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Anthelmintic activity of winter savory (*Satureja montana* L.) essential oil against gastrointestinal nematodes of sheep

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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, due to their numerous advantages. The present study aimed to evaluate the possibility of using winter savory (*Satureja montana* L.) EO against sheep GINs. The chemical composition of the tested oil was determined by gas chromatography-mass spectrometry (GC–MS). The efficacy of the tested oil was determined in vitro using the egg hatch test (EHT), and in vivo using the faecal egg count reduction test (FECRT) performed in two farms. Preliminary toxicity studies including clinical observation, haematological and biochemical blood analysis were also performed to evaluate the safety of the tested oil to the hosts. In addition, a coproculture study was carried out in the tested farms using the appropriate morphological keys.

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. No significant changes ($p > 0.05$) in the percentage representation of GIN genera were observed in the coproculture study, indicating that the treatment agent was not specific to a single genus.

Conclusion The anthelmintic potential showed 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. However, further studies should be conducted to increase efficacy in field conditions.

Keywords Anthelmintic resistance, Anthelmintic drug residues, Botanical anthelmintics, Integrated parasite control, In vitro test, In vivo test, Toxicity study, Chemical analyses, Coproculture

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Introduction

As a valuable source of meat, milk (and its products), wool and manure, sheep represent an important sector of the livestock industry worldwide [1]. This is especially true given the increasing demand for food for the growing human population [2]. 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 [3–5]. 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 hypoproteinemia resulting in reduced productivity, immunity and fertility, even death in cases of high parasite infection [1, 6]. All of this can lead to enormous economic losses, estimated at hundreds of millions of euros per year [7]. In addition, the occurrence of GIN infections is predicted to increase greenhouse gas emissions by up to 30% [8, 9].

Therefore, it is clear that the issue of controlling these parasites is a top priority, which requires the development of sustainable strategies. To date, heavy reliance has been placed on the use of commercial anthelmintics from different chemical classes (benzimidazoles, macrocyclic lactones and imidazothiazoles) in an attempt to maintain the number of parasites below levels that can cause disease, whether clinical or subclinical [9]. 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 [10, 11]. Annual costs due to AR development in Europe alone are estimated at €38 million and rising, with underdosing, overfrequent treatments, mass treatment, and single-drug regimens being the main risk factors [9, 12]. 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 [11, 13–15]. All of these justify 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 [15]. 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 [16]. 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 [16–18]. EOs represent plant secondary metabolites, i.e. volatile, aromatic and viscous components obtained from different parts of plants through well-regulated extraction processes [19, 20]. Therefore, they contain various bioactive substances with great pharmacological potential that can be exploited for various indications in veterinary medicine [18, 21–23]. Moreover, EOs from various plants have already been investigated for their anthelmintic activity against GINs in sheep, with some of them showing promising results [24, 25].

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 [26]. It is reported in many countries in this region including Serbia [27] and Italy [28]. *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. [29, 30]. In addition, winter savory is used in the food industry as a spice and flavoring agent [26]. 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 demonstrate the possibility of using *S. montana* EO against GINs in sheep. The hypothesis is that this EO has anthelmintic potential against these parasites, which can be demonstrated by regular in vitro and in vivo tests including the egg hatch test and the faecal egg count reduction test, while at the same time it is safe for the sheep.

Material and methods

Chemical composition

The essential oil of *Satureja montana* (L.) was obtained from the Institute of Field and Vegetable Crops, Novi Sad, Serbia. The steam distillation of the fresh aerial plant parts of *S. montana* L. was performed in a semi-industrial distillation unit according to the procedure described by Aćimović et al. [31]. 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 were 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. [32]. Data were acquired in scan mode (m/z range 35–400), with a 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. 2.0 d). 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 [33]. The relative amount of each component is expressed as a percentage of its peak area relative to the total peak area.

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 GINs, in an area with a typical Mediterranean climate, were used for the trials, where the animals were kept in free range (Farm 1) and in boxes (Farm 2) during the treatment.

In this region, extensive farming is still very widespread. With 6,707 sheep farms and 160,784 sheep, on a national scale, the region occupies the six positions for the number of farms and the seven position for the number of sheep. These data, updated as of 30 June 2024 were provided by the National Database (BDN) of the Zoot-technical Registry-CSN of the “G. Caporale” Institute of Teramo (Italy). The two farms, already monitored by the CREMOPAR research group, have never shown any signs of drug resistance so far.

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 the present study. However, animals with different faecal egg counts (up to 3640 EPG) were selected to simulate natural conditions. The animals selected for the study were all lactating females. They were fed with pasture and forage (barley and maize grains) with no changes during the trial. The hilly pasture (average altitude 390 m above sea level) is made up of spontaneous forage plants typical of the Mediterranean area, and the animals graze all together throughout the year regardless of sex and age. All the animals had not received anthelmintic treatments in the past six months. The last treatment was performed eight months before the trial with oral albendazole. The animals were fasted before and 2 h after the application of the treatments, as is usual when using commercial anthelmintics.

In vitro egg hatch test

Anthelmintic potential in vitro of *S. montana* EO was evaluated using the egg hatch test (EHT), with the recovery method described by Bosco et al. [6, 34] was

used to obtain GIN eggs. The faecal samples used for the in vitro tests came from the two sheep farms used for the in vivo tests. For this purpose, faecal samples ($n = 30$) were collected directly from the rectal ampulla of randomly selected sheep, transported to the laboratory at a temperature of 10 °C and processed within 2 h of collection. To isolate GIN eggs, faecal samples were pooled, homogenised and then filtered under running water through meshes of 1 mm, 250 µm, 212 µm and 38 µm size. Subsequently, the eggs retained at the last size were washed with distilled water and centrifuged at 52.36 rad/s, 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 [35, 36], 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 (40 µL) of approximately 150 eggs/well. The positive control was thiabendazole (TBZ, Sigma, Saint Louis, MO, USA) 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 a constant temperature of 27 °C for 48 h. 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.

In vivo faecal egg count reduction test

The field trial was conducted using the faecal egg count reduction test (FECRT) on two different farms. In each of them, the animals ($n = 36/\text{farm}$, 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.

To further describe the natural formulation used for this test, *S. montana* EO was mixed with sunflower oil in a ratio 1:4.5 to avoid the effect of the pure EO on the gastrointestinal mucosa. The quantity of the EO given to each tested animal was expressed in a given mg/kg of

body weight, and the final volume of the formulation (EO + sunflower oil) was 50 mL per animal. All treatments were administered once (single dose), directly into the rumen of the animals using a tube inserted through the oral cavity, pharynx and oesophagus (intraruminal application). For each group, a single tube was used and rinsed from one animal to another. 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 [37] 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 occurrences of natural-mixed GIN infections. On both farms, animals for the trial were randomly selected for each group and thus were different in worm burden to completely simulate the conditions in the practice.

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 h. 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. The reference values were used according to the Sajid et al. [38] with some modifications. 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 the kidney (urea, creatinine) and liver (aspartate aminotransferase, AST, and gamma-glutamyl transferase, GGT) function.

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 [39]. 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 larvae (L3) developed were identified using the morphological determination keys proposed by van Wyk and Mayhew [40]. The identification and percentages of each nematode genera were performed on 100 L3, identifying all larvae if the sample contained 100 or fewer L3. In this way, it was possible to determine the percentage of each GIN genus in the total number of identified larvae.

Statistical analyses

The inhibitions of egg hatchability (IH) in the in vitro test were calculated using the following formula [41, 42]:

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

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-hoc Tukey'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 [43].

The reductions in the faecal egg counts in the in vivo test were calculated using the following formula [41, 44]:

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

In this formula, T_1 and T_2 represent the average EPGs (arithmetic means) 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. The final results represent the mean value from both farms.

The results of the haematological and biochemical blood analyses were also analysed using two-way ANOVA. The post-hoc 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-hoc Tukey's test was performed ($p < 0.05$). Finally, the same tests were performed in the analysis of the results of the coproculture examination to evaluate the differences in the percentage of each GIN genera before and after the treatment in each group.

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

All statistical analyses were performed using GraphPad Prism 10.1.2. (GraphPad Holdings, LLC, San Antonio, CA, USA).

Results

Chemical composition

A total of 17 compounds belonging to different chemical groups were identified by GC–MS analysis (Table 1,

Figure 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%.

In vitro egg hatch test

In the EHT, *S. montana* EO showed 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. At all of the concentrations tested, the activity of the tested EO was significantly higher than that of the two negative controls ($p < 0.05$).

In vivo faecal egg count reduction test

In the field trial, *S. montana* EO showed anthelmintic activity with an overall average group EPG reduction of 15.7% and 33.0% in total at D7 and D14 post-treatment, respectively (Table 3). In contrast to the low activity on Farm 1 (Figure 2), 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, and the effect was significantly higher than the negative control on that Farm ($p < 0.05$) (Figure 3). However, there were no significant differences between the EPG values from the treated group and the negative control in total ($p > 0.05$). If each animal is observed separately, the median individual reduction was higher than the arithmetic average of

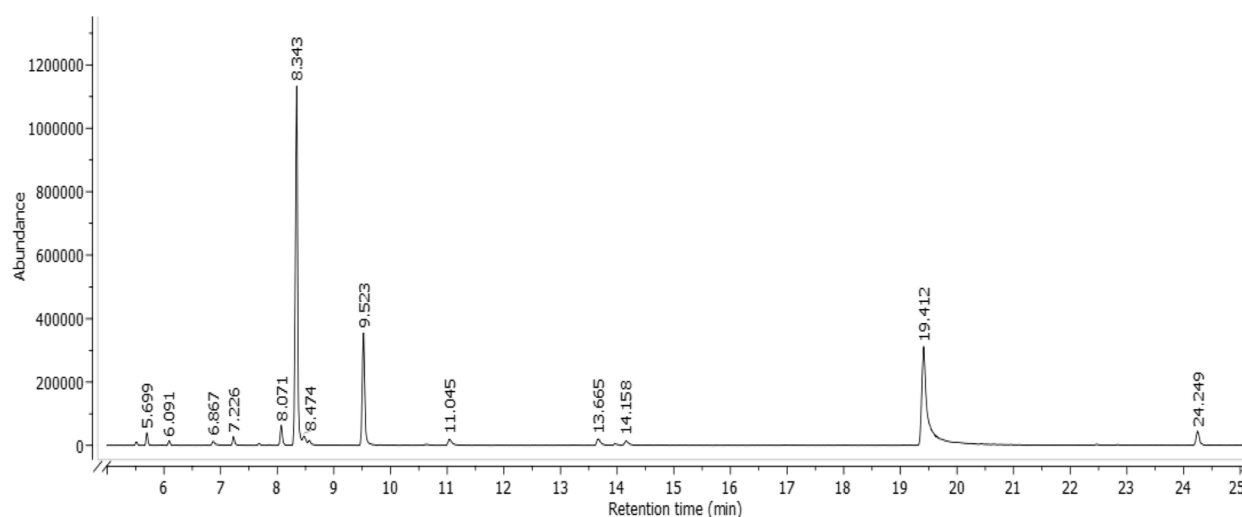
**Fig. 1** GC–MS chromatogram of the *Satureja montana* essential oil

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 ^B
12.5	70.0 \pm 2.65 ^C
3.125	61.0 \pm 2.00 ^D
0.781	56.7 \pm 1.53 ^D
0.195	48.0 \pm 1.00 ^E
0.049	22.0 \pm 1.00 ^F
0.025	20.0 \pm 1.00 ^F
0.0125	17.3 \pm 2.52 ^F
Control (+) ¹	96.3 \pm 1.53 ^A
Control (+) ²	95.0 \pm 1.00 ^A
Control (-) ¹	8.0 \pm 1.00 ^G
Control (-) ²	6.60 \pm 1.92 ^G

* Uppercase compares means between different concentrations and controls. Different letters indicate significant differences ($p < 0.05$). Control (+)¹—thiabendazole, 0.025 mg/mL; control (+)²—thiabendazole, 0.0125 mg/mL; control (-)¹—3% Tween 80, v/v; control (-)²—distilled water; EO—essential oil

the group level and reached 18.9% and 50.0% in total on D7 and D14 post-treatment, respectively. In the albendazole-treated group, there was significant total reduction of EPG ($p < 0.05$)(Table 3).

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, slightly increasing the levels of RBC, Hgb and HCT, while at the same time those levels decreased in the negative control group (Table 4). 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 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 before (D0) and after treatment (D7 and D14) are shown in Tables 6 and 7.

Discussion

The search for new anthelmintics requires the use of appropriate methods to test their efficacy. From this point of view, the EHT is used for studying the ovidal activity of drugs and it is suitable for an initial assessment of anthelmintic potential and forms a basis for other tests. Although the larval development test (LDT) has an advantage due to its higher sensitivity [45], the EHT is less labour-intensive and requires less time to perform [46], with both tests showing comparable and reliable results and having an advantage over other in vitro 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 [47]. At the same time, it is commonly used for the evaluation of the anthelmintic potential of EOs [25]. In the EHT performed in the present study (Table 2), the EO of *S. montana* showed ovidal 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 [48], and the obtained efficacy of 83.0% at the very high concentration of 50 mg/mL can be considered as low, the IC₅₀ value obtained in the present study is still high compared to oils from other studies

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 404.0 ^{Aa}
	FE _{CR} (group)	/	15.7%	33.0%
Albendazole, 3.8 mg/kg (Control +)	EPG	683.1 \pm 735.5 ^{Aa}	9.38 \pm 11.8 ^{Bb}	36.9 \pm 41.6 ^{Bc}
	FE _{CR} (group)	/	99.0%	95.1%
Sunflower oil, 50 ml (Control -)	EPG	914.0 \pm 821.9 ^{Aa}	914.4 \pm 784.8 ^{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 the group. Different letters indicate significant differences ($p < 0.05$); EPG—eggs per gram; EO – essential oil; FE_{CR} – faecal egg count reduction

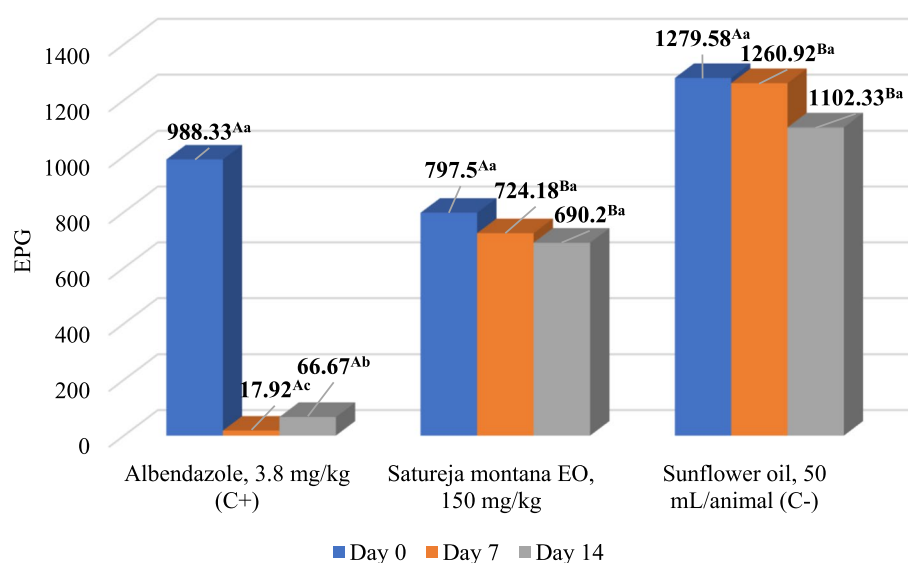


Fig. 2 Mean reduction of eggs per gram (EPG) of faeces of gastrointestinal nematodes in sheep treated with *Satureja montana* EO, albendazole and sunflower oil on Farm 1. Uppercase compares means between different groups at one time point; lowercase compares means of different time points within the group. Different letters indicate significant differences ($p < 0.05$); EPG - eggs per gram; EO - essential oil; C - control

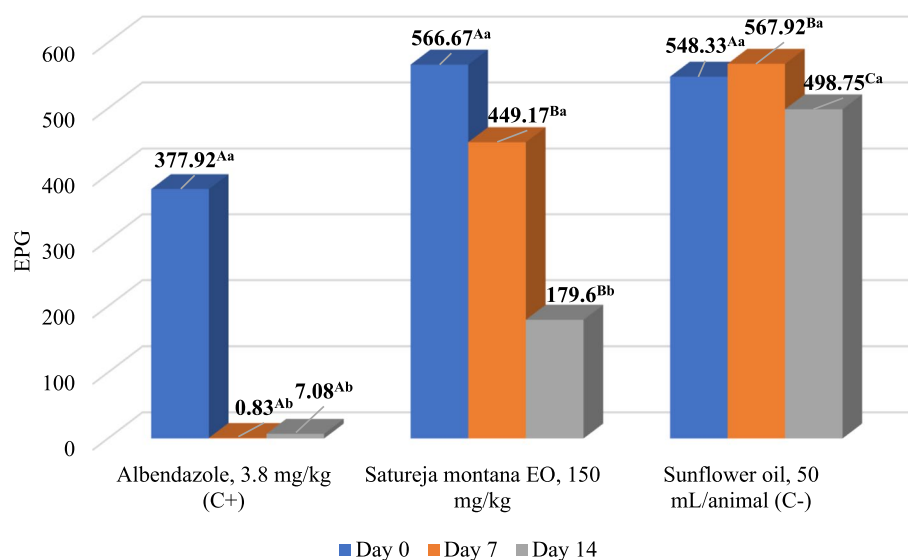


Fig. 3 Mean reduction of eggs per gram (EPG) of faeces of gastrointestinal nematodes in sheep treated with *Satureja montana* EO, albendazole and sunflower oil on Farm 2. Uppercase compares means between different groups at one time point; lowercase compares means of different time points within the group. Different letters indicate significant differences ($p < 0.05$); EPG - eggs per gram; C - control

[24, 25], indicating its anthelmintic potential. Indeed, this parameter is considered suitable for comparing the drug-inhibitory effect of different active substances [49].

The results obtained for two samples of *S. montana* EO in the present and the previous study [48] 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

Table 4 Effect (mean \pm standard deviation) of the application of *Satureja montana* EO on haematological blood parameters

Parameter	Reference values	Day	<i>Satureja montana</i> EO	Albendazole, C (+)	Sunflower oil, C (-)
WBC (K/uL)	4.0–12.0	0	9.61 \pm 2.0	9.59 \pm 1.6	8.78 \pm 2.9
		14	9.95 \pm 2.5	9.45 \pm 2.6	8.74 \pm 2.3
RBC (M/uL)	8.0–16.0	0	7.67 \pm 1.4	7.50 \pm 0.5	8.01 \pm 1.0
		14	7.74 \pm 0.9	7.66 \pm 0.6	7.45 \pm 1.1
Hgb (g/dL)	8.0–16.0	0	10.8 \pm 1.3	10.4 \pm 0.5	11.7 \pm 1.8
		14	11.4 \pm 0.9	11.1 \pm 0.8	11.0 \pm 1.7
HCT (%)	24.0–50.0	0	27.6 \pm 3.3	26.4 \pm 1.6	29.4 \pm 4.7
		14	28.0 \pm 1.9	27.3 \pm 1.8	27.4 \pm 4.2
MCV (fL)	23.0–48.0	0	36.4 \pm 3.9	35.2 \pm 1.7	36.8 \pm 2.4
		14	36.5 \pm 3.0	35.7 \pm 2.0	36.8 \pm 1.8
MCH (Pg)	9.0–12.0	0	14.3 \pm 1.4	13.9 \pm 0.6	14.6 \pm 0.8
		14	14.9 \pm 1.1	14.5 \pm 0.7	14.7 \pm 0.8
MCHC (g/dL)	31.0–38.0	0	39.2 \pm 0.9	39.5 \pm 1.0	39.7 \pm 0.8
		14	40.8 \pm 0.8	40.5 \pm 0.7	40.1 \pm 0.9
RDW (%)	-	0	16.0 \pm 1.0	15.5 \pm 0.5	15.9 \pm 0.7
		14	16.2 \pm 0.9	15.8 \pm 0.5	15.7 \pm 0.8
Pit (K/uL)	250—750	0	567.3 \pm 215.6	517.0 \pm 119.0	545.1 \pm 189.1
		14	522.2 \pm 179.9	468.2 \pm 142.0	483.0 \pm 207.7
MPV (fL)	-	0	11.5 \pm 2.1	11.3 \pm 1.5	11.1 \pm 1.3
		14	11.5 \pm 1.9	11.4 \pm 1.6	10.8 \pm 1.3
Pct (%)	-	0	0.57 \pm 0.2	0.60 \pm 0.6	0.58 \pm 0.2
		14	0.61 \pm 0.3	0.54 \pm 0.2	0.46 \pm 0.1
PDW (%)	-	0	6.22 \pm 8.4	5.43 \pm 4.5	5.83 \pm 8.0
		14	9.51 \pm 9.50	4.59 \pm 5.0	9.23 \pm 9.6
Lin (K/uL)	-	0	5.73 \pm 1.3	4.94 \pm 1.3	4.16 \pm 1.1
		14	5.97 \pm 1.9	5.41 \pm 2.5	4.73 \pm 1.6
Lin (%)	40—75%	0	59.5 \pm 6.9	51.8 \pm 12.0	52.8 \pm 12.3
		14	60.3 \pm 1.9	56.4 \pm 17.1	54.2 \pm 6.8
Gra (K/uL)	-	0	3.9 \pm 1.0	4.65 \pm 1.4	4.09 \pm 2.4
		14	4.0 \pm 1.3	4.04 \pm 1.7	4.01 \pm 1.2
Gra (%)	-	0	40.5 \pm 6.9	48.2 \pm 12.0	47.2 \pm 12.3
		14	39.7 \pm 10.8	43.6 \pm 17.1	45.8 \pm 6.8

* No statistically significant differences were found when comparing values on D14 compared to D0, nor when comparing values for different groups on the same day ($p > 0.05$)

Table 5 Effect (mean \pm standard deviation) of the application of *Satureja montana* essential oil on the biochemical blood parameters reflecting 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	0	31.0 \pm 8.74 ^{Aa}	14.1 \pm 3.03 ^{Aa}	180.3 \pm 61.10 ^{Aa}	71.8 \pm 7.62 ^{Aa}
	14	30.8 \pm 6.98 ^{Aa}	11.8 \pm 3.41 ^{Aa}	157.8 \pm 42.10 ^{Aa}	65.0 \pm 3.77 ^{Bb}
Albendazole (Control +)	0	27.6 \pm 9.45 ^{Aa}	12.4 \pm 2.75 ^{Aa}	173.3 \pm 82.72 ^{Aa}	72.0 \pm 8.86 ^{Aa}
	14	28.4 \pm 6.07 ^{Aa}	13.4 \pm 3.68 ^{Aa}	163.0 \pm 50.72 ^{Aa}	66.5 \pm 5.54 ^{Bab}
Sunflower oil (Control -)	0	29.8 \pm 7.87 ^{Aa}	12.4 \pm 2.84 ^{Aa}	189.7 \pm 72.63 ^{Aa}	72.5 \pm 7.82 ^{Aa}
	14	29.3 \pm 4.91 ^{Aa}	12.8 \pm 3.04 ^{Aa}	206.9 \pm 56.32 ^{Aa}	73.7 \pm 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

Table 6 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>Trichostrongylus</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
7	<i>Satureja montana</i> EO	53	26	18	3	100
	Albendazole (Control +)	56	21	15	8	100
	Sunflower oil (Control -)	46	29	22	3	100
14	<i>Satureja montana</i> EO	52	23	17	8	100
	Albendazole (Control +)	44	30	21	5	100
	Sunflower oil (Control -)	49	25	19	7	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 7 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>Trichostrongylus</i>	<i>Teladorsagia</i>	<i>Chabertia</i>	Total
0	<i>Satureja montana</i> EO	41	45	11	3	100
	Albendazole (Control +)	38	42	19	1	100
	Sunflower oil (Control -)	43	35	20	2	100
7	<i>Satureja montana</i> EO	39	43	15	3	100
	Albendazole (Control +)	42	38	20	0	100
	Sunflower oil (Control -)	38	41	16	5	100
14	<i>Satureja montana</i> EO	37	45	13	5	100
	Albendazole (Control +)	41	39	19	1	100
	Sunflower oil (Control -)	37	43	18	2	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

used, as they are sensitive to light, temperature and oxygen [50, 51]. However, not only the chemical composition of the EOs can influence their effect against GINs. Immunity of the animals is generally important for the host – GINs parasitism interaction [52] and thus factors such as genetic constitution, age and physiological status of the animals, management factors including nutrition, etc. are also important for the successful treatment and positive outcome.

On the other hand, in vivo FECRT is considered the method of choice for evaluating the field efficacy of anthelmintic compounds and the detection of anthelmintic resistance, as it is reliable, practical and is intended for all anthelmintic classes across all animal species. Furthermore, it can be performed for multiple parasitic species and do not require sacrificing the animals [47, 53]. Although even more reliable, the controlled efficacy test (CET) is not practicable in the field as it is labour-intensive, time-consuming and costly (animals are artificially infected, treated, then slaughtered and worm burden counted) [54]. For these

reasons, FECRT is the most commonly used method for evaluating the anthelmintic efficacy of drugs in practice [55]. Examination of the effect of *S. montana* EO in vivo was performed for the first time to our knowledge, and it showed limited activity with a total reduction of 33.0% on D14 after treatment (Table 3). 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% (Figure 3). A similar trend was also observed in our previous studies [35, 36] 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 median reduction was higher than that at the group level, reaching 50% in total on D14, suggesting that EO had an effect in most of the tested animals.

The lower efficacy in vivo of EOs in general can be attributed to their unstable nature as their ingredients are prone to degradation and evaporation, which causes limited bioavailability [51, 56]. On the other hand, the complexity of the gastrointestinal tract of ruminants that hinders oral applications is also involved [57]. For these reasons, the EO was administered in a specific, innovative way in the present study – intraruminally via a tube placed through the oral cavity, pharynx and oesophagus. This method of administration not only avoids the possible effect of the formulation on the mucous membranes of the upper parts of the gastrointestinal tract, but also the inactivation of the EO active ingredients in these parts. It also better ensures that the exact amount of EO can reach the target sites in the abomasum and intestine compared to peroral administration or administration via the feed, where a certain amount of the formulation can be spat out by the animals. However, the application of the formulation still can be hindered if the animals are keeping free range (not in the boxes) during the treatment due to the previously mentioned reasons.

The other way to solve the problem of the unstable nature of EOs, especially in ruminants, represent 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 [51, 58, 59]. 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 [60] or the use of lick blocks with herbal compounds [61] should also be considered. Ultimately, EOs, which have a certain but not required efficacy of $\geq 90\%$ [62], can still be a valuable component of an integrated approach to sustainable control of GINs along with other methods [63].

According to the results of GC–MS analysis (Table 1, Figure 1), 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. [64], the isolated carvacrol showed high anthelmintic potential in various in vitro tests, including the larval development test (LDT) and the adult worm motility test (AMT) in addition to the EHT. This indicates that this compound is effective against different parasite stages. 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. [65], 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* [66] and at 23.76% in *Thymus vulgaris* [67] as well as γ -terpinene at 20.15% in *Melaleuca alternifolia* [68] and at 11.42% in *Citrus aurantifolia* [43].

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 [35], 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 total 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 [65]. 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 [48]. These considerations could be useful for the future development of herbal anthelmintics.

Preliminary toxicity studies (Tables 4 and 5) indicate that the use of winter savory is safe in practice, at least as far as short-term side effects are concerned (14 days after treatment). Namely, no changes in feeding, defecation or animal behaviour were observed after the treatments. None of the tested haematological parameters were significantly altered ($p > 0.05$) and remained within the reference range, except for the parameters that were also outside this range before treatment. In fact, the administered *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. However, this can be attributed to the effect of the tested EO on the parasite load and the resulting improvement in the clinical aspects of the animals, including the degree of anaemia. For the biochemical parameters reflecting the effect of the administered EO on renal (urea and creatinine) and hepatic (AST and GGT) function, the absence of any statistical changes ($p > 0.05$) and, in the case of GGT, even significantly decreased values ($p < 0.05$), indicates that the administered EO did not affect their function. The same trend was also observed in our previous studies with the EOs of oregano [35] and peppermint [36], in which the same preliminary toxicity

studies were conducted, with no toxic effects observed in any of the animals tested at the administered dose (150 mg/kg bw, intraruminal). In other similar studies such as those conducted by Katiki et al. [69, 70], where the EO of lemongrass (*Cymbopogon schoenanthus*) was administered orally at doses of 180 and 360 mg/kg, and an encapsulated combination of anethole and carvone that was administered through the feed at doses of 20 and 50 mg/kg, the results showed that these plant products are safe for the lambs. However, although many of the EOs are currently considered safe for use in animals intended for human consumption [71], the exact studies confirming this fact are still scarce [25], suggesting that toxicity studies should be a regular part of studies aimed at evaluating the possibility of using plant products in animals.

As shown in Tables 6 and 7, the results of the coproculture study revealed the presence of four GINs genera in both farms, namely *Haemonchus*, *Trichostrongylus*, *Teladorsagia* and *Chabertia*, which are regularly found in this region [6, 48]. Apart from the differences in terms of genera representation between the two farms tested ($p < 0.05$), which is to be expected as these are two different farms and it is not important for the results obtained, no statistically significant difference in representation was observed for any of the genera before and after treatment in either farm ($p > 0.05$), which indicates that *S. montana* EO is not specific to a single genus. However, some other in vivo studies [42, 72] have shown that natural products, administered perorally, can have different effects on individual GINs observed on FECRT or CET, usually the highest one against *H. contortus* in comparison with e.g., *Trichostrongylus* spp. and *O. columbianum*. These results can be explained by the location of the gastrointestinal tract where various GINs are found, with the applied EOs acting mainly on *H. contortus* and other nematodes parasitizing in the abomasum, where the bioavailability of the EO active ingredients is higher after peroral administration compared to the small and large intestine. Further studies evaluating the efficacy against different GINs separately should also be conducted with *S. montana* EO.

Plant-based formulations have many advantages that can be exploited for their use in animals for various indications, including the control of GINs [73–75]. 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 [24, 25]. This may also contribute to their less susceptibility to resistance development in comparison with synthetic agents [43, 76]. 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 [13, 16, 77]. 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 [43], as in the case of *S. montana* in Serbia [78]. For these reasons, the possibility of using plant-based drugs against parasites is the subject of a large and increasing number of studies in recent times, attracting a growing number of researchers worldwide [25, 79–83], with promising results being obtained in most of these studies.

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 [12], some authors recommend a minimum efficacy of 90% in FECRT for new anthelmintic agents [62, 84]. 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 [85], pasture management and nutritional manipulations [86], biological control methods (direct – use of nematophagous fungi, bacteria or even other nematodes or indirect – use of deep buntle or earthworms) [87] and development of vaccines [88] 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 [11, 13, 89], 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 [90–93]. It has been shown that carvacrol, one of the main constituents of *S. montana* EO, can enhance the effect of drugs that are agonists of the nAChR (imidazothiazoles) or the agonists of GABA receptors (ivermectins and piperazine) [94, 95]. Studies with other plant species showed that the combination of e.g., ethanol extract of *Ananas comosus* with the fungal product *Clonostachys rosea* effectively reduced faecal egg and larva counts, larval development and the number of infective larvae (L3) in the pasture [60]. These examples have shown that various plant-based anthelmintics, including EOs and their active ingredients, may represent a valuable source in combination with commercial drugs, or other mentioned alternatives.

Conclusion

The results of the efficacy and preliminary toxicity tests conducted in this study indicate that the EO of *S. montana* has the potential to be used against GINs in sheep. The in vivo efficacy should be further improved by increasing the dose, different way of use including multiple application or the use of encapsulation technique. Nevertheless, the use of this oil, in association with other methods, may help to reduce the current frequency of treatments with chemotherapeutic agents to slow down the development of AR, and reduce the negative effects of these drugs. Further studies of *S. montana* EO with performing other testing, including those against individual GIN species (especially those that are resistant to existing drugs), should be performed.

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Involvement of a human participant or wild plant

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

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.

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Data availability

The data contained in this manuscript were part of the PhD thesis of the first and corresponding author, Dr. Filip Štrbac. Printed version of the dissertation is available in the library of Faculty of Agriculture, University of Novi Sad, Serbia, where the thesis was defended. In electronic form, it is available on the repository of the institution affiliated with the first author - Institute for Multidisciplinary Research, University of Belgrade, Serbia at the following link: <https://rimsi.imsi.bg.ac.rs/handle/123456789/2459>. Also, previous version of this manuscript is available online as a preprint on the following link: <https://www.researchsquare.com/article/rs-4576907/v1>.

Declarations

Ethics approval and consent to participate

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). The animals used in this study were obtained from a private source and the owners have given informed consent for their animals to be used in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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