots of Echinops kebericho (EK) were gathered from multiple locations **(Figure 1).**

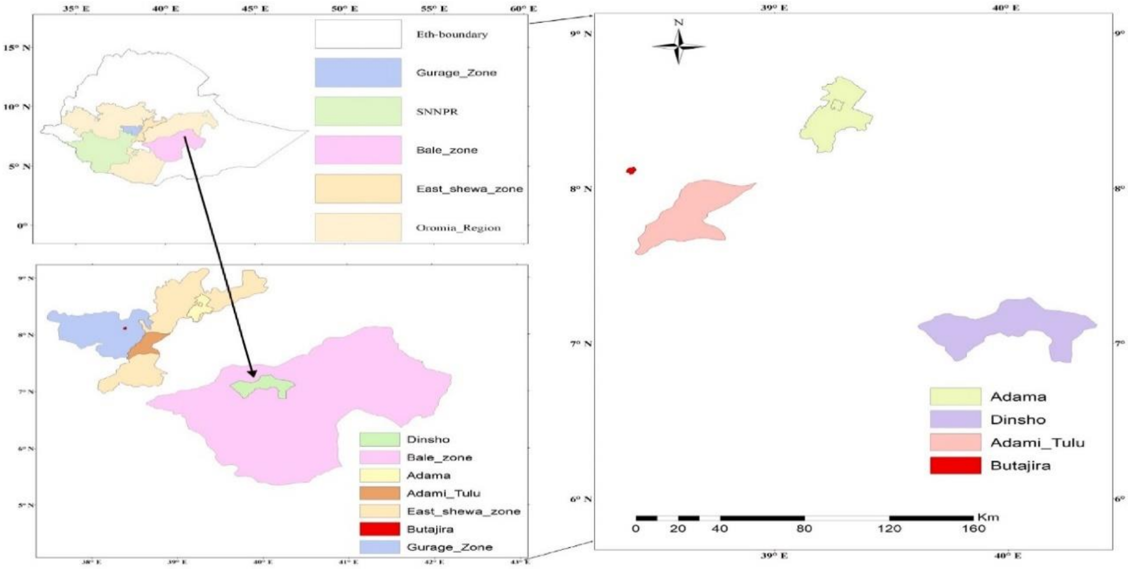

Figure 1. Map of the study area.

Preparation of extracts and analysis of anti-nutritional factors

Plant samples were first rinsed with distilled water to eliminate dust and other surface contaminants. They were then dried under shade at ambient conditions for 72 hours, during which the average temperature and relative humidity were 30 °C and 25%, respectively, as recorded by a digital hygrometer [18]. Once dried, the materials were milled to pass through a 1 mm screen (2 mm for samples intended for tannin analysis). The resulting powders were sealed in airtight plastic containers and stored at 4 °C in a cool, dark environment to minimize degradation of phenolic compounds.

For extraction, 150 g of each powdered sample was combined with 1500 mL of pure methanol in a glass flask, following the method of Kirby and Schmidt [19]. The mixture was agitated on a mechanical shaker at 150 rpm and 25 °C for 96 hours. After shaking, the solutions were filtered using a Buckner funnel fitted with Whatman No. 1 filter paper. The filtrates were then evaporated to dryness under reduced pressure at 50 °C using a rotary evaporator (Büchi Labortechnik, Germany) to remove the solvent and obtain concentrated extracts. The dried extracts were dissolved in methanol at a ratio of 1 mg to 10 mL and stored at 4 °C in light-resistant glass bottles until further analysis. Total phenolics and tannins were quantified using the procedure outlined by Makkar [20], with absorbance measured at 724 nm on a UV–Vis spectrophotometer (U-1800, High Technology Corporation, Tokyo, Japan).

For subsequent use as additives, each dried extract was further diluted in distilled water to prepare concentrations of 25, 50, and 75 mg/kg DM by dissolving 2.5, 5.0, and 7.5 mg of extract in 1000 mL of water, respectively [12]. All prepared solutions were refrigerated at 4 °C until needed.

Essential oil extraction was performed via steam distillation as described by Guenther [21]. Fresh leaves of CC, RO, TS, seeds of CS, and EK roots were collected from their respective locations. Approximately 250 g of fresh leaves were chopped and placed into a 1-L distillation flask containing 400 mL of distilled water. Hydro-distillation was carried out for 3 hours at 50 °C using a Clevenger-type apparatus. Seeds and roots were similarly processed after milling them into small pieces. Essential oil yield was expressed using the following formula:

$$\text{Oil content}\,\frac{\mathrm{V}}{\mathrm{W}}\,\% = \frac{Volume\ of\ extracted\ oil\ (ml)}{mass\ of\ sample\ (g)} \times 100 \qquad (1)$$

Proximate analysis of substrate feed

Maize stover (variety MH-130) was obtained from Adami Tulu Agricultural Research Center. The material was oven-dried at 60 °C for 48 h in a forced-air dryer, then milled to pass a 1-mm screen using a Wiley laboratory mill. The ground stover was stored in sealed plastic bags at ambient temperature until required for analysis or incubation.

The chemical composition of the maize stover was determined using standard procedures. Dry matter, ash, and crude protein were analyzed following AOAC methods [22]. Neutral detergent fiber (NDF) was measured using an Ankom 220 fiber analyzer according to Van Soest *et al*. [23], whereas acid detergent fiber (ADF) and acid detergent lignin (ADL) were quantified as described by Van Soest and Robertson [24].

The analyzed maize stover contained 8 % ash, 3.6 % crude protein, 76.2 % NDF, 47 % ADF, and 6.6 % ADL on a dry matter basis.

In vitro gas and methane production assessment

Collection of rumen fluid and fermentation procedures

Rumen contents were obtained from sheep at slaughter following the protocol of Wang *et al*. [25]. Each experimental day, rumen fluid was taken from two Arsi-Bale sheep at a local abattoir. The donor animals had been maintained on a grass-hay diet for one week prior to slaughter. The rumen fluid was collected early in the morning immediately after the animals were killed, transferred into a preheated (39 °C) thermos flask, and promptly transported to the laboratory. After pooling, the fluid was filtered through four layers of gauze and subsequently mixed with Menke's incubation buffer in a 1:2 (v/v) proportion. Preparation of the buffer solution followed the Menke and Steingass [26] procedure. Throughout the handling process, CO_2 was continuously supplied to maintain anaerobic conditions and preserve microbial viability.

In vitro fermentations were carried out in 100-mL glass syringes. Before incubation, 200 mg of dried maize stover (ground to 1-mm particle size) was weighed into each syringe. Two milliliters of the predetermined concentrations of plant extracts—derived from leaves of AN, AZ, CC, LL, MS, RO, TS; roots of EK; and seeds of CS—were added to the syringes containing maize stover, with each treatment prepared in triplicate across two independent incubation runs [12]. For each run, three control syringes (containing substrate but no extract) and three blanks (containing only rumen fluid plus buffer) were also included. All syringes were pre-warmed overnight at 39 °C, and their pistons were greased with vaseline to ensure smooth movement and prevent gas leakage.

A total of 30 mL of the rumen fluid–buffer mixture was then dispensed into each syringe under a CO_2 stream. The syringes were placed in a water bath at 39 °C and manually agitated every hour during the first 8 hours of incubation (including time zero) [27], and again at each subsequent measurement point [28]. Gas production was recorded at 0, 3, 9, 12, 24, 48, 72, and 96 hours. Final gas values were adjusted by subtracting the volumes recorded in the blank syringes and the initial gas readings for each time point.

Determination of total gas and methane production

Cumulative gas production was recorded by noting piston displacement at each designated time point, as described by Menke and Steingass [26]. Net gas production (expressed as ml per 200 mg DM) at any given incubation time t was computed using the following equation: $Gt = [(Vt - V_0 - G_0) \times 200] / WS$ where: Gt = net gas production at time t (ml/200 mg DM) Vt = syringe volume reading at time t (ml) V_0 = initial syringe volume reading (ml) G_0 = mean gas production from blank syringes (ml) WS = dry weight of incubated substrate (mg)

In vitro organic matter digestibility (OMD, %) and metabolizable energy (ME, MJ/kg DM) were predicted from 24-h gas production and substrate chemical composition using the established equations of Menke and Steingass [26].

$$\mathrm{OMD}(\%) = 14.88 + 0.889\mathrm{GP} + 0.45\mathrm{CP} + 0 : 0651\mathrm{XA} \tag{2}$$

In vitro organic matter digestibility (OMD, %) at 24 h was estimated using: OMD (%) = 14.88 + 0.889 GP + 0.45 CP + 0.0651 XA where: - GP = net gas production at 24 h (ml/200 mg DM) CP = crude protein content of the substrate (% DM) - XA = ash content (% DM)

Metabolizable energy (ME, MJ/kg DM) was calculated as: ME = 2.20 + 0.136 GP + 0.057 CP where GP and CP are as defined above.

Short-chain fatty acid (SCFA) productionwas estimated from 48-h gas volumes according to Getachew *et al*. [29]: SCFA (mmol/g DM) = 0.0239 × GP − 0.0601 where GP = net gas production at 48 h (ml/200 mg DM).

Methane measurement at 24 h followed the protocol of Fievez *et al*. [30]. After recording total gas volume at the end of incubation, a second syringe containing 4.0 ml of 10 M NaOH was connected to the incubation syringe. The NaOH was gently injected without loss of gas; the strong alkali absorbed all CO_2 (and any H_2S), leaving only CH_4 in the headspace. The residual gas volume was recorded as methane.

Net methane production was determined by subtracting the methane volume measured in the corresponding blank syringes. Methane proportion in the total gas was expressed as a percentage following Jayanegara *et al*. [31]:

$$\text{Methane concentration (\%)} = \left(\frac{\text{Net methane production}}{\text{Net gas production}} \times 100\right) \tag{3}$$

Statistical analysis

Anti-nutrient composition (total phenols, flavonoids, tannins, and essential oils) was analyzed using the PROC ANOVA procedure of SAS 9.4 [32]. The statistical model applied was:

$$Yij = \mu + \alpha i + eij \tag{4}$$

where Yij = observed value, μ = overall mean, αi = fixed effect of the ith plant species/variety, and eij = random residual error.

Treatment means were separated using the least squares means (LSMEANS) procedure, with differences declared significant at $P < 0.05$.

Total phenolic content was quantified from a gallic acid standard curve ($y = 0.0114x + 0.0563$, $R^2 = 0.9791$; 0–150 μg/mL) and reported as mg gallic acid equivalents (GAE) per gram of dry extract. Total flavonoid content was determined using a quercetin standard curve ($y = 10.031x + 0.0775$, $R^2 = 0.9613$; 0–125 μg/mL) and expressed as mg quercetin equivalents (QE) per gram of dry extract.

Data for total gas production, methane production, in vitro organic matter digestibility (IVOMD), metabolizable energy (ME), and short-chain fatty acids (SCFA) were analyzed as a 15 × 4 factorial arrangement (15 plant extracts × 4 inclusion levels, including the unsupplemented control) using the PROC GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) [32]. The model was:

$$Yijk = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ijk \tag{5}$$

where $Yijk$ = response variable, μ = overall mean, αi = fixed effect of the ith plant extract, βj = fixed effect of the jth dose level, $(\alpha\beta)ij$ = plant × dose interaction, and εijk = random residual error.

Mean separation was performed using LSMEANS, and differences were considered significant at $P < 0.05$.

Results and Discussion

Levels of anti-nutrients in medicinal plant extracts

Table 1 presents the levels of major secondary metabolites (total phenols, flavonoids, tannins, and essential oils) in the methanolic extracts of the tested medicinal plants. Considerable differences were found across species and accessions. Total phenolic content varied markedly, spanning from 26.1 to 471.7 mg GAE/g dry extract — a nearly 18-fold range. Among all entries, Acacia nilotica leaf extract displayed by far the highest ($P < 0.001$) total phenol concentration.

Table 1. Secondary metabolite profile of methanolic extracts from selected Ethiopian medicinal plants and varieties (values are means ± SE).

Plant Part & Species	Total Phenolics (TP, mg GAE/g dry extract)	Total Tannins (g/kg)	Condensed Tannins (g/kg)	Essential Oil (% fresh wt.)	Hydrolysable Tannins (g/kg)	Total Flavonoids (TF, mg QE/g dry extract)
Leaves						
AN	471.7 ± 3.5 (highest)	151.5 ± 1.2 (highest)	89.0 ± 0.9 (highest)	ND	62.5 ± 1.4	36.5 ± 1.2 (low)
AZ	52.8 ± 1.7 (low)	48.8 ± 0.9	25.0 ± 2.3	ND	23.8 ± 0.8	3.7 ± 0.1 (lowest)
CC-I	63.3 ± 2.0	45.0 ± 0.9	19.3 ± 0.9	0.34	25.7 ± 1.1	130.5 ± 1.8 (high)
CC-Java	131.8 ± 3.7	49.4 ± 2.6	19.8 ± 0.5	0.65	29.6 ± 0.8	143.6 ± 1.7 (highest)
CC-UA	123.0 ± 1.7	51.3 ± 1.1	43.2 ± 1.0	0.56	8.05 ± 1.1	129.0 ± 3.0
LL	92.0 ± 3.0	44.6 ± 1.0	19.2 ± 0.1	ND	25.3 ± 0.6	38.0 ± 1.0
MS	97.4 ± 1.5	25.9 ± 1.6	16.2 ± 1.4	ND	9.7 ± 0.8	65.5 ± 1.5
RO-I	64.0 ± 3.4	42.7 ± 0.4	29.8 ± 1.0	0.62	12.9 ± 0.1	47.0 ± 2.9

RO-II	58.1 ± 2.2	34.8 ± 0.2	19.3 ± 1.4	1.02 (highest)	15.5 ± 1.0	53.0 ± 1.0
RO-III	57.3 ± 1.6	33.3 ± 1.5	20.3 ± 2.0	0.76	12.9 ± 1.6	43.3 ± 1.5
TS	134.0 ± 2.6	39.9 ± 0.6	22.6 ± 2.6	0.45	17.3 ± 0.8	75.0 ± 1.8
Seeds						
CSB	26.0 ± 2.0	21.5 ± 0.6	13.9 ± 0.7	0.36	7.6 ± 1.4	5.7 ± 0.35
CST	27.4 ± 2.0	21.4 ± 0.5	12.6 ± 1.0	0.32	8.8 ± 0.5	9.6 ± 0.65
CSW	27.1 ± 2.0	23.7 ± 1.1	19.3 ± 0.9	0.42	4.4 ± 1.0	8.9 ± 0.95
Root						
EK	36.4 ± 1.5	25.4 ± 2.0	14.8 ± 0.9	0.10	10.6 ± 1.3	6.3 ± 1.0

Within each column, means bearing different superscript letters differ significantly (P < 0.05).
TP = total phenols; TF = total flavonoids; GAE = gallic acid equivalents; QE = quercetin equivalents; ND = not detected.

Plant abbreviations: AN = Acacia nilotica; AZ = Azadirachta indica; CSB = Coriandrum sativum Batu; CST = Coriandrum sativum Tulu; CSW = Coriandrum sativum Waltai; CC-I = Cymbopogon citratus-I; CC-Java = Cymbopogon citratus-Java; CC-UA = Cymbopogon citratus-Upper Awash; EK = Echinops kebericho; LL = Leucaena leucocephala; MS = Moringa stenopetala; RO-I = Rosmarinus officinalis-I; RO-II = Rosmarinus officinalis-II; RO-III = Rosmarinus officinalis-III; TS = Thymus schimperi.

Total flavonoid (TF) content ranged from 3.7 to 143.6 mg QE/g dry extract, representing a greater than 40-fold difference. The three Cymbopogon citratus accessions exhibited markedly higher flavonoid levels than all other tested materials, with the Java accession (CC-Java) recording the highest value (P < 0.001). In contrast, Azadirachta indica showed the lowest flavonoid concentration.

Total tannin content was highest (P < 0.001) in Acacia nilotica leaf extract and lowest in the three Coriandrum sativum seed varieties. Essential oil yield was greatest (P < 0.001) in Rosmarinus officinalis-II (1.02 % on fresh weight basis) and lowest in the root of Echinops kebericho (0.10 %).

In vitro total gas and methane production from maize stover supplemented with plant extracts

In vitro total gas production (TGP) and methane yields from maize stover incubated with the different crude plant extracts at varying inclusion levels are presented in **Table 2**.

At the lowest dose (25 mg/kg DM), none of the plant extracts caused any significant deviation (P > 0.05) in TGP compared with the unsupplemented control.

At the intermediate dose (50 mg/kg DM), most extracts significantly increased TGP (P < 0.001), with increments ranging from 2 % to 31 % depending on the plant species. The only exception was Acacia nilotica, which showed no stimulation. The greatest increases (31 %) were recorded with Azadirachta indica, Leucaena leucocephala, and Moringa stenopetala.

At the highest dose (75 mg/kg DM), all extracts markedly depressed TGP (P < 0.001) relative to the control.

A significant plant species × dose interaction (P < 0.05) was observed for total gas production, particularly evident at the 50 mg/kg DM inclusion level.

Table 2. In vitro total gas production and methane emission from maize stover supplemented with crude medicinal plant extracts at different inclusion levels (mg/kg DM) in south-eastern Ethiopia.

Source of Extract	**24 h Methane Volume (ml)**				**SEM**	**P value**	**48 h Total Gas Production (ml)**				**SEM**	**P value**
	Control	25 mg	50 mg	75 mg			Control	25 mg	50 mg	75 mg		
AN	12.0a	12.0a	11.0Cb	10.0Cc	0.25	<0.001	51.1a	52.0a	42.1Db	41.9b	1.6	0.003
AZ	12.0a	12.0a	12.0Ba	11.0Bb	0.13	<0.001	51.1b	52.0b	67.0Aa	42.0c	2.7	<0.001
CC I	12.0a	12.0a	12.3Ba	11.0Bb	0.17	0.002	51.0b	52.0b	55.0Ba	42.0c	1.5	<0.001
CC Java	12.0a	11.9a	9.3Ec	11.0Bb	0.40	<0.001	51.2b	52.0b	55.0Ba	42.0c	1.4	<0.001
CC UA	12.0	12.0	12.0B	12.3A	0.08	0.4	51.3b	52.2b	55.0Ba	42.0c	1.5	<0.001
CSB	12.0	12.0	12.0B	12.3A	0.07	0.5	51.1b	52.0b	55.0Ba	42.1c	1.5	<0.001
CST	12.0	12.0	12.3B	12.3A	0.11	0.6	51.1b	52.0b	55.0Ba	42.2c	1.5	<0.001
CSW	12.0	12.0	12.3B	12.3A	0.12	0.5	51.0b	52.0b	55.0Ba	42.2c	1.4	<0.001
EKM	12.0	12.2	12.0B	12.0A	0.08	0.44	51.0a	51.7a	52.1Ca	42.0b	1.2	<0.001
LL	12.0	12.0	12.3B	12.3A	0.11	0.6	51.1b	52.0b	67.0Aa	42.2c	2.7	<0.001

MS	12.0b	12.0b	14.0Aa	12.3Ab	0.26	<0.001	51.0b	52.0b	67.0Aa	42.0c	2.6	<0.001
RO I	12.0	12.1	12.7B	12.3A	0.13	0.2	51.1b	52.2b	55.0Ba	42.0c	1.4	<0.001
RO II	12.0	12.0	12.0B	11.7AB	0.08	0.4	51.0b	52.2b	55.0Ba	42.2c	1.6	<0.001
RO III	12.0	12.0	12.3B	11.7AB	0.15	0.3	51.1b	52.0b	55.0Ba	42.1c	1.5	<0.001
TS	12.0a	11.9a	10.0Db	11.7ABa	0.28	0.005	51.0b	52.2b	55.0Ba	42.0c	1.4	<0.001
SEM	0.02	0.02	0.09	0.12	–	–	0.01	0.05	0.61	0.07	–	–
P value	0.65	0.09	<0.001	<0.001	–	–	0.08	0.76	<0.001	0.51	–	–
P × D	0.82	0.72	<0.001	0.65	–	–	0.75	0.91	<0.001	0.61	–	–

Within each column, means denoted by different uppercase letters (plant species) differ significantly ($P < 0.05$); within each row, means with different lowercase letters (dose levels) differ significantly ($P < 0.05$). SEM = standard error of the mean; P × D = plant extract × dose interaction effect. Plant species codes are defined in the footnote to **Table 1.**

Overall, substantial methane suppression ($P < 0.001$) was achieved with Cymbopogon citratus-Java and Thymus schimperi extracts at the 50 mg/kg DM level. Relative to the control, these treatments lowered methane yield by 22.5% and 16.7%, respectively. A significant ($P < 0.001$) plant × dose interaction influenced methane volume specifically at this inclusion rate. No other extracts reduced methane at any dose, with the exception of Acacia nilotica at 50 mg/kg DM and Azadirachta indica, Rosmarinus officinalis-II, Rosmarinus officinalis-III, and Thymus schimperi at 75 mg/kg DM.

In vitro organic matter digestibility and metabolizable energy of maize stover supplemented with plant extracts

In vitro organic matter digestibility (IVOMD, %) and metabolizable energy (ME, MJ/kg DM) of maize stover incubated with varying levels of crude plant extracts are summarized in **Table 3**. At 50 mg/kg DM, extracts from Azadirachta indica, Cymbopogon citratus-Java, Leucaena leucocephala, Moringa stenopetala, and Thymus schimperi enhanced IVOMD ($P < 0.001$) by 8–16% compared to the unsupplemented control. No significant changes ($P > 0.05$) in IVOMD were noted at the 25 mg/kg DM dose across all extracts. A pronounced plant × dose interaction ($P < 0.001$) was evident at 50 mg/kg DM. However, at the highest inclusion (75 mg/kg DM), all extracts depressed IVOMD relative to the control, except for Cymbopogon citratus-I, Echinops kebericho, and Rosmarinus officinalis-I.

Table 3. In vitro organic matter digestibility (%) and metabolizable energy (MJ/kg DM) of maize stover incubated with crude medicinal plant extracts at different inclusion levels (24 h incubation).

Source of extracts	**Parameters and mean values**											
	IVOMD						**ME**					
	Control	**25**	**50**	**75**	**SEM**	**P value**	**Control**	**25**	**50**	**75**	**SEM**	**P value**
AN	50.0^{a}	50.1^{a}	48.0^{Ea}	44.0^{Bb}	0.73	<0.001	7.5^{a}	7.5^{a}	7.0^{Db}	6.5^{Cc}	0.12	<0.001
AZ	50.0^{b}	50.0^{b}	55.0^{Ba}	44.6^{Bc}	1.1	<0.001	7.5^{b}	7.5^{b}	8.1^{Ba}	6.5^{Cc}	0.17	<0.001
CC I	50.0	50.0	52.0^{C}	48.0^{A}	0.44	0.25	7.4^{a}	7.5^{a}	7.7^{Ba}	7.0^{Bb}	0.07	<0.001
CC Java	50.0^{b}	50.0^{b}	54.0^{Ba}	48.0^{Ab}	0.40	<0.001	7.5^{b}	7.5^{b}	7.9^{Ba}	7.0^{Bc}	0.07	<0.001
CC UA	50.0^{a}	50.0^{a}	52.0^{Ca}	47.0^{Ab}	0.54	<0.001	7.5^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.08	<0.001
CSB	50.2^{a}	50.0^{a}	52.0^{Ca}	46.0^{Ab}	0.64	<0.001	7.5^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.08	<0.001
CST	50.0^{a}	50.0^{a}	52.0^{Ca}	47.0^{Ab}	0.7	<0.001	7.5^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.07	<0.001
CSW	50.0^{a}	50.0^{a}	52.0^{Ca}	47.0^{Ab}	0.6	<0.001	7.4^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.08	<0.001
EKM	50.0	50.0	50.0^{C}	49.7^{A}	0.14	0.88	7.5	7.5	7.5^{C}	7.5^{A}	0.02	0.08
LL	50.2^{b}	52.0^{b}	55.0^{Ba}	48.0^{Ab}	0.80	<0.001	7.4^{b}	7.6^{b}	8.0^{Ba}	7.0^{Bc}	0.11	<0.001
MS	50.0^{b}	50.0^{b}	58.0^{Aa}	49.7^{Ab}	1.0	<0.001	7.5^{b}	7.5^{b}	8.6^{Aa}	7.0^{Bc}	0.15	<0.001
RO I	50.2	50.0	52.0^{C}	48.0^{A}	0.46	0.008	7.5^{a}	7.5^{a}	7.7^{Ca}	7.2^{Bb}	0.07	0.003
RO II	50.0^{a}	50.2^{a}	52.0^{Ca}	47.0^{Ab}	0.5	<0.001	7.5^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.07	0.002
RO III	50.0^{a}	50.0^{a}	52.0^{Ca}	47.0^{Ab}	0.6	<0.001	7.5^{a}	7.5^{a}	7.7^{Ca}	7.0^{Bb}	0.06	<0.001
TS	50.1^{b}	50.0^{b}	54.0^{Ba}	47.0^{Ac}	0.58	<0.001	7.5^{b}	7.5^{b}	7.9^{Ba}	7.1^{Bc}	0.05	<0.001
SEM	0.001	0.03	0.23	0.24	–	–	0.001	0.012	0.03	0.03	–	–
P value	0.089	0.086	<0.001	<0.001	–	–	0.68	0.59	<0.001	<0.001	–	–
P × D	0.075	0.082	<0.001	0.089	–	–	0.75	0.45	<0.001	0.069	–	–

Column means with different uppercase letters indicate statistically significant differences between plant species, whereas row means with differing lowercase letters reflect significant differences among the applied doses. SEM refers to the standard error of the mean. The plant species abbreviations are provided in the footnote of **Table 1**.

Incorporation of plant extracts at 25 mg/kg DM did not cause any notable changes in the estimated metabolizable energy (ME) of the maize stover across all species. At 50 mg/kg DM, extracts from Azadirachta indica (AZ), Cymbopogon citratus Java (CC-Java), Leucaena leucocephala (LL), Moringa stenopetala (MS), and Thyme schimperi (TS) significantly elevated the ME content compared to the control, with increases ranging between 6.7% and 26.7%. The most pronounced enhancement was observed in Moringa stenopetala at this dose, showing a 26.7% increase (P < 0.001). Conversely, the addition of Acacia nilotica extract at 50 mg/kg DM resulted in a 6.7% decrease in ME (P < 0.001). At the highest dose of 75 mg/kg DM, ME values generally declined by 6.7–13.3% for most plant extracts, except for Echinops kebericho (EKM), which did not alter the estimated ME at any of the tested doses.

Methane concentration and short-chain fatty acid production

Methane concentration (expressed as % of total gas at 24 h) and estimated short-chain fatty acid (SCFA) production from maize stover incubated with different doses of plant extracts are presented in **Table 4**.

The lowest methane concentrations were recorded when Cymbopogon citratus-Java (CC-Java; 23.3 %) and Thymus schimperi (TS; 25.0 %) extracts were added at 50 mg/kg DM. A highly significant plant species × dose interaction (P < 0.001) was observed for methane concentration at this inclusion level.

At the lowest dose (25 mg/kg DM), none of the extracts altered methane concentration compared with the control. In contrast, the highest dose (75 mg/kg DM) consistently increased methane percentage across all tested plant extracts.

Table 4. Methane concentration (% of 24-h total gas production) and estimated short-chain fatty acid production (mmol/L) of maize stover incubated with different doses (mg/kg DM) of medicinal plant extracts.

Source of Extract	**SCFA (mmol/g DM)**				**SEM**	**P value**	**CH_4 Concentration (%)**				**SEM**	**P value**
	Control	25 mg	50 mg	75 mg			Control	25 mg	50 mg	75 mg		
AN	0.84a	0.80a	0.77Eb	0.68Cc	0.02	0.003	31.5a	31.5a	30.8Ab	32.0Ba	0.14	<0.001
AZ	0.83b	0.85b	0.98Ba	0.70Cc	0.03	<0.001	31.4b	31.5b	30.7Ab	33.0Ba	0.65	<0.001
CC I	0.84b	0.85b	0.90Ca	0.80Bc	0.01	<0.001	31.5	31.5	31.5A	31.0B	0.19	0.14
CC Java	0.85b	0.85b	0.90Ca	0.77Bc	0.01	<0.001	31.5a	31.3a	23.3Cc	28.0Cb	1.0	<0.001
CC UA	0.85b	0.85b	0.90Ca	0.77Bc	0.007	<0.001	31.3b	31.4b	30.8Ab	35.0Aa	0.54	<0.001
CSB	0.85b	0.85b	0.90Ca	0.80Bc	0.01	<0.001	31.5b	31.3b	31.0Ab	35.0Aa	0.68	0.01
CST	0.85b	0.85b	0.90Ca	0.77Bc	0.01	<0.001	31.5b	31.4b	30.8Ab	35.0Aa	0.67	0.05
CSW	0.85b	0.84b	0.90Ca	0.77Bc	0.02	<0.01	31.3b	31.2b	30.8Ab	35.0Aa	0.62	0.02
EKM	0.85	0.85	0.85D	0.84A	0.01	0.072	31.5	31.4	31.8A	32.0B	0.09	0.92
LL	0.85b	0.85b	0.98Ba	0.76Bc	0.02	0.002	31.4b	31.5b	30.5Ab	34.0Aa	0.70	<0.001
MS	0.85b	0.83b	1.1Aa	0.77Bc	0.03	0.001	31.5b	31.6b	30.8Ab	34.0Aa	0.48	<0.001
RO I	0.85b	0.85b	0.90Ca	0.77Bc	0.04	<0.001	31.5b	31.2b	31.7Ab	34.0Aa	0.40	0.005
RO II	0.82b	0.84b	0.90Ca	0.77Bc	0.001	<0.001	31.3b	31.7b	30.9Ab	34.0Aa	0.55	0.04
RO III	0.83	0.83	0.94C	0.77B	0.06	<0.001	31.5	31.3	30.8A	32.0B	0.21	0.59
TS	0.85b	0.84b	0.94Ca	0.77Bc	0.02	<0.001	31.5a	31.5a	25.0Bc	28.0Cb	1.0	<0.001
SEM	0.41	0.02	0.06	0.07	–	–	0.001	0.90	0.25	0.34	–	–
P value	0.09	0.09	<0.001	<0.001	–	–	0.82	0.67	<0.001	0.003	–	–
P × D	0.56	0.068	<0.001	0.08	–	–	0.75	0.45	<0.001	0.52	–	–

Column means with different uppercase letters indicate significant differences among plant species, while row means with differing lowercase letters reflect significant differences among doses. Abbreviations used are: mmol = micromole; SEM = standard error of the mean; SCFA = short-chain fatty acids. The key for plant species is provided in the footnote of **Table 1**.

The highest SCFA concentration (1.1 mmol/g DM) was observed when maize stover was incubated with extracts of Moringa stenopetala, Leucaena leucocephala, and Azadirachta indica at 50 mg/kg DM ($P < 0.0001$). For the other plant species, SCFA levels increased by 9.3% to 29.4% at the same dose. Treatment with extracts at 25 mg/kg DM did not produce any notable change in SCFA concentration across all species. In contrast, at 75 mg/kg DM, SCFA concentrations were significantly reduced ($P < 0.001$) for all plant species, indicating that higher doses of the extracts negatively impacted fermentation and fiber digestibility.

Phytochemical composition of the medicinal plants

The total phenolic content recorded for Acacia nilotica in the present study aligns closely with the findings of Melesse *et al*. [33], who documented elevated phenolic levels in Ethiopian Acacia nilotica leaf powder. However, a considerably lower value (136.5 ± 2.5 mg GAE/g) was obtained from ethanol extracts of the same species [34]. For Azadirachta indica, Bhatta *et al*. [35] reported a higher phenolic concentration (108 g/kg DM) in mature and over-mature leaves collected in India, possibly due to differences in agro-climatic conditions and sampling sites. The total phenolic content observed here for Thymus schimperi is comparable to that reported by Safaei-Ghomi *et al*. [36] for Iranian Thymus caramanicus, whereas a lower value was noted for the same species in northern Ethiopia [37]. Similarly, the three Cymbopogon citratus accessions examined in this study exhibited markedly higher total phenols, flavonoids, and tannins than those reported by Uraku *et al*. [38] for West African lemongrass. Across all tested materials, the three Coriandrum sativum seed varieties consistently displayed the lowest contents of total phenols, flavonoids, and tannins.

Discrepancies between the present results and previous reports can largely be attributed to genetic variation among accessions, differences in soil characteristics, geographical origin, prevailing climatic conditions, and the choice of extraction solvent. As highlighted by Kumar *et al*. [39], agro-climatic factors strongly influence phytochemical profiles; plants often synthesize greater amounts of secondary metabolites under environmental stress. For instance, Kaplan *et al*. [40] demonstrated increased flavonoid, anthocyanin, and mucilage production in response to high-temperature stress, while Kumar *et al*. [39] noted that cooler conditions tend to favor phenolic accumulation and vice versa.

Total gas and methane production

The marked suppression of total gas production observed with Acacia nilotica extract at 50 mg/kg DM and with all plant extracts at 75 mg/kg DM is likely attributable to their high total phenolic and tannin contents, which can inhibit rumen microbial activity when present at elevated concentrations. This finding is consistent with Bhatta *et al*. [35], who reported reduced gas yields from total mixed rations incubated with foliage rich in phenolics and tannins. Both hydrolysable and condensed tannins exhibit strong protein-binding capacity and can become toxic to rumen microbes at higher doses [41], potentially by disrupting cell membrane integrity, blocking nutrient uptake, and impairing microbial proliferation [42].

The pronounced methane suppression achieved with Cymbopogon citratus-Java and Thymus schimperi extracts at 50 mg/kg DM can be linked to their elevated flavonoid and essential oil contents. These results corroborate earlier studies showing that flavonoids reduce populations of protozoa and methanogenic archaea in vitro [43, 44]. Essential oils and flavonoids have also been shown to exert direct inhibitory effects on methanogens [45, 46]. For instance, Broudiscou *et al*. [47] recorded 8–14 % lower methanogenesis when screening 13 flavonoid-rich plant extracts on a hay–barley diet, while Bodas *et al*. [48] observed up to 15 % methane reduction with certain plant species without compromising digestibility, total gas, or volatile fatty acid production.

Overall, the antimethanogenic effects noted in the present study with extracts rich in phenols, flavonoids, and essential oils are consistent with the known ability of these secondary metabolites to interfere with methanogen activity and hydrogen transfer pathways in the rumen.

In contrast, the methane reductions seen with Acacia nilotica at 50 mg/kg DM and with Azadirachta indica, Rosmarinus officinalis-II, Rosmarinus officinalis-III, and Thymus schimperi at 75 mg/kg DM appear to be a secondary consequence of depressed total gas production and organic matter digestibility rather than a specific antimethanogenic action. This aligns with the principle that low-digestibility, slow-fermenting substrates with prolonged rumen retention time tend to favor higher methane yields per unit of digested feed [49].

In vitro organic matter digestibility and metabolizable energy

The enhancement of in vitro organic matter digestibility (IVOMD) observed when maize stover was supplemented with Azadirachta indica, Cymbopogon citratus-Java, Leucaena leucocephala, Moringa stenopetala, and Thymus schimperi extracts at 50 mg/kg DM agrees with earlier evidence that plant secondary metabolites can improve the nutritive value of low-quality roughages. For example, Akanmu *et al*. [12] recorded higher IVOMD of Eragrostis hay upon addition of Aloe vera extract, an effect they attributed to the presence of polysaccharide-degrading enzymes such as diastase and amylase. Similarly, the digestibility improvements noted in the present work are likely driven by phenolic compounds and other phytochemicals that promote the hydrolysis of complex structural carbohydrates in maize stover. Nasser *et al*. [50] likewise demonstrated a strong positive relationship between cumulative gas production and digestibility in Berseem hay, supporting the link between enhanced fermentation and greater nutrient availability observed here.

Methane concentration and short-chain fatty acids

In the present study, the addition of *Cymbopogon citratus* Java and *Thyme schimperi* extracts at 50 mg/kg DM significantly reduced methane concentration, indicating that these plant species have strong potential as in vitro methane mitigation agents. Methane expressed as a proportion of total gas can serve as a useful indicator for evaluating the effectiveness of a feed additive in suppressing methane production, with lower methane-to-gas ratios suggesting a greater potential for rumen methane reduction [10]. Similarly, Berhanu *et al*. [9] observed lower methane emissions from forage species that were rich in secondary metabolites. The methane-to-total gas production ratio (MTGR) provides an index for assessing the CH_4 reduction capacity of feed additives, reflecting the volume of methane produced per unit of organic matter degraded [12].

The reduction in methane by *Cymbopogon citratus* Java and *Thyme schimperi* at 50 mg/kg DM occurred without affecting total SCFA production. This effect may be attributed to the phenolic compounds in these plants, which can directly inhibit methanogenic microorganisms. SCFA production and composition, however, can be influenced by several factors. For example, Hristov *et al*. [51] reported that tannic acids can reduce total SCFA output, while inhibition of fiber degradation shifts SCFA profiles away from acetate, consequently decreasing hydrogen availability and methane formation. According to Bhatta *et al*. [35], phytochemical feed additives are most effective when they reduce methane production without negatively affecting VFA concentrations. This aligns with the current findings, which are consistent with previous reports showing up to a 20% reduction in methane concentration without altering SCFA levels when *Azadirachta indica* and *Autocarpus integrifolis* powders were included at 2.5% in mixed rations.

Conclusion

Overall, the present study revealed that leaf extracts of Acacia nilotica are particularly rich in total phenols and tannins, whereas high flavonoid concentrations were observed in Thyme schimperi and two varieties of Cymbopogon citratus (Java and Upper Awash). Notably, crude extracts from Cymbopogon citratus Java and Thyme schimperi were effective in lowering methane emissions without compromising the in vitro digestibility of maize (Zea mays) stover at a dose of 50 mg/kg DM. These findings highlight the potential use of these plant extracts as natural feed additives for methane mitigation. Further investigations are recommended to assess the stability of phenolic compounds during storage and to validate their effects under in vivo conditions in ruminant animals.

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