

Original Research Article

Antileishmanial Activity of Selected Turkish Medicinal Plants

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Abstract

Purpose: To determine the *in vitro* and *in vivo* anti-leishmanial activities of extracts obtained from *Centaurea calolepis*, *Phlomis lycia*, *Eryngium thorifolium*, *Origanum sipyleum* and *Galium incanum ssp. centrale*.

Methods: To estimate the cytotoxicity of plant extracts, WST-1 assay was used. Parasite inhibition in the presence of plant extracts (25 - 500 µg/ml) in comparison with control group and reference group (glucantime, 25 µg/ml) at 12 - 72 h were determined *in vitro* on *L. tropica* promastigotes. The *in vivo* leishmanicidal activity of the extracts was evaluated against *L. tropica*-infected mice with glucantime as reference drug.

Results: The chloroform extract of *Galium incanum ssp. centrale* showed the highest cytotoxicity with IC_{50} value of 0.0316 ± 0.005 µg/ml. *In vitro* parasite inhibition by the plant extracts ranged between 16.7 ± 0.01 % and 100 ± 0.00 % at 25 µg/ml concentration. The methanol extract of *Eryngium thorifolium* possessed the highest activity on promastigotes of *L. tropica* with 100 % inhibition at 25 µg/ml. The water and chloroform extracts of *C. calolepis* and water and methanol extracts of *E. thorifolium* at a dose of 100 mg/kg reduced parasitaemia in *L. tropica* infected mice.

Conclusion: Parasite viability results suggest that the methanol extract of *Eryngium thorifolium*, regarded as non-cytotoxic, is a promising candidate drug for treating *L. tropica* infection.

Keywords: Leishmaniasis, *Leishmania tropica*, *Eryngium thorifolium*, Promastigote, WI-38 Human fibroblasts

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INTRODUCTION

Leishmaniasis is a vector-borne disease affecting 400 million people around the world. This public health problem is endemic in 88 countries. After malaria, leishmaniasis is the most important parasitic disease caused by protozoan parasite

species from *Leishmania* genus [1]. Sodium stibogluconate and meglumine antimonite are the most favourable leishmanicidal agents. These pharmaceutical agents administered by injection usually cause pain at the site of injection. In addition to stiff joints and gastrointestinal disorders, they have also been

known to cause cardiotoxicity and severe hepatic and renal insufficiencies [2].

Natural herbal products have been used in the treatment of infectious disease for many years. The World Health Organisation (WHO) emphasized that plants used in traditional medicine should primarily be investigated against leishmaniasis [1]. All over the world, researchers started on this issue and successful results were achieved [3-5].

With this knowledge and as part of our outgoing studies on anti-parasitic activities of Turkish medicinal plants [6-8], in the present work, antileishmanial efficacies of extracts prepared from *Centaurea calolepis* Boiss., *Phlomis lycia* D. Don., *Eryngium thorifolium* Boiss., *Origanum sipyleum* L., *Galium incanum* ssp. *centrale* Ehrend. were examined on *Leishmania tropica* promastigote and amastigote forms. In addition to preliminary phytochemical screening analysis, cytotoxic activities of each plant extract were also determined by WST-1 cell proliferation assay.

EXPERIMENTAL

Plant material

The aerial parts of *Centaurea calolepis* Boiss., *Phlomis lycia*, *Eryngium thorifolium*, *Origanum sipyleum*, *Galium incanum* ssp. *centrale* were collected from various localities in Turkey. The plant species were identified by Dr. Cenk

Durmuskahya (Izmir Katip Celebi University, Faculty of Forestry, Department of Forest Engineering, Balatcik, Izmir Turkey) and voucher specimens were deposited in Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Turkey (The collection areas for the plants, voucher numbers, extracts and their antimicrobial or antiprotozoal effects as well as the phytochemical profile of the plants are listed in Table 1).

Preparation of plant extracts

The plant materials were ground into a fine powder after air-drying. The methanol and chloroform extracts were prepared by maceration under stirring for 48 h at 24 °C where the solvent/plant material ratio was 15:1. The water extracts were prepared by 2 % infusion and all the extraction solvents were filtered through Whatman filter paper no.1. The filtrates were evaporated to dryness under reduced pressure in a rotary evaporator at 40 °C. The residues were lyophilised and stored in screw capped vials at -20 °C until analysed.

Phytochemical analysis of plant extracts

Phytochemical screening tests for plant secondary metabolites such as tannins, terpenoids, saponins, flavonoids and alkaloids were conducted on the plant extracts as described by Trease and Evans [9] and Harborne [10].

Table 1: Medicinal plants investigated for their anti-leishmanial and cytotoxic activities

Plant name/ Family/ Voucher number	Collection area	Extract	Secondary metabolites antimicrobial/antiprotozoal activity	Reference
<i>Centaurea calolepis</i> Boiss./ Asteraceae/ 1447	Antalya, Elmalı, 1100 m	water, chloroform	Flavonoids, sesquiterpenes; antimicrobial, antiprotozoal	[11,12]
<i>Eryngium thorifolium</i> Boiss./ Umbellifera/ 1446	Muğla, Koycegiz, Kandil Mountain, 600 m	water, methanol	Flavonoids, saponins; Antiprotozoal	[13-15]
<i>Origanum sipyleum</i> L./ Labiatae /1445	Manisa, Spil Mountain, 1050 m	water, methanol	Triterpenes (ursolic acid, oleanolic acid); antimicrobial, antiprotozoal	[13,16,17]
<i>Galium incanum</i> SM. subsp. <i>centrale</i> Ehrend./Rubiaceae/ 1432	Izmir, Bozdağ, 1500 m	water, chloroform	Flavonoids, anthracenes, iridoids; antiprotozoal	[18,19]
<i>Phlomis lycia</i> D. Don/ Labiatae/ 1448	Antalya, Korkuteli, 550 m	water, chloroform	Iridoids, flavonoids, diterpenes; antimicrobial	[20,21]

Determination of cytotoxic effect (IC₅₀) of plant extracts

Plant extracts in a concentration range of 0.001 - 100 µg/ml were prepared by dilution with water and IC₅₀ values determined by using "xCELLigence Real-Time Cell Analyzer" in 96 h. A total of 2×10^6 /ml WI-38 human fibroblast cells were distributed for each cell line in the plates having 96 gold-coated wells, including the control group without plant extract. Each assessment was run in triplicate. IC₅₀ values of the plant extracts in each cell line were determined using a calorimeter with WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) test. Following the addition of WST-1, all extracts were kept for 4 h inside an incubator with 5 % CO₂ and 95 % humidity at 37 °C. The yellow-orange colored product, stable soluble formazan, was quantified at 450 nm and 620 nm reference interval using a Multiscan FC Thermo Scientific microplate reader [22].

Preparation of *Leishmania tropica* isolates

Following clinical diagnosis by dermatologist at Harran University Hospital, Urfa, *L. tropica* was isolated from the patients with cutaneous leishmaniasis infection in Sanliurfa province which is an endemic area for this infection. Skin lesions of the patients were cleansed with 70 % ethanol before sample aspiration. A 26-gauge needle with syringe containing 0.1-0.2 ml of sterile saline was then inserted intradermally into the outer border of the lesion. The syringe was rotated and the tissue fluids were gently aspirated and partly smeared on three slides [23]. These preparations were stained with Giemsa and examined under a light microscope.

The remaining aspiration material was inoculated immediately in Nicolle-Novy-McNeal (NNN) culture medium. The culture tubes were kept in an incubator at 25 °C for 7 days. *L. tropica* promastigote observed during microscopy were transferred to flasks containing RPMI-1640 medium (Biochrom AG), 10 % fetal calf serum (FCS), 200 U/ml of penicillin and 0.2 mg/ml of streptomycin and incubated at 25 °C for mass cultivation. Promastigotes were collected by centrifugation at 1500 rpm at 4 °C for 10 min and washed with sterile saline before analysis. *Leishmania* promastigotes were kept in liquid nitrogen at -196°C until the analysis. The identification of *Leishmania* species and isoenzyme analysis were conducted according to the procedure of the World Health Organisation Leishmania Reference Center [1].

In vitro antileishmanial assay

Concentrations of plant extracts (25, 50, 125, 250 and 500 µg/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 2 ml with RPMI medium for each well of a 24-well microplate. In all experiments, in order not to affect parasite growth rate, mobility or morphology, the final concentration of DMSO was not higher than 0.5 % (v/v) [24]. After haemocytometer counting, promastigotes were suspended to yield 1×10^6 cells/ml in each well. As a reference drug, a pentavalent antimonial compound, glucantime was prepared in sterile DMSO. The highest concentration of DMSO and RPMI medium were also used for untreated groups. Microplates were incubated at 25 °C. The number of parasites were counted with a haemocytometer (Thoma slide) under a light microscope in 12 - 72 h. [6,25,26]. All the *in vitro* experiments were run in triplicate and the results were expressed as mean percent inhibition in parasite number.

In vivo antileishmanial assay

Animals and experimental infection

Female Balb-C mice (20 - 25 g, 5 - 7 weeks old) were obtained from Ege University Experimental Animals Center. The mice were maintained on standard pelleted diet and water *ad libitum*. The study was approved by the Animal Ethics Committee, Ege University. Promastigotes were cultured in RPMI-1640 medium supplemented with 10 % FCS and collected on the 14th day of the culture. Ten milliliters of the culture fluid was centrifuged and a final dilution of 1×10^8 promastigotes/ml was prepared. The promastigote solution (15 µl) was injected subcutaneously into the right hind footpads of the mice [6]. The development of the lesion was measured weekly with a dial micrometer during the course of infection. The infection was well established and lesions were obvious.

Drug administration

The mice were divided into 13 groups (n = 7). Treatment trials were initiated 30 days after the establishment of infection. Ten extracts prepared from the plant materials (water and chloroform extracts of *Centaurea calolepis*, *Galium incanum* ssp. *centrale*, *Phlomis lycia* and water and methanol extracts of *Eryngium thurifolium* and *Origanum sipyleum*) were administered at a dose

of 100 mg/kg. Glucantime was used as a reference drug at a dose of 28 mg/kg [6]. As a negative control, sterile saline solution was administered to mouse in the untreated group. A placebo group was included in the study. All injections were administered intraslesionally at 15 μ l, five times with 3-day intervals. The size of developing lesions on the footpads of the mice was measured with a dial micrometer weekly during the course of infection and expressed as the differences in size between the infected and uninfected footpads. The parasite vitality was also investigated in the established infection. Needle aspiration samples were taken from infected lesions before and after the treatment. The samples were stained with Giemsa and examined under oil immersion with a light microscope to detect amastigotes. On the 60th day of infection, 50 mg tissue samples were taken from the edges of the lesions for biopsy. They were smeared on the slides, stained with Giemsa and examined on the microscope to search for amastigote form of *L. tropica*. Some part of biopsy samples were inoculated in NNN medium for investigation of the live promastigotes. Touch slides were also prepared from the samples for investigation of the amastigotes by PCR after DNA isolation.

Statistical analysis

The results of assessments were analysed using Student's t-test with Windows SPSS Version 15.0. $P > 0.05$ was taken as statistically significant.

RESULTS

Phytochemical analysis

The extraction yields in percentages and the results of preliminary phytochemical analysis of screened plants were demonstrated in Table 2. All the extracts except for water extracts of *C. calolepis* and *P. lycia*, gave positive results for terpenoids. Water extract of *C. calolepis* were positive for flavonoids, tannins and anthracenes. The chloroform extract of *P. lycia* possessed terpenoids and flavonoids. Water and methanol extracts of *O. sipyleum* and *E. thoriifolium* were positive for terpenoids, flavonoids and tannins. Anthracenes were present in the water extract of *E. thoriifolium*.

Cytotoxicity of plant extracts

The IC_{50} values of plant extracts were summarised in Table 3. The chloroform extract of *Galium incanum* ssp. *centrale* showed the highest cytotoxic activity at a concentration of 0.03615 ± 0.005 μ g/ml. Chloroform extract of *Centaurea calolepis* possessed greater cytotoxicity against WI-38 cell lines than its water extract with IC_{50} values of 12.6 ± 0.611 and 141.21 ± 0.168 μ g/ml respectively. Water and chloroform extracts of *Phlomis lycia* showed activity with an IC_{50} value of 139.19 ± 0.185 and 748.58 ± 0.311 μ g/ml respectively. Meanwhile, water and methanol extracts of *Eryngium thoriifolium* and water extract of *Galium incanum* ssp. *centrale* had proliferative activities on cell lines.

Table 2: Extraction yield and preliminary phytochemical analysis of plant extracts

Plant extract	Extraction yield (%)	Phytochemical profile
<i>Centaurea calolepis</i>		
water extract	20.47	Flavonoids, tannins, anthracenes
chloroform	4.53	Terpenoids
<i>Phlomis lycia</i>		
water extract	11.56	Flavonoids, tannins, anthracenes
chloroform extract	2.22	Terpenoids, flavonoids
<i>Eryngium thoriifolium</i>		
water extract	12.92	Terpenoids, flavonoids, tannins, anthracenes
methanol extract	6.22	Terpenoids, flavonoids, tannins
<i>Origanum sipyleum</i>		
water extract	15.63	Terpenoids, flavonoids, tannins, anthracenes
methanol extract	13.89	Terpenoids, flavonoids, tannins, anthracenes
<i>Galium incanum</i> ssp. <i>centrale</i>		
water extract	10.25	Terpenoids, tannins
chloroform extract	4.85	Terpenoids, alkaloids

Table 3: IC₅₀ values for plant extracts tested against WI-38 cell lines

Plant extract	IC ₅₀ (µg/ml)*
<i>Centaurea calolepis</i>	
water extract	141.21 ± 0.168
chloroform extract	12.6 ± 0.612
<i>Phlomis lycia</i>	
water extract	139.19 ± 0.185
chloroform extract	748.58 ± 0.311
<i>Eryngium thoriolium</i>	
water extract	proliferative
methanol extract	proliferative
<i>Origanum sipyleum</i>	
water extract	1588.3 ± 1.371
methanol extract	270.47 ± 0.503
<i>Galium incanum</i> ssp.	
<i>Centrale</i>	
water extract	proliferative
chloroform extract	0.03615 ± 0.005

*Results are expressed as mean ± standard deviation (SD) (n = 3)

***In vitro* anti-leishmanial activity**

All the plant extracts showed inhibitory activity ranging between 16.70 and 100 % at twelfth hour analysis at 25 µg/ml concentrations (Table 4). Results showed that all the extracts at 500 µg/ml concentration exhibited parasite inhibition percentages ranging between 61.7 % and 100 % at twelfth hour. The chloroform extract of *Centaurea calolepis*, *Phlomis lycia* and *Galium incanum* ssp. *centrale* were found to have higher activity than the water extracts of mentioned plants. As shown in Table 4, at twelfth hour, water extract of *Centaurea calolepis* reached 99.2 % parasite inhibition at a concentration of 500 µg/ml whereas the chloroform extract showed 100 % inhibition at 50 µg/ml concentration at 24th hour. Water extract (500 µg/ml) of *P. lycia* had 98.4 % inhibition at twelfth hour, whereas 99.4 % parasite inhibition was observed for the chloroform extract of *P. lycia* at 25 µg/ml. In comparison with the untreated group, except for twelfth hour analysis results of water extract of *Galium incanum* ssp. *centrale* and methanol extract of *Origanum sipyleum* ($p > 0.05$), all the extracts showed significant differences ($p < 0.01$). Methanol extract of *Eryngium thoriolium* with 100 % parasite inhibition at 25 µg/ml concentration possessed highest activity on promastigotes of *L. tropica*.

***In vivo* antileishmanial activity**

In the *in vivo* anti-leishmanial activity assays of plant extracts, the average measurement of the lesions on the footpads of mice in infected but

untreated group reached 3.32 ± 0.01 mm (Figure 1) whereas the reference glucantime group had an average of 1.71 ± 0.01 mm lesion size at the end of 8th week. All the extracts, except for water and chloroform extracts of *G. incanum* and chloroform extract of *P. lycia*, significantly decreased the lesion sizes of mice in treatment groups (Table 5). At the end of the 8th week, the mice in placebo group had 1.34 ± 0.01 mm average footpad size difference whereas the untreated and glucantime group had 3.32 ± 0.01 and 1.71 ± 0.01 mm respectively. The mice receiving water and chloroform extract of *C. calolepis* had 1.82 ± 0.02 and 1.79 ± 0.01 mm average footpad size respectively. The average footpad sizes observed for the mice treated with *E. thoriolium* water and methanol extracts were 1.79 ± 0.01 mm and 1.76 ± 0.01 mm respectively. It is evident from the results that the water and chloroform extracts of *C. calolepis* and water and methanol extracts of *E. thoriolium* exhibited *in vivo* activity against *L. tropica* infected mice whereas the rest of studied plant extracts were found to have insignificant activity as compared to untreated group ($p > 0.05$). Amastigotes (Figure 2) and promastigotes (Figure 3) were present in the samples taken from the lesion of mice treated with extracts of *G. incanum*, *O. sipyleum* and *P. lycia*. The parasites in the aspiration lesion samples of the group of mice treated with water extract of *E. thoriolium* and chloroform and water extract of *C. calolepis* were found to lose vitality on the 60th day of infection. As shown in Table 5, amastigotes were present in the smear preparations of samples taken from mice treated with methanol extract of *E. thoriolium*, whereas no promastigotes were observed in the NNN medium.



Figure 1: Cutaneous lesion on right hind mouse footpad of untreated group

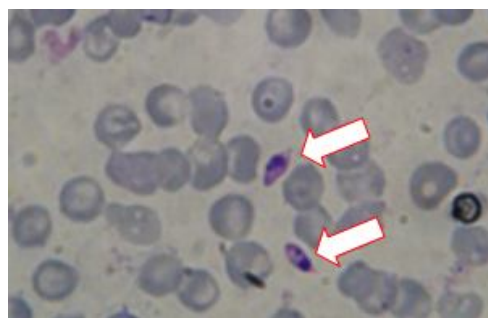


Figure 2: Amastigotes observed in needle aspiration samples

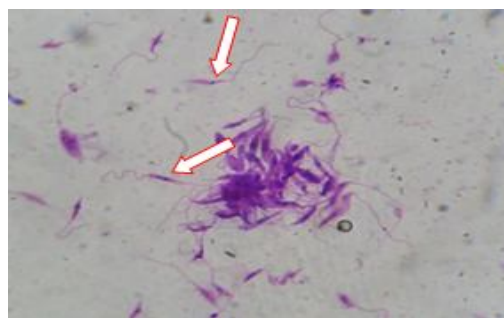


Figure 3: Promastigotes observed in NNN medium

Table 4: *In vitro* parasite inhibition percentages of plant extracts, glucantime and untreated groups

Plant extract	Time (h)	Parasite inhibition \pm SD (%)*				
		25 μ g/mL	50 μ g/mL	125 μ g/mL	250 μ g/mL	500 μ g/mL
<i>C. calolepis</i>						
Water	12	16.70 \pm 0.01	25.00 \pm 0.01	46.70 \pm 0.02	84.20 \pm 0.04	99.20 \pm 0.04
	24	14.30 \pm 0.03	35.70 \pm 0.01	62.90 \pm 0.04	93.60 \pm 0.03	99.40 \pm 0.04
	48-72	12.50 \pm 0.01	56.30 \pm 0.01	83.80 \pm 0.03	97.50 \pm 0.02	99.70 \pm 0.04
Chloroform	12	98.60 \pm 0.02	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	24	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	48-72	99.50 \pm 0.01	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
<i>P. lycia</i>						
Water	12	16.70 \pm 0.01	25.00 \pm 0.04	40.00 \pm 0.04	71.70 \pm 0.03	98.40 \pm 0.02
	24	42.90 \pm 0.02	48.60 \pm 0.03	52.90 \pm 0.01	78.60 \pm 0.01	99.30 \pm 0.01
	48-72	12.50 \pm 0.02	43.80 \pm 0.04	65.00 \pm 0.02	83.80 \pm 0.01	99.50 \pm 0.01
Chloroform	12	65.00 \pm 0.06	78.30 \pm 0.04	92.50 \pm 0.01	96.70 \pm 0.02	97.70 \pm 0.01
	24	78.60 \pm 0.02	93.60 \pm 0.04	97.10 \pm 0.02	98.00 \pm 0.01	98.70 \pm 0.02
	48-72	99.40 \pm 0.01	97.50 \pm 0.02	98.30 \pm 0.002	98.90 \pm 0.01	99.40 \pm 0.02
<i>Galium incanum ssp. centrale</i>						
Water	12**	16.70 \pm 0.01	25.00 \pm 0.05	41.70 \pm 0.04	45.00 \pm 0.04	61.70 \pm 0.08
	24	28.60 \pm 0.04	35.70 \pm 0.04	50.00 \pm 0.06	64.30 \pm 0.03	82.90 \pm 0.04
	48-72	12.50 \pm 0.07	25.00 \pm 0.03	68.80 \pm 0.06	85.00 \pm 0.04	95.00 \pm 0.08
Chloroform	12	45.00 \pm 0.04	53.30 \pm 0.06	75.00 \pm 0.02	85.00 \pm 0.02	95.20 \pm 0.06
	24	54.30 \pm 0.04	65.70 \pm 0.04	81.40 \pm 0.02	95.00 \pm 0.04	98.60 \pm 0.04
	48-72	71.30 \pm 0.01	85.00 \pm 0.02	96.90 \pm 0.01	99.00 \pm 0.04	99.70 \pm 0.03
<i>E. thorifolium</i>						
Water	12	25.00 \pm 0.01	41.70 \pm 0.04	51.70 \pm 0.02	78.30 \pm 0.04	97.40 \pm 0.02
	24	50.00 \pm 0.04	60.00 \pm 0.03	82.90 \pm 0.12	97.60 \pm 0.03	99.20 \pm 0.04
	48-72	80.00 \pm 0.04	87.50 \pm 0.04	99.20 \pm 0.05	99.40 \pm 0.04	99.60 \pm 0.02
Methanol	12	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	24	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	48-72	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
<i>O. sipyleum</i>						
Water	12	16.70 \pm 0.02	25.00 \pm 0.02	38.30 \pm 0.04	73.30 \pm 0.02	97.50 \pm 0.04
	24	14.30 \pm 0.04	28.60 \pm 0.02	52.90 \pm 0.04	81.40 \pm 0.01	98.40 \pm 0.02
	48-72	12.50 \pm 0.01	25.00 \pm 0.01	61.30 \pm 0.03	85.00 \pm 0.04	98.80 \pm 0.02
Methanol	12**	16.70 \pm 0.04	33.30 \pm 0.04	50.00 \pm 0.04	58.30 \pm 0.04	75.00 \pm 0.02
	24	28.60 \pm 0.02	50.00 \pm 0.01	58.60 \pm 0.06	75.70 \pm 0.01	87.10 \pm 0.02
	48-72	12.50 \pm 0.01	37.50 \pm 0.03	68.80 \pm 0.02	83.80 \pm 0.04	98.10 \pm 0.03
Untreated						
	12	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
	24	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
	48-72	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
Glucantime						
	12	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	24	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00
	48-72	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00	100.0 \pm 0.00

*Results are expressed as mean \pm SD ($p < 0.05$); **No significant difference compared to untreated group ($p > 0.05$)

DISCUSSION

In recent years, plant-derived products are gaining increasing scientific attention in the ongoing search for better leishmanicidal compounds [27]. Some researchers looking for new alternatives for the treatment of leishmaniasis have focused on microorganisms and plants [28]. *Faramea guainensis* was reported to have significant *in vivo* leishmanicidal activity on *Leishmania amazoensis* [29]. In India, *L. donovani*-infected mice were successfully treated with ethanolic extracts of *Alstonia scholaris* and *Swertia chirata* at a dose of 1 g/kg/day [30]. In a study conducted in Spain on antileishmanial activities of 12 plant species, *Inula montana*, *Bupleurum rigidum* and *Scrophularia scorodonia* were found to have promising potential [31]. In another study in Colombia, *Annona muricata* showed higher antileishmanial activity on *L. braziliensis* and *L. paramensis* than the reference drug, glucantime [32]. However, there are few studies in literature reporting the activity of plant extracts and plant derived compounds against *L. tropica* parasites. In a previous work on *Haplophyllum myrtifolium*, the extracts and pure compounds were reported to have both *in vivo* and *in vitro* activity on *L. tropica* [6]. The ethanol, water and n-hexane extracts from the leaves of *Arbutus unedo* were tested *in vitro* against *L. tropica* promastigotes and the ethanol extract of *A. unedo* leaves at the concentrations of 100, 250, 500 µg/ml were found to be more effective than the other extracts [33]. Extracts of the fruits of *M. azedarach* and *N. oleander* brought about 97 and 81.5 % inhibition of *L. tropica* promastigotes respectively [34]. In this study, selected Turkish medicinal plants from *Eryngium*, *Origanum*, *Centurea*, *Phlomis* and *Galium* genus were evaluated against *L. tropica*. In a previous investigation, Fokialakis et al reported that the dichloromethane extracts of *Eryngium ternatum*, *Origanum dictamnus* and *Origanum microphyllum* and methanol extract of *Eryngium amorginum* had significant *in vitro* anti-leishmanial activity against *L. donovani* [13]. Antiprotozoal and antimicrobial activities of Centaureae species growing in Turkey were investigated *in vitro* and the highest anti-leishmanial activities among the extracts were observed with the chloroform extract of *C. hierapolitana* [16].

A fraction obtained from the n-hexane extract of *Galium mexicanum* inhibited the growth of *L. donovani* parasite at a concentration of 333 µg/ml for the period of 72 hours [35]. The chloroform, n-hexane and water extracts of *Phlomis curdica* and *Phlomis leucophracta* were

Table 5: Lesion size and percent parasites observed in *in vivo* treatment groups

Group	P (%)*		Footpad size (mm) (mean ± SD, n=7)							
	A	P	1	2	3	4	5	6	7	8
CCA	85.71 ± 0.01	85.71 ± 0.01	1.37 ± 0.01	1.47 ± 0.01	1.56 ± 0.01	1.70 ± 0.01	1.85 ± 0.01	1.90 ± 0.01	1.85 ± 0.02	1.82 ± 0.02
CCC	57.14 ± 0.01	71.43 ± 0.01	1.37 ± 0.01	1.47 ± 0.01	1.56 ± 0.01	1.70 ± 0.01	1.80 ± 0.01	1.82 ± 0.01	1.80 ± 0.01	1.79 ± 0.01
ETA	71.43 ± 0.01	42.86 ± 0.01	1.37 ± 0.01	1.47 ± 0.01	1.56 ± 0.01	1.70 ± 0.01	1.80 ± 0.01	1.82 ± 0.01	1.80 ± 0.01	1.79 ± 0.01
ETM	14.29 ± 0.01	0.00 ± 0.01	1.37 ± 0.01	1.47 ± 0.01	1.60 ± 0.08	1.60 ± 0.01	1.86 ± 0.01	1.89 ± 0.01	1.81 ± 0.01	1.76 ± 0.01
GIA	100.0 ± 0.00	100.0 ± 0.00	1.36 ± 0.01	1.47 ± 0.01	1.60 ± 0.01	1.70 ± 0.01	1.92 ± 0.02	2.31 ± 0.01	2.56 ± 0.02	3.33 ± 0.01
GIC	100.0 ± 0.01	100.0 ± 0.00	1.37 ± 0.01	1.47 ± 0.01	1.57 ± 0.01	1.69 ± 0.01	1.93 ± 0.01	2.31 ± 0.01	2.55 ± 0.01	3.34 ± 0.01
OSA	85.71 ± 0.01	100.0 ± 0.00	1.36 ± 0.01	1.47 ± 0.01	1.57 ± 0.01	1.68 ± 0.01	1.87 ± 0.04	2.06 ± 0.08	2.30 ± 0.01	3.05 ± 0.05
OSM	100.0 ± 0.01	100.0 ± 0.00	1.36 ± 0.01	1.47 ± 0.01	1.57 ± 0.01	1.69 ± 0.01	1.91 ± 0.01	2.31 ± 0.01	2.54 ± 0.01	3.21 ± 0.15
PLA	100.0 ± 0.01	100.0 ± 0.00	1.37 ± 0.01	1.47 ± 0.01	1.57 ± 0.01	1.69 ± 0.01	1.89 ± 0.02	2.06 ± 0.08	2.30 ± 0.01	3.01 ± 0.04
PLC	100.0 ± 0.00	100.0 ± 0.00	1.36 ± 0.01	1.48 ± 0.01	1.57 ± 0.01	1.70 ± 0.01	1.91 ± 0.01	2.31 ± 0.01	2.56 ± 0.01	3.31 ± 0.01
RIG	0.00 ± 0.00	0.00 ± 0.00	1.38 ± 0.01	1.48 ± 0.01	1.57 ± 0.01	1.68 ± 0.01	1.82 ± 0.02	1.85 ± 0.01	1.79 ± 0.01	1.71 ± 0.01
CG	100.0 ± 0.00	100.0 ± 0.00	1.37 ± 0.01	1.47 ± 0.02	1.56 ± 0.01	1.68 ± 0.01	1.92 ± 0.01	2.31 ± 0.01	2.55 ± 0.01	3.32 ± 0.01
PG	0.00 ± 0.00	0.00 ± 0.00	1.34 ± 0.01	1.34 ± 0.01	1.34 ± 0.01	1.34 ± 0.00	1.36 ± 0.01	1.37 ± 0.01	1.34 ± 0.01	1.34 ± 0.01

*Significant lesion size difference compared to untreated group ($p < 0.05$); A = Amastigotes in aspiration sample (%), P = Promastigotes in NNN Medium (%); PG = placebo, RIG: glucantime, CG = untreated group; A = water, C: chloroform; M = methanol; ET = *E. thurifolium*; CC = *C. caliolepis*; PL = *P. lycia*; OS = *O. sipyleum*; GI = *G. incanum* supsp. centrale

reported to possess inhibitory activity against *L. donovani* amastigotes [21]. Thus, the present study constitutes the first report on antileishmanial activities of plant extracts of *Centaurea calolepis*, *Phlomis lycia*, *Eryngium thorifolium*, *Origanum sipyleum* and *Galium incanum* ssp. *centrale*.

The polarity and the nature of phytochemical compounds present in plant materials are the main factors that must be considered for selection of extraction solvent. The type of extractant may range from non-polar to polar solvent depending on the targeted bioactive components. In the present work, the sample preparation disparity in regarding the choice of solvent was based on the extraction yield and on our previous preliminary antiprotozoal investigations on the plant species. Water and chloroform extracts of *C. calolepis*, *G. incanum* ssp. *centrale* and *P. lycia* and the water and methanol extracts of *E. thorifolium* and *O. sipyleum* were comparatively investigated. *In vitro* studies have shown that all the investigated extracts exhibited 98.1-100 % inhibitory effect on *L. tropica* promastigotes at the highest dose of 500 µg/ml. Secondary metabolites such as alkaloids, flavonoids, saponins and terpenoids were known to possess antileishmanial activities [4,36]. Thus the inhibitory effect of the extracts could be due to presence of wide range of secondary metabolites with different polarities.

In the present study, the extracts of *Eryngium thorifolium* and *Centaureae calolepis* had both *in vitro* and *in vivo* effects on *L. tropica*. After the 60th day of infection, live promastigotes were not detected in the groups of mice treated with *E. thorifolium* methanol extract. This activity was explained by the mechanism of extract either by killing parasites or causing metabolic disorders to inhibit the reproduction of parasites.

To the best of our knowledge, this is the first study of the cytotoxic effect as well as the *in vivo* and *in vitro* antileishmanial activities of these plants.

CONCLUSION

Both the *in vivo* and *in vitro* results and parasite viability findings confirm that *E. thorifolium* methanol extract, which possesses terpenoids, flavonoids, anthracenes and tannins, is a potential source of new and selective antileishmanial agents.

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