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IN VITRO OVICIDAL EFFECT OF COMMON JUNIPER (*JUNIPERUS COMMUNIS L.*) ESSENTIAL OIL ON SHEEP GASTROINTESTINAL NEMATODES

IN VITRO OVICIDAN EFEKAT ETARSKOG ULJA KLEKE (JUNIPERUS COMMUNIS L.) NA GASTROINTESTINALNE NEMATODE OVACA

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ABSTRACT

The negative influence of gastrointestinal parasitism in sheep is growing today due to the development of anthelmintic resistance to commercial drugs. For this reason, researchers around the world are looking for new, alternative strategies for controlling these parasites. In this context, medicinal plants and their products are increasingly mentioned. The aim of this study was to evaluate *in vitro* ovicidal activity of common juniper (*Juniperus communis* L.) essential oil against sheep gastrointestinal nematodes. For that purpose, the egg hatch test was used, and the obtained results were analyzed by analysis of variance (ANOVA) followed by comparison with Tukey's test ($p<0.05$). Faecal samples were collected from two independent, natural-infected farms located in Eboli (SA), Italy, whereby the coproculture examination identified the presence of four genera of sheep gastrointestinal nematodes: *Haemonchus* (53%), *Trichostrngylus* (29.5%), *Teladorsagia* (14.5%) and *Chabertia* (3%). Main components of common juniper essential oil identified by GC-MS analysis were α-pinene (40.46%), sabinene (14.04%), myrcene (8.87%) and limonene (4.95%). *In vitro* ovicidal activity was evaluated at six different concentrations (50, 12.5, 3.125, 0.781, 0.195 and 0.049 mg/mL), whereby *J. communis* essential oil showed high activity with the inhibitory effect on hatchability of 81-96.75%, depending on the concentration. For all concentration tested, the effect was statistically significantly higher compared to the negative control, while for the three highest concentrations the effect was similar to the positive control. The obtained results suggested that *J. communis* essential oil have high anthelmintic potential. However, these results need confirmation in further field examination.

Keywords: essential oil, *Juniperus communis*, phytotherapy, nematodes, small ruminants.

SAŽETAK

Negativni uticaj gastrointestinalnih parazita kod ovaca u današnje vreme raste usled razvoja antihelmintičke rezistencije na komercijalne preparate. Iz tog razloga, istraživači širom sveta tragaju za novim, alternativnim strategijama u cilju kontrole ovih parazita. U tom kontekstu, lekovite biljke i njihovi proizvodi se sve češće spominju. Cilj ovog istraživanja je bio ispitati in vitro ovicidnu aktivnost esencijalnog ulja kleke (Juniperus communis L.) protiv gastrointestinalnih nematoda ovaca. Za ovu svrhu je upotrebljen egg hatch test, nakon čega su dobijeni rezultati obrađeni analizom varijanse i potom upoređeni Tukijevim testom ($p<0.05$). Uzorci fecesa su uzeti sa dve odvojene farme koje se nalaze u oblasti Eboli (SA), Italija, pri čemu je tokom ispitivanja koprokulture identifikovano prisustvo četiri roda gastrointestinalnih nematoda ovaca: Haemonchus (53%), Trichostrongylus (29.5%), Teladorsagia (14.5%) i Chaberita (3%). Najzastupljenije komponente etarskog ulja kleke identifikovane biohemijskim analizama su bile α-pinen (40.46%), sabinen (14.04%), mircen (8.87%) i limonen (4.95%). In vitro ovicidna aktivnost je testirana za šest različitih koncentracija (50, 12.5, 3.125, 0.781, 0.195 i 0.049 mg/mL), pri čemu je etarsko ulje kleke pokazalo visoku aktivnost sa inhibitornim efektom na izleganje larvi od 81-96.75%, u zavisnosti od koncentracije. Kod svih ispitivanih koncentracija efekat je bio statistički značajno veći u poređenju sa negativnom kontrolom, dok je kod tri najveće koncentracije efekat bio sličan pozitivnoj kontroli. Dobijeni rezultati ukazuju na to da etarsko ulje kleke ima visok antihelmintički potencijal. Međutim, potrebno je ove rezultate potvrditi u budućim ispitivanjima u terenskim uslovima.*

Ključne reči: esencijalno ulje, Juniperus communis, fitoterapija, nematode, mali preživari.

INTRODUCTION

Gastrointestinal nematodes (GINs) nowadays present a significant obstacle in the breeding of small ruminants (1). These parasites cause direct and indirect loses in different ways such as to impair of the health of the animals, reduction in food intake, low weight gain and milk production, lowered fertility and reduced work capacity (2). This is resulting in significant economic losses in meat, milk and wool production, as well as in sheep reproduction (3). Besides, anthelmintic resistance, which is spread worldwide, has developed as a consequence of frequent use, increased dosage and increased application rate of commercial anthelmintic drugs (1). These concerns have stimulated the search for alternative control methods such as a genetic selection of animals, pasture management, crop-livestock integration, nutrition adjustment, the use of fungi and bacteria in biological control, vaccine production and the use of herbal medicines (4)(5).

Essential oils (EOs) are natural, highly volatile metabolic secretions of plants whose role is still not fully understood by science (6). Actually, these plant products are a complex mixture of different compounds with great biotechnological and pharmaceutical potential (7). *Juniperus communis* L. (fam. *Cupressaceae*), also known as common juniper, is a very well known aromatic and medicinal herb with high therapeutic potential for the treatment of diseases in human and animals. It is a shrub or small evergreen tree with the largest natural range of any woody plant, mostly distributed throughout the cold and temperate regions of Northern Hemisphere (Europe, Asia and Northern America) (8)(9). This plant has a wide spectrum of pharmacological activity and is already widely used in folk medicine as antiseptic, diuretic, stomachic, cardiac and rheumatic drug (10). However, there are not too many data about using of *J. communis* in veterinary medicine.

The aim of this study was to evaluate the ovicidal activity of *Juniperus communis* essential oil against sheep gastrointestinal nematodes. These results can be significant for further in vivo research and for possible even wider therapeutical use in veterinary medicine.

MATERIAL AND METHODS

Essential oil and chemical analysis - Essential oil of *Juniperus communis* L. was purchased from producer BIOSS, Serbia in 2019. Qualitative and semi-quantitative chemical characterization of EO was performed by GC-MS, using Agilent Technologies 6890N gas chromatograph coupled with Agilent Technologies 5975B electron ionization mass-selective detector. The following technical conditions were used: injection volume of EO 1 µL; injector temperature 250 °C; split ratio 1:10; carrier gas helium; flow rate: 1 mL/min;; capillary column: HP-5 (30m×0.25mm, 0.25µm); temperature program 50-270 °C; ion source temperature 230 °C; electron energy 70 eV; quadrupole temperature 150 °C. The compounds were identified by comparison of mass spectra with data libraries (Wiley Registry of Mass Spectral Data, 7th ed. and NIST/EPA/NIH Mass SpectralLibrary 05) and confirmed by comparison of linear retention indices with literature data (11). Relative amounts of components, expressed in percentages, were calculated by normalization procedure according to peak area in total ion chromatogram.

Obtaining eggs and egg hatch test - The whole *in vitro* procedure was performed at the CREMOPAR research centre, Eboli (SA), Italy. Faecal samples were collected from two farms, Tobia Morena and Lullo Massimo, which are located at the same district. GIN eggs were recovered from faeces collected directly from the rectal ampulla of sheep with natural mixed infection. The faecal samples were processed within 2h of collection by using the recovery technique with some modifications (4). Firstly, faecal samples were homogenized and filtered under running water through sieves with a mesh size of 125, 63 and 38 µm, respectively. Next, the eggs retained on the last sieve were washed and centrifuged for 3 min at 1500 rpm with distilled water, after which the supernatant was discarded. In the end, centrifugation was performed using 40% sugar solution to float the eggs. Then, eggs were isolated in new tubes, mixed with distilled water and then centrifuged two more times in order to remove pallets and to get aqueous solution with eggs.

In vitro ovicidal activity of *Juniperus communis* was examined using the egg hatch assay, which is commonly used to test the efficacy of antiparasitic drugs and presence of parasitic resistance (12). 24-well plates, containing aqueous solutions of approximately 150 eggs/well, were used as proposed by literature with some modifications (13). A six different concentrations of *J. communis* EO (50, 12.5, 3.125, 0.781, 0,195 and 0.049 mg/mL) were emulsified in Tween 80 (3%, v/v) and completed with distilled water in a final volume of 0.5 mL/well. After incubation for 48h at the 27°C, the number of eggs and first-stage (L_1) larvae were counted under an inverted microscope and compared to the controls. The positive control was thiabendazole at a concentration of 0.025 mg/mL, and the negative control was Tween 80 (3%, v/v). The experiment was performed two times with two replicates each, and the results were expressed as the mean percentage of egg hatching.

Coproculture - In order to determine what exactly species of gastrointestinal nematodes were *in vitro* tested on the efficacy of *J. communis* EO, coproculture was performed. Faecal samples in a plastic box, covered by cellophane bag with holes, were left to incubation for 10 days (24-27°C). After, cellophane bag was removed and the warm water was added and left to incubation for 4h at room temperature. Next step were two filtrations by using large-hole and small-hole sieves to discard the supernatant and get a suspension with L_3 larvas. This suspension was put at Baerman

apparatus and left to incubation during 24h at room temperature. After centrifugation and discarding the supernatant, L_3 larvas were observed under an inverted microscope and identified using a morphological key (14).

Statistical analysis – As proposed by the literature (15), the mean percentage of egg hatching was calculated by using the following formula: IH (%) = Number of eggs / (number of eggs + number of larvae (L1)) x 100. Data on inhibition of hatchability (IH) were analyzed in order to compare values obtained for different concentrations with each other and with controls (+ and -). For this purpose, one-way analysis of variance (ANOVA) was applied followed by Tukey's test ($p<0.05$), using program GraphPad Prism 8.3.1.

RESULTS

The results of gas chromatography and mass spectrometry analyses showed a rich composition of *J. communis* EO, with a large number of potentially pharmacologically active substances. In total, 28 different substances were identified, whereby the most represented components were α-pinene (40.46%), sabinene (14.04%), myrcene (8.87%), limonene (4.95%), terpinen-4-ol (2.85%), β-pinene (2.70%), germacrene D (2.52%) δ-cadinene (2.46%), germacrene B (2.32%) and p-cymene (1.94%).

In vitro egg hatch test conducted in this study showed high activity of *J. communis* EO against GINs eggs. The inhibitory effect on hatchability varied from 81% to 96.75% and all concentrations tested showed a significantly higher efficacy compared to the negative control ($p<0.001$). For the three highest concentrations, the effect was similar to that of thiabendazole ($p>0.05$) (table 1).

The results of coproculture examination are shown in table 2. On both farms, four genera of sheep gastrointestinal nematodes were identified, *Haemonchus*, *Trichostrongylus*, *Teladorsagia* and *Chabertia*.

Table 1. Efficacy percentage (mean ± standard deviation) of Juniperus communis essential oil on egg hatching of sheep gastrointestinal nematodes

Treatment	Inhibition of hatchability
50 mg/mL	96.75 ± 1.71^{a}
12.5 mg/mL	95.5 ± 1.73^{ab}
3.125 mg/mL	94.75 ± 0.96^{ab}
0.781 mg/mL	91 ± 1.63^{b}
0.195 mg/mL	85.5 ± 0.58^{c}
0.049 mg/mL	81 ± 1.63^{c}
Thiabendazole, 0.025 mg/mL (+)	98 ± 0.82^{a}
Tween 80, 3% (-)	16.75 ± 5.56^{d}

**Values with different lowercase letters in the same column indicate significant differences ($p<0.05$)*

Table 2. Identified sheep GINs and their presence (%) on both examined farm

Farm	*Haemonchus* spp.	*Trichostrongylus* spp.	*Teladorsagia* spp.	*Chabertia* spp.
Tobia Morena	59	26	11	4
Lullo Massimo	47	33	18	2
Total	53	29.5	14.5	3

DISCUSSION

Parasitic nematodes of the gastrointestinal tract remain a major constraint associated with the breeding of sheep under grazing or browsing conditions (16). Among them, the most common parasites are *Haemonchus contortus*, *Trichostrongylus* spp., *Teladorsagia* spp., *Ostertagia ostertagi* and *Cooperia oncophora*. In addition, anthelmintic resistance in gastrointestinal nematodes has been developed and reported worldwide in multiple nematode and livestock species (17). In European countries, AR mainly concerns resistance against benzimidazoles and isolated cases of resistance against macrocyclic lactones (18). For these reasons, need to find new strategies for controlling these parasites have arisen, whereby essential oils of various plants are often mentioned in this context. However, although essential oils are widely used to prevent and treat human diseases, little is known about their use in veterinary medicine (19).

As mentioned before, common juniper is a well known medicinal herb that has long been used in a folk medicine. For this purpose, whole or different parts of the plant such as berries, fruit, bark or areal parts are used (20). In various studies, its antibacterial activity has been demonstrated against many bacterial species including both gram+ (*B. subtilis*, *S. auereus*) and gram- microorganisms (*P. aeruginosa*, *P. mirabilis*), although some species were resistant or poorly sensitive. Particularly high activity was observed against various fungi, especially dermatophytes and different *Candida* species. In these studies, α-pinene, β-pinene, sabinene, myrcene and limonene were also main compounds (21,22,23). There are also many reports about hepatoprotective, anti-inflammatory, antioxidant, antihyperlipidemic, analgesic, antimalarial, antihypercholesterolemic, anticataleptic, neuroprotective and many other activities of *Juniperus communis* (20). These medicinal properties can be used also for treating various diseases in animals.

Juniperus communis is also considered to be used as a nutraceutical in veterinary medicine (9). This and many other plants can be added into animal feed and be used in routine feeding practice as a preventive/treatment measure against many diseases. There are already some data about sporadic use of these plants for controlling internal parasites in ruminants, although most of this information are anecdotal and needs further verification. However, using of medicinal plants and their products as a feed supplements/additives instead of chemical drug may be considered a feasible approach in a controlling sheep GINs, which can also be important from an AR perspective (24). Nevertheless, addition further studies are needed to validate these assumptions.

In the present study, the high ovicidal anthelmintic potential of *J. communis* EO against sheep gastrointestinal nematodes was demonstrated. With slight differences, all concentrations tested were showed the high inhibitory effect on the egg hatching. It can be assumed that demonstrated efficacy is probably associated with its rich composition and synergistic effect of different compounds of EO tested. These compounds are already considered medically relevant because of their proven antimicrobial, anti-inflammatory, antioxidant, sedative, anticancer and many other properties (25, 26). This corresponds with previously mentioned activities of J. communis. More importantly in context of this study, these compounds are also represented in various essential oils with a proven in vitro anthelmintic effect against GINs of small ruminants. For example, limonene was well represented in *Citrus aurantifola* (56.37%) and *Eucalyptus staigeriana* (28.82%), α-pinene in *Rosmarinus officinalis* (14.76%), β-pinene in *Citrus aurantifolia* (11.86%), sabinene in *Anthemis nobile* (6.27%) and myrcene in *Lippia sidoides* (5.43%) essential oil (1, 4, 7, 27). Other components of *Juniperus communis* EO identified in this

study were also represented in these and similar studies in different percentages.

Egg hatch test, which is used in this study, is commonly used to test the efficacy of antiparasitic drugs and presence of parasitic resistance (12). This test is considered reliable and, like other *in vitro* tests, is advantageous for the choice of substances to be tested *in vivo* (13). The main advantages of the use of these tests prior to *in vivo* tests are the need for a smaller number of animals and time and cost savings. However, EOs that are effective in vitro are not necessarily effective to the same extent in the field conditions (7), which is especially true for ruminants because of their anatomical and physiological characteristics of the gastrointestinal tract. Therefore, *in vitro* assays are useful as a basis for the selection of potential active substances, and they can be used as the first step in a demonstrating the efficacy of certain medication based on which further *in vivo* testing can be planned (28). Thus, high ovicidal activity of *J. communis* EO against sheep GIN demonstrated in this study need to be confirmed in further *in vivo* testing.

CONCLUSION

This study showed the *in vitro* anthelmintic, ovicidal potential of *Juniperus communis* essential oil against sheep gastrointestinal nematodes. This is another confirmation of the possible importance of medicinal plants and their ingredients for veterinary medicine. That can be of particular importance nowadays because of the decreasing efficacy of commercial anthelmintics due to the development of anthelmintic resistance. However, these results need confirmation in further testing in field conditions.

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