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Assessment of the *In Vitro* antileishmanial and cytotoxic activity of *Nectaroscordum tripedale* fruit extract

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ABSTRACT

Plant extracts have been demonstrated to present remarkable biological and pharmacological properties, so that they are proper candidates for lead drug development. This study evaluates the invitro cytotoxic and antileishmanial effects of Nectaroscordum tripedale fruit extract against Leishmania tropica. Antileishmanial effects of the methanol extract of N. tripedale (5, 10, 25, 50, 100 µg/ml) on promastigote forms as well as their cytotoxic activities against J774 cells were examined using MTT assay for 72 h. Moreover, the antileishmanial activity of N. tripedale against amastigote stages of L. tropica was assessed at the concentrations of 5, 10, 25, 50, and 100 µg/ml in a macrophage model for 48 h. The obtained findings demonstrated that IC_{50} (50% inhibitory concentrations) value for the methanol extract was 61.6±3.05 µg/ml for promastigote forms and 32.6±2.15 µg/ml against amastigote forms. Regarding cytotoxic effects of N. tripedale extract, the findings demonstrated no significant cytotoxic effect in J774-A1 cells. According to the obtained results, N. tripedale might be a natural source of new anti-leishmanial agents in terms of use against cutaneous leishmaniasis.

Keywords: *Leishmania tropica*; J774 cells; extract; promastigote; amastigote

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INTRODUCTION

Leishmaniasis is a vector borne disease caused by protozoa parasites of the genus *Leishmania*. Leishmaniasis as a main Neglected Tropical Disease distributed in more than 90 countries with an incidence of 1.5 to 2.0 million people annually, and 350 million people under the risk of infection [1]. The disease shows different clinical manifestations including ulcerative skin lesions, mucocutaneous and visceral leishmaniasis [2]. At present, chemotherapy is the justuseful treatment for cutaneous leishmaniasis (CL). The drugs offered to treat CL are the pentavalent antimonials (e.g., sodium stibogluconate and meglumine antimoniate) as well as amphotericin B, and miltefosine and paromomycin. However, these drugs have produced severe side effects and toxicity [3, 4]. The concerns caused by these drugs, leading to most interest in searching substitute agents with higher efficacy and lower toxicity for the management of CL[5].

Currently, plant extracts have been demonstrated to present remarkable biological and pharmacological properties, so that they are proper candidates for lead drug development [6]. One of these interesting plants is *Nectaroscordum tripedale*L. from the Alliaceae family. The plant grows in various part of the world including Iran, and called

"Piazetabestaneh" or "Aneshk" in Persian [7]. In folk medicine, root, leaf, bark and fruit of *N. tripedale* have been broadly used for treatment and prevention of several diseases and illness such as rheumatic and joint pains, bladder and kidney stones [8]. However, modern medicine different pharmacological activities including laxative, expectorant diuretic, parasite repellent, appetizer, stimulant, muscle ache and joint pain reliever and sedative have related to *N. tripedale* [7, 8]. According to the literature and best of our knowledge, there is no study on antileishmanial effects of *N. tripedale*; therefore, the present study was designed to evaluate the antileishmanial and cytotoxic effects *N. tripedale* methanolic extract against promastigote and amastigote forms of *Leishmania tropica* and *L. major* on *in vitro* model.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents used were of the highest purity commercially available. Meglumine antimoniate (MA, Glucantime) as a control drug (1.5 g/ 5 mL) in the evaluation of antileishmanial effects was purchased from *Aventis*. Penicillin and streptomycin were obtained from Alborz Pharmacy, 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 prepared from Sigma-Aldrich.

Plant materials

The plants materials (fruits) were collected from the Khorramabad Mountains (Lorestan, Iran) in May 2014. The plant materials were identified by a botanist at the Razi Herbal Medicine Research Center, Khorramabad, Iran. A voucher specimen (LR12343) of the plant materials was deposited at the Herbarium of Agriculture and Natural Resource Research Center, Khorramabad, Iran.

Preparing of methanolic extract

Air-dried plant materials (200 gr) were extracted by methanol (80%) for 72 h at room temperature using the percolation method. Then, the extract was passed via filter paper (Whatman No.3, Sigma, Germany) to delete plant debris. Finally the isolated extract concentrated in vacuum at 50 °C using a rotary evaporator (Heidolph, Germany) and stored at -20 °C, until use [9].

Parasite and cell culture

Standard strains of *L. tropica* (MHOM/IR/2002/Mash2) and *L. major* (MRHO/IR/75/ER) were kindly prepared from Center for Research and Training in Skin Diseases and Leprosy (Tehran, 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. Murine macrophage cells (J774-A1) obtained from Pasteur Institute of Iran (Tehran, Iran). The cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS at 37°C in 5% CO_2.

Antipromastigote assay

The antipromastigote effects of *N. tripedale* methanolic extract was assessed by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) method based on tetrazolium salt reduction by mitochondrial dehydrogenases (10). Briefly, promastigotes from a logarithmic phase culture (10^6 cells/ml) were added into a 96-well microtiter culture plate. Then 100 µl of different concentrations of *N. tripedale* extract (5, 10, 25, 50, 100 µg/ml), and MA (2.5, 5, 10, 25, 50, 100 µg/ml) was added to each well and incubated at 24°C for 72 h. In the next step, 10 µl of MTT solution (5 mg/ml) was added into each well, incubated at 24°C for 3-4 h. The viability of promastigotes was measured using an ELISA reader (BioTek-ELX800) at 490 nm. The 50% inhibitory concentrations (IC_{50} values) were calculated by Probit analysis method using the software SPSS 17 for windows (SPSS Inc., Chicago). Promastigotes were cultured in complete medium with no drug used as control, and complete medium with no promastigote was used as blank. All experiments were repeated in triplicates.

Anti-amastigote assay

Antiamastigote effect of *N. tripedale* extract was determined according to the method described elsewhere [11]. Briefly, before adding the macrophages to the plates, 1 cm^2 cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International NY, USA). In the next step, 200 µL of macrophage cells (10^5/mL) were

incubated at 37˚C in 5% CO_2 for 2 h in DMEM. Then, 200 µL (10^6/mL) promastigotes of *L. tropica* in stationary phase were added to murine macrophages, so that proportion of *Leishmania*/macrophage was 10:1and 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 50 µL of various concentrations of *N. tripedale* extract (0-100 µg/mL) at 37˚C in 5% CO_2 for 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, 50% inhibitory concentrations (IC_{50} values) were measured for all the tested drugs by Probit test in SPSS software. All the tests were performed in triplicate.

Cytotoxic effects

In the present study, cytotoxic effects of *N. tripedale* extract was determined against J774-A1 cells by cultivating macrophages (5×10^5) with various concentrations of *N. tripedale* extract (0 to 500 µg/mL) in 96-well tissue culture plates at 37°C in 5% CO_2 for 48 h. The viability of macrophages was determined by colorimetric MTT assay and the results were displayed as percentage of dead cells compared to macrophages treated with MA and non-treated macrophages (100% of viability). Moreover, CC_{50} (cytotoxic concentration for 50% of cells) was calculated by Probit test in SPSS software [12].

Statistical analysis

All data represent the means ± standard deviations (SD) of three independent experiments. Data were analyzed by one-way ANOVA tests and Scheffe Post Hoc tests, using software SPSS 17 for windows (SPSS Inc., Chicago). Moreover, to compare the IC_{50} values of groups and control drug t-test was used and a *P*-value less than 0.05 was considered significant.

RESULTS

Anti-promastigote effects

Here we evaluated antipromastigote effects of *N. tripedale* methanolic extract against promastigotes forms of *L. tropica* using MTT assay. Figure 1 shows that *N. tripedale* methanolic extract significantly ($P<0.05$) reduced the proliferation rate of *L. tropica* promastigotes in a dose and time dependent manner compared with control group. The IC_{50} values of *N. tripedale* methanolic extract and MA were 61.6±3.05 and 88±3.1 µg/ml, respectively (Table 1).

Table 1. The IC_{50}, CC_{50} values, and selectivity index (SI) determined for the methanolic extract of *N. tripedale* and control drug (MA) against intra-macrophage amastigote forms of *L. tropica*.

Materials	IC_{50} values (µg/ml)		CC_{50} value (µg/ml)	Selectivity index (SI)
	Promastigote	Amastigote		
N. tripedale extract	61.6±3.05	32.6±2.15	367.3±14.6	11.3
Meglumine antimoniate	88±3.1	44±6.1	1012.6±13.6	24.3

Antiamastigote effects

Anti-amastigote effects of *N. tripedale* methanolic extract were tested using measuring the mean number of amastigotes in each macrophage infected by *L. tropica* promastigotes. The findings of the mean number of amastigotes in each macrophage demonstrated that *N. tripedale* methanolic extract is able to decrease significantly ($P<0.05$) the number of amastigotes in each macrophage compared with control group (Figure 2). The IC_{50} values of *N. tripedale* methanolic extract and MA for *L. tropica* amastigotes were 32.6±2.15 and 44±6.1 µg/ml respectively (Table 1).

Cytotoxic effects

In vitro cytotoxic activity of *N. tripedale* methanolic extract on J774-A1 cells were assessed by MTT assay. The findings demonstrated no significant cytotoxic effect in J774-A1 cells. The CC_{50} values for *N. tripedale* methanolic extract and also its SI value for amastigote forms of *L. tropica* were shown in Table 1.

DISCUSSION

Nowadays, ethno-pharmacology is known as one of the main sources in searching new therapies for a wide range of diseases such as infectious ones. Furthermore, an additional excellent reason for the ethno-pharmacological advance is to find new, effective, less expensive and simple therapies, limiting at the same time the cost of pharmaceutical research [6, 13]. The present study aims to evaluate the antileishmanial effects of *N. tripedale* methanolic extract against promastigote and amastigote forms of *L. tropica* as well as its cytotoxicity on J774-A1 cells on in vitro model. The obtained findings demonstrated that *N. tripedale* methanolic extract significantly reduced proliferation rate of promastigote of *L. tropica*. Similarly, this extract was able to significantly decrease the growth and number of amastigotes in the macrophages cells. The obtained results indicated that *N. tripedale* methanolic extract was more efficient against *L. tropicaa* amastigotes than promastigotes because of the IC_{50} values were higher in inducing antileishmanial activities in the promastigote model. Previous investigations have shown that the discrepancy in resistance of promastigote and amastigote stages in answer to treatment with the extracts is associated to morphological and biochemical features and sensitivity to the drugs in both the parasite forms [11, 14]. Regarding to antiparasitic effects of *N. tripedale*, Habib et al (2016) have reported that herbal extracts especially *N. tripedale* were effective in control of coccidiosis caused by the *Eimeria tenella* infection in broiler chickens [15].

Moreover, Mahmoudvand et al (2016) have demonstrated the potential of *N. tripedale* extract as a natural source for the production of new protoscolicidal drug for use during hydatid cyst surgery [16].

Recently, Mahmoudvand et al have shown the existence of terpenoids, flavonoids, tannins and fatty acids inphytochemical screening of *N. tripedale* extract [16]. In a study conducted by Cown (1999), antimicrobial activity of each of these compounds has been shown [17]; therefore antileishmanial activity of *N. tripedale* could be associated to these phytoconstituents; whereas the main antileishmanial mechanism of these constituents is poorly understood. However, previous studies on antimicrobial mechanisms of some of these components such as terpenoids demonstrated that they spread into pathogen and break cell wall structures, piercing into the inner of the cell and interacting with vital intracellular sites, cause DNA damage and disturb the fatty acid synthesis (18-22).
In the present study, the SI of greater than 10 for *N. tripedale* methanolic extract shows its protection to the mammalian cells and specificity to the parasite [23, 24]. In line with these findings, Mahmoudvand et al (2016) revealed that *N. tripedale* extract had no significant toxicity and could be consider safe for mice [16].

CONCLUSION

In conclusion, the findings of the present investigation exhibited proper antileishmanial activity of *N. tripedale* methanolic extract that might be a natural source for production of new antileishmanial drug against CL. However, more clinical surveys are mandatory to assess accurate biological effects of *N. tripedale* methanolic extract in animal models as well as volunteer human subjects.

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