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RESEARCH ARTICLE

Effect of *Hypericum thymbrifolium* BOISS. ET NOE, *Hypericum scabrum* L. and *Eryngium creticum* LAM. plant extracts on *Leishmania major, Leishmania tropica* and *Leishmania infantum/donovani* strains and their cytotoxic potential

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ABSTRACT

Leishmaniasis causes significant morbidity and mortality worldwide. In our country, there has been a significant increase in the number of cases of leishmaniasis in the last decade. In our study, the effects of *Hypericum thymbrifolium, Hypericum scabrum* and *Eryngium creticum* plant extracts were tested on *Leishmania major, Leishmania tropica* and *Leishmania infantum/donovani*, which were clinically resistant by not responding to Glucantime® therapy. Cytotoxicity of these extracts were evaluated by XTT method in the human fibroblast cell line. Possible active ingredients were detected by GC-MS analysis from plant extracts. Glucantime® resistance was detected at concentrations of 50 µg/mL and lower in 4 of the 7 strains tested. No living leishmania parasites were found in *leishmania* strains treated with plant extracts at concentrations of 100 µg/mL or higher. The concentrations of plant extracts included in the study on the WI-38 human fibroblast cell line were not cytotoxic. According to the GC-MS analysis, several active substances with biological activities and anti-parasitic effects, such as Thiophene, Germacrene-D, trans-Geranylgeraniol, Pyridine, and Maleimides, were identified. Based on the findings of the study, it is believed that these identified active substances when supported by in-vivo studies, will pave the way for future research and have the potential to be developed as anti-leishmania drugs.

Keywords: Anti-leishmanial activity; *H. thymbrifolium*; *H. scabrum*; *Eryngium creticum*.

INTRODUCTION

Leishmaniasis causes significant morbidity and mortality worldwide. In Turkey, there has also been a significant increase in leishmaniasis cases in the last decade. An estimated 700 000 to 1 million new leishmaniasis cases occur annually. About 95% of Cutaneous Leishmaniasis (CL) cases occur in the Americas, the Mediterranean basin, the Middle East and central Asia. It is estimated that 600 000 to 1 million new cases occur worldwide annually (Ok *et al*., 2002; Culha *et al*., 2006; WHO, 2023). The disease is endemic in developing tropics and economic globalization and increased travel in recent years have facilitated the parasite's access to people in developed countries. Therefore, leishmaniasis is now an important health problem for non-endemic regions too. In the absence of effective vaccines and vector control measures, the main line of defense against the disease is chemotherapy. Organic pentavalan antimony compounds are the first drug of choice in treatment. Clinical resistance, which has been reported quite frequently in recent years, is the biggest obstacle to successful treatment and control (Lira *et al*., 1999; Ashutosh *et al*., 2005, 2007). Resistance to pentavalan compounds is so common in India that primary care treatment in this region is based on either miltefosine or amphotersin B (El Fadili *et al*., 2005). In addition, the fact that anti-leishmanial drugs are highly toxic and expensive drugs is one of the difficulties of treatment.

In recent years, the use of herbal-derived drugs has been increasing gradually. Many plants are used as a treatment tool for various diseases and there are effects that will not be searched for synthetic drugs. The genus *Hypericum* L. (Hypericaceae) includes, at the most recent count, 469 species that are either naturally occurring on or which have been introduced to every continent in the world except Antarctica (Crockett *et al*., 2011). *H. perforatum* L. (Common St. John's wort), is the best known species among this genus and is known for the effects of plant extracts against mild to moderate depression, anxiety, scarring and ulcers (Crockett *et al*., 2011; Lyles *et al*., 2017; Nayak *et al*., 2017). Recent studies have revealed that chemical structures in the Guttiferae family called polycyclic polyprenylated acylphloroglucinols (PPAPs) have anti-bacterial, anti-depressant, anti-oxidant and anti-neurodegenerative effects (Zhou *et al*., 2014; Sun *et al*., 2016;). *Hypericum* is a member of the Guttiferae family and is rich in PPAP (Liu *et al*., 2014; Gao *et al*., 2016). In a study, PPAP were identified as the first time they were isolated from the aerial part of *Hypericum scabrum*. In the study,

it was reported that three of these compounds exhibit moderate hepatoprotective activity and one exhibits moderate antidepressive activity (Liu *et al.,* 2017).

In the literature, the *in vitro* effects of *Hypericum* species on *Leishmania amazonensis* were mentioned and this effect was compared with amphoterisin B (Dagnino *et al*., 2015).

Eryngium creticum LAM. which has a very common use in traditional treatment, contains many chemical compounds, mostly sesquiterpenes, monoterpenes, aldehydes, coumarins, sitosterols and sugars (Kikowska *et al*., 2016). With its biologically active compounds, *Eryngium creticum* LAM. has quite extensive medical effects. In traditional medicine, *Eryngium creticum* LAM. has been reported to be used mostly to treat snake and scorpion bites (Kikowska *et al*., 2016). In addition, antibacterial (Makki *et al*., 2015), antifungal (Ali-Shtayeh *et al*., 1999), antileishmanial (Fokialakis *et al*., 2006), hypoglycemic (Jaghabir, 1991) and antioxidant (Farhan *et al*., 2012) effects have been mentioned in studies. There were not enough studies on the antileishmanial effect of *Eryngium creticum* LAM. in the literature scans. The study by Fokialakis *et al*. (2006), used the *L. donovani* sensitive strain and reported its antileishmanial effect.

Our study, it was aimed to test the effects of *Hypericum thymbrifolium* BOISS. ET NOE, *Hypericum scabrum* L. and *Eryngium creticum* LAM. plant extracts on *L. major*, *L. tropica* and *L. infantum/donovani* isolates, which are clinically resistant by not responding to organic pentavalan antimony (Glucantime®) compound therapy, and to identify new active ingredient of with gas chromatography-mass spectrometry (GC-MS) analysis. These plant species were chosen because they have a wide range of biological activities and are widely used as traditional folk medicine in the treatment of some diseases among the public.

MATERIAL AND METHOD

Ethical approval

The study was approved with the decision of Hatay Mustafa Kemal University Non-Invasive Clinical Research Ethics Committee dated 26.12.2019 and numbered 09.

Study design

Leishmania major, *L. tropica* and *L. infantum* isolates used in our study were obtained from Parasite Bank in Hatay Mustafa Kemal University Faculty of Medicine.

Reference strains *L. tropica* MHOM/AZ/1974/SAF-K27, *L. major* MHOM/SU/1973/5ASKH, *L. donovani* MHOM/IN/1980/DD8 were used as controls. Reference strains were obtained from Parasite Bank in Manisa Celal Bayar University Faculty of Medicine.

Hypericum species from plant materials were collected during the flowering period, from natural populations in Sivas province, and *Eryngium creticum* from the natural population in Konya province.

In the study, methanol extracts of the aerial part of the plants were used.

DNA isolation of *Leishmania* isolates and typing by RT-PCR

Seven isolates kept in liquid nitrogen tank in the parasite bank were used in the study. This isolates were taken from patients with suspected CL who had not previously received a response to Glucantime® treatment.

The isolates, which were removed from the liquid nitrogen tank and rapidly thawed at 37°C, were first grown in NNN medium and then in RPMI 1640 and 15% Fetal Bovine Serum medium. DNA was isolated and typified by RT-PCR method.

DNA isolation from samples reaching the logarithmic phase was made in accordance with the manufacturer's recommendations using commercial DNA isolation kit (QIAamp DNA Mini Kit, Qiagen, Germany).

RT-PCR with ITS1 probe was used to determine the type in the samples. The ribosomal internal transcribed spacer 1 (ITS1) region of *Leishmania* parasites, which separates genes that encode ssu rRNA and 5.8S rRNA, was used in combination with the Forward primer; 5'-CTGGATCATTTTCCGATG-3', Reverse Primer; 5'-GAAGCCAAGTCATCCATCGC-3' primers Probe 1: 5'-CCGTTTATACAAAAAATATACGGCGTTTCGGTTT Fluo-3', Probe 2: 5'-LCRed-640-GCGGGGTGGGTGCGTGTGTG-Pho-3' original probes.

For PCR analysis, 1.5 µL H20 (PCR grade water), 1 µL Forward Primer, 1 µL Reverse Primer, 0.5 µL Probe1, 0.5 µL Probe 2, 12.5 µL QuantiTect Probe PCR Kit Master mix (Qiagen) and 5 µL of genomic DNA, a total volume of 25 µL was prepared (Toz *et al*., 2013). Melting analysis was performed on Rotor-Gene device and typing was performed. Reference strains *L. tropica* MHOM/AZ/1974/SAF-K27, *L. major* MHOM/SU/1973/5ASKH, *L. donovani* MHOM/IN/1980/DD8 were used as controls.

Determination of *in vitro* antimony resistance

In our study, the presence of antimony resistance in *L. major*, *L. tropica* and *L. infantum/donovani* promastigots were tested using micro-dilution method. Sterile plate with 96 wells was used in the study. The wells were planned as blank, negative control group and antimony compound experimental group. Meglumin antimony (Glucantime®, France) was used as an antimony compound. In the first stage, 100 µL was added from the RPMI 1640 (%10 FCS and %1 penicillin/streptomisin and %1 gentamicin) feeder to the wells that make up the blank and negative control group. 100 µL is also added to experimental group, meglumin antimonate (Glucantime®, France), calculated with a final volume concentration of 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL, 6.25 µg/mL. The liquid is remowed from nitrogen and produced in the feeder RPMI 1640 and calibrated at concentration of $1x10^8$ promastigote/mL added 100 µL to negative control and experimental group and it was incubated at 25°C. The parasites were kept at 25°C for 48 hours and the viability test was performed with XTT.

Preparation of plant extracts

Plant samples were collected at the flowering stage and dried. The samples were ground in the grinder and powdered. 10 g of powdered dry material was taken and 250 ml of methanol (96%) was added to it. Was shook in the shaker in room temperature for 24 hours. At the end of this period, methanol was taken into the collection container and 250 ml of methanol was added again on the solid part and the extraction was repeated 4 times. After the final extraction, the liquid parts were combined and filtered (Whatman, No.1). Methanol was flown in the rotary evaporator (Evaporator Buchi R-100, Vacuum pump V-300, Controller I-300) at 40°C and the extracts were stored at -20°C until the moment of analysis (Ozpinar *et al*., 2019).

Anti-leishmanial activity of plant extracts

In our study, the effect of *H. thymbrifolium*, *H. scabrum* and *E. creticum* against antimony-resistant *L. major*, *L. tropica* and *L. infantum/donovani* promastigots was tested using micro-dilution method. Sterile plate with 96 wells was used in the study. The wells were planned as blank, negative control group and experimental group consisting of each plant extract. Meglumin antimonate (Glucantime®, France) was used as a positive control. First, 100 µL was added from RPMI 1640 (10% FCS and %1 penicillin/streptomycin and 1% gentamycin) to the wells that make up the blank and negative control group. In the experimental group, the final volume concentrations were calculated to be 800 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL, and plant extracts were added separately to 100 µL. The liquid is remowed from nitrogen and produced in the feeder RPMI 1640 and calibrated at concentration of $1x10^8$ promastigote/mL added 100 µL to negative control and experimental group and it was incubated at 25°C. The

parasites were kept at 25°C for 48 hours and the viability test was performed with XTT (Ozbilgin *et al*., 2014)

Cytotoxicity of plant extracts

The XTT (The Cell Proliferation Kit, Merc, 11465015001) test was performed to investigate cytotoxicity of plant extracts on WI-38 human fibroblast cell line. The cells were prepared to be 1x105/mL WI-38 cells in the DMEM (Dulbecco's Modified Eagle Medium, fenol red'free) medium to be used in the experiment. Suspension was created by adding 10% FBS and 1% penicillin-streptomycin on top of DMEM. 100 µl (1x10^4 cell/well) was added to each well from the prepared cell suspension. After examining the distribution of the suspension with a microscope, 5% CO_2 of 37°C was incubated for 24 hours. Final volume concentrations at the end of incubation are 800 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL and 12.5 µg/mL were added to the wells in 2 µl. 200 µl DMEM (without phenol red) and 10% FBS and 1% penicillin-streptomycin suspension was added to the positive control and 2 µl DMSO was added to the negative control. The final volume in all wells was completed to 200 µl with a medium suspension of 198 µL. Then, it was incubated for 48 hours in an oven at 37°C with 5% CO_2 (Ozpinar *et al*., 2020a).

As a result of cytotoxicity studies, the data were evaluated and it was accepted that cytotoxicity values below 10% were not cytotoxic. Values between 10% and 25% were considered low, and values between 25% and 40% were considered intermediate levels. Values above 40% were considered high levels of cytotoxicity (Lorenzo-Morales *et al*., 2015)

XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazo-lium hydroxide) test

At the end of the cytotoxicity test incubation, a new plate with 96 wells was taken and 100 µL new plates were transferred from each well. 50 µL was added to each well from XTT. After incubation at 25°C for 24 hours, the plate was read at 450 nm with an ELISA reader and the absorbances were recorded. The viability rates were based on the following formula.

$$\text{Viability rate (\%)} = \frac{\text{Study sample absorbance} - \text{Blank absorbance}}{\text{Control sample absorbance} - \text{Blank absorbance}} \times 100$$

GC-MS analysis

GC-MS analysis of plant extracts was performed at Giresun University Central Research Laboratory Application and Research Center.

For GC-MS, the HP-5 MS IU capillary column (30 m X 250 µm X 0.25 µm) and the 7890A (Agilent) model GC-MS device with 5975C (Agilent) inert MSD mass detector were used. In GC-MS, an electron ionization system with 70 eV ionization energy and Helium (He) was used as carrier gas with 99.999% purity. The carrier gas entered the He column with a flow rate of 1.5 mL/min and a starting pressure of 17,897 psi. After keeping the oven temperature at the starting temperature of 50°C for 2 minutes, the rate of temperature rise in all stages was 5°C/min and was increased to 80°C (waiting 2 minutes at this temperature), 100°C (waiting 1 minute at this temperature), 150°C (waiting 1 minute at this temperature), 240°C (waiting 1 minute at this temperature) and 270°C (waiting 7 minutes at this temperature) respectively (Ozpinar *et al*., 2019).

Statistical analysis

The data obtained in our study were uploaded to the SPSS (Ver:22.0) program and One Way Anova and Tukey test was used to evaluate the data. If the p value was less than 0.05, the results were considered.

RESULTS

DNA isolation of Leishmania isolates and typing by RT-PCR

As a result of the RT-PCR analysis of 7 isolates belonging to suspected CL patients who had not previously received a response to glucantime treatment, it was determined that three of these isolates were *L. tropica*, two were *L. major* and two were *L. donovani/infantum*.

Determination of *in vitro* antimony resistance

The presence of antimony resistance was tested using the micro-dilution method to isolates that are replicated in the RPMI 1640 and 15% Fetal Bovine Serum feeder and reach the logarithmic growth phase. In the study, *Leishmania* isolates were treated with Glucantime® at 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL ve 12,5 µg/mL, 6,25 µg/mL concentrations and after 48 hours the viability rates of parasites were tested with XTT (Table 1, Figure1).

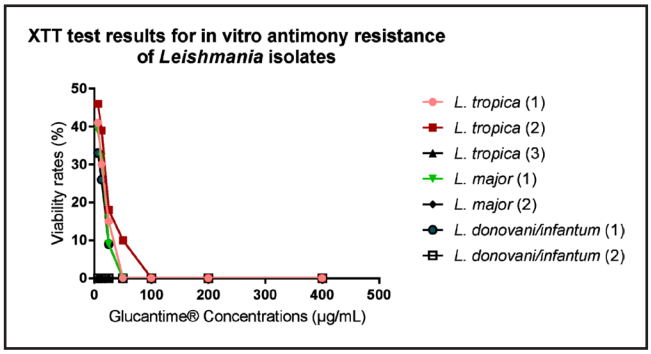

Figure 1. XTT test results for *in vitro* antimony resistance of Leishmania isolates.

Table 1. XTT test results for *in vitro* antimony resistance of *Leishmania* isolates

Isolates	Glucantime® Concentrations (µg/mL)						
	400	200	100	50	25	12.5	6.25
	Viability rates (%±SD)						
L. tropica (1)	0±0.00	0±0.00	0±0.00	0±0.00	15±0.54	30±0.58	41±0.59
L. tropica (2)	0±0.00	0±0.00	0±0.00	10±0.55	18±0.82	39±0.85	46±0.85
L. tropica (3)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
L. major (1)	0±0.00	0±0.00	0±0.00	0±0.00	9±0.48	32±0.56	39±0.58
L. major (2)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00
L. donovani/infantum (1)	0±0.00	0±0.00	0±0.00	0±0.00	9±0.46	26±0.55	33±0.58
L. donovani/infantum (2)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00

Table 2. Anti-leishmanial activity of *H. thymbrifolium* extract, XTT test results

Isolates	*H. thymbrifolium* concentrations (µg/mL)							
	800	400	200	100	50	25	12.5	6.25
	Viability rates (%±SD)							
L. tropica (1)	0±0.00	0±0.00	0±0.00	0±0.00	10±0.58	50±0.62	95±0.70	100±0.70
L. tropica (2)	0±0.00	0±0.00	0±0.00	0±0.00	11±0.55	41±0.58	88±0.69	100±0.71
L. major (1)	0±0.00	0±0.00	0±0.00	0±0.00	9±0.55	60±0.59	95±0.70	100±0.69
L. donovani/infantum (1)	0±0.00	0±0.00	0±0.00	0±0.00	10±0.55	40±0.58	96±0.70	100±0.69
DMSO	100±0.71							
NC	100±0.70							

*DMSO: Dimethyl sulfoxide; NC: Negative control.

As a result of antimony resistance analysis, it was observed that two *L. tropica*, one *L. major* and one *L. donovani/infantum* isolates maintained their vitality at least 8% after 48 hours at a concentration of 25 µg/mL. Viability rates were found to be 41% of *L. tropica* (1), 46% of *L. tropica* (2), 39% of *L. major* (1) and 33% of *L. donovani/infantum* (1) isolates at a concentration of 6.25 µg/mL. Viability rates of the same isolates at a concentration of 12.5 µg/mL were found as *L. tropica* (1) 30%, *L. tropica* (2) 36%, *L. major* (1) 32% and *L. donovani/infantum* (1) isolates 26%. The viability rates of parasites at a concentration of 25 µg/mL were determined as 15% for *L. tropica* (1), 18% for *L. tropica* (2), 9% for *L. major* (1) and 9% for *L. donovani/infantum* (1) isolates.

Since the study was aimed at testing the effects of *H. thymbrifolium*, *H. scabrum* and *E. creticum* plant extracts on *Leishmania* isolates with 5-value antimony resistance, four isolates with Glucantime® resistance (*L. tropica* (1), *L. tropica* (2), *L. major* (1) ve *L. donovani/infantum* (1)) presence were included in the study.

Anti-leishmanial activity of plant extracts

The extraction yields of the above-mentioned plants' aerial parts were determined as follows: *Hypericum thymbrifolium* with 9.81%, *Hypericum scabrum* with 10.21%, and *Eryngium creticum* with 6.8% after methanol extraction.

Antileishmanial activity of *H. thymbrifolium* methanol extract is shown in Table 2, Figure 2. Accordingly, no live leishmania isolates were found in the first four concentrations included in the study. The viability rates were found to be 10% in *L. tropica* (1) isolate, 11% in *L. tropica* (2) isolate, 9% in *L. major* (1) isolate, and 10% in *L. donovani/infantum* (1) isolate at 50 µg/mL concentration. In the group exposed to Glucantime®, this rate was 10% in *L. tropica* (2) isolation. When the data between the groups were statistically compared, it was found that there was no significant difference ($p>0.05$) between *L. tropica* (2) isolate treated with *H. thymbrifolium*

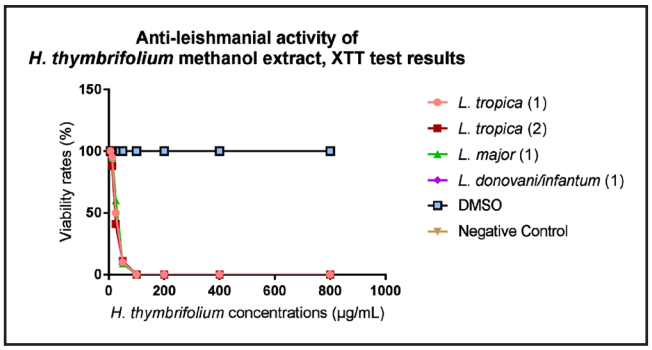

Figure 2. Anti-leishmanial activity of *H. thymbrifolium* methanol extract, XTT test results.

and Glucantime®. However, there was a significant difference ($p<0.05$) among the other groups. The viability rates of the other concentrations are presented in Table 2 and Figure 2.

Anti-leishmanial activity of *H. scabrum* methanol extract is shown in Table 3, Figure 3. Accordingly, no live leishmania isolates were found in the first four concentrations included in the study. The viability rates were found to be 19% in *L. tropica* (1) isolate, 20% in *L. tropica* (2) isolate, 5% in *L. major* (1) isolate and no live parasites were found in the *L. donovani/infantum* (1) isolate at 50 µg/mL concentration. In glucantim at the same concentration, the viability rate in *L. tropica* (2) isolate was 10%. When the intergroup data were statistically compared, it was determined that the difference between *L. donovani/infantum* (1) isolate and Glucantim was insignificant ($p>0.05$) and that the difference between other groups was significant ($p<0.05$). At 25 µg/mL concentration, the viability rates were 55% in *L. tropica* (1) isolate, 62% in *L. tropica* (2)

Table 3. Anti-leishmanial activity of *H. scabrum* extract, XTT test results

Isolates	*H. scabrum* concentrations (µg/mL)							
	800	400	200	100	50	25	12.5	6.25
	Viability rates (%±SD)							
L. tropica (1)	0±0.00	0±0.00	0±0.00	0±0.00	19±0.55	55±0.61	96±0.71	100±0.70
L. tropica (2)	0±0.00	0±0.00	0±0.00	0±0.00	20±0.56	62±0.70	88±0.70	100±0.70
L. major (1)	0±0.00	0±0.00	0±0.00	0±0.00	5±0.34	30±0.55	95±0.71	100±0.71
L. donovani/infantum (1)	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	10±0.55	96±0.71	100±0.73
DMSO	100±0.78							
NC	100±0.71							

*DMSO: Dimethyl sulfoxide; NC: Negative control.

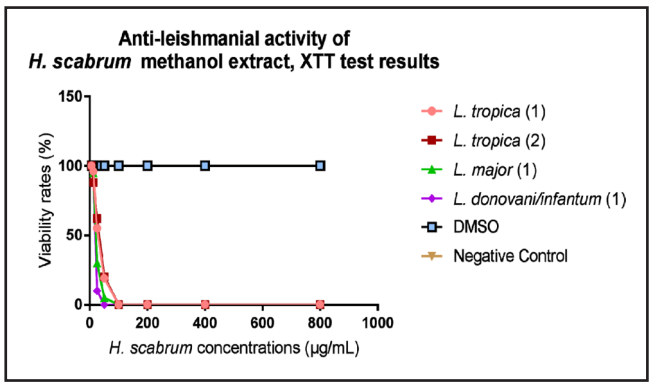

Figure 3. Antileishmanial activity of *H. scabrum* methanol extract, XTT test results.

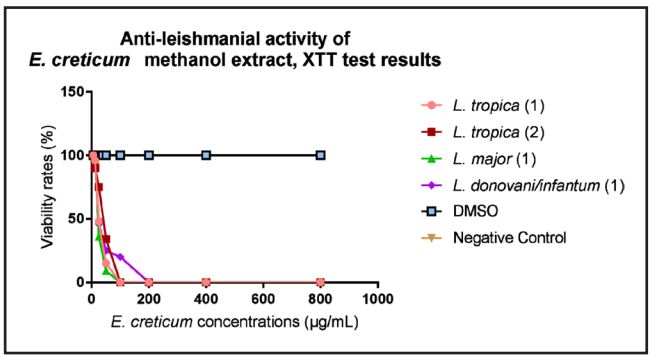

Figure 4. Antileishmanial activity of *E. creticum* methanol extract, XTT test results.

Table 4. Anti-leishmanial activity of *E. creticum* extract, XTT test results

Isolates	*E. creticum* concentrations (µg/mL)							
	800	400	200	100	50	25	12.5	6.25
	Viability rates (%±SD)							
L. tropica (1)	0±0.00	0±0.00	0±0.00	0±0.00	15±0.55	48±0.58	96±0.68	100±0.78
L. tropica (2)	0±0.00	0±0.00	0±0.00	0±0.00	34±0.56	75±0.61	90±0.69	100±0.74
L. major (1)	0±0.00	0±0.00	0±0.00	0±0.00	9±0.52	36±0.55	96±0.71	100±0.76
L. donovani/infantum (1)	0±0.00	0±0.00	0±0.00	20±0.55	25±0.52	46±0.55	96±0.70	100±0.76
DMSO	100±0.71							
NC	100±0.71							

*DMSO: Dimethyl sulfoxide; NC: Negative Control.

isolate, 10% in *L. major* (1) isolate, and 30% in *L. donovani/infantum* (1) isolate. In this concentration, it was statistically seen that the difference between Glucantim and *L. donovani/infantum* (1) isolate was insignificant (p>0.05).

Anti-leishmanial activity of *E. creticum* methanol extract is shown in Table 4, Figure 4. Accordingly, it is seen that no live *leishmania* isolates were found in the first four concentrations taken in the study. In addition, it was found to be 15% in *L. tropica* (1) isolate, 34% in *L. tropica* (2) isolate, 9% in *L. major* (1) isolate, 25% in *L. donovani/infantum* (1) isolate at 50 µg/mL concentration. In Glucantim, the rate was 10% in *L. tropica* (2) isolation. The difference between all isolates and Glucantim was significant when the intergroup data were statistically compared (p>0,05).

The difference between *H. thymbrifolium* and *E. creticum* at 50 µg/mL concentration in *L. major* (1) isolate was insignificant (p>0.05), while the data between other plant extracts and isolates were significant (p<0.05). At a concentration of 25 µg/mL, the difference between *H. thymbrifolium* and *E. creticum* in *L. tropica* (1) isolate, and the difference between *H. thymbrifolium* and *H. scabrum* in *L. tropica* (2) isolate was insignificant (p>0.05), while data between other plant extracts and isolates were significant (p>0.05). At a concentration of 12.5 µg/mL, while the difference between *H. thymbrifolium* and *H. scabrum* in *L. tropica* (1) isolate and the difference between *H. scabrum* and *E. creticum* was insignificant (p>0.05), the data between other plant extracts and isolates were significant (p>0.05).

Cytotoxics of plant extracts

It was determined that the working concentrations of the plant extracts we used in the study did not have any cytotoxic effects on the WI-38 human fibroblast cell line. *H. thymbrifolium*, *H. scabrum* and *E. creticum* plant extracts were found to have 95% or more of viability rates in all concentrations taken in the study.

GC-MS results

GC-MS findings of *H. thymbrifolium, H. scabrum* and *E. creticum* plant extracts are shown in Table 5-7.

Table 5. *H. thymbrifolium* GC-MS results

Components	RT	Methanol (%)
2-Propenoic acid	5.021	0.38
2-Furancarboxaldehyde	6.263	0.38
2-Furanmethanol	6.732	0.52
Thiophene	13.404	0.63
2,3-dihydro-3,5-di 4H-Pyran-4-one	16.928	5.08
Benzoic Acid	17.975	1.86
2,3-Dihydro-Benzofuran	19.011	2.79
5-Hydroxymethylfurfural	19.555	2.92
DL-Proline	25.677	2.51
Germacrene-D	29.459	0.34
Naphthalene	29.688	0.60
2(4H)-Benzofuranone	29.940	0.53
Dodecanoic acid	30.735	0.38
Spathuleno	31.113	1.14

*RT: Retention time.

Table 6. *H. scabrum* GC-MS results

Components	RT	Methanol (%)
Ethanone	14.268	0.65
Benzoic acid	15.212	2.97
4H-Pyran-4-one	17.054	1.37
Isopropyl phenyl ketone	27.542	1.74
Naphthalene	28.435	0.27
Dodecanoic acid	30.867	0.36
Spathulenol	31.147	0.33
Hexadecanoic acid	37.773	0.90
Palmitic acid	38.792	8.78
Furo[2,3-b]quinolin-4(9H)-one	40.091	1.28
4,1-herbertenolide	40.354	0.46
9-Octadecenoic acid	40.600	0.79
9,12-Octadecadienoic acid	41.527	1.70
(1RS,2SR)-2-methyl-2-4'-methylpen-1-carbaldehyde	42.116	2.01
trans-Geranylgeraniol	43.587	0.73
Tetracosane	46.093	0.65
Geranyl-linalol	46.242	0.78
Octadecane	49.137	0.28
Ent-beyer-15-en-18-ol	57.383	1.25

*RT: Retention time.

Table 7. *E. creticum* GC-MS results

Components	RT	Methanol (%)
Acetic acid	2.582	5.57
2-Tetradecene	16.355	0.63
1-Tridecene	16.355	0.63
2-Nitrocyclododecanone	16.767	0.56
2-Methylenecyclohexane	16.767	0.56
3-Hexadecene	22.632	0.64
1-Pentadecene	22.632	0.64
1-Hexadecene	22.632	0.64
2(3H)-Furanone	24.720	8.23
Decalactone	24.720	8.23
Beta-Selinene	25.207	9.50
Spathulenol	27.730	1.68
-Caryophyllene oxide	27.879	0.83
Chloroacetic acid	28.016	0.76
1H-Indene	29.601	0.74
Beta-Eudesmol	29.601	0.74
Caryophyllenol I	30.334	0.51
Pyridine	30.912	0.63
1H-Pyrrole-2,5-dione (Maleimides)	31.633	0.58
Benzoic acid	31.684	0.88
trans-Z-alpha-Bisabolene epoxide	31.827	2.38
2-Octynoic acid	33.235	0.44
2-Pentadecanone	33.950	2.46
Hexadecanoic acid	36.376	1.30
Pentafluoropropionic acid	38.259	2.19
9-Octadecenamide	40.387	1.19
2(1H)-Naphthalenone	43.357	0.36
Eicosane	46.807	1.48
Tricosane	46.807	1.48

*RT: Retention time.

DISCUSSION

Leishmaniosis is a vector-mediated disease caused by protozoan parasites belonging to the genus *Leishmania*. An estimated 700 000 to 1 million new cases occur annually according to World Health Organization reports (WHO, 2023). With the increase of people's travel to endemic regions and the increasing number of migrants from Syria, CL has started to be seen in non-endemic regions in Turkey. In this respect, leishmaniosis is now an important health problem for non-endemic regions (Inci *et al*., 2015; Korkmaz *et al*., 2015). The development of resistance to the drug of *leishmania* parasites and insecticides used in vector control of sand flies greatly reduces the chances of treatment of the disease. In addition, many vertebrate animals are reservoir hosts for the disease, and climate change, travel to endemic regions and migrations increase the prevalence of the disease. Considering these conditions, it is seen that researchers have been focusing on new treatment approaches and vaccine studies for leishmaniasis especially in recent years (Kumar, 2013).

Leishmaniasis has long been treated with antimonial drugs, first trivalent antimonials and later with the less toxic pentavalent antimonials. The limited arsenal of available anti-leishmanial therapies coupled with the lack of an effective human vaccine make antimonials a first-line treatment still today despite their harmful side effects (Papadopoulou *et al*., 1994; Ouellette *et al*., 2004). Resistance has become rampant in the field, rendering these drugs virtually ineffective against visceral leishmaniasis in some regions of the world (Leprohon *et al*., 2015; Rijal *et al*., 2003). In a study, resistance was investigated in 20 CL patients who did not respond to meglumine antimoniate treatment, and a 40% rate of resistant leishmanial isolates was detected under *in vitro* conditions (Rojas *et al*., 2006). In another study, *Leishmania donovani* clinical isolates were characterized in vitro for their sensitivity to sodium stibogluconate within a macrophage culture system. The study revealed that 78% of patient isolates exhibited varying degrees of resistance (Mukhopadhya *et al*., 2011). In our study, resistance was detected in 4 (57%) out of 7 clinical isolates. It is known that the development of resistance can vary depending on the *Leishmania* species and the region where the patient is located. Despite limited data in the literature, high resistance rates have been reported, and our findings are consistent with the literature.

Throughout history, humans have used many methods for the treatment of diseases by using natural resources and especially plants in line with the knowledge they learned from their ancestors. No matter how much medicine has improved in recent years, this tradition continues, especially in developing societies, due to easy transportation and economic reasons. Today, the location of herbal treatments is too large to ignore. For this reason, many researchers have sought new molecules of vegetable origin. Plants are an important source of molecules with anti-leishmanial activity. In recent years, herbal sources with anti-leishmanial activity are quite common (Sinha *et al*., 2000; Proulx *et al*., 2001; Lala *et al*., 2006; Georgopoulou *et al*., 2007).

In our study, the effects of *H. thymbrifolium, H. scabrum* and *E. creticum* plant extracts on *L. major, L. tropica* and *L. infantum/ donovani* isolates, which were found clinically resistant by not responding to the treatment of organic 5 valuable pentavalan antimony (Glucantim) compounds, were tested and new active ingredients of possible effect were identified by GC-MS analysis. Our study findings showed that all three plant extracts exhibited strong anti-leishmanial activity on all isolates. The anti-leishmanial drugs used today are highly toxic. It is an important finding that the plant extracts we used in the study did not have any cytotoxic effect on the WI-38 human fibroblast cell line in the study concentrations.

Species of the genus *Hypericum* have traditionally been used as antiseptic, diuretic, wound healing and antimicrobial agents in different parts of the World (Von Poser *et al*., 2006). In addition, it has been detected by researchers that *H.scabrum* and *H. polyanthemum* have anti-trichomonal activity on *Trichomonas vaginalis* protozoan (Cargnin *et al*., 2013; Ozpinar *et al*., 2020b). Furthermore, studies have shown that *H. lanceolatum* and *H. erectum* exhibit significant anti-plasmodial activity (Moon, 2010; Zofou *et al*., 2011), while *H. perforatum* samples show mild inhibitory activity against *Trypanosoma rhodesiense* protosoons (Orhan *et al*., 2013). Another study was conducted with 8 hypericum species and mentioned the anti-leishmanial effects of *H. carinatum*, *H. linoides* and *H. polyanthemum* species (Dagnino *et al*., 2015). In our study, *H. thymbrifolium*, *H. scabrum* species were studied with Glucantim-resistant leishmanias and strong anti-leishmanial activity was detected.

In a study conducted with methanol extract of the aerial part of *Eryngium foetidum* species, the presence of anti-leismanial effects on *L. tarentolae* promastigotes and *L. donovani* amastigotes was mentioned (Rojas-Silva *et al*., 2014). In another study, inhibition of *Eryngium thorifolium* on *L. tropica* promasticots was mentioned. The methanol extract of *Eryngium thorifolium* was found to have the highest activity among the other plant extracts studied on the promastigotes of *L. tropica* with 100% inhibition at a concentration of 25 µg/mL (Ozbilgin *et al*., 2014).

The extracts of aerial parts of *Eryngium creticum* from West Crete have been investigated for *in vitro* antiprotozoal activity. An evaluation of their activity was performed against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* and *L. donovani* promastigotes. The extracts were tested against promastigote cultures of *L. donovani*. IC50 (the concentration causing 50% of cell deaths) was 35 µg ml^{-1} and 38 µg ml^{-1} for methanolic extract and dichloromethanolic extract, respectively (Fokialakis *et al*., 2006).

In our study, it was observed that plant extract of *Eryngium creticum* has a strong anti-leishmanial effect in all four leishmania isolates.

When the GS-MS findings were examined, it was determined that plant extracts contained active substances with many biological activities. Thiophene's anti-leishmanial activity, one of the substances detected in the *H. thymbrifolium* extract, has been mentioned in previous studies. Researchers have found that some compounds derived from thiophene show anti-leishmanial activity 10 times more effective than pentamidine (Savornin *et al*., 1991). In addition, some other studies have shown that thiophene exhibits strong anti-leishmanial activity (Takahashi *et al*., 2011; Rodriguez *et al*., 2018). Another study mentioned the effects of Germacrene-D on leishmania strains (Moreira *et al*., 2019). When the results of *H. scabrum* GC-MS were examined, seven components were isolated from the stems and leaves of the *Croton lobatus* plant in a study and their anti-plasmodial activity was tested. One of these compounds is the trans-Geranylgeraniol compound, which was also detected in our study, and its anti-plasmodial activity has been detected by researchers (Attioua *et al*., 2007). When the findings of *E. creticum* GC-MS shown in Table 7 are examined, the larvacide effects of Caryophyllene oxide on anopheline mosquitoes larvae (Moussavi *et al*., 2015) and the anti-trypanosomal effect of the same compound on *Trypanosoma* protosoons (Moreno *et al*., 2018) were mentioned. In addition, many studies mentioning anti-leishmanial activities in substances derived from pyridine and pyridine are also available in the literature (Vieites *et al*., 2009; Caballero *et al*., 2012; Castera-Ducros *et al*., 2013; De Oliveira *et al*., 2020). In another study, it was mentioned that the maleimide compounds detected in our study are highly bioactive compounds and are compounds with many biological activities. Furthermore, the study investigated the anti-leishmanial activity of maleimides and concluded that some maleimids synthesized in the study could be developed as anti-leishmania drugs in the future (Fan *et al*., 2018).

CONCLUSION

In recent years, there has been a significant increase in CL cases worldwide and the currently used anti-leishmanial drugs are highly toxic. Therefore, many researchers are conducting studies to find new active compounds with strong anti-leishmanial activity and non-toxicity to human cells. In our study, we specifically used resistant leishmania isolates and a potent anti-leishmanial effect was observed against the selected leishmania isolates. According to the GC-MS analysis, several active compounds with biological activities and anti-parasitic effects, such as Thiophene, Germacrene-D, trans-Geranylgeraniol, Pyridine and Maleimides were identified. Although only a small fraction of these compounds has been reported in the literature for their anti-leishmanial activity, our study findings suggest that these identified active compounds may lead to further investigations with *in-vivo* studies.

Conflicts of interest

The authors declare no financial, or otherwise, conflicts of interest.

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