Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2024.295

RESEARCH ARTICLE

In Vitro Anthelmintic Potential of Selected Essential Oils against Gastrointestinal Nematodes of Sheep

Filip Štrbac[1*], Slobodan Krnjajić[1], Nataša Simin[2], Dejan Orčić[2], Laura Rinaldi[3], Vincenzo Musella[4], Fabio Castagna[4], Radomir Ratajac[5], Dragica Stojanović[6], Felwa Abdullah Thagfan[7], Fatma Mohamed Ameen Khalil[8] and Antonio Bosco[3*]

[1]Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11030 Belgrade, Serbia, [2]Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, 21102 Novi Sad, Serbia, [3]Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR, Via Federico Delpino 1, 80137 Naples, Italy, [4]Department of Health Sciences, University of Catanzaro Magna Græcia, Campus "S. Venuta", 88100, Catanzaro, Italy, [5]Scientific Veterinary Institute Novi Sad, Rumenački put 20, 21113 Novi Sad, Serbia, [6]Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad, Trg Dositeja Obradovića 8, 21102 Novi Sad, Serbia, [7]Department of Biology, College of Science, Princess Nourah bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia, [8]Unit of Health Specialties, Basic Sciences and Their Applications, Applied College, King Khalid University, Mohayil Asir Abha, 61421, Saudi Arabia
*Corresponding author: filip.strbac@imsi.bg.ac.rs (FŠ); antonio.bosco@unina.it (AB)

ARTICLE HISTORY (24-738)

Received:	November 15, 2024
Revised:	December 17, 2024
Accepted:	December 21, 2024
Published online:	December 27, 2024

Key words:
Anthelmintic resistance
Phytotherapy
Integrated parasite control
Egg hatch test
GC-MS
Coproculture

ABSTRACT

Gastrointestinal nematodes (GINs) represent major obstacle to modern sheep farming, where the exclusive use of commercial anthelmintics is no longer a sustainable option due to the development of resistance in nematodes and the problem of drug residues in animal products and the environment. The aim of this study was to investigate the *in vitro* phytotherapeutic properties of seven essential oils (EOs) against GINs in sheep. Their chemical composition was determined by gas chromatography-mass spectrometry (GC-MS) analysis. The anthelmintic potential was evaluated using the egg hatch test performed at eight concentrations for each oil (50, 12.5, 3.125, 0.781, 0.195, 0.049, 0.025 and 0.0125 mg/mL). Additionally, a coproculture examination was performed to identify the GIN genera present in the tested fecal samples. The tested EOs showed a dose-dependent (R^2 close to 1) ovicidal activity with basil - *Ocimum basilicum* L. (23.3-93.3%, IC_{50}=0.08 mg/mL), spearmint - *Mentha spicata* L. (13.0-92.7%, IC_{50}=0.07 mg/mL) and hyssop - *Hyssopus officinalis* L. (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 (*O. basilicum*) and the terpenoid ketones carvone (*M. spicata*) and pinocamphone (*H. officinalis*). Four genera of sheep GINs were identified on coproculture examination, i.e. *Haemonchus* 43%, *Trichostrongylus* 35%, *Teladorsagia* 17% and *Chabertia* 5%. The obtained results suggest that EOs of basil, spearmint 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.

To Cite This Article: Štrbac F, Krnjajić S, Simin N, Orčić D, Rinaldi L, Musella V, Castagna F, Ratajac R, Stojanović D, Thagfan FA, Khalil FMA and Bosco A, 2024. *In vitro* anthelmintic potential of selected essential oils against gastrointestinal nematodes of sheep. Pak Vet J, 44(4): 1053-1062. http://dx.doi.org/10.29261/pakvetj/2024.295

INTRODUCTION

Gastrointestinal nematodes (GINs) represent one of the most important problems and constraints for the livestock production in general, especially in grazing animals (Mavrot *et al.*, 2015; Khan *et al.*, 2023; Al-Saeed *et al.*, 2023; Abubakar *et al.*, 2024). In small ruminants, GINs are particularly important, with the most prevalent parasites belonging to the genera *Haemonchus*, *Cooperia*, *Teladorsagia*, *Trichostrongylus*, *Bunostomum*, *Chabertia*, *Nematodirus* and *Oesophagostomum* (Desalegn and Berhanu, 2023). 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 (Williams *et al*., 2021; Tachack *et al*., 2022). These include lower weight gains and productivity losses in the case of subclinical infections, but also the impairment of animal health and welfare in the case of clinical infections with signs of anaemia, diarrhoea, hypoproteinemia, oedema, anorexia which can lead to death of animals (Zajac and Garza, 2020; Desalegn and Berhanu, 2023; Maurizio *et al*., 2023). Therefore, GINs can affect sheep farming in different ways and cause high economic losses, estimated at several hundred million euros per year in Europe alone (Charlier *et al*., 2022).

The use of broad-spectrum anthelmintics (benzimidazoles, imidazothiazoles and macrocyclic lactones) are still the most important control measure to prevent damage caused by worms (Bresciani *et al*., 2017; Bosco *et al*., 2020; Szewc *et al*., 2021; Kandil *et al.*, 2024). 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 (Qamar and Alkheraije, 2023). This has led to decreased drug efficacy and additional economic losses estimated to the tens of millions of euros per year, with an upward trend in the future (Kaplan, 2020; Rose Vineer *et al*., 2020). 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 (Ahbara *et al*., 2021; Rafique *et al.*, 2022). In this context, the public demand for organic, chemical-free animal production should be considered (Zajac and Garza, 2020). Finally, the prices of these drugs are constantly increasing despite their decreasing efficacy (Lee *et al*., 2021). 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 (Reyes-Guerrero *et al*., 2021; de Agüero *et al*., 2023; Maurizio *et al*., 2023).

The use of plants with anthelmintic activity to control sheep GINs is considered a particularly promising option (Borges and Borges, 2016; Al-Hoshani *et al.*, 2024). 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 (Rizwan *et al*., 2021). 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 and viscous mixtures of volatile components produced by the secondary metabolism in different parts of aromatic plants (Cimino *et al*., 2021; Issa, 2024). 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 (Mucha and Witkowska, 2021; Nehme *et al*., 2021; Ratajac *et al.*, 2024). For example, previous studies and various reports indicate that they can be used for a range of indications in horses (Elghandour *et al*., 2023), ruminants (Kholif and Olafadehan, 2021), poultry and pigs (Zhai *et al*., 2018), dogs and cats (Štrbac *et al*., 2021), beekeeping (Bava *et al*., 2021; Bava *et al*., 2022; Castagna *et al*., 2022a), aquaculture (Dawood *et al*., 2022), 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 (André *et al*., 2018; Štrbac *et al*., 2022a). 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 (Fokou *et al*., 2020; Bava *et al*., 2023), suggesting the need for further studies. The aim of this study was to investigate the ovicidal activity of seven EOs from different plants *in vitro* and thus evaluate their anthelmintic potential for future treatments of sheep GINs.

MATERIALS AND METHODS

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).

Essential oils and chemical analyses: The EOs tested in the present study were obtained from the following two producers: BIOSS, Serbia–orange (*Citrus x 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–spearmint (*Mentha spicata* 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 1µL; 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 70eV; quadrupole temperature 150°C (Knežević *et al*., 2016). 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 (Adams, 2012). The relative amounts of the components, expressed in percentages, were calculated by the normalization procedure according to the peak area in the total ion chromatogram.

Egg hatch test: The anthelmintic potential of selected EOs was evaluated *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 fecal 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*. (2018, 2020) was used to isolate the eggs. Namely, after collection, the fecal samples were first pooled, homogenized and then filtered under running water through meshes of different sizes (1mm, 250μm, 212μm and 38μm). The eggs retained on the smallest-size sieve were then washed with distilled water and centrifuged at 1500rpm 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 similarly as in our previous *in vitro* study (Štrbac *et al*., 2022b) with some modifications. In the present study, all selected EOs were tested at eight different concentrations (50, 12.5, 3.125, 0.781, 0.195, 0.049, 0.025 and 0.0125 mg/mL), and the concentrations of EOs were prepared by dilution in compared to the micropipettes that were used in the previous study. Anyhow, 40μL of aqueous solution containing the GIN eggs was added to each plate in 24-well plates to obtain approximately 150 eggs/well. Subsequently, tested EOs were emulsified in 3% Tween 80 (v/v) and added in the required quantities to the wells, which were then topped up with distilled water to obtain a solution of 0.5mL/well with certain concentrations of EOs. 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 48h, after which Lugol`s solution was added to the wells to terminate the incubation. The eggs and first-stage larvae (L1) 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 (1986). The feces 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 feces was used for coprocultures. The developed third-stage (L3) larvae of GINs were identified using the morphological identification keys proposed by van Wyk and Mayhew (2013). 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 (Coles *et al*., 1992; Pinto *et al*., 2019):

$$\text{IH (\%)} = [(\text{number of eggs}) / (\text{number of larvae} + \text{number of eggs})] \times 100$$

A One-way Analysis of Variance (ANOVA) with post-hock Tuckey`s test was subsequently performed for the mutual comparison of the different concentrations in individual EO, as well as for the comparison with the controls. For the comparison of the efficacy of different oils, a two-way ANOVA with post-hock Bonferoni`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 (Ferreira *et al*., 2018).

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, Fig. 1-7). 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 abundance >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.

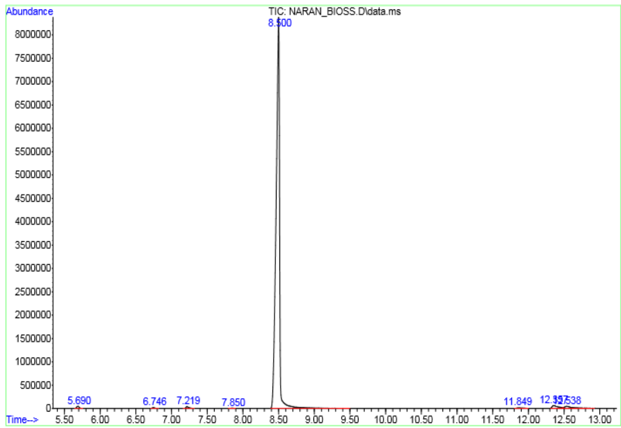

Fig. 1: GC–MS chromatogram of the *Citrus* × *sinensis* (L.) Osbeck essential oil.

Egg hatch test: The EHT showed a promising anthelmintic potential of the EOs tested *in vitro* (Table 2, Fig. 8-14). 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% (R^2=0.92). Their calculated IC_{50} values were 0.07, 0.08, 0.19, 0.31, 0.50, 0.53 and 2.52 mg/mL, respectively.

Coproculture: Four genera of sheep GINs were identified on coproculture examination, as shown in Fig. 15.

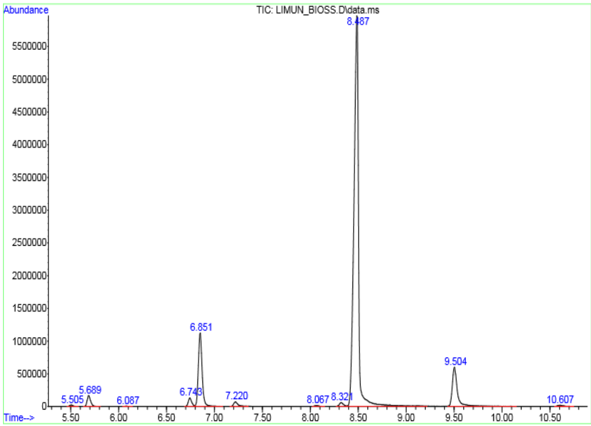

Fig. 2: GC–MS chromatogram of the *Citrus medica* L. essential oil.

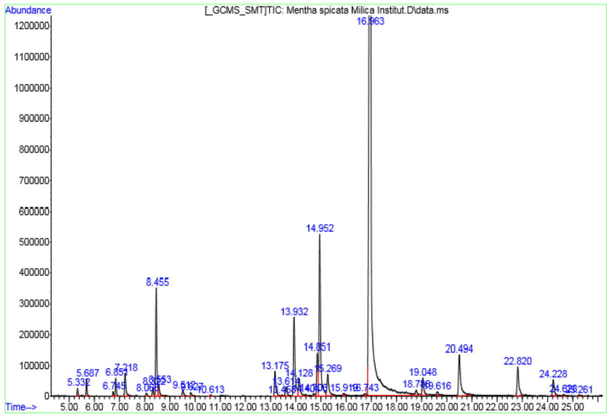

Fig. 3: GC–MS chromatogram of the *Mentha spicata* L. essential oil.

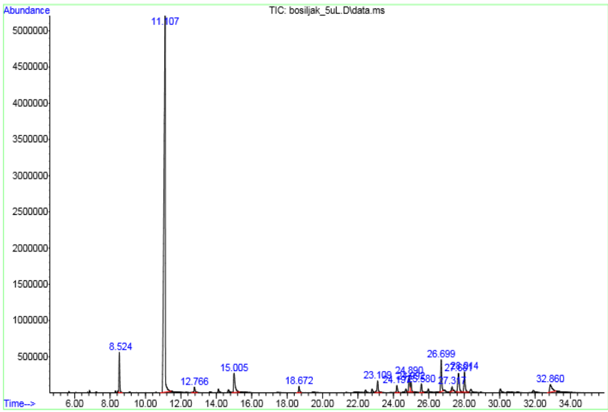

Fig. 4: GC–MS chromatogram of the *Ocimum basilicum* L. essential oil.

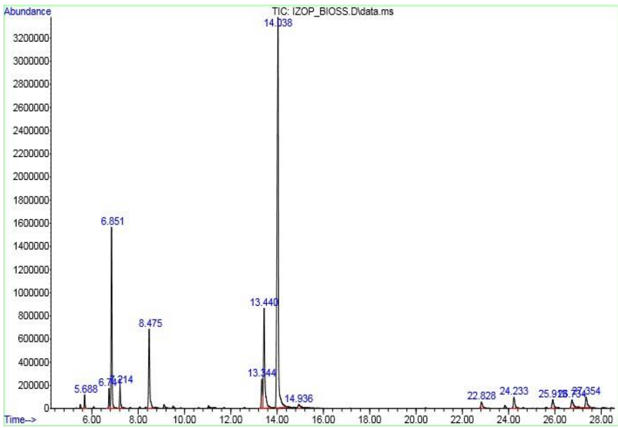

Fig. 5: GC–MS chromatogram of the *Hyssopus officinalis* L. essential oil.

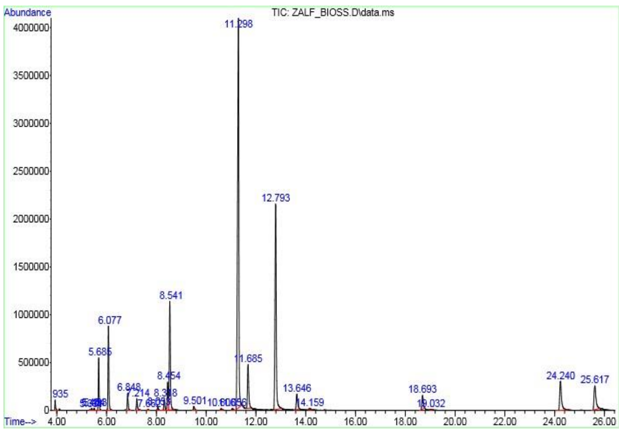

Fig. 6: GC–MS chromatogram of the *Salvia officinalis* L. essential oil.

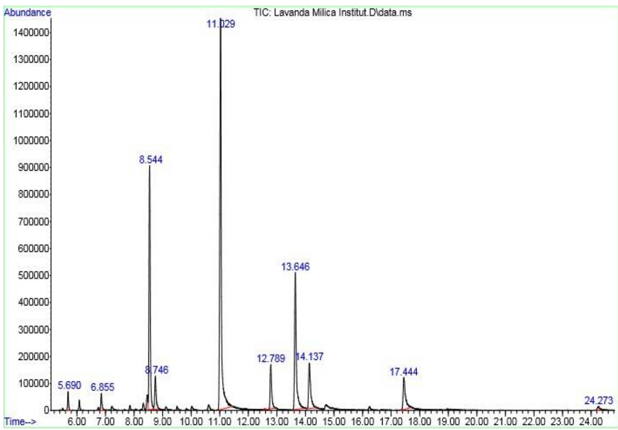

Fig. 7: GC–MS chromatogram of the *Lavandula angustifolia* L. essential oil.

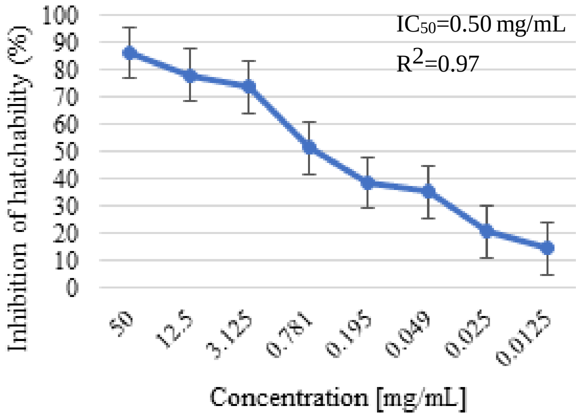

Fig. 8: Inhibition of egg hatchability of *Citrus* × *sinensis* (L.) Osbeck essential oil.

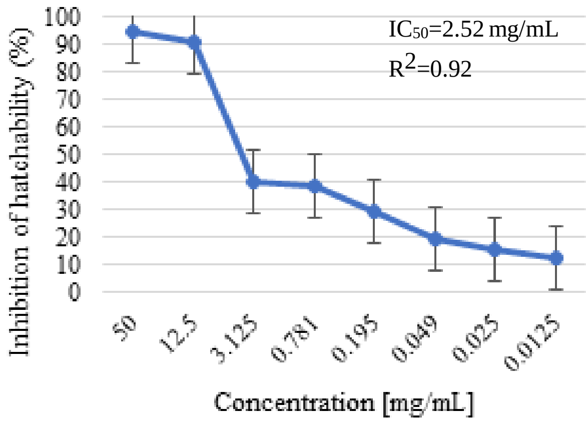

Fig. 9: Inhibition of egg hatchability of *Citrus medica* L. essential oil.

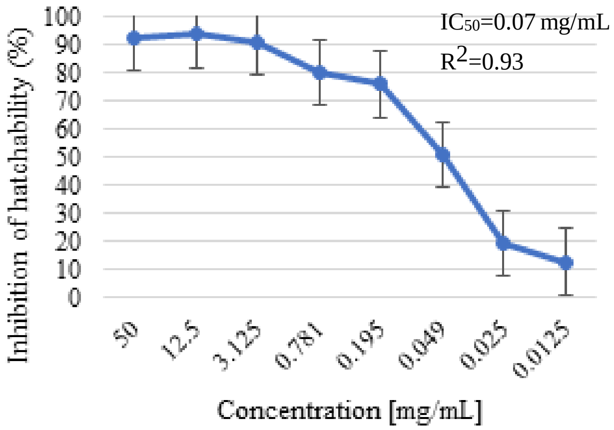

Fig. 10: Inhibition of egg hatchability of *Mentha spicata* L. essential oil.

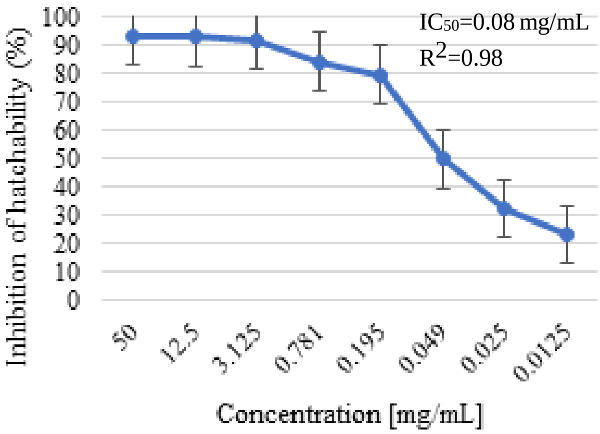

Fig: 11: Inhibition of egg hatchability of *Ocimum basilicum* L. essential oil.

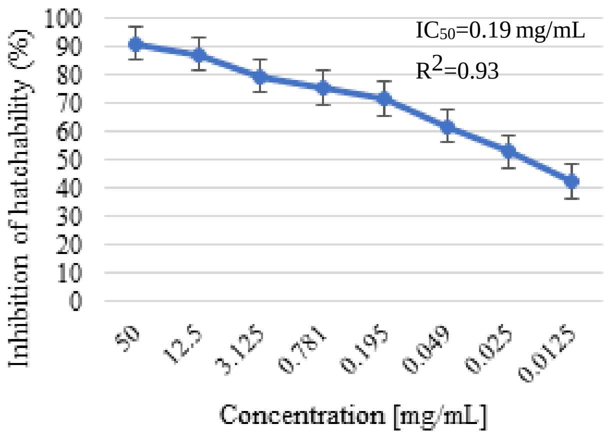

Fig. 12: Inhibition of egg hatchability of *Hyssopus officinalis* L. essential oil.

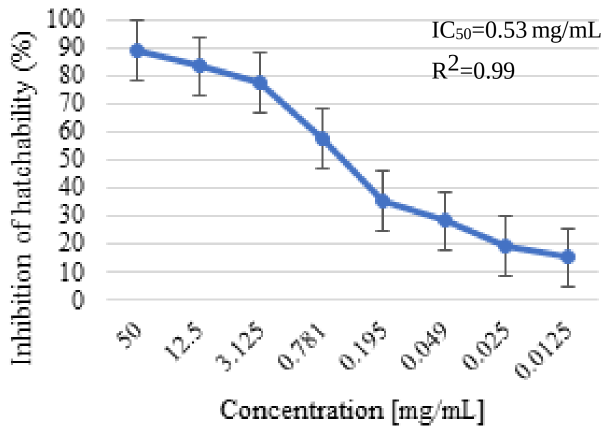

Fig. 13: Inhibition of egg hatchability of *Salvia officinalis* L. essential oil.

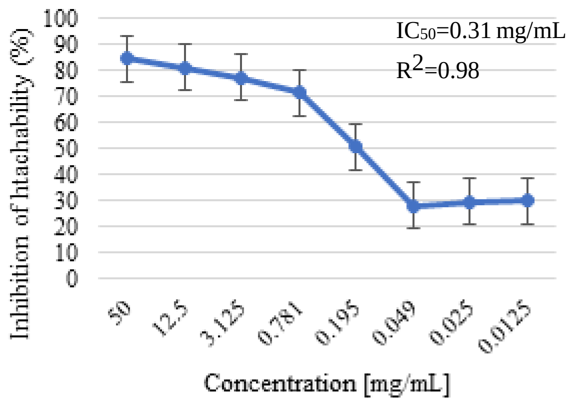

Fig. 14: Inhibition of egg hatchability of *Lavandula angustifolia* Mill. essential oil.

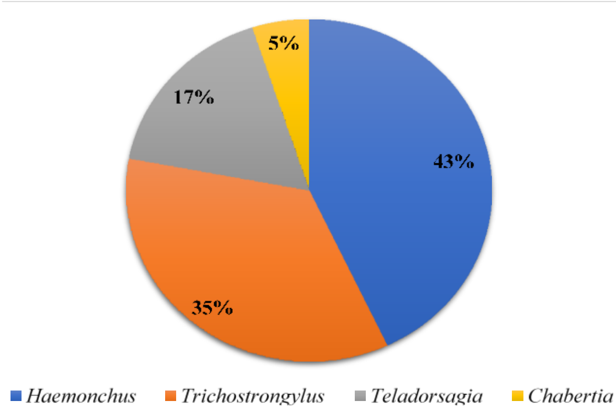

Fig. 15: Percentage (%) of GIN genera represented in fecal samples used for egg isolation.

DISCUSSION

Various tests can be used to evaluate the anthelmintic efficacy of standard anthelmintics and the detection of AR (Fissiha and Kinde, 2021). 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 (Ferreira *et al.*, 2018; Al-Saeed *et al.*, 2023). The two most commonly used *in vitro* methods for testing the efficacy of anthelmintics are the EHT and the larval development test (Babják *et al.*, 2021; Feyera *et al.*, 2022). 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 benzimidazoles and thus forms a basis for other tests (Babják *et al.*, 2021). 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 (Ferreira *et al.*, 2018; Babják *et al.*, 2021). Therefore, the efficacy of novel agents is confirmed after laboratory tests are complemented by field studies, with *in*

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	-	-	-
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

vitro tests providing a good basis for selecting the most promising anthelmintic agents (André *et al*., 2017; Soren and Yadav, 2020; Castagna *et al*., 2020; Ragusa *et al*., 2022; Osório TM *et al*., 2023).

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 (Fonseca *et al*., 2013; Ferreira *et al*., 2016). 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*, *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 (Berrouet *et al*., 2020). 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*. (2021) 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*. (2018), 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 also showed 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*. (2015), 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 regarding their ovicidal activity.

The anthelmintic potential of EOs derives from their chemical composition, i.e. the bioactive compounds that compose them (Valente *et al*., 2021), with the main compound usually being the most important (Dhifi *et al*., 2016). 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 (Katiki *et al*., 2017; Helal *et al*., 2020; Sousa *et al*., 2021; Aguiar *et al*., 2022), while pinocamphone has not yet been studied and is unknown for

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 (columns); lowercase compares means within all EOs at single concentration (rows). 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

this property. The anthelmintic activities of isolated EO ingredients can vary greatly (Katiki *et al.*, 2017), 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 (Štrbac *et al.*, 2022b), 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 a higher effect than isolated compounds (Camurça-Vasconcelos *et al.*, 2007; Ferreira *et al.*, 2016).

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 (Štrbac *et al.*, 2022b) 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. (Fokou *et al.*, 2020; Barra, 2009). The presence of certain animals and microorganisms may also be involved, as they stimulate the plants to produce oils in sight of defense mechanism (Butnariu and Sarac, 2018). Finally, the method used for extraction and the post-extraction process before use can also play a role (Fokou *et al.*, 2020). 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 (Camurça-Vasconcelos *et al.*, 2007; Sousa *et al.*, 2021; Štrbac *et al.*, 2022b).

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 (Feyaerts *et al.*, 2020; Cimino *et al.*, 2021). Although some of the EOs such as oregano (Štrbac *et al.*, 2023a) and peppermint (Štrbac *et al.*, 2023b) 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 (Cimino *et al.*, 2021; Sousa *et al.*, 2022). 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 (Piao *et al.*, 2020). Understanding the molecular mechanism of action of EOs activity is important for the development of the most appropriate formulation (Andrés *et al.*, 2012), but also for accurate risk assessment and remediation (Oro *et al.*, 2020).

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 (Borges and Borges, 2016; Ferreira *et al.*, 2018). On the other hand, EOs as herbal anthelmintics are often considered safer for the host than synthetic, chemical compounds (Kubkomawa *et al.*, 2020; Romero *et al.*, 2022). The environmental and public health aspects also favor herbal anthelmintics, as they are biodegradable (Veerakumari, 2015). Finally, the financial aspect also plays a role, as they tend to be cheaper than conventional treatments, especially in countries with developed biodiversity (Ferreira *et al.*, 2018; Kubkomawa *et al.*, 2020; Romero *et al.*, 2022).

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 (Maqbool *et al.*, 2017; Castagna *et al.*, 2021; Castagna *et al.*, 2022b; Al-Hoshani *et al.*, 2024; Kandil *et al.*, 2024), as mentioned earlier. Alternatively, they can be combined with the rational use of commercial drugs based on different refugia strategies (Höglund and Gustafsson, 2023), as some previous studies have shown that they can increase their efficacy (Miró *et* *al*., 2020). In any case, an integrated approach seems to be the backbone for the future management of GINs in small ruminants. However, despite the promising effect *in vitro* shown by the EOs in the present study, further studies are required to confirm the results obtained. These include, above all, *in vivo* field efficacy and toxicity trials for the most effective EOs such as basil, spearmint and hyssop to determine the most appropriate formulation, dose and application method, but also to evaluate their safety for animals and the environment. These steps are necessary before application in clinical practice is possible. In addition, studies aimed to evaluate the effect of the tested EOs against resistant GIN strains, as well as the anthelmintic effect of different combinations of these EOs, should also be performed.

Conclusions: The decreasing efficacy of commercially available drugs due to the development of anthelmintic resistance in nematodes, the problem of residues of these drugs in animal products and the environment, as well as other limitations associated with the exclusive use of these drugs necessitate the search for alternative solutions. In the present study, the high anthelmintic potential of seven plant EOs against GINs in sheep was demonstrated *in vitro*. According to the different parameters, basil, spearmint 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 and further investigations.

Funding: This research was co-funded by the Ministry of Education, Science, and Technological Development of the Republic of Serbia, rescript no. 451-03-1183/2021-14. The research was also co-funded by the Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Italy, as part of the „CREMOPAR" project. Authors extend their appreciation to Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R96), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia. In addition, the authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through large group Research Project under grant number RGP2/486/45.

Acknowledgements: We deeply thank Mario Parrilla for the technical support in the laboratory of the Regional Centre for Monitoring of Parasitosis (CREMOPAR), SS 18, località Borgo Cioffi, 84025 Eboli (SA), Italy. Authors extend their appreciation to Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2024R96), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia. In addition, the authors extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through large group Research Project under grant number RGP2/486/45.

Authors contribution: 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 procurement 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, RR, FAT, FMAK and approved by all co-authors.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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

REFERENCES

Abubakar M, Oneeb M, Rashid M, *et al*., 2024. *In vitro* anthelmintic efficacy of three plant extracts against various developmental stages of *Haemonchus contortus*. Pak Vet J 44:238-43. https://doi.org/10.29261/pakvetj/2024.174

Al-Hoshani N, Almahallawi R, Al-Nabati EA, *et al*., 2024. Anthelmintic effects of herbal mixture of selected plants of Apiaceae on *Strongylus vulgaris* and *Fasciola hepatica*. Pak Vet J 44:437-41. https://doi.org/10.29261/pakvetj/2024.14

Adams RP, 2012. Identification of essential oil components by gas chromatography/mass spectrometry, 4th ed., Allured Business Media: Carol Stream, IL, USA.

Aguiar AARM, Filho JVDA, Pinheiro HN, *et al*., 2022. *In vitro* anthelmintic activity of an R-carvone nanoemulsions towards multiresistant *Haemonchus contortus*. Parasitology 149:1631-41. https://doi.org/10.1017/S0031182022001135

Ahbara AM, Rouatbi M, Gharbi M, *et al*., 2021. Genome-wide insights on gastrointestinal nematode resistance in autochthonous Tunisian sheep. Sci Rep 11:9250. https://doi.org/10.1038/s41598-021-88501-3

Al-Saeed FA, Bamarni SSI, Iqbal KJ, *et al*., 2023. *In vitro* anthelmintic efficacy of *Haloxylon salicornicum* leaves extract using adult *Heamonchus contortus* worms. Pak Vet J 43:91-6. DOI: 10.29261/pakvetj/2022.091

André WPP, Cavalcante GS, Ribeiro WLC, *et al*., 2017. Anthelmintic effect of thymol and thymol acetate on sheep gastrointestinal nematodes and their toxicity in mice. Rev Bras Parasitol Vet 26:323–30. https://doi.org/10.1590/S1984-29612017056

André WPP, Ribeiro WLC, de Oliveira LMB, *et al*., 2018. Essential oils and their bioactive compounds in the control of gastrointestinal nematodes of small ruminants. Acta Sci Vet 46:1522. https://doi.org/10.22456/1679-9216.81804

Andrés MF, González-Coloma A, Sanz J, *et al*., 2012. Nematicidal activity of essential oils: A review. Phytochem Rev 11:371–90. https://doi.org/10.1007/s11101-012-9263-3

Babják M, Königová A, Dolinská MU, *et al*., 2021. Does the *in vitro* egg hatch test predict the failure of benzimidazole treatment in *Haemonchus contortus*? Parasite 28:62. https://doi.org/10.1051/parasite/2021059

Barra A, 2009. Factors affecting chemical variability of essential oils: A review of recent developments. Nat Prod Commun 4:1147–54.

Bava R, Castagna F, Piras C, *et al*., 2021. *In vitro* evaluation of acute toxicity of five *Citrus* spp. essential oils towards the parasitic mite *Varroa destructor*. Pathogens 10:1182. https://doi.org/10.3390/pathogens10091182

Bava R, Castagna F, Palma E, *et al*., 2022. Phytochemical profile of *Foeniculum vulgare* subsp. *piperitum* essential oils and evaluation of acaricidal efficacy against *Varroa destructor* in *Apis mellifera* by *in vitro* and semi-field fumigation tests. Vet Sci 9:684. https://doi.org/10.3390/vetsci9120684

Bava R, Castagna F, Palma E, *et al*., 2023. Essential oils for a sustainable control of honeybee varroosis. Vet Sci 10:308. https://doi.org/10.3390/vetsci10050308

Berrouet C, Dorilas N, Rejniak KA, *et al*., 2020. Comparison of drug inhibitory effects (IC50) in monolayer and spheroid cultures. Bull Math Biol 82:68. https://doi.org/10.1007/s11538-020-00746-7

Borges DGL and Borges FDA, 2016. Plants and their medicinal potential for controling gastrointestinal nematodes in ruminants. Nematoda 3:e92016. https://doi.org/10.4322/nematoda.00916

Bosco A, Maurelli MP, Ianniello D, *et al*, 2018. The recovery of added nematode eggs from horse and sheep feces by three methods. BMC Vet Res 14:7. https://doi.org/10.1186/s12917-017-1326-7

Bosco A, Kießler J, Amadesi A, *et al*., 2020. The threat of reduced efficacy of anthelmintics against gastrointestinal nematodes in sheep from an area considered anthelmintic resistance-free. Parasit Vectors 13:457. https://doi.org/10.1186/s13071-020-04329-2

Bresciani KDS, Coelho WMD, Gomes JF, *et al*., 2017. Aspects of epidemiology and control of gastrointestinal nematodes in sheep and cattle–Approaches for its sustainability. Rev Bras Cienc Agrar 40:664-9. https://doi.org/10.19084/RCA16028

Butnariu M and Sarac I, 2018. Essential oils from plants. J Biotechnol Biomed Sci 1:35-43. https://doi.org/10.14302/issn.2576-6694.jbbs-18-2489

Camurça-Vasconcelos ALF, Bevilaqua CML, Morais SM, *et al*., 2007. Anthelmintic activity of *Croton zehtneri* and *Lippia sidoides* essential oils. Vet Parasitol 288–94. https://doi.org/10.1016/j.vetpar.2007.06.012

Castagna F, Britti D, Oliverio M, *et al*., 2020. *In vitro* anthelminthic efficacy of aqueous pomegranate (*Punica granatum* L.) extracts against gastrointestinal nematodes of sheep. Pathogens 9:1063. https://doi.org/10.3390/pathogens9121063

Castagna F, Piras C, Palma E, *et al*, 2021. Green veterinary pharmacology applied to parasite control: Evaluation of *Punica granatum*, *Artemisia campestris*, *Salix caprea* aqueous macerates against gastrointestinal nematodes of sheep. Vet Sci 8:237. https://doi.org/10.3390/vetsci8100237

Castagna F, Bava R, Musolino V, *et al*., 2022a. Potential new therapeutic approaches based on *Punica granatum* fruits compared to synthetic anthelmintics for the sustainable control of gastrointestinal nematodes in sheep. Animals 2883. https://doi.org/10.3390/ani12202883

Castagna F, Bava R, Piras C, *et al*. 2022b. Green veterinary pharmacology for honey bee welfare and health: *Origanum heracleoticum* L. (Lamiaceae) essential oil for the control of the *Apis mellifera* varroatosis. Vet Sci 9:124. https://doi.org/10.3390/vetsci9030124

Charlier J, Rinaldi L, Musella V, *et al*., 2022. Initial assessment of the economic burden of major parasitic helminth infections to the ruminant livestock industry in Europe. Prev Vet Med 182:105103. https://doi.org/10.1016/j.prevetmed.2020.105103

Cimino C, Maurel OM, Musumeci T, *et al*., 2021. Essential oils: Pharmaceutical applications and encapsulation strategies into lipid-based delivery systems. Pharmaceutics 13:327. https://doi.org/10.3390/pharmaceutics13030327

Coles GC, Bauer C, Borgsteede FH, *et al*., 1992. World association for the advencement of veterinary parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 44:35–44. https://doi.org/10.1016/0304-4017(92)90141-u

Dawood MAO, Basuini MFE, Yilmaz S, *et al*., 2022. Exploring the roles of dietary herbal essential oils in aquaculture: A review. Animals 12:823. https://doi.org/10.3390/ani12070823

de Agüero VCG, Valderas-García E, del Palacio LG, *et al.,* 2023. Secretory IgA as biomarker for gastrointestinal nematodes natural infection in different breed sheep. Animals. 13:2189. https://doi.org/10.3390/ani13132189

Desalegn C and Berhanu G, 2023. Assessment of the epidemiology of the gastrointestinal tract nematode parasites in sheep in Toke Kutaye, West Shoa Zone, Ethiopia. Vet Med Res Rep 14:177-183. https://doi.org/10.2147/VMRR.S427828

Dhifi W, Bellili S, Jazi S, *et al*., 2016. Essential oils' chemical characterization and investigation of some biological activities: A critical review. Medicines 3:25. https://doi.org/10.3390/medicines3040025

Elghandour MMMY, Maggiolino A, García EIC, *et al*., 2023. Effects of microencapsulated essential oils on equine health: Nutrition, metabolism and methane emission. Life 13:455. https://doi.org/10.3390/life13020455

Ferreira LE, Benincasa BI, Fachin AL, *et al*., 2016. *Thymus vulgaris* L. essential oil and its main component thymol: anthelmintic effects against *Haemonchus contortus* from sheep. Vet Parasitol 228:70-6. https://doi.org/10.1016/j.vetpar.2016.08.011

Ferreira LE, Benincasa BI, Fachin AL, *et al*., 2018. Essential oils of *Citrus aurantifola*, *Anthemis nobile* and *Lavandula officinalis*: *in vitro* anthelmintic activities against *Haemonchus* contortus. Parasit Vectors 11:269. https://doi.org/10.1186/s13071-018-2849-x

Feyaerts AF, Luyten W and Dijck PV, 2020. Striking essential oil: tapping into a largely unexplored source for drug discovery. Sci Rep 10:2867. https://doi.org/10.1038/s41598-020-59332-5

Feyera T, Elliott T, Sharpe B, *et al*., 2022. Evaluation of *in vitro* methods of anthelmintic efficacy testing against *Ascaridia galli*. J Helminthol 96:e29, 1–12. https://doi.org/10.1017/S0022149X22000177

Fissiha W and Kinde MZ, 2021. Anthelmintic resistance and its mechanism: A review. Infect Drug Resist 14:5403–10. https://doi.org/10.2147/IDR.S332378

Fokou JBH, Dongmo PMJ and Boyom FF, 2020. Essential oil's chemical composition and pharmacological properties. In: El-Shemy, H. (Ed), Essential Oils, Oils of Nature. IntechOpen: London, UK. https://doi.org/10.5772/intechopen.86573

Fonseca ZAAS, Coelho WAC, André WPP, *et al*., 2013. Use of herbal medicines in control of gastrointestinal nematodes of small ruminants: efficacies and prospects. Rev Bras Hig Sanid Anim 7:233-49. https://doi.org/10.5935/1981-2965.20130021

Gaínza YA, Domingues LF, Perez OP, *et al*., 2015. Anthelmintic activity *in vitro* of *Citrus sinensis* and *Melaleuca quinquenervia* essential oil from Cuba on *Haemonchus contortus*. Ind Crops Prod 76:647-52. https://doi.org/10.1016/j.indcrop.2015.07.056

Helal MA, Abdel-Gawad AM, Kandil OM, *et al*., 2020. Nematocidal effects of a coriander essential oil and five pure principles on the infective larvae of major ovine gastrointestinal nematodes *in vitro*. Pathogens 9:740. https://doi.org/10.3390/pathogens9090740

Höglund J and Gustafsson K, 2023. Anthelmintic treatment of sheep and the role of parasites refugia in a local context. Animals 13:1960. https://doi.org/10.3390/ani13121960

Issa NA, 2024. Evaluation the antimicrobial activity of essential oils against veterinary pathogens, multidrug-resistant bacteria and Dermatophytes. Pak Vet J 44:260-5. https://doi.org/10.29261/pakvetj/2024.165

Kandil OM, Shalaby HA, Hassan NMF, *et al*., 2024. Comparative nematocidal efficacy of coriander oils against *Haemonchus contortus*. Int J Vet Sci 13:17-26. https://doi.org/10.47278/journal.ijvs/2023.062

Kaplan RM, 2020. Biology, epidemiology, diagnosis and management of anthelmintic resistance in gastrointestinal nematodes of livestock. Vet Clin North Am Food Anim Pract 36:17–30. https://doi.org/10.1016/j.cvfa.2019.12.001

Katiki LM, Barbieri AME, Araujo RC, *et al*., 2017. Synergistic interaction of ten essential oils against *Haemonchus contortus in vitro*. Vet Parasitol 243:47–51. https://doi.org/10.1016/j.vetpar.2017.06.008

Khan A, Jamil M, Ullah S, *et al*., 2023. The prevalence of gastrointestinal nematodes in livestock and their health hazards: A review. World Vet J 13:57-64. https://doi.org/10.54203/scil.2023.wvj6

Kholif AE and Olafadehan OA, 2021. Essential oils and phytogenic feed additives in ruminant diet: chemistry, ruminal microbiota and fermentation, feed utilization and productive performance. Phytochem Rev 20:1087-108. https://doi.org/10.1007/s11101-021-09739-3

Knežević P, Aleksić V, Simin N, *et al*., 2016. Antimicrobial activity of *Eucalyptus camaldulensis* essential oils and their interactions with conventional antimicrobial agents against multi-drug resistant *Acinetobacter baumannii*. J Ethnopharmacol 178:125–136. https://doi.org/10.1016/j.jep.2015.12.008

Kubkomawa HI, Nafarnda DW, Tizhe MA, *et al*., 2020. Ethno-veterinary health management practices amongst livestock producers in Africa: a review. Adv Agric Sci 6:1–006. ISSN: 2381-3911

Lee J, Joo H, Maskery BA, *et al*., 2021. Increases in anti-infective drug prices, subsequent prescribing, and outpatient costs. JAMA Netw Open 4:e2113963. https://doi.org/10.1001/jamanetworkopen.2021.13963

Maqbool I, Wani ZA, Shahardar RA, *et al*., 2017. Integrated parasite management with special reference to gastro-intestinal nematodes. J Parasit Dis 41:1-8. https://doi.org/10.1007/s12639-016-0765-6

Maurizio A, Perrucci S, Tamponi C, *et al*., 2023. Control of gastrointestinal helminths in small ruminants to prevent anthelmintic resistance: The Italian experience. Parasitology 150:1105-18. https://doi.org/10.1017/S0031182023000343

Mavrot F, Hertzberg H and Torgerson P, 2015. Effect of gastro-intestinal nematode infection on sheep performance: A systematic review and meta-analysis. Parasit Vectors 8:557. https://doi.org/10.1186/s13071-015-1164-z

Ministry of Agriculture, Fisheries and Food (MAFF), 1986. Grande-Bretagne, Manual of Veterinary Parasitological Laboratory Techniques. HM Stationery Off: London, UK.

Miró MV, Luque S, Cardozo P, *et al*. 2020. Plant-derived compounds as a tool for the control of gastrointestinal nematodes: Modulation of abamectin pharmacological action by carvone. Front Vet Sci 7:601750. https://doi.org/10.3389/fvets.2020.601750

Mucha W and Witkowska D, 2021. The applicability of essential oils in different stages of production of animal-based foods. Molecules 26:3798. https://doi.org/10.3390/molecules26133798

Nehme R, Andrés S, Pereira RB, *et al*., 2021. Essential oils in livestock: From health to food quality. Antioxidants 10:330. https://doi.org/10.3390/antiox10020330

Oro V, Krnjajić S, Tabaković M, *et al*., 2020. Nematicidal activity of essential oils on a psychrophilic *Panagrolaimus* sp. (Nematoda: Panagrolaimidae). Plants 9:1588. https://doi.org/10.3390/plants 9111588

Osório TM, Menezes LDM, Martins AA, *et al*., 2023. Essential oils against gastrointestinal nematodes in sheep *in vitro* and chemical composition of those plants. Ciênc Nat 45:e25. https://doi.org/ 10.5902/2179460X71665

Piao X, Sun M and Fengping Y, 2020. Evaluation of nematocidal action against *Caenorhabditis elegans* of essential oil of flesh fingered citron and its mechanism. J Chem 2020:1740938. https://doi.org/10. 1155/2020/1740938

Pinto NB, de Castro LM, Azambuja RHM, *et al*., 2019. Ovicidal and larvicidal potential of *Rosmarinus officinalis* to control gastrointestinal nematodes of sheep. Rev Bras Parasitol Vet 28:807–11. https://doi.org/10.1590/S1984-29612019060.

Qamar W and Alkheraije KA, 2023. Anthelmintic resistance in haemonchus contortus of sheep and goats from asia–a review of in vitro and in vivo studies. Pak Vet J 43(3): 376-387.

Rafique A, Mahmood MS, Abbas RZ, *et al*., 2022. Anthelmintic activity of *Moringa oleifera* and *Azadirachta indica* against gastrointestinal nematodes of wild sheep. J Hellenic Vet Med Soc 73:3989-96. https://doi.org/10.12681/jhvms.25876

Ratajac R, Pavličević A, Petrović J, *et al*., 2024. *In vitro* Evaluation of acaricidal efficacy of selected essential oils against *Dermanyssus gallinae*. Pak Vet J 44:93-8. https://doi.org/10.29261/ pakvetj/2023.123

Ragusa M, Miceli N, Piras C, *et al*., 2022. *In vitro* anthelmintic activity of *Isatis tinctoria* extracts against ewes' gastrointestinal nematodes (GINs), a possible application for animal welfare. Vet Sci 9:129. https://doi.org/10.3390/vetsci9030129

Reyes-Guerrero DE, Olmedo-Juárez A and Mendoza-de Gives P, 2021. Control and prevention of nematodiasis in small ruminants: background, challenges and outlook in Mexico. Rev Mex Cienc Pecu 12(Supl 3):186-204. https://doi.org/10.22319/rmcp.v12s3.5840

Rizwan HM, Sajid MS, Shamim A, *et al*., 2021. Sheep parasitism and its control by medicinal plants: A review. Parasitol United J 14:112-21. https://doi.org/10.21608/PUJ.2021.70534.1114

Romero B, Susperregui J, Sahagún AM, *et al*., 2022. Use of medicinal plants by veterinary practitioners in Spain: A cross-sectional survey. Front Vet Sci 9:2022. https://doi.org/10.3389/fvets.2022.1060738

Rose Vineer H, Morgan ER, Hertzberg H, *et al*., 2020. Increasing importance of anthelmintic resistance in European livestock: Creation and meta-analysis of an open database. Parasite 27:69. https://doi.org/10.1051/parasite/2020062

Soren AD and Yadav AK, 2020. Evaluation of *in vitro* and *in vivo* anthelmintic efficacy of *Cyperus compressus* Linn., a traditionally used anthelmintic plant in parasite-animal models. Future J Pharm Sci 6:126. https://doi.org/10.1186/s43094-020-00148-5

Sousa AIP, Silva CR, Costa-Júnior HN, *et al*., 2021. Essential oils from *Ocimum basilicum* cultivars: analysis of their composition and determination of the effect of the major compounds on *Haemonchus contortus* eggs. J Helminthol 95:e17. https://doi.org/ 10.1017/S0022149X21000080

Sousa VI, Parente JF, Marques JF, *et al*., 2022. Microencapsulation of essential oils: A review. Polymers 14:1730. https://doi.org/10.3390/ polym14091730

Szewc M, De Waal T and Zintl A, 2021. Biological methods for the control of gastrointestinal nematodes. Vet J 268:105602. https://doi.org/10.1016/j.tvjl.2020.105602

Štrbac F, Petrović K, Stojanović D, *et al*., 2021. Possibilities and limitations of the use of essential oils in dogs and cats. Vet J Rep Srp 21:238–65. https://doi.org/10.7251/VETJEN2101238S

Štrbac F, Bosco A, Pušić I, *et al*., 2022a. The use of essential oils against sheep gastrointestinal nematodes. In: Abbas, R.Z., Khan A., Liu, P., Saleemi, M.K. (Ed.), Animal Health Perspectives. Volume I, 2022a. Unique Scientific Publishers, Faisalabad, Pakistan, pp:86–94. https://doi.org/10.47278/book.ahp/2022.12

Štrbac F, Bosco A, Maurelli MP, *et al*., 2022b. Anthelmintic properties of essential oils to control gastrointestinal nematodes in sheep—*In vitro* and *in vivo* studies. Vet Sci 9:93. https://doi.org/10.3390/ vetsci9020093

Štrbac F, Krnjajić S, Maurelli MP, *et al*., 2023a. A potential anthelmintic phytopharmacological source of *Origanum vulgare* (L.) essential oil against gastrointestinal nematodes of sheep. Animals 13:45. https://doi.org/10.3390/ani13010045

Štrbac, F., Krnjajić, S., Stojanović, D., *et al*., 2023b. *In vitro* and *in vivo* anthelmintic efficacy of peppermint (*Mentha x piperita* L.) essential oil against gastrointestinal nematodes of sheep. Front Vet Sci 10:1232570. https://doi.org/10.3389/fvets.2023.1232570

Tachack EB, Oviedo-Socarrás T, Pastrana MO, *et al*., 2022. Status of gastrointestinal nematode infections and associated epidemiological factors in sheep from Córdoba, Colombia. Trop Anim Health Prod 54:171. https://doi.org/10.1007/s11250-022- 03170-2

van Wyk JA and Mayhew E, 2013. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: A practical lab guide. Onderstepoort J Vet Res 80:539. https://doi.org/10.4102/ ojvr.v80i1.539

Valente AH, Roode MD, Ernst M, *et al*., 2021. Identification of compounds responsible for the anthelmintic effects of chicory (*Cichorium intybus*) by molecular networking and bio-guided fractionation. Int J Parasitol Drugs Drug Resist 15:105–14. https://doi.org/10.1016 /j.ijpddr.2021.02.002

Veerakumari L. 2015. Botanical anthelmintics. Asian J Sci Technol 6:1881-1894. ISSN: ISSN: 0976-3376

Williams EG, Brophy PM, Williams HW, *et al*., 2021. Gastrointestinal nematode control practices in ewes: identification of factors associated with application of control methods known to influence anthelmintic resistance development. Vet Parasitol Reg Stud Reports 24:100562. https://doi.org/10.1016/j.vprsr.2021.100562

Zajac AM and Garza J, 2020. Biology, epidemiology, and control of gastrointestinal nematodes of small ruminants. Vet Clin North Am Food Anim Pract 36:73-87. https://doi.org/10.1016/j.cvfa.2019. 12.005

Zhai H, Liu H, Wang S, *et al*., 2018. Potential of essential oils for poultry and pigs. Anim Nutr 4:179–86. https://doi.org/10.1016/j.aninu. 2018.01.005