Anthelmintic Activity of *Nigella sativa* against *Caenorhabditis elegans*

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Received June 16, 2021; Revised July 29, 2021; Accepted August 22, 2021

Cite This Paper in the following Citation Styles

(a): [1] Emine Sen, Tugce Ogut, Abdullah Olgun, Ozgul Kisa , "Anthelmintic Activity of *Nigella sativa* against *Caenorhabditis elegans*," *Advances in Pharmacology and Pharmacy*, Vol. 9, No. 4, pp. 117 - 126, 2021. DOI: 10.13189/app.2021.090405.

(b): Emine Sen, Tugce Ogut, Abdullah Olgun, Ozgul Kisa (2021). Anthelmintic Activity of *Nigella sativa* against *Caenorhabditis elegans*. *Advances in Pharmacology and Pharmacy*, 9(4), 117 - 126. DOI: 10.13189/app.2021.090405.

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Abstract Increasing resistance against classical anthelmintic drugs makes discovering new anthelmintic compounds from natural plants important. *Nigella sativa* (*N. sativa*) is used as a medicinal plant overall the world and is known to have anthelmintic activity. *Caenorhabditis elegans* (*C. elegans*), a common cost-effective model organism that is easily maintained, is useful to determine the anthelmintic activity of new compounds derived from natural products. In our study we aimed to evaluate through toxicity assays the nematocidal activity of *N. sativa* on *C. elegans* during its larval and adult stages. Different concentrations of *N. sativa* oil (900, 450 and 270 mg/mL) were tested and toxicity assessments were done under stereomicroscope by counting the number of surviving nematodes. This study showed that *N. sativa* essential oil significantly decreases survival of *C. elegans* in both larval and adult stages at 900 mg/mL final concentration. Larval-stage worms were more sensitive to *N. sativa* essential oil than were adults. We recommend further studies on other effects of *N. sativa* on *C. elegans* after removing the toxic compound(s) from the extract. The further discovery of *N. sativa* essential oil compounds responsible for anthelmintic activity and determination of their mechanisms of toxicity can pave the way toward new medicines.

Keywords *Nigella sativa*, Anthelmintic, *Caenorhabditis elegans*, Medicinal Plants, Phytotherapy, Herbal Medicine

1. Introduction

Soil-Transmitted Helminth (STH) infections are caused by intestinal nematodes. Unfortunately, one-fourth of the general population worldwide is infected with STH. These diseases are most common in places like tropical and subtropical regions where fresh water and sanitation are deficient. They cause malnutrition, anemia, retardation of development, and mental problems, especially among school-aged children [1, 2]. Nematodes also infect farm animals and crops, thereby adversely influencing food production yields and causing economic losses and they may also infect domestic pets. Since parasitic infections affect humans in mostly under-developed countries major discoveries of new anthelmintic human medicines arise generally from the veterinary field in developed countries [3].

World Health Organization's list of essential medicines used in the treatment and control of STH contains four anthelmintic medications: mebendazole, albendazole, pyrantel pamoate, and levamisole [4]. The first two are benzimidazoles and are mainly used in combinations with different medications against rare tropical diseases. Their usage in combination with other medications is important in preventing the development of resistance. One drawback of combination therapy can be an increase in

adverse events, especially in children [2]. Resistance to current medications used to treat helminth infections in domesticated animals is a major issue for livestock as well as humans [5, 6]. Thus the discovery of new and safe drugs against pathogenic worms is an important research area for the pharmaceutical industry. Medicinal plants can be a fruitful source of new anthelmintic drugs. Plants synthesize multiple secondary metabolites that have diverse chemical and biological functions including defense against pathogens [7, 8] and human understanding of the use of plants for therapeutic purposes has accumulated over generations. This knowledge has been assimilated by ethnopharmacy and traditional medicine systems [8-10].

Nigella sativa (*N. sativa*) Linn. (family: Ranunculaceae), commonly known as black seed or black cumin, is one of the oldest medicinal plants, traditionally used in the Indian subcontinent, Arabian countries, and Europe as a natural remedy for symptoms including cough, headache, fever, dizziness, and eczema [11,12]. The seeds or their oil are used to increase milk production in lactating mothers, to relieve flatulence, and for diuresis and vermifuge. They are also consumed in the diet as components of food or spice [13-15]. *N. sativa* seeds contain saponin and fiber in addition to macronutrients and their fixed oil is rich with many essential and non-essential fatty acids and sterols. The essential oil contains secondary metabolites like nigellone, thymoquinone, thymol, carvacrol, and d-limonene [15].

Caenorhabditis elegans (*C. elegans*) is a microscopic, non-pathogenic, free-living soil nematode with hermaphrodite and male sexes. It is found in many parts of the world and survives by eating microorganisms – mainly bacteria – as a food source. It is used as a common model organism in many fields including genomics, cell biology, neuroscience, and human development. It provides many advantages like a short life cycle and lifespan, stereotypical development, small size, simplicity of propagation and maintenance, and a well-studied genome. An adult hermaphrodite worm contains approximately 1000 somatic cells. It generates hundreds of offspring. The worm can be maintained in the laboratory where it is grown on agar plates or liquid media with *Escherichia coli* (*E. coli* OP50) as a food source. Studies with *C. elegans* were started as early as the 1960s. It has similar biological mechanisms with all animal species, especially entozoa. Therefore, it provides an advantageous model organism for discovering new anthelmintic compounds [16-23].

N. sativa was reported to have anthelmintic activity in chickens. When combined with ivermectin, the *N. sativa*'s activity was found to be higher than that of either ivermectin or *N. sativa* used alone [24]. This suggests that the mechanism of *N. sativa*'s anthelmintic activity is different from that of ivermectin. *N. sativa* has been shown to have strong anthelmintic activity against

Fasciola hepatica in buffalo [25, 26], against *Hymenolepis nana* [27, 28] and *Aspiculuris tetrapetra* [28] in mice, and against helminths generally in sheep [24, 29].

Thymoquinone, one of the secondary metabolites of *N. sativa*, has been reported to have anthelmintic activity through mechanisms like surface tegumental damage [30] and protein carbonylation-type oxidative damage [31] in *Fasciola gigantica*. Condensed tannins of *N. sativa* have been shown to disturb the intestinal mucosa of adult worms [32], destroying them. *N. sativa* has also been reported to inhibit egg-laying and create biocidal effects in *Schistosoma mansoni* [33].

In this study, we aimed to test whether *C. elegans* is a suitable and cost-effective model to study the mechanisms of nematocidal activity of *N. sativa* or other natural products to facilitate the discovery of new anthelmintic compounds.

2. Methods

2.1. Materials

Wild type *C. elegans* strain N2 (from CGC) was used in this study. It was provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). *N. sativa* oil was purchased from Zade Vital Company in Turkey. Each capsule (900 mg/ml) contained 88% unsaturated fatty acids extracted by cold press production. 5-Fluoro-2-deoxyuridine (FUDR) was purchased from Sigma Chemical Company (St. Louis, MO).

2.2. Maintenance of *C. elegans*

Worms were maintained on nematode growth media (NGM) containing plates with an *Escherichia coli* OP50 lawn at 22 °C. Standard worm plate culture conditions and worm picking techniques were used [21].

2.3. Experimental Design

NGM plates were prepared by standard procedures. When needed, 5-fluorouracil was added into NGM at a final concentration of 40 microM to inhibit DNA synthesis and generation of new progeny. *E. coli* OP50 was heat-killed by incubating at 65 °C for 30 minutes and then used as a food source. *N. sativa* oil was dissolved in 50% ethanol to prepare the stock solution. In both larval and adult stage groups, 1% ethanol was used as a negative control and levamisole (8 mg/mL) as a positive control. In treatment groups *N. sativa* seed oil was tested at 900, 450, and 270 mg/mL final concentrations in adult stage groups and tested only at 900 mg/mL in larval stage groups (because it was the effective dose in adults). *N. sativa* oil was spread on NGM plates (Table 1). Each test plate

contained 50 adults of *C. elegans*. For the larval stage experimental group, doses mentioned in Table 2 were applied on petri dishes and then hypochlorite synchronized eggs (n = 100) were transferred to each petri dish. Experiments were performed in duplicate.

2.4. Anthelmintic, Toxicological and Life Span Assays

All groups were observed under microscope (ZEISS V8. Germany) at 24, 48, and 72 hours after the start of treatments for toxicological assessment. All worms were then followed daily and checked for viability until death with lifespan analysis. All phenotypes (dead worms, structural changes, etc.) were recorded. Only the plates used for adult worms contained 5-fluorouracil. Dead worms were removed from plates and recorded until all worms died. Worms having internal hatching and worms that were wounded and lost were censored. The experiments were duplicated. The experiments were performed at room temperature (21-23°C).

2.5. Statistical Analysis

One-way ANOVA and Tukey's HSD test ($p < 0.05$) were used to analyze the data from toxicological assessment. The data from the lifespan assays were plotted using Kaplan–Meier analysis. Statistical significances of lifespans were analyzed by log-rank test. IBM SPSS Statistics 20 program and OASIS: Online Application for the Survival Analysis of Lifespan Assays

Performed in Aging Research were used for calculations [34].

3. Results

3.1. Anthelmintic Properties of *N. sativa*

When we analyzed the survival of worms exposed to 900, 450, and 270 mg/mL seed oil for 24, 48, and 72 hours, we found that the number of surviving adult worms was significantly decreased in the 900 mg/mL group. For this reason, we tested only this dose instead of all three doses for larval stage analyses.

3.2. Toxicological Assessment

In toxicity studies, adults with abnormal features were observed at all doses starting at 24 hours; there were no structural changes but some worms demonstrated a hook-like bending (data not shown). At 24 hours adults showed a significantly changed survival only in the group treated with 900 mg/mL of *N. sativa* oil compared with the control group; this change was not significant in other groups. When the groups were compared within themselves. There was no difference between the 450 and 270 mg/mL doses, while there were significantly less surviving worms in the 900 mg/mL dose group compared with other groups.

Table 1. Experimental groups and their characteristics for adult stage *C. elegans* studies.

GROUPS	TREATMENT	FINAL CONCENTRATION
Negative Control	500 µL from 50% Ethanol (stock solution)	1% Ethanol
Positive Control	8 mg/mL Sitraks syrup (Levamisole)	8 mg/mL Levamisole
Concentration 1	0.5 ml 900 mg/mL <i>N. sativa</i> seed oil	900 mg/mL <i>N. sativa</i> seed oil
Concentration 2	0.25 mL 100% Ethanol. 0.25 mL 900 mg/mL <i>N. sativa</i> seed oil	450 mg/mL <i>N. sativa</i> seed oil
Concentration 3	0.35 mL 100% Ethanol. 0.15 mL 900 mg/mL <i>N. sativa</i> seed oil	270 mg/mL <i>N. sativa</i> seed oil

Table 2. Experimental groups and their characteristics for larval stage *C. elegans* studies.

Groups	Treatment	Final Concentration
Negative Control	500 µL from 50% Ethanol (stock solution)	1% Ethanol
Positive Control	8 mg/mL Sitraks syrup (Levamisole)	8 mg/mL Levamisole
Concentration	0.5 ml 900 mg/mL <i>N. sativa</i> seed oil	900 mg/mL <i>N. sativa</i> seed oil

Figure 1. Adult survival percentages after short-term *N. sativa* oil exposure*; compared to negative control **; compared to negative and positive control; ***; and compared to negative and positive control and 900 mg/ml *N. sativa* oil concentration ($p \leq 0.05$).

Figure 2. Larval survival percentage after short term *N. sativa* oil exposure*; compared to negative control**; and compared to negative and positive control ($p \leq 0.05$).

After 48 and 72 hours, the number of live adult worms was significantly decreased at all doses compared with the control group. At 72 hours, the 450 mg/mL dose was as effective as the 900 mg/mL dose (Figure 1). Similarly, the number and structural abnormalities of live larval worms was significantly decreased at all doses and treatment durations (24, 48, and 72 hours after administration) (Figure 2).

3.3. Life Span Analyses

Maximum lifespans of experimental groups were recorded as 22 days in the negative control group, 8 days in the 900 mg/mL group, 13 days in the 450 mg/mL group and 12 days in the 270 mg/mL group. Figure 3 and Table 3 show the results. According to statistical evaluations,

lifespans were decreased significantly in all experimental groups compared with the control group. Table 3 does not show the positive control group's data because all animals in this group died within the first 24 hours. In larval groups, maximum lifespans were 16 days in the negative control group (ethanol), 3 days in the positive control group (8 mg/mL levamisole), and 5 days in the 900 mg/mL group. Figure 4 and Table 4 show the results.

N. sativa seed oil at 900 mg/mL concentration was found to be more toxic than other doses (Figure 1. 2.) and decreased the lifespan of *C. elegans* more than the other doses (Figure 3. 4.). When we compared the adult and larval worms at the concentration of 900 mg/mL of *N. sativa* seed oil, we found the maximum lifespans to be 8 days in adult and 5 days in larval groups.

Table 3. Survival data of adult group's life span assay

Name	No. of subjects	Restricted Mean			Ages in days at % mortality					95% Median C.I.	Linear interpolation of mortality curve					Condition	Long Rank Test		
		Days	S.E.	95% C.I.	25%	50%	75%	90%	100%		25%	50%	75%	90%	100%		X ² -value	P-value	Bonferroni P-value
Negative control	200	11.57	0.38	10.82 ~ 12.32	7.00	12.00	16.00	19.00	22.00	11.0 ~ 12.0	6.89	11.25	15.65	18.07	22.00	Negative Control vs. 900 mg/mL <i>Nigella sativa</i> oil	354	≤0.001	≤0.001
																Negative control vs. 450 mg/mL <i>Nigella sativa</i> oil	297	≤0.001	≤0.001
																Negative control vs. 270 mg/mL <i>Nigella sativa</i> oil	299	≤0.001	≤0.001
900 mg/mL <i>Nigella sativa</i> oil	200	1.87	0.08	1.70 ~ 2.03	1.00	2.00	2.00	3.00	8.00	1.0 ~ 1.0	0.57	1.15	1.72	2.60	8.00	900 mg/mL <i>Nigella sativa</i> oil vs. Negative Control	354	≤0.001	≤0.001
																900 mg/mL <i>Nigella sativa</i> oil vs. 450 mg/mL <i>Nigella sativa</i> oil	31	≤0.001	≤0.001
																900 mg/mL <i>Nigella sativa</i> oil vs. 270 mg/mL <i>Nigella sativa</i> oil	90	≤0.001	≤0.001
450 mg/mL <i>Nigella sativa</i> oil	200	2.58	0.14	2.30 ~ 2.85	2.00	2.00	3.00	4.00	15.00	- ~ -	1.21	1.64	2.22	3.14	15.00	450 mg/mL <i>Nigella sativa</i> oil vs. negative control	297	≤0.001	≤0.001
																450 mg/mL <i>Nigella sativa</i> oil vs. 900 mg/mL <i>Nigella sativa</i> oil	31	≤0.001	≤0.001
																450 mg/mL <i>Nigella sativa</i> oil vs. 270 mg/mL <i>Nigella sativa</i> oil	25	≤0.001	≤0.001
270 mg/mL <i>Nigella sativa</i> oil	200	3.23	0.11	3.00 ~ 3.45	2.00	3.00	4.00	5.00	12.00	- ~ -	1.68	2.48	3.39	4.59	12.00	270 mg/mL <i>Nigella sativa</i> oil vs. negative control	299	≤0.001	≤0.001
																270 mg/mL <i>Nigella sativa</i> oil vs. 900 mg/mL <i>Nigella sativa</i> oil	90	≤0.001	≤0.001
																270 mg/mL <i>Nigella sativa</i> oil vs. 450 mg/mL <i>Nigella sativa</i> oil	25	≤0.001	≤0.001

Table 4. Survival data of larval group's life span assay

Name	No. of subjects	Restricted Mean			Ages in days at % mortality						Linear interpolation of mortality curve					Long Rank Test			
		Days	S.E.	95% C.I.	25%	50%	75%	90%	100%	95% Median C.I.	25%	50%	75%	90%	100%	Condition	X ² -value	P-value	Bonferroni P-value
Negative Control	400	8.94	0.24	8.47 ~ 9.41	6.00	8.00	14.00	15.00	16.00	8.0 ~ 8.0	5.50	8.00	13.50	14.57	16.00	Negative Control v.s Positive Control	625.65	≤0.001	≤0.001
																Negative Control vs. 900 mg/mL <i>Nigella sativa</i> Oil	544.67	≤0.001	≤0.001
																Positive Control vs. Negative Control	628.65	≤0.001	≤0.001
Positive Control	400	1.38	0.03	1.31 ~ 1.44	1.00	1.00	2.00	2.00	3.00	- ~ -	(1.71)	(0.29)	1.14	2.00	3.00	Positive Control vs. 900 mg/mL <i>Nigella sativa</i> Oil	132.39	≤0.001	≤0.001
																900 mg/mL <i>Nigella sativa</i> Oil vs. Positive Control	544.67	≤0.001	≤0.001
																Negative Control 900 mg/mL <i>Nigella sativa</i> Oil vs. Positive Control	132.39	≤0.001	≤0.001
900 mg/mL <i>Nigella sativa</i> Oil	400	2.26	0.07	2.13 ~ 2.40	1.00	2.00	3.00	4.00	5.00	- ~ -	(0.15)	1.38	2.75	4.00	5.00

Figure 3. Adult stage life span assay

Figure 4. Larval stage life span assay

4. Discussion

A very cost-effective, easily maintained and readily accessible model organism, *C. elegans* has been used for research in various fields of biology and medicine. Since it is a nematode, its use in the discovery of new anthelmintics is not surprising [3, 16-20]. The seeds of *N. sativa* yield an essential oil possessing anthelmintic activity used against cestodes (flatworms) in children; and the same oil's ameliorating effect on liver damage caused by *Schistosoma mansoni* in mice has been previously

reported [35], though the mechanisms of these effects and the responsible phytochemicals have not been identified. In this study, we aimed to prove the feasibility of *C. elegans* to investigate the mechanism of the anthelmintic activity of *N. sativa* oil. To this end, we tested the anthelmintic activity of *N. sativa* on *C. elegans*.

The crude alkaloid and water extracts of the *N. sativa* seeds have reportedly been effective against a variety of microorganisms, even drug-resistant ones [36], including *Staphylococcus albus*, *Escherichia coli*, *Salmonella typhi*, *Shigella niger*, and *Vibrio cholera*. Thymoquinone is one

of the main compounds of *N. sativa* essential oil and inhibits non-enzymatic lipid peroxidation [40]. Gram-negative bacteria have a cell wall with a thicker lipid layer than gram-positive bacteria, making them more vulnerable to thymoquinone's lipid peroxidation activity. The epidermis of *C. elegans* contains an extracellular matrix (ECM) consisting primarily of collagen, lipids, and glycoproteins and thymoquinone has been reported to affect the organism's lipid structures [41, 42]. We observed that *N. sativa* oil destroyed the structural integrity of *C. elegans* (Figure 1.2).

Oral administration of the seed oil at doses of up to 10 mL/kg in rats and mice did not cause any mortality or over-toxicity [43]. The LD50 value of thymoquinone is 2.4 g/kg (range 1.52–3.77) [44]. Thymoquinone protects against hepatotoxicity [44] and *N. sativa* seeds are sold to treat conditions that include liver diseases [45]. Considering these facts, *N. sativa* seed oil can be considered for the prevention and treatment of many diseases.

Levamisole has a strong anthelmintic effect on *C. elegans* as reported in many previous studies [44]. The sensitivity of *C. elegans* to levamisole has been reported to be about 60 minutes [46] and our experiment observed a similar effect. Additionally, levamisole (positive control) had the same toxicity when larval and adult stage worms were compared. Abnormalities observed in larval stage were like Pdp-7: tsp-15His and tsp-15 (sv15) mutant strains but in adult stage to tsp-15 (RNAi) phenotype (Figure 1.2).

We assessed both larval and adult animals to determine whether developmental periods affect the responses of *C. elegans* to *N. sativa* seed oil. *N. sativa* seed oil had the highest toxicity and lifespan shortening capability in *C. elegans* at 900 mg/mL concentration. At this concentration, the survival of adult and larval groups were 8 and 5 days, respectively. Larva were more sensitive, suggesting some protective mechanisms in adults.

This study demonstrated the anthelmintic and toxic activities of *N. sativa* oil against both larval and adult stages of *C. elegans*, finding the larval stage to be more sensitive. In further biological activity-guided purification studies investigating the secondary anthelmintic metabolite(s) of *N. sativa* oil, we can argue that *C. elegans* be used as a reliable, validated model.

Acknowledgments

Ozgul Kisa (Corresponding Author) was working at Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Altınbaş University when this study was performed.

This study presented as poster presentation at 12th International Symposium on Pharmaceutical Sciences (ISOPS-12) which hold in Ankara, Turkey between June

26-29, 2018.

Declaration of Interest

The authors declare no conflicts of interest.

Funding/Support

This study received no specific financial support.

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