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In vitro anthelmintic potential of selected essential oils against gastrointestinal nematodes of sheep

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Abstract

Background

Gastrointestinal nematodes (GINs) represent major obstacle to modern small ruminant farming due to their various negative effects on animal health, welfare and productivity. The exclusive use of commercial, synthetic anthelmintics is no longer a sustainable option due to the development of resistance in GINs and the problem of residues of these drugs in animal products and the environment. The aim of this study was to investigate *in vitro* the phytotherapeutic properties of seven different plant essential oils (EOs): *Citrus x sinensis* (L.) Osbeck, *Citrus medica* (L.), *Mentha spicata* (L.), *Ocimum basilicum* (L.), *Hyssopus officinalis* (L.), *Salvia officinalis* (L.) and *Lavandula angustifolia* Mill. against GINs in sheep.

Methods

First, the chemical composition (qualitative and semi-quantitative characterization) of the tested EOs was determined by gas chromatography–mass spectrometry (GC–MS) analysis. Their anthelmintic potential was evaluated *in vitro* using the egg hatch test, which was performed at eight different concentrations of each oil: 50, 12.5, 3.125, 0.781, 0.195, 0.049, 0.025 and 0.0125 mg/ml. Finally, a coproculture examination was performed to determine the genera of GIN against which the oils were tested.

Results

Some of the most frequently identified compounds in GC-MS analyses were limonene, myrcene, α- and β- pinene, γ-terpinene, sabinene, linalool, eucalyptol, terpinen-4-ol, camphor, etc., with their total number per oil varying from 8 (*C. sinensis*) to 42 (*O. basilicum*). The tested EOs showed a dose-dependent (R^2 close to 1) ovicidal activity, with *O. basilicum* (23.3–93.3%, IC_{50} = 0.08 mg/ml), *M. spicata* (13.0-92.7%, IC_{50} = 0.07 mg/ml) and *H. officinalis* (42.7–91.3%, IC_{50} = 0.19 mg/ml) being the most effective. The high activity of these oils could be attributed to the high content of the monoterpene alcohol linalool and the terpenoid ketones carvone and pinocamphone. The least effective were *S. officinalis* (15.0–89.0%, IC_{50} = 0.53 mg/ml) and C. medica 12.3–95.0% (IC_{50} = 2.52 mg/ml). Four genera of sheep GINs were identified on coproculture examination, i.e. *Haemonchus* 43%, *Trichostrongylus* 35%, *Teladorsagia* 17% and *Chabertia* 5%.

Conclusion

The results obtained suggest that EOs tested, especially wild mint, basil and hyssop, could represent a valuable alternative in the control of GINs in sheep, which could help to reduce the use of chemical drugs. Future field trials should be performed to confirm these findings.

Background

Gastrointestinal nematodes (GINs) represent one of the most important problems and constraints for the livestock production in general, especially in grazing animals (1, 2). In small ruminants, GINs are particularly important, with the most prevalent parasites belonging to the genera *Haemonchus*, *Cooperia*, *Ostertagia*, *Trichostrongylus*, *Bunostomum*, *Chabertia*, *Nematodirus* and *Oesophagostomum* (3). These parasites are common worldwide and can have various negative effects depending on different factors such as animal age, breed, parasite species and degree of parasitic infection (4, 5). These include lower weight gains and productivity losses in the case of subclinical infections, but also the imparment of animal health and welfare in the case of clinical infections with signs of anaemia, diarrhoea, hypoproteinemia and oedema, anorexia, etc. (3, 6, 7). Therefore, GINs can affect sheep farming in different ways and cause high economic losses, estimated at several hundred million per year in Europe alone (8).

The use of broad-spectrum anthelmintics (benzimidazoles, imidazothiazoles and macrocyclic lactones) are still the most important control measure to prevent damage caused by worms (9, 10, 11). However, the exclusive use of these drugs no longer seems to be the appropriate option for several reasons. Firstly, their overuse, underdosing or the use of only one drug without rotation have led to the development of anthelmintic resistance (AR), which is now a widespread problem. This has led to additional economic losses in the tens of millions of dollars per year, with an upward trend in the future (12, 13). In addition, the extensive use of chemical pharmaceuticals has led to greater food safety and public health concerns due to their residues in animal products and the environment (14). In this context, the public demand for organic, chemical-free animal production should be considered (6). Finally, the prices of these drugs are constantly increasing despite their decreasing efficacy (15). For these reasons, there is a broad consensus that alternative solutions need to be incorporated into practice, with many possibilities including genetic selection of animals, pasture management, biological control, vaccination and phytotherapy (7, 16, 17).

The use of plants with anthelmintic activity to control sheep GINs is considered a particularly promising option (18). Plants have long been used for various treatment purposes in humans and animals, as they are a great source of antimicrobial, antiparasitic and insecticidal agents (19). From this point of view, whole plants or their parts with anthelmintic properties can be used in animal nutrition, but phytotherapy also implies the various uses of herbal products such as essential oils (EOs) and aqueous or alcoholic extracts. EOs are liquid mixtures of volatile components produced by the secondary metabolism of aromatic plants (20). Due to the valuable pharmaceutical potential of their bioactive ingredients such as terpenes, terpenoids and phenylpropanoids, there is a growing interest in the use of EOs in veterinary medicine (21, 22). For example, previous studies and various reports indicate that they can be used for a range of indications in horses (23), ruminants (24), poultry and pigs (25), dogs and cats (26), beekeeping (27, 28, 29), aquaculture (30), etc.

As far as GINs in sheep are concerned, some EOs have already shown high anthelmintic potential against different parasite stages (eggs, larvae and adults). The list of plant species used to obtain oils is already very extensive and includes various members of the genera *Cymbopogon*, *Eucalyptus*, *Mentha*, *Lippia*, *Citrus*, *Origanum* and *Melaleuca*, among others (31, 32). Most studies were conducted *in vitro* and against *Haemonchus contortus* and date from recent years, indicating the novelty of the topics. However, many plant species with potentially strong anthelmintic activity remained unexplored. In addition, EOs extracted from the same plants may have different compositions and thus different effects due to various factors (33, 34), suggesting the need for further studies. The aim of this study was to investigate the ovicidal activity of eight EOs from different plants *in vitro* and thus evaluate their anthelmintic potential for future treatments of sheep GINs.

Methods

Essential oils and chemical analyses

The EOs tested in the present study were obtained from the following two producers: BIOSS, Serbia – orange (*Citrus sinensis* (L.) Osbeck), citron (*Citrus medica* L.), hyssop (*Hyssopus officinalis* L.) and sage (*Salvia officinalis* L.) as well as the Institute for Field and Vegetable Crops, Novi Sad, Serbia – wild mint (*Mentha spicata* L.), coriander (*Coriandrum sativum* L.), lavender (*Lavandula angustifolia* Mill.) and basil (*Ocimum basilicum* L.). Their chemical composition (qualitative and semiquantitative characterization) was determined by gas chromatography–mass spectrometry (GC–MS), using the following technical conditions: 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 (30 m × 0.25 mm, 0.25 μm); temperature program 50–270°C; ion source temperature 230°C; electron energy 70 eV; quadrupole temperature 150°C (35). 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 arithmetic retention indices (AI) with literature data (36). The relative amounts of the components, expressed in percentages, were calculated by the normalisation procedure according to the peak area in the total ion chromatogram.

Egg hatch test

The anthelmintic potential of selected EOs was evaluated *in vitro* using the egg hatch test (EHT), which examines the ovicidal activity of the active substances. The GIN eggs for this test were obtained from faecal samples collected directly from the rectal ampulla of sheep (n = 30) with natural-mixed infection from a farm located in southern Italy. The recovery method described by Bosco et al. (37) was used to isolate the eggs. After collection, the faecal samples were first pooled, homogenised and then filtered under running water through meshes of different sizes (1 mm, 250 μm, 212 μm and 38 μm). The eggs retained on the smallest-size sieve were then washed with distilled water and centrifuged at 1500 rpm for 3 minutes. After discarding the supernatant, a centrifugation with 40% sugar solution was performed to float the eggs, which were then isolated into new tubes and mixed with distilled water. Finally, two more centrifugations were performed to remove the pellets and obtain the aqueous solution containing the GIN eggs.

The EHT was performed at eight different concentrations (50, 12.5, 3.125, 0.781, 0.195, 0.049, 0.025 and 0.0125 mg/ml) for all selected EOs, as in our previously performed *in vitro* study (38). However, in comparison to that study, where micropipettes were used, a dilution method with emulsifier and distilled water was used to obtain different concentrations of EOs. An aqueous solutions containing GIN eggs were added to 24-well plates to obtain approximately 150 eggs/well. Subsequently, different concentrations of EOs were emulsified in 3% Tween 80 (v/v) and added to the wells, which were then topped up with distilled water to obtain a solution of 0.5 ml/well. The positive control was thiabendazole (0.025 and 0.0125 mg/ml) and the negative controls were 3% Tween 80 (v/v) and distilled H_2O. The plates were then incubated at 27°C for 48 h, after which Lugol`s solution was added to the wells to terminate the incubation. The eggs and first-stage larvae (L_1) were then counted under an inverted microscope to calculate the inhibitions of egg hatchability at different concentrations of the tested oils. The experiment was carried out in three replicates, and the values obtained were expressed as the arithmetic mean for each concentration.

Coproculture

To determine the GIN genera whose eggs were used in the experiment, a coproculture examination was performed according to the protocol developed by the UK Ministry of Agriculture, Fisheries and Food (39). The faeces of the animals used to obtain the GIN eggs were also collected to form a pool, and immediately after the eggs were extracted, the remaining part of the faeces was used for coprocultures. The developed third-stage (L_3) larvae of GINs were identified using the morphological identification keys proposed by van Wyk and Mayhew (40). The percentage of each genus was determined based on 100 L3s, and all larvae were identified if the sample contained 100 or fewer larvae. In this way, it was possible to determine the percentage of each genus in the total number of larvae identified.

Statistical analyses

Inhibitions of egg hatchability (IH) were calculated using the following formula (41, 42):

IH (%) = [(number of eggs)/(number of larvae + number of eggs)] × 100

A One-way Analysis of Variance (ANOVA) with post-hock Tuckey
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s test was performed to evaluate the differences between the means of the different EOs in a single concentration. In both cases, a p-value threshold of 0.05 was used. Finally, the half-maximal inhibitory concentration (IC_{50}) and the presence of a dose-dependent effect (R^2) in each EO were determined by nonlinear regression/logarithmic distribution (43).

Results

Chemical composition

The GC-MS analyses revealed the rich chemical composition of the tested EOs with compounds belonging to different chemical groups, and whose number varied from 8 (*C. sinensis*) to 42 (*O. basilicum*) (Table 1, Figures S1-7). Some of the most frequently identified compounds were limonene, myrcene, α- and β-pinene, γ-terpinene, sabinene (hydrocarbon terpenes), linalool, eucalyptol, terpinen-4-ol (alcohols), camphor (ketones) etc. The dominant compounds in each EO were limonene (96.1%), trans- (1.31%) and cis-limonene oxide (1.21%) in *C. sinensis*; limonene (74.6%), β-pinene (11.6%), γ-terpinene (8.66%) in *C. medica*; carvone (64.4%), trans-4-caranone (8.67%), limonene (4.37%) in *M. spicata*; linalool (62.8%), γ-muurolene (4.45%) and estragole (3.78%) in *O. basilicum*; cis-pinocamphone (47.6%), β-pinene (13.5%) and trans-pinocamphone (12.2%) in *H. officinalis*; α-thujone (38.8%), camphor (19.8%), eucalyptol (8.40%) in *S. officinalis* and linalool (37.5%), eucalyptol (17.3%), borneol (14.2%) in *L. angustifolia*. Due to the large number of compounds identified, only those with abudance > 1% in at least one oil are listed in the Table 1, with total number of identified compounds presented at the end of the table.

Table 1
Chemical composition (% of total peak area) of selected essential oils determined by gas chromatography-mass spectrometry analysis

AI	Compound	% of total peak area						
		CS	CM	MS	OB	HO	SO	LA
932	α-Pinene	0.42	**1.63**[a]	0.54	0.10	0.89	**3.16**	**1.03**
946	Camphene	-	0.06	-	0.03	0.12	**5.36**	0.58
971	Sabinene	0.18	**1.24**	0.16	0.04	-	0.02	0.18
976	β-Pinene	-	**11.6**	0.67	0.19	**13.5**	**1.16**	**1.09**
989	Myrcene	0.43	0.87	0.99	0.10	**2.08**	0.91	0.34
1023	p-Cymene	-	0.69	0.35	0.19	-	0.90	0.54
1027	Limonene	**96.1**	**74.6**	**4.37**	0.18	**7.79**	**2.22**	**1.06**
1029	Eucalyptol	-	-	0.53	**3.52**	-	**8.40**	**17.3**
1035	cis-Ocimene	-	-	-	-	0.28	-	**3.20**
1056	γ-Terpinene	-	**8.66**	0.32	0.02	0.25	0.36	0.27
1100	Linalool	-	-	-	**62.8**	0.43	0.20	**37.5**
1106	α-Thujone	-	-	-	-	-	**38.8**	-
1116	β-Thujone	-	-	-	-	-	**5.07**	-
1132	cis-Limonene oxide	**1.21**	-	-	-	-	-	-
1137	Trans-Limonene oxide	**1.31**	-	-	-	-	-	-
1143	Camphor	-	-	-	0.73	-	**19.8**	**4.76**
1152	Menthone	-	-	**1.28**	-	-	-	-
1156	pentyl-Benzene	-	-	-	-	**2.80**	-	-
1159	trans-Pinocamphone	-	-	-	-	**12.2**	-	-
1163	Isomenthone	-	-	0.50	-	-	-	-
1164	Borneol	-	-	-	-	-	**1.89**	**14.2**
1171	Menthol	-	-	**4.20**	-	-	-	-
1173	cis-Pinocamphone	-	-	-	-	**47.6**	-	-
1176	Terpinen-4-ol	-	-	**1.16**	0.46	-	0.34	**6.71**
1182	iso-Menthol	-	-	0.13	-	-	-	-
1191	α-Terpineol	-	-	0.12	0.51	-	-	**1.96**
1193	Dihydro carveol	-	-	**1.73**	-	-	-	-
1196	trans-4-Caranone	-	-	**8.67**	-	-	-	-
1197	Estragole	-	-	-	**3.78**	-	-	-
1204	trans-Dihydro carvone	-	-	**1.49**	-	-	-	-
1238	Pulegone	-	-	0.10	-	-	-	-
1244	Carvone	-	-	**64.4**	-	-	-	-
1255	Linalyl-acetate	-	-	-	-	-	-	**6.11**
1284	Bornyl acetate	-	-	-	-	-	**1.86**	-
1293	Menthyl acetate	-	-	**1.01**	-	-	-	-
1327	iso-Dihydro carveol acetate	-	-	**3.11**	-	-	-	-
1383	β-Bourbonene	-	-	**1.76**	-	**1.16**	-	-
1390	β-Elemene	-	-	-	**1.72**	-	-	-
1418	β- Caryophyllene	-	-	0.93	0.92	**1.56**	**3.95**	0.53
1434	α-trans-Bergamotene	-	-	-	**2.11**	-	-	-

AI	Compound	% of total peak area						
		CS	CM	MS	OB	HO	SO	LA
1436	γ-Elemene	-	-	-	**1.42**	-	-	-
1452	α-Humulene	-	-	-	**1.13**	0.16	**4.15**	
1459	allo-Aromadendrene	-	-	-	-	**1.52**	-	-
1479	γ-Muurolene				**4.45**			
1480	Germacrene D	-	-	-		**1.66**	-	-
1495	Bicyclogermacrene	-	-	-		**2.25**	-	-
1503	α-Bulnesene	-	-	-	**3.66**	-	-	-
1512	γ-Cadinene	-	-	-	**2.98**	-	-	-
1639	α-epi-Cadinol	-	-	-	**2.17**	-	-	-
Total number of identified compounds		8	10	34	42	35	27	27

* CS – *Citrus sinensis*; CM – *Citrus medica*; MS – *Mentha spicata*; CO – *Coriandrum sativum*; HO – *Hyssopus officinale*; SO – *Salvia officinalis*; LA – *Lavandula angustifolia*; OB – *Ocimum basilicum*; AI - arithmetic retention index; [a] Only the compounds present in more than 1% in at least one essential oil are presented in the table; [b] Compounds with abundance > 1% are written in bold.

Egg hatch test

The EHT showed a promising anthelmintic potential of the EOs tested *in vitro* (Table 2, Figures S8-14). The ovicidal activity varied depending on the EO used and the concentration, as follows: *M. spicata* 13.0-92.7% (R^2 = 0.93); *O. basilicum* 23.3–93.3% (R^2 = 0.98); *H. officinalis* 42.7–91.3% (R^2 = 0.93); *L. angustifolia* 28.0-84.7% (R^2 = 0.98); *C. sinensis* 14.7–86.3% (R^2 = 0.97); *S. officinalis* 15.0–89.0% (R^2 = 0.99) and *C. medica* 12.3–95.0%. Their calculated IC_{50} values were 0.07, 0.08, 0.19, 0.31, 0.50, 0.53 and 2.52 mg/ml, respectively.

Table 2
The inhibitory effect (mean ± standard deviation) of selected essential oils on the egg hatching of sheep gastrointestinal nematodes

Concentration [mg/ml]	*Citrus sinensis*	*Citrus medica*	*Mentha spicata*	*Ocimum basilicum*	*Hyssopus officinalis*	*Salvia officinalis*	*Lavandula angustifolia*
50	86.3 ± 2.08^{Acd}	95.0 ± 1.00^{Aa}	92.7 ± 0.58^{Aab}	93.3 ± 2.08^{Aab}	91.3 ± 2.52^{ABabc}	89.0 ± 1.00^{ABbcd}	84.7 ± 2.52^{Ad}
12.5	78.0 ± 2.00^{Bd}	91.0 ± 1.00^{Aab}	93.7 ± 0.58^{Aa}	93.0 ± 1.00^{Aab}	87.3 ± 5.69^{BCb}	83.3 ± 1.53^{BCbcd}	81.3 ± 1.53^{ABcd}
3.125	73.7 ± 2.52^{Bc}	40.0 ± 1.00^{Bd}	91.0 ± 1.00^{Aa}	91.7 ± 1.53^{ABa}	79.7 ± 1.53^{Cb}	77.3 ± 2.08^{Cbc}	77.3 ± 2.08^{BCbc}
0.781	51.7 ± 2.08^{Ce}	39.0 ± 1.00^{Bf}	80.3 ± 0.58^{Bab}	84.3 ± 3.06^{BCa}	75.7 ± 2.08^{Cbc}	57.7 ± 1.53^{Dd}	71.7 ± 1.53^{Cc}
0.195	38.7 ± 2.08^{Dd}	29.3 ± 1.53^{Ce}	76.0 ± 2.65^{Bab}	79.7 ± 1.53^{Ca}	71.7 ± 1.53^{Cb}	35.3 ± 2.52^{Ed}	50.7 ± 2.52^{Dc}
0.049	35.3 ± 4.16^{Dd}	19.7 ± 1.53^{Df}	51.0 ± 1.00^{Cc}	50.0 ± 5.29^{Dc}	62.0 ± 2.65^{Db}	28.0 ± 2.65^{Fe}	28.0 ± 1.00^{Ee}
0.025	20.7 ± 0.58^{Ed}	15.7 ± 3.06^{DEd}	19.7 ± 4.16^{Dd}	32.7 ± 2.08^{Ebc}	53.0 ± 4.36^{Ea}	19.3 ± 1.53^{Gd}	29.7 ± 1.53^{Ec}
0.0125	14.7 ± 1.53^{Ed}	12.3 ± 2.31^{EFd}	13.0 ± 1.00^{EFd}	23.3 ± 2.08^{Fc}	42.7 ± 2.08^{Fa}	15.0 ± 1.00^{Gd}	30.0 ± 1.00^{Eb}
Control (+)[a]	96.3 ± 1.53^{F}	96.3 ± 1.53^{A}	96.3 ± 1.53^{A}	96.3 ± 1.53^{A}	96.3 ± 1.53^{A}	96.3 ± 1.53^{H}	96.3 ± 1.53^{F}
Control (+)[b]	95.0 ± 1.00^{F}	95.0 ± 1.00^{A}	95.0 ± 1.00^{A}	95.0 ± 1.00^{A}	95.0 ± 1.00^{AB}	95.0 ± 1.00^{AH}	95.0 ± 1.00^{F}
Control (–)[a]	14.2 ± 3.34^{E}	14.2 ± 3.34^{DE}	14.2 ± 3.34^{DE}	14.2 ± 3.34^{G}	14.2 ± 3.34^{H}	14.2 ± 3.34^{G}	14.2 ± 3.34^{G}
Control (–)[b]	6.6 ± 1.92^{G}	6.6 ± 1.92^{F}	6.6 ± 1.92^{F}	6.6 ± 1.92^{G}	6.6 ± 1.92^{H}	6.6 ± 1.92^{I}	6.6 ± 1.92^{H}

* Uppercase compares means within different concentrations in one EO and controls; lowercase compares means within all EOs at single concentration. Different letters indicate significant differences ($p < 0.05$). Control (+)[a] - Thiabendazole, 0.025 mg/ml; Control (+)[b] - Thiabendazole, 0.0125 mg/ml; Control (–)[a] - Tween 80, 3% (v/v); Control (–)[b] - Distilled H_2O

Coproculture

Four genera of sheep GINs were identified on coproculture examination, as shown in Fig. 1.

Discussion

Various tests can be used to evaluate the anthelmintic efficacy of standard anthelmintics and the detection of AR (44). The use of *in vitro* tests in veterinary parasitology has numerous advantages over *in vivo* tests. Namely, these tests are rapid, inexpensive, reproducible, usually simple and reduce the number of animals needed for field trials, which can be expensive, laborious and time-consuming (43). The two most commonly used *in vitro* methods for testing the efficacy of anthelmintics are the EHT and the larval development test (45, 46). These methods are also extremely useful for screening substances of plant origin for their activity against helminths and are therefore commonly used for this purpose. The EHT is used to evaluate the ovicidal activity of an active substance and provides comparable, reliable and precise results for the evaluation of the efficacy of e.g., benzimidazoles and thus forms a basis for other tests (45). However, the results of EHT and *in vivo* tests such as FECRT do not always correlate with each other, as many factors can influence activity under field conditions (43, 45). Therefore, the efficacy of novel agents is confirmed after laboratory tests are complemented by field studies, with *in vitro* tests providing a good basis for selecting the most promising anthelmintic agents (47, 48, 49, 50).

In the present study, the tested EOs showed high, dose-dependent (R^2 values close to 1) ovicidal activity against GIN eggs, which can be compared in different ways. According to the criteria established by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) for evaluating the efficacy of various anthelmintics tested *in vitro*, compounds with those greater than 90% are considered effective in controlling nematodes, including GINs (51, 52). From this point of view, the three highest concentrations of *O. basilicum* and *M. spicata*, two of *C. medica* and one of *H. officinalis* EO reached the required efficacy, which was comparable to the positive control ($p > 0.05$). All tested concentrations (even the lowest) of *H. officinalis*, *C. sativum*, *L. officinalis* and *O. basilicum* showed significantly higher effects than both negative controls ($p < 0.05$). However, a commonly used pharmacological parameter for comparison of drug-inhibitory effects is the half-maximal inhibitory concentration (IC_{50}), which is precise as it counts all tested concentrations (53). According to this parameter, the most effective oils were *M. spicata*, *O. basilicum* and *H. officinalis* with IC_{50} values of 0.07, 0.08 and 0.19 mg/ml, respectively. These results largely correspond to the criteria mentioned above.

Some of the EOs tested in the present study were previously investigated *in vitro* for their anthelmintic effects against sheep GINs. Thus, Sousa et al. (54) tested the ovicidal activity of EOs from 12 different cultivars of *O. basilicum*, as well as their individual compounds (linalool, methyl chavicol, citral and eugenol) and their combinations, against the eggs of *H. contortus*. The effect of the EO samples was variable, with IC_{50} values ranging from 0.56 to 2.22 mg/ml, with the highest active variety consisting mainly of linalool and methyl chavicol. In fact, the isolated combination of these compounds in a ratio of 64:11 (similar to that of *O. basilicum* EO in the present study, along with other compounds) was the most potent combination with an IC_{50} of 0.44 mg/ml, indicating the great potential of these compounds for inclusion in anthelmintic formulations.

L. angustifolia EO was tested in a study by Ferreira et al. (43), where it showed ovicidal activity (IC_{50} = 0.316 mg/ml) that was higher than that of the other oils tested. Moreover, the concentration of 3.125 mg/ml and all higher concentrations were more than 90% effective, although the concentrations of 0.195 mg/ml and below were ineffective (< 10%). In the same study, *Lavandula* EO showed also larvicidal activity and had an effect on adult worm motility. The main compound of that sample was linalool acetate (36.0%), along with camphor (5.54%) and eucalyptol (4.87%). Finally, in a study by Gaínza et al. (55), the EO of *C. sinensis*, with limonene as the main constituent (96%), showed high ovicidal activity with IC_{50} and IC_{90} values of 0.27 and 0.99 mg/ml, respectively. To our knowledge, the rest of the EOs from our study were not tested against sheep GINs, at least not with regard to their ovicidal activity.

The anthelmintic potential of EOs derives from their chemical composition, i.e. the bioactive compounds that compose them (56), with the main compound usually being the most important (57). From this perspective, the acyclic monoterpene tertiary alcohol linalool, and the terpenoid ketones carvone and pinocamphone were the most abundant in the most effective EOs. The individual anthelmintic activity of linalool and carvone against sheep GINs is very well known and described (54, 58, 59, 60), while pinocamphone has not yet been studied and is unknown for this property. The anthelmintic activities of isolated EO ingredients can vary greatly (58), and therefore the components that make up the EO composition are critical to their pharmacological properties. Their percentage content is also important, especially that of the main compound. Thus, the EOs of *O. basilicum*, consisting of linalool at percentage 62.8%, showed a dominant activity compared to *L. angustifolia*, where linalool was also the main compound but in the percentage of 37.5%. This fact was already demonstrated in our previous study with eleven EOs (38), where the oils with a high percentage of one compound were the most effective (oregano, fennel etc). However, the presence of other compounds is also important due to synergistic effects, as previous studies have shown that whole EOs generally have the higher effect than isolated compounds (52, 61).

The results of these studies indicate that the number of compounds is not a decisive factor. While in the present study the three most effective oils (*O. basilicum*, *M. spicata* and *H. officinalis*) had the highest number of compounds, in the previous study (38) the number of compounds was inversely proportional to their efficacy. In general, the chemical composition of the EOs may vary depending on many factors related to the plant`s environment, such as soil properties (hydrology, pH, salinity), light, precipitation and season, but also age and part of the plant used for EO extraction, its genetic characteristics etc. (33, 62). The presence of certain animals and microorganisms may also be involved, as they stimulate the plants to produce oils in sight of defense mechanism (63). Finally, the method used for extraction and the post-extraction process before use can also play a role (33). All these factors can lead to differences in the chemical composition of EO extracted from different, but also from the same plant species, and consequently, to differences in their biological properties including anthelmintic activity, as shown in previous studies (38, 54, 61).

The problem of standardization of EO composition may hinder the commercialization of these products. Along with these, they possess some undesirable properties that are avoided to a certain extent in the pharmaceutical field: hydrophobicity and insolubility in water, instability due to hydrolysis and oxidation, and high volatility (20, 64). Although some of the EOs such as oregano (65) and peppermint (66) have already shown noticeable field efficacy against GINs with no side effects noted for the sheep, research efforts are currently focused on developing innovative formulations based on encapsulation techniques that can overcome these problems. Indeed, encapsulation can protect active ingredients that are sensitive to oxygen, light and moisture and prevent interactions with other compounds. In this way, the stability and bioavailability of EOs can be further increased, while reducing toxicity and volatility. In addition, it can enable controlled release, which is important for various uses of oils (20, 67). Nevertheless, there are still some unknowns related to the potential application of EOs against GINs, including their mechanism of action. So far, they are known to have significant negative effects on the survival, reproduction, development, behavior and metabolic pathways of nematodes (68). Understanding the molecular mechanism of action of EOs activity is important for the development of the most appropriate formulation (69), but also for accurate risk assessment and remediation (70).

With regard to the aforementioned disadvantages of the exclusive use of commercial anthelmintics in the control of sheep GINs, EOs have many advantages. On the one hand, the possibility of resistance development in nematodes is most likely lower due to the diversity of their ingredients with potentially different mechanisms of action (18, 43). On the other hand, EOs as herbal anthelmintics are often considered safer for the host than synthetic, chemical compounds (71, 72). The environmental and public health aspects also favor herbal anthelmintics, as they are biodegradable (73). Finally, the financial aspect also plays a role, as they tend to be cheaper than conventional treatments, especially in countries with developed biodiversity (43, 71, 72). Considering all these aspects, the use of herbal anthelmintics, including EO, seems to be a promising option that can help reduce the use of synthetic drugs, along with other alternative options (74, 75, 76), as mentioned earlier. Alternatively, they can be combined with the rational use of commercial drugs based on different refugia strategies (77), as some previous studies have shown that they can increase their efficacy (78). In any case, an integrated approach seems to be the backbone for the future management of GINs in small ruminants.

Conclusion

The decreasing efficacy of commercially available drugs due to the development of anthelmintic resistance in various GIN species, and other problems associated with the exclusive use of these drugs, necessitate the search for alternative solutions. In the present study, the high anthelmintic potential of eight plant EOs against GINs in sheep was demonstrated *in vitro*. According to the different parameters, basil, wild mint and hyssop were the most effective oils, indicating their potential for drug development. Therefore, we recommend these oils for future *in vivo* studies to confirm these results.

Declarations

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Author contributions

FŠ, RR and DS conceived of the presented idea, and the study was planned and designed in the cooperation with LR, AB, FC and VM. For the procerment of the material, NS, DO, RR and LR were responsible. The experiment was conducted as follows: chemical analyses – NS, DO; *in vitro* efficacy test – FŠ and AB; coproculture – AB and LR. The experimental part was supervised by DS, LR, RR, FC and VM. The results obtained were interpreted by FŠ, AB and NS and the statistical analyses were performed by FŠ. The original manuscript, including the preparation of tables and graphs, was written by FŠ, revised by AB, LR and RR and approved by all co-authors.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval - The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University of Naples (PG/2021/0130480, 16 December 2021).

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Competing interests - The authors declare that they have no competing interests.

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Figures

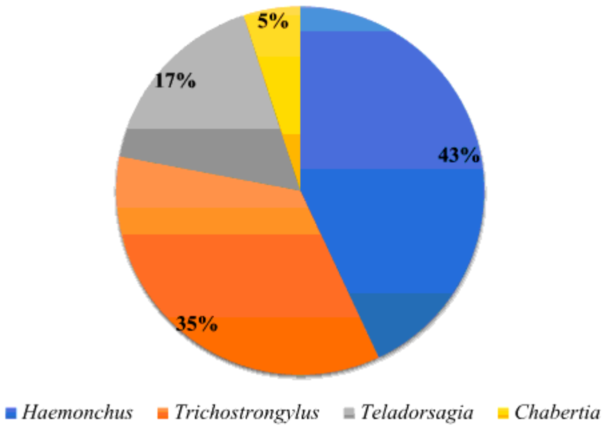

Figure 1

Percentage (%) of GIN genera represented in faecal samples used for egg isolation

Supplementary Files

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