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Original paper

Peganum harmala and *Nigella sativa*: anti-leishmanial activity against *Leishmania major* promastigotes and amastigotes: *in vitro* and *ex vivo* experiment

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ABSTRACT. Leishmaniosis is one of the most important vectors borne disease that is endemic in tropical and sub-tropical areas. There are many approved treatment for different types of leishmaniosis but all are with some adverse side effects that limited its uses. Here, we attempt to evaluate *in vitro* and *ex vivo* anti-leishmanial activities of *Peganum harmala* (*P. harmala*) and *N. sativa* (*Nigella sativa*) on promastigotes and amastigotes of *L. major*. The plants were extracted by maceration method and prepared in concentrations of 7.8, 3.9, 1.9, and 0.9 µg. *L. major* were cultured in RPMI-1640 medium alone and in J774 cell line separately. The extracts at different concentrations were assessed against promastigote (*in vitro* assay) and amastigotes (*ex vivo* assay) of *L. major* for 72 h at 22 and 37°C, respectively. In current work, *N. sativa* at highest concentration (7.8 µg/ml) showed 54.4 and 60% anti-leishmanial activity with IC50 of 5.3 and 3.278 µg/ml, respectively. Also, *P. harmala* at highest concentration (7.8 µg/ml) showed 68.9 and 58.6% anti-leishmanial activity with IC50 of 2.4 µg/ml for both of them, respectively. The SI value was 38.22 for *N. sativa*, 25.9 for *P. harmala*, 19.4 for Amphotericin B, and 16.33 for Glucantime. The results of our study indicated that *N. sativa* and *P. harmala* are effective against *L. major* promastigotes and amastigotes and could be consider as an alternative treatments for leishmaniosis. Therefore, it is recommended that further studies be performed to confirm the efficacy and evaluate the toxicity of the herbal extracts.

Keywords: anti-*Leishmania*, *Leishmania major*, herbal extract, *Nigella sativa*, *Peganum harmala*

Introduction

Leishmaniosis is one of the six major infectious diseases in the world mostly in tropical and sub-tropical areas that is transmitted by sand flies as vectors [1]. Leishmaniosis is a global endemic disease and public health problem in seven developing countries, including Afghanistan, Iran, Algeria, Peru, Brazil, Saudi Arabia, and Syria [1]. The World Health Organization (WHO) designated leishmaniosis as a neglected tropical disease with about 2 million new cases per year and more than 350 million people at risk [2]. The most common reasons for treating leishmaniosis are reducing the severity of the infected wound and the patient's mental and emotional concerns [3] and diminishing

the reservoir and transmission of the disease [2]. Glucantime, amphotericin B, miltefosine, sitamavine, azole, and itraconazole are the chemical treatment options used to treat leishmaniosis [4]. All of the mentioned antileishmanial drugs have serious shortcomings due to their low effectiveness, unreachability, high cost, high toxicity, increasing resistance, and some inevitable side effects [5]. Therefore, the development of cost-effective alternative therapeutic strategies has become a high priority [5].

To date, using a natural product has been one of the main effective choices in the treatment of parasitic diseases including leishmaniosis. Studies have suggested that a natural herbal compound does not have a high probability of causing adverse effects. They are sustainable, economical, available, and more importantly have immunomodulatory effects compared with common antileishmanial compounds [6]. *Nigella sativa* L. (*N. sativa*) is from Ranunculaceae family, which grows in the Southern Europe, North Africa, Middle East, and Western Asia. *N. sativa* has long been traditionally used as a natural medicine for antioxidant, neuroprotective, antitumor, anti-inflammatory, antiasthmatic, and antimicrobial properties. Recent studies have revealed antibacterial, antifungal, antiviral, and antiparasitic effects of *N. sativa* and its derivatives [7]. Moreover, a recent study demonstrated that *N. sativa* oil and its major constituent thymoquinone had inhibitory effects on *L. infantum* and *L. tropica* parasites [8]. *Peganum harmala* (*P. harmala*) is an endemic medicinal plant in Iran with a long history of use in traditional medicine. The alkaloid compounds well represent the diversity of antiprotozoal compounds found in *P. harmala*; among several alkaloids, harmaline is an important active alkaloid [9]. Antimicrobial antifungal, anti-protozoal [10–12], and anti-cancer [13] effects have been reported for *P. harmala*. In addition, some previous studies have shown the inhibitory effects of the extract of *P. harmala* seeds and its beta-carboline alkaloids on human DNA topoisomerase I enzyme [14]. Given that *N. sativa* and *P. harmala* have a brilliant history in the treatment of microbial infections and their antileishmanial effects on *L. major* have not been studied yet, this study attempted to investigate the antileishmanial effects of these two drugs against promastigote and amastigote stages of *L. major* in an *in vitro* study.

Materials and Methods

Preparation of extracts

The seeds of *N. sativa* were purchased from Medicinal Plants Store, Mazandaran Province, Northern Iran. The plant materials were identified by a botanist in the Botany Department, Mazandaran University of Medical Sciences, Sari, Iran. The dried plant materials of *N. sativa* and *P. harmala* (20 g) were ground and extracted by maceration method by natural grape vinegar, standardized on 3% acetic acid for 48 h at room temperature. The obtained extracts were individually filtered and concentrated in a rotary vacuum until a crude solid extract was achieved with a yield of ~20% and kept at 4°C until used for further work.

Parasite culture

L. major promastigotes (MRHO/IR/75/ER) was cultured in RPMI-1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml streptomycin, and 100 IU penicillin (Gibco, Paisley, Scotland, UK) at 24°C. The medium containing the parasite was monitored daily and sub-cultured regularly.

Evaluation of anti-promastigote activity (*in vitro*)

Activity evaluation of the anti-promastigote was performed in 96-well plates for 72 h. Initially, serial dilutions of *N. sativa* and *P. harmala* were prepared in 100 µl of RPMI-1640 medium at final concentrations of 7.8, 3.9, 1.9, and 0.9 µg. Amphotericin B and Glucantime, as positive controls, were evaluated at different concentrations (0.1 and 0.007 µg; 75 and 0.14 µg, respectively). Then, 100 µl of 1×10^5 promastigotes in stationary phase was added to each well. Three untreated wells were considered as negative controls. After incubating the plates at 24°C for 72 h, the number of promastigotes/ml was counted by a hemocytometer under light microscope (400× magnification) by combining 20 ml of each well content and 20 µl of 2% formaldehyde solution in phosphate-buffered saline (PBS; pH=7.2). The death rate (DR) of the promastigotes was calculated using the following formula: DR (%) = [(NC – DT)/NC]×100, where NC was the number of promastigotes in the negative control, and DT was the number of promastigotes in the each treated well. All the tests were done in triplicate. The final volume of DMSO in each well did not exceed 0.2% [15].

Table 1. The results of the effects of *N. sativa* and *P. harmala* on *L. major*

	Concentrations µg/ml	Promastigotes		Amastigotes		CC50 µg/ml	SI
		Effectiveness	IC_{50} µg/ml	Effectiveness	IC_{50} µg/ml		
Nigella sativa	0.9	38.7	5.3	17.2			
	1.9	43.8		31	3.27	124.9	38.2
	3.9	48.7		37.9			
	7.8	54.4		60			
Peganum harmala	0.9	39.6	2.4	24.1			
	1.9	49.4		39.3	2.4	62.1	25.9
	3.9	59.4		47.5			
	7.8	68.9		58.6			
Amphotericin B	0.007	12.2	0.001	25.6			
	0.001	16.5		43.8			
	0.003	19.2		59.5			
	0.006	46.2		62.8	0.001	0.021	19.4
	0.012	61.4		77.1			
	0.025	76.5		84.9			
	0.05	79.8		93.3			
	0.1	84.7		97			
Glucantime	0.14	12.5	19.95	9.8			
	0.29	14.57		12.3			
	0.58	17		16.7			
	1.17	27.4		22.1			
	2.34	30.2		25	33.09	540	16.3
	4.68	31.9		28.4			
	9.37	32.9		31.6			
	18.7	34.3		50.7			
	37.5	43.8		62.9			
	75	78.7		74.3			

***Evaluation of anti-amastigote activity* (*ex vivo assay*)**

The murine macrophage cell line J774A.1 was obtained from the Iranian National Cell Bank (Pasteur Institute, Tehran, Iran). The cells were grown in RPMI-1640 medium. Then, 2×10^5 cells were added to each well of a 96-well plate in a volume of 200 µl RPMI-1640 medium. The plates were incubated at 37°C and 5% CO_2 for 5 h. The supernatants were discarded and 200 µl of RPMI-1640 medium containing the promastigotes was added to each well at a ratio of 1:10 (cell: promastigote). After 24 h incubation at the same condition, the wells were washed by RPMI-1640 medium to remove the free parasites. After washing, different concentrations of drugs and compounds as described for the treatment of amastigote were added to each well in a volume of 200 µl of RPMI-

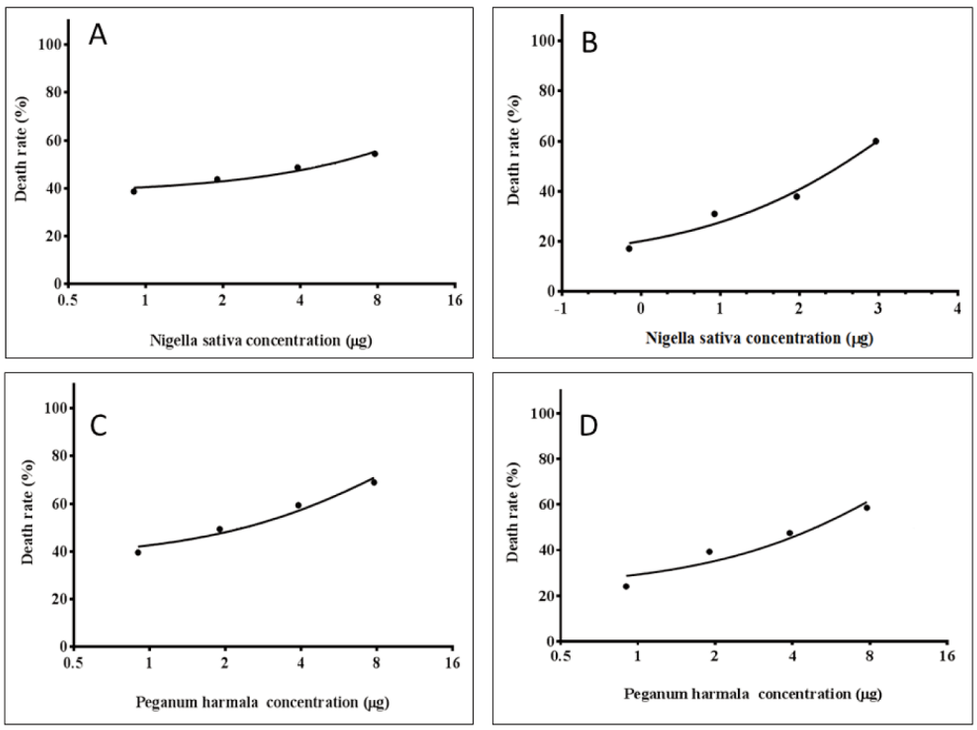

Figure 1. Antileishmanial effects of *N. sativa* and *P. harmala* in concentrations of 0.9, 1.9, 3.9, and 7.8 µg/ml. A, B: Cytotoxic effects of *N. sativa* against promastigotes and amastigotes of *L. major*; C, D: Cytotoxic effects of *N. sativa* against promastigotes and amastigotes of *L. major*.

1640 medium. The plates were incubated at 37°C and 5% CO_2 for 72 h. Also, 3 untreated wells were considered as negative controls. After 72 h, the supernatants were discarded and 50 ml of MTT solution (5 mg/ml stock solution in PBS) (Sigma, Lyon, France) was added to each well and the plates were incubated at 37°C and 5% CO_2 for 4 h. Then, 100 ml of dimethyl sulphoxide (DMSO) was added to each well. After 30 min under mild rotation, the optical absorbance of the wells was read using a scanning multi-well spectrophotometer (BioTek, Winooski, VT, USA) at a wave length of 570 nm. Cell death rate was determined by the following formula: 1-(AT/AC)×100, where AT was the mean absorbance of treated wells for each concentration of drugs or compounds, and AC was the mean absorbance of negative control wells [15].

Cytotoxicity assessment and selectivity index (SI)

The cytotoxicity of drugs or compounds was determined at several concentrations to the wells containing only the cells. The death rate was calculated for each concentration as described for the amastigotes. In addition, selectivity index (SI) was calculated by dividing CC_{50} by IC_{50} (amastigote) for each drug or compound. The SI>10 represents the safety of the medication [16].

Data analysis

GraphPad Prism v.6 was used to calculate IC_{50} and CC_{50} values. Statistical analysis was performed by two-tailed *t*-test using IBM SPSS v20 software (IBM Corp., Armonk, NY).

Results

The effects of N. sativa and P. harmala against promastigotes of L. major

The IC_{50} values obtained for *N. sativa* and *P. harmala* were 2.8 µg and 2.6 µg, respectively (Fig.1). The IC_{50} values for Amphotericin B and Glucantime were 0.007 and 19.95, respectively. There was a statistically significant difference between the IC_{50} of *N. sativa* and *P. harmala* and Amphotericin B and Glucantime (P<0.005). The results have been shown in Table 1.

The effects of N. sativa and P. harmala against amastigotes of L. major

The IC_{50} values obtained for *N. sativa* and *P. harmala* were 3.27 µg and 2.4 µg, respectively (Fig.

1). The IC_{50} values for Amphotericin B and Glucantime were 0.0011 and 33.09, respectively. There was a statistically significant difference between the IC_{50} of *N. sativa* and each of the other compounds, including *P. harmala* ($P<0.05$), Amphotericin B ($P<0.001$), and Glucantime ($P<0.001$). Moreover, the IC_{50} obtained for *P. harmala* showed a statistically significant difference in comparison with Amphotericin B and Glucantime ($P<0.001$). The results have been shown in Table 1.

Cell cytotoxicity and selectivity index

According to the CC_{50} values obtained for the drugs and herbal compounds, the SI value was 38.22 for *N. sativa*, 25.9 for *P. harmala*, 19.4 for Amphotericin B, and 16.33 for Glucantime.

Discussion

In current study, the effectiveness of the *N. sativa* and *P. harmala* extracts in concentrations of 0.9, 1.9, 3.9, 7.8, 0.9, 1.9, 3.9, and 7.8 µg/ml were assessed against *L. major* amastigote and promastigotes and the results were compared to Glucantime and Amphotericin B, as the positive controls. Many drugs and compounds have been studied for treating leishmaniosis, some of which have shown excellent results [17]. Despite various efforts and progresses, no definite anti-leishmanial drug is still available. Antimonials have been the first treatment choice for cutaneous leishmaniosis (CL) for nearly 65 years [18]. But in recent years, the search for a suitable alternative drug has been a health priority due to reduced effectiveness, side effects, toxicity, and resistance [19]. Plants and other natural products are important foundations of bioactive combinations; and they are a main potential basis of novel healing agents against several infectious diseases.

Arrabidaea chica [20], *Eugenia uniflora* [21], *Cymbopogon citratus* [22], *Zajuria multiflora* [23], and many other plants are being assessed for their anti-leishmanial effects by different cytotoxicity rates. However, no drugs have still been found that can replace chemical drugs. *P. harmala* and *N. sativa* are one of the most effective plants in Iranian natural and traditional medicine with broad ranges of efficacy [4,6]. Furthermore, significant anti-fungal, anti-leishmanial, breast cancer cell line, and anti-HIV-1 reverse transcriptase potential of *P. harmala* have been determined in different studies with the absence of any side effects, even at the top dose examined [8,11]. Tabari et al. [24] assessed the anti-trichomonal activity of alkaloid extract of *P. harmala* in concentrations of 5, 10, 15, 20, 30, 50, and 100 µg/ml against trichomoniasis in pigeon (*Columba livia domestica*) *in vitro* and *in vivo*; and the minimum inhibitory concentration (MIC) for *P. harmala* and metronidazole were calculated 15 and 50 µg/ml, respectively. In another study, Batiha et al. [11] evaluated the anti-piroplasmic potential of the methanolic extracts of *P. harmala* seeds and showed 60% efficacy against *Babesia microti* in mice.

N. sativa is another therapeutic plant that was effective on both promastigote and amastigotes with the IC50 values of 5.3 and 3.27 µg/ml, respectively. Furthermore, numerous studies have showed potent antifungal, antiparasitic, and antibacterial potentials of *N. sativa* and its derivatives such as ß-Pinene, O-Cymene, DL-Arabinose, Trans-4-methoxy thujane, Terpinen-4-ol, Longifolene, 10, 13-Eicosadienoic acid, methyl ester, E,E,Z-1,3,12-Nonadecatriene-5,14-diol, 1, 2-Benzenedicarboxylic acid, and bis (8-methylnonyl) ester against many pathogenic strains [25,26]. The SI rates for *N. sativa* and *P. harmala* extracts were calculated 38.2 and 25.9, respectively. Also, the SI rates for Amphotericin B and Glucantime were calculated as 19.4 and 16.3, that were lower than *N. sativa* and *P. harmala* extracts.

The results of this study showed that *N. sativa* and *P. harmala* are very effective in treating leishmaniosis infection and also their toxic effects are much less than those of Amphotericin B and Glucantime. A weaker anti-leishmanial activity was reported from the essential oil, methanolic extract as well as the chloroform extract of *N. sativa* seeds [27,28]. Similarly, a weaker anti-leishmanial activity has been reported from crude aqueus and hydroalcoholic extract from *P. harmala* [26]. *P. harmala* compounds include indole alkaloids (β-carboline), for exmaple harmaline (48.009%), harmine (38.440%), tetrahydroharmine (8.513%), tetrahydroharman (0.061%) and 6-methoxytetra-hydro-1-norharmanone (0.057%) that the main compound of *P. harmala* are alkaloids [29], which are known to be antiprotozoal [30], including harmalin, which is an important active alkaloid [32]. These alkaloids are isolated from the crushed seeds by extraction with acidified water [31]. Therefore, it may be concluded that extraction in acidic conditions may have resulted in the good activity of these two plants against the *Leishmania* parasite. Also, due to the reduced effectiveness of

conventional drugs and the toxicity of drugs, there is a strong need for a suitable alternative drug.

In this research, *N. sativa* and *P. harmala* were studied in different concentrations, which showed good effects coincidentally. Therefore, it is recommended that further studies be performed to confirm the efficacy and evaluate the toxicity of the drugs. If the results are acceptable, the herbs or compounds isolated from these plants could be used as an alternative to current drugs for the treatment of leishmaniosis.

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