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RESEARCH ARTICLE

Ovicidal Potential of Five Different Essential Oils to Control Gastrointestinal Nematodes of Sheep

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A B S T R A C T

The development of resistance to commercial anthelmintics, particularly in different species of gastrointestinal nematodes (GINs), requires the search for alternatives. Within that context, the aim of this study was to evaluate the *in vitro* ovicidal activity of five different essential oils (EOs): *Origanum vulgare*, *Satureja hortensis*, *Thymus vulgaris*, *Mentha x piperita* and *Helichrysum arenarium* against sheep GINs. For this purpose, the nematode eggs were collected from naturally infected sheep by GINs in two farms located in southern Italy. 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) for each EO. Gas chromatography-mass spectrometry chemical analyses of tested EOs, as well as coproculture examination of tested faecal samples, were also conducted. The results of EHT showed the greatest ovicidal activity of *O. vulgare* EO with a maximum effect on egg hatching (100%) for all tested concentrations. A similar effect was also shown by *S. hortensis* and *T. vulgaris* EOs with an activity of 99.3-100% and 98.5-100%, respectively. *M. piperita* EO showed medium, dose-dependent ovicidal activity with an inhibitory effect of 72.5-99.8% on the egg hatchability, while the least effective was *H. arenarium* EO with an activity of 59.8-69.3%. For the anthelmintic activities of the tested EOs are responsible their ingredients, above all carvacrol, thymol, p-cymene and γ-terpinene. The present study demonstrated the significant anthelmintic potential of the tested EOs and emphasizes the possible importance of medicinal plant products for the control of gastrointestinal parasites in sheep.

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INTRODUCTION

Infections caused by gastrointestinal nematodes (GINs) are considered as one of the most common infections of livestock (Mavrot *et al*., 2015). In sheep, clinical signs may vary from subclinical weight loss to lethal pathologies such as anaemia, diarrhea, severe protein loss and, in some cases, death. Moreover, other negative impacts such as reduced feed intake, anorexia and weakness often lead to low production and fertility, which resulting in significant economic losses (Macedo *et al*., 2010; Mavrot *et al*., 2015; Abbas *et al*., 2020). Although it is hard to quantify, these losses in, for example, the meat sheep industry across Europe amount to hundreds of millions €, with serious indications that the total losses due to gastrointestinal parasitism could be much higher (Mavrot *et al*., 2016). For these reasons, sheep GINs nowadays present a major problem faced by sheep producers worldwide (Kaplan, 2020).

Sheep GINs are important causes of infections and diseases in also Serbia and Italy. In Vojvodina, a lowland landscape in northern Serbia, the prevalence of GIN

genera is estimated by Pavlović *et al*. (2017). Infection occurred on 81.22% of tested animals with the most prevalent genera: *Nematodirus* 71.22%, *Ostertagia* 69.22%, *Trichostrongylus* 66.55%, *Haemonchus* 64.44% and *Chabertia* 60.11%. On the other hand, in eastern Serbia, where mountain landscape is predominated, infections were found in 74.56% of sheep in a study by Kulišić *et al*. (2013). The most prevalent GIN genera were *Haemonchus* (46.91%), *Oesophagostomum* (40.73%), *Trichostrongylus* (39.85%), *Nematodirus* (35.88%) and *Chabertia* (32.79%). In southern Italy (area of the present study), in the area of Mediterranean climate conditions, identification of sheep GINs was part of the study of Bosco *et al*. (2020). On 10 different farms, the following GIN genera was presented: *Haemonchus* (21-83%), *Trichostrongylus* (2-59%), *Chabertia* (0-48%), *Teladorsagia* (0-25%) and *Cooperia* (0-5%).

Synthetic anthelmintics were used for decades against sheep gastrointestinal nematodes (GINs) to reduce their damaging effects (Kaplan, 2020). However, inappropriate usage of commercial drugs, in terms of significantly increased frequency of anthelmintic treatments and application of higher doses of commonly used drugs, caused their declined effectiveness as a consequence of developed resistance (Ferreira *et al*., 2016; Pinto *et al*., 2019). For these reasons, researchers worldwide are now focused on devising new strategies in an attempt to control sheep GINs. That including genetic resistance control, pasture management, crop-livestock integration, nutrition adjustment, biological regulation - the use of fungi and bacteria, vaccine production and many others (Pinto *et al*., 2019).

As a significant natural resource of various compounds with different potential therapeutic effects, botanical anthelmintics are also considered as a valuable option to combat anthelmintic resistance (AR). Their medicinal properties have been known since ancient times and plants have been used as an element of folk medicine in many cultures (Ferreira *et al*., 2016; Ferreira *et al*., 2018). Essential oils (EOs) are aromatic, concentrated and complex mixtures of volatile nonpolar compounds extracted from plant material (Fayaz *et al*., 2019). These natural products have a long medicinal history that comes from their rich composition with a high number of compounds with possible pharmacological interest (Ishfaq *et al*., 2018). For some of these compounds and their binary, ternary and quaternary combinations, anthelmintic potential against sheep GINs was already demonstrated (Katiki *et al*., 2017).

Although the selection of the plant species appropriate for anthelmintic examinations and other pharmacological studies is not easy, it is an initial step in the development of new anthelmintic drugs. This step should be based on solid operational strategies and relevant ethnopharmacological/chemotaxonomic data (Ferreira *et al*., 2018). Within this context, *Origanum vulgare*, *Mentha x piperita*, *Thymus vulgaris*, *Satureja hortensis* and *Helichrysum arenarium* are well-known medicinal herbs with proven medicinal properties. These include antiseptic, antimicrobial, anti-inflammatory, anticancer activities and/or, on the other hand, different gastrohepatoprotective properties (Ratajac *et al*., 2016; Fierascu *et al*., 2018; Pljevljakušić *et al*., 2018; Singh *et al*., 2018). However, it is still little known about their anthelmintic activity.

In different studies so far, the anthelmintic efficacy of various EOs against small ruminant GINs has been examined. These mostly include exotic plants such as *Lippia sidoides*, *Croton zehntneri*, *Eucalyptus staigeriana*, *Zanthoxylum simulans*, *Melaleuca alternifolia* etc (Andre *et al*., 2018). However, the number of such studies, as well the number of tested EOs and the general knowledge of the anthelmintic activity of EOs are still limited thus requiring new studies. The mode of their nematicidal action is also still unclear; there are several reports within this topic. In a study by Andres *et al*. (2012), a list of suggested and proved mechanisms of nematicidal activity of EOs was provided. Depending on the ingredients that make up EO composition, these involve: interference with the neuromodulator octopamine or GABA-gated chloride channels, inhibition of AChE activity, disruption of the nematode cell membrane and changing its permeability, interaction with SER-2 tyramine receptor and triggering a signaling cascade etc. In this way, different constituents of EOs may cause different neurological and structural changes in nematodes that leads to paralysis and death.

Considering the possible great ethnobotanical and pharmaceutical significance of *O. vulgare*, *S. hortensis*, *T. vulgaris*, *M. piperita* and *H. arenarium* EOs and, on the other hand, the severity and wideness of the problem of AR, this study aimed to evaluate the *in vitro* ovicidal activity of these EOs against sheep GINs using the egg hatch test (EHT).

MATERIALS AND METHODS

Essential oils and chemical analysis: EOs of *Mentha* × *piperita* L. and *Helichrysum arenarium* (L.) Moench were purchased from BIOSS, Serbia, EO of *Origanum vulgar*e L. from Hippocratic Essentials P.C., Neo Perivoli, Greece, EO of *Thymus vulgaris* L. from Albert Vieille Sas, Vallauris, France, *Satureja hortensis* L. from Bio Salas Farago, Orom, Serbia in 2019. Chemical composition of tested EOs was determined by gas chromatography-mass spectrometry (GC-MS), whereby the following technical conditions were used: injection volume of EO 1 μL; injector temperature 250°C; split ratio 1:10; carrier gas helium; flow rate: 1 mL/min; capillary column: HP-5 (30m×0.25mm, 0.25μm); temperature program 50-270°C; ion source temperature 230°C; electron energy 70 eV; quadrupole temperature 150°C. The compounds were identified by comparison of mass spectra with data libraries (Wiley Registry of Mass Spectral Data, 7th ed. and NIST/EPA/NIH Mass Spectral Library 05) and confirmed by comparison of linear retention indices with literature data (Adams, 2012).

Coproculture: A pooled faecal culture for each farm was performed following the protocol described by the Ministry of Agriculture, Fisheries and Food (MAFF, 1986). Developed third-stage larvae (L_3) were identified using the morphological keys proposed by van Wyk and Mayhew (2013). Identification and percentages of each nematode genera were conducted on 100 L_3.

Extraction of eggs and egg hatch test: Faecal samples were collected directly from the rectal ampulla of naturally infected sheep by GINs located in two farms in Southern Italy. These samples were processed within 2h of collection using the recovery technique with some modifications (Bosco *et al*., 2018). Firstly, faecal samples were homogenized and filtered under running water through sieves with a mesh size of 1 mm, 250 µm, 212 µm and 38µm to separate the eggs from the faeces. Next, the GIN eggs retained on the last sieve were washed and centrifuged for 3 minutes at 1500 RCF with distilled water, after which the supernatant was discarded. In the end, centrifugation was performed using 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 and to get an aqueous solution with eggs.

The EHT was performed as proposed by Ferreira *et al*. (2018) with some modifications. 24-well plates, containing aqueous solutions of approximately 150 eggs/well, were used for this examination. Six different concentrations of each EO (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 incubation for 48h at the 27°C, the number of eggs and first-stage larvae (L_1) were counted using an inverted microscope and compared to the positive control (thiabendazole, 0.025 mg/mL) and the negative control (Tween 80 3%, v/v). The experiment was performed two times with two replicates each, and the results were expressed as the mean percentage of egg hatching.

Statistical analysis: The mean percentage of egg hatching was calculated by using the following formula (Macedo *et al*., 2019):

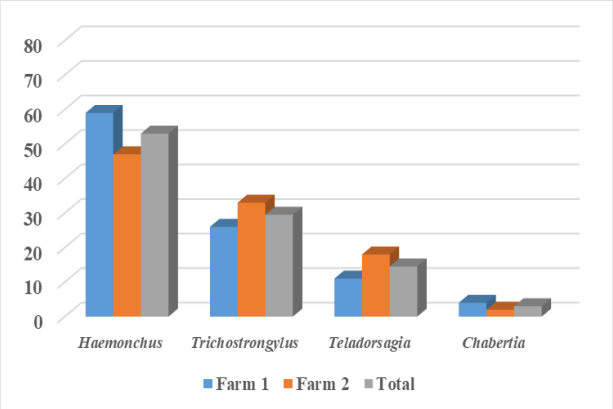
IH (%) = Number of eggs / (number of eggs + number of larvae (L_1)) x 100

Data on the inhibition of hatchability (IH) was analyzed by one-way ANOVA followed by Tukey's test ($p<0.05$) in order to compare values of different concentrations of the same EO with each other and with controls. Two-way ANOVA followed by Bonferroni's test ($p<0.05$) was used to compare the values of the same concentration of different EOs (Macedo *et al*., 2019). Nonlinear regression/logarithmic distributions were applied to calculate the half-maximal inhibitory concentration (IC_{50}) (Ferreira *et al*., 2018). All statistical procedures were performed using the software GraphPad Prism 8.3.2.

RESULTS

GC-MS analysis: The results obtained by GC-MS analysis are given in Table 1 in order of the arithmetic retention index (AI). Because of a very high number of the fully and tentatively identified compounds (51 in all the tested oils), only the compounds with abundance exceeding 1% in at least one oil are shown. The total numbers of identified compounds in *O. vulgare*, *S. hortensis*, *T. vulgaris*, *M. piperita* and *H. arenarium* were: 10, 13, 19, 21 and 30, representing 100, 100, 99.96, 96.73 and 92.13% of total identified compounds, respectively. The dominant compounds in EOs of *O. vulgare*, *S. hortensis* and *T. vulgaris* are monoterpene alcohols (carvacrol and thymol) and monoterpene hydrocarbons (p-cymene and γ-terpinene). *M. piperita* EO is rich in monoterpene ketones – piperiton and dihydrocarvone and alcohols α-terpineol and terpinen-4-ol. On the other hand, a specific characteristic of *H. arenarium* EO is a high amount of various sesquiterpens and α-pinene.

Coproculture: Coproculture examination identified the presence of four genera of sheep GINs on tested farms i.e. *Haemonchus* 53%, *Trichostrongylus* 29.5%, *Teladorsagia* 14.5% and *Chabertia* 3%. The percentage of represented GIN genera on each farm is shown in Graph 1.



Graph 1: Percentage (%) of represented sheep GIN genera on tested farms.

Egg hatch test: The EHT results showed a high ovicidal, anthelmintic potential of the tested EOs (Table 2). The greatest ovicidal potential was shown by *O. vulgare*, *S. hortensis* and *T. vulgaris* EOs with the inhibitory effect on hatchability of 100, 99.3-100 and 98.5-100%, respectively. The effects of all tested concentrations of these three EOs were similar to positive control, and there was not any significant difference between these oils ($P>0.05$). The *M. piperita* EO also showed solid ovicidal activity varying from 72.5-99.8% which was dose-dependent ($R^2 = 0.9834$). The least effective was *H. arenarium* EO (59.8-69.3%), although all obtained values significantly differ from negative control ($P<0.0001$).

DISCUSSION

As a consequence of the worldwide presence of various single and multi-drug resistant species of sheep GINs, anthelmintic resistance has recently become a global problem that seriously affects countries with small ruminant industries (Dolinská *et al*., 2014). Therefore, combating anthelmintic resistance requires reliable methods for its detection, as well as methods for testing the efficacy of anthelmintic agents. *In vitro* tests are used to select plant species, their secondary metabolites and ingredients that exhibit anthelmintic activity (Andre *et al*., 2017). These tests are the most optimal for the initial evaluation and verification of the anthelmintic potential of new vegetal compounds (Fonseca *et al*., 2013). Because of their advantages such as ease of application, low cost,

Table 1: Chemical composition of all tested essential oils determined by GC-MS analyses

AI*	Compound	% of total peak area				
		Origanum vulgare	*Satureja hortensis*	*Thymus vulgaris*	*Mentha x piperita*	*Helichrysum arenarium*
932	α-Pinene	0.20	1.22	1.76	0.20	28.04
972	Sabinene	-	-**	-	1.28	-
976	β-Pinene	0.43	-	1.55	0.12	0.21
990	β-Myrcene	0.20	1.32	1.40	0.84	-
1016	α-Terpinene	0.61	2.02	1.55	-	0.11
1023	*p*-Cymene	4.64	13.54	21.61	0.07	0.08
1028	Limonene	0.64	-	-	7.14	1.66
1030	1,8-Cineole	1.00	-	0.79	2.64	0.57
1057	γ-Terpinene	3.88	30.01	7.95	4.46	0.31
1100	Linalool	2.05	0.14	3.24	-	0.30
1152	Menthone	-	-	-	3.33	-
1163	Isomenthone	-	-	-	6.04	-
1164	Borneol	0.74	-	1.04	-	-
1174	Terpinen-4-ol	0.53	-	-	7.88	-
1186	α-Terpineol	0.60	-	-	9.77	0.33
1204	trans-Dihydrocarvone	-	-	-	14.56	-
1214	Isodihydrocarveol	-	-	-	6.25	-
1253	Piperitone	-	-	-	25.36	-
1292	Thymol	1.73	0.29	48.26	-	-
1303	Carvacrol	77.38	48.97	7.92	-	-
1364	Neryl acetate	-	-	-	-	3.05
1375	α-Copaene	-	-	-	-	3.09
1402	*iso*-Italicene	-	-	-	-	3.20
1418	trans-β-Caryophyllene	3.59	1.26	1.02	1.69	6.36
1442	sesquiterpene	-	-	-	-	2.09
1474	sesquiterpene	-	-	-	-	1.64
1479	γ-Curcumene	-	-	-	-	20.08
1482	ar-Curcumene	-	-	-	-	4.15
1485	β-Selinene	-	-	-	-	9.32
1493	α-Selinene	-	-	-	-	5.21
1499	sesquiterpene	-	-	-	-	1.33
1506	sesquiterpene	-	-	-	-	1.33
1512	β-Curcumene	-	-	-	-	1.89
1523	δ-Cadinene	-	-	-	-	1.53
1582	Caryophyllene oxide	-	-	-	2.14	-

*arithmetic retention index, *not detected.

Table 2: Efficacy (mean±standard deviation) of different essential oils tested on egg hatching of sheep gastrointestinal nematodes in total from both examined farms

Concentration (mg/mL)	Inhibition of hatchability (%)				
	Origanum vulgare	*Satureja hortensis*	*Thymus vulgaris*	*Mentha x piperita*	*Helichrysum arenarium*
50	100 ± 0^{Aa}	100 ± 0^{Aa}	100 ± 0^{Aa}	99.8 ± 0.50^{Aa}	69.3 ± 2.22^{Ab}
12.5	100 ± 0^{Aa}	100 ± 0^{Aa}	99.5 ± 0.58^{Aa}	99 ± 0.82^{Aa}	68.5 ± 2.89^{Ab}
3.125	100 ± 0^{Aa}	100 ± 0^{Aa}	100 ± 0^{Aa}	99 ± 0.82^{Aa}	68.3 ± 3.59^{Ab}
0.781	100 ± 0^{Aa}	100 ± 0^{Aa}	100 ± 0^{Aa}	94.8 ± 1.71^{Ab}	63.8 ± 1.26^{ABc}
0.195	100 ± 0^{Aa}	99.8 ± 0.50^{Aa}	100 ± 0^{Aa}	83 ± 1.63^{Bb}	59.8 ± 2.22^{Bc}
0.049	100 ± 0^{Aa}	99.3 ± 0.96^{Aa}	98.5 ± 0.58^{Aa}	72.5 ± 1.29^{Cb}	59.8 ± 0.96^{Bc}
IC_{50} (mg/mL)	very low	0.187	0.098	0.281	0.952
Thiabendazole, 0.025 mg/mL (+)	98 ± 0.82^{A}	98 ± 0.82^{A}	98 ± 0.82^{A}	98 ± 0.82^{A}	98 ± 0.82^{C}
Tween 80 (3%, v/v) (-)	16.8 ± 5.56^{B}	16.8 ± 5.56^{B}	16.8 ± 5.56^{B}	16.8 ± 5.56^{D}	16.8 ± 5.56^{D}

*Uppercase compares means within each column and lowercase within a row. Different letters indicate significant differences ($p<0.05$).

speed, high reproducibility and lack of experimental animals (protection of animal welfare), *in vitro* tests have been widely used in the screening of medicinal plants, often rather than *in vivo* tests (Ferreira *et al*., 2016). Among the others, the EHT is one of the most commonly used *in vitro* test for evaluating the anthelmintic potential of new anthelmintic agents such as EOs (Katiki *et al*., 2011; Ferreira *et al*., 2016; Ferreira *et al*., 2018; Macedo *et al*., 2019; Pinto *et al*., 2019; Štrbac *et al*., 2020a; Štrbac *et al*., 2020b). However, results of laboratory testing require confirmation in controlled field trials, which represent the next step in the development of an anthelmintic agent.

The World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) set up the criteria for evaluating the efficacy of different anthelmintic agents tested *in vitro* (Fonseca *et al*., 2013, Ferreira *et al*., 2016). According to these criteria, compounds with more than 90% efficiency are considered effective in controlling nematodes including GINs. In the present study, all tested concentrations of *O. vulgare*, *S. hortensis* and *T. vulgaris*, as well as most concentrations of the *M. piperita* EO were more than 90% efficacious, suggesting that these EOs represent a promising alternative for controlling GINs of sheep. Moreover, all tested concentrations of the first three EOs produced an inhibitory effect on hatchability higher than 98%, which is considered highly effective according to the previously mentioned criteria. Plants that showed some but not enough effectiveness, such as the *H. arenarium* EO, should also be considered. Although they may not be useful if applied independently, these plant products may still be a valuable part of an integrated approach designed to control parasites in the ruminant production system (Macedo *et al*., 2010).

Some of the EOs used in this study were already evaluated on their ovicidal potential against sheep GINs. Similar to the present study, Ferreira *et al.* (2016) evaluated the ovicidal activity of *T. vulgaris* EO against *Haemonchus contortus* in sheep, where it was also found to be highly effective. Unlike the present study, the inhibition effect on egg hatchability varied from 49.4-100% depending on concentration, with an IC_{50} of 0.436 mg/mL. Regardless of this, concentrations >0.781 mg/mL showed significant effects which were similar (P>0.05) to the positive control, as in the present study. The ovicidal effect of *M. piperita* EO against *H. contortus* and *Trichostrongylus* spp. in sheep were evaluated by Katiki *et al*., (2011), where it also showed significant, but inferior ovicidal activity compared to other EO tested (*Cymbopogon martini* and *Cymbopogon schoenanthus*) with and IC_{50} of 0.26 mg/mL, similarly like in the present study. However, it is important to note that the efficacy of plant EO can vary depending on many exogenous and endogenous factors, which can lead to different results even of the EOs obtained from the same plant species, as showed in our previously study (Štrbac *et al*., 2020b). There are no data about the ovicidal effects of the EOs of *O. vulgare*, *S. hortensis* and *H. arenarium* against GINs in sheep according to the knowledge of the authors of the present study.

The anthelmintic effect of EOs is most likely related to their chemical composition, primarily to their main components (Dhifi *et al*., 2016). As results of the present study showed, EOs of *O. vulgare*, *S. hortensis* and *T. vulgaris*, rich in carvacrol, thymol, p-cymene and γ-terpinene showed higher activity against sheep GINs compared to EOs of *M. piperita* and *H. arenarium*, rich in other compounds such as piperitone, dihidrocarvone, α-pinene and γ-curcumene. This indicates the dominant role of monoterpene alcohols and monoterpene hydrocarbons as anthelmintics. Indeed, some of these compounds were showed high anthelmintic potential against sheep GINs when tested individually. For example, isolated carvacrol and thymol showed high ovicidal potential in a study of Katiki *et al*. (2017). However, as many studies so far demonstrated, EO often shows higher anthelmintic activity in comparison with the single isolated compound, due to the synergistic effect among many different constituents of the whole EO.

Main components from the present study were also identified in different relative percentages in other EOs with proven anthelmintic activity. Thus, carvacrol was found in *Lippia gracilis* (61.7%) and *L. origanoides* (40.4%), thymol in *L. sidoides* (54.5%) (Chagas *et al*., 2018), γ-terpinene in *Melaleuca alternifolia* (20.15%) (Grando *et al*., 2016), α-pinene in *Juniperus communis* (40.46%) (Štrbac *et al*., 2020a) etc. However, each EO has a unique chemical composition with compounds belonging to a large number of chemical groups (hydrocarbons, alcohols, ketones, sesquiterpenes) with different possible mechanisms of action, which all lead to their different anthelmintic efficacy. Therefore, any new anthelmintic study of EOs is unique and may be significant within the context of finding new means in combating anthelmintic resistance. Moreover, simultaneous *in vitro* testing of several EOs provides the possibility to compare them and to select suitable ones for further *in vivo* examinations.

Despite the fact that medicinal plants have already long been used to combat parasitism in both human and veterinary practice, it seems that their great potential is still underutilized in modern medicine. There are several possible explanations for this; most evidence of the anthelmintic effects of medicinal plants is based on anecdotal observations without scientific validation (Muthee *et al*., 2018), which is required before their adoption as a novel method for parasite control. On the other hand, the anthelmintic activity of plants was found to be lower than that reported for synthetic anthelmintics in various *in vivo* studies (Macedo *et al*., 2010). However, nowadays research is looking to overcome these problems; there is a growing number of controlled studies that aim to scientifically verify and validate the anthelmintics potential of plants (Muthee *et al*., 2018) and novel techniques such as nanotechnology offer the possibility of increasing their efficiency in field conditions (Bilia *et al*., 2014).

Regardless of the above limitations, the use of botanical anthelmintics has many advantages (Ferreira *et al*., 2018). These include low costs, easy access in countries with developed biodiversity and environmental acceptability. Moreover, the natural origin of botanical anthelmintics most likely contributes to their low toxicity to host animals as well as the small amount of residues in meat and milk. Finally, a wide number of different compounds and their synergism allows a great deal of possible medical effects and may also contribute to reduced susceptibility to pharmacological resistance. Therefore, the use of bioactive plants and their secondary metabolites offers wide possibilities for sustainable control of nematode infections in ruminants.

Conclusions: The development of AR requires devising new strategies. The high anthelmintic potential of EOs demonstrated in this study suggests that these plant products may play a significant role in the control of sheep GINs. This specifically refers to *Origanum vulgare*, *Satureja hortensis* and *Thymus vulgaris* EOs, which represent promising candidates for further *in vivo* examinations. Therefore, the present study may be of great pharmaceutical and veterinary interest in the context of the search for new anthelmintic agents.

Authors contribution: FŠ, RR and DS made substantial contributions to the basic idea, while the conduct of the research was made possible by IP and LR; RR and SK were responsible for the procurement of EOs and the experiment was designed by FŠ, AB and LR; EHT and coproculture were conducted by FŠ, AB and AA with great advisory assistance from RR, SK, DS and LR, while chemical analyses were performed by NS and DO; For interpreting the results and drawing conclusions were responsible FŠ, RR, IP and NS, while the final version of the manuscript was drafted by FŠ with the assistance of all co-authors who revised the manuscript.

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