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Evaluation of antileishmanial activity and cytotoxicity of the extracts of *Berberis vulgaris* and *Nigella sativa* against *Leishmania tropica*

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ABSTRACT

Background & objectives: Leishmaniasis is a major public health problem, and the alarming spread of parasite resistance underlines the importance of discovering new therapeutic products. The present study aims to investigate the *in vitro* antileishmanial activity and cytotoxicity of the ethanolic extract of *Berberis vulgaris* fruits and chloroform extract of *Nigella sativa* seeds against *Leishmania tropica*.

Methods: In this study, antileishmanial activity of *B. vulgaris* and *N. sativa* extracts on promastigote and amastigote stages of *L. tropica* in comparison to meglumine antimoniate (MA) was evaluated, using MTT assay and macrophage model, respectively. MTT test was also used to assess the cytotoxicity of extracts on murine macrophages. The significance of differences was determined by analysis of variances (ANOVA) and student's *t*-test using SPSS software.

Results: The results showed that ethanolic extract of *B. vulgaris* (IC_{50} 4.83 µg/ml) and chloroform extract of *N. sativa* (IC_{50} 7.83 µg/ml) significantly reduced the viability of promastigotes of *L. tropica* in comparison to MA (IC_{50} 11.26 µg/ml). Furthermore, extracts of *B. vulgaris* (IC_{50} 24.03 µg/ml) and *N. sativa* (IC_{50} 30.21 µg/ml) significantly decreased the growth rate of amastigotes in each macrophage as compared with positive control (p <0.05). Our findings also revealed that extracts of *B. vulgaris* and *N. sativa* had no significant cytotoxicity against murine macrophages.

Conclusion: The *B. vulgaris* and *N. sativa* extracts exhibited an effective leishmanicidal activity against *L. tropica* on *in vitro* model. Further, works are required to evaluate the exact effect of these extracts on *Leishmania* species using a clinical setting.

Key words Amastigote; *Berberis vulgaris;* black seed; European barberry; *Nigella sativa;* promastigote

INTRODUCTION

Leishmaniasis has been identified as a major public health problem in tropical and sub-tropical areas, where the infection is transmitted by the bite of a female sandfly. It is endemic in 98 countries and territories, affecting 12 million people and approximately threatens 350 million people around the world[1]. Cutaneous leishmaniasis (CL) is the most common type of leishmaniasis affecting 1.5 million people annually, worldwide. About 90% of the cases are reported from countries such as Iran, Afghanistan, Pakistan, Iraq, Saudi Arabia, Syria and Peru[2–3].

Due to the non-avaialability of potential effective vaccine, drug treatment is the the only control option against leishmaniasis[4–5]. The first choice treatment of CL is pentavalent antimony compounds including meglumine antimoniate (MA) and sodium stibogluconate (SSG), which are widely prescribed despite their toxicity, high cost, difficulty in administration and emergence of resistant parasites[6–8]. These factors emphasize the urgent need for development of new effective treatment alternatives.

Natural products are valuable sources commonly used as alternative medicines to treat a wide range of diseases such as infectious diseases[9]. European barberry, *Berbesis vulgaris* L. (*Berberidaceae)*, grows in Asia and Europe. *B. vulgaris* called "*Zereshk*" in Persian is native to south-east of Iran. Previously, various studies have been carried out on chemical composition of *B. vulgaris* which indicated that the most important constituents of this plant are isoquinoline alkaloids such as berbamine, palmatine and particularly berberine[10]. Different parts of this plant including root, leaf, bark and fruit have widely been used as folk medicine for the preventation and treatment of various diseases including cardiovascular, gastrointestinal, respiratory, skin, renal and infectious diseases[10]. Furthermore, antibacterial, antifungal and antiparasitic effects of *B. vulgaris* and its derived compounds against some pathogenic strains have also been shown[10–12].

Nigella sativa L. commonly known as black seed from *Ranunculaceae* family grows in the southern Europe, North Africa, Middle East and Western Asia. *Nigella sativa* called "*Siah Daneh*" in Persian has long been traditionally used as a natural medicine for treatment of many acute as well as chronic conditions including hypertension, diabetes, cough, bronchitis, headache, eczema, fever and dizziness[13]. Reviews have reported that *N. sativa* has antioxidant and neuroprotective effects in addition to many other therapeutic activities such as antitumor, immunopotentiation, anti-inflammatory, antiasthmatic and antimicrobial properties[14]. Moreover, studies have revealed antibacterial, antifungal, antiviral and antiparasitic effects of *N. sativa* and its derivatives[13–15].

This study was aimed to evaluate the antileishmanial effects of ethanolic extract of *B. vulgaris* fruits and chloroform extract of *N. sativa* seeds against promastigote and amastigote stages of *L. tropica* using colorimetric assay [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide)]—MTT and macrophage model, respectively. In addition, in this survey, the inhibition of infection in macrophages (infectivity rate) and cytotoxicity effects of both the extracts on murine macrophage cells were investigated.

MATERIAL & METHODS

Chemicals

Meglumine antimoniate (MA, Glucantime) as control drug was purchased from Rhône, Poulenc, France and penicillin and streptomycin were procured from Alborz Pharmacy, Karaj, Iran and were stored at room temperature (25°C) until testing. 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, MA (USA). All other chemicals and solvents were of analytical grade.

Parasite and cell culture

Leishmania tropica standard strain (MHOM/IR/2002/Mash2) was kindly prepared by the Center for Research and Training in Skin Diseases and Leprosy, Tehran, Iran. The parasite was cultured in NNN medium, sub-cultured in RPMI-1640, supplemented with penicillin (200 IU/ml), streptomycin (100 µg/ml), and 15% heat-inactivated FCS. Murine macrophages were collected from male BALB/c mice (4–8 wk old) by injecting 2–5 ml of cold RPMI-1640 medium into mouse peritoneal cavity and then aspirated macrophages were washed twice and re-suspended in RPMI-1640 medium. The experimental procedures carried out in this survey were in compliance with the guidelines of the Kerman University of Medical Sciences, Kerman, Iran for the care and use of laboratory animals.

Preparation of extracts

The *B. vulgaris* fruits were collected from Baft district in September 2012, Kerman province, Iran. 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, Iran. The dried plant materials of *B. vulgaris* and *N. sativa* (100 g) were ground and extracted by percolation method by ethanol and chloroform for 72 h at room temperature, respectively. The extracts were passed through filter paper (Whatman No. 3, Sigma, Germany) to remove plant debris, then concentrated in vacuum at 50°C using a rotary evaporator (Heidolph, Germany) and stored at –20°C, until testing.

Antiproliferation effects of extracts against promastigote forms

Antileishmanial effects of *B. vulgaris* and *N. sativa* extracts on promastigotes were evaluated by colorimetric cell viability MTT assay using the method described by Mahmoudvand *et al*[8]. Briefly, 100 µl of the promastigotes (10^6 cells/ml) harvested from logarithmic growth phase were added into a 96-well microtiter plate. Then 100 µl of various concentrations (0–100 µg/ml) of each extract was added to each well and incubated at 25°C ± 1°C for 72 h. After incubation, 10 µl of MTT solution (5 mg/ml) was added into each well and were incubated at 25°C for 4 h. Promastigotes were cultured in complete medium with no drug used as positive control, and complete medium with no promastigotes and drugs as blank. All the experiments were repeated thrice. Finally, absorbance was measured by an ELISA reader (BioTek-ELX800) at 490 nm. We also measured the 50% inhibitory concentrations (IC_{50} value) of each extract by Probit test in SPSS software.

Cytotoxicity of extracts in intramacrophage amastigotes

In this study, for assessment of cytotoxicity effect of *B. vulgaris* and *N. sativa* extracts against intra-macrophage amastigotes of *L. tropica,* we used murine macrophages which were collected from male BALB/c mice according to the method described by Carrio *et al*[16]. Also similar to promastigote stage, all the experiments were repeated thrice. 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 In-

ternational NY, USA). In the next step, 200 µl of the cells (10^6 cells/ml) was placed in each well. After 2 h of incubation at 37°C in 5% CO_2 promastigotes in stationary phase were added to murine macrophages and again incubated in similar condition for 24 h. Free parasites were removed by washing with RPMI-1640 medium and infected macrophages were treated with various concentrations (0–100 µg/ml) of *B. vulgaris* and *N. sativa* extracts (50 µl) at 37°C in 5% CO_2 for 72 h. At the end, dried slides were fixed with methanol, stained by Giemsa and studied under a light microscope. Also macrophages containing amastigotes without extract and macrophages with no parasite and extract were considered as positive and negative controls, respectively. Anti-intramacrophage amastigotes activity of extracts was assessed by the mean infection rate (MIR) of each macrophage and also by counting the number of intra-macrophage amastigotes in each macrophage by examining 100 macrophages (% amastigotes viability). Also the IC_{50} values of each extract was calculated by Probit test in SPSS software.

Inhibition of infection in macrophages

In order to evaluate the inhibitory effect of the *B. vulgaris* and *N. sativa* extracts on the *L. tropica* incursion of macrophages, promastigotes were pre-incubated in both extracts (5 µg/ml), for 2 h at room temperature. Then promastigotes were washed with RPMI-1640 medium and incubated with murine macrophages for 4 h. After washing the cells again, the macrophages were stained by Giemsa and examined by a light microscope to evaluate the percentages of infected macrophages by counting 100 macrophages.

Cytotoxicity effect of extracts on murine macrophages

Cytotoxicity effect of the *B. vulgaris* and *N. sativa* extracts was evaluated by cultivating macrophages (5×10^5) with various concentrations (0–1000 µg/ml) of both the extracts in 96-well microtiter plates, at 37°C in 5% CO_2 for 72 h. Cell viability was evalutaed by colorimetric MTT assay, and results were displayed as the percentage of dead cells compared to macrophages treated with MA and non-treated macrophages (100% of viability). In addition, the IC_{50} values of each extract were calculated by Probit test in SPSS software.

Statistical analysis

Data analysis was carried out by using SPSS software and ANOVA (analysis of variances) test to determine the possible significant different effects among the extracts and control drug. Also Student's *t-test* was used to compare the IC_{50} values of extracts and control drug and $p < 0.05$ was considered as significant.

RESULTS

Antiproliferation effects of extracts against promastigotes

In the evaluation of antiproliferation effects of ethanolic of *B. vulgaris* fruits and chloroform extracts of *N. sativa* seeds against promastigote forms of *L. tropica*, it was be observed that both the extracts significantly ($p < 0.05$) inhibited the growth rate of promastigotes in a dose-dependent response compared with control drug (Fig. 1). Similarly, the IC_{50} values for *B. vulgaris* and *N. sativa* extracts against promastigotes of *L. tropica* were 4.83 and 7.83 µg/ml, respectively, while it was 11.26 µg/ml for MA (Table 1).

Cytotoxicity effects of extracts against intramacrophage amastigotes

Similar to the promastigote stage, a high cytotoxicity effect was demonstrated for amastigotes after 72 h incubation. Further, results indicated that the anti-intramacrophage amastigote effects of *B. vulgaris* and *N.*

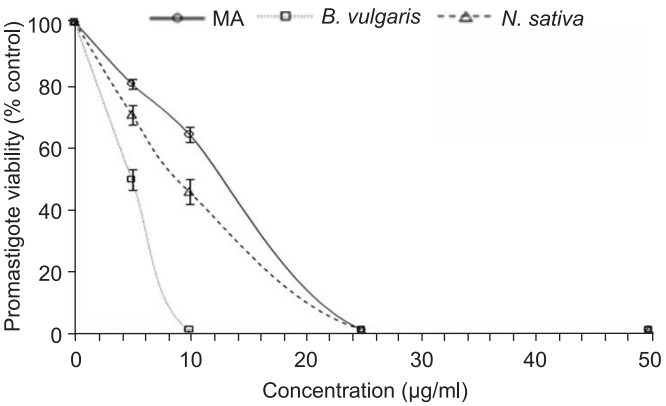

Fig. 1: The viability of *Leishmania tropica* promastigotes in the presence of various concentrations of the meglumine antimoniate (MA), *B. vulgaris* and *N. sativa* after 72 h incubation. Data are expressed as the mean ± SD (n = 3).

Table 1. Comparison of the mean IC_{50} values among meglumine antimoniate (MA), *B. vulgaris* and *N. sativa* against the growth rate of promastigote and intramacrophage amastigote forms of *Leishmania tropica* and also murine macrophages

Chemicals	IC_{50} (µg/ml)		
	Promastigotes	Amastigotes	Murine macrophages
MA	11.2 ± 0.57	33.83 ± 1.52	436 ± 3
B. vulgaris	4.8 ± 2.08	24.03 ± 1.15	326.3 ± 2.5
N. sativa	7.3 ± 1.52	30.21 ± 1.4	447.6 ± 3.6

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

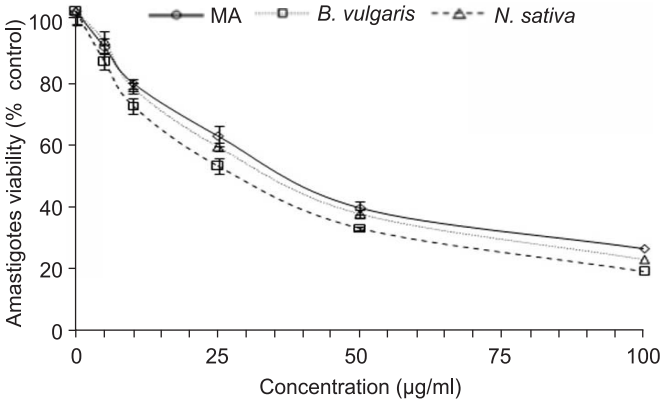

Fig. 2: The effect of different concentrations of the meglumine antimoniate (MA), *B. vulgaris* and *N. sativa* on the mean number of amastigotes in each macrophage (amastigotes viability %) in comparison with infected macrophages with no treatment as positive control. Data are expressed as the mean ± SD (n = 3).

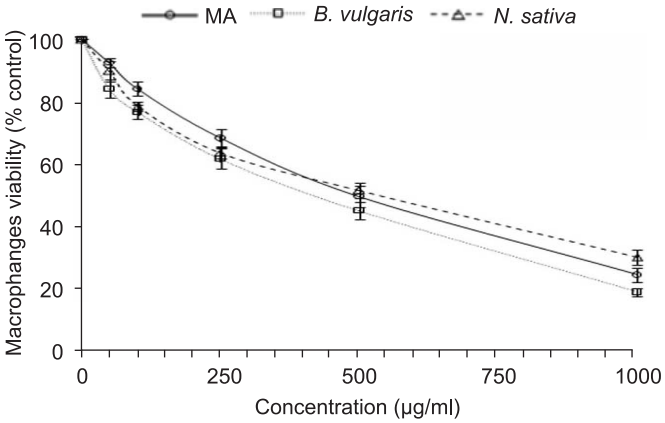

Fig. 3: The cytotoxic effects of the meglumine antimoniate (MA), *B. vulgaris* and *N. sativa* on peritoneal macrophages isolated from Balb/c mice. Data are expressed as the mean ± SD (n = 3).

sativa extracts were based on a dose-dependent manner, so that both the extracts were compeletly able to inhibit the growth rate of amastigote forms within macrophage cells at different concentrations of ≥100 µg/ml (Fig. 2). The IC_{50} values for *B. vulgaris* and *N. sativa* against amastigote forms of *L. tropica* were 24.03 and 30.21 µg/ml, respectively, whereas it was 33.83 µg/ml for MA (Table 1).

Inhibition of infection in macrophages

Findings exhibited that promastigotes with no extract of *B. vulgaris* or *N. sativa* had ability to infect 76% of the macrophages, whereas those promastigotes treated with the extracts of *B. vulgaris* or *N. sativa* significantly (p <0.05) were able to infect only 18.3 or 23.6% of the murine macrophages, respectively (Table 2).

Cytotoxicity effects of extracts on murine macrophages

Results of cytotoxicity effects of extracts on murine macrophages by MTT assay indicated a dose-dependent response, *i.e.* with increasing concentrations of both the extracts, the cytotoxicity effect was enhanced (Fig. 3). Also the IC_{50} values for *B. vulgaris* and *N. sativa* against murine macrophages were 326.3 and 447.6 µg/ml, respectively, while this value was 436 µg/ml for MA as control drug (Table 1).

Table 2. Inhibition of the infection in murine macrophages after treatment of promastigotes of *Leishmania tropica* with meglumine antimoniate (MA), *B. vulgaris* and *N. sativa*

Chemicals	Percentage of infected macrophages by non-treated promastigotes	Percentage of infected macrophages by treated promastigotes	Infectiveness reduction (%)
MA	79.3 ± 1.52	28.3 ± 1.15	64
B. vulgaris	79.3 ± 1.52	18.3 ± 1.52	77
N. sativa	79.3 ± 1.52	23.6 ± 0.57	70

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

DISCUSSION

Plant-derived components and plant extracts are valuable sources for traditional medicines due to having less side-effects, low cost and high availability and are mainly used to treat a wide range of disease conditions, including microbial infections. In the present study, we evaluated antileishmanial effects of ethanolic extract of *B. vulgaris* and chloroform extract of *N. sativa* against promastigote and amastigote forms of *L. tropica* as preclinical phase. Our results showed that *B. vulgaris* extract had potent antileishmanial activity on promastigotes of *L. tropica,* and at concentration of ≥10 µg/ml totally inhibited the promastigotes growth after 72 h of incubation. Moreover, it was able to significantly reduce the growth and number of amastigotes in the murine macrophages. Our finding also indicated that *B. vulgaris* extract was more effective against *L. tropica* promastigotes than amastigotes since the IC_{50} values were higher in inducing leishmanicidal effects in the amastigote-macrophage model. It should be mentioned that the differential susceptibility of promastigote and amastigote stages in response to treatment with the extracts is related to morphological and biochemical features and sensitivity to the drugs in both the parasite forms[17]. We have reported the inhibitory effects of methanolic root extracts of *B. vulgaris* against promastigotes of *L. tropica* and *L. infantum* in comparison to their main component of berberine[18].

It is important to note the distinction between fruit and root extracts. Often, *B. vulgaris* is mentioned without making a distinction between the fruit and the root[10]. The results here show that the fruits of *B. vulgaris* could inhibit both promastigotes and amastigotes of *L. tropica* more efficiently than the root of the plant (IC_{50} of methanolic root extract against promastigotes and amastigotes were 16.1 and 39.4 µg/ml, respectively)[19]. Considering the lack of berberine in the fruit extract of *B. vulgaris*, its antileishmanial activity could be attributed to the other components of the plant such as organic acids, especially its polyphenols. There are several studies which indicate polyphenols potentially inhibit amastigote and promastigote forms of different species of *Leishmania*[19–20].

So far, several studies have been carried out on antimicrobial effects of *B. vulgaris* and bioactive compounds of this plant that showed a good activity against some microorganisms[10]. The study carried out by Kaneda *et al*[11] revealed that berberine, most important compound of *B. vulgaris*, significantly suppressed the growth of *Entamoeba histolytica, Giardia lamblia* and *Trichomonas vaginalis* in BI-S-33 medium, and caused morphological changes in their structure. Furthermore, in the survey conducted by Sheng *et al*[21] it was observed that in chloroquine resistant malaria, a combination of berberine and pyrimethamine showed better results in the elimination of parasites and it was more effective than other drugs such as tetracycline or cotrimoxazole.

Vennerstrom *et al*[12] also reported that berberine derivatives significantly reduce the parasite burden in liver or ulcer size in golden hamsters infected with *L. donovani* and *L. braziliensis* compared with control drug (meglumine antimoniate). The effects of *B. vulgaris* on the experimental lesion of CL caused by *L. major* on BALB/c mice were also investigated by some researchers[22–23]. These indicated that *B. vulgaris* extract significantly reduced the size of lesion in infected mice. Recently, in a study conducted by Rouhani *et al*[24] it has been shown that aqueous extract of *B. vulgaris* at the concentration of 4 mg/ml indicated potent scolicidal activity against protoscoleces of hydatid cysts after 5 min incubation. The present findings demonstrated that *B. vulgaris* ethanolic extract is highly effective against *L. tropica* probably due to the presence of bioactive compounds especially polyphenols. It should be noted that the mechanism of action of polyphenols against parasites especially *Leishmania* species is not clear and need to be further explored.

N. sativa, black seed, for several decades has been used as a natural product to treat many diseases[25]. In this study, the extract of *N. sativa* showed potent antileishmanial effects on promastigotes of *L. tropica,* so that at concentration of ≥25 µg/ml the extract completely eliminated the promastigote forms. Also it is capable to significantly suppress the growth rate and number of amastigotes in the murine macrophages. Previous study conducted by Agrawal *et al*[14] demonstrated antimicrobial and antihelminthic effects of oil extract of *N. sativa*. Also results of a survey conducted by Mahmoud *et al*[15] showed that *N. sativa* oil can significantly decrease the number of *Schistosoma mansoni* worms and eggs in liver and intestine of patients. Nilforoushzadeh *et al*[26] indicated that combination of honey and *N. sativa* extract in patients with CL receiving glucantime is more effective to treat and improve the clinical signs as compared to honey alone. 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[27–28]. It seems that antimicrobial effects of *N. sativa* extract are attribiuted to bioactive ingredients particulary thymoquinone and other important components[29].

In the present study, we demonstrated that various extracts of *B. vulgaris* and *N. sativa* showed no significant cytotoxicity effect on murine macrophage cells. Similar to our findings, Peychev[30] reported that the oral administration of *B. vulgaris* is moderately toxic in mice (LD_{50} = 2.6 ± 0.22 g/kg b.w. in mice). Furthermore, 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 [31–32]. Therefore, it could be suggested that the extracts of *B. vulgaris* and *N. sativa* are safe for mammalian cells, considering that at high concentrations exhibited significant cytotoxicity in the host cells.

In conclusion, our findings confirmed that extracts of *B. vulgaris* and *N. sativa* had potent antileishmanial activity against promastiogote and amastigote stages of *L. tropica* on *in vitro* model, whereas, these extracts exhibited no significant cytotoxicity effect on murine macrophage cells. Further clinical studies are required to evaluate the exact effect of these extracts and particularly their main components on other *Leishmania* species in animal models as well as the volunteer humans as new therapeutic agent against leishmaniasis.

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