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ORIGINAL ARTICLE

In vitro efficacy of ethanolic extract of *Artemisia absinthium* (Asteraceae) against *Leishmania major* L. using cell sensitivity and flow cytometry assays

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Abstract Leishmaniasis is one of the most neglected human diseases with an estimated global burden ranking second in mortality and fourth in morbidity among the tropical infections. Chemotherapy involving the use of drugs like glucantime is the mainstay treatment in endemic areas of Iran. Drug resistance is increasingly prevalent, so search for alternative therapy is gathering pace. Medicinal herbs, like wormwood *Artemisia*, have chemical compounds effective against a number of pathogens. In this study, the efficacy of ethanol extract from *Artemisia absinthium* (Asteraceae) against *Leishmania major* L. was investigated in vitro. The outcome of different effective doses (1–40 mg/ml) of ethanol extracts from this medicinal herb, *A. absinthium,* on a standard Iranian parasite strain of *L. major* was examined. The *L. major* promastigote cell sensitivity and mortality or viability effects due to the addition of herbal extract were measured using the MTT assay and the flow cytometry technique, respectively. There was complete agreement between the two assays. The lethal concentration (LC50) was measured as 101 mg/ml. Some contrasting relationships between the medicinal herb concentrations and the viability of parasites were observed; so that there was an increased multiplication of the parasite at low concentrations of the drug, but an anti-parasitic apoptotic effect was seen at high concentrations of *A. absinthium*. It was concluded that there might be one or more chemical constituents within the herbal extract of wormwood which at high concentration controlled cell division and affected the relevant activity within the only one giant mitochondrion in this flagellate parasite. At low doses, however, it showed the opposite effect of leading to mitotic cell divisions.

Keywords Wormwood · *Artemisia* · Viability assay · MTT · *Leishmania* · IC50 · LC50

Introduction

Species of *Leishmania* are vector-borne flagellate protozoan parasites of humans and other vertebrate hosts that

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lead to the conditions known as leishmaniases (Azizi et al. 2012a, b). These are classified as one of the most neglected tropical diseases with an estimated global burden of disease ranking second in mortality and fourth in morbidity among the tropical infections (Kedzierski 2011). These are a range of diseases that affect the skin, mucosa, and/or internal organs. They are manifested in severity from skin scars to serious disfigurement and fatal systemic infection (WHO 2010). Transmission to the vertebrate host is by *Phlebotomus* sand flies from a reservoir host to humans (Moemenbellah-Fard et al. 2003; Azizi et al. 2011).

Current treatments for leishmaniasis rely on chemotherapy, such as pentavalent antimonial drugs like sodium stibogluconate and meglumine antimonate which have been prescribed for over 70 years, to ameliorate disease and on vector control to reduce pathogen transmission. Many side effects are associated with the use of these drugs. Resistance to these drugs is also increasing and alternative search strategies should be looked for (Croft et al. 2006; Hadighi et al. 2006; Manzano et al. 2013). About 1.5 million new cases of leishmaniasis are reported every year and there is a critical need to develop better therapies (St. George et al. 2006).

Medicinal plants are indispensible sources of diverse types of bioactive organic compounds. One of these shrubs, *Artemisia absinthium* L. (*Asteraceae/Compositae*), commonly known as wormwood, is an erect, medium-sized herb with greenish silvery leaves and white twigs with strong aroma. About 400 species are described globally of which some 30 species are present in Iran only two of which are native to this country. They are known to have diverse antiparasitic (Rocha et al. 2005; Sen et al. 2007; Abdel-Sattar et al. 2010), antibacterial (Ramezani et al. 2004; Valdes et al. 2008; Ahameethunisa and Hopper 2010), antifungal (Kordali et al. 2005), antioxidant and antidepressant (Mahmoudi et al. 2009; Ali et al. 2013) and cytotoxic (Tariku et al. 2011) effects. Their efficacy could be due to the presence of an endoperoxide bridge at the heart of artemisinin, a bitter substance derived from extracts of wormwood (Krishna et al. 2008).

To the best of authors' knowledge, there is no corroborative report on the effect of wormwood extracts on *Leishmania major* within the scope of this investigation so far. The aim of this study was to find the effective dose of hydro-alcoholic extract from *A. absinthium* on the standard Iranian strain of *L. major* and to determine the efficacy of different doses of this medicinal herb on the parasite.

Materials and methods

Plant identification and preparation

In May 2010, aerial parts (stem, leaves and flowers) of wormwood, *A. absinthium*, or Afsanthin as it is known in Iran, during blossoming stage were collected from the foothills of Darab, 260 km to the east of the capital city of Shiraz in the southern Iranian Fars province (30°45′N, 50°45′E, at an altitude of about 780 m a.s.l.). A voucher specimen is deposited in the herbarium of Eram botanical garden at Shiraz University, Shiraz, Iran. The plants were air dried in the shade at ambient temperature. Morphological features of *A. absinthium* were determined using Flora Iranica key (Rechinger 1965–2008) and a Stereomicroscope. The height of this plant measures about 30–60 cm with tubular corolla of flowers on the shoot extremities.

Isolation of extracts

The wormwood plants were ground into very fine powder with an electric stainless steel grinder. Air-dried plant material (200 g) from the aerial parts of *A. absinthium* was then percolated three times with 80 % ethanol solvent and subjected to hydro-distillation for 3 h in a Clevenger type glass apparatus model Soxhlet. After extraction, the resultant samples were dried over a rotary evaporator to delete water and kept in amber vial at 4 °C prior to the viability assays. The sample yielded 23.7 g of solvent extract on a dry weight basis of 200 g.

Parasite culture

Promastigotes of *L. major* (MRHO/IR/75/ER) were cultured in the dark in heat-sterilized brain heart infusion (BHI) medium with 10 % fetal calf serum (FCS), 200 mg streptomycin and 200 K unit of penicillin antibiotics at a temperature of 25 ± 2 °C in an atmosphere of 5 % CO_2 in an incubator. Once promastigote culture reached its log or exponential phase, subculture samples were used for further studies.

Promastigote viability using the MTT assay

Leishmania major viability was assessed using the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay based on tetrazolium salt reduction to formazan crystals by mitochondrial dehydrogenases (Meerloo et al. 2011). Inoculums of 2×10^6 ml^{-1} promastigote cells were seeded into each of the 96-well ELISA plates. Subcultures to be assayed were incubated with the MTT reagent. Viable promastigotes can metabolize the MTT reagent into purple-color formazan. An increase in the absorbance at 600 nm, due to the formation of the formazan, indicates active mitochondria and thus viable promastigote cells. Absorbance data were acquired at 600 nm using a microplate enzyme-linked immunosorbent assay (ELISA) reader (Bio-Rad®).

Using dilution method, the solvent extract from *A. absinthium* was added to the fourth well onward with concentrations of 100, 200, 400, 600, 800 and 1,000 µg/ml into two plates (for measurements at 24 and 72 h). Two other plates received concentrations of 1, 2, 5, 10, 20 and 40 mg/ml. About 15 µl of *A. absinthium* without cell was poured into well number I (negative control). In well number II (positive control), 40 µl cells and 110 µl BHI without *A. absinthium* was inserted. In well number III (negative control), only 15 µl BHI without cell and plant extract was poured in. The remaining volumes of these wells were filled with 20 µl cell and BHI so that their final volumes reached 150 µl.

The plates were then incubated at 25 °C for 48 and 72 h. The number of live promastigotes of *Leishmania* in each well was counted after 24, 48 and 72 h with a Neobar slide on an inverted compound microscope. Then 0.3 g MTT powder with 600 λ was dissolved in PBS for one hour, filtered through 0.22 mesh filter and after 48 and 72 h 15 µl/mg of MTT reagent was added to each parasite-containing well. Four hours after incubation at 26 °C, 150 µl (equivalent to the volume in original culture medium) of acidic isopropanol (prepared by a mixture of 50 ml of two molar HCl acid in a 2.5 l isopropanol solution) was added to each well in order to dissolve the formazan crystals. The plates were incubated in the dark for 15 min. The photosensitive absorbance of plates were obtained by subtraction of optical densities at 540 and 720 nm wavelengths using an ELISA microplate reader (Bio-Rad®) since the relative number of viable cells was directly related to the light absorption rate of samples (Meerloo et al. 2011). The percentage cell viability was calculated for control and *A. absinthium*-exposed cells using the following formula:

$$\% \text{ Cell Viability} = [(A_t - A_b) / (A_c - A_b)] \times 100$$

where A_c is the absorbance of control well, A_t the absorbance of *A. absinthium*-treated well, and A_b the absorbance of blank well. Eventually, the data were expressed in terms of IC_{50} (50 % inhibition concentration of promastigote cell growth) with a concentration of *A. absinthium* which inhibited the growth of 50 % of promastigote cells.

Flow cytometry procedure

This was based on Ferreira-da-Silva et al. (2010) with slight modifications. The parasites were washed at 200 g centrifugal force. Parasites (1×10^7 cells) were exposed for two hours to different concentrations (1, 2, 4, 6, 8, 10, 20, 50, 100, 200 and 400 λ) of herbal extract. A test tube with parasite without propidium iodide (PI) as control for calibration, a tube with parasite as negative control stained later with 15 λ PI, and a tube with parasite exposed to 0.2 % saponin as positive control stained later with 15 λ PI were also considered. Furthermore, two test tubes with 4 and 8 % dimethyl sulphoxide (DMSO) with parasites were used. The samples were transferred to dark chamber in flow cytometry apparatus (4-colored BD FACS Calibur model, BD Biosciences Co, USA) for data acquisition and analysis with 10 000 cells. The Cell Quest PRO software was used to analyze the data. In this study, the LC_{50} was measured as 101 mg/ml.

Data analysis

Statistical analyses of the differences in mean values between different experimental groups were done with Student's *t* test. The significance of difference was measured by analysis of variance (ANOVA) and with a confidence interval of 95 %, *P*-values of 0.05 or less were considered significant.

Results

Inhibition of parasite growth by *A. absinthium*

In this study, the *L. major* promastigote cell sensitivity and mortality or viability effects due to the addition of the herbal extracts were measured using the MTT assay and the flow cytometry technique, respectively. There was complete agreement between the two assays. The cytotoxic efficacy of *A. absinthium* based on the LC_{50} value using the viability rate of *L. major* promastigotes was investigated with the MTT method. The result of MTT method showed the inhibitory effects on parasite growth in vitro. The lethal effects of all tested extracts were measured. It was found that there was an inverse relationship between the medicinal herb concentrations and the viability of parasites, so that there was an increased replication of the parasite at lower concentrations of the drug, but an anti-parasitic effect was seen at higher concentrations of *A. absinthium*. The data obtained from MTT absorbance showed that after 72 h all *L. major* promastigotes were killed due to lack of food. Low doses (1–2 mg) of this herbal extract caused replication of cells, but at high (16.6–40 mg) doses 50–100 % of parasite cells were dead (Fig. 1).

Leishmanicidal activity using flow cytometry

The exposed promastigotes to different extract concentrations were treated with PI stain and the results were noted (Figs. 2, 3). The percentages of apoptotic dead cells as well as necrotic deaths were examined after two hours using the flow cytometry. It was found that the extract concentration

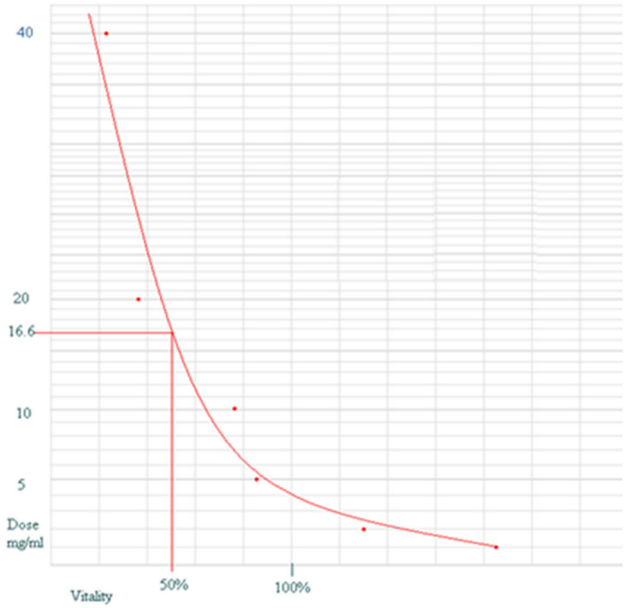

Fig. 1 Vitality curve of *Leishmania major* promastigotes exposed to different concentrations of *Artemisia absinthium* ethanol extracts after 48 h post-MTT assay (LC_{50} = 16.6 mg/ml)

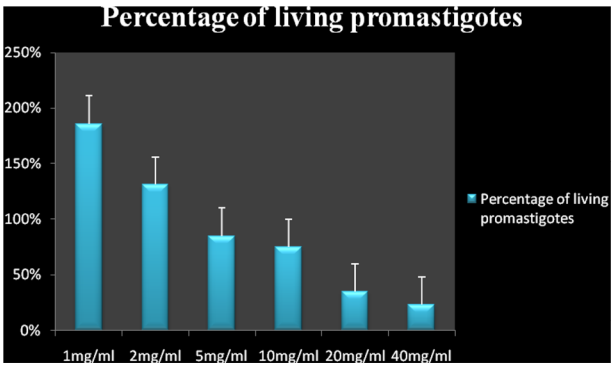

Fig. 2 Histogram exhibiting the percentage growth of living promastigotes of *Leishmania major* 48 h after exposure to different concentrations of *Artemisia absinthium* extracts

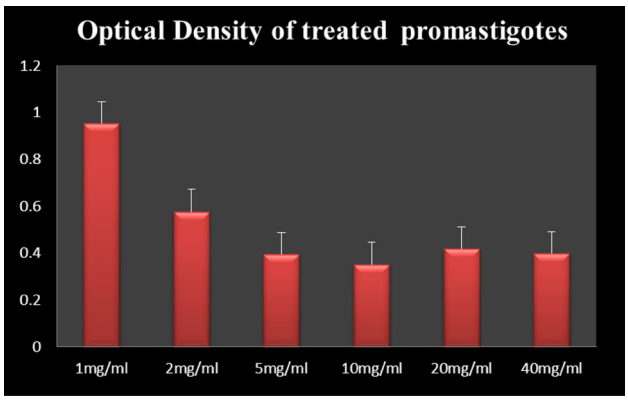

Fig. 3 Histogram of average optical density of treated promastigotes of *Leishmania major* 72 h after exposure to different concentrations of *Artemisia absinthium* extracts

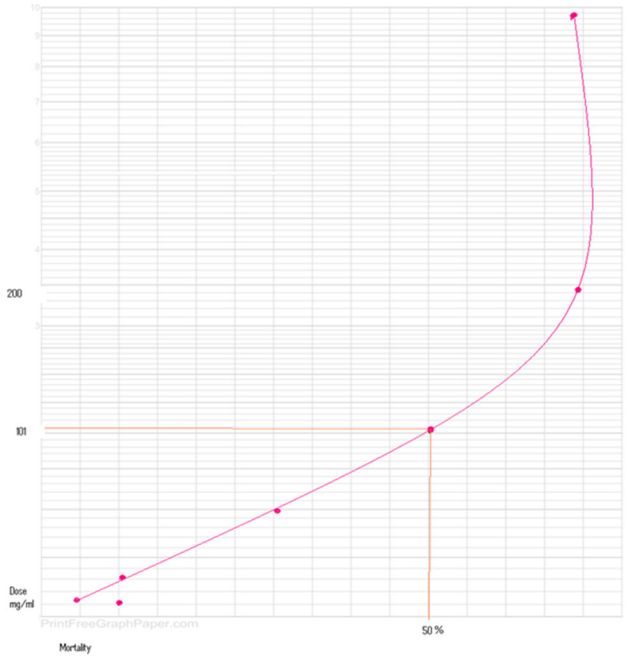

Fig. 4 Mortality curve of *Leishmania major* promastigotes exposed to different concentrations of *Artemisia absinthium* ethanol extracts after two hours using flow cytometry method (LC_{50} = 101 mg/ml)

at which 50 % of cells were dead (LD_{50}), was 101 mg/ml (Fig. 4).

Discussion

Leishmaniasis, after malaria, is the second most important vector-borne disease in Iran (Fakoorziba et al. 2011; Azizi et al. 2012a, b; Moemenbellah-Fard et al. 2012), though occasionally other malignant infections are also superimposed (Moemenbellah-Fard et al. 2009; 2014). There is a shortage of cheap and effective chemotherapeutic agents for the treatment of leishmaniasis. Drug resistance as well as multiple side effects is associated with the use of these chemical agents. The efficacy of different concentrations of herbal extracts of wormwood, *A. absinthium,* on *L. major* promastigotes in the logarithmic phase of growth was investigated using MTT colorimetric assay at 48 and 72 h. Using the PI stain, the mortality rates on exposure to different doses of these extracts after 2 h were monitored using a flow cytometry apparatus. So flow cytometry technique was used to measure mortality or viability effects of the extracts, but the result of MTT method exhibited inhibitory effects on parasite growth. It was shown here that there was complete agreement between the two methods.

Several studies have attempted to demonstrate the potential anti-*Leishmania* effects of various herbal extracts in the past. It has previously been stated that various

extracts from the leaves of different wormwood plants had anti-*Leishmania* activity in vitro (Hatimi et al. 2001; Ganguly et al. 2006). The latter report found IC_{50} values ranged from 0.21 to 0.58 mg/ml for *Absinthium indica*, while another report on *A. absinthium* against *L. major* demonstrated an IC_{50} value of 0.28 mg/ml (Kheiri Manjili et al. 2012). Essential oils from *A. absinthium* demonstrated growth inhibitory effects at certain concentrations against promastigotes of two different *Leishmania* species (Tariku et al. 2011). Similarly, the growth inhibitory activities of 11 different *Artemisia* species were shown against the promastigotes of *L. major* (Emami et al. 2012). Anti-*Leishmania* effects due to certain essential oils derived from cultivated *A. absinthium* were further exhibited in a recent paper (Bailen et al. 2013). The IC_{50} of *L. major* promastigote cells was also determined to be 25 and 50 µg/ml for *Artemisia sieberi* and artemisinin, respectively (Heydari et al. 2013). The outcome from MTT colorimetric assay in the present study indicated that this medicinal herb (*A. absinthium*) was mitogenic at low doses of 1-2 mg and increased the mitotic divisions of parasites. At high concentrations, however, an inverse or inhibitory effect on the parasite was noted. The IC_{50} was calculated to be 16.6 mg which was in line with that of others (Emami et al. 2012; Heydari et al. 2013).

The flow cytometry studies were in perfect conformity to these findings and exhibited that after 2 h exposure with the herbal medicine of wormwood, 50 % of parasitized cells underwent apoptosis with a dose of 101 mg/ml. It was thus concluded that there was one or more chemical compound within the herbal extract of wormwood which at high concentration control cell division and affect the relevant biochemical activity within mitochondria. At low concentrations, however, it showed the opposite effect. It led to mitotic cell divisions.

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