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Original article

Effect of Some Essential Oils on *in vitro* Ruminal Fermentation of Alfalfa Hay

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Abstract. *Study Objectives:* This study aimed to investigate the effects of essential oils derived from the leaves of *Laurus nobilis* (LNEO), *Myrtus communis* (MCEO), *Lavandula stoechas* (LSEO), *Artemisia annua* (AAEO), and *Thymbra spicata* (TSEO) on the rumen fermentation parameters (gas production, methane emission, organic acids, and the number of protozoa) of dry alfalfa hay. *Methods:* The components of all essential oils were analyzed. The effects of adding the same dose (60 mg/L) of each essential oil to the rumen fluid on in vitro rumen digestion were determined by in vitro gas production. *Results:* The addition of *Lavandula stoechas, Artemisia annua, Myrtus communis,* and *Laura nobilis* essential oils decreased the total gas and methane formation (ml), organic matter digestion (OMD), ruminal ammonia nitrogen levels, and the metabolic energy (ME) values. On the other hand, the *Thymbra spicata* essential oil did not affect any parameter except the ruminal ammonia nitrogen level. The rumen protozoa numbers were unaffected by the addition of *Thymbra spicata* essential oil. The total volatile fatty acids (TVFA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) amounts in the in vitro fermentation fluid of alfalfa hay were low in all groups. *Conclusion:* It was determined that the active ingredients of LSEO, AAEO, MCEO, and LNEO may have a regulatory effect on ruminal fermentation. We think that more studies using different feed types and combinations of essential oils are required to reveal the effects of these essential oils on ecology and the physiology of the digestive system.

Key words: Essential Oil, In Vitro Gas Production, Methane

Introduction

As the global need for food increases, the current resources are depleting; this has led researchers to make changes in ruminant feeding systems. The use of feed additives has had positive results on greenhouse gas emissions (1-2). In parallel with the increase in ruminant animals' worldwide, ruminant-originated greenhouse gas (methane, carbon dioxide, and nitrogen dioxide) emission has also increased. Studies have shown that cattle produce 60-160 kg/year of methane (CH_4) per animal, sheep, and goats 4 kg/year depending on the type of feed, particle size, and the dry matter rate (3). On the other hand, the energy loss caused by methane gas is 2-12% of the gross energy taken with feed (4). Feed additives with defined effectiveness in increasing feed use efficiency by changing the characteristics of ruminal fermentation to inhibit ruminal methanogenesis are required (5). Intensive research has been carried out to prevent the loss of energy received through rations via methane and reduce greenhouse gas emissions. After the

prohibition of using antibiotics in feeding animals, the focus has shifted to methods that reduce methane production without leaving residues in animal products. Among these, the effects of plants and plant extracts containing essential oils on the formation of methane have been extensively investigated (6-9).

The current study aims to identify the effects of plant essential oils considered for use as feed additives for ruminants on methane gas formation and in vitro organic substance digestion using the in vitro gas production technique.

Materials and Methods

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Plant material

The plant essential oils used in the study were extracted from the leaves of *Laurus nobilis*, *Myrtus communis*, *Lavandula stoechas*, *Artemisia annua* and *Thymbra spicata* gathered during the flowering stage of the plants and dried at 35°C by hydrodistillation.

Chemical analysis of dried alfalfa

Alfalfa, which was harvested during the vegetation period and subsequently dried, was analysed for dry matter (DM), crude ash (CA), crude protein (CP), and crude fat (CF) using the official methods of analysis of the Association of Official Analytical Chemists (10). The composition of the neutral-detergent fibres (NDF), acid-detergent fibres (ADF), and acid detergent lignin (ADL) were analysed as described by Van-Soest et al. (11).

Analyses of the composition of essential oils by GC-MS

The dried plant material was chopped and placed in a beaker, and then steam distillation was used to extract the essential oil. Steam distillation is based on the principle of applying pressure to the plant materials using steam, creating droplets of oil and water together, and then evaporating the water from the droplets in the beaker. The chemical components of the volatile oil were determined using a Thermo Scientific ISQ Single Quadrupole model gas chromatograph and a TG-Wax MS-A model, 5% phenyl polysilphenylene-siloxane column of 0.25 mm inner diameter, 30 m length, and 0.25 µm film thickness. Helium (99.9%) was used as the carrier gas at a flow rate of 1 mL/minute. The ionization energy was 70 eV and the mass range (m/z) was set from 1.2 to 1200 amu. The scan mode was used for data collection. The temperature of the mass spectrometry (MS) transfer line was 250°C, the MS ionization temperature was 220 °C, the temperature of the injection port was 220 °C, and the column temperature was initially 50°C and increased up to 220°C at a rate of 3°C/minute. The structure of each component was described using the Xcalibur software and mass spectra.

Determination of in vitro gas and methane production

The in vitro gas production technique was used to determine the gas and methane production of the essential oils used in the study (12). The rumen fluid obtained from three awassi breed sheep was obtained before the morning feeding and filtered through a four-layer muslin and mixed with a 1:2 buffer solution. In this technique, the same dose (60 mg/l) of each essential oil (LNEO, MCEO, LSEO, AAEO, TSEO) was prepared in glass syringes of 100 ml and incubated with 200 ± 10 mg alfalfa and a mixture of solutions of buffer + micromineral + micromineral + reduction + resazurin (20 ml) and rumen fluid (10 ml). Gas production of samples obtained from each group in the study was implemented in four replications. Four syringes were used blindly in calculations.

In the study, the total amount of gas (ml) produced in each syringe was determined by reading the syringes after 24 hours of incubation. The methane content in the total gas was determined using the Infrared methane measuring device (Sensor, Europe GmbH, Erkrath, Germany).

Determination of in vitro degradability parameters

The effects of laurel volatile oil on the *in vitro* metabolizable energy (ME), organic matter digestibility (OMD), and net energy lactation (NE_L)values were calculated using the formulae indicated below (12-13).

ME (MJ /kg DM) = 2.20 + 0.136 × GP + 0.057 × CP + 0.0028597 x EE^2

OMD (g/kg DM) = 14.88 + 0.889 × GP+ 0.45 × CP + 0.0651 × CA

GP= 24 h net gas production (ml/200 mg), CP=Crude protein (g/kg DM), CA= Crude ash (g/kg DM), EE = Ether extract (g/kg DM).

Determination of total protozoa count

At the end of the incubation period, the content of the glass syringes was used to count the number of protozoa. At the end of 24 h, 1 ml of the content was filtered from each syringe and added 49 ml of a diluent (mixture of 20 ml of 37% formalin, 150 ml of glycerin, and 820 ml of distilled water) to prepare ready-to-count 50-ml sample aliquots as described by Boyne et al. One ml of each aliquot was placed in the chamber of a Macmaster's slide to count the number of protozoa per cubic centimeter (14).

Statistical analysis

The statistical analysis of the raw data was performed using the SPSS 17.0 software bundle. The statistical significance between the groups was determined by the One Way Analysis of Variance (ANOVA). When significance was detected, the multiple comparison test, the "Tukey Multiple Range Test", was used. $p<0.05$ were considered statistically significant.

Results

The chemical composition of essential oils obtained by distillation has been presented in Table 1.

The analysis showed that gamma-terpinene (35.27%) was the main component of *Thymbra spicata*, Eucalyptol (43.82%-38.44%) of *Laurus nobilis* and *Myrtus communis*, Fenchone (46.12%) of *Lavandula stoechas* and Linalool (38.97%) of *Artemisia annua* essential oil.

The effect of the study after 24 hours of incubation on in vitro gas production, ammonia nitrogen, and protozoa numbers has been presented in Table 2. The results revealed significant differences in gas production after different EO applications. *Artemisia annua* and *Lavandula stoechas* decreased the gas production by 54% compared to the control group ($p<0.01$). The total gas and methane production (ml), organic matter digestion (OMD), and metabolic energy (ME) values were low in the groups that contained essential oils other than *Thymbra spicata* essential oil ($p<0.01$). The protozoa levels increased substantially in the groups except for *Thymbra spicata* ($p<0.01$), and the ruminal ammonia levels substantially decreased in all groups compared to the control group ($p<0.05$).

It was identified that the effect of adding *Laurus nobilis*, *Myrtus communis*, *Lavandula stoechas*, *Artemisia annua,* and *Thymbra spicata* plant essential oils to the rumen fluid on the characteristics of ruminal fermentation was significantly decreasing the TVFA, acetic, propionic, and butyric acid contents of all essential oils as shown in Table 3 ($p<0.05$). It was found that *Lavandula stoechas* and *Thymbra spicata* were the essential oils most effective on ruminal fermentation.

Discussion and Conclusion

Laurus nobilis, *Myrtus communis*, *Lavandula stoechas*, *Artemisia annua,* and *Thymbra spicata* plants are seen along the entire Mediterranean coast. In terms of the proportion of the main component of the plants essential oils, the order is eucalyptol (42.82% for *Laurus nobilis*, 38.44% for *Myrtus communis*, 16.13% for *Lavandula stoechas*), γ-terpinene (35.27% for *Thymbra spicata*), fenchone (46.12% for *Lavandula stoechas*), linalool (38.97% for *Artemisia annua*, 11.32% for *Lavandula stoechas*), and carvacrol (25.04% for *Thymbra spicata*). The essential oil rates and chemical components of plants gathered from similar locations in previous studies are similar to a significant portion of the components identified in our study (6,15-16). However, in their study of the same plant in three different locations, Cook et al., (2007) observed differences in

Table 1. Chemical composition [%] of the investigated essential oils

	TSEO	LNEO	MCEO	AAEO	LSEO
α -Pinene	1.34	6.18	25.13	3.99	7.31
α -Thujene	3.45	1.47	2.40		3.28
Camphene		14.02		3.09	6.45
Myrcene	3.27		0.9	1.29	2.84
α -Terpinene	6.46	1.99	8.20		4.09
D-Limonene	0.84				0.64
Eucalyptol		43.82	38.44	16.13	
Chamazulene				0.37	6.38
γ -Terpinene	35.27	0.66			3.25
P-Cymene	13.30	2.34	4.13	0.63	2.02
Terpinolene		0.22			0.32
Fenchone				46.12	
Geranyl acetate			3.18		
Linalool				11.32	38.97
Linalyl Acetate		3.18	5.42	2.25	4.70
Caryophyllene			1.88	0.84	
3-Cyclohexen-1-ol	2.06	1.55	1.47	2.28	
Terpineol			2.83	1.40	
Borneol	0.18	0.33		0.30	
β -Bisabolene	0.30			1.54	
Carvone				0.82	
Benzaldehyde					0.58
Geraniol			1.47		
1-Phenylaziridine			1.52		
β -Caryophyllene oxide				2.24	
Spathulenol			0.46		1.68
Thymol	5.25				
Carvacrol	25.04				4.46
Sabinene		12.57			4.45
Trans-Sabinene hydrate	0.34	1.01			1.09
β - Pinene	0.70	3.33			
4-carvomenthenol		2.71	0.78		
Norbornan			0.41	2.74	
Eugenol		2.84			2.32
Terpinen-4-ol	0.73				3.83
Total	98.53	98.22	98.62	97.35	98.66

the composition of essential oils between regions and plant organs that did not seem directly related to climatic conditions, but that they may have been related to local differences (17).

This study identified that methane levels (ml) were significantly lower in the *Laurus nobilis*, *Myrtus communis*, *Artemisia annua* and *Lavandula stoechas* groups compared to the control group (P<0.01) (Table 2). This result, which is consistent with previous studies reporting that the use of essential oils reduces the methane emissions, has been attributed to the high Eucalyptol (43.82% for *Laurus nobilis*, 38.44% for *Myrtus communis*, 16.13% for *Lavandula stoechas*), Fenchone (46.12% for *Lavandula stoechas*), α–Pinene (25.13% for *Myrtus communis*) and linalool levels (38.97% for *Artemisia annua*, 11.32% for *Lavandula stoechas*) in the essential oils used (18-19).

It has been stated that the anti-methanogenic and antiprotozoal effects of plant species and their extracts may differ due to their different structures and doses (20). Protozoa participate in the reduction of CO_2 to CH_4. Hence, the antiprotozoal effect of plant and plant extracts reduces the production of methane as some methanogens bind to the protozoa (21).

Table 2. LNEO, MCEO, LSEO, AAEO, TSEO oils on the in vitro digestion parameters of alfalfa hay

Groups	Gas	CH_4(ml)	CH_4(%)	ME	OMD	NH_3-N	Protozoa
CONTROL	42.20^{a}	7.07^{a}	16.76	8.87^{a}	66.03^{a}	30.5^{a}	2.15^{b}
TSEO	42.60^{a}	7.48^{a}	17.57	8.93^{a}	66.38^{a}	21.8^{b}	2.10^{b}
LNEO	26.40^{bc}	4.37^{bc}	16.58	6.73^{bc}	51.98^{bc}	27.9^{ab}	3.00^{a}
MCEO	28.40^{b}	5.34^{b}	18.74	7.00^{b}	53.75^{b}	25.6^{ab}	2.98^{a}
AAEO	23.20^{c}	4.26^{bc}	18.49	6.30^{c}	49.13^{c}	23.6^{b}	3.48^{a}
LSEO	23.20^{c}	3.58^{c}	15.27	6.29^{c}	49.13^{c}	23.6^{b}	3.54^{a}
SEM	1.428	0.429	1.171	0.193	1.271	0.898	0.131
Sig.	**	**	NS	**	**	*	**

LNEO, MCEO, LSEO, AAEO, TSEO: Groups added 60 mg/L of volatile oil. SEM: Standard errors of means, NS: non-significant, TGP: Total gas production (24h ml/ 0.2 g DM), CH_4: methane production as a percentage of total gas production, ME: Metabolic energy as MJ/kg DM, OMD: Organic matter digestibility as %.

Table 3. The effect of LNEO, MCEO, LSEO, AAEO, TSEO volatile oils ruminal fermentation characteristics.

Groups	TVFA (mmol/L)	AA	PA	BA	OFA	AA/PA
CONTROL	97.29±4.69^{a}	47.17±1.55^{a}	25.23±1.52^{a}	19.27±1.26^{a}	5.60^{a}	1.88^{d}
TSEO	54.69±4.67^{c}	25.94±1.42^{c}	10.35±0.96^{c}	12.86±2.21^{bc}	5.53^{a}	2.55^{bc}
LNEO	69.97±1.36^{b}	36.15±0.71^{b}	15.79±0.71^{b}	14.81±1.96^{ab}	3.21^{b}	2.30^{cd}
MCEO	69.35±3.36^{b}	35.18±3.63^{b}	16.85±0.86^{b}	13.96±1.12^{b}	3.35^{b}	2.10^{cd}
AAEO	61.11±2.20^{bc}	35.37±0.41^{b}	11.60±0.76^{c}	11.42±1.41^{bc}	2.71^{b}	3.09^{b}
LSEO	53.37±3.02^{c}	33.19±0.82^{b}	8.88±0.93^{c}	8.79±1.22^{c}	2.49^{b}	3.87^{a}
SEM	3.018	1.333	1.079	0.838	0.336	0.144
Sig.	**	**	**	*	*	**

TVFA: (as mmol/L, in rumen fluid) total volatile fatty acids comprise of acetate + propionate + butyrate + iso butyrate + valerate + iso-valerate; OFA: other fatty acids comprise of iso-butyrate + valerate + iso-valerate; AA: acetic acid, PA: propionic acid, BA: butyric acid, AA/PA: acetate / propionate. SEM: Standard errors of means.

However, contrary to the studies conducted, although there was an increase in the number of rumen protozoa in the groups in which the essential oils of *Laurus nobilis*, *Myrtus communis, Artemisia annua,* and *Lavandula stoechas* were administered, the lack of a reducing effect on CH_4 (ml) in our study is consistent with studies indicating that in vitro methanogenesis is not primarily related to the density of the protozoa population (22-23).

Plant extracts and doses that do not adversely affect ruminal fermentation and the likelihood of feed spoilage with a favorable effect on reducing the ammonia concentration in the rumen should be selected. Protozoal growth, low ammonia concentration, and a parallel decrease in digestibility by plant secondary compounds have been recorded in various studies (P. K. Patra et al., 2016), and the positive effect of *Thymbra spicata* essential oil used in the study that lowers the rumen ammonia level without any effect on the protozoal population suggests that this may be due to the inhibition of ammonia producing bacteria. This result is consistent with the studies of Mandal 2016 and Onel 2017 (24-25). On the other hand, unlike the results of in vitro studies, studies conducted in cattle and sheep have shown that essential oils do not affect the levels of bacterial NH_3-N (26-27). However, the highest value of gas and methane production being in the group in which *Thymbra spicata* essential oil

was added could be due to the main components of the essential oil γ–*Terpinene (35.27%)* and *carvacrol (25.04%)*. Although the effects of *Artemisia annua* decreased the NH_3-N in our study, in vivo studies reported that the addition of 30-50 g/kg of *Artemisia montana* to the ration increased ruminal volatile fatty acids (VFA) and NH_3-N concentrations in sheep (28). In this study, the total volatile fatty acid (TVFA) molarity (53-97 mmol/L) produced during the fermentation in the ration administered to the animals was at the ideal level for the normal rumen ecosystem (7). All essential oils used in the study decreased the acetate, propionate, and butyrate levels, and this reduced the TVFA molarity in the fermentation fluid.

Possibly due to the natural structure, activity, and concentration of their active compounds, the 60 mg/l doses of five different essential oils studied in the current investigation have exhibited different effects on in vitro rumen fermentation. In particular, it has been determined that the essential oils of *Laurus nobilis*, *Myrtus communis*, *Artemisia annua* and *Lavandula stoechas* have an important potential as regulators of in vitro rumen fermentation due to their capacity to stimulate gas production by minimizing methanogenesis and protozoa. In this context, in vitro and long-term in vivo studies are required to support the effects of the plant essential oils used in this study on rumen microflora and fermentation gases in terms of the dose-adaptation duration.

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Conflicts of interest: The authors declare that there is no conflict of interest about this manuscript.

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