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Short Communication

Antileishmanial Potential of Crude Plant Extracts Derived from Medicinal Plants in Palestine

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Abstract

Herbal and traditional medicine is commonly and widely used in Palestine. There has been no ethno pharmacological study to document the usefulness of traditional or medicinal plants from Palestine against leishmaniasis, a spectrum of severe parasitic diseases that occur worldwide and is caused by protozoa of the genus *Leishmania*. The aim of the present study was to collect and analyze some of the traditionally used medicinal plants from Palestine against *Leishmania major* parasites that cause cutaneous leishmaniasis.

Plant materials were collected during spring and summer of the year 2011, identified and the voucher numbers were kept at Al-Quds University Gardens (AQUG). The whole plant (except roots), flowers, fruits or seeds were collected, washed with distilled water, air dried in the shade for 20 days and then powdered in an electric grinder. For each plant species, alcoholic and dimethyl sulfoxide extracts were tested *in vitro* against *L. major* promastigotes and their antileishmanial activities were evaluated by Alamar Blue bioassay.

Twenty plant species belonging to 14 families were examined for their *in vitro* anti-parasitic effect against *L. major*. Among the total crude extracts tested; five were found to have various levels of activities (20%), some extracts having significant antileishmanial activity with IC₅₀ values ranging from 8.83 to 100 µg/mL. The most active crude extracts were from the shoots of *Artemisia inculta* and *Malva sylvestris* with activity of 84.1%, IC₅₀ = 8.8 µg/mL. And 90.1%, IC₅₀ = 19.5 µg/mL respectively.

The results demonstrate that the crude extracts of *Artemisia inculta* and *Malva sylvestris* showed promising antileishmanial activity, further and extensive studies should be carried out; particularly bio-guided fractionation to identify the active fraction and further chemical characterization of structure

ABBREVIATIONS

AQUG: Al-Quds University Gardens; DMSO: Dimethyl Sulfoxide; EtOH: Ethanol; FBS: Fetal Bovine Serum; PBS: Phosphate Buffered Saline; IC₅₀: 50% Inhibitory Concentration

INTRODUCTION

Through much of human history, plants have been used in medical treatments; such traditional medicine is still widely practiced today. Moreover, a huge number of novel drug components have been isolated from natural plant sources, where many of these plants and their extracts were used in traditional medicine [1,2]. This natural secretes, in the form of herbal remedies, could be explored based on information collected from local residents and traditional practitioners in different parts of the world [3-5]. Investigations carried out on folk medicinal plants with a potential of being curative have provided many clinical drugs for various infectious diseases. It has been reported that many plant-derived compounds

regarded as important drugs currently in use, the majority of these compounds were derived from traditional medicines [6-9]. Leishmaniasis is a disease caused by different species of *Leishmania* parasites and is transmitted by the bite of a female phlebotomine sand fly. Depending on the parasite species, three different forms of the disease existed, ranging from the mild self-limiting cutaneous form, but causing disfigurement of the skin and lifelong scars, to more severe and life threatening mucocutaneous and visceral forms. Leishmaniasis is a disease with a large worldwide distribution, endemic in 98 countries where about 1/3 of the cutaneous cases occur in the Americas, the Mediterranean basin and western Asia with 70-75% of the cases registered in Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, Costa Rica, Peru and northern Sudan [10]. Incidence of the disease is increasing worldwide due to the expansion of international travel, especially among countries that are at war and/or where there are no effective vaccines for humans [11,12]. Additional problems are the emergence of strains resistant to

first-line drugs as well as increasing cases of co-infection with HIV/AIDS [13-16].

Chemotherapy of parasitic diseases including leishmaniasis is still challenging, the drug efficacy is mostly limited by the inability of the pharmaceuticals to reach its target in a sufficient concentration and for a sufficient duration. The available drugs currently in use for the treatment of leishmaniasis include the followings: pentavalent antimonials; N-methylglucamine and antimoniate are considered the first-line treatment and, as a second option, amphotericin B or pentamidine. However, these drugs are disadvantaged by emergence of resistant parasites, parenteral administration, lethal side effects, high price and low availability especially in low-income and developing countries. The absence of vaccines and other effective prophylactic measures indicates the need for new therapies against leishmaniasis to cure people in endemic areas. Natural products of plant origin are potential preparations have been used for centuries to treat empirically parasitic diseases including leishmaniasis for people around the world which stimulating clinical and laboratory research [17,18].

Palestine is distinguished for its availability of medicinal plants because of the unique geographical location and biodiversity; these plants have been used for a long period of time to treat various illnesses. The Palestinian mountains are rich in plant species; about 2953 species are found with more than 700 species being mentioned in published ethnobotanical data as medicinal herbs or as botanical pesticides [19-21].

In the present study, we investigated the *in vitro* antileishmanial activity of crude extracts from 20 medicinal plants from various regions in Palestine. These plants were used by the local people to treat many infectious diseases and have never evaluated for their activity against *Leishmania* parasites

MATERIALS AND METHODS

Selection and collection of plant materials

The list of medicinal plants in Palestine was reviewed with the aid of local traditional practitioners and botanists. Literature survey has been performed for specific reports on traditional, medical and therapeutic importance of Palestinian medicinal plants. Plant names were selected based on information available in the literature about their anti-leishmanial, anti-protozoal, anti-parasitic, antimicrobial or anti-oxidant activities, other plants were selected based on their uses in the Palestinian traditional medicine to treat bacterial, fungal or parasitic infections. Plant materials were collected during spring and summer of the year 2011, identified by a botanist and the voucher numbers were kept at Al-Quds University Gardens (AQUG) and available upon request. Based on the traditional reports on the plant part used medicinally [17,22,23], the whole plant (except roots), flowers, fruits or seeds were collected, washed with distilled water, air dried in the shade for 20 days and then powdered in an electric grinder. The list of plant names used in this study can be found in Table (1).

Preparation of the crude extracts

Aqueous and organic extractions were done for each plant; each powdered plant material was extracted by maceration of

plant powder in absolute ethanol and dimethyl sulfoxide (DMSO) separately for 72 hours at room temperature with gentle shaking. The quantity of solvent used for each extraction was 10 times the quantity of plant material. The filtrate obtained through Whatman No. 1 filter paper was concentrated under reduced pressure in a rotary evaporator at 30 °C. The extraction yields were calculated and the plant crude materials were dissolved in their respective solvents to a concentration of 160 mg/mL. All crudes were kept at room temperature and protected from light until further processing.

Preparation of *Leishmania major* promastigotes

Promastigotes of *L. major* (1×10^6 parasites/well) were cultured in micro plates with 96 wells (Corning) containing Schneider's medium with 10% heat inactivated fetal bovine serum (FBS), 100 IU/mL Penicillin and 100 µg/mL streptomycin. Promastigotes were then washed 3 times with phosphate-buffered saline (PBS) by centrifugation at 1500 rpm for 10 min at room temperature.

In vitro test for anti-leishmanial activity

The *in vitro* test was performed using Alamar Blue bioassay [24] and it includes the following: cultured promastigotes at the log phase (1×10^6 parasites/mL) were seeded in 125 µl Schneider's medium in 96-well flat-bottom micro-plate, and then, 1 µl of each crude extract dissolved in DMSO and EtOH were mixed separately in 124 µl culture medium and transferred into the well. The final concentration of EtOH and DMSO was less than 0.1% (v/v) as this concentration will not affect the parasite growth rate, mobility and morphology. Amphotericin B (0.5 µg/mL) was used as drug positive control while parasites only in culture media were used as growth control. Negative control was cultured in media only. Each crude extract was tested in triplicate. The micro plate was incubated at 26°C in 5% CO₂ for 24 h in which 10% Alamar Blue (Sigma) was added to each well and the plates were incubated at 26°C for another 24 h. Optical density values (test wavelength 450 nm; reference wavelength 630 nm) were measured using a micro plate reader. The decrease of fluorescence (which indicated inhibition) was expressed as the percentage of the fluorescence of the control cultures.

Determination of the 50% effective concentration (IC₅₀)

The IC₅₀ values at the 95% confidence interval were calculated using sigmoid dose-response curves (Graph Pad Prism version 5.01 software Inc., San Diego, CA)

Phytochemical analysis

The crude extracts that have anti-leishmanial activity were screened for the presence of different phytochemicals; alkaloids, anthocyanins and betacyanin, quinones, flavonoids, phenols, saponins, tannins, sterols, triterpenoids, terpenoids and acids, following the standard methods of analysis [25-27]

RESULTS

Twenty plant species belonging to 14 families were examined for their *in vitro* antiparasitic effect against *L. major* using the Alamar Blue bioassay method. Local names in Palestine, their

scientific names and medicinal and traditional uses are listed in Table (1). Antileishmanial activities and IC₅₀ results are listed in Table (2). Among the total crude extracts tested; five were found to have various levels of activities (20%), some extracts having significant antileishmanial activity with IC₅₀ values ranging from 8.83 to 100 µg/mL. Extracts with IC₅₀ less than 100 µg/mL, were considered active. One prominent extract, *Artemisia inculta* of the ASTERACEAE (COMPOSITAE) family, was the most potent (activity 84.1% and IC₅₀ = 8.8 ± 2.3 µg/mL). This was considered as promising activity, as shown in Table (2). Others have moderate to very little activities with IC₅₀ values between 19.5 - 100 µg/mL., these include extracts of *Malva sylvestris* of MALVACEAE family

with leishmanicidal activity of 90.1% and IC₅₀ of 19.5 ± 16.3 µg/mL (Table 2). On the other hand, three plant extracts including *Trigonella berythea*, *Carthamus tinctorius*, and *Paronychia argentea* showed very low or even negligible activities with IC₅₀ values between 37.01 - 77.84 µg/mL. Three plant extracts, *Sinapis arvensis*, *Crataegus aronia*, and *Calotropis procera* may be considered inactive with IC₅₀ > 100 µg/mL. Twelve plant extracts including *Coridothymus capitatus*, *Arum palaestinum*, *Carum carvi*, *Dittrichia viscosa*, *Punica granatum*, *Rosmarinus officinalis*, *Nigella ciliaris*, *Hibiscus sabdariffa*, *Ficus carica*, *Citrullus colocynthis*, *Origanum majorana*, and *Pimpinella anisum* were found inactive.

Table 1: Selected plant species used in this study with scientific and popular names, plant parts used and their medical importance.

No	Plant scientific name	English name	Parts used	AQUG voucher number	Medical importance	Antimicrobial activities Reference
1	<i>Artemisia inculta</i> Delile	White Wormwood	Shoots	PS-Ai10	Anti- <i>Helicobacter pylori</i>	[40]
2	<i>Coridothymus capitatus</i> (L.) Rchb.f.	Capitate Thyme	Leaves	PS-Cc19	Antioxidant	[41]
3	<i>Sinapis arvensis</i> L.	Mustard/wild	Shoots	PS-Sa11	Antibacterial	[19]
4	<i>Arum palaestinum</i> Boiss	Arum	Leaves	PS-Ap20	Anticancer	[42]
5	<i>Malva sylvestris</i> L.	Common Mallow	Leaves	PS-Ms50	Antileishmanial, Antibacterial, Anti-inflammatory	[43]
6	<i>Carum carvi</i> L.	Caraway	Seeds	PS-Cc12	Treatment of Gastrointestinal disorders, increase flow of breast milk	[44]
7	<i>Trigonella berythea</i> Boiss. & Blanche	Fenugreek	Seeds	PS-Tb21	Anti-diabetic activity	[45]
8	<i>Carthamus tinctorius</i> L.	Safflower	Flowers	PS-Ct33	Treatment of dysmenorrhea, amenorrhea, postpartum abdominal pain.	[46]
9	<i>Paronychia argentea</i> Lam.	Silvery Whitlow	Flowers	PS-Pa15	Aphrodisiac, Diuretic	[47]
10	<i>Dittrichia viscosa</i> (L) Greuter	Inula	Leaves	PS-Dv22	Antidiabetic, antiphlogistic, antiviral, antifungal, antibacterial, and antiseptic properties	[48]
11	<i>Crataegus aronia</i> (L) Bosc ex DC	Hawthorn, Azarole	Leaves	PS-Ca55	Antioxidant, treatment of cardiovascular diseases	[49]
12	<i>Punica granatum</i> L.	Pomegrante	Peel	PS-Pg70	Anticancer, anti-inflammatory, treatment of Osteoarthritis, traditional remedies against diarrhea, dysentery and intestinal parasites.	[50]
			Seeds		Antidiarrhoeal activity, antiparasitic, antioxidant	[4]
13	<i>Rosmarinus officinalis</i> L.	Rosemary	Shoots	PS-Ro80	Anti-inflammatory, antitumor, antioxidant, antimicrobial, Antileishmanial, and Antitrypanosomal	[51]
14	<i>Nigella ciliaris</i> DC.	Black cumin	Seeds	PS-Nc30	Anticancer, anti-oxidant	[21]
15	<i>Hibiscus sabdariffa</i> L.	Roselle	Flowers	PS-Hs17	Treatment of Melanoma	[52]
16	<i>Ficus carica</i> L.	Fig tree	Leaves	PS-Fc40	Antimicrobial, antifungal, antioxidant, antiviral, anti-inflammatory	[53]
			White sap			
17	<i>Citrullus colocynthis</i> (L) Schrader	Colocynth	Leaves	PS-Cc18	Antidiabetic	[54]
			Fruits		antimicrobial	[55]
18	<i>Origanum majorana</i> L.	Sweet-Marjoram	Leaves	PS-Om60	Antimicrobial, antioxidant	[56]
19	<i>Calotropis procera</i> (Aiton) Dryand.	Apple of Sodom	Leaves	PS-Cp22	Anti-inflammatory	[57]
20	<i>Pimpinella anisum</i> L.	Anis	Seeds	PS-Pa02	Antiviral, antioxidant, muscle relaxant, analgesic and anticonvulsant activity, hypoglycemic and hypolipidemic effect	[58]

Abbreviations: AQUG: Al-Quds University Gardens; PS: Palestine

Table 2: *In vitro* antileishmanial activity and cytotoxicity of the plant extracts used in this study.

No.	Plant Scientific name	Family name	AQUG voucher number	Solvent ^(a)	Parts used	Antileishmanial activity	
						^(b) Activity (%)	IC50, µg/ml Average ± Standard Deviation
1	<i>Artemisia inculata</i> Delile	ASTERACEAE (COMPOSITAE)	PS-Ai10	DMSO	Shoots	84.1	8.83± 2.3
2	<i>Coridothymus capitatus</i> (L.) Rchb.f.	LAMIACEAE (LABIATAE)	PS-Cc19		Leaves		
3	<i>Sinapis arvensis</i> L.	BRASSICACEAE (CRUCIFERAE)	PS-Sa11	Ethanol	Shoots	20	>100
4	<i>Arum palaestinum</i> Boiss	ARACEAE	PS-Ap20		Leaves		
5	<i>Malva sylvestris</i> L.	MALVACEAE	PS-Ms50	DMSO	Leaves	90.1	19.50±16.3
6	<i>Carum carvi</i> L.	APIACEAE (UMBELLIFERAE)	PS-Cc12		Seeds		
7	<i>Trigonella berythea</i> Boiss. & Blanche	FABACEAE (LEGUMINOSAE)	PS-Tb21	Ethanol	Seeds	30	77.84± 46.2
8	<i>Carthamus tinctorius</i> L.	ASTERACEAE (COMPOSITAE)	PS-Ct33	DMSO	Flowers	82.1	37.01± 0.001
9	<i>Paronychia argentea</i> Lam	CARYOPHYLLACEAE	PS-Pa15	DMSO	Shoots	74.5	77.80± 46.3
10	<i>Dittrichia viscosa</i> (L.) Greuter	ASTERACEAE (COMPOSITAE)	PS-Dv22		Leaves		
11	<i>Crataegus aronia</i> (L.) Bosc ex DC	ROSACEAE	PS-Ca55	DMSO	Leaves	31.9	>100
12	<i>Punica granatum</i> L.	PUNICACEAE	PS-Pg70		Peel		
					Fruits		
13	<i>Rosmarinus officinalis</i> L.	LAMIACEAE (LABIATAE)	PS-Ro80		Shoots		
14	<i>Nigella ciliaris</i> DC.	RANUNCULACEAE	PS-Nc30		Seeds		
15	<i>Hibiscus sabdariffa</i> L.	MALVACEAE	PS-Hs17		Leaves		
16	<i>Ficus carica</i> L.	MORACEAE	PS-Fc40		Leaves		
					White sap		
17	<i>Citrullus colocynthis</i> (L.) Schrader	CUCURBITACEAE	PS-Cc18		Leaves		
					Fruits		
18	<i>Origanum majorana</i> L.	LAMIACEAE (LABIATAE)	PS-Om60		Leaves		
19	<i>Calotropis procera</i> (Aiton) Dryand.	APOCYNACEAE	PS-Cp22	Ethanol	Leaves	3	>100
20	<i>Pimpinella anisum</i> L.	APIACEAE (UMBELLIFERAE)	PS-Pa02		Seeds		

Abbreviations: AQUG: Al-Quds University Gardens; PS: Palestine; DMSO: Dimethyl Sulfoxide

Among the active extracts, four were extracted with DMSO and only one with ethanol. Therefore, DMSO extracts were generally more active than the ethanol ones. The results of the qualitative phytochemical analysis of the five active extracts showed that anthocyanins and betacyanins are found in only one extract, *Paronychia argentea*, which also contains phenols, saponins, tannins, and acids. Flavonoids were found only in the most active extract, *Artemisia inculata*, which also contain phenols, saponins, and triterpenoids. *Trigonella berythea* contains saponins, tannins, terpenoids, and acids. *Carthamus tinctorius* which has moderate activity contains saponins, tannins, terpenoids, and acids. Although none of the studied extracts contained alkaloids, quinones, or sterols, each of the studied plant extract contained at least two classes of secondary metabolites. The detailed phytochemical composition is shown in Table (3). The presence of these phytoconstituents is thought to be responsible for antileishmanial activity

DISCUSSION

Medicinal plants have been known throughout history as most appropriate sources of active chemicals and their derivatives to be used as templates for designing and developing

more effective compounds, preferably with fewer side effects. Most plants that have medicinal properties in Palestine have not yet been thoroughly evaluated for their biological activities. *In vitro* screenings of various medicinal plants currently used in traditional medicine are essential and important first steps to prove the efficacy and safety of these plants in the treatment of infectious diseases, especially leishmaniasis, in poor and developing countries.

This study is designed to obtain preliminary results on the antileishmanial effects of selected medicinal plants from Palestine on *L. major*. Our results strongly suggest that *Artemisia inculata* and *Malva sylvestris* could be promising for treatment of leishmaniasis demanding a search for new chemotherapeutic agents. However, further studies need to be carried out, in order to isolate, purify and characterize active ingredients in pure forms and to understand the mechanisms of action and to evaluate the highly active crudes for further drug development. Toxicity against human cells should be done once active materials have been purified. The cytotoxicity was not carried out at this level for two reasons; firstly the plants that showed activity against *Leishmania* promastigotes are edible and traditionally used as medicinal plants for the treatment of various illnesses, and

Table 3: Phytochemical composition of the five active plant extracts.

Plant sample	Phytochemical composition										
	Alka- loids	Anthocy- anines & Betacyanin	Qui- nones	Flavo- noids	Phe- nols	Sapon- ins	Tan- nins	Sterols	Triterpe- noids	Terpe- noids	Acids
<i>Artemisia inculta</i> Delile				+	+	+			+		
<i>Malva sylvestris</i> L											
<i>Trigonella berythea</i> - Boiss. & Blanche						+	+				+
<i>Carthamus tinctorius</i> L						+				+	+
<i>Paronychia argentea</i> Lam		+			+	+	+				+

secondly, the extracts are solely in crude and not in pure form and the active ingredient that confers toxicity for *Leishmania* is mixed up with many others that may have toxicity against human cells; therefore, cytotoxicity tests at this level may lead to exclusion of active crude extracts that may have toxic ingredients. The most active extracts come from the use of DMSO as extraction solvent, suggesting that the less polar compounds are responsible for the observed activity. Plants found active were also extracted with dichloromethane with almost the same activity, however dichloromethane is recommended since it has a boiling temperature much less than DMSO and hence easily evaporated and removed.

Natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans and other parasites [28-30]. The most active extract, *Artemisia inculta*, contains polyphenolic compounds like flavonoids and triterpenoids (Table 3) which are believed to have the ability to inhibit trypanosomal and leishmanial infections without significant toxicity to mammalian cells [31-33]. Different *Artemisia* species have been reported to have *in vitro* and *in vivo* activity against various *Leishmania* species; *Artemisia annua* leaves extract was proved to have leishmanicidal activity against *L. donovani* which causes visceral leishmaniasis [34,35]. The second promising plant is *Malva sylvestris*, reported in this study to have antileishmanial activity for the first time; it is an edible plant and people in Palestine used it extensively.

The activity of these plants is believed to be structure-dependent, according to literature flavonoids bind to C-terminal nucleotide-binding domain (NBD2) of the P-glycoprotein-like transporter in *L. tropica* which is involved in parasite multidrug resistance [36,37]. Flavonoids also inhibit important enzymes or proteins; flavonoids quercetin inhibits DNA topoisomerases, promoting site specific DNA cleavage resulting in the growth inhibition of *L. donovani* promastigotes and amastigotes [38]. In other parasites, flavonoids inhibit the synthesis of heat shock proteins (Hsp90, Hsp70, and Hsp27); these factors are important to protect virulent parasites from the effects of host immune responses [39].

Based on susceptibility tests using Alamar Blue Bioassay, phytochemicals are routinely evaluated for antileishmanial activity. For crude extracts, activity is considered to be significant if IC₅₀ values are below 20 µg/mL and moderate when 20 < IC₅₀ < 100 µg/mL. Therefore, the activity recorded with *Artemisia*

inculta, *Malva sylvestris*, *Coridothymus capitatus*, *Trigonella berythea*, *Carthamus tinctorius*, and *Paronychia argentea* against *L. major* can be considered important. Previous reports documented leishmanicidal activity of *Artemisia* species from Iran against *L. major* promastigotes [35]. The genus *Artemisia* L. (*Astraceae*) is a large, heterogeneous and widely distributed throughout the world. These species are perennial, biennial and annual herbs or small shrubs.

There was no toxicity mentioned in the literature or concerns about the use of these medicinal plants. The samples were not assayed on intracellular amastigote forms, which should be done the next step of evaluation and validation of these plants. Further analysis still to be done on the active crudes; bio-guided fractionation should also be conducted and may lead to the isolation of the major components in the active crude.

CONCLUSION

Our study investigated twenty selected crude plant extracts and their antileishmanial activity. Among them, *Artemisia inculta* and *Malva sylvestris* exhibited promising results that may lead to the development of effective and affordable antileishmanial drugs. In developing countries, these results provide an alternative way to use plant-based remedies that might be safer, cheaper, and less toxic than existing prescription medicines. This is an area rich in possibilities, and the world's flora represents an enormous source of material for testing. However, further studies are needed, particularly bio-guided fractionation to identify the active fraction and further chemical characterization of structure. This research belongs to the global effort carried by researchers around the world to locate compounds with antileishmanial activity by validating natural products as genuine sources for drug discovery

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