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Leishmanicidal and Cytotoxic Activity of Algerian Medicinal Plants on *Leishmania major* and *Leishmania infantum*

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ABSTRACT

Introduction: Leishmaniasis is a severe disease that presents a real public health problem worldwide. Antileishmanial therapy remains expensive with intolerable side effects; therefore, it is essential to develop tolerable antileishmanial medications with a selective efficacy. **Methods:** In this study, the leishmanicidal activities of seven Algerian plant extracts, selected based on either ethnobotanical or chemotaxonomical data, were screened for their antileishmanial activity against promastigotes and amastigotes of cutaneous leishmaniasis agent *Leishmania major* (MON 25), and visceral leishmaniasis agent *Leishmania infantum* (MON 1). The cytotoxic activity against human monocytes THP1 was also determined. **Results:** In both species, amastigotes showed more sensitivity to the extracts than promastigotes. *Erica arborea* flower (IC_{50}=43,98 μg/mL), *Marrubium vulgare* leaves (IC_{50}=45,84 μg/mL) and *Artemisia herba-alba* Asso aerial parts (IC_{50}=55,21 μg/mL) had an almost similar inhibitory effect on *L. major* promastigote. *Marrubium vulgare* leaves (IC_{50}=35,63 μg/mL) was most effective against *L. infantum* promastigotes. Besides, these extracts exhibited low selectivity indices. The best results were obtained with *M. vulgare* on both *L. major* and *L. infantum* promastigotes (IC_{50}s of 45,84 µg/ml and 35,63 µg/ml), and amastigotes (IC_{50}s of 32,15 µg/ml and 18,64 µg/ml). The selectivity index was above two (2.34 *for L. major* and 3.01 *for L. infantum*), calculated based on the acceptable cytotoxic effect of *M. vulgare* on human macrophage cell line (CC_{50}=107,45 µg/ml). **Conclusion:** Out of the seven methanol extracts tested against promastigotes of *L. major* and *L. infantum*, three showed promising activity with potent leishmanicidal effect and acceptable selectivity indices on *L. major* and *L. Infantum*.

INTRODUCTION

Leishmaniases are parasitic diseases of humans and animals. The causative agents, various species of the protozoa belonging to the genus *Leishmania*, are transmitted through the infective bite of sandflies. More than 20 *Leishmania* species infect humans, presenting a broad spectrum of clinical manifestations [1, 2]. The parasites are endemic to 98 countries, with 2 million cases reported annually and approximately 350 million persons at risk of infection. Despite being a serious public health threat, leishmaniases remain neglected diseases worldwide [3].

In Algeria, more than seven million inhabitants are at risk of infection. In the country, two clinical forms, *i.e.*, cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) are prevalent. Cutaneous leishmaniasis is a significant public health problem in Algeria, and among the countries most affected by the disease in the Old World, this country ranks the second after Afghanistan [3, 4].

Three species are prevalent in Algeria; *Leishmania infantum* in the north of the country is responsible for cutaneous and visceral leishmaniasis, *Leishmania killicki* CL occurs in single foci of Ghardaia in the central south of Algeria, and *Leishmania major* CL accounts for most cutaneous form cases. *Leishmania major* predominates in extremely different foci and ecosystems and infects humans, rodents, and sandflies [5-7].

Presently, a vaccine is not available, and treatment is the only approach to fight against the disease. Current treatments include pentavalent antimonials, liposomal amphotericin B, and miltefosine which are all sharing some limitation to some extent, including high toxicity, unaffordable treatment

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costs and growing resistance [8, 9, 10]. Currently, there is a need for the development of safer and more effective drugs against leishmaniasis, especially in Algeria, where Glucantime® is the primary treatment [7].

In recent years, attempts to develop new antileishmanial medications from plants, and natural products have received tremendous attention [9, 11, 12]. Medicinal plants currently used for primary healthcare in developing countries are a promising source of valuable bioactive compounds.

Algeria is a vast country with various ecosystems characterized by flora rich in medicinal and aromatic plants, due to its climatic and topographic diversity [13]. Algeria is one of the notable Arab countries in terms of plant diversity and is home to 3,164 species of vascular plants [14].

In Algeria, the therapeutic potentials of the plants used by folk medicine were highlighted by several ethnopharmacological surveys carried out in different areas such as the central Sahara, the central parts of the North, Northwest, and Northeast of the country [15-20].

To investigate the antileishmanial activity, we used methanolic extracts of seven plant species collected from the central North and central West of Algeria, including Algiers, Blida, and M'Sila. The plants were selected based on previous reports of their medicinal properties reflected in the medical literature. The antileishmanial activity of the plant extract, demonstrated as the half-maximal inhibitory concentration (IC_{50}), was evaluated against intramacrophagic amastigotes and promastigotes of *L. major* and *L. infantum*, the causative agents of CL and VL in Algeria. Also, 50% cytotoxic concentration on human macrophages (CC_{50}) was used to determine the selectivity index (SI).

MATERIAL AND METHODS

Study area. We collected the plants from two areas, Mitidja (Algiers and Blida) and M'Sila. The Mitidja plain in northern Algeria is the largest sub-coastal plain covering approximately 1400 Km^2. On the northern side, the plain is separated from the Mediterranean sea by the Sahel ride, and on the south, by the Blida Atlas mountain - The Mitidja has a Mediterranean climate with a cold, humid winter. The M'Sila Province, also known as Hodna region, is situated in the central part of northern Algeria and covers a total area of 18,718 km^2. The area is characterized by an ecological diversity represented by two principal ecosystems: the steppe (Chott el Hodna, a wetland of international importance as defined by the Ramsar Convention, and reserve of El Mergueb) and the forest (Maadid and Ouanougha forest in the north and the Djebel Messaad forest in the south) [17].

Plant material. The aerial parts of the plant were washed, dried at room temperature in the dark and then finely ground to a powder. For the preparation of the methanolic extract, 20 g of powdered aerial parts were extracted with 300 ml aqueous methanol 85% using a soxhlet apparatus at 65°C for 6 h. After filtration with Wattman paper, the solution was evaporated in a vacuum (rotary evaporator Buchi R210) and then kept in small amber bottles at -20°C until used.

Vegetal extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Gillingham, U.K.) and dissolved in phosphate-buffered saline (PBS) at a concentration of 100 mg/ml and then stored at -70°C until further use, maintaining the final DMSO concentration at a maximum of 0.05% w/v.

Parasite culture. The strains *L. major* (MHOM/DZ/-2009/LIPA100/09), and *L. infantum* (MHOM/DZ/1985-/LIPA80) were cultured at 26°C in RPMI 1640 medium (SIGMA, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (SIGMA, St. Louis, MO, USA), 100 µg of streptomycin/mL and 100 U of penicillin/mL.

Reference drug. As a drug of reference, we used Amphotericin B (Sigma, St. Louis, MO, USA), Potassium antimonyl tartrate (SbIII) (Sigma-Aldrich, St. Louis, MO, USA), and gluconate antimoniate of meglumine (Glucantime® or SbV). The drugs were diluted in sterile distilled water. A PBS solution containing DMSO with a final concentration of 0.05% was used as the negative control.

Antileishmanial assay on promastigote. Promastigotes were distributed in 96-well plates at 10^5 cell density per well and incubated at 26°C for 72 h in the presence of various concentrations of plant extracts ranging from 10 µg/ml to 100 µg/ml. All assays were conducted three times in quadruplicate along with negative control (parasites) and with the reference drug (SbIII and Amphotericin B). First, screening was done with a final concentration of 200 µg/ml to select only the effective product on *Leishmania* strains. Viability was evaluated by parasite motility after 72 h, and cell density was determined using a hemocytometer. The survival rate was calculated according to the formula: percentage survival = (mean number of viable parasites in treated group/mean number of viable untreated parasites) × 100.

Intramacrophage susceptibility assay. After the initial screening, the effect of the selected plants on *L. major* and *L. infantum* growth in a human leukemia monocyte cell line (THP-1 cells) was evaluated according to the method previously described [21]. The THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 IU of penicillin/ml, and 100 µg of streptomycin/ml. Then, the THP-1 cells in the log phase of growth were differentiated by incubation for 2 days in a medium containing 20 ng of PMA/ml. Macrophages were infected with stationary-phase *L. major* and *L. infantum* amastigotes at a parasite/macrophage ratio of 5:1 for 4 h at 37°C with 5% CO_2. Non-internalized parasites were removed, and methanolic plant extract and reference drug (SbV and Amphotericin B) was added to the media followed by 3 days of incubation. Infected cells were then washed and stained with Giemsa. The parasitic index (PI) was calculated by the equation, % PI = (percentage of infected macrophages × number of intracellular parasites/macrophage in treated wells) / (percentage of infected macrophages × number of intracellular parasites / macrophage in untreated wells) × 100, and then the 50% inhibitory concentration (IC_{50}) value was determined by nonlinear regression analysis [22].

***In vitro* cytotoxicity.** The inhibition of macrophages viability was assessed on THP1 cells. Briefly, 5×10^5 cells of THP-1 cells were differentiated by incubation in RPMI 1640 supplemented with 10% FBS containing 20 ng of PMA/ml

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in 96-well culture plates. After 48 h, non-adherent macrophages were removed, and methanolic plant extract was added to the media followed by incubation for 2 days at 37°C. SbIII and Amphotericin B were used as positive control respectively at 100 µg/mL and 10 µg/mL. Cell viability was assessed by measuring the reduction of 2 mg/mL MTT, and absorbance was measured at 570 nm by using a multiwell scanning spectrophotometer. Background absorbance was subtracted from total absorbance to calculate formazan production. The results were expressed as the mean percentage reduction of macrophage viability compared to that in the untreated control wells, and the 50% cytotoxic concentration (CC_{50}) was calculated by nonlinear regression analysis as described [22]. The selectivity index was calculated using the ratio between CC_{50} and IC_{50} for all evaluated products.

Statistical analysis. Each experiment was performed in quadruplicates and results expressed as the mean ± standard error of the mean (SEM). The 50% inhibitory concentration (IC_{50}) values for each extract were determined by nonlinear regression analysis using GraphPrism Software, version 5.0.

RESULTS

In this study, the *in vitro* leishmanicidal activities of seven medicinal plants used in folk medicine in the north of Algeria were evaluated. Table 1 shows the data of the plants, including the family, the names, the investigated parts, local names, and their traditional usages. The results of antileishmanial activity, cytotoxic effects on human THP1 macrophages, and selectivity of methanolic extracts studied are summarized in table 2 for *L. major* and table 3 for *L. infantum*.

Out of seven methanol extracts tested against promastigote forms of *L. major* and *L. infantum*, four including, *Erica arborea* (flowers), *Artemisia herba-alba* Asso (aerial parts), and two species of *Marrubium*, *M. vulgare* (leaves) and *M. deserti* (leaves) showed a significant leishmanicidal activity and reliable selectivity indices (Tables 2 and 3). The most efficient methanolic extract on both species promastigotes of *L. major* and *L. infantum* after 72 h belonged to *M. vulgare*. Moreover, it had the best effect on amastigotes of *L. infantum* (IC_{50}=18.64±1.05 µg/ml), and an acceptable selectivity index (SI=2.34) as presented in table 3.

Among the four active extracts, *M. deserti* was effective only against *L. major* promastigotes. The most efficient extract against *L. major* promastigotes was *E. arborea* (IC_{50}=43.98±1.36 µg/ml) after *M. vulgare* (IC_{50} =45.84±0.53 µg/ml), and the most efficient extract against *L. infantum* promastigotes was *M. vulgare* (IC_{50}=32.15±1.5 µg/ml). Moreover, *E. arborea* flower extract (CC_{50}=89.96±0.68 µg/ml) was toxic for THP1 cells. *Artemisia herba-alba* Asso aerial parts (CC_{50}=131.5±1.38 µg/ml) showed the lowest cytotoxic effect among the extracts.

Table 1. List of the Algerian medicinal plants extracted by methanolic method and tested for their antileishmanial activity and medicinal properties

Family	Plant name/family	Local name	Extracted parts	Medicinal properties
Ericaceae	*Erica arborea*	Khalnedj	Flower	Kidneys diseases : bedwetting, urolithiasis
Lamiaceae	*Ajuga iva*	Chendgoura	Aerial parts	Antidiabetic, antihypertensive, leishmanicidal, digestive disorders, eczema
Lamiaceae	*Ballota hirsuta*	Meriout	Aerial parts	Digestive disorders
Lamiaceae	*Marrubium vulgare*	Merriouet sahraui	Leaves	Leishmanicidal, antidiabetic, digestive disorders
Asteraceae	*Artemesia herba alba* Asso	Chih	Aerial parts	Antidiabetic, antispasmodic, carminative, eczema
Lamiaceae	*Marrubium supinum*	Merriouet	Aerial parts	Antihypertensive
Lamiaceae	*Marrubium deserti*	Merriouet	Aerial parts	Antidiabetic, leishmanicidal, digestive disorders

Table 2. *In vitro* Leishmanicidal activity of medicinal plant extract on *L. major* promastigotes and amastigotes forms and cytotoxicity on THP1 Macrophages

Plant species	IC_{50}[b]±SD[a] (µg/ml) Promastigotes	CC_{50}[c]±SD (µg/ml) Macrophages	Selectivity index	IC_{50}±SD (µg/ml) Amastigotes
Erica arborea	**43.98±1.36**	**89.96±0.68**	**2.04**	**36±0.75**
Ajuga iva	**<200**	-	-	-
Ballota hirsuta	**<200**	-	-	-
Artemisia herba-alba Asso	**55.21±1.32**	**131.5±1.38**	**2.38**	**37.87±0.83**
Marrubium vulgare	**45.84±0.53**	**107.45±2.54**	**2.34**	**32.15±1.50**
Marrubium supinum	**<200**	-	-	-
Marrubium deserti	**53.49±1.06**	**95.7±1.28**	**1.78**	**42.15±2.03**
SbIII[d]	**3.59±0.56**	-	-	-
SbV[d]	-	-	-	**20.44±1.02**
Amphotericin B[d]	**0.2±0.01**			**0.2±0.01**

[a]SD, standard deviation.
Bold data indicate the extract selected as active.
[b]IC_{50}: concentration of drug that caused 50% of growth inhibition
[c]CC_{50}: concentration of drug that caused 50% of cytotoxicity
[d]Antileishmanial reference drugs.

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Table 3. *In vitro* Leishmanicidal activity of medicinal plant extract on *L. infantum* promastigotes and amastigotes forms and cytotoxicity on THP1 Macrophages

Plant species	IC_{50}[b]±SD[a] (µg/ml) Promastigotes	CC_{50}[c]±SD (µg/ml) Macrophages	Selectivity index	IC_{50}±SD (µg/ml) Amastigotes
Erica arborea	**61.27±0.72**	**89.96±0.68**	**1.46**	**53.93±0.26**
Ajuga iva	**<200**	-	-	-
Ballota hirsuta	**<200**	-	-	-
Artemisia herba-alba Asso	**77.97±1.48**	**131.5±1.38**	**1.86**	**68.25±1.32**
Marrubium vulgare	**35.63±1.06**	**107.45±2.54**	**3.01**	**18.64±1.05**
Marrubium supinum	**<200**	-	-	-
Marrubium deserti	**<200**	-	-	-
SbIII[d]	**1.34±0.56**	-	-	-
SbV[d]	-	-	-	**13.78±1.07**
Amphotericin B[d]	**0.2±0.01**			**0.2±0.01**

[a]SD, standard deviation.
Bold data indicate the extract selected as active.
[b]IC_{50}: concentration of drug that caused 50% of growth inhibition
[c]CC_{50}: concentration of drug that caused 50% of cytotoxicity
[d]Antileishmanial reference drugs.

The potent extracts on promastigotes forms were further tested against intracellular amastigotes of *L. major* and *L. infantum*, and four showed a significant effect (Table 2 and 3). In particular, the extract from *M. vulgare* leaves displayed the most promising activity on both species with IC_{50}=32.15 µg/ml for *L. major* and 18.64. µg/ml for *L. infantum*.

The selectivity index values obtained for both *Leishmania* species was above two. The aerial parts of *A. herba-alba* Asso and *M. vulgare* leaves showed the lowest SI on *L. major* (SI=2.38), and *L. infantum* (SI=3.01).

Amphotericin B inhibited 50% growth of both parasitic stages at a concentration around 0.2 µg/ml, while potassium antimonyl tartrate (SbIII) was active on *L. major* and *L. infantum* promastigotes with an IC_{50}s of 3.59±0.56 µg/ml and 1.34±0.56 µg/ml, respectively). However, Gluconate antimoniate of meglumine (Glucantime®) was less active against *L. major* and *L. infantum* amastigotes (IC_{50}s: 20.44±1.02 µg/ml and 13.78±1.07 µg/ml, respectively).

DISCUSSION

Medicinal plants have historically proven their values as the origin of ingredients with therapeutic potentials and nowadays still represent a valuable source for the identification of novel medications. A survey on plant-derived pure compounds used as drugs in countries hosting WHO-Traditional Medicine Centers indicated that, out of 122 compounds identified, 80% were used for the same or related ethnomedical purposes and were derived from only 94 plant species [23, 24].

Algeria is characterized by a high diversity in ecology, landscape and bioclimate spanning from the Mediterranean coast to the Tell Atlas mountains, the steppe, and the great Sahara desert. In Algeria, plants are traditionally used by herbalists and botanists to treat many disorders [17].

Here, we investigated the leishmanicidal and cytotoxic effect of methanolic extracts obtained from seven medicinal plants on the endemic species of *Leishmania* in Algeria, i.e., *L. major* and *L. infantum*. Our results showed that not all the extracts had the same effect on both species; some like extracts of *E. arborea* and *A. herba-alba* also showed a significant effect on *L. major*, without affecting *L. infantum*. In the case of extracts from *M. deserti*, the aerial part showed good antileishmanial activity against promastigotes of *L. major* and not *L. infantum*, although the selectivity index was low. The rest of the extracts from aerial parts of *Ballota hirsuta*, and *Marrubium supinum* did not affect promastigotes (IC_{50}> 200 µg/ml) (Table 2 and 3).

The *Marrubium* genus, commonly known as horehound or hoarhound, belongs to the *Lamiaceae* family and include more than 30 different species of flowering plants native to temperate regions of Europe, northern Africa, and Asia [25]. Today, in the North Africa (Algeria, M'Sila region) *Marrubium* plants are used in folk medicine to treat coughs and also as antispasmodics in acute and chronic bronchitis to treat asthma and in general for respiratory infections. They are also used for the treatment of anorexia and dyspepsia. The species *M. vulgare* (white horehound or common horehound, locally named "Marriout"), the representative of the genus *Marrubium*, is widely distributed in the area of M'Sila. This plant is currently used by traditional healers, alone or in combination with other herbs such as elecampane (*Inula helenium L.*) and licorice (*Glycyrrhiza glabra L.*) to treat bronchitis, coughs, and colds. The leaves and young flowering stems are used as antiseptic, antispasmodic, antidiabetic, diuretic, and for preparing expectorants, and tonics [26].

The *in vitro* antiprotozoal activity of several Turkish *Lamiaceae* revealed a significant activity for *Marrubium* extracts [27]. In our studies, among the three *Marrubium* species tested for antileishmanial activity, *M. deserti* and *M. vulgare* exhibited a significant effect. The labdane diterpenoid and methoxylated flavones reported from the lipophilic extract of *Marrubium* species could be responsible for the antileishmanial activity of *Marrubium* species on *L. major* and *L. infantum* [25, 27, 28].

Our results also showed that the extracts from some plants, such as *E. arborea and A. herba- alba* were more effective on *L. major* than *L. infantum*. This is the first report on the antileishmanial activity of *E. arborea* flower. This plant is used in folk medicine to treat nocturnal enuresis, urolithiasis, and hypertension [19, 29]. Moreover, the leaves

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of *E. arborea* from Morocco contains some bioactive compounds of antioxidant and anti-inflammatory properties, which can have an antileishmanial effect as well [29, 30].

In our study, *A. herba alba* had more effect on *L. major* than *L. infantum*. Crude extracts from *Asteraceae* plants, in particular of the genus *Artemisia*, were previously investigated against protozoan parasites. The well-known and potent antimalarial drug, artemisinin, which originates from the Chinese herb *Artemisia annua*, is currently used as an artemisinin combination therapy for treating malaria [31, 32]. *Artemisia* is a heterogeneous genus consisting of more than 500 diverse species occurring in the temperate areas throughout the world but mainly in Europe, Asia, and North America [33]. Traditionally, *A. herba-alba* is used for the treatment of a variety of ailments such as cold, diabetes, and bronchitis and gastrointestinal diseases. Essential oils from a wide range of *Artemisia* species and some of their components have shown to possess ethnopharmacological properties, particularly against infectious diseases [34]. Artemisia plants contain chemical components such as sesquiterpenes, monoterpenes lactones, flavonoids, coumarins, sterols, and polyacetylenes [35]. Previously, a preliminary study described the antileishmanial activity of *A. herba-alba* essential oil from Morocco with an IC_{50} of 2 μg/mL against *L. major* [36]. However, essential oil of *A. herba-alba* and *A. campestris* from Tunisia inhibited *L. infantum* promastigotes growth in a dose-dependent manner with IC_{50} values of 68 μg/mL and 44 μg/mL, respectively. The antileishmanial activity of both products is mediated by cell apoptosis induction and cell cycle arrest at the sub-G0/G1 phase [37].

The evaluation of the antileishmanial activity of seven plant extracts from two areas Mitidja and M'Sila of Algeria against *L. major* and *L. infantum* revealed that four extracts had promising antileishmanial phytochemical constituents. In particular, the extract of *M. vulgare* was the most promising and should be further investigated using Bioactivity-guided isolation of the active constituents. Moreover, the evaluation of antileishmanial activity confirms the ethnobotanical usages of some plant extracts.

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CONFLICT OF INTEREST

The authors declare that there are no issues regarding this article to be perceived as a conflict of interest.

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