ISSN: 2383-2568 *Quarterly of International Archives of Health Sciences 2015;2(2):69-74*

Methanolic Extract's Activity of *Artemisia absinthium*, *Vitexagnus-castus and Phytolacaamericana* Against *Leishmania major; in vitro* and *in vivo*

ARTICLE INFO

Article Type
Original Research

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How to cite this article
Khanjani Jafroodi S, Farazmand A, Amin M, Doroodgar A, Shirzadi M.R, Razavi M.R. Methanolic Extract's Activity of Artemisia absinthium, Vitexagnus-castus and Phytolacaamericana Against Leishmania major; in vitro and in vivo. International Archives of Health Sciences. 2015;2(2):69-74.

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Article History
Received: December 16, 2014
Accepted: May 20, 2015
ePublished: June 6, 2015

ABSTRACT

Aims Leishmaniasis is the most prevalent vector- borne parasitic disease in Iran. Drug treatment is the best way to treat leishmaniasis, while the common drugs are not efficient enough and inevitable side effects limit using these drugs. The aim of this study was to analyze in vitro and in vivo activity of the methanolic extract of *Artemisia absinthium*, *Vitex agnus-castus* and *Phytolaca americana* Against *Leishmania major.*

Materials & Methods The methanolic extracts of *Artemisia absinthium*, *Vitex agnus-castus* and *Phytolaca americana* were prepared by cold percolation method. The inhibitory concentration 50 (IC50) of the plant extracts was determined against *L. major* promastigotes followed by efficacy evaluation of the extracts against amastigotes and in vivo assay in the BALB/c animal model. The data was analyzed with SPSS 19 software using Student's T test and ANOVA.

Findings *Artemisia absinthium* had the highest amount of active compounds against promastigotes of *L. major* (IC50=159.45) and antiprolifrative activity of *Artemisia absinthium* on both forms of *L. major* (extracellular promastigotes and intracellular amastigotes) was the highest (MI=33%). *Vitex agnus-castus* had the least toxic effect for macrophages (8%). All extracts limited the progression of lesion size versus control group, however, only inhibitory effect of *Artemisia absinthium* extract was statistically significant.

Conclusion *Artemisia absinthium* is the most effective growth inhibitor of amastigotes in animal lesions and it is safe for drug application in human and animals.

Keywords Leishmaniasis; *Leishmaniamajor; Artemisia absinthium; Vitex agnus-castus* extract

CITATION LINKS

[1] Cutaneous ... [2] Leishmaniasis.Cutaneous ... [3] Leishmaniasis, Burden of Disease, Magnitude of ... [4] Therapy of cutaneous ... [5] Miltefosine efficiently eliminates Leishmania major amastigotes from infected murine dendritic cells ... [6] leishmaniasis worldwide and global estimates of ... [7] A review of natural products with antileishmanial ... [8] Antileishmanial activity in Israeli ... [9] Brazilian flora extracts as source of novel antileishmanial and ... [10] Use of antimony in the treatment of Leishmaniasis: Current status and future ... [11] Artemisinins: Mechanisms of action and potential for ... [12] Plant extracts: Search for new alternatives to treat microbial ... [13] Natural products as ... [14] Chemical composition and antimicrobial activity of Vitexagnus-castus L. fruits and ... [15] Volatile components of four Ethiopian Artemisia species extracts and their in vitro antitrypanosomal and ... [16] Major components of Spanish cultivated Artemisia absinthium populations: Antifeedant, antiparasitic, and ... [17] Antileishmanial activities associated with plants used in the ... [18] Rapid colorimetric assay for cellular growth and survival: Application to ... [19] Activity of antileishmanial agents against amastigotes in human monocyte-derived macrophages and in ... [20] In vitro activities of iboga alkaloid congenerscoronaridine and 18-methoxycoronaridine against ... [21] In vitro antileishmanial activity of extracts of ... [22] The site of Leishmania major infection determines disease severity and ... [23] Artemisinin triggers induction of cell-cycle arrest and apoptosis in ... [24] Efficacy of artemisinin in experimental visceral ... [25] Antipromastigote activity of an ethanolic extract of leaves of ... [26] In vitro evaluation of antileishmanial activity and toxicity of essential oils of Artemisia absinthium and ... [27] Ethnopharmacological and biotechnological significance of ... [28] Efficacious topical treatment for murine cutaneous leishmaniasis with ethanolic formulations of ... [29] Antibacterial, phytotoxic, insecticidal and cytotoxic potential of ... [30] Determination of the chemical composition and antioxidant activity of the essential oil ... [31] Composition and antimicrobial activity of the essential oil of Artemisia absinthium from Croatia and ...

Introduction

Leishmaniasis is an important parasitic disease, caused by species of flagellate protozoan genus, Leishmania, and transmitted by the bite of sand flies. The common forms of the disease are cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL). In spite of being mostly self-healing, CL leaves permanent scars on the skin, which may lead to long term social stigmas for the patient even after full healing. On the other hand, VL is the progressive form of the disease that can be lethal in human. Two phases of Leishmania life cycle are characterized as promastigote and amastigote. The promastigote of the parasite enters the human body. Promastigotes are up taken by macrophages and afterwards they are transformed into the amastigote of Leishmania as the intracellular form [1, 2].

The disease is endemic in 88 countries, mostly reported in the developing countries. More than 90% of CL cases occur in Afghanistan, Algeria, Brazil, Pakistan, Peru, Saudi Arabia, Syria and Iran. Incidence ratio of leishmaniasis in Iran is about 30 in each 100,000 people per year [3].

Since there is no vaccine to prevent the disease, drug treatment is the only way to treat leishmaniasis [4, 5]. The first line treatment for leishmaniasis is application of pentavalent antimonial compounds, Amphotericin B and Miltefosine, that can bring about serious side effects and resistance to the drugs, which has become an important issue [6]. Therefore, determination and development of new medicinal agents are essential for alternative treatment. On the other hand, according to World Health Organization (WHO) reports, leishmaniasis is endemic in those developing countries that have limited access to effective drugs [6]. For this reason, introducing plants with antileishmanial effects can be very useful, as plants are valuable, safe and inexpensive source of antimicrobial agents.

Numerous studies in the world have been focused on search for natural products with leishmanicidal effects [7-9]. However, many current drugs are toxic and resistance development to some drugs, like antimonial compounds, has been reported [10, 11]. Considering the fact that adverse effects of phytotherapeutic agents are less common compared to that of synthetic drugs, plant materials are likely to provide a valuable source of new medicinal agents for protozoa caused lesions [12]. Therefore, extracts derived from plants material suggest novel possibilities to obtain new compounds that are active against Protozoa e.g., Leishmania. Nevertheless, if the effectiveness of phytopharmaceuticals is proved versus chemical drugs in experimental analyses and research laboratories, they can be used efficiently for disease treatment [13, 14].

Previous studies suggest that many plant extracts contain effective compounds such as quinines, alkaloids, terpenes, saponins, phenol derivatives and other metabolites, which are reported to have antimicrobial, antiparasitic and specifically antileishmanial activity [14-16]. So, the aim of this study was to analyze *in vitro* and *in vivo* activity of the methanolic extract of *Artemisia absinthium*, *Vitex agnus-castus* and *Phytolaca americana* Against *Leishmania major.*

Materials & Methods

Plant collecting and preparation of the extracts

Artemisia absinthium, *Vitex agnus-castus* and *Phytolaca americana* were collected from Tehran province, Iran, in August 2012. Botanical identification was carried out by botanists in the Department of Biology of Tehran University, Iran, and voucher specimens were kept in the herbarium.

The extracts were prepared by cold percolation method. The different parts of each plant were dried at room temperature and ground within a blender. 50g of each powder was macerated in 300ml of methanol 80% in water for 48 hours in room temperature with shaking. The crude extracts were obtained following filtration using Whatman No. 1 filter paper. The solvent was evaporated in reduced pressure at below 40°C in a rotary evaporator [17]. All extracts were dried and weighed, then dissolved in a few drops of DMSO. The primary analyses indicated that the fruit extract of *Phytolaca americana* show more efficacy than the leaf extract of the plant.

Parasitic assays

Antipromastigote: *L. major* promastigotes, strain MROH/IR/75/IR, provided from Pasteur Institute of Iran were used for *in vitro*

assay. The promastigotes were maintained in NNN (Novy-MacNeal-Nicollem) medium, at 25°C. PBS (pH=7.2) was used as liquid phase of the culture medium. *L. major* promastigotes were cultured in RPMI 1640 medium (Sigma-Aldrich Chemicals; Germany), to reach logarithmic phase. Then, the plant extracts were prepared and added to a 96 well tissue-culture microplate with serial dilution from 31.25µg/ml to 1000µg/ml. After Neubar chamber counting, 2×10^6promastigotes/ml, was added to each well. The plates were incubated at 25°C for 48 hours. The viability of promastigotes was assessed using tetrazolium-dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) with a colorimetric method [18, 19]. Briefly, after incubation, 25µl of MTT solution (0.5mg/ml) was added to each well and incubated for 3-4 hours at 37°C. Then, 100µl of DMSO was added to dissolve MTT formazan. Optical Density (OD) was measured using an ELISA plate reader (BioTek Company; USA) at 570nm. The controls were dissolved in DMSO without any drugs. Miltefosine was used as the standard drug.

Antiamastigote: The macrophage J774 cells were provided from Pasteur institute of Iran and were cultured in RPMI 1640 (Sigma-Aldrich Chemicals; Germany), supplemented with Heat-inactivated Fetal Bovine Serum 10% (Sigma-Aldrich Chemicals; Germany), penicillin (100U/ml) (Sigma-Aldrich Chemicals; Germany) and streptomycin (100µg/ml) (Sigma-Aldrich Chemicals; Germany). They were incubated at 37°C in a humidified CO_2 5% incubator (Thermo Scientific; USA). The antiamastigote activity of the plant extract was assessed according to previous methods [20]. Briefly, the cells (4×10^5macrophages/well) were cultured in a 24-well microplate containing glass cover slips. After 4 hours, non-adherent cells were removed and plate was incubated at 37°C and CO_2 5% for 24 hours. The macrophages were infected with *L. major* promastigotes at a ratio of 7:1 parasite/macrophage and after 4 hours of incubation, unattached promastigotes were removed by washing with PBS. After overnight incubation at 37°C and CO_2 5%, the plant extracts were added to the plates followed by 48 hours incubation. Then the medium containing drugs were renewed and incubated for 60 hours. Cover slips were washed with PBS, fixed in methanol, stained with Giemsa 10% (Sigma-Aldrich Chemicals; Germany) solution and examined with light microscope. The number of infected macrophages in 100 macrophages (IR; infection rate) and "the number of amastigotes in experimental culture in 100 macrophages/the number of anastigotes in control culture in 100 macrophages" (MI; multiplication index) was counted [21].

Toxicity for macrophages: The toxicity of extracts for macrophage cells was assessed by the MTT colorimetric assay. The J774 macrophage (2×10^5cells/well) was added in a 96-well microplate. After 24 hours incubation at 37°C and CO_2 5%, the cells were exposed to plant extracts with IC_{50} concentrations of each plant. Following 60 hours incubation at 37°C, 20µl of MTT (0.5mg/ml) was added to each well and the plate was incubated for 4 hours. The optical density was measured at 570nm. Cell viability was determined using "Average of absorbance in duplicate drug wells-average blank wells/Average absorbance control wells" [21].

Animal experiments: Inbred balb/c mice, aged 4-6 weeks were provided from Pasteur institute of Iran, department of laboratory animals. The mice were divided into five groups, each group of 5 mice. Acclimatization time was two weeks. The mice were infected by 2×10^6 stationary phase promastigotes of *L. major*, through intradermal injection of parasites at the tail base. After three weeks leishmanial nodules and ulcers were visible and were confirmed by a direct smear microscopy. The treatment was started one week later [22].

The ointments were prepared in Eucerin (ointment base) from methanolic extracts, in 50% w/w. Each test groups was treated with ointments, containing *Artemisia absinthium, Vitex agnus-castus* or *Phytolacca americana* extracts. One group was treated with ointment base and the last group received no drug as the control group. Vertical and horizontal diameters of the lesions were measured by Kulis Vernier in the beginning of the study. Measurement and photography were done daily for four weeks. The mean of both vertical and horizontal measurements was calculated [22].

Statistical analysis: The means and standard deviation were determined at least in three

independent experiments. IC_{50} values for antipromastigote assay were calculated with MasterPlex software 2010. In order to assess the antileishmanial activity of plant extracts on balb/c mice lesions, caused by infective *L. major* stationary phase promastigotes, the diameter of lesions was measured during 4 weeks after beginning of the treatments. The data was analyzed with SPSS 19 software. The mean of each experiment was compared with control group using Student's T test and ANOVA.

Findings

***In vitro* leishmanicidal activity:** *Artemisia absinthium* had the highest amount of active compounds against promastigotes of *L. major* (IC_{50}=159.45) and antiprolifrative activity of *Artemisia absinthium* on both forms of *L. major* (extracellular promastigotes and intracellular amastigotes) was the highest (MI=33%). *Vitex agnus-castus* had the least toxic effect for macrophages (8%). Miltefosine as a standard drug with IC_{50} of 5.3µg/ml showed an efficient antileishmanial activity on promastigotes and more than 80% inhibition on amastigote proliferation (Figure 1).

Figure 1) IC_{50} values of *Vitex agnus-castus*, *Artemisia absinthium* and *Phytolacca americana* for antipromastigote assay, multiplication index and toxicity of plant extracts for macrophage cells

IC_{50} for antipromastigote assay (µg/ml)	**Multiplication Index for antiamastigote assay** (%)	**Toxicity for macrophage cell line** (%)
Vitex agnus-castus Leaf (Verbenaceae Family)		
234.15	36	8
Artemisia absinthium Leaf (Asteraceae Family)		
159.45	33	10
Phytolacca americana Fruit (Phytolacaceae Family)		
171.1	35	12
Miltefosine		
5.3	20	22

***In vivo* leishmanicidal activity:** All extracts limited the progression of lesion size versus control group, however, only inhibitory effect of *Artemisia absinthium* extract was statistically significant (p<0.05; Figure 2).

Discussion

CL is one of the most common parasitic diseases. It usually produces lesions on the exposed parts of the body. Although cutaneous leishmaniasis can be healed without any treatment, it can cause serious problems [1, 6]. In the present study, it is showed that methanolic extracts of *Artemisia absinthium, Vitex agnus-castus and Phytolaca americana* exert potent inhibitory effects on *Leishmania* promastigotes. The IC_{50} values of extracts were below 0.3mg/ml and they caused more than 60% lethality for amastigotes inside macrophages.

Figure 2) The means and standard deviations of leishmania lesions in balb/c mice during 30 days after treatment. The treatment began 4 weeks after infection applying the ointments containing *Phytolacca americana*, *Vitex agnus-castus* and *Artemisia absinthium* methanolic extracts. The lesion size in different groups of mice measured daily for 30 days repeated 3 times.

Day 0	**Day 7**	**Day 14**	**Day 21**	**Day 30**	**p value**
Artemisia absinthium					
4.4±2.4	6.8±2.6	8.1±2.8	9.1±2.8	9.9±2.4	<0.05
Vitex agnus-castus					
4.9±1.9	7.5±2.6	9.9±2.4	11.5±3.1	13.1±2.8	>0.05
Phytolacca americana					
5.3±2.4	8.4±2.2	11.3±2.6	13.6±2.5	15.3±2.6	>0.05
Ointment base					
4.3±2.6	7.9±2.4	11.0±2.6	14.7±2.8	18.3±2.6	>0.05
Control					
4.5±2.0	8.2±1.7	11.8±2.1	15.1±2.4	18.7±1.9	>0.05

Sen *et al.* evaluated the effect of Artemisinin (the sesquiterpene lactone isolated from Artemisia species) against visceral leishmaniasis (*L. donovani*) and have revealed IC_{50} values of 160µM for promastigote and 22µM for amastigote [23]. They have also shown that the antileishmanial activity of Artemisinin was mediated by apoptosis. In addition, it is suggested that Artemisinin helps to restore Th_1 cytokines, which is the protective immune response against leishmaniasis in human [24]. Several studies on antileishmanial activity of various Artemisia species are reported. Ganguli *et al.* have revealed the potential leishmanicidal activity of ethanolic extract of *Artemisia indica* on several strains of Leishmania with IC_{50} values varied from 0.21 to 0.43µg/ml [25]. El-On *et al.* have evaluated the antileishmanial effects of methanolic extract of Artemisia herba-alba on *L. major* promastigotes and amastigotes. IC_{50} values for both were reported to be higher

than 250µg/ml [8]. Tariku *et al.* fractionated *Artemisia absinthium* and the antileishmanial activity of two fractions containing monoterpene camphor and sesquiterpene lactone were tested on *L. donovani* and *L. aethiopica*. Both components were effective against promastigote and amastigote forms of Leishmania [26]. According to previous studies, fractionated components of *Artemisia absinthium* may exert a powerful effect against cutaneous leishmaniasis caused by *L. major* [24-26].

The other potentially efficient components of the extracts against *L. major*, are alkaloids, triterpenoids and saponins [11, 27]. Therefore, it is likely that the antileishmanial activity of these plant extracts is mediated by similar compounds. However, further studies, including fractionation of the plant extracts are needed to find out the respective components causing antileishmanial activity.

In most cases, plant extracts are evaluated under *in vitro* conditions, especially on promastigotes and few *in vivo* experiments are reported. In current study, the efficacy of the plant extracts was assessed in *L. major* infected balb/c mice. The extracts have inhibited the progression of leishmanial lesions, during four weeks and the results were statistically significant only for *Artemisia absinthium*, versus control group. Topical treatment with ointment was applied to find a painless and safe method. This method can reduce the toxic side effects of drugs and has been reported previously for cutaneous leishmaniasis treatment [28].

The results showed that the effect of plant extracts in various stages, promastigote and amastigote or *in vivo* may vary. This finding about Leishmania, protozoan with several stages of life cycle is very important because the infective form of the organism exists only in a limited period during its life cycle. The effectiveness of therapeutic agents on this stage of cycle is the matter of great interest and requires more research. The results of this experiment have shown an effective antileishmanial activity of the experimental plant extracts, while indicating their valuable chemicals to develop new drugs for CL treatment. In recent studies several plant extracts exhibit various antimicrobial effects, like anticancer [15, 29], antifungal, antibacterial and antiparasitic activity [14-17, 30, 31].

Conclusion

Artemisia absinthium is the most effective growth inhibitor of amastigotes in animal lesions and it is safe for drug application in human and animals. The toxicity of all plant methanol extracts for J774 macrophage cell line is less than Miltefosine.

Acknowledgements: We are grateful to the department of Zoonosis, center for disease control of Ministry of Health, Treatment and Medical Education for their valuable assistance in this study.

Ethical Permission: This research was carried out under ethical permission of Pasteur Institute of Iran ethical committee.

Conflict of Interests: We certify that here is no conflict of interest in this manuscript.

Funding Sources: None declared by authors.

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