

The phytotherapy properties of winter savory (*Satureja montana* L.) essential oil against gastrointestinal nematodes of sheep

Filip Štrbac

`filip.strbac@imsi.bg.ac.rs`

Institute for Multidisciplinary Research, University of Belgrade

Slobodan Krnjajić

Institute for Multidisciplinary Research, University of Belgrade

Radomir Ratajac

Scientific Veterinary Institute Novi Sad

Laura Rinaldi

Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR

Vincenzo Musella

Department of Health Sciences, University of Catanzaro Magna Græcia

Fabio Castagna

Department of Health Sciences, University of Catanzaro Magna Græcia

Dragica Stojanović

Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad

Nataša Simin

Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad

Dejan Orčić

Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad

Antonio Bosco

Department of Veterinary Medicine and Animal Production, University of Naples Federico II, CREMOPAR

Research Article

Keywords: anthelmintic resistance, gastrointestinal nematodes, *satureja montana*, phytotherapy, essential oils, integrated control

Posted Date: July 10th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4576907/v1>

License:  This work is licensed under a Creative Commons Attribution 4.0 International License.
[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background: The increasing difficulties in combating anthelmintic resistance in gastrointestinal nematodes (GINs) of sheep worldwide and the residues of chemical drugs in animal products and the environment necessitate the search for alternatives. Previous studies have shown that plant essential oils (EOs) could be valuable anthelmintic agents. The aim of this study was to evaluate the possibility of using winter savory (*Satureja montana* L.) EO against sheep GINs through performing *in vitro* and *in vivo* efficacy tests and preliminary toxicity studies.

Methods: The chemical composition of the *S. montana* EO was determined by gas chromatography-mass spectrometry (GC-MS). For the evaluation of anthelmintic potential *in vitro*, the Egg Hatch Test (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). Field efficacy was evaluated using the Faecal Egg Count Reduction Test (FECRT) on two farms where the oil was applied intraruminally to sheep at a mean dose of 150 mg/kg. The toxic effect of the oil was evaluated by physical observation of the tested animals and by collecting blood samples for further haematological and biochemical analyses. Coproculture examination of faecal samples from the tested sheep before and after treatment was also performed to identify the GIN genera present.

Results: Main compounds of the *S. montana* oil identified by GC-MS analysis were *p*-cymene (42.8%), carvacrol (28.1%) and γ -terpinene (14.6%). The *in vitro* EHT showed a dose-dependent ($R^2=0.94$) anthelmintic potential of the tested oil with ovicidal activity varying from 17.0-83.3% and determined IC_{50} value of 0.59 mg/ml. The field efficacy reached 33% (at group level) and 50% (at individual level) at D14 after treatment. *In vivo* efficacy was significantly higher in farm 2 (FEC above 65% at group level, $p<0.05$) where sheep were kept in pens during treatment. No toxic effects were observed either in the physical observation of the test animals or in their liver and kidney function.

Conclusion: The moderate efficacy on EHT and FECRT, without adverse effects on the sheep, suggests that *S. montana* EO is suitable for the control of sheep GINs as part of an integrated parasite management. Further studies should be conducted to further increase efficacy.

INTRODUCTION

As a valuable source of meat, milk (and its products), wool and manure, sheep represent an important sector of the livestock industry worldwide (Desalegn and Berhanu, 2023). This is especially true given the increasing demand for food for the growing human population (van Dijk et al., 2021). However, modern sheep farming is facing an increasing problem of infections caused by gastrointestinal nematodes (GINs), which are considered as a major obstacle to sustainable sheep production from both a health and economic perspective (Tachack et al., 2022; Maurizio et al., 2023). Depending on the level of worm burden, these parasites can have various negative effects on sheep. These include reduced appetite and feed intake, weight loss, anaemia, diarrhoea and hypoproteinaemia resulting in reduced productivity, immunity and fertility even death in cases of high parasite infection (Desalegn and Berhanu, 2023; Bosco

et al., 2020). All of this can lead to enormous economic losses, estimated at hundreds of millions of euros per year (Charlier et al., 2020). In addition, the occurrence of GIN infections is predicted to increase greenhouse gas emissions by up to 30% (Fox et al., 2018; Rose Vineer et al., 2020).

Therefore, it is clear that the issue of controlling these parasites is a top priority, which requires the development of suitable strategies. To date, heavy reliance has been placed on the use of commercial anthelmintics from different chemical classes (benzimidazoles, macrocyclic lactones and levamisoles) in attempt to maintain the number of parasites below levels that can cause disease, whether clinical or subclinical (Rose Vineer et al., 2020). However, the extensive and inappropriate use of these drugs has led to the emergence and development of anthelmintic resistance (AR) in nematodes to all major groups of drugs, sometimes simultaneously to several different classes (Ahbara et al., 2021; de Agüero et al., 2023). The estimated annual cost due to AR development in Europe alone is amounted to €38 million and rising, with underdosing, overfrequent treatments, mass treatment, and single-drug regimens being the main risk factors (Rose Vineer et al., 2020; Kaplan, 2020). In addition, the exclusive use of chemical drugs is associated with the occurrence of residues in animal products and in the environment, leading to growing concerns about food safety and public health concerns (de Agüero et al., 2023; Castagna et al., 2022a; Ragusa et al., 2022). All of these justifies the search for alternative solutions and their utilization in the future management of GIN infections.

Medicinal plants have been used worldwide for thousands of years as a valuable source for the treatment of various diseases in humans and animals (Varshney et al., 2022). Although these practices have been replaced by modern medicine, interest in phytotherapy has increased recently for many reasons. They are generally considered to be effective while being safer and cheaper than chemical medicines (Romero et al., 2022). For these reasons, herbal medicines may be a suitable option (especially for the treatments in organic livestock) and generally help to reduce the use of commercial antibiotics and antiparasitics (Shao et al., 2021; Romero et al., 2022; Bava et al., 2023). EOs represent plant secondary metabolites, i.e. volatile and aromatic components obtained from plants through well-regulated extraction processes (Napoli and Di Vito, 2021). Therefore, they contain various bioactive substances with great pharmacological potential that can be exploited for various indications in veterinary medicine (Ebani and Mancianti, 2020; Mucha and Witkowska, 2021; Bava et al., 2023; Ratajac et al., 2024). Moreover, EOs from various plants have already been investigated for their anthelmintic activity against GIN in sheep, with some of them showing promising results (André et al., 2018; Štrbac et al., 2022b).

In this context, winter or mountain savory (*Satureja montana* L. *sensu lato*) is a perennial, aromatic wild shrub that grows along or near the Adriatic coast, but also in the Pyrenees (Aćimović et al., 2022). It is reported in many countries in this region including Serbia (Aćimović et al., 2019) and Italy (Tomaselli et al., 2021). *S. montana* is a well-known medicinal plant with a high biological potential that has been used in traditional medicine. Thus, its antimicrobial, antioxidant, digestive, antidiuretic and other properties have been used for the traditional treatment of various ailments such as infectious diseases, diarrhea, muscle pain, nausea, etc. (Maccelli et al., 2020; Matejić et al., 2020). In addition, winter savory is used in

the food industry as a spice and flavoring agent (Aćimović et al., 2022). However, the anthelmintic potential of this plant against gastrointestinal nematodes in sheep has not yet been fully explored. Therefore, the aim of this study was to evaluate the possibility of using *S. montana* EO against gastrointestinal nematodes in sheep.

MATERIAL AND METHODS

2.1. Chemical composition

The essential oil of *Satureja montana* (L.) was obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. The chemical composition (qualitative and semi-quantitative characterization) of the tested oil was determined by gas chromatography-mass spectrometry (GC-MS) at the Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Serbia. Analyses was performed using an Agilent Technologies gas chromatograph coupled with an Agilent Technologies 5975B electron ionization mass-selective detector using the technical conditions as described by Knežević et al. (2016). Data were acquired in scan mode (m/z range 35–400), with solvent delay of 2.30 min, and processed using Agilent Technologies MSD ChemStation software (revision E01.01.335) combined with AMDIS (ver. 2.64) and NIST MS Search (ver. 2.0d). 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). The relative amount of each component is expressed as a percentage of its peak area relative to the total peak area.

2.2. Experimental animals

The remaining analyses were carried out at the Regional Center for Monitoring Parasitic Infections (CREMOPAR, Campania region, southern Italy). Two farms in this region with a previously identified high prevalence of GIN were used for the trials, where the animals were kept in free range (Farm 1) and in the boxes (Farm 2). Thus, sheep with natural mixed infection, mainly a mixture of Lacaune and Bagnolese dairy breeds, of similar age (2 ± 0.5 years), grazing season and body weight (b.w.) of 50 ± 5 kg were used for these studies. However, animals with different worm burdens (up to 3640 EPG) were selected to simulate natural conditions. They were fed with pasture and forage (barley and maize grains) with no changes during the trial. The animals were fasted before and 2h after the application of the treatments, as is usual when using commercial anthelmintics.

2.2. In vitro test

Anthelmintic potential *in vitro* of *S. montana* EO was evaluated using the Egg Hatch Test (EHT), while the recovery method described by Bosco et al. (2018, 2020) was used to obtain GIN eggs. For this purpose, faecal samples ($n = 30$) were collected directly from the rectal ampulla of randomly selected sheep and processed within 2h of collection. To isolate GIN eggs, faecal samples were pooled, homogenised and then filtered under running water through meshes of 1 mm, 250 μ m, 212 μ m and 38 μ m. Subsequently,

the eggs retained at the last size were washed with distilled water and centrifuged at 1500 RPM for 3 min, and the supernatant was discarded. The eggs were then floated by centrifugation with 40% sugar solution, isolated into new tubes and mixed with distilled water. Two more centrifugations were performed to remove the pellets, after which an aqueous solution containing GIN eggs was obtained.

As in our previous studies (Štrbac et al., 2023a; Štrbac et al., 2023b), 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) of the tested oil. Twenty-four well plates were used for the experiments, whereby different oil concentrations were emulsified in Tween 80 (3%, v/v) and added to wells containing aqueous solutions of approximately 150 eggs/well. The positive control was thiabendazole at the two lowest concentrations used for EO, and the negative controls were emulsifier and distilled water. All wells were completed with distilled water to obtain a final volume of 0.5 ml/well, and incubated at 27°C for 48h. The incubation was stopped with Lugol's solution, after which the eggs and first-stage larvae (L₁) were counted under an inverted microscope. The experiment was performed in three replicates, and the values obtained were expressed as the arithmetic mean of the inhibition of egg hatchability for each concentration.

2.3. In vivo test

For this test, *Satureja montana* EO was diluted in sunflower oil (1:4.5) to avoid the effects of the pure oil on the gastrointestinal mucosa. The field trial was conducted using the faecal egg count reduction test (FECRT) on two different farms. In each of them, the animals (n = 36/farm, 72 in total) were divided into three groups which were treated as follows:

G1: *Satureja montana* EO, 150 mg/kg (n = 12)

G2: Albendazole, 3.8 mg/kg (n = 12), positive control

G3: Sunflower oil, 50 ml (n = 12), negative control

All treatments were administered directly into the rumen of the animals using a tube inserted through the mouth and oesophagus (intraruminal application). Individual faecal samples were collected rectally at Day 0, D7 and D14 after treatments and stored at 4°C before processing. For counting of eggs, novel Mini-FLOTAC technique (Cringoli et al., 2017) was used with a detection limit of 5 eggs per gram (EPG) of faeces, and using a sodium chloride flotation solution (specific gravity = 1.200).

The farms were chosen based on previously determined occurrence of natural-mixed GIN infections. On both farms, animals for the trial were randomly selected for each group and thus were differ in worm burden to completely simulate the conditions in the practice. As the EPGs of the selected animals varied greatly, the FECRT was performed at both group (by comparing average group EPGs after and before treatments in each group) and individual level (by calculating the EPG reductions for each examined animal separately), as further described in subsection 2.6. In this way, it was possible to see the effect of the tested essential oil on reducing the number of GIN eggs in animal faeces in more detail and to compare it with the controls.

2.4. Clinical observation, haematological and biochemical blood analyses

All treated animals were observed clinically at the sampling times (D0, D7 and D14) for the presence of adverse effects of the administered EO, with particular attention paid to their feed, defecation, and behaviour. In addition, blood samples were taken from randomly selected animals from each group (two farms, $n = 6/\text{group}$, 36 in total) at D0 and D14 to assess the effects of the EO on blood parameters. For haematological parameters, samples were placed in EDTA-containing vacuum tubes and processed within 2–4 hours. This evaluation reflected the potential presence of toxic effects of the applied EO, but also its impact on reducing signs of anaemia caused by blood-sucking nematodes. In contrast, blood samples were collected for biochemical analyses in empty vacuum tubes and subsequently analysed, and these analyses reflected the effects of EO on kidney (urea, creatinine) and liver (aspartate aminotransferase, AST, and gamma-glutamyl transferase, GGT) function.

2.5. Coproculture examination

For the identification of GIN genera present on the tested farms, coproculture examination was performed following the protocol developed by the UK Ministry of Agriculture, Fisheries and Food (1986). For each of the groups for the *in vivo* test (*S. montana* EO, albendazole and sunflower oil), an equal amount of faeces was collected to form a pool for coproculture study. These were performed for each of the sampling time points (D0, D7 and D14) to evaluate the effects of the treatments on the percentage representation of the nematode genera. Third-stage (L_3) larvae developed were identified using the morphological determination keys proposed by van Wyk and Mayhew (2013). The identification and percentages of each nematode genera were performed on 100 L_3 , identifying all larvae if the sample contained 100 or fewer L_3 . In this way, it was possible to determine the percentage of each GIN genus in the total number of identified larvae.

2.6. Statistical analyses

The inhibitions of egg hatchability (IH) in the *in vitro* test were calculated using the following formula (Coles et al., 1992; Pinto et al., 2019):

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

To evaluate the significance of the differences ($p < 0.05$) between the values obtained for different EO concentrations with each other and with controls, a One-way Analysis of Variance (ANOVA) with post-hock Tuckey's test was performed. In addition, a nonlinear regression/logarithmic distribution was applied to determine the half-maximal inhibitory concentration (IC_{50}) and to evaluate the presence of a dose-dependent effect (Ferreira et al., 2018).

The reductions in the faecal egg counts in the *in vivo* test were calculated using the following formula (Coles et al., 1992, Macedo et al., 2019):

$$\text{FECR (\%)} = [1 - (T_2/T_1 \times C_1/C_2)] \times 100$$

In this formula on the group level, T_1 and T_2 represent the average EPGs (arithmetic mean $\pm$ standard deviation) before (D0) and after treatment (D7 or D14) in the *S. montana* EO or albendazole group, while C_1 and C_2 represent the average EPGs before (D0) and after treatment (D7 or D14) in the group that received sunflower oil. Results were analysed using a two-way ANOVA followed by Tukey's test to evaluate the presence of significant differences ($p < 0.05$) in EPG values obtained within one group and farm on different time points, to compare EPGs in different groups at the same time point and farm, but also to compare EPGs between two farms. At the individual level, the formula was applied for each animal separately in the *S. montana* EO or albendazole group, where T_1 and T_2 represents the EPGs of certain animal before (D0) and after treatment (D7 or D14), and the correction (C_1 and C_2) was the same as at the group level. Efficacy in both the EO and positive control groups was expressed as the median value of individual reductions. At both group and individual level, the final results represent the mean value from both farms.

Finally, the results of the blood analyses were also analysed using two-way ANOVA. The post-hock Sidak's test ($p < 0.05$) was used to compare the values of individual parameters in the same group on D0 and D14. In contrast, for the comparison of the values of individual parameters obtained in different groups on the same day, the post-hock Tukey's test was performed ($p < 0.05$).

RESULTS

3.1. Chemical composition

A total of 17 compounds belonging to different chemical groups were identified by GC-MS analysis (Table 1). The most abundant group of compounds were hydrocarbon monoterpenes, i.e. p-cymene (42.8%), γ -terpinene (14.6%), β -caryophyllene (2.46%), α -terpinene (2.32%), limonene (1.52%) and α -pinene (1.16%). However, the a phenolic terpenoid carvacrol (28.1%) and the alcoholic terpenoids borneol (1.27%) and linalool (1.20%) were also represented. The rest of the compounds were present in less than 2%.

Table 1
Chemical composition (% of total peak area) of the *Satureja montana* (L.) essential oil identified by gas chromatography-mass spectrometry analysis

AI	Compound	% of total peak area
925	α -Thujene	0.33
932	α -Pinene	1.16^a
947	Camphene	0.44
976	β -Pinene	0.66
990	β -Myrcene	0.96
1005	α -Phellandrene	0.18
1016	α -Terpinene	2.32
1024	p-Cymene	42.8
1027	Limonene	1.52
1030	1,8-Cineole	0.73
1057	γ -Terpinene	14.6
1088	α -Terpinolene	0.17
1100	Linalool	1.20
1164	Borneol	1.27
1176	Terpinen-4-ol	0.78
1302	Carvacrol	28.1
1418	β -caryophyllene	2.46
Total % of identified compounds		99.7
*AI - arithmetic retention index; ^a - compounds with abundance > 1% are written in bold		

3.2. In vitro egg hatch test

In the EHT, *S. montana* EO showed moderate ovicidal activity with an inhibition of egg hatchability that varied from 17.3 to 83.0%, depending on the concentration used (Table 2). The effect was dose-dependent ($R^2 = 0.94$) with a determined IC_{50} value of 0.59 mg/ml (Graph 1). At most of the concentrations tested, the activity of the tested EO was significantly higher than that of the two negative controls ($p < 0.05$).

Table 2
The inhibitory effect (mean $\pm$ standard deviation) of different concentrations of the *Satureja montana* L.) essential oil on egg hatching of gastrointestinal nematodes in sheep

Concentration of EO [mg/ml]	Inhibition of hatchability (%)
50	83.0 $\pm$ 2.00 ^A
12.5	70.0 $\pm$ 2.65 ^B
3.125	61.0 $\pm$ 2.00 ^C
0.781	56.7 $\pm$ 1.53 ^C
0.195	48.0 $\pm$ 1.00 ^D
0.049	22.0 $\pm$ 1.00 ^E
0.025	20.0 $\pm$ 1.00 ^{EF}
0.0125	17.3 $\pm$ 2.52 ^{EF}
Control (+) ^a	96.3 $\pm$ 1.53 ^G
Control (+) ^b	95.0 $\pm$ 1.00 ^G
Control (-) ^a	14.2 $\pm$ 3.34 ^F
Control (-) ^b	6.6 $\pm$ 1.92 ^H

*Uppercase compares means between different concentrations and controls. Different letters indicate significant differences ($p < 0.05$). Control (+)^a - thiabendazole, 0.025 mg/mL; control (+)^b - thiabendazole, 0.0125 mg/mL; control (-)^c - 3% Tween 80, v/v; control (-)^d - distilled water; EO - essential oil.

3.3. In vivo faecal egg count reduction test

Similar to EHT, *S. montana* EO also showed moderate anthelmintic activity in the field trial with an average group EPG reduction of 15.7% and 33.0% in total at D7 and D14 post-treatment, respectively (Table 3). However, the effect was significantly higher on Farm 2 ($p < 0.05$), where the EPG reduction at group level reached 68.3% at D14 after treatment. Also, the average EPG value at this time point was significantly lower when compared to the pre-treatment value ($p < 0.05$) (Graph 2). The average individual reduction was higher than that of the group level and reached 18.9% and 50.0% on D7 and D14 post-treatment, respectively. In the albendazole-treated group, there was significant group reduction of EPG ($p < 0.05$). However, the average individual efficacy at D14 was below 95%.

Table 3

Average (arithmetic mean $\pm$ standard deviation) reduction of eggs per gram of gastrointestinal nematodes in sheep treated with *Satureja montana* essential oil – in total from both examined farms

Treatment		Day 0	Day 7	Day 14
<i>S. montana</i> EO, 150 mg/kg	EPG	682.1 $\pm$ 632.9 ^{Aa}	586.7 $\pm$ 552.9 ^{Aa}	434.9 $\pm$ 404.9 ^{Aa}
	FECR _(group)	/	15.7%	33.0%
	FECR _(individual)	/	18.9%	50.0%
Albendazole, 3.8 mg/kg (C+)	EPG	683.1 $\pm$ 735.5 ^{Aa}	9.38 $\pm$ 11.8 ^{Bb}	36.9 $\pm$ 41.6 ^{Bc}
	FECR _(group)	/	99.0%	95.1%
	FECR _(individual)	/	97.5%	93.4%
Sunflower oil, 50 ml (C-)	EPG	914.0 $\pm$ 821.9 ^{Aa}	914.4 $\pm$ 748.8 ^{Aa}	800.5 $\pm$ 680.3 ^{Aa}

* Uppercase compares means between different groups at one time point; lowercase compares means of different time points within one group. Different letters indicate significant differences ($p < 0.05$); EPG - eggs per gram; EO – essential oil.

3.4. Clinical observation, haematological and biochemical blood analyses

No side effects were observed during clinical observation of the examined animals. The haematological analyses showed that there were no significant changes in the values of the tested parameters after any of the applications ($p > 0.05$). Moreover, the applied *S. montana* EO, as well as albendazole, reduced to some extent the signs of anaemia observed in the animals by slightly increasing the levels of RBC, Hgb and HCT (Additional file 1). In the biochemical analyses, no significant increase was observed in any of the parameters tested ($p > 0.05$). In fact, the mean value of GGT at D14 was significantly lower than the pre-treatment value ($p < 0.05$) (Table 4).

Table 4

Effect of the application of *Satureja montana* essential oil on the biochemical blood parameters indicating renal and hepatic function in the tested animals

Treatment	Day	Urea (mg/dl)	Creatinine (mg/dl)	AST (UI/l)	GGT (UI/l)
<i>S. montana</i> EO	Day 0	31.0 ± 8.74 ^{Aa}	14.1 ± 3.03 ^{Aa}	180.3 ± 61.10 ^{Aa}	71.8 ± 7.62 ^{Aa}
	Day 14	30.8 ± 6.98 ^{Aa}	11.8 ± 3.41 ^{Aa}	157.8 ± 42.10 ^{Aa}	65.0 ± 3.77 ^{Bb}
Albendazole (C+)	Day 0	27.6 ± 9.45 ^{Aa}	12.4 ± 2.75 ^{Aa}	173.3 ± 82.72 ^{Aa}	72.0 ± 8.86 ^{Aa}
	Day 14	28.4 ± 6.07 ^{Aa}	13.4 ± 3.68 ^{Aa}	163.0 ± 50.72 ^{Aa}	66.5 ± 5.54 ^{Bab}
Sunflower oil (C-)	Day 0	29.8 ± 7.87 ^{Aa}	12.4 ± 2.84 ^{Aa}	189.7 ± 72.63 ^{Aa}	72.5 ± 7.82 ^{Aa}
	Day 14	29.3 ± 4.91 ^{Aa}	12.8 ± 3.04 ^{Aa}	206.9 ± 56.32 ^{Aa}	73.7 ± 5.30 ^{Aa}

* Uppercase compares means between values at different time points within one treatment group and lowercase values at the same time points between different treatment groups. Different letters indicate significant differences ($p < 0.05$); AST - aspartate aminotransferase, GGT - gamma-glutamyl transferase; EO - essential oil.

3.5. Coproculture examination

Four genera of sheep GINs were identified on both tested farms during the coproculture examination. In total, they were represented on D0 as follows: *Haemonchus* 45%, *Trichostrongylus* 32%, *Teladorsagia* 19% and *Chabertia* 4%. After treatment, the percentages changed slightly, but with no difference between the groups ($p > 0.05$). Similarly, changes observed before and after treatment were not statistically significant in any of the tested groups ($p > 0.05$). The percentages of each GIN genera in each *in vivo* treatment group in both farms after treatment (D7 and D14) are shown in Tables 5 and 6.

Table 5
Distribution of sheep nematode third-stage larvae (L₃) genera (%) at different time points (D0, D7 and D14) on Farm 1

Day	Group	<i>Haemonchus</i>	<i>Trichonstrogylus</i>	<i>Teladorsagia</i>	<i>Chabertia</i>	Total
0	<i>Satureja montana</i> EO	51	22	23	4	100
	Albendazole (Control +)	42	26	28	4	100
	Sunflower oil (Control -)	48	21	30	1	100
	Mean	47.0 ± 4.58	23.0 ± 2.65	27.0 ± 3.61	3.00 ± 1.73	100
7	<i>Satureja montana</i> EO	53	26	18	3	100
	Albendazole (C+)	56	21	15	8	100
	Sunflower oil (C-)	46	29	22	3	100
	Mean	51.7 ± 5.13	25.3 ± 4.04	18.3 ± 3.51	4.67 ± 2.89	100
14	<i>Satureja montana</i> EO	52	23	17	8	100
	Albendazole (C+)	44	30	21	5	100
	Sunflower oil (C-)	49	25	19	7	100
	Mean	48.3 ± 4.04	26.0 ± 3.61	19.0 ± 2.00	6.67 ± 1.53	100

* No statistically significant differences were found when comparing the values of different treatments within one time point, nor comparing the values obtained at different time points within one treatment ($p < 0.05$); EO – essential oil.

Table 6
Distribution of sheep nematode third-stage larvae (L₃) genera at different time points (D0, D7 and D14)
on Farm 2

Day	Group	<i>Haemonchus</i>	<i>Trichonstrogylus</i>	<i>Teladorsagia</i>	<i>Chabertia</i>	Total
0	Satureja montana EO	41	45	11	3	100
	Albendazole (Control +)	38	42	19	1	100
	Sunflower oil (Control -)	43	35	20	2	100
	Mean	40.7 ± 2.52	40.7 ± 5.13	16.7 ± 4.93	2.00 ± 1.00	100
7	Satureja montana EO	39	43	15	3	100
	Albendazole (C+)	42	38	20	0	100
	Sunflower oil (C-)	38	41	16	5	100
	Mean	39.7 ± 2.08	40.7 ± 2.52	17.0 ± 2.65	2.67 ± 2.52	100
14	Satureja montana EO	37	45	13	5	100
	Albendazole (C+)	41	39	19	1	100
	Sunflower oil (C-)	37	43	18	2	100
	Mean	38.3 ± 2.31	42.3 ± 3.06	16.7 ± 3.21	2.67 ± 2.08	100

* No statistically significant differences were found when comparing the values of different treatments within one time point, nor comparing the values obtained at different time points within one treatment ($p < 0.05$); EO – essential oil.

DISCUSSION

The search for new anthelmintics requires the use of appropriate methods to test their efficacy. From this point of view, the EHT, which is used to study *in vitro* the ovicidal activity of drugs, offers comparable, reliable and precise results and has an advantage over other tests due to its higher sensitivity. It is suitable for an initial assessment of anthelmintic potential and forms a basis for other tests. For this reason, it is the most commonly used *in vitro* test for the evaluation of the anthelmintic activity of drugs, i.e. benzimidazoles, and for the detection of resistance (Babják et al., 2021). In the EHT performed in the present study, the EO of *S. montana* showed moderate ovicidal activity varying from 17.3–83.0%, with an IC₅₀ value of 0.59 mg/ml. Although the maximum inhibitory effect (100%) on hatching of GINs eggs was

observed in our previous study (Štrbac et al., 2022a), the IC_{50} value obtained in the present study is still high compared to oils from other studies (André et al., 2018; Štrbac et al., 2022b), indicating its anthelmintic potential. Indeed, this parameter is considered suitable for comparing drug-inhibitory effect of different active substances (Berrouet et al., 2020).

The results obtained for two samples of *S. montana* EO in present and the previous study (Štrbac et al., 2022a) indicate a high variability in the efficacy of EOs, since both samples were obtained from the same producer and had the same chemical composition. However, in addition to the known factors that can influence the presence and abundance of the compounds (geographical origin of the plant, light, rainfall, soil conditions, age and part of the plant, genetic characteristics, presence of certain organisms and microorganisms, etc.) and thus their effect, many other factors related in post-extraction period may also be involved. That especially refers to the way the EOs are stored and the length of time before they are used, as they are sensitive to light, temperature and oxygen (Fokou et al., 2020; Cimino et al., 2021). The experimental parameters may also influence the results, as the method used to obtain the different concentrations was different, with the dilution method being used in the present study and highly sensitive micropipettes being used in the last one.

On the other hand, *in vivo* FECRT is considered the gold standard for the evaluation of drug efficacy and the detection of AR and is intended for all anthelmintic classes (Babják et al., 2021). It is the most commonly used method for evaluating the anthelmintic efficacy of drugs in practice (Denwood et al., 2023). Examination of the effect of *S. montana* EO *in vivo* was performed for the first time to our knowledge, and it also showed moderate activity with a total reduction of 33.0% on D14 after treatment. The overall efficacy of the entire formulation (EO + sunflower oil for dilution) was 40.0%. While in Farm 1 the applied oil was less effective, in Farm 2 it significantly reduced the EPG on D14 after treatment and reached an efficacy of 68.3%. A similar trend was also observed in our previous studies (Štrbac et al., 2023a; Štrbac et al., 2023b) conducted with oregano and mint EOs, where these oils were also much more effective in farm 2. This can be explained by the differences in animal husbandry (free range in comparison with the boxes), which affect the manipulation of the animals during treatment, but may also have an influence on the gastrointestinal tract of animals in general due to differences in their feeding. However, the average individual reduction was higher than that at the group level, reaching 50% in total on D14, suggesting that EOs had an effect in most of the tested animals. Preliminary toxicity studies indicate the safety of the use of winter savory in practice.

The lower efficacy *in vivo* of EOs in general, including *S. montana*, can be attributed to their unstable nature as their ingredients are prone to degradation and evaporation, which causes limited bioavailability (Cimino et al., 2021; Nehme et al., 2021). On the other hand, complexity of gastrointestinal tract of ruminants that hinder per oral applications is also involved (Hoste et al., 2008). Both of this can be improved by the use of encapsulation techniques that can protect the main active ingredients that are sensitive to various factors (oxygen, light and moisture), and prevent interaction with other compounds. In this way, the stability and bioavailability of EOs can be further increased, while reducing toxicity, volatility, odour and taste. Also important, encapsulation enables a controlled release of the active

ingredients of EOs along the gastrointestinal tract (Cimino et al., 2021; Sousa et al., 2022; Yammine et al., 2023). As the dose tested did not cause any adverse effects in the animals, increasing the dose or multiple applications during several consecutive days could also be an option to improve efficacy under field conditions. Alternatively, other forms of EO application such as drenching (Ahmed et al., 2014) or the use of lick blocks with herbal compounds (Junkuszew et al., 2015) should also be considered.

According to the results of GC-MS analysis, the most important compounds responsible for the anthelmintic properties of winter savory are p-cymene, carvacrol and γ -terpinene. In a study by André et al. (André et al., 2016), the isolated carvacrol showed high anthelmintic potential in various *in vitro* tests, including the larval development test (LDT) and the adult worm motility test (AWMT) in addition to the EHT. This indicates that this compound is effective against different parasite stages (eggs, larvae and adult worms). Moreover, in the same study, its acetylated derivative reduced the group EPG by 65.9% at D16 after treatment in the FECRT. Its strong anthelmintic activity was also confirmed in a study by Katiki et al. (2017), in which it showed one of the highest ovicidal effects against *H. contortus* eggs compared to other compounds. P-cymene and γ -terpinene were not individually tested for their anthelmintic activity against GINs, but were present in some EOs with this property: p-cymene at 22.56% in *Alpinia zerumbet* (Macedo et al., 2013) and at 23.76% in *Thymus vulgaris* (Ferreira et al., 2016) as well as γ -terpinene at 20.15% in *Melaleuca alternifolia* (Grando et al., 2015) and at 11.42% in *Citrus aurantifolia* (Ferreira et al., 2018).

Not only the presence but also the percentage of the compounds in the EOs is important for the pharmacological effects. Thus, these three compounds were also the main constituents of the previously tested oregano EO (Štrbac et al., 2023a), but in a different ratio: carvacrol 76.21%, p-cymene 12.57% and γ -terpinene 2.63%. However, oregano showed a superior effect with an average group EPG reduction of 43.21% and 60.13% at D7 and D14 after treatment, respectively. This could indicate a superior activity of carvacrol compared to p-cymene, although this statement needs to be confirmed by individual testing and comparison of these compounds. Indeed, individual compounds can differ considerably in their anthelmintic activity (Katiki et al., 2017). Moreover, the dominance of one compound and its appropriate abundance (at least > 50%) with the simultaneous presence of other compounds but in lower percentages, may lead to high activity, as shown by the results of a previously conducted study with eleven EO tested *in vitro* (Štrbac et al., 2022a). These considerations could be useful for the future development of herbal anthelmintics.

Plant-based formulations have many advantages that can be exploited for their use in animals for various indications, including the control of GINs (Bava et al., 2021; Castagna et al., 2021; Castagna et al., 2022b). The compounds that compose them have a wide variety of pharmacological effects, belong to different chemical groups with possibly different mechanisms of action, and have therefore already shown the effect against various parasite stages of GINs (André et al., 2018; Štrbac et al., 2022b). This may also contribute to their less susceptibility to resistance development in comparison with synthetic drugs (Borges and Borges, 2016; Ferreira et al., 2018). Botanical drugs are also considered safer than synthetic, whereby environmental aspect and public health also favouring natural drugs due to their

biodegradability, to challenge the residue problem (Veerakumari, 2015; Castagna et al., 2022a; Romero et al., 2022). Finally, the price of plant drugs is also considered reasonable and even cheaper than that of commercial drugs, so the procurement of these medicines should not be a problem, especially in countries with developed biodiversity (Ferreira et al., 2018), as in the case of *S. montana* in Serbia (Šubarević et al., 2015).

Novel anthelmintics can be used in different ways to control sheep GINs. They can be used independently, but only if they achieve sufficient efficacy under field conditions. Although a threshold of 95% is used to assess efficacy and the development of potential resistance to commercial anthelmintics in ruminants (Kaplan, 2020), some authors recommend a minimum efficacy of 90% in FECRT for new anthelmintic agents (Geurden et al., 2022). Thus, *S. montana* as well as EOs from other studies, are not yet suitable for independent use in the doses, formulations and route of administration examined. However, other alternative methods such as genetic selection of animals that are naturally resistant to nematodes (Tsukahara et al., 2021), pasture management and nutritional manipulations (Yoshihara et al., 2023), biological control methods (direct – use of nematophagous fungi, bacteria or even other nematodes or indirect – use of deep buntle or earthworms) (Szewc et al., 2021) and development of vaccines (Liu et al., 2023) also have some limitations despite their numerous advantages. Finally, the exclusive use of commercial drugs is also no longer suitable due to the development and spread of resistance, as well as residue issues that concerns public health and environment (Castagna et al., 2022a; Maestrini et al., 2022; de Agüero et al., 2023), as already mentioned.

Therefore, there is a broad consensus that integrated parasite management, which refers to the use of several appropriate methods of parasite control, represent the most appropriate solution for the future management of GINs in sheep (Maqbool et al., 2017; Tariq et al., 2017; Mondragón-Ancelmo et al., 2019). In this system, various plant-based anthelmintics, including EOs, may represent a valuable source in combination with other mentioned alternatives. They also can be combined with commercial drugs whose use should be based on target treatments or target selective treatments (Maurizio et al., 2023). Thus, the combination of the ethanol extract of *Ananas comosus*, drenched weekly for 6 weeks using a stomach tube (100 mg/kg), with 1 g of the fungal product *Clonostachys rosea* given daily, effectively reduced faecal egg and larva counts, larval development and the number of infectious larvae (L₃) in the pasture (Ahmed et al., 2014). Carvacrol has also been shown to enhance the effect of drugs agonists of nAChR (imidazothiazoles) or agonists of GABA receptors (ivermectins and piperazine), and can therefore be considered for use with these drugs to achieve a synergistic effect (Trailović et al., 2015; Marjanović et al., 2020). In contrast, the results of a study by Mesquita-Sousa et al. (2023) showed that the combination of *Citrus aurantium* var. *Dulcis* EO and albendazole has antagonistic effect against benzimidazole resistant *Haemonchus contortus*, which is also possible and should be considered.

CONCLUSION

Novel anthelmintic measures are needed due to problems associated with the development of anthelmintic resistance as well as the concerns about residues of commercial drugs in animal products

and the environment. From this point of view, botanical anthelmintics have numerous advantages that make them a valuable alternative. The results of the efficacy and safety tests performed in the present study indicate that *S. montana* EO may be used in the control of GINs in sheep as part of an integrated parasite management. A consecutive application over several days, an increase in dose, a different route of administration or an encapsulation form of the tested oil should also be investigated to further increase its efficacy under field conditions.

Declarations

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).

Consent for publication

Not applicable

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Involvement of a human participant or wild plant

The authors declare that neither human participants nor protected wild plants were involved in this study.

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' contributions

FŠ, RR, DS and SK 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, SK, RR and LR were responsible. The experiment was conducted as follows: chemical analyses – NS, DO; *in vitro* and *in vivo* efficacy tests – FŠ, AB; preliminary toxicity studies – FŠ, AB; coproculture – AB and LR. The experimental part was supervised by LR, SK, DS, FC and VM. The results obtained were interpreted by FŠ, AB, RR, DS

and NS and the statistical analyses were performed by FŠ. The original manuscript was drafted by FŠ, revised by AB, LR and NS and approved by all co-authors.

Acknowledgements

We deeply thank Mario Parrilla for the technical support in the laboratory.

References

1. Aćimović M, Todosijević M, Varga A, Kiproviski B, Tešević V, Čabarkapa I, Sikora V (2019) Bioactivity of essential oils from cultivated winter savory, sage and hyssop. *Lek Sirov* 39:11–17. <https://doi.org/10.5937/leksir1939011A>
2. Aćimović M, Šovljanski O, Pezo L, Travičić V, Tomić A, Zheljazkov VD, Četković G (2022) Variability in biological activities of *Satureja montana* Subsp. *montana* and Subsp. *variegata* based on different extraction methods. *Antibiotics* 11:1235. <https://doi.org/10.3390/antibiotics11091235>
3. Adams RP (2012) Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th edn. Allured Business Media, Carol Stream, IL, USA
4. Ahbara AM, Rouatbi M, Gharbi M, Rekik M, Haile A, Rischkowsky B, Mwacharo JM (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>
5. Ahmed M, Laing MD, Nsahlai IV (2014) A new control strategy for nematodes of sheep using chlamydospores of a fungus, *Clonostachys rosea* f. *rosea*, and an ethanolic extract of a plant, *Ananas comosus*. *Biocontrol Sci Technol* 24:860–871. <https://doi.org/10.1080/09583157.2014.897304>
6. André WPP, Ribeiro WLC, Cavalcante GS, dos Santos JML, Macedo ITF, de Paula HCB, de Moraes SM, de Melo JV, Bevilaqua CML (2016) Comparative efficacy and toxic effects of carvacryl acetate and carvacrol on sheep gastrointestinal nematodes and mice. *Vet Parasitol* 218:52–58. <https://doi.org/10.1016/j.vetpar.2016.01.001>
7. André WPP, Ribeiro WLC, de Oliveira LMB, Macedo ITF, Rondon FCR, Bevilaqua CML (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>
8. Babják M, Königová A, Dolinská MU, Kupčinskas T, Vadlejch J, von Samson-Himmelstjerna G, Petkevičius S, Várady M (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>
9. Bava R, Castagna F, Piras C, Palma E, Cringoli G, Musolino V, Lupia C, Perri MR, Statti G, Britti D, Musella V (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>
10. Bava R, Castagna F, Ruga S, Nucera S, Caminiti R, Serra M, Bulotta RM, Lupia C, Marrelli M, Conforti F, Statti G, Britti D, Palma E (2023) Plants and their derivatives as promising therapeutics for

- sustainable control of honeybee (*Apis mellifera*) Pathogens. Pathogens 12:1260.
<https://doi.org/10.3390/pathogens12101260>
11. Berrouet C, Dorilas N, Rejniak KA, Tuncer N (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>
 12. Borges DGL, Borges FDA (2016) Plants and their medicinal potential for controlling gastrointestinal nematodes in ruminants. Nematoda 3:e92016. <http://dx.doi.org/10.4322/nematoda.00916>
 13. Bosco A, Maurelli MP, Ianniello D, Morgoglione ME, Amadesi A, Coles GC, Cringoli G, Rinaldi L (2018) The recovery of added nematode eggs from horse and sheep faeces by three methods. BMC Vet Res 14:7. <https://doi.org/10.1186/s12917-017-1326-7>
 14. Bosco A, Kießler J, Amadesi A, Varady M, Hinney B, Ianniello D, Maurelli MP, Cringoli G, Rinaldi L (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>
 15. Castagna F, Piras C, Palma E, Musolino V, Lupia C, Bosco A, Rinaldi L, Cringoli G, Musella V, Britti D (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>
 16. Castagna F, Bava R, Musolino V, Piras C, Cardamone A, Carresi C, Lupia C, Bosco A, Rinaldi L, Cringoli G, Palma E, Musella V, Britti D (2022a) Potential new therapeutic approaches based on *Punica granatum* fruits compared to synthetic anthelmintics for the sustainable control of gastrointestinal nematodes in sheep. Animals 12:2883. <https://doi.org/10.3390/ani12202883>
 17. Castagna F, Bava R, Piras C, Carresi C, Musolino V, Lupia C, Marrelli M, Conforti F, Palma E, Britti D, Musella V (2022b) Green veterinary pharmacology for honey bee welfare and health: *Origanum heracleoticum* L. (Lamiaceae) essential oil for the control of the *Apis mellifera* varroaosis. Vet Sci 9:124. <https://doi.org/10.3390/vetsci9030124>
 18. Charlier J, Rinaldi L, Musella V, Ploeger HW, Chartier C, Rose Vineer H, Hinney B, von Samson-Himmelstjerna G, Băcescu B, Mickiewicz M, Mateus TL, Martinez-Valladares M, Quealy S, Azaizeh H, Sekovska B, Akkari H, Petkevicius S, Hektoen L, Höglund J, Morgan ER, Bartley DJ, Claerebout E (2020) 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>
 19. Cimino C, Maurel OM, Musumeci T, Bonaccorso A, Drago F, Souto EMB, Pignatello R, Carbone C (2021) Essential oils: Pharmaceutical applications and encapsulation strategies into lipid-based delivery systems. Pharmaceutics 13:327. <https://doi.org/10.3390/pharmaceutics13030327>
 20. Coles GC, Bauer C, Borgsteede FH, Geerts S, Klei TR, Taylor MA, Waller PJ (1992) World association for the advancement 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](https://doi.org/10.1016/0304-4017(92)90141-U)
21. Cringoli G, Maurelli MP, Levecke B, Bosco A, Vercruysse J, Utzinger J, Rinaldi L (2017) The Mini-FLOTAC technique for the diagnosis of helminth and protozoan infections in humans and animals. *Nat Protoc* 12:1723–1732. <https://doi.org/10.1038/nprot.2009.235>
 22. de Agüero VCG, Valderas-García E, del Palacio LG, Giráldez FJ, Balaña-Fouce R, Martínez-Valladares M (2023) Secretory IgA as biomarker for gastrointestinal nematodes natural infection in different breed sheep. *Animals* 13(13):2189. <https://doi.org/10.3390/ani13132189>
 23. Denwood MJ, Kaplan RM, McKendrick IJ, Thamsborg SM, Nielsen MK, Levecke B (2023) A statistical framework for calculating prospective sample sizes and classifying efficacy results for faecal egg count reduction tests in ruminants, horses and swine. *Vet Parasitol* 314:109867.
<https://doi.org/10.1016/j.vetpar.2022.109867>
 24. Desalegn C, Berhanu G (2023) Assessment of the epidemiology of the gastrointestinal tract nematode parasites in sheep in Toke Kutaye, West Shoa Zone, Ethiopia. *Vet Med (Auckl)* 14:177–183. <https://doi.org/10.2147/VMRR.S427828>
 25. Ebani VV, Mancianti F (2020) Use of essential oils in veterinary medicine to combat bacterial and fungal infections. *Vet Sci* 7:193. <https://doi.org/10.3390/vetsci7040193>
 26. Ferreira LE, Benincasa BI, Fachin AL, França SC, Contini SSHT, Chagas ACS, Beleboni RO (2016) *Thymus vulgaris* L. essential oil and its main component thymol: Anthelmintic effects against *Haemonchus contortus* from sheep. *Vet Parasitol* 228:70–76.
<https://doi.org/10.1016/j.vetpar.2016.08.011>
 27. Ferreira LE, Benincasa BI, Fachin AL, Contini SHT, França SC, Chagas ACS, Beleboni RO (2018) Essential oils of *Citrus aurantifolia*, *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>
 28. Fokou JBH, Dongmo PMJ, Boyom FF (2020) Essential oil's chemical composition and pharmacological properties. In: El-Shemy H (ed) *Essential oils - Oils of nature*. Chapter 2, IntechOpen: London, UK. <https://doi.org/10.5772/intechopen.86573>
 29. Fox NJ, Smith LA, Houdijk JGM, Athanasiadou S, Hutchings MR (2018) Ubiquitous parasites drive a 33% increase in methane yield from livestock. *Int J Parasitol* 48:1017–1021.
<https://doi.org/10.1016/j.ijpara.2018.06.001>
 30. Geurden T, Smith ER, Vercruysse J, Yazwinski T, Settje T, Nielsen MK (2022) World association for the advancement of veterinary parasitology (WAAVP) guideline for the evaluation of the efficacy of anthelmintics in food-producing and companion animals: general guidelines. *Vet Parasitol* 304:109698. <https://doi.org/10.1016/j.vetpar.2022.109698>
 31. Grando TH, de Sá MF, Baldissera MD, Oliveira CB, de Souza ME, Raffin RP, Santos RCV, Domingues R, Minho AP, Leal MLR, Monteiro SG (2015) In vitro activity of essential oils of free and nanostructured

- Melaleuca alternifolia and of terpinen-4-ol on eggs and larvae of Haemonchus contortus. J Helminthol 90:377–382. <https://doi.org/10.1017/S0022149X15000401>
32. Hoste H, Torres-Acosta JF, Alonso-Diaz MA, Brunet S, Sandoval-Castro C, Adote SH (2008) Identification and validation of bioactive plants for the control of gastrointestinal nematodes in small ruminants. Trop Biomed 25:56–72
33. Junkuszew A, Milerski M, Bojar W, Szczepaniak K, Le Scouarnec J, Tomczuk K, Dudko P, Studzińska MB, Demkowska-Kutrzepa M, Bracik K (2015) Effect of various antiparasitic treatments on lamb growth and mortality. Small Rumin Res 123:306–313. <https://doi.org/10.1016/j.smallrumres.2014.11.019>
34. 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>
35. Katiki LM, Barbieri AME, Araujo RC, Veríssimo CJ, Louvandini H, Ferreira JFS (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>
36. Knežević P, Aleksić V, Simin N, Svirčev E, Petrović A, Mimica-Dukić N (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>
37. Liu H, Zhang Y, Liu F, Ye L, Liu X, Wang C, Hu M (2023) Progress and challenges for developing vaccines against gastrointestinal nematodes of ruminants. Veterinary Vaccine, 2023: 100041. <https://doi.org/10.1016/j.vetvac.2023.100041>
38. Maccelli A, Vitanza L, Imbriano A, Frascchetti C, Filippi A, Goldoni P, Maurizi L, Ammendolia MG, Crestoni ME, Fornarini S, Menghini L, Carafa M, Marianecchi C, Longhi C, Rinaldi F (2020) Satureja montana L. essential oils: Chemical profiles/phytochemical screening, antimicrobial activity and O/W nanoemulsion formulations. Pharmaceutics 12:7. <https://doi.org/10.3390/pharmaceutics12010007>
39. Macedo ITF, de Oliveira LMB, Camurça-Vasconcelos ALF, Ribeiro WLC, dos Santos JML, de Moraes SM, de Paula HCB, Bevilaqua CML (2013) In vitro effects of Coriandrum sativum, Tagetes minuta, Alpinia zerumbet and Lantana camara essential oils on Haemonchus contortus. Rev Bras Parasitol Vet 22:463–469. <https://doi.org/10.1590/S1984-29612013000400004>
40. Macedo ITF, de Oliveira LMB, Andre WBP, Filho JVA, dos Santos JML, Rondon FCM, Ribeiro WLC, Camurça-Vasconcelos ALF, de Oliveira EF, de Paula HCB, Bevilaqua CML (2019) Anthelmintic effect of Cymbopogon citratus essential oil and its nanoemulsion on sheep gastrointestinal nematodes. Rev Bras Parasitol Vet 28:522–527. <https://doi.org/10.1590/S1984-29612019065>
41. Maestrini M, Forzato C, Mancini S, Pieracci Y, Perrucci S (2022) In vitro anthelmintic activity of sea buckthorn (Hippophae rhamnoides) berry juice against gastrointestinal nematodes of small ruminants. Biology 11:825. <https://doi.org/10.3390/biology11060825>

42. Maqbool I, Wani ZA, Shahardar RA, Allaie IM, Shah MM (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>
43. Marjanović ĐM, Zdravković N, Milovanović M, Trailović JN, Robertson AP, Todorović Z, Trailović SM (2020) Carvacrol acts as a potent selective antagonist of different types of nicotinic acetylcholine receptors and enhances the effect of monepantel in the parasitic nematode *Ascaris suum*. *Vet Parasitol* 278:109031. <https://doi.org/10.1016/j.vetpar.2020.109031>
44. Matejić J, Stefanović N, Ivković M, Živanović N, Marin P, Džamić A (2020) Traditional uses of autochthonous medicinal and ritual plants and other remedies for health in Eastern and South-Eastern Serbia. *J Ethnopharmacol* 261:113186. <https://doi.org/10.1016/j.jep.2020.113186>
45. Maurizio A, Perrucci S, Tamponi C, Scala A, Cassini R, Rinaldi L, Bosco A (2023) Control of gastrointestinal helminths in small ruminants to prevent anthelmintic resistance: the Italian experience. *Parasitology* 150:1105–1118. <https://doi.org/10.1017/S0031182023000343>
46. Mesquita-Sousa D, Campos NRCL, Pereira JRF, Gomes MN, Silva CR, Cutrim-Junior JAA, Brito DRB (2023) Evaluation of a combination of *Citrus aurantium* var. *Dulcis* essential oil and albendazole for the treatment of sheep gastrointestinal nematodes. *Vet Parasitol* 318:109929.
<https://doi.org/10.1016/j.vetpar.2023.109929>
47. Ministry of Agriculture, Fisheries and Food (MAFF) (1986) *Grande-Bretagne, Manual of Veterinary Parasitological Laboratory Techniques*; HM Stationery Off: London, UK
48. Mondragón-Ancelmo J, Olmedo-Juárez A, Reyes-Guerrero DE, Ramírez-Vargas G, Ariza-Román AE, López-Arellano ME, de Gives PM, Napolitano F (2019) Detection of gastrointestinal nematode populations resistant to albendazole and ivermectin in sheep. *Animals* 9:775.
<https://doi.org/10.3390/ani9100775>
49. Mucha W, 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>
50. Napoli E, Di Vito M (2021) Toward a new future for essential oils. *Antibiotics* 10:207.
<https://doi.org/10.3390/antibiotics10020207>
51. Nehme R, Andrés S, Pereira RB, Jemaa MB, Bouhallab S, Ceciliani F, López S, Rahali FZ, Ksouri R, Pereira DM, Abdennebi-Najar L (2021) Essential oils in livestock: From health to food quality. *Antioxidants* 10:330. <https://doi.org/10.3390/antiox10020330>
52. Pinto NB, de Castro LM, Azambuja RHM, Capella GDA, de Moura MQ, Terto WD, Freitag RA, Jeske ST, Villela MM, Cleff MB, Leite FBL (2019) Ovicidal and larvicidal potential of *Rosmarinus officinalis* to control gastrointestinal nematodes of sheep. *Rev Bras Parasitol Vet* 28:807–811.
<https://doi.org/10.1590/S1984-29612019060>
53. Ragusa M, Miceli N, Piras C, Bosco A, Castagna F, Rinaldi L, Musella V, Taviano MF, Britti D (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>

54. Ratajac R, Pavličević A, Petrović J, Stojanov I, Orčić D, Štrbac F, Simin N (2024) In vitro Evaluation of acaricidal efficacy of selected essential oils against *Dermanyssus gallinae*. *Pak Vet J* 44:93–98. <http://dx.doi.org/10.29261/pakvetj/2023.123>
55. Romero B, Susperregui J, Sahagún AM, Diez MJ, Fernández N, García JJ, López C, Sierra M, Diez R (2022) Use of medicinal plants by veterinary practitioners in Spain: A cross-sectional survey. *Front Vet Sci* 9:1060738. <https://doi.org/10.3389/fvets.2022.1060738>
56. Rose Vineer H, Morgan ER, Hertzberg H, Bartley DJ, Bosco A, Charlier J, Chartier C, Claerebout E, de Waal T, Hendrickx G, Hinney B, Höglund J, Ježek J, Kašný M, Keane OM, Martínez-Valladares M, Mateus TL, McIntyre J, Mickiewicz M, Munoz AM, Phythian CJ, Ploeger HW, Rataj AV, Skuce PJ, Simin S, Sotiraki S, Spinu M, Stuen S, Thamsborg SM, Vadlejch J, Varady M, von Samson-Himmelstjerna G, Rinaldi L (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>
57. Shao Y, Wang Y, Yuan Y, Xie Y (2021) A systematic review on antibiotics misuse in livestock and aquaculture and regulation implications in China. *Sci Total Environ* 798:149205. <https://doi.org/10.1016/j.scitotenv.2021.149205>
58. Sousa VI, Parente JF, Marques JF, Forte MA, Tavares CJ (2022) Microencapsulation of essential oils: A review. *Polymer* 14:1730. <https://doi.org/10.3390/polym14091730>
59. Szewc M, De Waal T, Zintl A (2021) Biological methods for the control of gastrointestinal nematodes. *Vet J* 268:105602. <https://doi.org/10.1016/j.tvjl.2020.105602>
60. Štrbac F, Bosco A, Maurelli MP, Ratajac R, Stojanović D, Simin N, Orčić D, Pušić I, Krnjajić S, Sotiraki S, Saralli G, Cringoli G, Rinaldi L (2022a) 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>
61. Štrbac F, Bosco A, Pušić I, Stojanović D, Simin N, Cringoli G, Rinaldi L, Ratajac (2022b) The use of essential oils against sheep gastrointestinal nematodes. In: Abbas RZ, Khan A, Liu P, Saleemi MK (eds) *Animal Health Perspectives*, vol 1. Unique Scientific, pp 86–94. <https://doi.org/10.47278/book.ahp/2022.12>
62. Štrbac F, Krnjajić S, Maurelli MP, Stojanović D, Simin N, Orčić D, Ratajac R, Petrović K, Knežević G, Cringoli G, Rinaldi L, Bosco A (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>
63. Štrbac F, Krnjajić S, Stojanović D, Ratajac R, Simin N, Orčić D, Rinaldi L, Ciccone E, Maurelli MP, Cringoli G, Bosco A (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>
64. Šubarević N, Stevanović O, Petrujkić B (2015) Use of phytotherapy as a form of ethnoveterinary medicine in the area of Stara planina mountain in Serbia. *Acta Med Hist Adriat* 13:75–94

65. Tachack EB, Oviedo-Socarrás T, Pastrana MO, Pérez-Cogollo LC, Bernavides YH, Pinto CR, Garay OV (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>
66. Tariq KA (2017) Anthelmintics and emergence of anthelmintic resistant nematodes in sheep: need of an integrated nematode management. *Int J Vet Sci Anim Husb* 2:13–19
67. Trailović SM, Marjanović ĐM, Trailović JN, Robertson AP, Martin RJ (2015) Interaction of carvacrol with the *Ascaris suum* nicotinic acetylcholine receptors and gamma-aminobutyric acid receptors, potential mechanism of antinematodal action. *Parasitol Res* 114:3059–3068. <https://doi.org/10.1007/s00436-015-4508-x>
68. Tsukahara Y, Gipson TA, Hart SP, Dawson L, Wang Z, Puchala R, Sahlu T (2021) Genetic selection for resistance to gastrointestinal parasitism in meat goats and hair sheep through a performance test with artificial infection of *Haemonchus contortus*. *Animals* 11:1902. <https://doi.org/10.3390/ani11071902>
69. Tomaselli V, Silletti G, Forte L (2021) A new association of *Satureja montana* L. subsp. *montana* dominated garrigues in Puglia (SE Italy). *Plant Sociol* 58:1–14. <https://doi.org/10.3897/pls2021582/01>
70. Varshney B, Malik S, Singh A, Mehta N (2022) Role of medicinal plants and herbs in veterinary medicine. In: Singh A (ed) *Advanced Phytochemicals and Plant-Based Drug Discovery*. IGI Global, pp 32–48. Chap. 3
71. van Dijk M, Morley T, Rau ML, Saghai Y (2021) A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. *Nat Food* 2:494–501. <https://doi.org/10.1038/s43016-021-00322-9>
72. van Wyk JA, 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://hdl.handle.net/10520/EJC134539>
73. Veerakumari L (2015) Botanical anthelmintics. *Asian J Sci Technol* 6:1881–1894
74. Yammine J, Chihib NE, Gharsallaoui A, Ismail A, Karam L (2023) : Advances in essential oils encapsulation: development, characterization and release mechanisms. *Polym Bull* 81:1–46. *Polymer Bulletin* (2024) 81:3837–3882. <https://doi.org/10.1007/s00289-023-04916-0>
75. Yoshihara Y, Saiga C, Tamura T, Kinugasa T (2023) Relationships between sheep nematode infection, nutrition, and grazing behavior on improved and semi-natural pastures. *Vet Anim Sci* 19:100278. <https://doi.org/10.1016/j.vas.2022.100278>

Figures

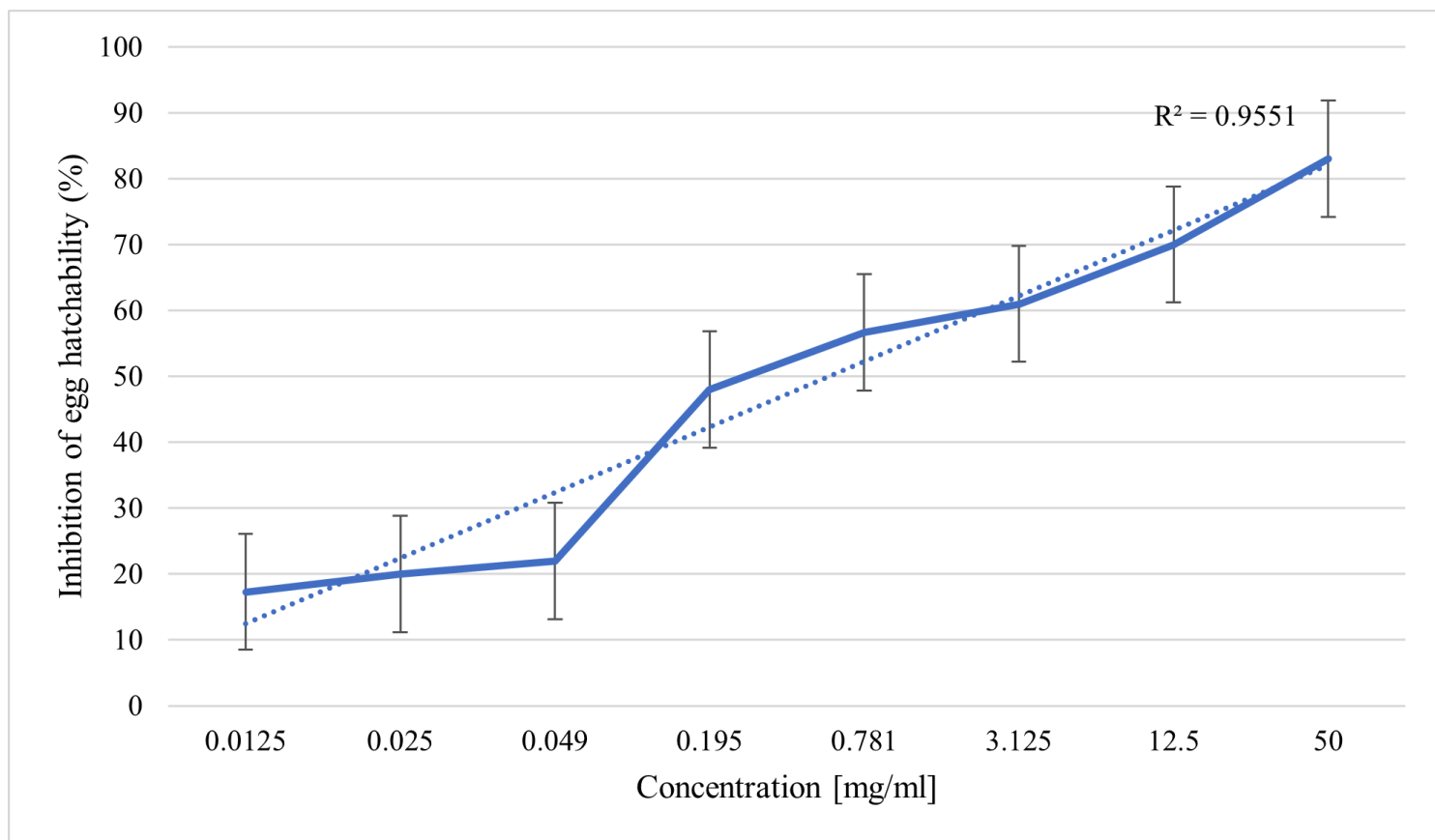


Figure 1

Graph 1. Dose-response curve of the effect of *Satureja montana* essential oil on the inhibition of egg hatchability of gastrointestinal nematodes in sheep

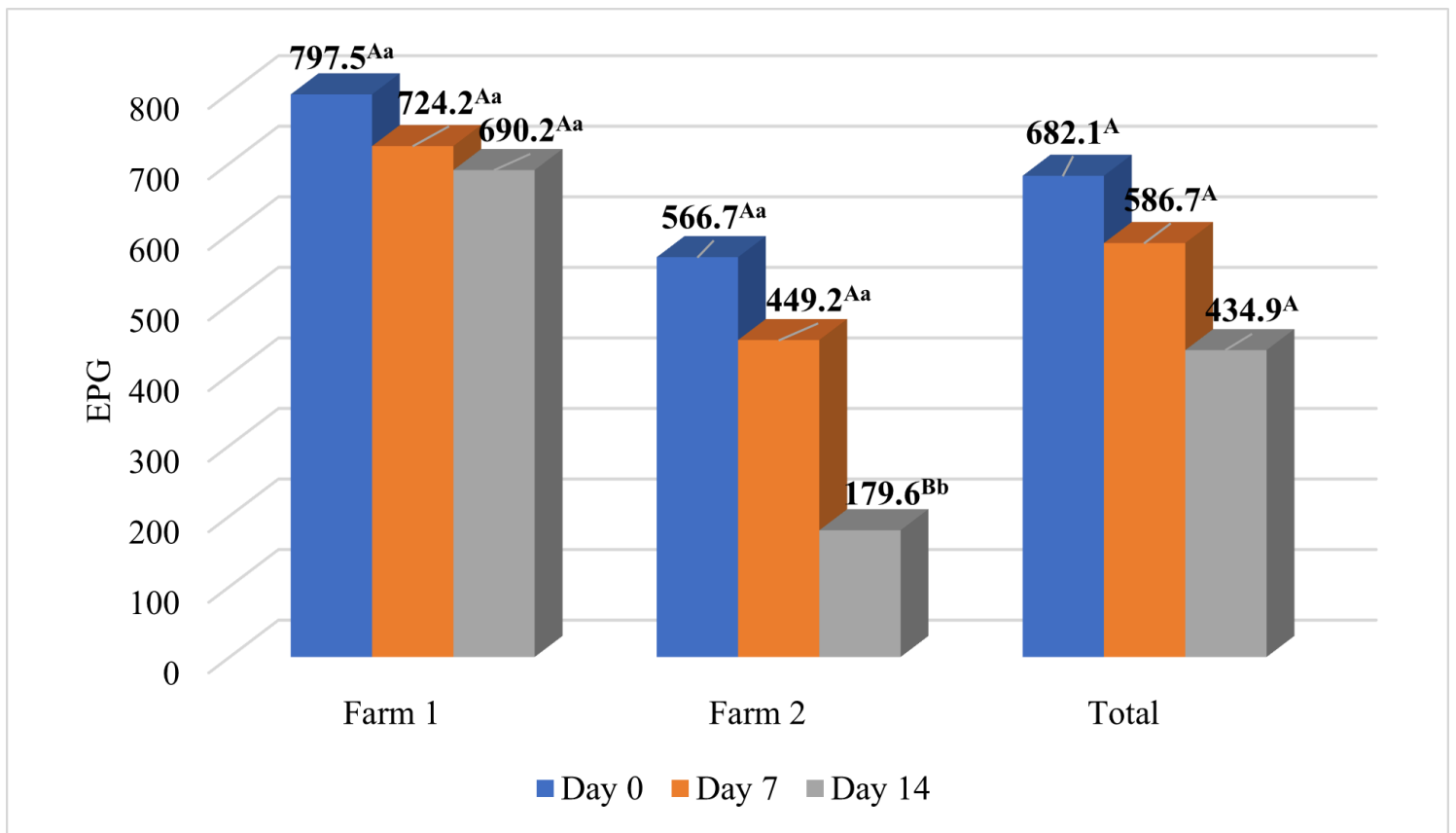


Figure 2

Graph 2. Mean reduction of eggs per gram of gastrointestinal nematodes in sheep – per farm and overall in a group of animals treated with *Satureja montana* essential oil

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)