VETERINARSKI PREGLED / VETERINARY REVIEW, Vol 2, No1, 2021
ISSN: 2683-5932; ISSN(Online): 2683-5924; UDK 636.09
Štrbac et al., pp 49-59

IN VITRO OVICIDAL ACTIVITY OF MIXTURE OF LINALOOL AND ESTRAGOLE AGAINST GASTROINTESTINAL NEMATODES OF SHEEP

IN VITRO OVICIDNA AKTIVNOST SMEŠE LINALOOLA I ESTRAGOLA PROTIV GASTROINTESTINALNIH NEMATODA OVACA

Filip Štrbac[1], Antonio Bosco[2], Alessandra Amadesi[2], Laura Rinaldi[2], Giuseppe Mangieri, Dragica Stojanović[1], Nataša Simin[3], Dejan Orčić[3], Ivan Pušić[4], Slobodan Krnjajić[5], Radomir Ratajac[4]

[1]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Serbia
[2]Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Naples, Italy
[3]Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Serbia
[4]Scientific Veterinary Institute Novi Sad, Novi Sad, Serbia
[5]Institute for Multidisciplinary Research, University of Belgrade, Serbia
**Corresponding author:* strbac.filip@gmail.com

ABSTRACT

Based on the results in different studies conducted so far, plant essential oils (EOs) are recently been marked as a possible solution for the problem of anthelmintic resistance (AR). However, their efficacy against a wide range of livestock parasites is due to active ingredients. In that context, the aim of this study was to evaluate the *in vitro* ovicidal effect of a mixture of linalool and estragole (14% : 86%, respectively) against sheep gastrointestinal nematodes (GINs) using the egg hatch test (EHT). The study was conducted using faecal samples of naturally infected sheep by GINs from two different farms located in Southern Italy. Coproculture of tested faecal samples was performed, as well as chemical analyses of the tested mixture. The egg hatch test (EHT) was performed at six different concentrations (50, 12.5, 3.125, 0.781, 0.195 and 0.049 mg/mL) and the obtained values were compared to the positive control (thiabendazole, 0.025 mg/mL) and the negative control (3% Tween 80, v/v). The tested binary combination showed high and dose-dependent ovicidal activity varied from 29.5% to 100%. The inhibitory effect on egg hatchability was similar ($p>0.05$) to the positive control (98.0%) at concentrations of 50 mg/mL (100%), 12.5 mg/mL (100%) and 3.125 (99.8%). Moreover, all tested concentrations showed significantly higher ($p<0.001$) activity compared to the negative control. The obtained results suggest that the combination of linalool:estragole has the potential to be used against sheep GINs. These also highlight the possible role of different combinations of certain constituents of essential oils in combating AR. However, these findings need confirmation in further *in vivo* studies.

Key words: *in vitro*, plant essential oils, linalool, estragole, gastrointestinal nematodes,

anthelmitical resistance

SAŽETAK

Na osnovu rezultata do sada sprovedenih istraživanja, etarska ulja biljaka su *označena kao jedna od mogućih opcija za rešenje problemarezistencije na antihelmintike. Pri tome, za njihovu efikasnost protiv različitih parazita domaćih životinja odgovorni su njihovi aktivni sastojci. U tom kontekstu, cilj ovog istraživanja je bio ispitati ovicidnu efikasnost smeše* linaloola i estragola (14% i 86%, tim redom) protiv gastrointestinalnih nematoda (GIN) ovaca *koristeći test izleganja jaja (egg hatch test (EHT)). U studiji su korišćeni uzorci fecesa ovaca prirodno inficiranih GIN sa dve različite farme u južnoj Italiji. Koprokultura testiranih uzoraka je sprovedena, kao i hemijske analize testirane smeše. EHT je sproveden za šest različitih koncentracija (50; 12,5; 3,125; 0,781; 0,195 i 0,049 mg/mL), pri čemu su dobijeni rezultati upoređeni sa pozitivnom (tiabendazol, 0.025 mg/mL) i negativnom (3% Tween 80) kontrolom. Testirana binarna kombinacija je pokazala snažnu, dozno*-zavisnu ovicidnu aktivnost koja je varirala *između 29,5% i 100%. Inhibitorni efekat na izleganje larvi je bio sličan (p<0,05) pozitivnoj kontroli (98,0%) pri koncentracijama od 50 mg/mL (100%), 12,5* mg/mL (100%) i 3,125 mg/mL (99,8%). Pri tome, kod svih testiranih koncentracija efekat je *bio značajno veći (p<0,001) u poređenju sa negativnom kontrolom. Dobijeni rezultati ukazuju na to da ispitana smeša linaloola i estragola ima potencijal da se koristi protiv GIN ovaca. Takođe, ova studija ukazuje na moguću ulogu različitih sastojaka biljnih etarskih ulja* u borbi protivrezistencije na antihelmintike. *Međutim, ovi nalazi zahtevaju dodatnu potvrdu u budućim* in vivo ispitivanjima.

Ključne reči: in vitro, etarska ulja biljaka, linalool,estragol, gastrointestinalne nematode, *rezistencija na antihelmintike*

INTRODUCTION

The intensive use of anthelmintics in the past few decades, for the control of various nematode infections in livestock, has resulted in the development of anthelmintic resistance (AR) (1,2). This is especially pronounced in sheep gastrointestinal nematodes (GINs), which are associated with low body development, decrease in production and reproductive indexes and increase in mortality in the sheep flocks (3). Unfortunately, various single- and multiple-drug resistant species of these parasites are already common throughout the world (1). The increased resistance led to reduced efficacy of most commonly used anthelmintics, such as benzimidazoles and macrocyclic lactones (3), consequently causing major economic losses (4). For these reasons, infections caused by GINs became nowadays a major concern and obstacle facing sheep farmers around the world (3,5).

The worldwide increased difficulty to control GIN infection in sheep, as well other nematode infections in livestock, requires devising new strategies and alternative approaches. These include the use of medicinal plants and their products known as plant secondary metabolites (PSM) with anthelmintic activity (5). Among them are essential oils (EOs) - aromatic, concentrated and complex mixtures of volatile nonpolar compounds extracted from plant material (6). These plant products were already shown a wide range of medicinal properties such as antibacterial, antifungal, antiparasitic, anti-inflammatory and antiseptic effect, therefore increasing interest in their use in veterinary medicine (7,8). Furthermore, some EOs have already shown anthelmintic potential against GINs of sheep, which include EOs of *Mentha piperita* (9), *Thymus vulgaris* (10), *Lavandula officinalis* (4), *Rosmarinus officinalis* (11), *Eucalyptus citriodora* (12), *Juniperus communis* (13), *Achillea millefolium* (14) and some others (15).

The anthelmintic effect of EOs is related to their chemical composition, i.e. presence of many bioactive compounds (16). Therefore, some studies examined and demonstrated the anthelmintic potential of isolated compounds from essential oils. That includes cinnamaldehyde, anethole, carvone, carvacrol, thymol, eugenol, vanillin and linalool and others (17, 18, 19, 20). In some cases, the anthelmintic activity of their binary, ternary and quaternary combinations against sheep GINs was also evaluated, whereby it was demonstrated that some of these components are showing synergic properties and higher activity when formulated in combination (17). For some of these combinations, it was concluded that they represent promising formulations for sustainable parasite control based on the activity they showed. For example, cinnamaldehyde:carvone was tested *in vitro* (17) and anethole:carvone was tested both *in vitro* and *in vivo* (17,21), whereby their high anthelmintic potential was demonstrated.

Linalool ($C_{10}H_{18}O$) is a monoterpene alcohol widely present as a major constituent of plant EOs with extensive use. Linalool *per se* is considered non-toxic for mammals, and its comprehensive range of bioactive properties was demonstrated in many studies so far. That refers to its anti-inflammatory, anticancer, anti-hyperlipidemic, antimicrobial, antiparasitic, analgesic and neuroprotective effects besides all (22). On the other hand, estragole (methyl chavicol, $C_{10}H_{12}O$) is a relatively non-toxic volatile ether which is an isomer of anethole. Estragole is also an important constituent of many EOs with widespread application in folk medicine and aromatherapy mostly for antimicrobial, antiparasitic, antispasmodic, immunostimulant and local anesthetic properties (23,24). Since both compounds expressed antiparasitic effects individually, it was assumed that the mixture of these compounds could have even stronger anthelmintic properties, which can be likely attributed to their synergism.

A combination of linalool and estragole in a similar ratio used in the present study was found in some chemotypes of basil EO (*Ocimum basilicum* L.), mostly with other compounds present in smaller percentages (25,26,27). However, it is important to note that the chemical composition of basil EO may vary depending on various endogenous and exogenous factors. It may contain monoterpenes derivates (linalool, camphor, limonene, 1,8-cineole, geraniol) and phenylpropanoid derivates (chavicol, methyl chavicol - estragole, eugenol, methyl eugenol, methyl-cinnamate). Nevertheless, basil is a plant very famous for its many therapeutic properties and it has long been used for curing a lot of diseases and conditions such as strong aching, pyrexia, headaches, coughs, acne, diarrhea, constipation, kidney malfunction, infective diseases and worm diseases as well (25). In addition, antimicrobial activities against *E. coli* and *S. aureus*, as well antiparasitic against L_3 of *Aedes aegypti* for mentioned chemotypes that contain a similar ratio of linalool and estragole were scientifically validated (26,27).

The aim of this study was to evaluate the anthelmintic potential of binary combination linalool:estragole, against GINs of sheep.

MATERIAL AND METHODS

Sample – the binary mixture

A mixture of linalool:estragole was obtained from the producer “Alekpharm”, Belgrade, Serbia. Confirmation of the presence of these compounds and determination of their ratio in the mixture was done by gas chromatography-mass spectrometry (GC-MS) analyses (13,14), which is widely used in plant essential oil compounds identification. The chemical analyses were performed at the Faculty of Sciences, University of Novi Sad, Serbia.

Obtaining eggs and the egg hatch test

The whole *in vitro* procedure was performed at the Regional Center for Parasitic Diseases (CREMOPAR), located in Eboli (Salerno Province, Campania Region), Italy. *In vitro* ovicidal potential of the tested synergistic combination was evaluated by using the egg hatch test (EHT), which is commonly used to test the efficacy of antiparasitic drugs and the detection of parasitic resistance. Faecal samples of sheep with natural mixed infection from two different farms in southern Italy were collected directly from the rectal ampulla, whereby samples were processed within 2h of collection using the recovery technique with some modification (30). Firstly, faecal samples were homogenized and filtered under running water through four sieves of different mesh sizes (1 mm, 250 μm, 212 μm and 38 μm) to separate the eggs from the faeces. Next, the GIN eggs retained on the last sieve were washed and centrifuged for 3 min at 1500 relative centrifugal force with distilled water, after which the supernatant was discarded. In the end, centrifugation was performed using a 40% sugar solution to float the eggs which are then isolated in new tubes, mixed with distilled water and then centrifuged two more times to remove pellets. In this way, an aqueous solution with eggs necessary for the egg hatch test was obtained.

The EHT was performed with different concentrations used as proposed by Ferreira et al. (2018) with some modification (4). 24-well plates, containing aqueous solutions of approximately 150 eggs/well, were used for this experiment. Six different concentrations of binary combination of linalool:estragole (50, 12.5, 3.125, 0.781, 0,195 and 0.049 mg/mL) were emulsified in Tween 80 (3%, v/v) and completed with distilled water in a final volume of 0.5 mL/well. After an incubation of 48h at 27°C, the number of eggs and first-stage (L_1) larvae were counted under an inverted microscope and the results were expressed as the mean percentage of egg hatching for each concentration. The obtained values were compared to the positive control (thiabendazole, 0.025 mg/mL), and the negative control (3% Tween 80, v/v).

Coproculture

To determine the genera of GINs *in vitro* tested on the efficacy of linalool:estragole binary combination, a pooled faecal culture for each farm was performed at CREMOPAR center following the protocol described by the Ministry of Agriculture, Fisheries and Food (28). Developed third-stage larvae (L_3) were identified using the morphological keys proposed by the used literature (29). Identification and percentages of each nematode genera were conducted on 100 L_3.

Statistical analysis

The mean percentage of egg hatching was calculated using the following formula (31):

IH (%) = Number of eggs / (number of eggs + number of larvae (L_1)) x 100

Data on the inhibition of hatchability (IH) was analyzed by one-way ANOVA followed by Tukey's test ($p<0.05$) to compare values obtained for different concentrations with each other and with controls (+ and -) (31). Half maximal inhibitory concentration (IC_{50}) was determined by nonlinear regression (4). All statistical procedures were performed by using the program GraphPad Prism 8.3.2.

RESULTS

Chemical analyses

The results of GC-MS analyses confirmed the presence of linalool and estragole (methyl chavicol) in the tested sample and their ratio was 14:86, respectively.

Egg hatch test

The results of the conducted EHT showed high activity of linalool:estragole combination against GIN eggs. The inhibitory effect on egg hatchability varied from 29.5% to 100% (table 1) and the effect was dose-dependent (R^2= 0,9528). All tested concentrations showed significantly higher activity ($p<0.001$) compared to the negative control and, for the three highest concentrations, the effect was high and similar to that of thiabendazole ($p>0.05$). Calculated half-maximal inhibitory concentration (IC_{50}) was 0.9804 mg/mL.

Table 1. Efficacy percentage (mean ± standard deviation) of linalool:estragole binary combination on egg hatching of sheep gastrointestinal nematodes

Concentration (mg/mL)	**Inhibition of hatchability (%)**
50	100 ± 0^a
12.5	100 ± 0^a
3.125	99.8 ± 0.5^a
0.781	47.0 ± 20.45^b
0.195	29.5 ± 1.29^b
0.049	29.5 ± 2.65^b
Thiabendazole, 0.025 mg/mL	98.0 ± 0.82^a
3% Tween 80 (v/v)	16.8 ± 5.56^c

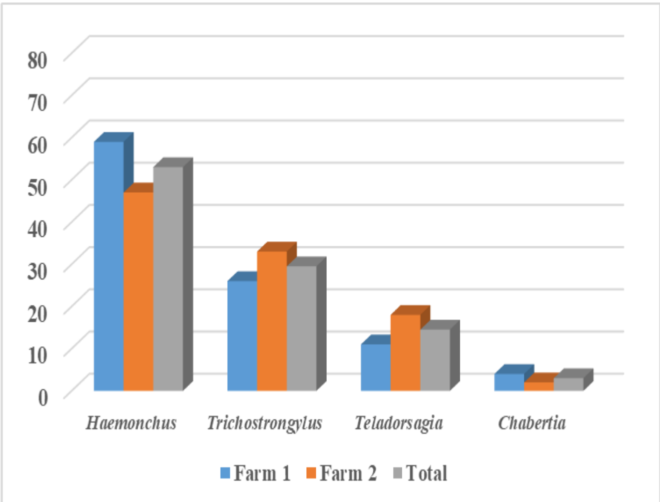
**Values with different lowercase letters indicate significant differences ($p<0.05$)*

Coproculture

In both examined farms, the coproculture identified the presence of four GIN genera: *Haemonchus* (53%), *Trichostrongylus* (29.5%), *Teladorsagia* (14.5%) and *Chabertia* (3%).

Results of the coproculture on each farm are shown in graph 1.

Graph 1. Percentage (%) of presented sheep genera on each tested farm



DISCUSSION

Recently, it was estimated that infections caused by GINs nowadays cost the European sheep industry hundreds of million € (32). However, it is difficult to assess the exact losses because these parasites can cause them in different ways. Therefore, due to the costs of anthelmintic-resistant nematode infections, there is a wide consensus on the need to enhance and implement early detection of AR, which includes the use of appropriate *in vitro* and *in vivo* methods (33). These methods are also widely used for testing the efficacy of different anthelmintic agents. In that context, *in vitro* tests are suitable for an initial evaluation and for the selection of compounds that have anthelmintic activity (19,34). Moreover, their advantages such as ease of application, low cost, speed, high reproducibility and lack of experimental animals make these tests widely used in the screening of medicinal plants (10). Among them, one of the most commonly used tests for evaluating the anthelmintic potential of EOs and their compounds is the EHT (4,9,10,11,12,13,14,17), whereby this test is considered accurate and reliable for this purpose. Therefore, EHT is important in the first step of the development of a new anthelmintic agent, after which different *in vivo* and toxicity tests may be conducted.

As a result of AR, as well as the public health concern due to the use of chemical therapeutics in livestock, there is a growing interest in alternative strategies for treating GINs. Within that context, many researchers agree that bioactive constituents produced by medicinal herbs to control GINs are a promising alternative to conventional anthelmintic drugs (35). In the present study, a binary combination of linalool:estragole showed high, dose-dependent ovicidal potential against ovine gastrointestinal nematode eggs. Namely, for the three highest concentrations (3.125-50 mg/mL) of the tested mixture, inhibition of egg hatchability was in the range of 99.75-100%, i. e. expressed the same activity as an effective anthelmintic. According to the criteria set up by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.), developed for the evaluation of different anthelmintic agents tested *in vitro*, these activities are considered highly effective (10,34) Although the rest three concentrations showed lower activity, the effect was still significantly higher than negative control suggesting that the mixture of linalool and estragole exhibit the anthelmintic potential even at very small doses. Furthermore, the results of this study are also important because anthelmintic activity was shown against important nematode genera such as *Haemonchus*, *Trichostrongylus*, *Teladorsagia* and.

According to the knowledge of the authors of the present study, there are no literature data about examining the anthelmintic potential of linalool:estragole binary combination and this is the first evaluation. Katiki et al. (2017) evaluated the ovicidal activity of isolated linalool against *Haemonchus contortus* eggs, where it showed a high inhibition of hatchability with IC_{50} of 0.29 mg/mL (17). In the study of Helal et al. (2020), the tested nematode species were similar as in the present study and pure linalool expressed a significant inhibitory activity against the motility of L_3 of sheep GINs at the highest concentrations tested for all examined species (*H. contortus*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* and *Trichostrongylus vitrinus*) except *Cooperia oncophora*, and also induced considerable structural damage to larvas (36). For isolated estragole, there are no data about the anthelmintic activity. However, linalool and estragole are common ingredients of *Ocimum basilicum* EO and often the two most represented compounds of this EO. The recent research showed that EOs from different *Ocimum basilicum* cultivars exhibited a significant effect on egg-hatch inhibition of *H. contortus* with a mean IC_{50} varying from 0.56-2.22 mg/mL. Interestingly, the cultivar constituted mainly of linalool and methyl chavicol (estragole) was the cultivar with the highest egg-hatching inhibition, thus showing its high anthelmintic potential (37). Based on this and other results, linalool:estragole combination was chosen for the evaluation of its ovicidal effects against sheep GINs in the present study.

Furthermore, in another study, our research team also evaluated the *in vivo* activity of this combination (same ratio of compounds) using the faecal egg count reduction test (FECRT) (38). The combination was perorally administrated to the sheep at the single dose of 100 mg/kg, whereby it reduced the number of counted nematode eggs by 24.91% and 25.90% at flock level on Days 7 and 14 after treatment, respectively. Individually observed, linalool:estragole combination average reduced FEC for 51.88% (Day 7 after treatment). Moreover, no side and toxic effects were observed on the animals after the administration of the tested combination. The results of this and the present study suggest the potential use of linalool:estragole combination in the future management of GINs infections as a valuable additional mean, in order to reduce the use of commercial anthelmintic and slowing development of anthelmintic resistance.

Linalool and estragole and their derivates were also important compounds in other EOs with proven anthelmintic activity against sheep GINs. Thus, linalool-acetate, an acetate ester of linalool, was a major compound of *Lavandula officinalis* EO (35.97%), which showed high ovicidal (IC_{50} = 0.316 mg/mL) and larvicidal (IC_{50} = 0.280 mg/mL) activity against *H. contortus* (4). Linalool *per se* was a major component of *Arisaema franchetianum* EO that was also exhibited ovicidal and larvicidal activity also against *H. contortus* with IC_{50} values of 1.63 mg/mL and 1.10 mg/mL, respectively (39). However, in that study isolated linalool did not show high activity in every assay and this result suggests that linalool is probably more effective in the presence of other bioactive ingredients due to synergism. On the other hand, estragole was one of the major components in both tested samples of *Croton zehntneri* EO (14.95% and 21.84%) which showed high anthelmintic potential with IC_{50} values in the EHT of 0.55 and 0.74 mg/mL, respectively (20).

The exact mode of action and high anthelmintic efficacy of different EOs is still not fully understood. It was suggested that bioactive ingredients of the EOs penetrate the cuticle of the nematodes by transcuticular diffusion, altering the mechanisms of locomotion and causing cuticular lesions (15). Other reports suggested the involvement of EOs components in interrupting the nematode nervous system (40) and/or in inhibition of AChE activity (41,42). Furthermore, bioactive compounds may disrupt the cell membrane of the nematode and change its permeability (40). On the other hand, high ovicidal activities shown in the EHT suggested abilities of EO compounds to penetrate the gelatinous matrix and act on nematode eggs. It is important to note that knowing and understanding the mode of action of the EOs and their bioactive ingredients are of practical importance for nematode control because it may give useful information on the most appropriate formulation and delivery means (43). For comparison, benzimidazoles selectively bind with high affinity to parasite β-tubulin and inhibit microtubule polymerization. This lead to the destruction of cell structure and consequent death of the parasite (44)

Although all compounds may be important for the biological properties of EOs, their efficacy mostly comes from their main ingredient or a few of them and their synergism (15,16). That fact offers a possibility for a use of binary, ternary and quaternary combinations of main components of the same or different EOs in an attempt to find the most active combination for control of nematodes (17). There is a large number of EOs bioactive constituents and a practically unlimited number of their combinations, so it is important to find the most suitable ones for the development of formulations. Besides high efficacy against parasites, those combinations should have low toxicity for hosts and small residues in meat and milk. Moreover, it is considered that in comparison to commercial drugs, these natural formulations would have higher environmental acceptability and lower susceptibility to anthelmintic resistance (4,45). Therefore, the use of plant-based formulations as medicine offers an attractive alternative for controlling livestock parasites and combating anthelmintic resistance.

CONCLUSION

Plant EOs are valuable natural sources that can play a significant role in combating AR, which is especially important in the case of sheep GINs. The results of the present study showed that a mixture of two constituents of EOs – estragole and linalool - may also greatly affect these parasites, thus it represents a good basis for the development of new formulations for the prevention and treatment of infections caused by nematodes. That may highly increase the possibility to find valuable means for controlling livestock parasites.

ACKNOWLEDGEMENTS

This work was part of the STSM (Short Term Scientific Mission) titled “The methodology of the diagnostics of parasitic infections and methods for evaluating the efficacy of antiparasitic drugs” of COST Action COMBAR (Combatting Anthelmintic Resistance in Ruminants), number CA16230. Special thanks to the STSM Coordinator Maria Martinez-Valladares, who approved this STSM; to the Grant Holder Smaragda Sotiraki, who approved the financial support from the COST network for this STSM; and to Johannes Charlier, MC Chair. We also deeply thank Mario Parrilla for the tehnical support in the laboratory.

LITERATURE

1. *Dolinská M., Ivanišinová O., Königová A., Várady M. Anthelmintic resistance in sheep gastrointestinal nematodes in Slovakia detected by in-vitro methods. BMC Veterinary Research 2014, 10:233.*

2. *Kaplan R.M. Biology, epidemiology, diagnosis and management of anthelmintic resistance in gastrointestinal nematodes of livestock. Veterinary Clinics of North America Food Animal Practice 2020, 36(1):17-30.*

3. *Ramos F., Portella L.P.,Rodrigues F.D.S., Reginato C.Z., Cezar A.S., Sangioni L.A., Vogel F.S.F. Anthelminthic resistance of gastrointestinal nematodes in sheep to monepantel treatment in central region of Rio Grande do Sul, Brazil. Pesquisa Veterinária Brasileira 2018, 38(1):48-52.*

4. *Ferreira L.E., Benincasa B.I., Fachin A.L., Contini S.H.T., França S.C, Chagas A.C.S., Beleboni R.O. Essential oils of Citrus aurantifola, Anthemis nobile and Lavandula officinalis: in vitro anthelmintic activities against Haemonchus contortus. Parasites & Vectors 2018, 11:269.*

5. *Chagas A.C.S., Figuerido A.., Politi F.A.S., Moro I.J., Esteves S.N., Bizzo H.R., Gama P.E., Chaves F.C.M. Efficacy of essential oils from plants cultivated in Amazonian Biome against gastrointestinal nematodes of sheep. Journal of Parasitic Diseases 2018, 42(3):357-364.*

6. *Fayaz M.R., Abbas Z.R., Abbas A., Khan M.K., Raza M.A., Israr M., Khan J.A., Mahmood M.S., Saleemi M.K., Rehman T., Zaman M.A., Sindhu Z.D. Potential of botanical driven essential oils against Haemonchus contortus in small ruminants. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 2019, 18(6):533-543.*

7. *Ebani V.V., Mancianti F. Use of essential oils in veterinary medicine to combat bacterial and fungal infections. Veterinary Sciences 2020, 7(4):193.*

8. *Vučinić M., Nedeljković-Trailović J., Trailović S., Ivanović S., Milovanović M., Krnjajić D. Mogućnost primene etarskih ulja u veterinarskoj medicini s posebnim osvrtom na etarsko ulje origana. Veterinarski Glasnik 2012, 66(5-6):407-416.*

9. *Katiki L.M., Chagas A.C.S., Bizzo H.R., Ferreira J.F.S., Amarante A.F.T. Anthelmintic activity of Cymbopogon martinii, Cymbopogon schoenanthus and Mentha piperita essential oils evaluated in four different in vitro tests. Veterinary Parasitology 2011, 183(1-2):103-108.*

10. *Ferreira L.E., Benincasa B.I., Fachin A.L., França S.C., Contini S.S.H.T., Chagas A.C.S., Beleboni R.O. Thymus vulgaris L. essential oil and its main component thymol: Anthelmintic effects against Haemonchus contortus from sheep. Veterinary Parasitology 2016, 228:70-76.*

11. *Pinto N.B., de Castro L.M., Azambuja R.H.M., Capella G.D.A., de Moura M.Q., Terto W.D., Freitag R.A., Jeske S.T., Villela M.M., Cleff M.B., Leite F.P.L. Ovicidal and larvicidal potential of Rosmarinus officinalis to control gastrointestinal nematodes of sheep. Brazilian Journal of Veterinary Parasitology 2019, 28(4):807-811.*

12. *Araújo-Filho J.V., Ribeiro W.L.C., André W.P.P., Cavalcante G.S., Rios T.T., Schwinden G.M., Rocha L.O.D., Macedo I.T.F., Morais S.M., Bevilaqua C.M.L., Oliveira L.M.B. Anthelmintic activity of Eucalyptus citriodora essential oil and its major component, citronellal, on sheep gastrointestinal nematodes. Brazilian Journal of Veterinary Parasitology 2019, 28(4):644-651.*

13. *Štrbac F., Bosco A., Amadesi A., Rinaldi L., Stojanović D., Simin N., Orčić D., Pušić I., Krnjajić S., Ratajac R. In vitro ovicidal effect of common juniper (Juniperus communis L.) essential oil on sheep gastrointestinal nematodes. Veterinary Review 2020, 1(1):152-159.*

14. *Štrbac F., Bosco A., Amadesi A., Rinaldi L., Stojanović D., Simin N., Orčić D., Pušić I., Krnjajić S., Ratajac R. In vitro ovicidal activity of two chemotypes of yarrow (Achillea millefolium L.) essential oil against ovine gastrointestinal nematode eggs. Archives of Veterinary Medicine 2020, 13(2):59-76.*

15. *André W.P.P., Ribeiro W.L.C., de Oliveira L.M.B., Macedo I.T.F., Rondon F.C.R., Bevilaqua C.M.L. Essential oils and their bioactive compounds in the control of gastrointestinal nematodes of small ruminants. Acta Scientiae Veterinarie 2018, 46:1522.*

16. *Dhifi W., Bellili S., Jazi S., Bahloul N., Mnif W. Essential oils` chemical characterization and*

investigation of some biological activities: a critical review. Medicines 2016, 3(4):25.
17. Katiki L.M., Barbieri A.M.E., Araujo R.C., Veríssimo C.J., Louvandini H., Ferreira J.F.S., Synergistic interaction of ten essential oils against Haemonchus contortus in vitro. Veterinary Parasitolology 2017, 243:47-51.
18. André W.P.P., Ribeiro W.L.C., Cavalcante G.S., Dos Santos J.M.L., Macedo I.T.F., de Paula H.C.B., de Freitas R.M., de Morais S.M., de Melo J.V., Bevilaqua C.M.L. Comparative efficacy and toxic effects of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes and mice. Veterinary Parasitology 2016, 218:52-58.
19. André W.P.P., Cavalcante G.S., Ribeiro W.L.C., Dos Santos J.M.L., Macedo I.T.F., de Paula H.C.B., de Morais S.M., de Melo J.V., Bevilaqua C.M.L. Anthelmintic effect of thymol and thymol acetate on sheep gastrointestinal nematodes and their toxicity in mice. Brazilian Journal of Veterinary Parasitology 2017, 26(3):323-330.
20. Camurca-Vasconcelos A.L.F., Bevilaqua C.M.L., Morais S.M., Maciel M.V., Costa C.T.C., Macedo I.T.F., Oliveira L.M.B., Braga R.R., Silva R.A., Vieira L.S. Anthelmintic activity of Croton zehntneri and Lippia sidoides essential oils. Veterinary Parasitology 2007, 148:288-294.
21. Katiki L.M., Araujo R.C., Ziegelmeyera L., Gomesa A.C.P., Gutmanisa G., Rodriguesa L., Buenoa M.S., Veríssimoa C.J., Louvandinic H.,Ferreirad J.F.S.,Amarantee A.F.T. Evaluation of encapsulated anethole and carvone in lambs artificially- and naturally-infected with Haemonchus contortus. Experimental Parasitology 2019, 197:36-42.
22. Pereira I., Severino P., Santos A.C., Silva A.M., Souto E.B. Linalool bioactive properties and potential applicability in drug delivery systems. Colloids and Surfaces B: Biointerfaces 2018, 171:566-578.
23. Leal-Cardoso J.H., Matos-Brito B.G., Lopes-Junior J.E.G., Viana-Cardoso K.V., Sampaio-Freitas A.B., Brasil R.O., Coelho-de-Souzaand A.N., Albuquerqu A.A.C. Effects of estragole on the compoundaction potential of the rat sciatic nerve. Brazilian Journal of Medical and Biological Research 2004, 37(8):1193-1198
24. Oliveira C.B.S., Meurer Y.S.R., Medeiros T.L., Pohlit A.M., Silva M.V., Mineo T.W.P., Andrade-Neto V.F. Anti-toxoplasma activity of estragole and thymol in murine models of congenital and noncongenital toxoplasmosis. Journal of Parasitology 2016, 102(3):369-376.
25. Poonkodi K. Chemical composition of essential oil of Ocimum basilicum L. (basil) and its biological activities - an overview. Journal of Critical Reviews 2016, 3(3):56-62.
26. Sastry K.P., Kumar R.R., Kumar A.N., Sneha G., Elizabeth M. Morpho-chemical description and antimicrobial activity of different ocimum species. Journal of Plant Development 2012, 19:53-64
27. Nour AH, Nour AH, Yusoff MM, Jessinta S. Bioactive compounds from basil (Ocimum basilicum) essential oils with larvicidal activity against aedes aegypti larvae. 3rd International Conference on Biology, Environment and Chemistry IPCBEE, Bangkok, Thailand, 2012.
28. Ministry of Agriculture, Fisheries and Food (MAFF), Grande-Bretagne. Manual of veterinary parasitological laboratory techniques. H.M. Stationery Off, London, UK, 1986.
29. van Wyk J.A., Mayhew E. 2013. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: a practical lab guide. The Onderstepoort Journal of Veterinary Research 2013, 80(1):E1-E14.
30. Bosco A., Maurelli M.P., Ianniello D., Morgoglione M.E., Amadesi A., Coles G.C., Cringoli G., Rinaldi L. The recovery of added nematode eggs from horse and sheep faeces by three methods. BMC Veterinary Research 2018, 14(1):7.
31. Macedo I.T.F, de Oliveira L.M.B., Andre W.B.P., Filho J.V.A., dos Santos J.M.L., Rondon F.C.M., Ribeiro W.L.C., Camurça-Vasconcelos A.L.F., de Oliveira E.F., de Paula H.C.B., Bevilaqua C.M.L. Anthelmintic effect of Cymbopogon citratus essential oil and its nanoemulsion on sheep gastrointestinal nematodes. Brazilian Journal of Veterinary Parasitology 2019, 28(3):522-527.
32. Mavrot F., Hertzberg H., Torgerson P. 2016. First assessment of production losses due to nematode infection in European dairy cattle and meat sheep. In: Mavrot F. Livestock nematode infection in a changing world: investigating the European situation. University of Zurich, Vetsuisse

Faculty, p. 70-130.
33. Bosco A., Kießler J., Amadesi A., Varady M., Hinney B., Iannielo D., Maurelli M.P., Cringoli G., Rinaldi L. The threat of reduced efficacy of anthelmintics against gastrointestinal nematodes in sheep from an area considered anthelmintic resistance-free. Parasites & Vectors 2020, 13(1):457.
34. Fonseca Z.A.A.S., Coelho W.A.C., Andre W.P.P., Bessa E.N., Ribeiro W.L.C., Perreira J.S., Ahid S.M.M. Use of herbal medicines in control of gastrointestinal nematodes of small ruminants: efficacies and prospects. Revista Brasileira de Higiene e Sanidade Animal 7(2):233-249.
35. Zeineldin M., Abdelmegeid M., Barakat R., Ghanem M. A Review: Herbal Medicine as an Effective Therapeutic Approach for Treating Digestive Disorders in Small Ruminants. Alexandria Journal of Veterinary Sciences 2018, 56(1):33-44.
36. Helal M.A., Abdel-Gawad A.M., Kandil O.M., Khalifa M.M.E., Cave G.W.V., Morisson A.A., Bartley D.J., Elsheikha H.M. Nematocidal Effects of a coriander essential oil and five pure principles on the infective larvae of major ovine gastrointestinal nematodes in vitro. Pathogens 2020, 9:740.
37. Sousa A.I.P., Silva C.R., Costa-Júnior H.N., Silva N.C.S., Pinto J.A.O., Blank A.F., Soares A.M.S., Costa-Júnior L.M. Essential oils from Ocimum basilicum cultivars: analysis of their composition and determination of the effect of the major compounds on Haemonchus contortus eggs. Journal of Helminthology 2021, 95:e17.
38. Štrbac F., Bosco A., Rinaldi L., Amadesi A., Stojanović D., Simin N., Orčić D., Pušić I., Krnjajić S., Ratajac R. 2021. In vivo potential of thyme (Thymus vulgaris L.) essential oil and synergistic combination of linalool:estragole to control sheep gastrointestinal nematodes. Proceedings of 28th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Dublin, Republic of Ireland, 19-22 July, 2021, pp 517.
39. Zhu L., Dai J., Yang L., Qui J. Anthelmintic activity of Arisaema franchetianum and Arisaema lobatum essential oils against Haemonchus contortus. Journal of Ethnopharmacology 2013, 148(1):311-316.
40. Oka Y., Nacar S., Putievsky E., Ravid U., Yaniv Z., Spiegel Y. Nematicidal activity of EOs andtheir components against the root-knot nematode. Phytopathology 2000, 90:710–715.
41. Lee S.E., Lee B.H., Choi W.S. Fumigant toxicity of volatile natural products from Korean spices and medicinal plants towards the rice weevil. Pest Management Science 2001, 57:548–553.
42. Priestley C.M., Williamson E.M., Wafford K.A., Sattelle D.B. Thymol, a constituent of thyme essential oil, is a positiveallosteric modulator of human $GABA_A$ receptors and ahomooligomeric GABA receptor from Drosophila melanogaster. British Journal of Pharmacology 2003, 140:1363–1372.
43. Andres M.F., González-Coloma A., Sanz J., Burillo J., Sotomayor P.S. Nematicidal activity of essential oils: a review. Phytochemistry Reviews 2012, 11(4):371-390.
44. Abongwa M., Martin R.J., Robertson A.P. A brief review on the mode of action of antinematodal drugs. Acta Veterinaria (Beograd) 2017, 67(2):137-152.
45. Demeler J., Gill J.H., Himmelstjerna G.V.S., Sangster N.C. The in vitro assay profile of macrocyclic lactone resistance in three species of sheep trichostrongyloids. International Journal for Parasitology: Drugs and Drug Resistance 2013, 3:109–118.