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Thymus kotschyanus Essential Oil Components and Their Effects on *in vitro* Rumen Fermentation, Protozoal Population and Acidosis Parameters

Research Article

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ABSTRACT

This study aimed to identify chemical composition of *Thymus kotschyanus* essential oil (TKEO) and to evaluate the effects of different doses of TKEO on *in vitro* gas production, fermentation parameters, acidosis and protozoal population using a completely randomized design with four replicates. Two diets (D_1: 100% forage and D_2: 30% forage+70% concentrate) were incubated with buffered rumen fluid as fermentation substrates. The plant materials were dried and hydro-distilled in order to obtain their essential oil (EO). The essential oil was analyzed by capillary GC/MS. Gas production ($GP_{144\ h}$) was recorded up to 144 h of incubation. After 24 h, the parameters of apparent *in vitro* dry matter digestibility (AIVDMD), true *in vitro* dry matter digestibility (TIVDMD), organic matter digestibility (OMD), partitioning factor (PF) and microbial biomass (MB) were estimated. The main components of TKEO were 25.77% geraniol and 14.85% thymol. Asymptotic $GP_{144\ h}$ decreased by TKEO. Addition of TKEO also decreased $GP_{24\ h}$, AIVDMD, TIVDMD and OMD. However, PF and MB increased. Acidosis test was not affected by EO. Inclusion of TKEO decreased the total number of protozoal population. These results indicated that TKEO could potentially be used to modulate rumen fermentation therefore further *in vivo* research is needed to determine the optimal doses.

KEY WORDS acidosis, essential oil, *in vitro* digestibility, microbial biomass, partitioning factor.

INTRODUCTION

Due to emergence of antibiotic resistance bacteria, many researches have been directed toward using natural compounds as feed additives. Natural compounds such as tannins, saponins and essential oils (EOs) have the potential to modulate rumen fermentation (Benchaar *et al*. 2008). Gram positive and negative bacteria are susceptible to the antimicrobial activities of EOs due to their terpenoids and phenolic constituents (Calsamiglia *et al*. 2007). One of fourteen species of thymus which is widely grown in Iran is *Thymus kotschyanus* (Mozafarian, 1996). It has been reported that the main components of *Thymus kotschyanus* EO (TKEO) are carvacrol, p-cymene, γ-terpinene and L-Borneol (Khanavi *et al*. 2013).

However, the differences in chemical composition of EOs lead to varying responses from study to study (Talebzadeh *et al*. 2012).

Therefore, the objective of this study was to evaluate the effect of TKEO on the fermentation parameters, protozoal population and rumen acidosis and screening of its potency to modulate fermentation.

MATERIALS AND METHODS

Essential oils

Aerial parts (leaves, flowers and stems) of plant materials of *Thymus kotschyanus* were collected from locality in Kermanshah province located in the west of Iran. Plants materials were hydro-distillated for 3 h, using an all-glass Clevenger-type apparatus, according to the method recommended by British Pharmacopoeia (1988). The extracted oil was stored in tightly sealed vials at 4 ˚C until further uses. The components of oil were analyzed by GC-MS as describe by Talebzadeh *et al*. (2012).

Fermentation substrates

Stock solutions were prepared by dissolving the essential oil in absolute ethanol (w/v). Equal volumes of ethanol (1% v/v) were added to control bottles. The volume of ethanol used (10 mg/mL) does not seem to affect rumen fermentation (Morgavi *et al*. 2004). Representative samples of two diets meaning D_1: 100% roughage and D_2: 30% roughage + 70% concentrate (Table 1) oven-dried and ground through a one mm screen and were used as fermentation substrates. The TKEO was used at the rates of 0, 250, 500, 750 and 1000 µg/mL.

In vitro rumen fermentation

The rumen contents were collected from both the liquid and the solid phase and handled properly (Makkar, 2010) from three ruminally fistulated mature Sanjabi sheep (45±4.5 kg BW), which fed on diet containing of alfalfa hay, barley grain, wheat bran, soybean meal and mineral supplements, before morning feeding. The experiment was conducted according to method of Lopez *et al*. (2010). For kinetics measurements, 200 mg of fermentation substrate was weighed into a 120 mL Wheaton vial (Wheaton Science Products, Millville, NJ). The vials were subsequently filled with 30 mL of incubation medium. In brief, the incubation medium was composed of 1282.5 mL of bicarbonate buffer solution (70 g $NaHCO_3$ and 8 g NH_4HCO_3 in 2 liter distilled water), 1282.5 mL of macromineral solution (12.4 g KH_2PO_4, 11.4 g Na_2HPO_4 and 1.2 g $MgSO_4.7H_2O$ in 2 liters distilled water), 6.5 mL of a micromineral solution (1.0 g $MnCl_2.4H_2O$, 1.32 g $CaCl_2.2H_2O$), 0.1 g $CoCl_2.6H_2O$, 0.8 g $FeCl_3.6H_2O$ in 100 mL distilled water, 6.5 mL of resazurine solution (0.1 g resazurine in 100 mL distilled water), 2565 mL distilled water, 260 mL reducing solution (1.875 g cysteine-HCl and 1.875 g of $Na_2S.9H_2O$ in 200 mL distilled water, 12 mL of 1 N sodium hydroxide solution (dissolve 4 g sodium hydroxide in 100 mL distilled water for 1 N sodium hydroxide) and finally, 1350 mL of filtered rumen fluid were added to the medium (rumen fluid diluted to 20% v/v; i.e. the final ratio of rumen fluid to medium was 1:4); whilst, keeping the mixture stirring and flushing with CO_2. Thereafter, different doses of TKEO were added to respective vials, incubated in four replicates at 39 ˚C for 144 h. The pressure of gas produced in each vial was recorded using a pressure transducer (Testo 512; Testo Inc., Germany). These recorded pressures were used to estimate the generated gas volumes (Lopez *et al*. 2010). The gas volume was recorded at 1, 4, 7, 10, 13, 16, 19.5, 23, 26, 29, 32, 36, 40, 45, 50, 55, 61, 69.5, 80, 96, 120 and 144 h after incubation.

Data for gas production were fitted to the generalized Mitscherlich model of France *et al*. (2000) as:

$$GP= A \times (1-\exp(-c\times(T-L)))$$

Where:
GP (mL/g OM): denotes the cumulative gas production at time T.
A (mL/g OM): asymptotic gas produced.
C: rate of gas production.
L: lag time.

The apparent *in vitro* dry matter digestibility (AIVDMD), true *in vitro* dry matter digestibility (TIVDMD), partitioning factor (PF), the ratio of DM truly degraded (mg) to gas volume (mL) and microbial biomass (MB) were measured according to procedure described by Blümmel *et al*. (1997). Briefly, about 500 mg of the substrate weighed in 120 mL serum bottles (n=4) and inoculated with 40 mL of buffered rumen fluid. After 24 h incubation, bottles contents were transferred to weighed tubes and centrifuged at 20000 *g* for 30 min at 4 ˚C. The supernatant was discarded. To measure AIVDMD, tubes containing digestion residues were oven-dried at 60 ˚C for 48 h; thereafter, the dried tubes were weighed.

To measure TIVDMD, the residues in tubes were transferred to 600 mL beaker, by rinsing twice with 20 mL of neutral detergent solution and were boiled for 1 h. The solutions were filtered using sintered glass crucibles and the residues were dried (100 ˚C, 10 h) and then weighed.

The ratio of DM truly degraded (mg) to gas volume (mL) at 24 h incubation was expressed as the partitioning factor. The mass difference of original residue and NDS-boiled residue was taken as a rough estimate of MB.

The control of lactic acidosis

The pH value and produced gas in a carbohydrate challenged system used to screen potential of TKEO against acidosis as describe by Hutton *et al*. (2010). In brief, the rumen fluid samples were taken from fistulated sheep, three hours post-feeding and transported immediately to the laboratory under anaerobic condition (sparged CO_2).

Table 1 Chemical composition of diets (fermentation substrate; g/kg DM)

	DM	OM	ASH	CP	EE	NDFom	ADFom
Diet 1	930	926.7	73.3	130	32.1	456.4	387.3
Diet 2	920	937.6	62.4	155.2	18.3	330.8	182.9

DM: dry matter (g/kg fresh weight); OM: organic matter; CP: crude protein; EE: ether extract; NDFom: neutral detergent fiber and ADFom: acid detergent fiber.

The pH of the pooled rumen fluid was measured. The control tubes were composed of 10 mL rumen fluid and 0.1 g Oaten chaff as the main substrate of gas producer. Uncontrolled tubes were included rumen fluid, Oaten chaff and 1 g glucose as pH reducer. Positive control consist of substrate of uncontrolled tube plus 100 µL virginiamycin in which was considered as the antibiotic control, because of its potent and selective inhibition of lactate producing bacteria (based on the work by Hutton *et al*. 2010). Thyme control acidosis tubes were containing substrate of the uncontrolled tube plus 100 µL TKEO with different dosses (meaning, 0, 250, 500, 750 and 1000 µg/mL). The tubes incubated in shaking incubator (constantly at 50 rpm) at 39 ˚C for 6 h. The tubes were removed from the incubator at 2 h intervals and placed in a 39 ˚C water bath. The gas produced was recorded using a pressure transducer (Testo 512; Testo Inc., Germany). After the final gas pressure reading, stopper was removed and the pH in the liquid phase of each tube was recorded.

Protozoal population

For protozoal counts, after 24 h incubation in a separate runs, whole bottle contents were preserved by diluting with an equal volume of formalin solution (185 mL formaldehyde/one liter distilled water). Total numbers and generic composition of ciliate protozoa were determined as method of Dehority (1993).

Statistical analysis

A completely randomized design was used to analyze *in vitro* data using one-way analysis of variance (ANOVA) of SAS 9.2 (SAS, 2002). For all analyses, specific orthogonal contrasts were used to test linear (L), quadratic (Q) and cubic (C) effects of EOs doses on parameters. Treatment means were compared using Duncan's test (all pairwise multiple comparison procedures). All statements of significance were based on a probability of $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of experimental diets

The chemical composition of diets which used as fermentation substrates is shown in Table 1.

GC/MS analysis of essential oils

The results showed that geraniol (26%) and thymol (15%) were the main constituents of TKEO (Table 2).

Kinetics of gas production

In general, asymptotic gas production (A) of diets were influenced by adding TKEO (Table 3). "A" value decreased by TKEO in the D1 (L, Q and C; $P<0.001$) at more than 500 µg/mL; however, in D2 only at the dose of 1000 µg/mL gas production declined (L and Q; $P<0.001$, C; $P<0.05$). Fermentation rate (C) in both diets, also was diminished (L; $P<0.001$) and the maximum effect was observed at the rate of 1000 (µg/mL). Presence of the TKEO in both diets prolonged lag time, significantly.

Fermentation parameters and digestibility

Incorporation of TKEO to the diets decreased (L; $P<0.001$) IVGP, AIVDMD, TIVDMD and OMD (Table 4) and lowest values were observed at the level of 1000 µg/mL. Contrary, PF, MB and EMB were increased (L; $P<0.001$). The greatest values for PF and EMB were observed at the rate of 1000 µg/mL.

The control of lactic acidosis

The results of the TKEO effects on lactic acidosis showed that, this essential oil did not have a positive effect on pH (Table 5), which means TKEO did not protect against acidosis.

Protozoal concentration

At the end of incubation only seven groups of protozoa (*Isotricha* spp., *Dasytricha* spp., *Entodinium* spp., *Diplodinium* spp., *Epidinium ecaudatum*, *Entandrophragma caudatum* and *Ophryo scolecidae*) were observed (Table 6). The results showed that TKEO had a strong antiprotozoal effect; inclusion of TKEO into the both diets, scaled down the number of *Isotricha* spp., linearly (L; $P<0.001$). *Dasytricha* spp., and *Entodinium* spp., had the lowest values at the high doses of EO (L; $P<0.001$).

Population of *Diplodinium* spp., was affected linearly and quadratically and the lowest number was observed at 1000 µg/mL of TKEO. Population of *Epidinium* in both diets did not have a systematic trend; nevertheless, *Ophryo scolecidae* population in both diets were decreased. On the other hand, incorporation of TKEO to the diets, decreased the total population of protozoa linearly and quadraticly (L, $P<0.001$; Q, $P<0.05$). Generally, there were fewer numbers of *Epidinium ecaudatum*, *Entandrophragma caudatum* and *Ophryo scolecidae* compared to other genera and the minimum count of total protozoa was observed at 1000 µg/mL of TKEO.

Table 2 The essential oil profile of *Thymus kotschyanus*

Peak No.	Components	%	Peak No.	Components	%
1	α-thujene	0.94	24	Carvacrol methyl ether	0.57
2	α-pinene	1.63	25	Geraniol	25.77
3	Camphene	1.28	26	Geranial	1.4
4	Sabinene	0.43	27	Thymol	14.85
5	β-pinene	0.5	28	Carvacrol	2.6
6	β-myrcene	1.4	29	Bicycloelemene	0.09
7	α-phellandrene	0.12	30	Geranyl acetate	8.55
8	δ-3-carene	0.03	31	α-copaene	0.04
9	α-terpinene	0.97	32	β-bourbonene	0.26
10	p-cymene	3.31	33	β-elemene	0.14
11	Limonene	0.39	34	trans caryophyllene	4.49
12	1,8-cineol	2.95	35	α-humulene	0.25
13	E-β-ocimene	2.38	36	Germacrene D	2.39
14	γ-terpinene	5.34	37	Bicyclogermacrene	0.48
15	cis-sabinene hydrate	0.53	38	β-bisabolene	0.33
16	α-terpinolene	0.07	39	δ-cadinene	0.1
17	Linalool	4.01	40	trans-α-bisabolene	1.29
18	Camphore	0.73	41	Geranylbutanoate	0.56
19	1-borneol	1.73	42	Spathulenol	0.1
20	4-terpineol	0.53	43	Geranylisoalerate	0.33
21	α-terpineol	2.32	44	Caryophyllene oxide	0.38
22	Nerol	2.05	45	tau-cadinol	0.13
23	Neral	1.16	46	Geranylhexanoate	0.10

Table 3 Effects of *Thymus kotschyanus* essential oil (TKEO) on kinetics of *in vitro* gas production[1]

Parameter[2]	TKEO dose (mg/mL)						Significance of the contrast			
	0	250	500	750	1000	SEM	L	Q	C	CD
Diet 1 (roughage)										
A	428.03^{a}	401.49^{a}	381.13^{a}	319.07^{b}	105.76^{c}	17.02	**	**	**	NS
C	0.031^{a}	0.026^{a}	0.025^{a}	0.025^{a}	0.013^{b}	0.001	**	NS	*	NS
L	3.22^{b}	1.77^{c}	1.19^{d}	0.61^{e}	4.00^{a}	0.30	NS	**	**	*
Diet 2 (roughage+concentrate)										
A	467.11^{a}	430.98^{a}	416.69^{a}	410.24^{a}	179.06^{b}	16.93	**	**	*	NS
C	0.048^{a}	0.040^{ab}	0.035^{b}	0.035^{b}	0.026^{c}	0.002	**	NS	NS	*
L	1.23^{b}	0.65^{d}	0.67^{d}	0.99^{c}	4.64^{a}	0.32	**	**	**	**

[1] There is no comparison between diets on estimated parameters.
[2] Parameters were estimated using the generalized Mitscherlich model: GP= A × (1-exp(-c×(T-L))); A: asymptotic gas production; L: lag time and c: the rate of fermentation.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
CD: control compare to other treatments.
SEM: standard error of mean.
NS: non significant.
** (P≤0.01) and * (0.01≤P≤0.05).
L: linear; Q: quadratic and C: cubic.

Phenolic components

The main components of TKEO in current study were geraniol and thymol, while, Khanavi *et al*. (2013) reported that carvacrol was the main constituent of TKEO. However, thymol and eugenol were isolated from the extracted essential oils of *Thymus kotschyanus* aerial parts by Mohammed and Al-Bayati, (2009).

The major components of the essential oil of *Thymus daenensis* were thymol (39.91%) and carvacrol (29.93%); whereas *Thymus lancifolius* which contained carvacrol (25.55%) and thymol (20.79%; Jaberi *et al*. 2015). It has been reported that thymol and carvacrol were the main phenolic components of *Thymus vulgaris* (Leung and Foster, 1996). Thymol was also the main constituent of *Thymus capitatus* (Patra and Yu, 2012) and *Thymus zygis* (Martínez *et al*. 2006).

According to a study carried out by Raal *et al*. (2004), geranyl acetate (up to 46.4%), linalyl acetate (up to 31.4%), geraniol (up to 30.3%) and myrcene (up to 20.2%) were determined to be the major constituents of *Thymus serpyllum* essential oil. These results indicated that different species of TKEO containing various components and amount of phenolic materials. Stage of harvesting, climatic and seasonal conditions may affect the composition of essential oils (Daferera *et al*. 2000).

It was demonstrated that the effect of each plant species is depended on the nature, concentration and activity of its compounds. Different factors, such as origin, botanical activity, condition of cultivation and growth, harvesting part of the plant used, might influence the concentration and activity of secondary metabolites within a given plant species (Wenk, 2003).

Table 4 Effects of different doses of *Thymus kotschyanus* essential oil (TKEO) on the fermentation parameters and digestibility

Parameter	TKEO (mg/mL)					SEM	Significance of the contrast			
	0	250	500	750	1000		L	Q	C	CD
Diet 1 (roughage)										
GP (24 h)	218.08^{a}	208.21^{b}	118.72^{c}	59.84^{d}	54.15^{e}	1.78	**	**	**	**
AIVDMD	0.27^{a}	0.17^{b}	0.15^{c}	0.13^{d}	0.12^{d}	0.01	**	**	*	NS
TIVDMD	0.57^{a}	0.55^{b}	0.40^{c}	0.29^{d}	0.28^{e}	0.03	**	**	**	**
OMD	0.59^{a}	0.57^{b}	0.44^{c}	0.35^{d}	0.34^{e}	0.03	**	**	**	**
PF	3.03^{c}	3.08^{c}	3.92^{b}	5.73^{a}	6.01^{a}	0.35	**	*	**	*
MB	77.93^{b}	78.16^{b}	88.72^{a}	90.47^{a}	88.96^{a}	1.60	**	NS	*	NS
EMB	0.83^{c}	0.88^{c}	1.72^{b}	3.53^{a}	3.81^{a}	0.35	**	*	**	*
Diet 2 (roughagae+concentrate)										
GP (24 h)	271.18^{a}	259.74^{b}	211.93^{c}	126.12^{d}	83.38^{e}	19.80	**	**	**	*
AIVDMD	0.45^{a}	0.39^{b}	0.37^{b}	0.32^{c}	0.24d	0.02	**	*	*	NS
TIVDMD	0.67^{a}	0.65^{a}	0.60^{b}	0.48^{c}	0.37^{d}	0.03	**	**	*	*
OMD	0.67^{a}	0.66^{b}	0.58^{c}	0.45^{d}	0.39^{e}	0.03	**	**	**	*
PF	2.86^{d}	2.91^{d}	3.30^{c}	4.43^{b}	5.16^{a}	0.25	**	**	*	*
MB	76.84^{c}	79.55^{c}	100.71^{b}	121.03^{a}	106.49^{b}	4.59	**	*	*	NS
EMB	0.66^{d}	0.71^{d}	1.10^{c}	2.23^{b}	2.96^{a}	0.25	**	**	*	*

GP: gas production after 24 h of incubation (mL g^{-1} OM); AIVDMD: apparent *in vitro* dry matter digestibility (g kg^{-1} DM); TIVDMD: true *in vitro* dry matter digestibility (g kg^{-1} DM); OMD: organic matter digestibility (g kg^{-1} OM); PF: partitioning factor; MB: microbial biomass (mg) and EMB: efficiency of microbial biomass.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
CD: control compare to other treatments.
SEM: standard error of mean.
NS: non significant.
** (P≤0.01) and * (0.01≤P≤0.05).
L: linear; Q: quadratic and C: cubic.

Table 5 Effects of different doses of *Thymus kotschyanus* essential oil (TKEO) on the control of lactic acidosis

Treatments	Parameters	
	GP (mL)	pH
Controlled acidosis	28.10^{h}	5.50^{a}
Uncontrolled acidosis	38.22^{d}	4.14^{d}
Antibiotic controlled acidosis	33.10^{g}	5.00^{b}
Thyme control acidosis (µg/mL)		
0	41.47^{b}	4.04^{fg}
250	39.12^{c}	4.02^{g}
500	38.34^{c}	4.07^{e}
750	37.21^{e}	4.05^{ef}
1000	34.35^{f}	4.17^{c}
Thyme aerial (0.1 g)	46.24^{a}	4.15^{cd}
SEM	1.02	0.08
P-value	< 0.0001	< 0.0001

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of mean.
Positive control= 10 mL strained rumen liquor plus 0.1 g oaten chaff + 100 µL virginiamycin.
Control= 10 mL strained rumen liquor plus 0.1 g oaten chaff.
Uncontrolled acidosis= control plus 1 g D-glucose.

Kinetics of gas production

Similar to the present study, incorporation of *Zataria multiflora* which contained phenolic components of thymol and carvacrol, decreased asymptotic gas production and fermentation rate but the lag time was increased, significantly (Talebzadeh *et al*. 2012; Taghavi-Nezhad *et al*. 2014).

In the current study, decline in gas production was 6-75% (TKEO) in forage based substrate and 8-62% (TKEO) in the forage plus concentrate diet. In agreement with our results, Macheboeuf *et al*. (2008) have mentioned various doses of thymol and carvacrol from five natural EOs and three pure constituents, were also reduced gas production at the end of the incubation period. Lag time was increased due to addition of 1000 µg/mL TKEO by 85%. Singh *et al*. (2012) showed that geraniol has strong antibacterial and antifungal activity. It has been also reported that geraniol showed antimicrobial effects characteristics (Chen and Viljoen, 2010).

Carvacrol and thymol in their chemical structures have a phenolic group (Benchaar *et al*. 2007a), which may have similar effects of tannins at high doses. Effect of phenolic constituents of EOs on digestion of soluble fractions of substrate (Aharoni *et al*. 1998) is parallel with decreasing the attachment of microbes to insoluble fractions of diets (McAllister *et al*. 1994). Wallace *et al*. (2002) reported EOs mainly containing thymol which decreased colonisation and digestion of readily fermentable substrates without affecting fibrous substrates.

In vitro fermentation characteristics ($GP_{24\,h}$)

Gas production is the result of fermentation of carbohydrates to volatile fatty acids (Makkar, 2010).

Table 6 Effects of *Thymus kotschyanus* essential oil (TKEO) dose on the ruminal protozoa concentration (log_{10}/mL)

	TKEO dose (mg/mL)					SEM	Significance of the contrast			
	0	250	500	750	1000		L	Q	C	CD
Diet 1 (roughage)										
Isotricha spp.	4.40^{a}	4.37^{a}	4.16^{b}	4.03^{c}	3.95^{c}	0.044	**	NS	NS	NS
Dasytricha spp.	4.81^{b}	4.77^{c}	4.85^{a}	4.71^{d}	4.57^{e}	0.023	**	**	*	**
Entodinium spp.	5.06^{a}	4.92^{b}	4.81^{c}	4.77^{d}	4.71^{e}	0.029	**	**	NS	NS
Diplodinium spp.	4.57^{b}	4.56^{bc}	4.68^{a}	4.51^{cd}	4.46^{d}	0.018	*	**	NS	**
Epidinium caudatum	4.15^{a}	3.74^{c}	3.89^{b}	3.51^{d}	3.70^{c}	0.052	**	*	NS	**
Epidinium ecaudatum	3.98^{a}	3.98^{a}	3.82^{b}	3.89^{b}	3.84^{b}	0.018	*	NS	NS	*
Ophryoscolecidae	4.04^{a}	3.75^{b}	3.75^{b}	3.65^{bc}	3.42^{c}	0.058	**	NS	NS	ns
Total	5.45^{a}	5.35^{b}	5.34^{b}	5.23^{c}	5.15^{d}	0.024	**	NS	NS	*
Diet 2 (roughagae+concentrate)										
Isotricha	4.37^{a}	4.33^{a}	4.27^{a}	4.09^{b}	3.77^{c}	0.054	**	*	NS	NS
Dasytricha	4.77^{c}	4.86^{a}	4.80^{b}	4.63^{d}	4.53^{e}	0.028	**	**	**	NS
Entodinium	5.00^{a}	4.92^{b}	4.74^{c}	4.76^{c}	4.68^{d}	0.027	**	**	NS	**
Diplodinium	4.51^{c}	4.61^{a}	4.56^{b}	4.51^{c}	4.38^{d}	0.019	**	**	NS	NS
Epidinium caudatum	3.74^{a}	3.32^{c}	3.89^{a}	3.74^{a}	3.51^{b}	0.052	NS	NS	**	**
Epidinium ecaudatum	3.93^{a}	3.74^{b}	3.65^{c}	3.82^{b}	3.82^{b}	0.024	NS	**	*	*
Ophry oscolecidae	3.94^{a}	3.42^{ab}	3.77^{a}	3.75^{a}	2.36^{b}	0.195	*	NS	NS	NS
Total	5.37^{a}	5.36^{a}	5.28^{b}	5.21^{c}	5.09^{d}	0.024	**	**	NS	NS

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
CD: control compare to other treatments.
SEM: standard error of mean.
NS: non significant.
** (P≤0.01) and * (0.01≤P≤0.05).
L: linear; Q: quadratic and C: cubic.

Carvacrol and thymol decreased *in vitro* gas production (Benchaar *et al*. 2007a), therefore, decrease in fermentation resulted in lower gas production. In our experiment, suppression in $GP_{24\ h}$ was observed due to presence of the EO in which highest decline was recorded at 1000 (ug/mL) for TKEO (69-75%) along with diets. In agreement with current study, Talebzadeh *et al*. (2012) found that the essence of *Zataria multiflora* totally decreased GP_{24} and inhibited growth and enzyme activity of an isolated fungus from rumen of sheep. Debashis *et al*. (2015) also emphasized thyme at the rate of 600 ppm decreased gas production, OM and DM degradability. Decline in fermentation parameters after incorporation of thyme to the medium may be a result of their constituent such as thymol in which has more antimicrobial activity rather than other EO components due to the presence of a hydroxyl group in the phenolic structure and altered integrity of bacterial membrane followed by reduction in glucose uptake by bacteria (Devi *et al*. 2010).
Following decrease in gas production, OM digestibility decreased by 42% (Table 4). This may reflect the negative effect of the essential oil on degradation of OM (Benchaar *et al*. 2007b). Reduction in DM and OM digestibility has been reported due to supplementation of diets with herbal plant EOs (Sobhy and Samir, 2010). Similar to our results, in an *in vitro* trial; Martínez *et al*. (2006) showed that two EOs of thyme, one rich in carvacrol and the other in thymol, decreased *in vitro* DM degradability of diet (C:F, 70:30). Carvacrol, thymol and eugenol resulted in a decrease in GP compared with the control, which was consistent with a reduction in IVDMD (Benchaar *et al*. 2007a).

Using a dual-flow continuous culture fermenter maintained at constant pH, Castillejos *et al*. (2006) reported that at 500 mg/L, thymol reduced DM digestibility compared to the control. The negative effect of oil tested in the present experiment on digestion could have been partly due to the inhibitory effect of EO on the cellulolytic bacteria or ruminal fungi (McIntosh *et al*. 2003). Patra and Yu (2012) declared that total gas, methane production and apparent degradability of DM and NDF decreased linearly with increasing doses of EOs, however, true DM and NDF degradability were not affected by several EOs.

In this study, addition of TKEO (at>500 ug/mL) led to an increase in PF and MB. The PF is considered as an index of efficiency of microbial biomass synthesis (Blümmel *et al*. 1997). This was paralleled with estimated EMB (Table 4). Proportional to the amount of substrate degraded, lower gas production (higher PF) is indicator of greater microbial biomass synthesis (Blümmel *et al*. 2003). TKEO (at>500µg/mL) increased PF, probably a greater amount of digested organic matter were directed towards the growth of microbial cell rather than towards volatile fatty acids (VFA) production (Taghavi-Nezhad *et al*. 2011). Sallam *et al*. (2009) found that amount of PF decreased at the presence of the EOs of thymus and vulgare but increased with inclusion of *Zingiber officinale* and *Nigella sativa*. Increasing PF as the effect of EOs extracted from thyme and savory is indicative of the efficiency of fermentation and microbial protein production (Blümmel *et al*. 1997). However, the antimicrobial activity of EOs is inconsistent with increasing the volume of microbial biomass

(Macheboeuf *et al*. 2008). Talebzadeh *et al*. (2012) showed that using different levels of EO from *Zingiber multiflora* led to the occurrence of PF above the theoretical range and the greatest value for PF (5.6) was observed at 600 µg/mL of EO.

Some EOs have antimicrobial effects in a dose dependent manner (Macheboeuf *et al*. 2008). At different doses, they may possess specific inhibitory or stimulatory effects on microorganisms (Greathead, 2003). Some of EOs has an inhibitory effect on the growth of rumen protozoa (Cardozo *et al*. 2006), amino acid fermenting bacteria (Taghavi-Nezhad *et al*. 2014) and methanogenic *archaea* (Calsamiglia *et al*. 2007). These could be an explanation for increased MB, which is the main determinant of PF (Eugéne *et al*. 2004; Iqbal, 2008).

Several mechanisms have been proposed to explain the antimicrobial properties of EOs, with chemical structures and physical properties being thought to be important to determine their antimicrobial potency (Burt, 2004). The presence and relative position of a hydroxyl group in the phenolic structures of EOs (e.g., thymol and eugenol) were proposed to influence the antimicrobial potency of EOs (Burt, 2004; Devi *et al*. 2010).

Acidosis

The oil tested in this study did not affect occurrence of acidosis as much as virginiamycine. It is reported in several studies that *Streptococcus bovis* (main bacteria causes' rumen acidosis) was partly resistant against some EOs compare to other bacteria (McIntosh *et al*. 2003). Evans and Martin (2000) studied the effect of thymol on ruminal microorganisms and found that thymol was a potent inhibitor of lactate production by *Streptococcus bovis* or *Selenomonas ruminantium*. In contrast with this study, Hutton *et al*. (2012) found that several diterpenes isolated from an Australian plant, *Eremophila glabra*, had antimicrobial activity against *Streptococcus bovis*.

Protozoal concentration

The antiprotozoal effect of TKEO was most likely due to the phenolic structure of its main active compounds (Table 2). Such structure can lead to demolition of cell membrane, inhibition of enzymes and lack of substrates and metal ions which are essential for cell metabolism (Goel *et al*. 2005).

In an experiment on the antiprotozoal effects of oregano and thyme EOs against *Trypanosoma cruzi*, Santoro *et al*. (2007) demonstrated that due to hydrophobicity of EOs, they entered to the cell membrane and interfered with the metabolic reactions. Similar to the results of this study, Talebzadeh and Alipour (2013) found that ajowan EO supplementation decreased the total concentration of protozoa by more than 85 percent; and Talebzadeh *et al*. (2012) also confirmed an antiprotozoal effect of *Zingiber multiflora* EO which contained thymol and carvacrol. The numbers of holotrichs and spirotrichs declined with increasing dose of peppermint oil and the reduced number of protozoa was also reflected in terms of anti-protozoal activity as measured by ^{14}C-radio-isotopic technique (Agarwal *et al*. 2009). Patra and Yu (2012) also mentioned that EOs activity is mainly related to their strong activity against protozoa. In addition to that, Lin *et al*. (2013) demonstrated that population of protozoa and methanogen bacteria declined following the addition of either mixture of EOs of several herbal plants or the mixture of principal components of EOs.

Several researches have shown the high relationship between *Entodinium caudatum* and methane production in the rumen (Morgavi *et al*. 2010). According to the result of Table 6, following incorporation of TKEO to the medium, concentration of *Entodinium caudatum* reduced. Methane production was not measured in the present study, however, it can be suggested that defaunation property of TKEO may lower methane production.

CONCLUSION

The results of this study showed that TKEO has the potential to modulate ruminal fermentation. Since the doses of TKEO suppressed the extent of degradability (IVADMD, IVTDMD and IVTOMD), lower levels of the TKEO should be inquired to identify a suitable dose without adverse effect on feed degradability. To investigate its usefulness in ruminant diets, effects on other ruminal microorganisms must be studied.

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REFERENCES

Agarwal N., Shekhar C., Kumar R., Chaudhary L.C. and Kamra D.N. (2009). Effect of peppermint (*Mentha piperita*) oil on *in vitro* methanogenesis and fermentation of feed with buffalo rumen liquor. *Anim. Feed Sci. Technol*. **148,** 321-327.

Aharoni Y., Gilboa N. and Silanikove N. (1998). Models of suppressive effect of tannins. Analysis of the suppressive effect of tannins on ruminal degradation by compartmental models. *Anim. Feed Sci. Technol*. **71,** 251-267.

Benchaar C., Calsamiglia S., Chaves A.V., Fraser G.R., Colombatto D., McAllister T.A. and Beauchemin K.A. (2008). A review of plant derived essential oils in ruminant nutrition and production. *Anim. Feed Sci. Technol*. **145,** 209-228.

Benchaar C., Chaves A.V., Fraser G.R., Wang Y., Beauchemin K.A. and McAllister T.A. (2007a). Effects of essential oils and their components on *in vitro* rumen microbial fermentation. *Canadian J. Anim. Sci*. **87,** 413-419.

Benchaar C., Petit H.V., Berthiaume R., Ouellet D.R., Chiquette J. and Chouinard P.Y. (2007b). Effects of essential oils on digestion, ruminal fermentation, rumen microbial populations, milk production, and milk composition in dairy cows fed alfalfa silage or corn silage. *J. Dairy Sci.* **90,** 886-897.

Blümmel M., Karsli A. and Russell J.R. (2003). Influence of diet on growth yields of rumen microorganisms *in vitro* and *in vivo*: influence on growth yield of variable carbon fluxes to fermentation products. *Br. J. Nutr.* **60,** 625-634.

Blümmel M., Makkar H.P.S. and Becker K. (1997). *In vitro* gas production: a technique revisited. *J. Anim. Physiol. Anim. Nutr*. **77,** 24-34.

British Pharmacopoeia (1988). Her Majesty's Stationery Office. London.

Burt S. (2004). Essential oils: Their antibacterial properties and potential applications in foods: a review. *Int. J. Food Microbiol*. **94,** 223-253.

Calsamiglia S., Busquet M., Cardozo P.W., Castillejos L. and Ferret A. (2007). Invited review: essentiol oil as modifiers of rumen microbial fermentation. *J. Dairy Sci*. **90,** 2580-2595.

Cardozo P.W., Calsamiglia S., Ferret A. and Kamel C. (2006). Effects of alfalfa extract, anise, capsicum and a mixture of cinnamaldehyde and eugenol on ruminal fermentation and protein degradation in beef heifers fed a high concentrate diet. *J. Anim. Sci*. **84,** 2801-2808.

Castillejos L., Calsamiglia S. and Ferret A. (2006). Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in *in vitro* systems. *J. Dairy Sci*. **89,** 2649-2658.

Chen W. and Viljoen A.M. (2010). Geraniol: a review of a commercially important fragrance material. *South African* J. Bot. **76,** 643-651.

Daferera D.J., Ziogas B.N. and Polissiou M.G. (2000). GC–MS analysis of essential oils from Greek aromatic plants and their fungi toxicity on *Penicillum digitatum*. *J. Agric. Food Chem*. **48,** 2576-2581.

Dehority B.A. (1993). Laboratory Manual for Classification and Morphology of Rumen Ciliate Protozoa. CRC Press, Boca Raton, FL, USA.

Debashis R., Tomar S.K. and Kumar V. (2015). Rumen modulatory effect of thyme, clove and peppermint oils *in vitro* using buffalo rumen liquor. *Vet. World*. **8(2),** 203-207.

Devi K.P. Nisha S.A. Sakthivel R. and Pandian S.K. (2010). Eugenol (an essential oil of clove) acts as an antibacterial agent against *Salmonella typhi* by disrupting the cellular membrane. *J. Ethnopharmacol*. **130(1),** 107-115.

Eugéne M., Archimede H. and Sauvant D. (2004). Quantitative meta-analysis on the effects of defaunation of the rumen growth, intake and digestion in ruminants. *Livest. Prod. Sci*. **85,** 81-97.

Evans J.D. and Martin S.A. (2000). Effects of thymol on ruminal microorganisms. *Curr. Microbiol*. **41,** 336-340.

France J., Dijkstra J., Dhanoa M.S., Lopez S. and Bannink A. (2000). Estimating the extent of degradation of ruminant feeds from a description of their gas production profile observed *in vitro*: derivation of models and other mathematical considerations. *Br. J. Nutr*. **83,** 143-150.

Goel G., Puniya A.K., Aguliar C.N. and Singh K. (2005). Interaction of gut microflora with tannins in feeds. *Naturwissenschaften*. **92,** 497-503.

Greathead H. (2003). Plant and plant extract for improving animal productivity. *Proc. Nutr. Soc*. **62,** 279-290.

Hutton P.G., Durmic Z., Ghisalberti E.L., Flematti G.R., Duncan R.M., Carson C.F., Riley T.V. and Verco P.E. (2012). Inhibition of ruminal bacteria involved in lactic acid metabolism by extracts from Australian plants. *Anim. Feed Sci. Technol*. **176,** 170-177.

Hutton P.G., Nagaraja T.G., White C.L. and Vercoe P.E. (2010). Screening plants for the antimicrobial control of lactic acidosis in ruminant livestock. Pp. 159-189 in *In vitro* Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies. P.E. Vercoe H.P.S. Makkar and A.C. Schlink, Eds., Springer, Dordrecht, Netherlands.

Iqbal F.M., Cheng Y.F., Zhu W.Y. and Zeshan B. (2008). Mitigation of ruminant methane production: current strategies, constraints and future options. *World J. Microbiol. Biotchnol*. **24,** 2747-2755.

Jaberi S., Moein M., Jafari A. and Karami F. (2015). Chemical compositions of two different *Thymus* species essential oils. *Trends Pharmacol. Sci*. **1(2),** 27-37.

Khanavi M., Vatandoost H., Khosravi Dehaghi N., Sanei Dehkordi A., Sedaghat M.M., Hadjiakhoondi A. and Hadjiakhoondi F. (2013). Larvicidal activities of some Iranian native plants against the main malaria vector, *Anopheles stephensi*. *Acta. Med. Iran*. **51,** 141-147.

Leung A.Y. and Foster S. (1996). Encyclopedia of Common Natural Ingredients: Used in Food, drugs and Cosmetics. A Wiley Inter science Publication-John Wiley and Sons, Inc., New York.

Lin B., Lu Y., Salem A.Z.M., Wang J.H., Lianga Q. and Liu J.X. (2013). Effects of essential oil combinations on sheep ruminal fermentation and digestibility of a diet with fumarate included. *Anim. Feed Sci. Technol*. **184,** 24-32.

Lopez S., Makkar H.P.S. and Soliva C.R. (2010). Screening plants and plant products for methane inhibitors. Pp. 191-231 in *In vitro* Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies. P.E. Vercoe H.P.S. Makkar and A.C. Schlink, Eds., Springer, Dordrecht, Netherlands.

Macheboeuf D., Morgavi D.P., Papon Y., Mousset J.L. and Arturo-Schaan M. (2008). Dose-response effects of essential oils on *in vitro* fermentation activity of the rumen microbial population. *Anim. Feed Sci. Technol*. **145,** 335-350.

Makkar H.P.S. (2010). *In vitro* screening of feed resources for efficiency of microbial protein synthesis. Pp. 107-144 in *In vitro* Screening of Plant Resources for Extra-Nutritional Attributes in Ruminants: Nuclear and Related Methodologies. P.E. Vercoe H.P.S. Makkar and A.C. Schlink, Eds., Springer, Dordrecht, Netherlands.

Martínez S., Madrid J., Hernández F., Megías M.D., Sotomayor J.A. and Jordán M.J. (2006). Effect of thyme essential oils (*Thymus hyemalis* and *Thymus zygis*) and monensin on *in vitro*

ruminal degradation and volatile fatty acid production. *J. Agric. Food Chem*. **54,** 6598-6602.

McAllister T.A., Bae H.D., Jones G.A. and Cheng K.J. (1994). Microbial attachment and feed digestion in the rumen. *J. Anim. Sci*. **72,** 3004-3018.

McIntosh F.M., Willams P., Lose R., Wallance R.J., Beever D.A. and Newbold C.J. (2003). Effects of essential oils on ruminal microorganisms and their protein metabolism. *Appl. Environ. Microbiol*. **69,** 5011-5014.

Mohammed M.J. and Al-Bayati F.A. (2009). Isolation and identification of antibacterial compounds from *Thymus kotschyanus* aerial parts and *Dianthus caryophyllus* flower buds. *Phytomedicine*. **16,** 632-637.

Morgavi D.P., Boudra H., Jouany J.P. and Michalet-Doreau B. (2004). Effect of stability of gliotoxin, an *Aspergillus fumigates* toxin, on *in vitro* rumen fermentation. *Food Addit. Contam*. **21,** 871-878.

Morgavi D.P., Forano E., Martin C. and Newbold C.J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal*. **4(7),** 1024-1036.

Mozafarian V.A. (1996). Dictionary of Iranian Plant Names. Farhang Mosavar, Tehran, Iran.

Patra A.K. and YU Z. (2012). Effects of essential oils on methane production, fermentation, abundance and diversity of rumen microbial populations. *Appl. Environ. Microbiol*. **78(12),** 4271-4280.

Raal A., Paaver U., Arak E. and Orav A. (2004). Content and composition of the essential oil of *Thymus serpyllum* growing wild in Estonia. *Med. Kaunas*. **40,** 795-800.

Sallam S.M.A., Nasser M.E.A., Araujo R.C. and Abdalla A.L. (2009). Methane emission *in vivo* by sheep consuming diet with different levels of eucalyptus essential oil. Em: FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health, Vienna, Austria.

Santoro G.F., das Graças Cardoso M., Guimarães L.G., Salgado A.P., Menna-Barreto R.F. and Soares M.J. (2007). Effect of oregano (*Origanum vulgare*) and thyme (*Thymus vulgaris*) essential oils on *Trypanosoma cruzi* (Protozoa: Kinetoplastida) growth and ultrastructure. *Parasitol. Res*. **100,** 783-790.

SAS Institute. (2002). SAS®/STAT Software, Release 9.2. SAS Institute, Inc., Cary, NC. USA.

Singh D., Kumar T.R.S., Gupta V.K. and Chaturvedi P. (2012). Antimicrobial activity of some promising plant oils, molecules and formulations. *Indian J. Exp. Biotechnol*. **50(10),** 714-717.

Sobhy M.A.S. and Samir A.M.A. (2010). Effect of different levels of citrus essential oil and its active component on rumen microbial fermentation and methane production *in vitro*. *Cuban J. Agric. Sci*. **44(4),** 367-370.

Taghavi-Nezhad M., Alipour D., Flythe M.D., Zamani P. and Khodakaramian G. (2014). The effect of essential oils of *Zataria multiflora* and *Mentha spicata* on the *in vitro* rumen fermentation, and growth and deaminative activity of amino acid-fermenting bacteria isolated from Mehraban sheep. *J. Anim. Prod. Sci*. **54(3),** 299-307.

Taghavi-Nezhad M., Alipour D., Torabi-Goudarzi M., Zamani P. and Khodakaramian G. (2011). Dose response to carvone rich essential oils of spearmint (*Mentha spicata*): *in vitro* ruminal fermentation kinetics and digestibility. *J. Agric. Sci. Technol*. **13,** 1013-1020.

Talebzadeh R., Alipour D., Saharkhiz M.J., Azarfar A. and Malecky M. (2012). Effect of essential oils of *Zataria multiflora* on *in vitro* rumen fermentation, protozoal population, growth and enzyme activity of anaerobic fungus isolated from Mehraban sheep. *Anim. Feed Sci. Technol*. **172,** 115-124.

Talebzadeh R. and Alipour D. (2013). The effect of ajowan (*Carum copticum*) essential oils on eukaryotic ruminal microorganisms of Mehraban sheep. *Iranian J. Microbiol*. **4,** 418-421.

Wallace R.J., McEwan N.R., McInotosh F.M., Teferedegne B. and Newbold C.J. (2002). Natural products as manipulators of rumen fermentation. *Asian Australas J. Anim*. **15,** 1458-1468.

Wenk C. (2003). Herbs and botanicals as feed additives in monogastric animals. *Asian-Australas J. Anim*. **16,** 282-289.