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ORIGINAL ARTICLE

Leishmanicidal and cytotoxic activities of *Nigella sativa* and its active principle, thymoquinone

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Abstract

Context: Leishmaniasis is a complex disease with a broad spectrum of clinical presentations.
Objective: We evaluated the anti-leishmanial effects of *Nigella sativa* L. (Ranunculaceae) against *Leishmania tropica* and *Leishmania infantum* with an *in vitro* model.
Materials and methods: Antileishmanial effects of essential oil and methanolic extract of *N. sativa* (0–200 μg/mL) and thymoquinone (0–25 μg/mL) on promastigotes of both species and their cytotoxicity activities against murine macrophages were evaluated using the MTT assay at 24, 48, and 72 h. Moreover, their leishmanicidal effects against amastigotes were investigated in a macrophage model, for 48 and 72 h.
Results: The findings showed that essential oil (*L. tropica* IC_{50} 9.3 μg/mL and *L. infantum* IC_{50} 11.7 μg/mL) and methanolic extract (*L. tropica* IC_{50} 14.8 μg/mL and *L. infantum* IC_{50} 15.7 μg/mL) of *N. sativa*, particularly thymoquinone (*L. tropica* IC_{50} 1.16 μg/mL and *L. infantum* IC_{50} 1.47 μg/mL), had potent antileishmanial activity on promastigotes of both species after 72 h. In addition, essential oil (*L. tropica* IC_{50} 21.4 μg/mL and *L. infantum* IC_{50} 26.3 μg/mL), methanolic extract (*L. tropica* IC_{50} 30.8 μg/mL and *L. infantum* IC_{50} 34.6 μg/mL), and thymoquinone (*L. tropica* IC_{50} 2.1 μg/mL and *L. infantum* IC_{50} 2.6 μg/mL) mediated a significant decrease in the growth rate of amastigote forms of both species. Thymoquinone (CC_{50} 38.8 μg/mL) exhibited higher cytotoxic effects against murine macrophages than the other extracts.
Conclusion: *N. sativa*, especially its active principle, thymoquinone, showed a potent leishmanicidal activity against *L. tropica* and *L.infantum* with an *in vitro* model.

Keywords

Black cumin, Leishmania infantum, Leishmania tropica

History

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Introduction

Leishmaniasis has been identified as a major public health problem in tropical and sub-tropical areas, where infection is transmitted by the bite of a female sand fly. It is endemic in 98 countries and territories, affecting 12 million people and threatening approximately 350 million around the world (WHO, 2010). Cutaneous leishmaniasis (CL) is the most common type of leishmaniasis which annually affects 1.5 million people worldwide. About 90% of cases are reported from countries such as Iran, Afghanistan, Pakistan, Iraq, and Saudi Arabia. Visceral leishmaniasis (VL) is the most severe form of leishmaniasis in the world, which is responsible for an annually estimated 500 000 cases globally (Desjeux, 2004). At present, there is no efficacious vaccine against different forms of leishmaniasis, therefore, chemotherapy is the only choice against leishmaniasis. The existing treatments are associated with adverse effects and the efficacy of anti-leishmanial drugs is controversial (Santos et al., 2004).

The first choice of treatment for CL and VL is pentavalent antimony compounds including meglumine antimoniate (MA) and sodium stibogluconate (SSG), which are widely prescribed despite their toxicity, high cost, difficult administration route, and emergence of resistance (Croft et al., 2006). Recent studies have shown that plant extracts and plant-derived compounds, due to having less side effects, low cost, and high availability, are valuable sources that are commonly used to treat a wide range of disease conditions including infectious diseases (Rocha et al., 2005; Rojas-Silva et al., 2014). Thus, plant medicines would be needed for developing new, effective treatment alternatives.

Nigella sativa L. (Ranunculaceae) is commonly known as black seed and is grown in the Middle East, Eastern Europe, and Western and Middle Asia. It is traditionally used as a natural treatment for a number of diseases and conditions

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including asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza worldwide (Ali & Blunden, 2009). Various pharmacological activities such as antioxidant, anti-inflammatory, anticancer, and antimicrobial effects have been related to *N. sativa* or its active principles which include thymoquinone, thymohydroquinone, dithymoquinone, carvacrol, *p*-cymene, and thymol (Ali & Blunden, 2009; Randhawa & Al-Ghamdi, 2002). Moreover, in previous studies, antibacterial, antifungal, antiparasitic, and antiviral activities of this plant have been proven (Agrawal et al., 1979; Khan et al., 2003; Mahmoud et al., 2002; Morsi, 2000; Salem & Hossain, 2000). The aim of this study was to evaluate anti-leishmanial effects of essential oil and methanolic extract of *N. sativa* seeds and also its active principle, thymoquinone, against *Leishmania tropica* and *Leishmania infantum* species using an *in vitro* model.

Materials and methods

Chemicals

MTT powder [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide)], fetal calf serum (FCS), and RPMI 1640 medium with L-glutamine were purchased from Sigma-Aldrich, St Louis, MO. Meglumine antimoniate (MA, Glucantime) as a control drug was purchased from Rhône, Poulenc, France. Penicillin and streptomycin were prepared from Alborz Pharmacy, Karaj, Iran, and were stored at room temperature (25 °C) until testing. All other chemicals and solvents were of analytical grade.

Parasite strains

Leishmania tropica standard strain (MHOM/IR/2002/Mash2) was prepared from Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). *Leishmania infantum* standard strain (MCAN/IR/07/Moheb-gh) was obtained from Laboratory for Leishmaniasis, Department of Medical Parasitology, Tehran University of Medical Sciences, Iran. The parasites were cultured in NNN medium and subcultured in RPMI 1640 supplemented with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 15% heat-inactivated FCS.

Isolation of murine macrophage cells

Murine macrophages were collected from male BALB/c mice (4–8 weeks old) by injecting 2–5 mL of cold RPMI-1640 medium into mouse peritoneal cavity and then aspirated macrophages were washed twice and resuspended in the RPMI 1640 medium. The experimental procedures carried out in this survey were in compliance with Guidelines of Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals in line with Animal Ethics Committee (protocol no. 90/83).

Collecting the plant materials

Seeds of *N. sativa* were collected from rural regions of Bam district in September 2012, Kerman province, southeast of Iran. The plant materials were identified by a botanist in Botany Department, Shahid Bahonar University of Kerman. Voucher specimen (KF 575) was deposited at Herbarium of Pharmacognosy Department of Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Isolation of essential oil

Crushed seeds were extracted with light petroleum (BP 40–60 °C) using a Soxhlet apparatus. The solvent was removed under vacuum and the brownish residue was steam distilled. Finally, extraction of the aqueous distillate with *n*-hexane and removal of the solvent produced essential oil. The essential oil was stored in sealed vials at 2–8 °C for gas chromatography/mass spectrometry (GC/MS) analysis.

Preparing methanolic extract

The dried plant materials (100 g) were grinded and extracted by percolation method by methanol for 72 h at room temperature. The solvents were removed in a rotary evaporator and, after filtering, the extracts were concentrated to dryness and stored at −20 °C, until testing.

Preparing thymoquinone

Thymoquinone was obtained from Sigma-Aldrich (St. Louis, MO) and was dissolved in the dimethyl sulfoxide (DMSO). The final concentration of DMSO never exceeded 1% either in control or treated samples.

Gas chromatography/mass spectrometry (GC/MS) analysis of essential oil

GC analysis

In this study, GC analysis was carried out by a Hewlett-Packard 6890 (Hewlett-Packard Company, Palo Alto, CA) with a HP-5MS column (30 m × 0.25 mm, film thickness 0.25 mm). The column temperature was maintained at 60 °C for 3 min and programmed to 220 °C at a rate of 5 °C per min, and kept constant at 220 °C for 5 min. Injector and interface temperatures were 220 °C and 250 °C, respectively. The flow rate of helium as a carrier gas was 1 mL/min CF. The percentages were calculated by electronic integration of FID peak areas without the use of response factors correction. Linear retention indices for all components were determined by coinjection of the samples with a solution containing homologous series of C8–C22 *n*-alkanes.

GC/MS analysis

GC/MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with fused silica capillary DB-5 column (30 m × 0.25 mm, film thickness 0.25 mm) coupled with a TRACE mass (Manchester, UK). Helium was used as a carrier gas with an ionization voltage of 70 eV. Ion source and interface temperatures were 220 °C and 250 °C, respectively. The mass range was from 40 to 400 u. Oven temperature program was the same given above for the GC.

Identification of the EO components

The components of the EO were identified by comparison of their relative retention time and mass spectra with those of

standards Wiley 2001 library data of the GC/MS system or with those of reported in the literature data (Adams, 2004).

Antileishmanial effects against promastigote forms

Antileishmanial effects of EO, methanolic extract, and thymoquinone on promastigote stage of both species were evaluated by colorimetric cell viability MTT assay using the method described elsewhere (Mahmoudvand et al., 2014a; Valadares et al., 2011). Briefly, 100 µL of the promastigotes of both species (10^6 cells/mL) harvested from logarithmic growth phase was added to a 96-well tissue culture plate. Then, 100 µL of various concentrations (0–25 µg/mL) of thymoquinone and extracts (0–200 µg/mL) was added to each well and incubated at 25 °C ± 1 °C for 24, 48, and 72 h. After incubation, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated at 25 °C for 4 h. Promastigotes were cultured in the complete medium with no drug used as a positive control and the complete medium with no promastigotes and drugs as blank. Finally, the absorbance was measured by an ELISA reader (BioTek-ELX800, BioTek Instruments, Winooski, VT) at 490 nm. Also, 50% inhibitory concentrations (IC_{50} values) were measured for all the tested extracts by Probit test in SPSS software (SPSS Inc., Chicago, IL).

Effects on intramacrophage amastigotes

In order to evaluate the inhibitory effects of essential oil, methanolic extract and thymoquinone of *N. sativa* seeds against intramacrophage amastigotes of *L. tropica* and *L. infantum,* murine macrophages which were collected from male BALB/c mice according to the method described by Carrio et al. (2000) were used. Also, similar to the promastigote stage, all the experiments were repeated in triplicate. Initially, before adding the murine macrophages to the plates, 1 cm^2 cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International, Rochester, NY). In the next step, 200 µL of murine macrophage cells (10^5/mL) were incubated at 37 °C in 5% CO_2 for 2 h. Then, 200 µL (10^6/mL) promastigotes of both species in stationary phase were separately added to murine macrophages, so that proportion of *Leishmania*/macrophage was 10:1 and incubated again in a similar condition for 24 h. Free parasites were removed by washing with RPMI 1640 medium and the infected macrophages were treated with various concentrations (0–500 µg/mL) of extracts (50 µL) at 37 °C in 5% CO_2 for 48 and 72 h. At the end, the dried slides were fixed with methanol, stained by Giemsa and studied under a light microscope. Also, the macrophages containing amastigotes without extract and those with no parasite and extract were considered positive and negative controls, respectively. Activity of anti-intramacrophage amastigotes of the extracts was evaluated by counting the number of amastigotes in each macrophage by examining 100 macrophages (% amastigotes viability) in comparison with those obtained with positive control. Also, IC_{50} values of the extracts and thymoquinone were calculated by Probit test in SPSS software (SPSS Inc., Chicago, IL).

Inhibiting infection in murine macrophages

To evaluate inhibitory effect of *N. sativa* on promastigotes of *L. tropica* and *L. infantum* invasion of macrophages, promastigotes of both species were pre-incubated in essential oil, methanolic extract (5 µg/mL), and also thymoquinone (1 µg/mL) for 2 h at room temperature. Then, the promastigotes were washed with the RPMI 1640 medium and incubated with murine macrophages for 4 h. After washing the cells again, the macrophages were stained by Giemsa and studied by a light microscope to evaluate percentages of the infected macrophages by counting 100 cells (Valadares et al., 2011).

Cytotoxic effects on murine macrophage cells

Cytotoxic effects of *N. sativa* seeds against murine macrophage cells were assessed by cultivating macrophages (5×10^5) with various concentrations of essential oil, methanolic extract (0–500 µg/mL), and thymoquinone (0–50 µg/mL) in 96-well tissue culture plates at 37 °C in 5% CO_2 for 72 h. The cell viability was evaluated by colorimetric MTT assay and the results were displayed as percentage of dead cells compared with macrophages treated with MA (100 µg/mL) and non-treated macrophages (100% of viability). Moreover, CC_{50} (cytotoxic concentration for 50% of cells) was evaluated by probit analysis (Mahmoudvand et al., 2014b).

Statistical analysis

In this study, all the tests were performed in triplicate and IC_{50} and CC_{50} values were directly determined by probit test in SPSS software (SPSS Inc., Chicago, IL). Selectivity index (SI), calculated based on the equation of CC_{50} for murine macrophage cells/IC_{50} for amastigote forms of both species, was used to compare toxicity and activity of essential oil, methanolic extracts, and thymoquinone as described by Weninger et al. (2001). Also, *t*-test was used to compare IC_{50} values of extracts and control drug and $p < 0.05$ was considered as significant.

Results

GC-MS analysis of *N. sativa* essential oil

In this survey, essential oil of *N. sativa* obtained by Soxhlet extraction using GC-MS was analyzed. Table 1 lists the identified compounds and percentage obtained by GC/MS. The main components were thymoquinone (42.4%), *p*-cymene (14.1%), carvacrol (10.3%), longifolene (6.1%) and 4-terpineol (5.1%).

Leishmanicidal effects against promastigotes

In studying antiproliferation effects of essential oil, methanolic extract and thymoquinone of *N. sativa* seeds against promastigote forms of *L. tropica* and *L. infantum*, it could be observed that all the extracts, particulary thymoquinone, significantly ($p < 0.05$) inhibited growth of promastigotes in a dose-dependent response compared with the control drug. So that thymoquinone at concentrations of $\geq$1.5 µg/mL inhibited above 50% the parasite growth. However, essential oil in comparison with methanolic extract exhibited higher

leishmanicidal effects to promastigotes of both species. As shown in Table 2, IC_{50} values for essential oil and thymoquinone against promastigotes of *L. tropica* and *L. infantum* were significantly lower than those of MA when compared with IC_{50} values of methanolic extract. Moreover, the results indicated that *L. tropica* promastigotes were more susceptible to essential oil, methanolic extract, and thymoquinone of *N. sativa* seeds than promastigotes of *L. infantum*. However, the difference in antileishmanial effects of extracts and thymoquinone against promastigotes of both species was not statistically significant ($p > 0.05$).

Effects on intramacrophage amastigotes

Similar to the promastigote stage, *N. sativa* and its derivatives demonstrated high inhibitory effects with intramacrophage amastigotes of both species of *Leishmania* after 48 and 72 h. Further results indicated that anti-amastigote effects of essential oil and methanolic extract were mediated in a dose-dependent manner. Table 3 shows the IC_{50} values of essential oil, methanolic extract, and thymoquinone against amastigote forms of both species of *Leishmania*. These results showed that the thymoquinone and subsequently essential oil of *N. sativa* had more inhibitory effects against amastigotes of both *Leishmania* species than its methanolic extract. Meanwhile, MA used as a positive control, caused a significant reduction in viability of the two *Leishmania* species.

Table 1. Essential oil composition of *N. sativa* seeds identified by GC-MS.

No	Compound	Percentage
1.	Camphene	0.06
2.	t-Anethole	2.3
3.	β-Pinene	0.03
4.	α-Pinene	0.04
5.	γ-Terpinene	0.4
6.	β-Myrcene	0.05
7.	α-Terpinene	0.01
8.	Limonene	1.7
9.	β-Phellandrene	≤0.01
10.	1,8-Cineole	0.16
11.	Sabinene	1.3
12.	ρ-Cymene	15.1
13.	α-Terpinolene	0.01
14.	2-Heptanal	≤0.01
15.	ρ-Cymene-8-ol	0.42
16.	Carvacrol	12.3
17.	Longipinene	0.4
18.	Camphor	1.5
19.	Linaloolcis	0.21
20.	Sabinenehydrate	0.2
21.	Longifolene	6.1
22.	Bornylacetate	0.53
23.	Thymol	1.2
24.	4-Terpineol	5.1
25.	Borneol	0.14
26.	Carvone	1.5
27.	Thymoquinone	42.4
28.	2-Tridecanone	0.8
29.	Thujone	0.08
30.	ρ-Anisaldehyde	0.1
31.	2-Undecanone	0.21
32.	Unknown peak	3.1
	Total	96.7

Inhibiting infection in murine macrophages

Leishmania tropica and *L. infantum* promastigotes not treated were able to infect 78 and 81% of the murine macrophages, respectively. When promastigote forms of *L. tropica* were treated with essential oil, methanolic extract, and thymoquinone and later used to infect macrophages, they were only able to infect 27.3, 35.6, and 13% of the murine macrophages, respectively (Table 4), while promastigote forms of *L. infantum* treated with aforementioned extracts and thymoquinone were able to infect only 33.6, 39.3, and 16.3% of murine macrophages, respectively (Table 5).

Cytotoxic effects of *N. sativa* on murine macrophages

Evaluation of cytotoxic effects of essential oil, methanolic extract, and thymoquinone of *N. sativa* seeds on murine macrophages by the MTT assay showed a dose-dependent response; with increasing concentrations of both extracts, cytotoxic effects were shown. In this stage, thymoquinone

Table 3. Comparison of the mean IC_{50} values among with the essential oil and methanolic extract *N. sativa* seed and its active principle, thymoquinone against the growth rate of intramacrophage amastigote forms of different *Leishmania* species.

	IC_{50} (μg/mL)			
	L. tropica		*L. infantum*	
Plant extracts	48 h	72 h	48 h	72 h
Essential oil	35.3 ± 2	21.4 ± 2.15	41.7 ± 1.15	26.3 ± 2.0
Methanolic extract	55.2 ± 2.15	30.8 ± 2.52	67.3 ± 2.51	34.6 ± 3.1
Thymoquinone	4.9 ± 0.05	2.1 ± 0.05	5.5 ± 0.05	2.6 ± 0.1
MA	64.6 ± 3.05	33.3 ± 3.05	77.6 ± 02.15	39.4 ± 3.05

Data are expressed as the mean ± SD ($n = 3$).

Table 2. Comparison of the mean IC_{50} values among with the essential oil and methanolic extract of *N. sativa* seed and its active principle, thymoquinone against the growth rate of promastigote forms of different *Leishmania* species.

	IC_{50} (μg/mL)					
	L. tropica			*L. infantum*		
Plant extracts	24 h	48 h	72 h	24 h	48 h	72 h
Essential oil	53.3 ± 2.51	20.4 ± 2	9.3 ± 2.08	62.1 ± 3.05	25.7 ± 1.15	11.7 ± 1.15
Methanolic extract	128.6 ± 4.3	38.2 ± 2.15	14.8 ± 2.52	136.7 ± 4.1	56.3 ± 2.51	15.7 ± 2.51
Thymoquinone	9.1 ± 1.51	3.6 ± 0.05	1.16 ± 0.05	12.8 ± 1.15	4.2 ± 0.05	1.47 ± 0.05
MA	78.8 ± 3.05	36.4 ± 3.05	16.5 ± 1.15	84.2 ± 3.05	42.2 ± 02.15	17.6 ± 2.1

Data are expressed as the mean ± SD ($n = 3$).

Table 4. Inhibition of the infection in murine macrophages after treatment of *Leishmania tropica* promastigotes with the essential oil, methanolic extract, and thymoquinone of *N. sativa* seeds.

Chemicals	Percentage of infected macrophages by non-treated promastigotes	Percentage of infected macrophages by treated promastigotes	Infectiveness Reduction
Essential oil	78.3 ± 2.52	27.3 ± 1.15	65 ± 2.52
Methanolic extract	78.3 ± 2.52	35.6 ± 1.52	55 ± 2.15
Thymoquinone	78.3 ± 2.52	13 ± 1.17	83 ± 3.1

Data are expressed as the mean ± SD ($n = 3$).

Table 5. Inhibition of the infection in murine macrophages after treatment of *Leishmania infantum* promastigotes with the essential oil, methanolic extract, and thymoquinone of *N. sativa* seeds.

Chemicals	Percentage of infected macrophages by non-treated promastigotes	Percentage of infected macrophages by treated promastigotes	Infectiveness Reduction
Essential oil	81.3 ± 3.51	33.6 ± 1.15	59 ± 2.52
Methanolic extract	81.3 ± 3.51	39.3 ± 1.52	52 ± 2.08
Thymoquinone	81.3 ± 3.51	16.3 ± 1.52	80 ± 3.51

Data are expressed as the mean ± SD ($n = 3$).

Table 6. Comparison of the mean CC_{50} values among with essential oil, methanolic extract, and thymoquinone of *N. sativa* seeds on murine macrophage cells and their selectivity index (SI) against intramacrophage amastigote forms of different *Leishmania* species.

		SI[a]			
		L. tropica		*L. infantum*	
Plant extracts	CC_{50} (μg/mL)	48 h	72 h	48 h	72 h
Essential oil	444.3 ± 4.1	12.6	20.7	10.6	16.8
Methanolic extract	791.4 ± 3.6	14.3	25.7	11.7	22.9
Thymoquinone	38.8 ± 2.8	7.9	18.4	7	14.9
MA	1012.6 ± 3.6	15.6	30.4	13	25.7

[a]Selectivity index (CC_{50}/IC_{50} value of amastigote forms).

showed a more cytotoxic effect on murine macrophages as compared with essential oil or methanolic extract of *N. sativa*. Table 6 shows the CC_{50} value for essential oil, methanolic extract, and thymoquinone against murine macrophages and subsequently their SI_s (CC_{50}/IC_{50}) for amastigote forms of both species. SI of greater than 10 for essential oil and methanolic extract of *N. sativa* showed their safety to the macrophages and specificity to the parasite according to Weninger et al. (2001).

Discussion

Findings of present study showed *in vitro* growth inhibition of promastigote and amastigote stages of *L. tropica* and *L. infantum* by essential oil and methanolic extract of *N. sativa* seeds and also its active principle, thymoquinone. Plant extracts and plant-derived compounds have been used as a valuable source of folk medicine since ancient times (Rocha et al., 2005). With the advent of industrial and synthetic antimicrobial agents in the middle of last century, lack of interest in plants as a natural and valuable source for antimicrobial drugs was caused (Cowan, 1999). Recently, with the emergence of some limitations in the use of these drugs, the situation has shifted and field of ethnobotanical research has been expanded (McCutcheon et al., 1992). At present, standard drugs for the treatment of leishmaniasis are pentavalent antimonials such as meglumine antimoniate (Desjeux, 2004). The use of these compounds is limited due to high cost, toxicity, long-term treatment, and emergence of drug resistance (Croft et al., 2006). For these reasons, development of new, effective, and safe antileishmanial drugs from natural resources is an urgent need. In the present study, *N. sativa* as well as its active principle, thymoquinone, was found as a natural source for producing new antileishmanial drugs. A previous study conducted by Agrawal et al. (1979) proved that *N. sativa* oil had anticestodal and antinematodal activities comparable with those of piperazine. Also, results of a survey conducted by Mahmoud et al. (2002) showed that *N. sativa* oil (2.5 and 5.0 mL/kg, orally for 2 weeks) could significantly decrease the number of *Schistosoma mansoni* worms in the liver and reduce the total number of ova deposited in both liver and intestine. In addition, Okeola et al. (2011) revealed that *N. sativa seeds* had a strong antioxidant property and might be a good phytotherapeutic agent against *Plasmodium* infection in malaria. In the case of antileishmanial effects of *N. sativa,* Nilforoushzadeh et al. (2010) indicated that combination of honey and *N. sativa* extract in patients with CL receiving glucantime was more effective in treating and improving clinical signs than honey alone. Currently, Fattahi Bafghi et al. (2011) reported that alcoholic extract of *N. sativa* exhibited a significant anti-cutaneous leishmanial activity in BALB/c mice. In contrast, in some studies, it has been proven that *N. sativa* shows no significant effect on treatment of balantidiasis in equines and *Cryptosporidium parvum* infection in calves (Khan et al., 2013; Nasir et al., 2013). So far, no study has been conducted on antileishmanial activity of essential oil of *N. sativa.* Thus, in this investigation, for the first time, leishmanicidal effects of essential oil of *N. sativa* were evaluated. In line with previous findings (Ali & Blunden, 2009; Randhawa & Al-Ghamdi, 2002), it was found that the main component of *N. sativa* essential oil was thymoquinone (42.4%) when being analyzed by GC/MS. It was shown that the most biological activity of *N. sativa* seeds was due to thymoquinone, the major component of essential oil, which was also present in the fixed oil (Ali & Blunden, 2009). Similarly, it was presented that thymoquinone as a major component of *N. sativa* seeds had a highly potent antileishmanial activity on both *Leishmania* species. Therefore, better anileishmanial effects of *N. sativa* essential oil could be related due to having higher content of thymoquinone than its methanolic extract. Previous study also showed potent antileishmanial effects of other components of essential oil such as carvacrol, *p*-cymene, thymol, carvone, limonene, and terpinene against *L. chagasi* (Escobar et al., 2010) and *L. amazonensis* (Monzote et al., 2014). However, these components exhibited significantly ($p < 0.05$) lower antileishmanial activity than thymoquinone against *Leishmania* spp.

Since exact mechanisms of antimicrobial activity of thymoquinone are not clear, further studies are required to elucidate these mechanisms. However, it has been proven that *N. sativa* oil can inhibit DNA synthesis by inhibiting histone deacetylase (HDAC) enzyme interacting with the chromosomes (Suthar et al., 2010). In addition, it has an immunopotentiating effect through stimulating phagocytic activity and phagocytic index of peritoneal macrophages or activating lymphocytes in streptozotocin (STZ)-induced diabetic hamsters (Fararh et al., 2004). Various studies have shown that administration of *N. sativa* seed extract and its components as oral or intraperitoneally represents a low level of cytotoxicity in rats and mice (Badary, 1998; El Daly, 1998; Khanna et al., 1993; Salem & Hossain, 2000). Similar to these results, the present finding showed that essential oil and methanolic extract of *N. sativa* had no significant cytotoxic effect whereas thymoquinone indicated higher cytotoxic effect on murine macrophages. In addition, $SI_s \geq 10$ of the extracts showed their safety to the macrophages and specificity to the parasite (Weninger et al., 2001). Therefore, it can be suggested that the *N. sativa* seeds derivatives are safe for mammalian cells.

In conclusion, essential oil and methanolic extract of *N. sativa* and its active principle, thymoquinone, showed potent antileishmanial effects against *L. tropica* and *L. infantum* species in the *in vitro* model. In addition, further clinical studies are required to evaluate exact biological activity of *N. sativa* in animal models as well as volunteer human subjects as a new therapeutic agent against leishmaniasis.

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Declaration of interest

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References

Adams RP. (2004). *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*. Carol Stream, IL: Allured Publishing Corporation.

Agrawal R, Kharya MD, Shrivastava R. (1979). Antimicrobial and anthelmintic activities of the essential oil of *Nigella sativa* Linn. *Indian J Exp Biol* 17:1264–5.

Ali B, Blunden G. (2009). Pharmacological and toxicological properties of *Nigella sativa*. *Phytother Res* 17:299–305.

Badary OA, Al-Shabanah OA, Nagi MN, et al. (1998). Acute and subchronic toxicity of thymoquinone in mice. *Drug Dev Res* 44: 56–61.

Carrio MC, Reiva C, Gallego M, et al. (2000). *Leishmania infantum*: Stage specific activity of pentavalent antimony related with the assay conditions. *Exp Parasitol* 95:209–14.

Croft SL, Sundar S, Fairlamb AH. (2006). Drug resistance in leishmaniasis. *Clin Microb Rev* 19:11–26.

Cowan MM. (1999). Plant products as antimicrobial agents. *Clin Microb Rev* 12:564–82.

Desjeux P. (2004). Leishmaniasis: Current situation and new perspectives. *Comp Immunol, Microbiol Infect Dis* 27:305–18.

Escobar P, Milena Leal S, Herrera LV, et al. (2010). Chemical composition and antiprotozoal activities of Colombian *Lippia* spp essential oils and their major components. *Mem Inst Oswaldo Cruz* 105:184–90.

El Daly ES. (1998). Protective effect of cysteine and vitamin E, *Crocus sativus* and *Nigella sativa* extracts on cisplatin induced toxicity in rats. *J Pharm Belg* 53:87–95.

Fararh KM, Atoji Y, Shimizu Y, et al. (2004). Mechanisms of the hypoglycaemic and immunopotentiating effects of *Nigella sativa* L. oil in streptozotocin-induced diabetic hamsters. *Res Vet Sci* 77:123–9.

Fattahi Bafghi A, Vahidi AR, Anvari MH, et al. (2011). The *in vivo* antileishmanial activity of alcoholic extract from *Nigella sativa* seeds. *Afr J Microbiol Res* 5:1504–10.

Khan A, Khan MS, Avais M, et al. (2013). Prevalence, hematology, and treatment of balantidiasis among donkeys in and around Lahore, Pakistan. *Vet Parasitol* 196:203–5.

Khan MA, Ashfaq MK, Zuberi HS, Zuberi AH. (2003). The *in vivo* antifungal activity of the aqueous extract from *Nigella sativa* seed. *Phytother Res* 17:183–6.

Khanna T, Zaidi FA, Dandiya PC. (1993). CNS and analgesic studies on *Nigella sativa*. *Fitoterapia* 5:407–10.

Mahmoud MR, El-Abhar HS, Saleh S. (2002). The effects of *Nigella sativa* oil against the liver damage induced by *Schistosoma mansoni* in mice. *J Ethnopharmacol* 79:1–11.

Mahmoudvand H, Sharifi I, Fasihi Harandi M, et al. (2014a). Anti-*leishmania* effects of methotrexate (MTX) alone and in combination with meglumine antimoniate (MA) against Iranian isolate of sensitive and MA-resistant *Leishmania tropica*: An *in-vitro* assay. *Asian Pacific J Trop Med* 7:412–20.

Mahmoudvand H, Sharififar F, Sharifi I, et al. (2014b). *In vitro* inhibitory effect of *Berberis vulgaris* (Berberidaceae) and its main component, berberine against different *Leishmania* species. *Iranian J Parasitol* 9:28–36.

McCutcheon AR, Ellis SM, Hancock REW, Tower GNH. (1992). Antibiotic screening of medicinal plants of the British Columbian native peoples. *J Ethnopharmacol* 37:213–23.

Monzote L, García M, Pastor J, et al. (2014). Essential oil from *Chenopodium ambrosioides* and main components: Activity against *Leishmania*, their mitochondria and other microorganisms. *Exp Parasitol* 136:20–6.

Morsi NM. (2000). Antimicrobial effect of crude extracts of *Nigella sativa* on multiple antibiotics-resistant bacteria. *Acta Microbiol Poland* 49:63–74.

Nasir A, Avais M, Khan MS, et al. (2013). Treating *Cryptosporidium parvum* infection in calves. *J Parasitol* 99:715–17.

Nilforoushzadeh MA, Hejazi SH, Zarkoob H, et al. (2010). Efficacy of adding topical honey-based hydroalcoholic extract *Nigella sativa* 60% compared to honey alone in patients with cutaneous leishmaniasis receiving intralesional glucantime. *J Skin Leishmaniasis* 1:26–31.

Okeola VO, Adaramoye OA, Nneji CM, et al. (2011). Antimalarial and antioxidant activities of methanolic extract of *Nigella sativa* seeds (black cumin) in mice infected with *Plasmodium yoelli nigeriensis*. *Parasitol Res* 108:1507–12.

Randhawa MA, Al-Ghamdi MS. (2002). A review of the pharmacotherapeutic effects of *Nigella sativa*. *Pak J Med Res* 41:77–83.

Rocha LG, Almeida JR, Macedo RO, Barbosa-Filho JM. (2005). A review of natural products with antileishmanial activity. *Phytomedicine* 12:514–35.

Rojas-Silva P, Graziose R, Vesely B, et al. (2014). Leishmanicidal activity of a daucane sesquiterpene isolated from *Eryngium foetidum*. *Pharm Biol* 52:398–401.

Salem ML, Hossain MS. (2000). Protective effect of black seed oil from *Nigella sativa* against murine cytomegalovirus infection. *Int J Immunopharmacol* 22:729–40.

Santos DO, Coutinho CE, Madeira MF, et al. (2004). Leishmaniasis treatment – A challenge that remains: A review. *Parasitol Res* 103: 1–10.

Suthar MP, Patel PN, Shah TG, Patel RK. (2010). *In vitro* screening of *Nigella sativa* seeds for antifungal activity. *Int J Pharm Appl Sci* 1: 84–91.

Valadares DG, Duarte MC, Oliveira JS, et al. (2011). Leishmanicidal activity of the *Agaricus blazei* Murill in different *Leishmania* species. *Parasitol Int* 60:357–63.

Weninger B, Robledo S, Arango GJ, et al. (2001). Antiprotozoal activities of Colombian plants. *J Ethnopharmacol* 78:193–200.

World Health Organization. (2010). *Control of the Leishmaniasis. Technical Report Series 949*. Geneva: WHO; 5–12.