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Research article

ANTIHELMINIC ACTIVITY OF CARVACROL, THYMOL, CINNAMALDEHYDE AND P-CYMEN AGAINST THE FREE-LIVING NEMATODE *CAENORHABDITIS ELEGANS* AND RAT PINWORM *SYPHACIA MURIS*

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In the present study we tested the dose andh time dependence of the antinematodal effects of carvacrol and tyhmol on *Caenorabditis elegans,* and the efficacy of carvacrol, thymol, p-cymene and cinnamaldehyde,which were administrated in the drinking water of rats naturally infected with the pinworm *Syphacia muris*. The control treatment of the infected rats was carried out with piperazine. Thymol caused a dose and time-dependent mortality in adult *C. elegans*. The value of the Median Lethal Concentration (LC_{50}) of thymol was 117.9nM after 24h and 62.89 nM after 48h of exposure. Carvacrol exhibited a higher antinematodal efficiency than thymol. The LC_{50} of carvacrol, after 24 hours of exposure, was 53.03 nM, while after 48 hours it was 33.83 nM. On the other hand, piperazine showed an extremely high efficacy against *S. muris* infection in rats. Piperazine, at a dose of 625 mg/kg bw, administered in drinking water continuously for 10 days, eliminates the infection completely. However, none of the investigated active ingredients of essential oils were effective against *S. muris*. The reason for the lack of efficiency may be due to their pharmacokinetic properties. A relatively low amount of, orally administered, active ingredients of essential oils reaches the distal segments of the gastrointestinal tract, where *S. muris* inhabits the gut (colon and cecum). The obtained results, on *C. elegans*, indicate a clear dose and time-dependent antinematodal effect of thymol and carvacrol. However, for clinical application, it is necessary to examine the efficacy of microencapsulated formulations with a controlled release of active ingredients of essential oils in certain parts of the gastrointestinal tract.

Key words: *C. elegans, S. muris,* carvacrol, thymol, piperazine

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INTRODUCTION

Nematodes are the most diverse of all animals, over 28000 nematode species have been described, of which over 16000 are parasitic [1]. Current antiparasitic pharmacotherapy is facing several important issues. Reports relating to resistance of different parasitic nematodes to antiparasitic drugs are becoming common, while the increase of drug doses often causes a pronounced toxicity [2]. Furthermore, the rigorous standards for maximum residue levels in animal tissues limit the use of drugs with long withdrawal periods. For the pharmacotherapy of parasitic infections, it is important to introduce some new drugs, with new mechanism of action, which would be effective and at the same time safe for the host. Essential oils (EOs) are among the class of natural products that have an anthelmintic activity and may be an alternative treatment for the control of gastrointestinal nematode infections [2,3]. Plants produce EOs as organic products of secondary metabolism. Their active principles, based on the pharmacological characteristics are potentially the most serious alternative drugs. These herbal medicines could replace the classical antiparasitic drugs but their effectiveness must still be tested, both *in vitro* and *in vivo*.

We decided to investigate the antinematodal effects of selected active ingredients of EOs in the free-living nematode *Caenorabditis elegans*, and *in vivo* in rats naturally infected with the pinworm *Syphacia muris*.

The free-living nematode *Caenorhabditis elegans* may offer a convenient alternative experimental model to search for new compounds that specifically kill nematodes. *C. elegans* is a useful experimental model to study anthelmintics and offers a throughput that is not possible with parasitic species. For example, it is possible to apply certain concentrations of test substances directly to *C. elegans* and measure the dose and time dependence of the effect. The majority of marketed anthelmintics are effective against *C. elegans* [4,5] and the use of this experimental model has been instrumental in improving the understanding of the mechanism of action of several anthelmintic compounds. Therefore it might also be a powerful screening system for anthelmintic lead compounds, as has been suggested over 30 years ago [6].

Rodent pinworm are oxyurid nematodes of the genera *Syphacia*. *Syphacia muris* (commonly referred to as pinworms) is a nematode which may be found in the intestinal tract of the laboratory rat (*Rattus norvegicus*) and is the most prevalent pinworm in rats. The eggs are very light and will aerosolize, resulting in a widespread environmental contamination [7]. Most pinworm infections are asymptomatic, but complications attributed to infestations include decreased weight gain, behavioral changes and an altered immune response [7,8]. The life cycle of *S. muris* is direct and completed in 7 to 8 days, making this particular pinworm ideal for epidemiologic studies. Adult worms of *S. muris* inhabit the cecum and colon and female worms migrate to the anus and deposit all their eggs in the perianal region of the host before dying. The eggs embryonate within a few hours, at which point they are considered infective [9]. These parasites are detected routinely within rodent facilities and their persistence, despite

control measures, indicates deficiencies in the diagnostics and eradication. Many institutions never truly eradicate pinworms, presumably due to a failure to identify the presence of the organism or because of ineffective treatment. However, a number of treatments are available and have proven to be efficacious [10].

In the present study we tested the dose and time dependence of the antinematodal effects of carvacrol and thymol on the *C. elegans* and the efficacy of the active ingredients of essential plant oils: carvacrol, thymol, p-cymene and cinnamaldehyde, against pinworm infection in rats. The control treatment of infected rats was carried out with piperazine, a well-known antinemtodal drug.

Carvacrol and thymol are monoterpenic phenols. Together with p-cymene they are present in the essential oils of plants from the *Lamiaceae* family. On the other hand cinnamaldehyde was isolated from cinnamon essential oil [11]. Pharmacological studies of carvacrol showed that it possesses an antiparasitic effect [2,12]. Furthermore studies have shown that thymol demonstrated its efficacy against experimental murine cystic echinococcosis and is a potential alternative for the treatment of human hydatid disease [13]. P-cymene, in a blend of active ingredients of four plants EOs, shows the ability to reduce infection burdens of soil transmitted helminths in pigs [14], while cinnamaldehyde has potent *in vitro* antinematodal activity [11].

MATERIALS AND METHODS

Ethical approval

All experimental procedures in the study were approved by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia - Veterinary Directorate (permit N°323-07-05566/20I5-05/1), according to the Serbian Animal Welfare Protection Law, and Directive 2010/63/EU.

C. elegans

C. elegans, N2 wild-type was a gift from Prof. Dr Alan P. Robertson (Iowa State University, IA, USA). Nematode Growth Median was used for the cultivation of *C. elegans* (NGM, US Biological, Life Sciences, USA). After culturing on the NGM plates for six days, the worms were collected by cutting the medium into squares 3x3mm and transferred to glass tubes with 2ml of 0.1M NaCl. The tubes were placed in a shaker until complete dissolution of the substrate and release of the worms. Then tubes were centrifuged for 4 minutes at 2500 r/min, and 400μl of supernatant were transferred to a second test tube. This was followed by another 1 minute of centrifugation, at 2000 r/min. After that 20 μl of a suspension of nematodes were inoculated on the Petri dish (diameter 3cm) with 2.5 ml NGM substrate and increasing concentrations of thymol (0, 1, 10, 30, 100, 300 i 1000 nM) and carvacrol (0, 1, 10, 100, 1000, 10000, 100000nM). The titer of worms was 19-34/20 μM and each concentration was tested on five Petri dishes. The five Petri dishes without the added test substances were untreated controls.

The plates inoculated with *C. elegans* were placed in a thermostat (Memmert IN30, Germany) at 20° C for 24 and 48 h. After incubation, the plates were observed under an inverted microscope (Motic AE 31, PRC) and the movement and pharynegeal pumping of *C. elegans* were recorded with a camera (Motic 5 MP, NRK) on the hard disk of a PC, for later analysis. The survival rate of the adult *C. elegans* was determinated in the medium with thymol and carvacrol, as well as in the untreated control medium. The lethality was determined by the cessation of movement and pharyngeal pumping. *C. elegans* was considered dead when it did not move and did not respond to repeated touching with a probe. Mortality was calculated for each treatment after 24 and 48 hours and expressed as percentage.

Housing of rats and study design

Adult Wistar rats (100-150g), used in the present study, were obtained from the Military Medical Academy Breeding Farm, Belgrade, Serbia. The animals were housed in groups of five, in home cages on autoclaved wood shaving bedding under standard conditions: temperature of 21±1ºC, relative humidity of 55-60% and 12/12 h light/dark cycle. Food and water (medicated or control) were provided *ad libitum*.

Cages with wood shavings and water bottles were changed daily with a strict, aseptic, technique. All cages and implements were washed in a mechanical washer and autoclaved prior to entry into the room.

Rats were free of ecto- and endoparasites other than *Syphacia muris* on in-house screening tests. All animals in the study were maintained in an isolated quarantine area apart from the control group. The rats were randomly divided into 10 groups. For each dose of the test substances (carvacrol, thymol, p-cymene, cinnamaldehyde and piperazine), as well as for the control, groups of 10 rats were formed. The first group was the control (n=10) non-medicated group. All other groups of rats were treated by the addition of the drug or active ingredients of EOs in the drinking water. Rats of the second group (n=10) were treated for 10 days with piperazine 5 mg/ml in drinking water. The third group (n=10) was treated for 7 days with thymol in dose of 10 mg/ml. The forth group (n=10) of rats was treated for 7 days with p-cymene in dose of 10 mg/ml, the fifth group (n=10) included rats treated with cinnamaldehyde in dose of 10 mg/ml in drinking water during 7 days. The sixth, seventh, eight and tenth group (n=10) of rats were treated with carvacrol in doses of 0.06, 0.6 mg/ml; 6.0 and 10 mg/ml during 7 days.

Every day, at the same time (6:00pm), the amount of consumed water was measured and recorded. Based on water consumption the exact dosage of applied substances was calculated per kg of body weight of the rats.

At the end of the treatment 2 rats, from each group, including the control group, were sacrificed. In order to determine the presence of *Syphacia muris* larvae and adult parasites, microscopic examination of the contents of colon and cecum was done.

Drugs

Substances used in this study were: carvacrol (Sigma-Aldrich), absolute ethanol (Zorka Pharma, Serbia), thymol (Sigma-Aldrich), p-cymene (Sigma-Aldrich), cinnamaldehyde (Sigma-Aldrich) and piperazine (Sigma-Aldrich). Carvacrol, thymol, cinnamaldehyde and p-cymene were diluted with the ethanol-aqueous solvent, with the final concentration of ethanol in tap water of 0.1 %v/v. When tested, the 0.1% of ethanol in tap water did not alter the drug responses. Piperazine was dissolved in tap water. All substances used in the experiment were administrated per os in drinking water.

Experimental methods

Perianal cellophane tape testing

After the acclimatization period, a cellophane tape test was performed to confirm the infection in rats. During the trial, for each group of rats, the test was done on the 0, 4^{th} and 7^{th} day of the treatment (for piperazine treated rats 0, 4^{th}, 7^{th} and 10^{th} day) and three days after the treatment, at the same time, at 6 pm. For each rat, a single piece of cellophane tape was firmly applied to the anal region 3 consecutive times at each time point. The tape length varied from 25 to 30 mm. Tapes were attached to standard 25 mm × 75 mm microscope slides and systematically examined by using a 4x microscope objective. The same person performed all of the microscopic examinations. The animals were included in the study only if they had an *S. muris*-positive tape test on the day the study began.

Fecal flotation and microscopic examination of smears

Fecal flotation was performed before the start of the treatment to confirm the infection and at the end of treatment. The flotation of feces is a well-known method for detecting *Syphacia muris* eggs in which a solution of salt or sugar is used to concentrate the ova at the surface of the suspension [15]. The samples were processed with a saturated solution of NaCl and transferred to a flat-bottomed vial, on top of which a slide was placed. After 5 min, the slides were removed from the bottle and the material adhering to the slide was stained with Lugol solution and analyzed under a light microscope.

The microscopic examination of smears was performed at the end of the treatment (in sacrificed rats).

We used these methods for detecting eggs, larvae and adult *Syphacia muris* in the presented investigation.

Detection of S. muris in the contents of the cecum and colon

The rats were sacrificed by an overdose of thiopentone sodium applied intravenously into the tail vein. A total of 20 rats were euthanized, 2 rats from each tested group including the control. The gastrointestinal tract (GIT) was exposed and the cecum

and colon were placed in a culture dish. The guts were opened under a dissecting microscope and the contents were examined by using saline to facilitate worm identification. *S. muris* has a round esophageal bulb and small cervical alae allowing easy identification.

Statistical analysis

The mortality of *C. elegans* was calculated for each Petri dish after 24 and 48 hours and expressed as a mean percentage (%±SD) for five dishes. Prism 5.0 (GraphPad Software, San Diego, CA) was used to estimate the LC_{50} in the dose-response investigation by non-linear regression.

RESULTS

Thymol caused a dose and time-dependent mortality of adult *C. elegans*. The value of the Median Lethal Concentration of thymol (LC_{50}) was 117.9nM after 24h (95% Confidence Intervals 114.0 to 121.9nM) and 62.89nM (95% Confidence Intervals 59.61 to 66.34nM) after 48h of exposure (Figure 1).

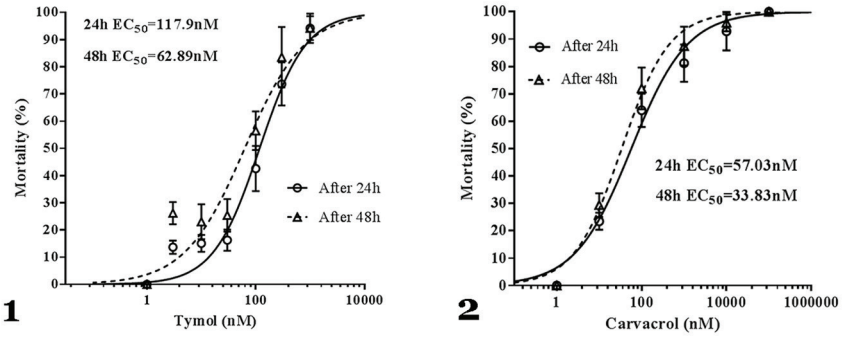

Figure 1. A sigmoidal dose-response curves of thymol nematicidal effects in *C. elegans* after 24 and 48 hours of exposure. Each point represents the mean mortality (%±SD) in 5 Petri dishes.
Figure 2. A sigmoidal dose-response curves of carvacrol nematicidal effects in *C. elegans* after 24 and 48 hours of exposure. Each point represents the mean mortality (%±SD) in 5 Petri dishes.

Carvacrol exhibited a higher antinematodal efficiency than thymol. LC_{50} of carvacrol for adult *C. elegans,* after 24 hours of exposure, was 53.03 nM (95% Confidence Intervals 53.65 to 60.61nM). After 48 hours of exposure the LC_{50} was 33.83 nM (95% Confidence Intervals 32.09 to 35.66 nM) (Figure 2).

The mortality of *C. elegans* was not recorded in the control Petri dishes.

By analyzing the video of treated nematodes in the period between 24 and 48 hours it was observed that the pharyngeal pumping in *C. elegans* slows down and stops before the termination of the movement.

The doses of the test substances per kg of body weight of the rats were determined by calculating the daily consumption of drinking water: piperazine 625 mg/kg; thymol

1670 mg/kg; p-cymene 1250 mg/kg; cinnamaldehyde 1120 mg/kg; carvacrol 6.6, 72, 750 and 1250 mg/kg.

Table 1 shows the results of the cellophane tape test and the fecal flotation test in the control and treated rats. At the beginning of the study both tests were positive for the presence of *S. muris* infection in all rats. The microscopic examination showed that the pinworm eggs are thick-shelled, football shaped with one side slightly flattened. The egg often contains a fully developed embryo. The size of eggs is approximately 75 x 30 μm (Figure 3). In all rats treated with piperazine, the cellophane tape test was negative after 7 and 10 days of treatment. Also, the flotation test was negative on the 10th day of treatment. However, both tests were positive in all of the other rats treated with the active ingredients of essential plant oils.

Table 1. Results of the parasitological examination

Group/day	Cellophane tape testing				Fecal flotation test	
	0	4th	7th	10th	0	7th
Control	+	+	+	+	+	+
Piperazine 5 mg/ml	+	+/-	–	–	+	–*
Thymol 10 mg/ml	+	+	+	+	+	+
P-cymene 10 mg/ml	+	+	+	+	+	+
Cinnamaldehyde 10 mg/ml	+	+	+	+	+	+
Carvacrol 0.06 mg/ml	+	+	+	+	+	+
Carvacrol 0.6 mg/ml	+	+	+	+	+	+
Carvacrol 6.0 mg/ml	+	+	+	+	+	+
Carvacrol 10 mg/ml	+	+	+	+	+	+

+ positive test; – negative test; +/- positive test in a small number of animals; * 10th day

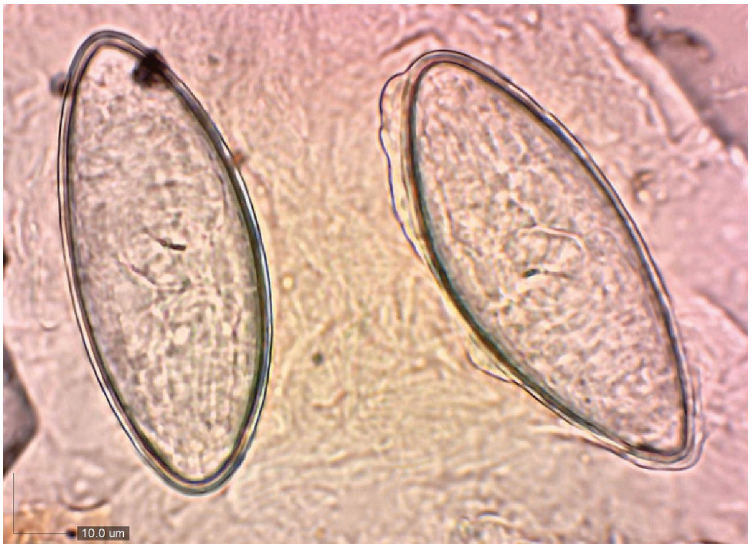

Figure 3. Eggs of *Syphacia muris* isolated from a rat via a perianal tape test.

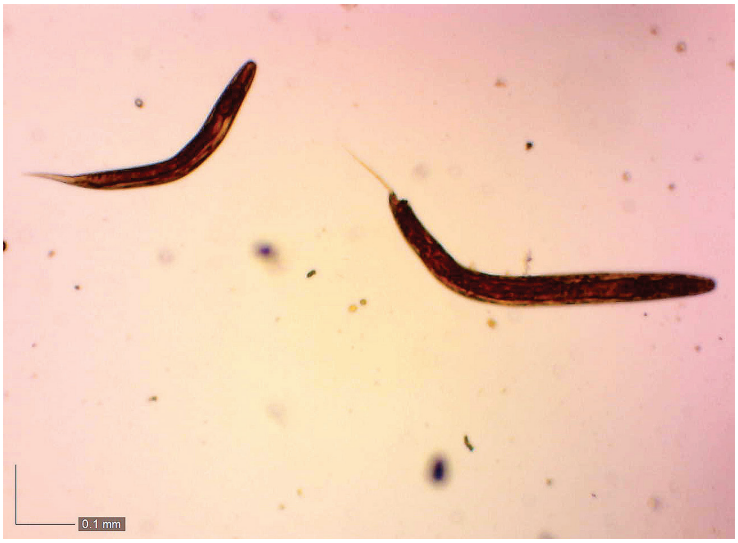

Figure 4. *Syphacia muris* adult, from the cecum of rats.

The microscopic examination of smears at the end of the treatment (in sacrificed rats) revealed the presence of different developmental forms of *S. muris,* predominantly in the cecum and less in the colon of all of the examined rats, except in the rats which had received piperazine. Adult males are about 1 mm in size, and the females about 3 mm (Figure 4).

In control and treated animals during the treatment were not recorded any disturbances of the digestive system, nor any other clinical disorders.

DISCUSSION

The obtained results confirmed that the *C. elegans* can be successfully used as a model for testing the effects of various substances with potential antinematodial properties. The effect of thymol and carvacrol, based on our results is dose and time dependent, whereby carvacrol exhibited a higher efficiency (LC_{50}=53.03nM). These results are consistent with data of Lei et al. [16]. These authors did not examine the dose and time dependency of the nematocidal effects of carvacrol and thymol, but they proved that 670μM (100μM/ml) of thymol and carvacrol cause a mortality of 99% and 100% in *C. elegans,* respectively. The test was performed in a liquid medium, PBS (Phosphate-buffered saline). Similar results were obtained by Abdel-Rahman et al. [17] The authors examined the toxicity of carvacrol and thymol for *C. elegans* in a liquid medium, M9 buffer solution. LC_{50} values amounted to 3 and 8 μg/ml, for carvacrol and thymol, respectively. Based on the research described above, it is evident that the test is more sensitive when the investigated substances are incorporated in an NGM, as opposed to testing in a liquid medium. The reason for this could be a more intense pharyngeal

pumping in the NMG medium than in the liquid buffer medium, but this assumption should be tested in future studies.

The nematicidal effect of carvacrol is due, in part, to its interaction with nicotinic acetylcholine receptors (nAChR) in the neuromuscular system of nematodes. We have previously shown that carvacrol acts as a nAChR antagonist in *Ascaris suum* [2]. This effect could be an explanation for motility disorders and its termination, which is observed in *C. elegans* after the exposure to carvacrol. On the other hand, in the period between 24 and 48 hours of incubation with carvacrol, the pharyngeal pumping slows down and stops before the termination of movement. There is a possibility that carvacrol acts with the homomeric nAChR, called ACR-16, in the pharynx. The existence of this receptor was proven by Abongwa et al. [18], but the precise role, in the contractions of the pharynx, has not been examined yet. This could be a significant new drugable target for anthelmintic treatment and it deserves future research.

In accordance with the obtained results, piperazine showed an extremely high efficacy against *S. muris* infection in rats. Piperazine, in the dose of 625mg/kg bw, administered in drinking water continuously, for 10 days, with daily changes of the bedding, eliminates the infection completely. One hundred percent efficiency was confirmed by the perianal cellophane tape test, the fecal flotation test and by the microscopic examination of smears. These results were expected and are in accordance with the already published findings [19, 20]. However, none of the active ingredients of essential plant oils were effective against *S. muris*. This is contrary to previously published data about clinical antinematodal efficacy of carvacrol and thymol. The efficacy of carvacrol acetate (250 mg/kg) was evaluated in 30 sheep, naturally infected with gastrointestinal nematodes, by the fecal egg count reduction test (FECRT). The larvae recovered from fecal cultures of sheep, before treatment, were identified as *Haemonchus spp.* (90%), *Trichostrongylus spp.* (7%) and *Oesophagostumum spp.* (3%). Carvacrol acetate reduced the number of eggs in feces (epg) by 65.9%, 16 days after the treatment, which was significantly different from the negative control [12]. A similar clinical study was published for thymol [21]. The anthelmintic effect of thymol was evaluated in thirty crossbred sheep of both genders. Each sheep received thymol in a dose of 250mg/kg, and the feces were collected directly from the rectum of the animals on days 0, 7 and 14. The larvae recovered from fecal cultures of the sheep, before treatment, were identified as *Haemonchus spp.* (90%), *Trichostrongylus spp.* (7%) and *Oesophagostumom spp.* (3%). Thymol reduced the sheep egg count per gram of faeces (epg) by 59.8%. Contrarily, cinnamaldehyde was not effective in reducing the *A. suum* infection in pigs *in vivo* [11]. Data on the clinical antinematodal efficacy of p-cymene was not found in the literature.

The reason for the lack of efficiency of the tested active ingredients of EOs may be due to their pharmacokinetic properties. Investigations in pigs have shown, that after oral administration, an almost complete absorption of unchanged carvacrol, thymol and cinnamaldehyde occurs in the stomach and the small intestine. On the other hand, the most intensive biotransformations of carvacrol, thymol and cinnamaldehyde, *in*

vitro, are recorded in the cecum [22]. The presented data indicates that a relatively low amount, of orally administered active ingredients of EOs, reaches the distal segments of the gastrointestinal tract. Therefore, this may be the reason for the absence of an antinematodal effect, because *S. muris* parasites in the colon and cecum.

During the investigation we did not observe any clinical symptoms of disorders in the digestive tract of treated rats which could be brought into connection with the effect of the active ingredients of essential plant oils. This is consistent with data that oregano and thyme oil have the advantage of inhibiting the growth of potential pathogens in while only moderately influencing beneficial members of the intestinal microbiota. This difference in sensitivity may strengthen the microbiota and contribute to improved animal health [23].

The obtained results, on *C. elegans,* indicate a clear dose and time-dependent antinematodal effect of thymol and carvacrol. However, for the clinical application of active ingredients of EOs, it is necessary to examine the efficacy of their microencapsulated formulations and the possibility of using nanocarriers. The controlled release of active ingredients of EOs in certain parts of the GIT would allow higher concentrations, better antinematodal efficiency and exclude the need to increase the applied dose.

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Authors' contributions:

All authors participated in the design of the study, performed the statistical analysis, conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests:

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ANTIHELMINTIČKO DEJSTVO KARVAKROLA, TIMOLA, CINAMALDEHIDA I P-CIMENA PROTIV SLOBODNOŽIVUĆE NEMATODE *CAENORHABDITIS ELEGANS* I PARAZITSKE NEMATODE PACOVA *SIPHACIA MURIS*

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U prezentiranom ispitivanju testirali smo doznu i vremensku zavisnost antinematodnih efekata karvakrola i timola na *Caenorabditis elegans* i efikasnost karvakrola, timola, p-cimena i cinamaldehida davanih u vodi za piće pacovima prirodno inficiranim parazitskom nematodom *Syphacia muris*. Kontrolni tretman inficiranih pacova sproveden je piperazinom. Timol je izazvao dozno i vremenski zavisni mortalitet adultnih *C. elegans*. Vrednost srednje letalne koncentracije timola (LC_{50}) bila je 117.9 nM posle 24h i 62.89 nM posle 48h ekspozicije. Karvakrol je ispoljio višu antinematodnu efikasnost od timola. Vrednost LC_{50} karvakrola posle 24h bila je 53.03 nM, a posle 48h isnosila je 33.83 nM. Sa druge strane piperazin je ispoljio visoku efikasnost protiv infekcije *S. muris* u pacova. Piperazin u dozi od 625 mg/kg tm primenjen u vodi za piće kontinuirano 10 dana, u potpunosti eliminiše infekciju. Međutim, ni jedan od aktivnih sastojaka esencijalnih biljnih ulja nije bio efikasan protiv *S. muris*. Razlog izostajanja efikasnosti može da bude posledica njihovih farmakokinetičkih karakteristika. Relativno mali deo oralno aplikovanih aktivnih sastojaka esencijalnih ulja dolazi do distalnih segmenata gastrointetsinalnog trakta gde parazitira *S. muris* (kolon i cekum). Dobijeni rezultati na *C. elegans* ukazuju jasan dozno i vremenski zavistan antinematodni efekat timola i karvakrola. Međutim, za njihovu kliničku aplikaciju neophodno je ispitati efikasnost mikroinkapsiliranih formulacija sa kontrolisanim oslobađanjem aktivnih sastojaka esencijalnih ulja u određenim delovima digestivnog sistema.