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The effect of *Nepeta glomerulosa* Boiss. *(Lamiaceae)* essential oil (NGEO) on *in vitro* gas production and ruminal fermentation

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Abstract The study aimed to evaluate the chemical composition and the effects of *Nepeta glomerulosa* Boiss. (*Lamiaceae*) essential oil (NGEO) on *in vitro* gas production and ruminal fermentation. The essential oil (EO), obtained by steam distillation from *Nepeta glomerulosa* Boiss. (0, 150, 300, and 450 mg/L), was investigated in an *in vitro* culture medium using sheep rumen fluid and artificial saliva. A fattening diet was used as the substrate in the culture medium and gas production was measured. The profile of NGEO was determined by GC-mass analysis. The 1, 8-cineole (23.2%), α-pinene (15.3%), limonene (9.1%), and β-pinene (3.5%) were the major components in NGEO. Ammonia nitrogen and total volatile fatty acids (TVFA) concentrations did not change when NGEO was added to the culture medium, whereas TVFA tended to increase at the higher concentration of NGEO ($P<0.1$). The pH value of the culture medium linearly and quadratically decreased with increasing NGEO ($P<0.05$). The potential of gas production (b_{gas}; linear, and quadratic, $P<0.05$) increased with increasing NGEO, however, the constant rate decreased linearly and quadratically ($P<0.05$). Dry matter (DMD) and organic matter degradability (OMD) were increased (linear and quadratic) with increasing NGEO in the culture medium. The partitioning factor (PF), microbial mass yield (MMY), and efficiency of microbial mass synthesis (EMMS) linearly and quadratically decreased when the concentration of NGEO increased. It seems that NGEO affected the fermentation process *in vitro* partly via improving TVFA production or by increasing DMD and OMD. Further *in vitro* and *in vivo* studies are needed to confirm that NGEO in the diet has no adverse effects on the health and production in ruminants.

Keywords: culture medium, gas production, GC-mass, ruminal fermentation

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Introduction

For many years, promoters (ionophores and antibiotics) have been used to improve ruminant growth performance (McGuffey et al., 2001). However, the European Commission has banned the use of these products because of the possibility of harming the health of consumers (Calsamiglia et al., 2007). Recently, the consumption of these agents has considerably decreased in Iran.

Essential oils (EOs) are aromatic compounds obtained from commonly medicinal plants, which can be used as natural additives in animal feeds because of their antibacterial, antifungal, and antioxidant characte-

ristics (Calsamiglia et al., 2007). Currently, nutritionists are applying the herbal extracts as modifiers instead of antibiotics to improve ruminal fermentation parameters (Demirtaş et al., 2018). The EOs contain effective compounds that can be extracted by steam distillation (Benchaar et al., 2007). It has been reported that most EOs have strong antimicrobial characteristics due to compounds such as terpenoid and phenolic compounds (Helander et al., 1998; Fraser et al., 2007). The aerial parts of *Nepeta glomerulosa* Boiss. produced about 0.25% of a clear yellowish EO with a pleasant odor (Sajjadi and Ghassemi, 1999). The most abundant component in NGEO is *α*-pinene (18.3%, Sajjadi and Ghassemi, 1999) which has been reported to have inhibitory effects on *in vitro* growth of some gram-positive bacteria (Karting et al., 1991; Aronen, 1997) and fungal activity *in vitro* (Xing-dong and Hua-li, 2014).

To the best of our knowledge, there is no information available on relationships between NGEO and rumen fermentation parameters in a laboratory culture medium. Therefore, this study was conducted to investigate the effects of NGEO on ruminal fermentation, *in vitro* gas production, dry matter and organic matter degradability using ruminal fluid obtained from sheep feeding on a fattening diet.

Material and methods

Plant collection, extraction and GC/MS protocol

Nepeta glomerulosa Boiss. was collected during the flowering stage from the village of Revenj, Torbat-e Jam, Iran in spring 2018 (Figure 1). Plant samples, in tightly-closed plastic bags, were immediately transferred to the laboratory. The *Nepeta glomerulosa* Boiss. essential oil (NGEO) was obtained from the dry whole plant through water distillation by a Clevenger apparatus for 4 h. The obtained NGEO was subsequently dried by anhydrous sodium sulfate and then used for gas chromatography/mass spectrometry analysis. Analysis of NGEO was performed by gas chromatography-mass spectrometry (GC–MS) (Agilent Technologies, Palo Alto, CA, USA), fitted with an FID detector Agilent 5975C mass selective detector (MSD) and quadrupole EI mass analyzer. A BP-1 capillary column (30 m length, ID 0.25 mm and film thickness of 0.25 mm) was used as the stationary phase. Oven temperature was set from 60 °C to 275 °C. Injector and detector temperatures were 280 °C. Helium was considered as carrier gas with a flow rate of 0.8 mL/min. A volume of 0.1 µL of the NGEO with a split ratio of 1:50 was injected into the apparatus (Cui et al., 2018).

Figure 1. *Nepeta glomerulosa* Boiss. Collected from Revenj village, Torbat-e Jam, Iran

In vitro techniques

Four levels of NGEO (0, 150, 300, and 450 mg/L) were added to the culture medium prepared from sheep rumen fluid and artificial saliva (1:2 ratio). The incubated substrate was formulated according to NRC (2007). A 200 mg sample of the experimental diet (Table 1) was incubated in 60 mL glass syringes to determine the *in vitro* gas production according to Menke and Steingass (1988). Before the morning feeding, ruminal fluid was collected from two Baluchi male sheep (30±3.5 kg), strained through four layers of cheesecloth, and trans-

Table 1. Ingredients and chemical compositions of the diet used in the culture medium

Ingredients	% in DM
Corn grain, ground	21
Barley grain, ground	21
Soybean meal, 44%CP	8
Wheat bran	11
Wheat grain, ground	6
Salt	0.6
Sodium bicarbonate	0.9
Vitamin-mineral complex[1]	0.8
Calcium carbonate	0.7
Alfalfa hay	30
Chemical compositions	
Ash	7.6
Crude protein	16.2
Neutral detergent fiber	24.9
Metabolizable energy (Mcal/kg DM)	2.74
Calcium	0.8
Phosphorus	0.4

[1]Contained: 40,0000 IU of vitamin A, 100,000 IU of vitamin D, 100 mg of vitamin E, 10 mg vitamin B_1, 20 mg vitamin B_2, 3% Ca, 1.2% P, 4% Na, 11000 Mg, 2000 mg Zn, 12 mg Se, 2000 mg Mn, 1000 mg Cu, 60 mg I, 3000 mg Fe, 60 mg Co.

ferred immediately to the laboratory. After adding 30 mL rumen fluid and artificial saliva (1:2 ratio) to the syringes, they were incubated in a water bath at 39 °C for 3, 6, 9, 12, 24, 48, 72, and 96 h. Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979), i.e. $Y = b(1 - e^{-ct})$, in which, the *Y* is the cumulative gas production at time of "t", *b* the potential gas production (mL/200 mg DM), *c* the fractional rate of gas production (%/h) and *t* the time of incubation (h).

In another run, a medium, similar to the one for gas production, was used to measure total volatile fatty acids (Barnett and Reid, 1957) and NH_3-N (Komolong et al., 2001). The content of each syringe (96 h incubation) was filtered through polyester cloth (45 µ), dried in the oven (60 °C, 48 h), and then calculated for dry matter (DM) and subsequently organic matter (OM) degradability (after ashing). The pH of the culture medium was measured immediately using a pH meter (Metrohm, 691, Metrohm AG, Switzerland) after 24 h incubation. The partitioning factor (PF) was determined (Makkar, 2010) using to the following equation:

PF (mg OM truly degraded/mL gas)=TOMD/IVGP= c-(a-b)/IVGP

in which, TOMD is the true organic matter degradability; *c,* was the OM weighed into the syringe; *a,* the weight of undegraded feed; and *b,* the weight of ash. The microbial mass yield (MMY) and efficiency of microbial mass synthesis (EMMS) were determined (Blümmel et al., 1997) using to the following equation:

MMY (mg)=mg substrate truly degraded-[NG (mL)×2.2]

where, NG is mL of net gas production at 24 h of incubation and 2.2 is the stoichiometric coefficient.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (2002) in a completely randomized design based on the following statistical model:

$Y_{ij} = \mu + T_i + e_{ij,}$

where, Y_{ij} is the general observation; µ, the general mean; T_i, the effect of treatment; and e_{ij}, the residual error term.

The comparison between means was performed using the Duncan's test. The level of significance was set at $P<0.05$, and trends were considered when $0.05<P<0.1$.

Results

The results from the GC-MS analysis revealed the presence of 36 major components, representing 98.15% of the total oil (Table 2). The major components were 1, 8-cineole (23.2%), followed by *α*-pinene (15.3%), limonene (9.1%), *β*-pinene (5.35%), linalool (3.5%), trans-*α*-bergamotene (3.4%), camphene (2.9%) and humulene epoxide (2.5%).

There were no significant differences between treatments for ammonia nitrogen (NH_3-N), however, TVFA tended to increase linearly by increasing NGEO (Table 3). The pH value was also decreased linearly when NGEO was added to the culture medium (Table 3).

There were significant differences in potential gas production (b_{gas}) and fractional rate of gas production

Table 2. Composition of the essential oil of *Nepeta glomerulosa* Boiss

Compound	Proportion (%)	RI
α-pinene	15.3	927
Camphene	2.9	950
β-pinene	5.35	970
Dehydro-1,8-cineole	1.8	983
Myrcene	tr	986
α-terpinene	tr	1014
p-cymene	1.5	1025
Limonene	9.1	1029
1,8-cineole	23.2	1030
trans-β-ocimene	2.7	1044
γ-terpinene	0.9	1058
Unidentied	2.2	1060
Linalool	3.5	1099
α-campholenal	0.5	1123
trans-limonen oxide	1.5	1135
trans-pinocarveol	1.8	1131
Citronellal	0.5	1150
Borneol	1.5	1161
Terpinen-4-ol	2.5	1172
α-terpineol	1.1	1187
Myrtenol	0.4	1191
Verbenone	0.2	1198
Geraniol	0.3	1255
Bornyl acetate	1.8	1282
Carvacrol	2.2	1294
α-Copaene	0.3	1360
Geranyl acetate	1.9	1381
trans *α*-bergamotene	3.4	1436
cis-*β*-farnesene	1.5	1440
α-humulene	2.0	1445
Germacrene-D	0.5	1460
β-selinene	0.9	1484
β-bisabolene	1.0	1508
β-sesquiphellandrene	1.1	1520
Humulene epoxide	2.5	1608
σ-cadinol	0.3	1640

RI: Retention indices on HP-5 capillary column. tr: trace (<0.05%).

Table 3. Effect of *Nepeta glomerulosa* Boiss. essential oil (NGEO) on fermentation parameters

Fermentation parameters	NGEO levels (mg/L)							
	0 (control)	150	300	450	SEM	Linear	Quadratic	Cubic
pH	6.73[a]	6.75[a]	6.74[a]	6.57[b]	0.02	<0.05	0.009	0.35
Ammonia nitrogen (mg/dl)	31.48	30.19	33.55	31.23	2.33	0.88	0.89	0.54
TVFA (mmol/l)	32.12	36.00	39.50	40.62	3.55	0.09	0.71	0.69

TVFA: total volatile fatty acids.
[a,b]: within row, means with common superscript do not differ (P>0.05).

(c_{gas}) with NGEO addition (Table 4). After 24 h of incubation, a linear tendency (P<0.1) in reduced gas production was observed. However, a linear increase in the volume of gas production was observed after 96 h incubation. Addition of NGEO significantly decreased the PF, MMY, and EMMY (Table 5), whereas, all doses of NGEO linearly increased (P<0.05) the DMD and OMD (Table 5).

Discussion

The EO obtained from *Nepeta glomerulosa* Boiss. contained a complex mixture dominated by monoterpene hydrocarbons (62.8%), oxygenated monoterpenes (19.7%), and sesquiterpene hydrocarbons (15.4%). It has been reported that 1, 8-cineole (a phenolic compound) is an active component in *Salvia officinalis* which is effective against *Escherichia coli* and *Staphylococcus aureus* (Alekish et al., 2017). Our findings are in contrast with the results obtained by Sajjadi and Ghassemi (1999) who found thirty-five compounds in NGEO, of which the most abundant components were *α*-pinene (18.3%), followed by 1,8-cineole (13.9%), limonene (9.7%), linalool (4.8%), trans-*β*-ocimene (4.7%) and humulene epoxide (4.2%), respectively. They also reported that NGEO contained 10 monoterpene hydrocarbons (44.0%), 14 oxygenated monoterpenes (34.4%), 7 sesquiterpene hydrocarbons (11.2%), and 3 oxygenated sesquiterpenes (5.1%). In another study, oxygenated monoterpenes were the major constituents in EO of *Nepeta crassifolia* and *Nepeta nuda* from northwest of Iran (Narimani et al., 2017).

The reduction in pH value could be due to the tendency to increase TVFA in the culture medium. Using different doses of EOs can have different effects on ruminal fermentation parameters *in vitro* (Castillejos et al., 2008). Castillejos et al. (2006) reported that the addition of some EO active compounds to the culture medium at high level resulted in a significant increase in TVFA concentration, which is inconsistent with our study. Newbold et al. (2004) observed that deamination was inhibited in a culture medium when ruminal fluid was obtained from animals receiving 1000 mg EOs/day or 110 mg EOs/sheep/day. A similar result was reported by McIntosh et al. (2000) who found that EOs inhibited the deamination of amino acids by 25%. However, in our study, no differences were found in NH_3-N concentration as a product of protein deamination following the use of NGEO, which is in agreement with Castillejos et al. (2007). An increase in pH value following a decline in TVFA reflected a decrease in the fermentation of the diet ascribed by the antimicrobial activity of phenolic compounds in medicinal plants (Fraser et al., 2007). Consistent with our results, Newbold et al. (2004) found that TVFA concentration tended to be higher at 6 h after feeding in sheep supplemented with 110 mg/d of a blend of EOs. Generally, variations in rumen TVFA production by EOs supplementation are more evident at low ruminal pH, suggesting the undissociated hydrophobic form of the EOs active molecules which is more active against the cell membrane of ruminal microorganisms (Cardozo et al., 2005; Spanghero et al., 2008). Effects of EOs on ruminal fermentation are desirable if

Table 4. Effect of *Nepeta glomerulosa* Boiss. essential oil (NGEO) on gas production parameters

Gas production (mL)	NGEO levels (mg/L)							
after	0 (control)	150	300	450	SEM	Linear	Quadratic	Cubic
12 h	41.05[ab]	40.58[b]	42.37[a]	39.47[c]	0.48	0.19	0.02	0.007
24 h	47.35[a]	45.18[b]	49.30[a]	47.82[a]	0.96	0.08	0.60	0.001
48 h	55.32[bc]	52.50[c]	56.65[ab]	59.05[a]	0.98	0.004	0.02	0.07
72 h	58.82[bc]	58.38[c]	61.70[b]	66.97[a]	1.02	<0.0001	0.02	0.70
96 h	59.32[bc]	58.65[c]	62.50[b]	70.67[a]	1.08	0.04	0.84	0.73
b_{gas} (mL)	57.16[bc]	55.71[c]	59.85[b]	66.42[a]	1.04	<0.0001	0.002	0.50
c_{gas} (%/h)	0.101[a]	0.102[a]	0.095[a]	0.067[b]	0.002	<0.0001	0.002	0.17

b_{gas}: potential gas production; c_{gas}: fractional rate of gas production.
[a,b,c]: within row, means with common superscript (s) do not differ (P>0.05).

Table 5. Effect of *Nepeta glomerulosa* Boiss. essential oil (NGEO) on estimated laboratory parameters

Parameter	NGEO levels (mg/L)				SEM	Linear	Quadratic	Cubic
	0 (control)	150	300	450				
DMD (%)	77.39^{b}	83.50^{a}	81.21^{a}	84.22^{a}	1.01	0.02	0.0007	0.72
OMD (%)	80.97^{b}	85.72^{a}	85.75^{a}	85.91^{a}	1.22	0.02	0.09	0.39
PF (mg/mL)	3.10^{ab}	3.15^{a}	2.95^{b}	2.62^{c}	0.05	<0.0001	0.002	0.65
MMY (mg)	53.27^{ab}	55.62^{a}	47.16^{b}	29.21^{c}	2.45	<0.0001	0.01	0.91
EMMS (%)	28.98^{ab}	30.12^{a}	25.54^{b}	15.81^{c}	1.32	<0.0001	0.001	0.92

DMD: dry matter degradability; OMD: organic matter degradability; PF: partitioning factor; MMY: microbial mass yield; EMMS: efficiency of microbial mass synthesis.

^{a,b,c}: within row, means with common superscript (s) do not differ (P>0.05).

they increase or do not change TVFA concentration; and decrease NH_3-N concentration and methane yield (Benchaar and Greathead, 2011; Bodas et al., 2012).

It seems that at lower incubation times (12 h), a high level of EO (450 mg/L) had inhibitory effects on fermentation parameters in the culture medium, while at higher incubation times (48 and 72 h) the microorganisms were adopted to the increased NGEO level. It has been reported that the inclusion of EO at a moderate or high level, can be toxic to methanogenic bacteria that are active in gas production (Wallace, 2004; Helander et al., 1998). A decreased in gas production at earlier times of incubation (up to 24 h) can be ascribed to the antibacterial effect exerted by NFEO mainly, 1, 8-cineole against bacteria, as Benchaar et al. (2007) reported that phenolic compounds revealed antimicrobial activity due to their hydroxyl groups. On the other hand, the increase in potential gas production and consequently OM digestibility following administration of NGEO could be related to the ability of some strains of bacteria to degrade the phenolic compounds and utilize them as an energy source (Salem et al., 2011). Furthermore, it has been reported that monoterpene hydrocarbons slightly inhibited the growth and metabolism of bacteria and, sometimes, stimulated the activity of rumen microbes (Benchaar et al., 2008).

Our results are inconsistent with Nooriyan Soroor and Rouzbehan (2014) who reported that *Heracleum persicum* (containing flavonoids) increased PF, MMY, and EMMS *in vitro*. Similar results were also observed when 100 or 150 µL eucalyptus oil was added to the *in vitro* medium (Sallam et al., 2009). In contrast to our results, higher microbial protein synthesis along with lower methane production as a result of the inclusion of *Sesbania sesban*, a medicinal plant, was reported by Goel et al. (2008). Recently, Besharati et al. (2020) found an increase in PF, MMY, and EMMY with the addition of cinnamon EO to the culture medium containing pomegranate seeds in comparison to the control.

In the present study, the lowest PF, MMY, and EMMS were observed at 450 mg/L NGEO, which indicates the negative effect of NGEO on *in vitro* ruminal fermentation. However, Bettaieb et al. (2016) reported that the addition of Myrtle EO; a plant rich in α-pinene and limonene; at medium (40 and 80 µL/50 mL) and high (120 µL/50 mL) concentrations decreased TOMD but increased PF. Gas production is the result of fermentation of carbohydrates to acetate, propionate, and butyrate, hence improvement in TVFA concentration may suggest higher feed digestibility which is supported by the significant difference in OMD compared to control. In our study, despite the increase in potential gas production at high NGEO level (450 mg/L), the PF, MMY, and EMMY decreased compared to the control. So, as we expected an inverse relationship between gas volume or TVFA and MMY was detected. Therefore, the gas produced from anaerobic ruminal fermentation can be associated with loss of energy and reduction of fermentation efficiency (Jahani-Azizabadi et al., 2011). An increase in gas yield is not suitable on all occasions. The PF is a better index to evaluate the fermentation efficiency relation to ruminant requirements and limitations (Makkar, 2010; Jahani-Azizabadi et al., 2011). To identify the best level of NGEO, two criteria were considered in the present study. First, a decrease in gas production and second, an increase in PF. It seems that 150 mg/L NGEO was the best level with regards to these criteria. Overall, the results of many studies investigating a wide range of EOs and their components at various dose rates and in different diets, have been inconsistent. The varied responses among EO products evidently reflect the differences in chemical structure, which influences their effects on microbial activity.

Conclusions

Of the compounds evaluated, 1, 8-Cineole was the major component and monoterpene hydrocarbons were dominant in NGEO. Although potential gas production,

DM and OM digestibility significantly increased at highest level of NGEO inclusion, PF, MMY, and EMMS decreased at 450 mg/L. Inclusion of NGEO up to 450 mg/L did not have a deleterious effect on *in vitro* ruminal fermentation (pH, ammonia nitrogen, and TVFA) and digestibility; however, with respect to PF, MMY, and EMMY, the lowest level of NGEO (i.e. 150 mg/L) was suitable for manipulating the ruminal fermentation. Further researches are needed to investigate these effects on rumen fermentation and microbial protein synthesis *in vivo*.

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