

Original Research

Survival of Nematode Larvae after Treatment with Eugenol, Isoeugenol, Thymol, and Carvacrol

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Abstract

Background: Helminthiasis inflict annual losses on the meat and dairy livestock industries. The commonest species of ruminant parasites are the nematodes: *Strongyloides papillosus* and *Haemonchus contortus*, which lay eggs in the intestine and enter the feces. There, the eggs develop into larvae, which when voided with the feces crawl onto plants. **Methods:** In our experiment, we evaluated the survivability of the noninvasive and invasive (L_{1–2} and L₃, respectively) larvae of *S. papillosus*, *H. contortus* (L₃), and *Muellerius capillaris* (L₁) *in vitro* by subjecting each to natural compounds present in the essential oils of many plants. In the experiment, we used aqueous emulsions of eugenol, isoeugenol, thymol, and carvacrol. **Results:** Administering 1% concentrations of those compounds killed 100% of the nematode larvae following 24 h of exposure. Thymol, eugenol, and isoeugenol at a concentration of 0.1% also caused high larvae mortality (over 96%). **Conclusions:** Continuous usage of synthetic anthelmintic drugs in veterinary medicine has led to the parasites developing resistance, thus, a search for novel nematocidal drugs is required. Eugenol, isoeugenol, thymol, and carvacrol are promising compounds against nematodes. However, additional research is required regarding peculiarities in their actions toward the bodies of mammals and parasitic nematodes.

Keywords: nematode larvae mortality; flavoring; essential oil; nematocidal activity; migrating larva; plant extract

1. Introduction

Pollution of the environment by various chemical drugs, including antiparasitic, is a global problem. Against the background of their extensive application in agriculture, including veterinary medicine, researchers have observed an increase in toxic loading on natural ecosystems and agroecosystems. Moreover, they have observed the presence of residual amounts of those drugs in products with animal origins. Researchers have also recorded resistance by parasites to many active compounds in various synthetic drugs [1]. An important aspect of this issue is the high price of anthelmintic drugs. Combating nematodes is economically important in various spheres of animal farming in many countries worldwide. Therefore, many scientists devote their best efforts to studying alternative ways of fighting parasites, including helminths [2–6], parasitic protozoa [7,8], and ectoparasites [9,10].

One such method is using compounds from natural sources, including medicinal plants [11–13], their essential oils [14,15], and any individual compounds found in them [16–19]. Many of them have a broad range of uses in various spheres of human activity, including agriculture, medicine, and cosmetology. Such compounds exert an array of beneficial properties, including having low toxicity and being easily metabolized [20,21]. In addition, many are cheap and available. Therefore, medicinal plants, essen-

tial oils, and their constituents are potentially interesting as an alternative to modern synthetic antiparasitic drugs [22–25]. The purpose of our research was to study the effect of eugenol, isoeugenol, carvacrol, and thymol on the survival of ruminant nematode larvae *in vitro*.

2. Materials and Methods

The effect of aromatic compounds extracted from essential oils was studied *in vitro* in 2022–2023, in a Laboratory at the Department of Parasitology and Veterinary–Sanitary Expertise of the Dnipro National Agrarian and Economic University (Ukraine). Fecal samples were collected from naturally infected ruminants (goats) by the Clinical–Diagnostic Center of Veterinary Medicine at the Dnipro State Agrarian–Economic University.

Coprological examinations of the feces were performed using the McMaster technique. The larvae of *Haemonchus contortus* (Rudolphi, 1803), and *Strongyloides papillosus* (Wedl, 1856) were cultivated in goat feces for 10 days at a temperature of 18–22 °C. *Muellerius capillaris* (Mueller, 1889) emerges into the environment at the larval stage. Thus, *H. contortus* (L₃), *S. papillosus* (L₁, L₂, L₃), and *M. capillaris* (L₁) larvae were isolated from the feces after 10 days. Since the *M. capillaris* (L₁) larvae are active and resistant to environmental factors, we observed their strong mobility in the control after 10 days. The nematode larvae were extracted using the Baermann test [26].



Table 1. Uses of the additives with aromatic properties that were utilized to determine the survivability among nematodes.

Name	Formula	Use
Thymol CAS 89-83-8	C ₁₀ H ₁₄ O	Thymol is used to make menthol. In medicine, it is used as an anti-worm drug when treating hookworm infection, trichuriasis, and other helminthiases. In beekeeping, it is used against Acari. It is a good antiseptic drug for disinfecting the mouth, fauces, and nasopharynx. It is broadly used in stomatology. It is used in the pharmaceutical industry as a preservative component in many drugs.
Carvacrol CAS 499-75-2	C ₁₀ H ₁₄ O	It is used as a food aroma ingredient and also as a preservative. Additionally, it is used in cosmetology as a flavoring.
Eugenol CAS 97-53-0	C ₁₀ H ₁₂ O ₂	Eugenol is used in the food industry as an aroma ingredient and a flavoring in tea, meat, cakes, perfumery, aroma ingredients, and essential oils. In medicine, it is a local antiseptic and anesthetic agent. Eugenol is also used as an attractant to lure and collect bees. This compound is present in some insecticides, fungicides, and herbicides in the agriculture of the European Union. Eugenol is used in many household items as an aroma ingredient.
Isoeugenol CAS 97-54-1	C ₁₀ H ₁₂ O ₂	Isoeugenol is used similarly to eugenol. It has been approved for cleaning or safety in an occupational or industrial setting (e.g., industrial cleaning supplies or laundry detergent, eye wash, spill kits). Home air fresheners, including candles with a fragrance.

During the *in vitro* experiment, we used first–third-stage (L₁, L₂, L₃) *S. papillosus*, third-stage (L₃) *H. contortus* larvae, and first-stage (L₁) *M. capillaris* larvae. Species-specific nematode larvae were identified according to their morphological traits [27–30]. In the identification process, particular attention was paid to the larvae body sizes, shape and size of the tail end, presence of intestinal cells, their numbers, the number of rows where the cells were located, shape of the cells, and the shape and size of the esophagus [31,32].

For the experiment, we used four compounds (Table 1), which were extracted from the essential oils of medicinal plants: eugenol (manufactured by Carlo Erba Reagents, Italy), isoeugenol (Acros Organics, Belgium), thymol (Carl Roth, Karlsruhe), and carvacrol (Acros Organics, Belgium). When preparing the aqueous emulsion, we used 0.01 mL of eugenol per 1 mL of a 10% solution of polysorbate-80 in water.

To study the effects of the experimental compounds on nematode larvae, the parasites previously placed in water (4 mL) were put into 10 mL test tubes and centrifuged for 4 min at 1500 rpm. Then, the supernatant was removed, and the larvae and sediment were evenly stirred and placed in 1.5 mL plastic test tubes, 0.1 mL in each [33].

Next, various concentrations of the aqueous emulsions of the studied compounds (1%, 0.1%, 0.01%, and 0.001%) were added to the larval culture consecutively (10–60 larvae/sample on average). The experiment was performed at 22 °C for 24 h. During the experiment, we counted live and dead specimens, taking into account two factors: immobility and destruction of the larvae intestines.

According to the results of the experiment, we calculated the mean and standard deviations ($\bar{x} \pm SD$). Significant differences between samples were evaluated by Analysis of Variance (ANOVA), using the Bonferroni correction. To determine the differences between the samples within one line in Table 2, we used Tukey’s test.

3. Results

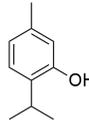
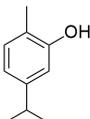
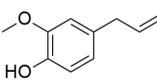
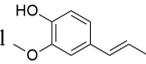
The aromatic compounds, extracted from essential oils of medicinal plants—eugenol, isoeugenol, carvacrol, and thymol—appeared to have effects on the nematode larvae (Table 2). After using 1% solutions of all four compounds, we found no viable nematodes.

A sufficiently strong effect on the nematode larval stages was also exhibited by the isoeugenol, eugenol, and thymol 0.1% emulsion solutions. When using the 0.1% solutions, we observed a mortality rate of over 96% in the studied nematode larvae at various stages of development. Carvacrol also had notable anthelmintic properties against noninvasive stages of the nematode larvae, including L_{1–2} of *S. papillosus*, and L₁ of *M. capillaris*. Emulsion solutions of those compounds at 0.1% concentrations also resulted in a higher level of mortality by those larvae. However, the infective larvae of third-stage nematodes were more resistant to the action of this carvacrol concentration.

Solutions of eugenol, isoeugenol, thymol, and carvacrol at 0.01% had no significant effects on various stages of the larvae development. Nonetheless, noninfective larvae of the studied nematodes were partially affected. At 0.01%, the greatest effect on the noninfective stages of *S. papillosus* was exerted by thymol and carvacrol emulsions, and the greatest effect on *M. capillaris* was exerted by eugenol. A subsequent reduction in concentration of the studied compounds caused death in no more than 11.5% of the nematode larvae at various development stages.

During the experiment, we identified a lethal concentration, which caused 50% of the nematode larvae to die. For L_{1–2} of *S. papillosus*, thymol exhibited the lowest LC₅₀. For the noninfective stage of the development of *M. capillaris*, eugenol demonstrated the lowest LC₅₀. For L₃ of *S. papillosus*, the lowest LC₅₀ was observed for both thymol and carvacrol, while thymol showed the lowest for *H. contortus*.

Table 2. Mortality of the larvae of *S. papillosus*, *H. contortus*, and *M. capillaris* (%) during the 24 h laboratory experiment under the influence of thymol, carvacrol, eugenol, and isoeugenol ($\bar{x} \pm SD$, n = 5).

Substance	Nematode species	Nematode larvae mortality (control), %	Nematode larvae mortality in 1% solution, %	Nematode larvae mortality in 0.1% solution, %	Nematode larvae mortality in 0.01% solution, %	Nematode larvae mortality in 0.001% solution, %	LC ₅₀ , %
 Thymol	L ₁₋₂ of <i>S. papillosus</i>	7.9 ± 2.3 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	47.8 ± 12.7 ^c	7.2 ± 2.9 ^a	0.0138 ± 0.0223
	L ₃ of <i>S. papillosus</i>	0.0 ± 0.0 ^a	100.0 ± 0.0 ^b	97.7 ± 3.1 ^b	14.6 ± 5.7 ^c	0.0 ± 0.0 ^a	0.0483 ± 0.0050
	L ₃ of <i>H. contortus</i>	2.7 ± 2.9 ^a	100.0 ± 0.0 ^b	96.4 ± 3.5 ^c	14.6 ± 7.8 ^d	3.0 ± 2.8 ^a	0.0489 ± 0.0066
	L ₁ of <i>M. capillaris</i>	1.3 ± 1.4 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	25.4 ± 9.1 ^c	1.4 ± 1.9 ^a	0.0397 ± 0.0075
 Carvacrol	L ₁₋₂ of <i>S. papillosus</i>	6.4 ± 6.9 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	41.6 ± 22.4 ^c	11.3 ± 5.5 ^a	0.0229 ± 0.0347
	L ₃ of <i>S. papillosus</i>	0.0 ± 0.0 ^a	100.0 ± 0.0 ^b	89.7 ± 10.8 ^b	23.4 ± 3.3 ^c	0.0 ± 0.0 ^a	0.0461 ± 0.0087
	L ₃ of <i>H. contortus</i>	6.1 ± 3.5 ^a	100.0 ± 0.0 ^b	43.8 ± 18.2 ^c	17.7 ± 5.6 ^d	7.9 ± 2.6 ^a	0.1993 ± 0.2897
	L ₁ of <i>M. capillaris</i>	6.4 ± 6.2 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	29.4 ± 2.5 ^c	9.2 ± 2.8 ^a	0.0363 ± 0.0023
 Eugenol	L ₁₋₂ of <i>S. papillosus</i>	7.9 ± 2.3 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	11.9 ± 3.2 ^a	11.5 ± 4.1 ^a	0.0489 ± 0.0019
	L ₃ of <i>S. papillosus</i>	0.0 ± 0.0 ^a	100.0 ± 0.0 ^b	98.3 ± 3.7 ^b	2.3 ± 2.2 ^a	0.0 ± 0.0 ^a	0.0547 ± 0.0028
	L ₃ of <i>H. contortus</i>	0.0 ± 0.0 ^a	100.0 ± 0.0 ^b	99.3 ± 1.6 ^b	8.2 ± 7.8 ^c	1.3 ± 3.0 ^{ac}	0.0513 ± 0.0049
	L ₁ of <i>M. capillaris</i>	0.3 ± 0.7 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	51.2 ± 7.2 ^c	1.1 ± 1.5 ^a	0.0098 ± 0.0013
 Isoeugenol	L ₁₋₂ of <i>S. papillosus</i>	2.7 ± 3.1 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	9.2 ± 1.0 ^c	3.1 ± 3.0 ^a	0.0504 ± 0.0005
	L ₃ of <i>S. papillosus</i>	0.0 ± 0.0 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	1.5 ± 2.1 ^a	0.0 ± 0.0 ^a	0.0543 ± 0.0010
	L ₃ of <i>H. contortus</i>	4.2 ± 3.2 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	8.9 ± 2.1 ^a	5.4 ± 2.6 ^a	0.0506 ± 0.0011
	L ₁ of <i>M. capillaris</i>	2.2 ± 5.0 ^a	100.0 ± 0.0 ^b	100.0 ± 0.0 ^b	8.4 ± 8.1 ^a	2.0 ± 4.5 ^a	0.0509 ± 0.0044

Note: different letters indicate values which reliably differed one from another within one line of the table according to the results of comparison using the Tukey test with Bonferroni correction ($p < 0.05$).

4. Discussion

In the literature, much data exist on the effects of eugenol on *H. contortus*. The study by Pessoa *et al.* [34] revealed that essential oil from *Ocimum gratissimum* L., the main component of which is eugenol, has ovicidal properties against *H. contortus*. The influence of the essential oil and eugenol in the 0.5% concentration emulsion resulted in the maximal inhibition of the egg development. The same results were reported by Anthony *et al.* [35] and Pandey *et al.* [36]. Thus, eugenol is active not only against eggs but also third-stage larvae, as observed in our experiment.

Inhibition of the emergence of *H. contortus* (L₁) first-stage larvae from eggs by eugenol was also studied by Sousa *et al.* [37]. The authors observed the best results after combining 11% eugenol and 64% linalool. Subject to eugenol alone, the eggs of this nematode species did not die as quickly (IC₅₀ = 1.39 mg/mL). Comparative analysis of the results by Sousa *et al.* [37] with our experiments showed a significant difference in the effects of eugenol on the eggs and third-stage larvae of *H. contortus*. Perhaps this difference in impact was observed due to the peculiarities of the experiments: the stage of development of the nematodes, the temperature of the experiment (27 °C in the experiment of Sousa *et al.* [37] and 18–22 °C in our experiment), exposure (2 days in the Sousa experiment *et al.* [37] and 24 in ours), etc.

Eugenol is one of the main components identified in the extract, as well as essential oil from *Syzygium aromaticum* [38–40]. Carrillo-Morales *et al.* [40] recorded an inhibition rate of 99.87% against the emergence of *H. contortus* from the eggs, subject to methanol extract at a concentration of 1.25 mg/mL.

The ovicidal effects of eugenol, thymol, and carvacrol were reported by Katiki *et al.* [41]. A comparison of those compounds revealed that the best results were produced by carvacrol, which is also different from our results, whereby carvacrol produced the lowest effect on the larvae of the third-stage *H. contortus* in our experiment. As with the LC₅₀ (%), its larvicidal properties in relation to *H. contortus* were the highest (0.1993), compared to the other compounds. However, this parameter was almost the same as in the study of the ovicidal properties, equaling 0.11. A significant difference was seen between thymol and eugenol during an *in vitro* egg hatch assay, whereby this parameter was much greater (0.13 and 0.57, respectively) than when studying the effects of those compounds on the larvae (0.0489 and 0.0513, respectively). Perhaps, such a difference in the results suggests that this was affected by other experimental conditions, such as temperature, or by using a multidrug-resistant strain of *H. contortus* during the *in vitro* egg hatch assay.

Helal *et al.* [42] confirmed that eugenol has an inhibiting effect on *H. contortus* third-stage larvae. However, by comparing this result of influence with the inhibiting properties of other compounds included in the coriander oil, the

strongest effect on larvae mobility was attained by linalool. A combination of coriander oil and linalool had a synergic anthelmintic effect on larvae mobility.

Thymol is one of the main components in the plant extracts from the *Thymus* genus (Lamiaceae). According to Elandalousi *et al.* [43], extracts from *T. capitatus* can inhibit eggs from hatching when applied at a concentration close to 2 mg/mL. However, the LC₅₀ of the ethanol extract from *T. capitatus* was 0.368 mg/mL, while the aqueous extract was only 6.344 mg/mL ($p < 0.05$).

André *et al.* [44] studied the effect of thymol on the different stages of *H. contortus* development: eggs, larvae, and mature specimens. According to the *in vitro* egg hatch assay, a thymol concentration of 0.5 mg/mL inhibited 98% of the larvae from hatching. Thymol at a concentration of 8 mg/mL can inhibit 100% of the larvae from developing and 100% decrease in the mobility of adult worms.

André *et al.* [45] studied the anthelmintic properties of carvacrol using tests on egg hatching and larvae development, and by assessing the mobility of adult *H. contortus*. André and co-authors found that carvacrol inhibited the larvae from hatching by 97.7% when administered at a dose of 1.0 mg/mL. A carvacrol concentration of 2 mg/mL inhibited 100% of larvae from developing, whereas 200 µm/mL inhibited the mobility of the adult worms by 58.3% following a 24-hour exposure.

A strong effect of carvacrol on nematodes was reported by Abidi *et al.* [46]. Essential oil from *Origanum majorana*, of which carvacrol is one of the main constituents (35.65%), displayed ovicidal activity in all tested concentrations (1, 2, 4, and 8 mg/mL), the highest dose (8 mg/mL) of which produced an inhibitive effect on egg hatching of over 80%.

The inhibiting properties of various essential oil components were studied by Zhu *et al.* [47]. They found that carvacrol was a main component in the essential oils from *Arisaema franchetianum* and *A. lobatum* and was lethal to the larvae of *H. contortus*.

Ferreira *et al.* [48] also analyzed the anthelmintic properties of thymol, which is one of the main constituents of the essential oil from *Thymus vulgaris*. This oil and its main constituent were quite effective against three stages of the *H. contortus* development. Oil from *Thymus vulgaris* and thymol can inhibit the eggs from hatching by 96.4–100.0%. Larvae inhibition accounted for 90.8–100% and inhibition of mobility equaled 97.0–100.0%. In the experiment by Ferreira *et al.* [48], who used mature specimens, the mobility of *H. contortus* was completely inhibited within the first 8 h. By contrast, the same effects against *H. contortus* were not observed in a subsequent *in vivo* experiment [48]. Another *in vivo* experiment conducted by Imani-Baran *et al.* [49] also indicated that crude powder and crude aqueous extract from *Trachyspermum ammi* (the main component of thymol) have dose-dependent anthelmintic effects against gastrointestinal nematodes in donkeys (*Equus asinus*). However, such in-

effectiveness can be corrected through technical improvements that increase the bioavailability of the oil. Despite some differences in the experiments with thymol, carvacrol, eugenol, and isoeugenol, the results of numerous studies allow us to assume that those compounds can be further researched in relation to preparing drugs with anthelmintic properties.

Here, controlling the adults of the studied nematode species was the primary objective since targeting the sexually mature stages in parasitic vertebrates is very difficult. Hence, we targeted the larval stages that are found in the environment. This research topic remains relevant not only for the species we studied but also for other nematodes that are also parasites in vertebrate animals. It is also advisable to study the effects of eugenol, isoeugenol, carvacrol, and thymol solutions on them. Such a direction in parasitology can help solve a number of issues related to the resistance of *H. contortus* and other nematodes to anthelmintic drugs, and also the problems associated with toxic loading in natural ecosystems.

5. Conclusions

Administering eugenol, isoeugenol, carvacrol, and thymol using *in vitro* conditions exerted antiparasitic properties against the nematode larvae of *S. papillosus*, *H. contortus*, and *M. capillaris*. Of the compounds we tested, following extraction from essential oils of medicinal plants (eugenol, isoeugenol, carvacrol, and thymol), the strongest effect against various larval stages of *S. papillosus*, L₃ *H. contortus*, and L₁ *M. capillaris* was exhibited by thymol. Subject to its emulsion at a concentration of 0.01%, a total of 47% of the *S. papillosus* first-second stage larvae died. The indicators in the nematode larvae of other species were also not significantly different when using eugenol and isoeugenol. The invasive larvae of *H. contortus* were the least affected by the carvacrol emulsion, while its LC₅₀ was the highest (0.1993%).

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author.

Author Contributions

OB and VB designed the research study, analyzed the data, wrote the manuscript. OB performed the research. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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