academicJournals

Vol. 8(49), pp. 6557-6562, 19 December,2013
DOI: 10.5897/AJAR2013.7485
ISSN 1991-637X ©2013 Academic Journals
http://www.academicjournals.org/AJAR

African Journal of Agricultural Research

Full Length Research Paper

The effect of *Origanum onites* L., *Rosmarinus officinalis* L. and *Schinus molle* L. on *in vitro* digestibility in lamb

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Accepted 4 December, 2013

The objective of this study was to evaluate the effects of different levels of aromatic plants (AP);"*Origanum onites L.* (ORE), *Rosmarinus officinalis L.* (ROS) and *Schinus molle L.* (SHN)" on *in vitro* gas production (GP), organic matter digestibility (OMD) and net energy lactation (NEL) using an *in vitro* gas-production method. Two rumen-fistulated sheep were used in the experiment. The sheep were fed with 60% of alfalfa hay and 40% of concentrate feed twice daily. Five different levels of ORE, ROS, and SHN were added to the concentrate (CON) to produce 200 mg KM (C - without , AP; 1 - 0.02 mg AP + 198 mg CON; 2 - 0.04 mg AP + 196 mg CON; 3 - 0.06 mg AP + 194 mg CON; 4 - 0.08 mg AP + 192 mg CON; 5 - 0.10 mg AP + 190 mg CON). The volume of gas produced was recorded at 2, 4, 8, 12, and 24 h after incubation. The results showed that, GP, OMD and NEL contents decreased significantly as the level of ORE, ROS, and SHN added to CON increased. It was concluded that, ORE, ROS, and SHN are potential supplements that alter rumen fermentation. To obtain exact results, the findings obtained under *in vitro* conditions should be supported by *in vivo* studies.

Key words: Ruminant, aromatic plants, feed digestibility, gas production, organic matter digestibility.

INTRODUCTION

In Europe, the utilisation of such plant extracts as essential oils (EOs) in livestock production has expanded following the ban of the use of antibiotic growth promoters, including iontophores, in livestock nutrition (OJEU; 2003; Regulation (EC) 1831/2003). EOs are naturally occurring volatile components that can be extracted from plants by different distillation methods, particularly steam distillation (Greathead, 2003). Different parts of the plants can be used to obtain EOs, including the flowers, leaves, seeds, roots, stems, and bark. For many centuries, EOs have been used for their essence, flavour, antiseptic and/or preservative properties (Chaves et al., 2008).

The antimicrobial properties of EOs have been related to a number of small terpenoid and phenolic compounds (Helander et al., 1998). EOs that is particularly rich in phenolic compounds has been shown to possess the high levels of antimicrobial activity against both Gram-positive and -negative bacteria (Fraser et al., 2007).

Recent studies have shown that, at low concentrations, some EOs, their active components or mixtures of EOs have the potential to modify rumen N metabolism by reducing the degradation of proteins and ammonia production in the rumen (McIntosh et al., 2003; Molero et al., 2004; Newbold et al., 2004; Busguet et al., 2005 a,b; Chaves et al., 2008). Busguet et al. (2006) showed that, at 3.000 mg/L, capsicum oil, carvacrol, carvone, cinnamaldehyde, cinnamon oil, clove bud oil, eugenol, fenugreek and oregano oils resulted in a 30 to 50% reduction in the ammonia N concentration.

Additionally, these authors reported that, the careful selection and combination of these extracts may allow

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Table 1. The nutrient composition of CON and AP.

Samples	DM (%)	CA (%)	CP (%)	EE (%)	CF (%)	NFE (%)	ME (kcal/kg)
CON	89.38	4.8	15.15	4.39	6.27	58.03	2725
ORE	88.77	7.69	9.49	3.60	18.20	49.79	2078
ROS	91.01	5.17	6.12	10.99	26.25	42.48	2153
SHN	88.62	3.49	6.34	7.59	16.69	54.51	2397

DM:Dry matter, CA:crude ash, CP:crude protein, EE:ether extract ,CF:crude fiber, NFE:nitrogen free extract, ME:metabolic energy.

Table 2. Essential oil ratios of aromatic plants.

Aromatic plant	**Essential oil ratio** (% DM)
ORE	1.35
ROS	0.60
SHN	0.23

the manipulation of rumen microbial fermentation.

The objective of the present study was to evaluate the effects of increasing levels of aromatic plants (AP) on *in vitro* gas production (GP), organic matter digestibility (OMD), and net energy lactation ((NEL) using an *in vitro* gas-production method.

MATERIALS AND METHODS

Experimental material and procedures

Two rumen-fistulated sheep (Tahirova breed, East Friesian 75% × Kivircik25%) were used in the test. Sheep were fed with 60% of alfalfa hay and 40% concentrate feed twice daily as described by Steingass and Menke (1986). The feed material consisted of fattening concentrate (CON), and the AP consisted of *Origanum onites* L. (ORE), *Rosmarinus officinalis* L. (ROS) and *Schinus molle* L. (SHN). Five different levels of ORE, ROS, and SHN (all of them called aromatic plant; AP) were added to the concentrate (CON) to produce 200 mg KM (C - without , AP; 1 - 0.02 mg AP + 198 mg CON; 2 - 0.04 mg AP + 196 mg CON; 3 - 0.06 mg AP+194 mg CON; 4 - 0.08 mg AP+192 mg CON; 5 - 0.10 mg AP+190 mg CON). The results of the crude nutrient analysis of CON and each AP are presented in Table 1.

The rates of ORE, ROS, and SHN EOs used in the study are presented in Table 2, and the EOs components are presented in Table 3.

Chemical analyses

The concentrate feed and AP were grounded through a 1 mm screen in preparation for the chemical analysis. The dry matter (DM), crude protein (CP), ether extract (EE), crude ash (CA) and crude fibre (CF) were analysed according to Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten, VDLUFA (Naumann and Bassler, 1993). The metabolisable energy (ME) was calculated based on the chemical composition (Anonymous, 1991).

The rumen fluid was collected before the morning feeding from two ruminally fistulated lambs. The estimates of GP were obtained using the method of Menke and Steingass (1988). A buffer solution (macro and microminerals) was prepared on the day prior to the analysis and incubated in a waterbath at 39°C under a continuous CO_2 stream (DLG, 1981). Incubations were terminated after 24 h for the OMD and NEL estimations of the concentrate and AP mixtures. The volumes of gas produced were recorded at 2, 4, 8, 12, and 24 h after inoculation, and the (GP) results were applied to calculate OMD and NEL using the following equations.

$OMD\ (\%) = 0.889 \times GP + 0.448 \times CP^{*} + 0.651 \times CA^{*} + 14.88\ ^{*}$in % DM. (Menke ve Huss, 1987).

$NEL\ (MJ/kg\ DM) = 3.95 + 0.3305 \times GP - 0.0023 \times GP^{2} + 0.0535 \times CP + 0.0132 \times EE^{2} - 0.0336 \times CF - 0.1073 \times CA$ (Aiple, 1993).

GP: 24-h cumulative GP in DM.

The EOs from 10 g of dry plant materials were extracted by hydro-distillation for 3 h using a Clavenger-type apparatus, according to the European Pharmacopoeia (1975), with three replications. The GC analyses were performed at the Central Laboratory of Aegean University using a Carlo Erba Fractovap Series 2350 gas chromatograph equipped with a flame ionisation detector. A glass column (3 m long, 3.18 mm internal diameter) packed with 3% OV-1 50 chromosorb 80/100-mesh was used. The carrier gas was N_2 at a flow rate of 25 ml/min. Each GC run lasted for 20 min. The oven temperature was isothermal at 110°C, and the injector and detector temperatures were 225 and 250°C, respectively.

Statistical analysis

The data obtained were evaluated using the GLM procedure of SPSS V10 software. Duncan's test was employed for the comparison of the differences between the group averages (Efe et al., 2000).

RESULTS

As shown in Figure 1A and B, the highest and lowest GP

Table 3. The chemical composition of essential oils.

ORE		ROS		SHN	
Compounds	**Percent composition (%)**	**Compounds**	**Percent composition (%)**	**Compounds**	**Percent composition (%)**
Carvacrol	69.10	Borneol	26.16	α-Phellandrene	27.60
Thymol	10.70	Camphor	23.54	β- Phellandrene	21.95
P-Cymene	4.00	1,8-cineole	14.34	Myrcen	8.50
Borneol+ α-Terpineol	3.00	α-Terpineol	11.99	(+)-Spathulenol	6.30
γ-Terpinene	2.50	Limonene	11.86	α-Eudesmol	5.45
		Bornyl acetate	7.56	P-Cymene	5.20
		α-Pinene	2.48	β-Eudesmol	3.40
				α-Pinene	3.27
Unknown	10.70		2.00		18.33
Total compounds	100		100		100

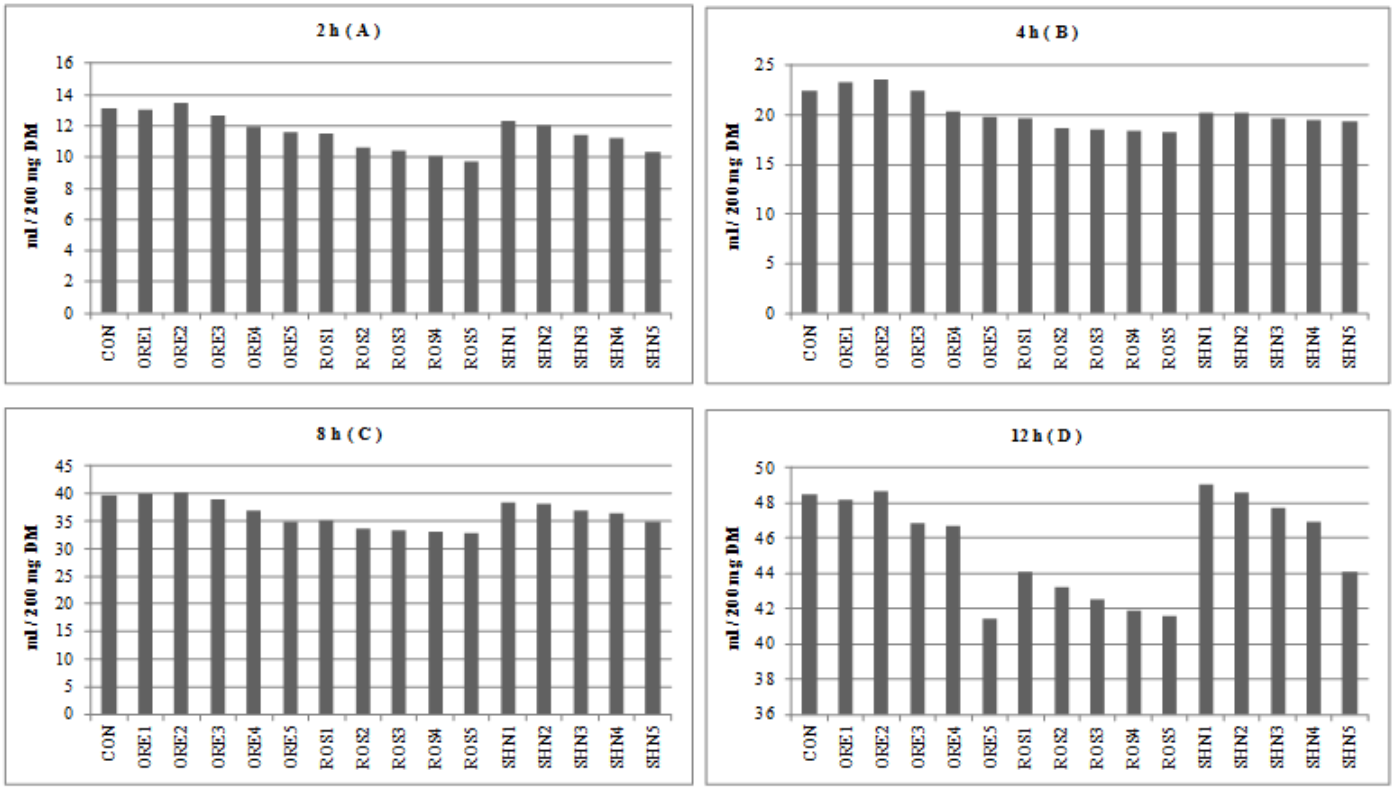

Figure 1. Incubation periods of GP, 2 h (A), 4 h (B), 8 h(C), and 12 (D) h.

values were found after 2 and 4 h of incubation, respectively, for ORE_2 and ROS_5. The GP values determined after 8 h of incubation (Figure 1C) showed a significant difference between the treatments except for ORE_1, ORE_2, ORE_3, and SHN_1 ($P < 0.01$). After 12 h, the highest GP was found in SHN_1 (49.06 ± 0.28), whereas the lowest value was determined in ORE_5 (41.45 ± 0.38) (Figure 1D). The values for GP, OMD, and NEL determined under *in vitro* conditions are presented in Table 4. The highest total GP was found in ORE_2 (60.28 ± 0.37), and the lowest was found in ORE_5 (52.63 ±0.35). The OMD and NEL values decreased significantly

Table 4. GP, OMD, and NEL contents of CON and APs.

Sample	24-h GP (ml/200 mg DM)	OMD (%)	NEL (MJ/kg DM)
CON	$60.13^{ab} \pm 0.52$	$79.47^{ab} \pm 0.47$	$8.01^{a} \pm 0.03$
ORE_1	$59.66^{ab} \pm 1.26$	$79.06^{ab} \pm 1.12$	$7.97^{ab} \pm 0.08$
ORE_2	$60.28^{a} \pm 0.37$	$79.61^{a} \pm 0.33$	$8.02^{a} \pm 0.02$
ORE_3	$59.21^{abc} \pm 0.20$	$78.66^{abc} \pm 0.18$	$7.96^{ab} \pm 0.01$
ORE_4	$58.08^{bcdef} \pm 0.35$	$77.66^{bcdef} \pm 0.32$	$7.89^{abcd} \pm 0.02$
ORE_5	$52.63^{g} \pm 0.35$	$72.81^{g} \pm 0.31$	$7.48^{f} \pm 0.03$
ROS_1	$57.52^{cdef} \pm 0.20$	$77.15^{cdef} \pm 0.17$	$7.85^{bcde} \pm 0.01$
ROS_2	$57.05^{def} \pm 0.27$	$76.74^{def} \pm 0.24$	$7.82^{cde} \pm 0.01$
ROS_3	$56.82^{ef} \pm 0.31$	$76.53^{ef} \pm 0.28$	$7.81^{cde} \pm 0.02$
ROS_4	$56.08^{f} \pm 0.37$	$75.88^{f} \pm 0.33$	$7.75^{e} \pm 0.03$
ROS_5	$53.51^{g} \pm 0.24$	$73.59^{g} \pm 0.22$	$7.55^{f} \pm 0.02$
SHN_1	$60.25^{a} \pm 0.22$	$79.58^{a} \pm 0.20$	$8.02^{a} \pm 0.01$
SHN_2	$59.44^{abc} \pm 0.31$	$78.86^{abc} \pm 0.28$	$7.97^{ab} \pm 0.02$
SHN_3	$59.00^{abcd} \pm 0.90$	$78.47^{abcd} \pm 0.80$	$7.94^{abc} \pm 0.06$
SHN_4	$58.71^{abcde} \pm 0.35$	$78.21^{abcde} \pm 0.31$	$7.93^{abcd} \pm 0.02$
SHN_5	$56.72^{ef} \pm 0.26$	$76.45^{ef} \pm 0.23$	$7.80^{de} \pm 0.02$
P	0.001	0.001	0.001

[abc:] Means with different letters in the same column are statistically significant ($p < 0.01$), CON: concentrate, ORE; *O. onites*, ROS; *R. officinalis*, SHN;*S. molle*, 1,2, 3, 4, 5; aromatic plant level in concentrate (200 mg).

($P < 0.01$) in ROS_1, ROS_2, ROS_3, ROS_4, ROS_5, ORE_5, and SHN_5.

DISCUSSION

The highest GP values were found in the ORE_2 and SHN_1 groups at 60.28 ± 0.37 and 60.25 ± 0.25 ml/200 mg KM, respectively. Although the increase was not significant, the groups supplemented AP had slightly higher GP values than the control group. Similarly, no significant difference was found for OMD and NEL ($P < 0.01$), indicating that supplementing CON with ORE and SHN does not positively affect OMD in the rumen. Busquet et al. (2005a,b) found that, cinnamaldehyde and garlic oil had no effect on DM, OM, NDF, and ADF digestibility or on the total VFA concentration, and the authors suggested that, these additives did not modify the overall diet fermentability. Castillejos et al. (2005) determined that 1.5 mg/l BEO (Crina ruminants) supplemented to high concentrate and coarse feed rations (that is, 100 forage and 900 concentrate versus 600 forage and 400 concentrate) did not affect DM, OM, NDF, ADF, and CP digestion, though BEO did increase the total VFA concentration (122.8 mM versus 116.2 mM). In another study, Newbold et al. (2004) observed a reduction in the *in situ* DM degradation of soya-bean meal after 8 and 16 h of incubation when 110 mg/d EO_s (a mixture of thymol, guaiacol, and limonene) was added to the diet of sheep. However, the mixture had no effect on the DM degradability of rapeseed meal and hay.

In the present study, the lowest total GP, OMD, and NEL values were found in ORE_5. Examining the effects of carvacrol on rumen fermentation, Garcia et al. (2007) reported that, the addition of carvacrol reduced *in vitro* DM, CP, and neutral-detergent fibre (NDF) digestion. The effects induced by 250 mg/l carvacrol on DM digestion after 72 h of incubation were comparable to those of monensin, whereas a greater reduction was obtained when carvacrol was supplemented at a concentration of 500 mg/l. The researchers explain that, the reduced CP potential degradability by the supplementation was mainly caused by a reduction of the slowly degradable fraction. Indeed, the GP-reducing effect that occurred in the early periods of incubation in ORE_3, ORE_4, ORE_5, SHN_3, SHN_4 and SHN_5 (Figure 1A, B, and C) was found in the ORE_5 and SHN_5 groups at the end of the incubation. ORE_5, SHN_5 and all ROS groups affected the rapidly and slowly degraded fractions.

Castillejos et al. (2006) reported that, the effect of thymol on *in vitro* DM, OM, NDF, and ADF digestion varies according to the level used, determining that DM, OM, NDF, and ADG digestion did not change with the addition of 5 mg/l thymol yet decreased with the use of 500 mg/l thymol. In another study, Benchaar et al. (2007) found that, although 400 mg/l carvacrol and 200 mg/l thymol reduced GP and NDF digestibility in an *in vitro* 24 h batch culture environment, 200 mg/l oregano and thyme oil caused a reduction of NDF digestibility while not affecting GP digestibility.

In agreement with the findings of Garcia et al. (2007), Castillejos et al. (2006) and Benchaar et al. (2007), in the

present study, it was determined that, GP, OMD, and NEL were not affected in the groups supplemented with low amounts of ORE (ORE_1, ORE_2, ORE_3, and ORE_4), whereas these parameters are significantly ($P < 0.01$) reduced at high levels of (ORE_5) addition.

Helander et al. (1998) reported that, the capacity of carvacrol and thymol to degrade the outer membrane of Gram-negative bacteria and observed the release of membrane lipopolysaccharides and increased permeability of the plasma membrane. Therefore, the small molecular weight of these compounds may allow them to be active in Gram-positive and Gram-negative bacteria.

The ORE EOs used in this research consists of 69.10% carvacrol and 10.70% thymol as the principal components. The increasing carvacrol and thymol concentrations in the ORE-supplemented groups caused decreases in GP, OMD, and NEL by increasing the antimicrobial effect. According to Helander (1998) and Covan (1999), this is a result of the antimicrobial effect of carvacrol and thymol on rumen microorganisms.

In all groups in which ROS was added to CON, GP decreased significantly ($P < 0.01$) from the 2^{nd} h of incubation, and OMD and NEL decreased accordingly. Smith-Palmer et al. (1998) report that, the bacteriostatic concentrations of ROS EOs against *S. aureus* and *L. monocytogenes* range from 0.02 to 0.04%, bacteriocidal concentrations are less or equal to 1% and that concentrations over 1% can be used for inhibiting Gram-negative bacteria. In another study, Santoyo et al. (2005) reported that, ROS EOs shows antimicrobial activity against Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), a yeast (*Candida albicans*) and a fungus (*Aspergillus niger*) and that the effects increase as the camphor, borneol, and verbenone contents in the composition of the EOs increase.

Pattnaik et al. (1997) stated that, 1,8-cineole and camphor exhibit strong antimicrobial effects. However, Hayouni et al. (2008) reported that, the antimicrobial activity of *S. officinalis* EOs is related to the 1,8-cineole, α / β-thujone and borneol content in the oil.

It is believed that the reducing effect of ROS addition on GP, OMD, and NEL becomes more evident with increasing rates of ROS and is related to the presence of 26.16% borneol and 23.54% camphor in the composition of the ROS essential oil. Similarly, Santoyo et al. (2005), Pattnaik et al. (1997), and Hayouni et al. (2008) reported that the antimicrobial effect is related to the borneol and camphor concentrations in ROS essential oil.

It was observed in this study that, in the SHN-including groups of CON, GP, OMD, and NEL decreased proportionally with the amount of ROS addition. Having determined the α-phellandrene and germacrene D contents of SHN EOs as 6.94, 6.54, 3.53, and 20.77%, respectively, Deveci et al. (2010) reported that these compounds show potential in terms of antimicrobial and repellent activity. With regard to sensitivity to SHN EOs, Hayouni et al. (2008) determined that *Enterococcus faecalis* ATCC 2912, *E. coli* ATCC 25922 and *E. coli* (clinical strain 1) are very sensitive, followed by *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6539, *E. coli* (clinical strain 2) and *Salmonella anatum*. The researchers reported that the SHN EOs they used contained 35.86 % α-phellandrene and 29.30 % β- phellandrene as the principal components and that these compounds are related to the antimicrobial activity of the oil. Similar to the EO used by Hayouni et al. (2008), the SHN EOs used in this study contained 27.60 % α-phellandrene and 21.95 % β-phellandrene as the principal components. Thus, the decrease of GP, OMD, and NEL in SHN_5 may be related to the increase in the α-phellandrene and β- phellandrene contents, in accordance with the increase in the amount of SHN added to CON.

It is reported that, there is a high level of correlation ($r = 0.82$) between the amount of gas created with the *in vitro* incubation of feed with rumen liquid for 24 h and the level of digestion of organic substances (Menke et al., 1979). The increasing amount of EOs and antimicrobial effect was correlated with the increase in the levels of ORE, ROS, and SHN used in this study, and lower GP, OMD, and NEL were determined in an inversely proportional manner with this increase. In a previous study by Soycan-Onenc (2008) it was determined, that varying levels of ORE, ROS and SHN addition to barley decreased total GP, OMD, and ME and that, this result was associated with a decrease or inhibition of the activities of amylolytic bacteria in the rumen. In addition, Oh et al. (1967, 1968) observed that, high levels of different plant EOs decreased the production of gas and total VFA in the *in vitro* fermentations of mixed ruminal microorganisms, suggesting that, high levels resulted in a general inhibition of rumen microbial fermentation.

Conclusions

In this study, it was found that, GP, OMD, and NEL decreased significantly as the level of ORE, ROS, and SHN added to CON increased. It was concluded that, ORE, ROS, and SHN are potential supplements to alter rumen fermentation. To obtain exact results, the findings obtained under *in vitro* conditions should be supported by *in vivo* studies.

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