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Effect of Methanolic Extracts of *Artemisia aucheri* and *Camellia sinensis* on *Leishmania major* (In Vitro)

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Aim: Infections caused by protozoa of the genus *Leishmania* are major worldwide health problems with high endemicity in developing countries. The incidence of leishmaniasis has increased in the absence of a vaccine. Usual drugs for treatment of the disease have many side effects; therefore, there is an urgent need to find new effective alternatives. The plant kingdom is a valuable source of new medicinal agents.

Methods: In this randomized, one-blind clinical trial, the in vitro leishmanicidal effects of *Artemisia aucheri* and *Camellia sinensis* on *Leishmania major* were evaluated. The methanolic extracts were prepared by percolation method. The extracts were dried and redissolved in PBS+DMSO 1% solvent. *L. major* cells treated with five concentrations (150, 300, 450, 600, and 750 µg/ml) of the extracts and an untreated control group were used in the study. The number of promastigotes in each concentration was calculated using a hemocytometer slide at time zero and at 24, 48, and 72 hours after being harvested.

Results: Methanolic extract of *A. aucheri* inhibited the parasite multiplication at doses of 150, 300 and 450 µg/ml at 48 and 72 hours of culture. Doses of 600 and 750 µg/ml showed the same effect at 24, 48 and 72 hours of culture ($P< 0.05$). Methanolic extract of *C. sinensis* showed inhibition of parasite multiplication when administered at doses of 150, 300, 450, 600 and 750 µg/ml at 72 hours ($P<0.05$).

Conclusion: These results provide a new perspective on drug development against *Leishmania*. The extract of *A. aucheri* at 750 µg/ml is strikingly potent against *Leishmania*, inhibiting the growth of promastigotes of *L. major* after 72 hours.

Key Words: *Leishmania*, *Artemisia aucheri*, *Camellia sinensis*, medicinal plant, promastigote

Artemisia aucheri ve *Camellia sinensis*'in metanolik ekstrelerinin In vitro şartlarda *Leishmania major*'a Etkisi

Giriş ve Amaç: *Leishmania* grubunun yol açtığı enfeksiyonlar özellikle gelişmekte olan ülkelerde ciddi bir problemdir. Aşı yokluğunda enfeksiyon sıklığıda artmıştır. Tedavi amacıyla kullanılan ilaçların yan etkileri son derece fazladır, bu nedenle, etkin alternatiflere acilen gereksinim vardır. Yeni ilaçlar için bitkiler en önemli kaynaktır.

Yöntem ve Gereç: Bu randomize tek-kör çalışmada *Artemisia aucheri* ve *Camellia sinensis*'in *Leishmania major* üzerindeki etkileri araştırılmıştır. Metanolik ekstreler perkolasyon yçntemi ile hazırlandı. Ekstreler kurutuldu ve % 1'lik PBS+DMSO'da çözüldü. *L. major* hücreleri ekstrelerin beş farklı konsantrasyonuna (150, 300, 450, 600, and 750 µg/ml) maruz bırakıldı ve sonuçlar tedavi edilmemiş kontrol grubu ile karşılaştırıldı. Her konsantrasyondaki promastigot sayısı 0, 24, 48,ve 72 saat sonra hemositometre kullanılarak hesaplandı.

Bulgular: *A. aucheri*'nin metanolik ekstresi 48 ve 72. saatteki kültürlerde 150, 300 ve 450 µg/ml dozlarında parazitin çoğalmasını inhibe etti. 600 ve 750 µg/ml dozlar kültürün 24, 48 ve 72. saatlerinde aynı etkiyi gösterdi($p< 0.05$). *C. sinensis*'in metanolik ekstreleri 150, 300, 450, 600 ve 750 µg/ml dozlarda ve sadece 72 satte parazitin çoğalmasını inhibe etti($p<0.05$).

Sonuç: Bu veriler *Leishmania*'ya karşı ilaç geliştirilmesi için yeni bir bakış açısı göstermiştir. *A. aucheri* ekstresi 750 µg/ml dozunda is strikingly potent against *Leishmania*'ya karşı son derece etkilidir ve 72 saatte *L. major* promastigotlarının gelişimini inhibe eder.

Anahtar Sözcükler: *Leishmania*, *Artemisia aucheri*, *Camellia sinensis*, Tıbbi Bitkiler, Promastigote

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Introduction

Leishmaniasis is a disease caused by hemoflagellate protozoa species of the genus *Leishmania* (1). The reservoirs of the disease are rodents, dogs, sanguine marsupials and other wild animals. The disease is transmitted by mosquitoes of the genera Lutzomyia

and Phlebotomus. According to the World Health Organization, 88 countries are affected by leishmaniasis, with approximately 350 million people at risk. Comprehending 12 million infected cases, the incidence is increasing worldwide with 1-2 million new cases registered annually, despite efforts being made to fight the disease (2). Leishmaniasis encompasses three clearly distinguishable clinical manifestations: generalized visceral infection (visceral leishmaniasis or "Kala-azar"), cutaneous leishmaniasis (oriental button), and mucocutaneous leishmaniasis (ulceration of the skin and hyper-development of the mucous membrane) (3). Leishmaniasis can be controlled mainly by vector and reservoir control and infected case management. The first two approaches are either impractical or expensive, leaving only the case control as the most effective strategy (2).

The best drug for the treatment of leishmaniasis is the pentavalent antimonies, which have been in use for more than 50 years. High failure rates and relapses are observed, particularly in treating co-infected patients, using these drugs (4). Clinical reports indicate that a large proportion of the cases are becoming unresponsive to chemotherapy. Variable efficacy, toxicity, requirement of long courses of parenteral administration and drug resistance, or combinations of these factors, have been reported. Pentavalent antimonies are still the first choice among drugs used for the treatment of leishmaniasis, despite cardiac and renal toxicity. There is also restriction in prescribing these drugs for systemic use (5).

Folk medicine is often a good source of bioactive substances potentially useful against many diseases, including leishmaniasis. These plants have offered many substances that are claimed to be promising for the synthesis of new classes of antileishmanial chemotherapeutic drugs (6). Most people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs, and more plants are discovered to be the main source of drug therapy in traditional medicine. The recognition and validation of traditional medical practices and the search for plant-derived drugs could lead to new strategies for leishmaniasis control.

Phytomedicine originated from China with the *Artemisia annua*, a plant that was traditionally used as an antipyretic (7). Artemisinin has been shown conclusively to pose anti-malarial activity. It is effective against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum* (8). The *Artemisia aucheri* is an indigenous plant very abundant in the north of Iran (9).

Trypanocidal action of green tea catechins (*Camellia sinensis*) against two different developmental stages of *Trypanosoma cruzi* was reported (2). Green tea is an audiometric local plant and is also very abundant in northern Iran. This study intended to verify ethnopharmacological use of selected traditionally administered plants of Iran, especially plants that are employed for treatment of leishmaniasis and similar diseases. Ethnobotanical information thus serves as a basis for the selection of the plants of choice.

Materials and Methods

Collection of plants

The aerial parts of *A. aucheri* were collected from Sari city and green leaves of *C. sinensis* from Lahijan city in July 2004. Both cities are in the north of Iran. The plants were authenticated by the Botany Division of the Pharmacognosy Department, Mazandaran University of Medical Sciences, Iran. A voucher specimen is kept in the herbarium of the Department.

Extraction

Shade-dried and powdered parts of the plants were placed in a glass percolator with methanol (Merck) and were allowed to stand at room temperature for about 48 h (in dark place). The percolates were collected and subjected to fractionation (10). The methanolic extracts were concentrated under vacuum using rotavaporator at 40ºC, then dissolved in dimethyl sulfoxide (DMSO) + phosphate buffered saline (PBS) and diluted with RPMI-1640 medium to the highest concentration. Required quantities of the extract were weighed, dissolved in DMSO + PBS, crushed in a pestle mortar and taken to final concentrations using sterile triple distilled water. DMSO standard solutions of the appropriate extract were diluted with lyses buffer (1000 v/v) to yield corresponding sample solutions.

Parasite culture

Promastigotes of *Leishmania major* (strain) were maintained by RPMI-1640 medium supplemented with

10% bovine serum (FBS) at 26 ºC. *L. major* promastigote (1×10^6 parasites/ml) were incubated at 26 ºC for 24, 48, and 72 h in fresh medium, in absence or presence of several concentrations (150, 300, 450, 600, 700 mg/ml) of the methanolic extracts. Intact live *L. major* promastigotes in the stationary growth phase were added to the plate well. A negative control (without plant extracts), and positive control (with Glucantime: 150 mg/ml) were included in the study.

Statistical analysis

All experiments were performed in triplicate. The mean and standard error of at least three experiments were determined. Statistical analysis of the differences between mean values obtained from experimental groups was done by means of Student's *t*-test. P values of 0.05 or less were considered significant.

Results

Inhibition of parasite growth

Different parts (stems, leaves) of the selected plant species were extracted and five concentrations (Tables 1 and 2) were used to assess their antileishmanial activity. The methanolic extracts of *A. aucheri* and *C. sinensis* were capable of completely abrogating the cellular growth of *L. major* promastigotes.

Antileishmanial activity

Tables 1 and 2 show the time course of the viability of *L. major* promastigotes in the absence or presence of methanolic extracts of *A. aucheri* and *C. sinensis*. 750 µg/ml methanolic extract of *A. aucheri* was able to kill about 25% of both developmental stages of the parasite after 72 h. Corroborating this result, microscopic observations showed mainly complete lyses of promastigotes after treating with 750 mg/ml of *A. aucheri* extract for 72 h. The results of the antileishmanial screenings are shown in Tables 1 and 2.

Discussion

There is a general lack of effective and inexpensive chemotherapeutic agents for treating protozoa parasitic diseases, including leishmaniasis (3). Pentavalent antimonial drugs are the first-line treatment for leishmaniasis in most affected areas, with amphotericin B and pentamidine being used as alternative drugs (10). These agents are not active orally and require long-term parenteral administration. They also have important side effects and are expensive. In addition, resistance to these compounds has become a serious problem. Therefore, new drugs are urgently required (11). In this sense, new drugs of herbal origin from the north of Iran discovered through ethnopharmacological studies have shown interesting leishmanicidal activities. Extracts from the

Table 1. Effect of different concentrations of *Camellia sinensis* methanolic extract in DMSO 1% and PBS buffer on the number of *Leishmania major* promastigotes in comparison to controls.

Extract concentrations and controls	Number of parasites (hours)			
	Beginning of culture	24 h	48 h	72 h
Culture medium (Negative control)	390000	410000	410000	570000
DMSO+PBS buffer	410000	400000	410000	520000
Glucantime	450000	110000	100000	60000
(Positive control)				
150 mg/ml extract	440000	360000	350000	200000
300 mg/ml extract	460000	390000	260000	240000
450 mg/ml extract	450000	220000	220000	200000
600 mg/ml extract	461000	300000	190000	120000
750 mg/ml extract	460000	290000	200000	120000

Table 2. Effect of different concentrations of *Artemisia aucheri* methanolic extract in DMSO 1% and PBS buffer on the number of *Leishmania major* promastigotes in comparison to controls.

Extract concentrations and controls	Number of parasites (hours)			
	Beginning of culture	24 h	48 h	72 h
Culture medium (Negative control)	390000	410000	410000	570000
DMSO+PBS buffer	410000	400000	410000	520000
Glucantime (Positive control)	450000	110000	100000	60000
150 mg/ml extract	560000	90000	50000	50000
300 mg/ml extract	590000	90000	50000	50000
450 mg/ml extract	610000	100000	40000	30000
600 mg/ml extract	590000	50000	40000	30000
750 mg/ml extract	460000	50000	30000	10000

aerial parts of *A. aucheri* and leaves of *C. sinensis* displayed in vitro activity at concentrations of 150, 300, 450, 500, 750 µg/ml against promastigotes of *L. major*, leading to a complete disruption of the nucleus, followed by cell lyses. In the present work, a novel pharmacological activity was obtained from the methanolic extract of these plants. Our initial observation is that the crude extracts of *A. aucheri* and *C. sinensis* strongly inhibited the in vitro growth of *L. major* promastigotes. A bioassay-guided fractionation of the antileishmanial activity is then recommended. The methanolic extract showed inhibitory activity against developmental stages of *L. major*; after 24, 48, 72 h of culture in fresh medium incubation, the parasites showed irreversible damages, suggesting a metabolic injury. These results were corroborated by lyses of promastigote cells. *A. aucheri* contains 0.3-0.5% artemisinin and other sesquiterpenelactone derivatives. These compounds have antihelmintic activity. Thus, the effect of *A. aucheri* on *L. major* in this research may be due to these compounds. *C. sinensis* has alkaloids such as caffeine and tannins (catechins). Tannins have antimicrobial, antiviral, and antiprotozoal activity. Therefore, the effect of *C. sinensis* on *L. major* observed in this research may well be because of these compounds.

Our results revealed a novel pharmacological activity against *L. major* and suggest that these methanolic extracts have the potential of being used as topical application in wound healing. The results presented here provide motivation for further exploration of these compounds, particularly as antileishmanial agents. Laboratory synthesis and the possibility of modifying the chemical structure of methanolic extracts are important advantages for the development of new antileishmanial agents using these plants.

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