In vitro and *in vivo* activities of *Haplophyllum myrtifolium* against *Leishmania tropica*

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SUMMARY

This study aimed to evaluate the *in vitro* and *in vivo* leishmanicidal activity of an endemic Turkish plant and compare its efficacy with a reference drug. In addition to the *in vitro* activities of the ethanol, acidified and alkaloid extracts and furoquinoline alkaloids skimmianine and γ-fagarine, *in vivo* antileishmanial activitiy of the acidified extract of *Haplophyllum myrtifolium* Boiss. (Rutaceae) were investigated against *Leishmania tropica* (*L. tropica*), a causative agent of cutaneous leishmaniasis. All the extracts and pure compounds showed *in vitro* inhibitory activity against the promastigotes of. *L. tropica*. The *in vitro* 50% inhibitory concentrations of γ-fagarine, acidified extract, ethanol extract, skimmianine and alkaloid extract against promastigotes were determined as 8.7, 9.4, 10.9, 25.7 and 25.8 μg/ml respectively. *In vivo* results of *Haplophyllum myrtifolium* acidified extract showed that this plant has a limited effect on decreasing the lesion size of experimental mice infected with *Leishmania tropica*. To the best of our knowledge, this is the first time both the *in vitro* and *in vivo* antileishmanial activity of *Haplophyllum mrytifolium* have been reported in the same research.

KEY WORDS: Antileishmanial activity, Experimental leishmaniasis, *Haplophyllum myrtifolium, Leishmania tropica*, Plant extract

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INTRODUCTION

Leishmaniasis is an important global public health problem. The most common transmission of the *Leishmania* parasite occurs through the bites of the infected female sand flies. Transmission is also possible by parenteral, sexual and occupational (contaminated needle) routes or by blood transfusion (Otero *et al.,* 2000, Singh, 2006a). The clinical forms of leishmaniasis are visceral, cutaneous, mucocutaneous and post kala azar dermal leishmaniasis (Mandell *et al.,* 1998, Singh 2006b). The cutaneous leishmaniasis (CL) and sporadic cases of visceral leishmaniasis (VL) have been widely observed in southeastern Anatolia, the Mediterranean and recently Aegean regions of Turkey (Ok *et al.,* 2002).

The invasion capability of the parasite into the mononuclear phagocytic system cells of the host makes the treatment of leishmaniasis quite difficult. Since there is no vaccine for this parasite, there is an urgent need for effective drugs to replace those in current use. The first choice of drugs in the treatment are the pentavalent antimonials, such as sodium stibogluconate and meglumine antimonate (Rocha *et al.,* 2003). The parasite has gained resistance to these drugs due to

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their prolonged use in certain regions of the world (Singh 2006a). Furthermore, parasites were reported to be isolated from the cutaneous scars years after antimonial therapy and clinical cure (Schubach *et al.,* 1998). In addition, both the first choice of treatment drugs and alternative drugs, such as amphotericin B and pentamidine, have unpleasant side effects that include pain at the site of injection, gastrointestinal problems, stiff joints, cardiotoxicity, and in some cases, hepatic and renal insufficiency (Chan-Bacab *et al.,* 2001). Therefore researchers have directed their attention to the use of natural products, especially those of plant derivatives.

A plant-screening program based on the ethnomedical knowledge of the local population for potential leishmanicides was iniated by 1984 in French Guiana (Rocha *et al.,* 2005). Essential oil from *Croton cajucara* (Rosa *et al.,* 2003), a coumarin from *Esenbeckia febrifuga* (Napolitano *et al.,* 2004), bacterial diterpenes from *Premna schimperi* and *P. oligotricha* (Habtemariam, 2003) and parthenolide from the *Tanacetum parthenium* (Tiuman *et al.,* 2005) are several plant products with different chemical structures that display antileishmanial activity. Leishmanicidal activity of natural products including several ferns and *Betula* constituents were examined and several pterosin and atisene compounds were found to be active (Takahashi *et al.,* 2004).

The seventy species of *Haplophyllum* are spread from the Mediterranean to Eastern Siberia and these species have been shown to contain many secondary metabolites such as alkaloids, coumarins, lignans, and flavonoids with a large spectrum of biological activities (Joseph, 2005, Sağlam *et al.,* 2003). They have a broad spectrum of pharmacological action and are used in Sudanese and Mongolian folk medicine as antipyretics and for the treatment of diarrhea (Ali *et al.,* 1992). Thus, in the present study, we investigated the potential leishmanicidal effect of *H. myrtifolium*, an endemic plant of Turkey.

MATERIAL AND METHODS

Plant material

Haplophyllum myrtifolium Boiss. was collected from the vicinity of Honaz, Denizli located in the Aegean region of Turkey on June 11, 1993. The plant was identified by M.A. Önür (Ege University, Faculty of Pharmacy, Department of Pharmacognosy, Izmir, Turkey). A voucher specimen, No 1151, was deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Ege University.

Preparation of the plant extracts

The extracts were obtained from both the aerial and underground parts of the plant. The dried and finely powdered plant material (33.2 kg) was dissolved in ethanol at room temperature. The solvent was evaporated to yield the ethanol extract (2.684 kg). The dark syrupy residue was acidified with 2% hydrochloric acid and filtered. The filtrate was basified to pH 9-10 with 25% aqueous ammonium hydroxide and extracted with chloroform. Evaporation of the organic solvent *in vacuo* furnished 76.08 g of the alkaloid extract. The aqueous part was then acidified with 2% hydrochloric and extracted with chloroform to obtain the acidified extract (110 g).

As previously described (Sağlam *et al.,* 2003), the alkaloid extract was subjected to fractionation and the known furoquinoline alkaloids γ-fagarine (238 mg) and skimmianine (13.8 mg) were obtained by crystallization from methanol. The compounds were identified with the aid of the determination of thin-layer chromatography, UV, NMR and mass spectroscopy. In the mass spectrum of skimmianine (4,7,8-trimethoxyfuro [2,3-b]quinoline) the $[M]^+$ at m/z 259, indicated the molecular formula $C_{14}H_{13}O_4N$ whereas the $[M]^+$ at m/z 229 for γ-fagarine indicated the molecular formula as (4,8-dimethoxyfuro[2,3-b]quinoline) $C_{13}H_{11}O_3N$.

The extracts and pure compounds were kept under -80ºC until analysis.

Cultivation of parasites

L. tropica promastigotes (Lab code: EP127) were isolated from a patient with cutaneous leishmaniasis in Aydın-Turkey. The promastigotes were grown in NNN medium and then cultured in RPMI-1640 medium (Biochrom AG) supplemented with 10% fetal calf serum (FCS).

Antileishmanial assays

25, 50, 250 and 500 µg/ml concentrations of ethanol, acidified and alkaloid extracts and the pure compounds skimmianine (0.0965-1.9305 µ-

mol/ml) and γ-fagarine (0.1092-2.1834 µmol/ml) were prepared for *in vitro* experiments. The extracts were dissolved in dimethyl sulfoxide (DMSO) and diluted in RPMI medium containing 10% FCS. The final volume was adjusted to 2000 µl with RPMI medium for each well of a 24-well microplate. In all experiments, the final concentration of DMSO was less than 0.5% (v/v) as this concentration does not affect parasite growth rate, mobility or morphology (Zhai *et al.,* 1999). After haemocytometer counting, promastigotes were suspended to yield $1x10^6$ cells/ml in each well. As a reference drug, a pentavalent antimonial compound, glucantime was prepared in sterile DMSO at 20 µg/ml concentration. The highest concentration of DMSO and RPMI medium were also used for control groups. Microplates were incubated at 25°C. The number of parasites were counted with a haemocytometer under a light microscope after 6, 12, 24 and 48 hours. In order to keep in line with previous works in the same field, 48 hours were chosen to express the effect of the extracts and compounds (Napolitano *et al.,* 2004, Takahaski *et al.,* 2004, Navarro *et al.,* 2003). All the *in vitro* experiments were run in triplicate and the results were expressed as the percent inhibition in parasite number. The drug concentration required for 50% inhibition *in vitro* (IC_{50}) was calculated with a parametric statistical procedure (Finney Probit Analysis Programme) with the associated 95% confidence interval (Finney, 1971).

Animals and experimental infection

Male BALB/c mice obtained from Ege University Experimental Animals Center, were bred and raised in conventional animal housing. The 8-16 weeks old mice weighed 20-25 g. Promastigotes were cultured in RPMI-1640 medium (Biochrom AG) supplemented with 10% FCS and collected on the 14^{th} day of the culture. 10 ml culture fluid was centrifuged and a final dilution of $1x10^8$ promastigotes/ml was prepared. 20 µl of the promastigote solution was injected subcutaneously into the left hind footpads of the mice (Girginkardeşler *et al.,* 2001, Souza-Neto *et al.,* 2004). The development of the lesion was measured with a dial micrometer weekly during the course of infection and expressed as the difference in size between the infected footpads and uninfected footpads. The needle aspiration samples of infected lesions before and after treatment were stained with Giemsa and examined under oil immersion with a light microscope.

Drug treatment

The infection was well established and lesions were obvious. Eight weeks after the inoculation, the treatment was initiated. Glucantime was used as a reference drug at a dose of 28 mg/kg per mouse intralesionally (n=4). The acidified extract of *H. myrtifolium,* which has the highest activity in *in vitro* studies, was administered intralesionally at a dose of 50 mg/kg per mouse (n=4) (Fournet *et al.*, 1996). As a control, sterile saline was administered to animals (n=2). 40 µl of the extract and sterile saline injections were done five times at intervals of 5 days, by a tuberculin injector.

RESULTS

In vitro antileishmanial effect of H. myrtifolium

The extracts and the two furoquinoline alkaloids skimmianine and γ-fagarine showed significant inhibitor activity against the promastigotes of *L.*

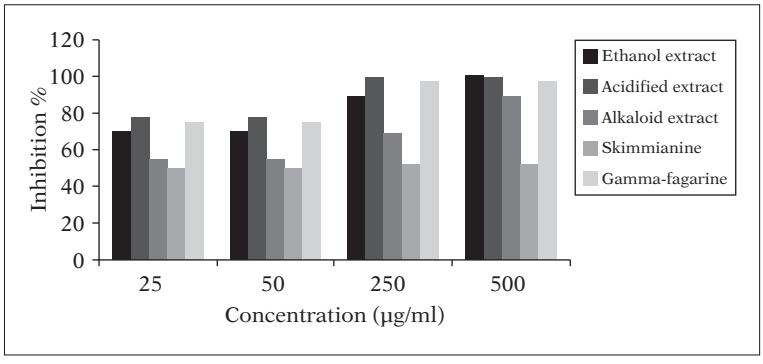

FIGURE 1 - *The comparison of the in vitro inhibition percentages.*

tropica (Figure 1). Even with a concentration of 25 µg/ml, all the extracts and pure compounds had an inhibition higher than 50%. Table 1 shows that the IC_{50} of extracts and pure compounds ranged between 8.7 to 25.8 µg/ml. γ-fagarine was found to be a more active than skimmianine. The acidified extract showed the highest antileishmanial activity of 70% at 25 µg/ml. The alkaloid extract was the weakest one showing 55% inhibition at 25 µg/ml. The ethanol extract was the only one possessing 100% inhibition at 500 µg/ml. The alkaloid extract and skimmianine were found to possess similar IC_{50} values as 25.8 and 25.7 respectively. DMSO and RPMI-culture controls were found to be inactive in all experiments. The reference drug glucantime was found to have 100% inhibition after 48 hours.

In vivo antileishmanial effect of H.mrytifolium

Cutaneous ulcers as swelling, tightness and redness were observed in all mice in 4-12 weeks after the infection. There was no sloping and crustaceous wound formation or any second lesion in various parts of the body except the infected left footpads of mice. The amastigotes were seen in smear preparations of cutaneous ulcers of all mice. On the 10^{th} day of treatment, the footpad swelling sizes of animals were found to have decreased and to have become softer in groups treated with the reference drug and *Haplophyllum* extract compared to the control group. On the 20^{th} day of treatment, the sizes decreased more in animals treated with glucantime, but there was no significant change in the plant extract injected group.

At the end of the treatment, the sizes of infected and uninfected footpads of mice were the same in glucantime treated animals. In animals treated with *Haplophyllum* extract, the decrease in lesion sizes was smaller than in animals treated with glucantime, and there was only an inhibition at the beginning of the treatment period during the first two weeks.

However, the lesions of the control mice which

TABLE 1 - *In vitro efficacy of Haplophyllum myrtifolium extracts and pure compounds.*

Sample	*(IC_{50})* (µg/ml)*	*Doses (µg/ml)*	*Number of promastigotes ($x10^4$)*	*% Inhibition*
Ethanol extract	(10.9)	25	30	70
		50	32	68
		250	11	89
		500	0	100
Acidified extract	(9.4)	25	23	77
		50	23	77
		250	1	99
		500	1	99
Alkaloid extract	(25.8)	25	45	55
		50	46	54
		250	30	70
		500	10	90
Skimmianine	(25.7)	25	50	50
		50	50	50
		250	48	52
		500	49	51
Gamma-fagarine	(8.7)	25	25	75
		50	25	75
		250	3	97
		500	2	98

*IC_{50} values were calculated according to a parametric statistical procedure (Finney Probit Analysis Programme) with the associated 95 % confidence interval (Finney, 1971).

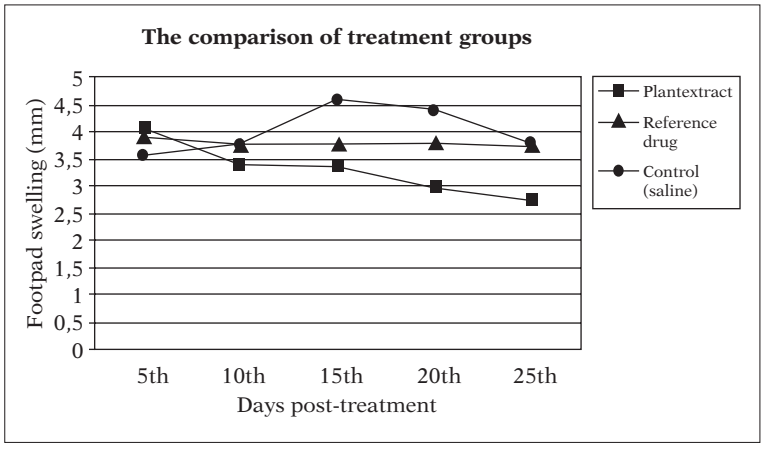

FIGURE 2 - *The treatment curves of experimental mouse groups* (*Reference drug, plant extract and saline were injected intralesionally five times at intervals of 5 days).*

received sterile saline continued to increase and self-healing was not observed in this mice group (Figure 2). One week after the last injection, there were no amastigotes in smear preparations of glucantime treated lesions, while there was no significant difference in amastigote counts of plant extract treated lesions between pretreatment and post treatment counts.

DISCUSSION

It is known that natural products have a future in the search for new and selective agents for the treatment of leishmaniasis. A major advantage of this product screening is the structural diversity of substances that could make natural products a source of novel lead compounds against leishmaniasis. Sixteen species of Turkish plants and a total of 58 extracts had promising biological activities particularly against *Leishmania* and *Plasmodium* (Taşdemir *et al.,* 2005). In addition, the activity of crude plant extracts of nine species of Rutaceae against the trypomastigote form of *Tyrpanosoma cruzi* was evaluated at 4 mg/ml (Mafezoli *et al.*, 2000).

Recently, 101 plants described in literature with antileishmanial activity were reviewed and three of them belonging to the Rutaceae family showed activity against *L. braziliensis, L. amazonensis and L. donovani* (Rocha *et al.,* 2003). Plants of the Rutaceae family showed 50% inhibition at a 100 µg/ml concentration on promastigote and amastigote of *Leishmania* parasites (Plock *et al.,* 1997). All these results directed our attention to *Haplophyllum*, a genus of the Rutaceae family which is widely available in a CL endemic region of Turkey.

The two quinolin-4-one alkaloids derived from the bark of *Dictyoloma peruviana* (Rutaceae) showed total lysis of the promastigotes of *L.amazonensis* but a minor activity on promastigotes of *L. braziliensis* at 100 µg/ml concentration (Chan-Bacab *et al.,* 2001). The 70% and 77% inhibition at the 25 µg/ml dose of ethanol and acidified extracts respectively are promising results to investigate this plant in further studies.

Diphyllin isolated from *Haplophyllum bucharicum* Litv. displayed a moderate antiproliferative activity towards *Leishmania* promastigotes (Di Giorgio *et al.,* 2005). Skimmianine and γ-fagarine isolated from *Haplophyllum myrtifolium* showed also significant activity in this study. In some experimental models, γ–fagarine isolated from *Ravenia spectabilis* showed mild to significant in vitro antibacterial activity (Sohrab *et al.,* 2004). Some evidence was offered that the sister chromatid exchanges (SCEs)-inducing activity of the tincture was mainly due to the presence of γ-fagarine (Schimmer *et al.*, 1989). Furthermore, γ-fagarine isolated from *Zanthoxylum* sp. (Rutaceae) exhibited strong *in vitro* activity against the malaria parasite, *Plasmodium falciparum* (Randrianarivelojosia *et al.,* 2003). γ-fagarine and skimmianine have been isolated from other sources of the Rutaceae family such as *Acronychia laurifolia* (Cui *et al.*, 1999), *Zanthoxylum dimorphopyllum* (Mai *et al.,* 2001) and *Dictamnus dasycarpus* (Du *et al.*, 2005). γ-fagarine with IC_{50} as 8.7 µg/ml was found to be the most active product in the present study. The examination of crude methanol extracts of three

Sudanese plants had revealed a considerable *in vitro* antileishmanial activity on *L. major* (Khalid FA *et al.,* 2005). Similarly, the ethanol extract of *H. myrtifolium* also presented a considerable activity against *L. tropica* in our study.

Most studies directed to the detection of secondary plant metabolites with leishmanicidal activity have been directed by using the promastigote form of the parasite because of its easier maintenance under *in vitro* conditions. As regards the *in vitro* antileishmanial activity of *Zanthoxylum* sp. (Rutaceae). it has been reported that intralesional administration of isolated products of this plant did not significantly reduce the parasite burden while the reference drug reduced the parasite loads in the lesion by 91% (Ferreira *et al.,* 2002). It is known that in some cases, intracellular amastigotes are more resistant and that the different forms of *Leishmania* parasites may show different activities in *in vivo* and *in vitro* test conditions.

In this study, when compared with the reference drug, it is concluded that the *in vitro* efficacy of *Haplophyllum* is hopeful, but the acidified extract has a limited *in vivo* activity at 50 mg/kg concentration. Further studies are planned to investigate higher doses and the other extracts and isolated compounds of *H. mrytifolium* on animals experimentally infected with *L. tropica*.

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