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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Effect of *Cyclotrichium niveum* Essential Oil on Rumen Microbial Fermentation and *in vitro* Digestibility of Barley

Cyclotrichium niveum Esansiyel Yağının Rumen Mikrobiyal Fermantasyonuna ve Arpanın *in vitro* Sindirilebilirliğine Etkisi

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Abstract

The digestibility of feed in ruminants and the volatile fatty acids (VFA) resulting from digestion directly affect animal performance. It has long been studied on the use of essential oils as an alternative to antibiotics to ensure optimum conditions for the rumen environment. *Cyclotrichium niveum* (mountain mint) essential oil is rich in antimicrobial agents. In this study different doses (0, 50, 100 and 150 mg/L of culture fluid) of *Cyclotrichium niveum* essential oil was incubated 24 hours with barley in diluted ruminal fluid takes from fistulated cows fed with a 40:60 concentrate:forage diet in order to assess the effects on rumen microbial fermentation and *in vitro* true digestibility of barley. True digestibility of barley was determined in ANKOM Daisy Incubator. All doses of *Cyclotrichium niveum* significantly (P<0.001) decreased the *in vitro* dry matter (DM), organic matter (OM), neutral detergent fiber (NDF) digestibility of barley. *Cyclotrichium niveum* essential oil negatively affected end-products of rumen fermentation; the value of the measured rumen volatile fatty acids (VFA: acetate, propionate, butyrate, isobutyric acid, valerate and isovalerate) significantly decreased compared to the control group (P<0.001). Carbon dioxide and methane gases created as a result of incubation in rumen fluid were calculated using volatile fatty acid values formed in rumen fluid. The addition of *Cyclotrichium niveum* essential oil significantly reduces the CO_2 in rumen fluid (P<0.001). Methane (CH_4) gas from rumen fluid was significantly decreased in treatment groups compared the control group (P <0.001) While end-products of rumen fermentation parameters significantly decreased with all doses of *Cyclotrichium niveum* essential oil, rumen pH has increased (P<0.05). The results show that when the *Cyclotrichium niveum* essential oil incubated with barley significantly inhibited the rumen microbial activity and overall fermentation process of rumen.

Keywords: *Cyclotrichium niveum*, Digestibility, Barley, Essential Oil, Methane, Mountain mint

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Özet

Ruminantlarda yemin sindirilebilirliği ve sindirim sonucu ortaya çıkan uçucu yağ asitleri (UYA) hayvan performansını doğrudan etkiler. Rumen ortamı için optimum koşulları sağlamak amacıyla, antibiyotiklere alternatif olarak uçucu yağların kullanımı üzerinde uzun zamandır çalışılmaktadır. *Cyclotrichium niveum* (dağ nanesi) esansiyel yağı antimikrobiyal ajanlar açısından zengindir. Bu çalışmada rumen mikrobiyal fermantasyonu ve arpanın *in vitro* gerçek sindirilebilirliği üzerindeki etkilerini değerlendirmek için farklı dozlardaki (0, 50, 100 ve 150 mg / L kültür sıvısı) *Cyclotrichium niveum* esansiyel yağı, 40:60 konsantre: kaba yem ile beslenen fistüle edilmiş ineklerden alınan seyreltilmiş ruminal sıvı içinde 24 saat süreyle arpa ile inkübe edilmiştir. Arpanın gerçek kuru madde (KM), organik madde (OM) ve nötral deterjan lif (NDF) sindirilebilirlikleri ANKOM Daisy İnkübatörde belirlenmiştir. Tüm *Cyclotrichium niveum* dozları arpanın *in vitro* kuru madde (KM), organik madde (OM), nötral deterjan lif (NDF) sindirilebilirliğini önemli ölçüde azaltmış ($P<0.001$), *Cyclotrichium niveum* esansiyel yağının rumen fermantasyonu son ürünlerini olumsuz etkilemiştir. Ölçülen rumen uçucu yağ asitleri (UYA: asetat, propiyonat, bütirat, izobütirik asit, valerat ve izo valerat) değerleri kontrol grubuna göre önemli ölçüde azalmıştır ($P <0.001$). Rumen sıvısında inkübasyon sonucu oluşan karbondioksit ve metan gazları rumen sıvısında oluşan uçucu yağ asiti değerleri kullanılarak hesaplanmıştır. *Cyclotrichium niveum* uçucu yağının eklenmesi rumen sıvısındaki CO_2'i önemli ölçüde azaltmıştır ($P <0.001$). Rumen sıvısından ölçülen metan (CH_4) gazı deneme gruplarında kontrol grubuna göre anlamlı olarak azalmıştır ($P <0.001$), rumen fermantasyonunun son ürünleri, *Cyclotrichium niveum* uçucu yağının tüm dozlarıyla önemli ölçüde azalırken, rumen pH'ı artmıştır ($P <0.05$). Sonuçlar, arpa ile inkübe edilen *Cyclotrichium niveum* uçucu yağının rumen mikrobiyal aktivitesini ve rumen genel fermantasyon sürecini önemli ölçüde inhibe ettiğini göstermektedir.

Anahtar Kelimeler: *Cyclotrichium niveum*, Sindirilebilirlik, Arpa, Esansiyel Yağ, Metan, Dağ nanesi

1. Introduction

With the banning of antibiotics in animal production since 2006, today, plant extracts have started to gain importance as alternative feed additives and growth stimulants to antibiotics. Efforts are being made to determine the benefits of herbal extracts and extract oils and to be a suitable alternative for the future (Kamel, 2007). Plants that can produce secondary metabolites such as sarsaponin, tannin, thymol, which affect microbiological activity in rumen are considered as alternative feed additives that have valuable potential for use (Benchaar et al., 2015).

Essential oils (EOs) obtained from plants have antimicrobial effects on gram (+) and gram (—) bacteria. Due to these effects, EOs have reduced protein breakdown in the rumen, control of pathogenic microorganisms, increasing the activity and nitrogen absorption of digestive enzymes and reducing environmental pollution caused by fertilizers (Wallace et al., 2004; Cardozo et al., 2005; Chaves et al., 2008; Patra and Yu, 2012; Önenç et al., 2016; Belanche et al., 2019).

There are many studies on the modulator effects of essential oils on rumen digestibility. The variability of these studies results depends on the variability of the chemical compositions of the plant used in general, the dosage used, the rumen pH and the type of diets used in the experiments. (Cardozo et al., 2005; Calsamiglia et al., 2007). Looking at the rumen fermentation effect of the dose ;while EOs are ineffective or selectively effective on the rumen microbial ecosystem at low and medium doses, they show a general inhibitory effect on rumen fermentation when used at high doses (Yadeghari et al., 2015), referring that EOs have a dose response effect on rumen fermentation.

Cyclotrichium niveum (mountain mint) is a one-year-old herbaceous plant with dense white trichome, strong mint fragrance and a height of 20-50 cm belonging to *Lamiaceae* family. Its active ingredients are pulegone (31.9%), germacrene-D (16.45%), menthone (15%), isomenthone (9%) and spathulenol (3.5%). It has been shown in many studies that *Cyclotrichium niveum* essential oil is rich in antimicrobial agents (Gulcin et al., 2008; Alim et al., 2009; Gürsoy et al, 2009). Due to its antimicrobial properties, it was expected that might be effective in manipulating rumen fermentation by affecting microorganisms in the rumen. In other words, they are thought to be effective in promoting microbial protein synthesis, preventing widespread breakdown of proteins, controlling methane-producing bacteria, stimulating the proliferation of cellulose-digesting bacteria and suppressing acid-causing bacteria. Despite all these expectations any study investigating the effect of *Cyclotrichium niveum* essential oil on rumen fermentation has not been acrossed. In this study, the potential for use of *Cyclotrichium niveum* (Boiss.) Manden & Scheng (mountain mint) as a feed additive for ruminant animals and its effective dose were investigated by *in vitro*.

2. Material and Methods

The amount of *Cyclotrichium niveum (*mountain mint) to be used in the study was collected by senior taxonomists Dr. Ahmet Zafer Tel in August ideal time for collected and dried in a cool environment then extracted by steam distillation methods for EOs. The oil was taken in dark glass bottles and stored at 4 ° C until use for incubation and chemical analyse. GC-MS analysis of EOs obtained after dilution with hexane in the ratio of 1:20, Agilent30m ∗ 0.250mm * 0.25mμ in brand GC-MS device In size DB-5MS column, 1ml / min. helium gas flow rate, 1:50 split rate, 250 °C inlet temperature, 1 μl injector volume, starting at 60 °C, 3 °C / min. Heating brought to 300 °C and waiting for 5 minutes the analysis was then terminated.

The effects of *Cyclotrichium niveum* on *in vitro* true digestibilities of barley were evaluated using ANKOM Daisy Incubator and Technology. The ruminal inoculum, used for *in vitro* incubations, was collected from fistulated cow fed 60:40 feed forage: concentrate diet. The ruminal inoculum collection was carried out 3 hours (h) post morning meal. Then the ruminal inoculum was strained through various layers of cheese cloth and kept at 39 °C under a CO_2 atmosphere. Barley is grounded using a 1 mm sieve for chemical analysis and *in vitro* incubation. Weighed about 0.5 g of ground barley in filter bags (F57) and closed with heat stamping device and incubated in a Daisy^{II} incubator (ANKOM Technology Corp., Macedon, NY) with rumen fluid, buffer in a 1:4 ratio. *Cyclotrichium niveum* in an amount to meet the dosage of 50,100,150 ppm for 24 h under anaerobic conditions at

39.8 ˚C. In the experiment, nutrient analysis of barley and residues were determined according to the methods specified in AOAC (2007). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analysed with the ANKOM 200 fibre analyser using reagents described by Van Soest and Wine (1975) and Van Soest (1963), respectively (Table 1).

Table 1. Chemical Composition of Barley (DM basis)

Ingredient	**%**
Dry matter	90.30
Organic matter	86.64
Crude protein	11.89
Ash	3.66
Crude fat	1.66
ADF	8.84
NDF	20.09
Nitrogen-free extract	66.51

Determination of the volatile fatty acid (VFA) content in the rumen liquid was conducted by using a gas chromatograph with a semi-capillary FFAP column (Hewlett-Packard, Wardbronn, Germany), at temperature range of 45-230°C.

Carbon dioxide (CO_2) and methane (CH_4) gases formed by in vitro fermentation were calculated with the following formula using the values of volatile fatty acids obtained from the rumen fluids come off after 24 hours of incubation of barley (Blümmel et al., 1999).

Carbon dioxide, CO_2 = Acetic acid / 2 + Propionic acid / 4 + 1.5 x Butyric acid

Methane, CH_4 = (Acetic acid + 2 × Butyric acid) − CO_2

(The concentration of UYA has been taken as mmol)

The obtained data were subjected to variance analysis in accordance with the trial model using the SAS (1998) package program. Comparison of means is provided by using Duncan Multiple Comparison Test.

All experimental procedures were approved by the Ethic Committee of Research Council of Çukurova University.

3. Result and Discussion

The results showed that *Cyclotrichium niveum* essential oil significantly affects the *in vitro* true digestibility of barley (dry matter digestibility (DMD), organic matter digestibility (OMD) and neutral detergent fiber digestibility (NDFD)) (Table 2). The *in vitro* digestibility of barley was decreased (linear (DMD) and quadratic (OMD, NDFD) response, $P < 0.01$; $P < 0.05$; $P < 0.001$ respectively by *Cyclotrichium niveum* essential oil. The *in vitro* DMD was 70.70% in the control group. The values of DMD measured as 62.99% (with a 10.9% reduction), 59.13% (with a 16.36% reduction) and 58.39% (with a 17.41% reduction) in group of 50 ppm, 100 ppm and 150 ppm doses respectively. Interestingly, the lowest values in *in vitro* OMD and NDFD were observed at the 50 ppm dose group. It constitutes the belief that there is a negative effect of *Cyclotrichium niveum* EO on starch digesting bacteria in rumen fluid. It suggests that this feature of the EO can be used with carbohydrates that are easily digested and causes acidosis in the rumen environment. Thus, risk of acidosis can be reduced with *Cyclotrichium niveum* EO. There are many data on the effect of EOs on rumen digestibility and fermentation assorted depending on the type, composition, dose of EOs and also rumen pH and animal diet type (Cardozo et al.,2004; Malecky et al., 2009). Assuming that not all end products of rumen fermentation are due to substrate destruction, it becomes difficult to explain the reason for the change observed in rumen fermentation. Because whether the changing in digestibility

is a result of *Cyclotrichium niveum* EO direct utilization by rumen microorganisms or the consequence of its indirect impact on substrate degradation and fermentation are not clear.

Table 2. Effect of Cyclotrichium niveum on in vitro true digestibility of barley

	Doses(mg L^{-1})				SEM	P
	0	**50**	**100**	**150**		
DMD (%)	70.704[a]	62.998[b]	59.138[b]	58.390[b]	4.4132	**
OMD (%)	57.708[a]	47.894[b]	52.434[ab]	54.616[ab]	5.1153	*
NDFD (%)	30.108[a]	18.378[c]	24.838[b]	25.252[b]	3.0393	***

Each letter (a and b) shows that the doses differ from each other at the level of p<0.001 (***), p <0.01(**) and p<0.05 (*).DMD: dry matter digestibility OMD: organic matter digestibility NDFD: NDF digestibility

Volalite fatty acids (VFAs) produced in the rumen form the major source of energy to the ruminant. Therefore, the optimum VFA ratios are very important for the animal performance. There are many studies investigating the effect of EOs on VFA. Some of them have observed that EOs increase VFAs rates (Benchaar et al., 2008) or not effected (Kim et al., 2019; Matloup et al., 2017; Kamalak et al., 2011; Tager and Krause 2011). Similarly with the results of our study, there are studies that significantly decrease VFA rates (Castillejos et al., 2005; Busquet et al., 2006); *Cyclotrichium niveum* oil supplementation significantly decreased total VFA production, propionic acid, butyric acid, acetic acid concentration with increasing level of the *Cyclotrichium niveum* (P<0.001, P<0.05) (Table 3). These results be concluded that *Cyclotrichium niveum* EO supplementation significantly inhibited microbial activity and overall fermentation process.

Table 3. Effect of Cyclotrichium niveum on in vitro ruminal fermentation of barley

	Doses(mg L^{-1})				SEM	P
	0	**50**	**100**	**150**		
Volatile fatty acids						
TVFA	68.30[a]	63.01[b]	55.58[c]	52.55[d]	1.0444	***
Acetate	35.83[a]	34.47[a]	30.25[b]	26.51[c]	0.9052	***
Propionate	17.25[a]	15.85[b]	13.88[c]	15.87[b]	0.5214	***
Butyrate	11.01[a]	9.15[b]	8.03[c]	6.88[d]	0.3480	***
Isobutyric acid	1.22[a]	1.18[ab]	1.15[b]	1.11[c]	0.0156	*
Valerate	1.54[a]	1.50a[b]	1.48[bc]	1.44[c]	0.0229	*
Isovalerate	1.46[a]	0.85[b]	0.80[bc]	0.75[c]	0.0252	**
A:P	2.08[a]	2.18[a]	2.18[a]	1.68[b]	0.0924	***
pH	6.1[b]	6.2 [ab]	6.2 [ab]	6.4 [a]	0.06	*
Gases						
CO_2	38.75[a]	34.93[b]	30.33[c]	27.55[d]	0.7857	***
CH_4	19.11[a]	17.8533[b]	15.67[c]	12.73[d]	0.6232	***

Each letter (a,b,c) shows that the doses differ from each other at the level of p <0.01(**) and p<0.05(*).p<0.001 (***) A:P: acetate: propionate ratio, TVFA: Total volatile fatty acids

The amount of gases such as CO_2 and CH_4 formed as a result of digestion in the rumen is usually related with an increase in digestion and given information for microorganism activity in the rumen (Hungate et al., 1954). Nasab et al. (2018) observed that the plant with main component pulegone (38.83%), significantly reduced gas production in their study. This result is also compatible with our results. In our study *Cyclotrichium niveum* essential oil significantly affected CH_4 production (P<0.001). These results consistent with decrease of acetate which is methane production precursors. Because of their antimicrobial properties EOs could be used selectively restrain rumen methanogenesis. Methane has global warming potential 21 times compared to CO_2 (Bodas et al., 2012) depending on diet composition and feed intake, enteric methane losses be regarded as 2–12% of gross energy intake in ruminants (Boadi et al, 2004). For these reasons' reduction of CH_4 emissions via EOs application

could be beneficial both for the animals (improved feed efficiency and productivity) and for the environment (mitigation of greenhouse effects) (Benchaar and Greathead 2011).

Many studies recorded the reduction in CH_4 production by EO (Nasab et al., 2018; Wang et al., 2009). The decrease in the amount of gases observed in this study may indicate that microorganisms in the incubation environment and therefore fermentation parameters are negatively affected by essential oil.

The rumen pH is very important balance factor for sustain the rumen optimum fermentation. Changes in pH may more or less affect the types and activities of the microorganism community in the rumen, the formation and absorption of protein and fatty acids, especially VFAs. *Cyclotrichium niveum* EO increased ruminal pH at all included doses ($p<0.05$), this result made think that *Cyclotrichium niveum* EO might be protecting effect on rumen acidosis.

4. Conclusion

The effects of *Cyclotrichium niveum* oil can be attributed to many reasons, these are; restriction of some protozoal populations; restriction of the bacteria producing the methane production precursors, such as acetate producing bacteria and redirecting rumen fermentation towards the pathways producing more propionate as a H_2 sink.

From the study results, it may be concluded that *Cyclotrichium niveum* EO supplementation significantly inhibited rumen microbial activity and overall fermentation process of rumen. *Cyclotrichium niveum* EO inclusion had significant negative effects on digestibility parameters, VFAs and gases. Therefore, before use for livestock industry, more investigations are required to determine the effect of *Cyclotrichium niveum* EO on animal performance and economics aspect of supplementation.

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