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EFFECTS OF PLANT EXTRACTS ON *IN-VITRO* GAS PRODUCTION KINETICS AND RUMINAL FERMENTATION OF FOUR FIBROUS FEEDS: TOWARDS SUSTAINABLE ANIMAL DIETS

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ABSTRACT

This study was conducted to determine the effects of increasing doses of plant extracts of moringa, thyme, and rosemary on *in-vitro* gas production (IVGP) of four fibrous feeds (kikuyu grass, alfalfa hay, oat hay, and corn stover). The extracts were applied at doses of: 0 (control), 6 (low), 12 (medium), and 18 (high) ml/g dry matter (DM). IVGP was assessed using three fistulated goats and different incubation periods. At the end of incubation, dry matter degradability (DMd), gas yield (GY24), metabolizable energy (ME), short chain fatty acids (SCFA), and microbial crude protein (MCP) production were determined. The results showed that the increasing doses of the evaluated extracts had neither linear nor quadratic effect on gas production (GP) of all fibrous feeds ($P > 0.05$). The addition of plant extracts increased GP at all incubation periods ($P \leq 0.05$), being higher for thyme. Moringa, thyme, and rosemary extracts altered ruminal fermentation parameters, including ME, MCP, SCFA, the partitioning factor at 72 h of incubation (PF72), and DMd, being higher for thyme

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followed by rosemary and moringa. In conclusion, the addition of thyme extract increased GP, ME, MCP, SCFA, PF72, and DMd compared with rosemary and moringa plant extracts. However, additional *in-vivo* studies should be conducted to confirm these effects.

Keywords: Gas production, goats, Rosemary, moringa, plant secondary metabolites, thyme.

INTRODUCTION

Today, there is a trend towards improving degradation of fibrous feeds in ruminants using feed additives, especially, plant secondary metabolites (PSMs) (Ku-Vera et al., 2020; Karásková et al., 2015). PSMs are a generic term used for more than 30,000 different substances that are exclusively produced by plants (Kholif et al., 2018). PSMs were often referred to as anti-nutritional factors, but they have gained growing interest in ruminant nutrition in recent years (Ku-Vera et al., 2020). In general, PSMs such as tannins, saponins, and flavonoids affect the attachment of rumen microorganisms to fibrous feeds (Zeru et al., 2022), create a stable extract feed complex (Karásková et al., 2015), and alter the fiber structure, which could stimulate microbial colonization (Kholif et al., 2018). Several studies have reported on the use of numerous plants with a wide spectrum of PSMs to improve forage fermentation in the rumen (Ku-Vera et al., 2020; Kholif et al., 2018; Karásková et al., 2015). In this regard, it has been described that moringa (*Moringa oleifera*) (Shah et al., 2017), thyme (*Thymus vulgaris*) (Khorrami et al., 2015), and rosemary (*Salvia rosmarinus*) (Cobellis et al., 2015) stimulate the activity of rumen microbiota (Shah et al., 2017; Khorrami et al., 2015).

Moringa is a perennial slender softwood tree that belongs to the Moringaceae family, showing a relatively high phenolic and flavonoid content (Kholif et al., 2018; Zeru et al., 2022). Thyme, which is a flowering plant in the Lamiaceae family, is rich in flavonoids, saponins, alkaloids, and tannins (Gunal et al., 2013). Rosemary originates from the Mediterranean region; it belongs to the Lamiaceae family and also contains diverse metabolites such as carnosol, carnosic acid, and rosmarinic acid (Kong et al., 2022). Many *in-vitro* and *in-vivo* studies have been conducted to investigate the effects of moringa, thyme, and rosemary in livestock nutrition, but the results have been inconsistent (Karásková et al., 2015; Zeru et al., 2022). Moreover, little is known about the effects of these plant extracts on gas production kinetics and ruminal fermentation parameters in goats. Therefore, this study aimed to determine the modulator activity of moringa, thyme, and rosemary extracts on *in-vitro* gas production and ruminal fermentation parameters of four fibrous feeds (kikuyu grass, alfalfa hay, oat hay, and corn stover).

MATERIALS AND METHODS

Preparation of plant extracts

Pre-dried *Moringa oleifera* seeds were ground in a Willey Mill (Arthur Hill Thomas Co., Philadelphia, PA), and 6-g samples were placed in cellulose cartridges for extraction of ether extract, replicating the procedure until 100 mL of the desired oil was obtained. Thyme (*Thymus Vulgaris*) and rosemary (*Rosemarinus officinalis*) plants were dehydrated at 60 ° C, for 48 h. Subsequently, the samples were placed in an ultrasonicator (20 kHz and 50 W) with distilled water and CO_2 as the supercritical fluid for extraction (250 bar, 55 ° C) for 15 minutes.

Laboratory analysis

Forage samples were analyzed for dry matter (DM, method no. 934.01), organic matter (OM, method no. 942.05), crude protein (CP, method no. 976.05) and ether extract (EE, method no. 920.39), according to the Association of Official Analytical Chemists (AOAC, 1997). Contents of neutral detergent fiber (NDF), and acid detergent fiber (ADF) were determined according to Van Soest et al. (1991) using an Ankom 200 Fiber Analyzer (Ankom Technol. Co. Macedon, NY, USA) according to Robinson et al. (1999). NDF was assayed with α-amylase and sodium sulfite. Both NDF and ADF were expressed without residual ash.

The total phenolic content was determined by Folin-Ciocalteu reagent (Adesegun et al., 2007), and the results were expressed as gallic acid equivalent (GAE). The aluminum chloride colorimetric method was used for the determination of the total flavonoid content as described by Es-Safi et al. (2007). All measurements were performed in triplicate, and data are the means of the measurements performed.

In-vitro gas production

All the experimental procedures were approved by the Animal Experimental Guidelines of the Universidad Autónoma del Estado de Mexico (project code UAEMex 4335/2017). Three non-lactating goats (45 ± 5 kg body weight, mean

± SD) with rumen fistula were used to collect rumen fluid. Goats were fed the total mixed rations at 08:00 and16:00 h daily, with free access to water. Briefly, about 3 L of rumen content from the individual donor was collected before morning feeding.

Plant extracts of moringa, thyme, and rosemary were added at doses of: 0 (control), 6 (low), 12 (medium), and 18 (high) mL/g DM to four fibrous feeds (kikuyu grass, alfalfa hay, oat hay, and corn stover), and their effects on in-vitro gas production (IVGP) were determined as described by Theodorou et al. (1994). Briefly, 0.999 g DM of substrate was placed into glass flasks bottles of 125 mL in triplicate in two batches and repeated for three incubation runs, with 10 mL of ruminal liquid and 90 mL of buffer solution. The cumulative GP (mL/g DM) was recorded at 3, 6, 9, 12, 24, 36, 48, 60, and 72 h post incubation at 39 °C. To measure gas production kinetics, data (mL/g DM) were fitted with the NLIN procedure of SAS according to the Krishnamoorthy et al. (1991), using the model: GP= b $(1-e^{-ct})$. Where GP= Gas production (mL gas/ g DM); b= total gas production (mL gas / g DM); c= degradation rate compared with the time (hours); t= time (h). Dry matter degradability (DMd), metabolizable energy (ME), short chain fatty acids (SCFA), and microbial crude protein (MCP) production were also estimated as described by Blümmel et al. (1997) and Getachew et al. (2002).

Design and statistical analyses

For IVGP data, a completely randomized design (CRD) with a 3×4×4 factorial arrangement was used. Least-square means (LSM) were calculated and significant differences between the means were determined using the Tukey's test. A polynomial contrasts analysis was employed to determine the linear and quadratic effects of the extracts. Differences of LSM were considered to be significant at P ≤ 0.05, and that of P ≤ 0.10 was considered as a tendency. The statistical model was as follows:

$$Y_{ijkl}= \mu+A_i + B_j+ Ck+ AB_{ij} + AC_{ik}+ BC_{jk} + E_{ijk}$$

Where: Y_{ijkl} = variable response; μ = average general; A_i = effect plant extract; B_j = forage species; Ck = effect of doses; ABC_{ijk} = Effect of ABC interaction at level i, j, k; and E_{ijkl} = random error.

RESULTS

Chemical composition and PSMs of the substrates

The highest CP content was observed in alfalfa hay, followed by kikuyu grass (Table 1). Corn stover had the highest NDF content, while alfalfa hay had the lowest content. The highest ADL content was found in corn stover, while the lowest value was obtained in kikuyu grass. Total

Table 1. Chemical composition of the four fibrous plants (g/kg DM) and total phenolic and flavonoid contents in the plant extracts under study.

Item[a] (g/kg DM)	Forages				Plant extracts		
	Oat hay	Alfalfa hay	Corn stover	Kikuyu grass	Rosemary	Thyme	Moringa oleifera
DM [b]	919	903	925	201	none	none	none
OM	947	865	932	879	none	none	none
CP	91	169	54	151	none	none	none
EE	22	25	11	27	none	none	none
NDF	580	416	700	653	none	none	none
ADF	364	328	440	351	none	none	none
ADL	65	76	80	43	none	none	none
NFC	254	179	167	48	none	none	none
Calcium	1.2	15	1.0	2.5	none	none	none
Phosphorus	3.5	3.0	3.0	2.5	none	none	none
Total Phenols[c]	ND	ND	ND	ND	309±1.38	299±1.57	2865±0.67
Flavonoids[d]	ND	ND	ND	ND	258±1.80	260± 1.10	0.44±0.07

[a] DM, dry matter; OM, organic matter; CP, crude protein; ADF, acid detergent fiber; NDF, neutral detergent fiber; NFC, non-fiber carbohydrates.
[b] Expressed as g/kg FM
[c] Expressed as mg gallic acid equivalent/g Fresh weight
[d] Expressed as mg quercetin eq/g Fresh weight
ND, not determined

phenolic content was higher in moringa, followed by rosemary and thyme. With respect to total flavonoid content, similar levels were observed in the extracts of thyme and rosemary, while moringa extract presented lower values.

In-vitro gas production (IVGP)

Alfalfa hay showed the highest gas production (C) (P < 0.0001, Table 2), followed by oat hay, kikuyu grass, and corn stover. Regarding plant extracts, moringa showed the greatest (P < 0.0001) lag time. There was an interaction between extract and dose (P < 0.0001) for GP at incubation periods of 6, 12, 24, 48, and 72 h. Moreover, the substrate interaction per dose for fractions b and c was significant (P < 0.0001).

In-vitro rumen fermentation profile

The highest DMd value was obtained in kikuyu grass (P < 0.001), followed by oat hay and alfalfa hay (Table 3). Likewise, ME and MCP values were higher for the kikuyu grass (P < 0.001) compared with the other forages. The addition of thyme extract resulted in a 26% increase in DMd (P < 0.05; Table 3). Both thyme and rosemary extracts showed a lower partitioning factor at 72 h of incubation (PF72) compared to that of moringa (P < 0.05). Thyme had higher values of ME, MCP, and SCFA (P < 0.05). The interactions between extract and dose were significant for PF72, SCFA, and ME (P < 0.0001). The interaction of extract by substrate per dose showed a significant difference (P < 0.0001; Table 3) in terms of GP (at incubation periods of 6, 12, 24, 48 and 72 h), PF72, SCFA, and ME.

Table 2. ***In-vitro*** **gas production of four feed species and four different doses of plant extracts added (mg/g DM).**

	Gas production parameters[a]			*In-vitro* gas production (mL/g DM)				
Item	**b**	**c**	**Lag Time**	**GP6**	**GP12**	**GP24**	**GP48**	**GP72**
Substrate								
Kikuyu grass	211.2[a]	0.022[c]	1.29[c]	17.93[b]	51.14[b]	77.5[b]	128.4[a]	161.1[a]
Corn stover	199.9[a]	0.015[d]	3.49[a]	10.45[d]	24.58[d]	51.54[c]	96.42[c]	124.4[b]
Oat straw	177.7[b]	0.031[b]	-0.043[d]	29.53[a]	61.79[a]	88.00[a]	131.7[a]	159.5[a]
Alfalfa hay	146.6[c]	0.034[a]	2.37[b]	14.84[c]	44.90[c]	73.86[b]	110.78[b]	129.6[b]
SEM	2.48	0.001	0.11	0.42	0.87	1.12	1.43	1.59
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Extract								
Moringa	172.9[b]	0.027[a]	3.65[a]	13.01[c]	39.74[b]	67.17[b]	112.75[b]	134.9[b]
Thyme	223.1[a]	0.023[b]	0.221[c]	26.11[a]	53.90[a]	87.05[a]	140.37[a]	173.2[a]
Rosemary	155.6[c]	0.026[a]	1.45[b]	15.43[b]	43.13[b]	63.93[b]	97.31[c]	122.8[c]
SEM	2.481	0.0004	0.1054	0.416	0.868	0.1165	1.432	1.589
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Doses								
0	178.98	0.0259	1.6280	18.41	45.03	72.83	116.17	142.02
6	183.91	0.0251	1.8984	17.91	45.26	72.21	116.97	144.52
12	186.54	0.0256	1.7841	18.06	45.99	72.66	116.81	143.90
18	186.03	0.0254	1.8013	18.35	46.07	73.18	117.30	144.21
SEM	0.2481	0.0004	0.1054	0.4169	0.8685	1.1165	1.43.29	1.58.98
P-value	0.6746	0.9436	0.8264	0.9692	0.9620	0.9913	0.9933	0.9406
Lineal	0.2694	0.8039	0.5908	0.7629	0.6866	0.9550	0.8704	0.6689
Quadratic	0.8450	0.5758	0.4446	0.7492	0.9024	0.8409	0.8895	0.6797
Overall								
SEM	0.212	0.004	0.002	0.014	0.032	0.040	0.054	0.065
P-value	0.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Ext × S	0.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Ext × Doses	0.0001	0.0374	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
S × Doses	0.0001	0.0003	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001
Ext × S × Doses	0.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

[a] b, the asymptotic gas production (mL/g DM); c, the rate of gas production (h^{-1}); Lag time, the initial delay before gas production begins (h).

Table 3. *In-vitro* rumen fermentation profile of four feed species and four different doses of plant extracts added.

Item	DMD (mg/100 mg DM)	PF_{72} (mg DMD:mL gas)	GY_{24} (mL gas/g DMD)	SCFA (mmol/gDM)	MCP (mg/g DM)	ME (MJ/kg DM)
Substrate						
Kikuyu grass	65.53^{a}	88.37^{c}	86.60^{bc}	0.34^{b}	621.28^{a}	19.95^{a}
Corn stover	48.56^{d}	102.05^{b}	75.45^{c}	0.22^{c}	462.93^{d}	8.86^{d}
Oat straw	55.54^{b}	120.17^{a}	104.04^{a}	0.39^{a}	516.70^{b}	11.23^{c}
Alfalfa hay	51.91^{c}	97.02^{b}	96.90^{ab}	0.32^{b}	486.69^{c}	15.67^{b}
SEM	0.533	3.27	3.06	0.01	5.04	0.119
P-value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Plant Extract						
Moringa	49.89^{b}	269.96^{a}	13.435^{b}	0.2945^{b}	469.40^{b}	13.63^{b}
Thyme	66.69^{a}	21.63^{b}	132.33^{a}	0.3827^{a}	628.67^{a}	14.70^{a}
Rosemary	49.57^{b}	14.12^{b}	126.48^{a}	0.2795^{b}	467.637^{b}	13.45^{b}
SEM	3.198	19.63	18.35	0.057	30.20	0.71
P-value	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Doses						
0	55.81	100.4	89.74	0.319	526.0	13.94
6	56.00	101.8	88.35	0.316	528.3	13.90
12	54.94	100.2	92.58	0.319	517.5	13.93
18	54.81	105.3	92.33	0.317	515.9	13.96
SEM	0.27	1.68	1.57	0.005	2.59	0.06
P-value	0.274	1.683	1.574	0.005	2.589	0.061
Lineal	0.285	0.672	0.719	0.985	0.219	0.992
Quadratic	0.256	0.513	0.513	0.967	0.233	0.973
Overall						
SEM	0.181	0.619	0.095	0.006	1.83	0.025
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ext × S	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ext × Doses	0.3377	<0.0001	0.0054	<0.0001	0.4563	<0.0001
S × Doses	0.0002	<0.0001	0.0001	<0.0001	0.0003	<0.0001
Ext × S × Doses	0.282	<0.0001	0.004	<0.0001	0.342	<0.0001

DMD, dry matter degradability; ME, metabolizable energy; PF96, the partitioning factor at 96 h of incubation; GY24, gas yield at 24 h; SCFA, short chain fatty acids; MCP, microbial CP production.

DISCUSSION

Chemical composition and PSMs of substrates

The chemical composition of the substrate used in the present study was consistent with that described in previous reports (García-González et al., 2008). However, it should be noted that forage plants have variations in chemical composition depending on the soil, climatic conditions, and stage of maturity (Dijkstra et al., 2005). The chemical composition of the feed is an essential factor for predicting the true digestibility of dry matter or organic matter in *in-vitro* gas production (García-González et al., 2008). Our results showed that moringa exhibited higher contents of PSMs, which agrees with Gunal et al. (2013) and Shah et al. (2017).

In-vitro gas production (IVGP)

In-vitro fermentation techniques have been widely applied to evaluate the effects of feed additives on rumen fermentation and methane production (Zeru et al., 2022). Forages with higher levels of degradability tend to have higher gas production (Dijkstra et al., 2005). Water-soluble carbohydrates act as an energy source for ruminal microorganisms during digestion (Theodorou et al., 1994). In this sense, our results showed that oat hay, kikuyu grass, and alfalfa had a higher GP compared to corn stover. It has been reported that the inclusions of plant extracts with high PSMs can affect gas production (Kong et al., 2022; Ku-Vera et al., 2020; Khorrami et al., 2015). In the present study, the addition of thyme, rosemary, and moringa extracts affected the *in-vitro* gas production kinetics, with the highest ME, MCP, SCFA, and DMd values being recorded with thyme. These findings agree with previous studies that have reported that the addition of plant extracts with high PSMs result in higher *in-vitro* gas production due to a faster rate of substrate degradation in the rumen (Akanmu & Hassen, 2017; García-González et al., 2008). Moreover, Morgavi et al. (2000) indicated that the higher GP resulting from the addition of moringa extracts could be explained by the high PSMs, which may support fibrolytic microbes in the rumen by increasing the proximity between substrates and microbes, and thus causing a faster fermentation rate and subsequent degradation of substrates. However, it has been suggested that the different effects of PSMs in gas production could be justified by the combined inhibitory and stimulatory action of PSMs on some rumen microorganisms (Karásková et al., 2015). Moreover, several factors, such as basal diet, donor, and time, have interaction effects on *in-vitro* experiments, which should be considered when comparing the results of different studies. However, none of these studies considered the effect of plant extracts on goats.

In-vitro fermentation profile of the rumen

Our results also showed that corn stover had the lowest DMd of all the evaluated forages. This can be explained by the hemicellulose content of forages, which is totally fermented by microorganisms (Theodorou et al., 1994; Getachew et al., 2002). In this sense, Dijkstra et al. (2005) reported that forage degradation is mainly determined by the characteristics of the cell wall. In the present study, corn stover and alfalfa hay had a greater amount of NDF and ADF, which could explain the lower DMd, MCP, and ME values in these substrates.

The addition of moringa, thyme, and rosemary extracts enhanced the digestibility of the forages. Likewise, Kolif et al. (2018) observed greater digestibility of the diet supplemented with moringa extract (at levels of 10, 20, and 40 mL). However, Khorrami et al. (2015) found no changes in digestibility with the addition of thyme and cinnamon extracts (500 mg / kg DM) in steers. Previous reports have shown that the addition of moringa could exert a negative effect on ruminal fermentation and digestibility of nutrients due to the large number of PSMs in moringa (Kholif et al., 2018; Shah et al., 2017). In contrast, Kolif et al. (2018) observed a positive effect of moringa extract on ruminal digestion, being in agreement with other studies that have indicated that moringa and thyme extracts increase *in-vitro* digestibility of OM and DM (Akanmu and Hassen, 2017; Dey et al., 2014). In fact, it has been well documented that herbal extracts with PSMs can enhance the efficiency of ruminal microorganisms to degrade forage components (Ku-Vera et al., 2020; Khorrami et al., 2015). However, it has been described that high levels of PSMs may act as an antimicrobial agent on the rumen microbiota, particularly cellulolytic bacteria; e.g. rosemary, which is associated with negative effects on DMd (Ku-Vera et al., 2020; Durmic et al., 2014). Similarly, Gunal et al. (2013) reported a decrease in DMd and MCP using *in-vitro* batch culture with high doses of rosemary oil (500 mg/L). Furthermore, a more recent study conducted by Ku-Vera et al. (2020) revealed that the effect of plant extracts on *in-vitro* fermentation is associated with the physiological stage of the plant, the parts of the plant used, the composition of the soil, temperature, water stress, as well as the dose of the extracts, extraction method, and the animal species.

In the present study, the doses of thyme, moringa, or rosemary extracts added to the feed showed no significant effect on *in-vitro* rumen fermentation kinetics. Probably, the dose of the extract is the main limiting factor for the effects of PSMs on ruminal microorganisms (Karásková et al., 2015; García-González et al., 2008), and thus the amount and type of PSMs in our study did not affect the microbial population. In this sense, Kong et al. (2022) has reported that the effects of PSMs on *in-vitro* fermentation are level- and source-dependent.

CONCLUSION

Increasing doses of moringa, thyme, and rosemary extracts had no effect on ruminal degradation or GP parameters. The addition of thyme extracts favorably affected DMd, GY24, SCFA, MCP, and ME, which indicates that doses of 6, 12, and 18 μL / g DM can improve degradation efficiency of forages. However, additional *in-vivo* studies are required to evaluate the sustainability of various plant extracts in improving ruminant production.

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