

## ***In vitro* anti-leishmanial activity of *Sarcopoterium spinosum* against *Leishmania tropica***

**Hüseyin CAN<sup>1</sup>, Hüsnüye KAYALAR<sup>2</sup>, Buket BOZKURT<sup>2</sup>, Şengül CAN<sup>3</sup>, Mert DÖŞKAYA<sup>4</sup>, Seray TÖZ<sup>4</sup>**

<sup>1</sup>Ege University, Faculty of Science, Department of Biology, Molecular Biology Section, İzmir; <sup>2</sup>Ege University, Faculty of Pharmacy, Department of Pharmacognosy, İzmir; <sup>3</sup>Celal Bayar University, Faculty of Business, Department of Business, Operations Management-Marketing Section, Manisa; <sup>4</sup>Ege University, Faculty of Medicine, Department of Parasitology, İzmir, Turkey.

**Abstract:** Complex clinical symptoms such as ulcerative skin lesions, destructive mucosal inflammation, and disseminated visceral infection can reveal in leishmaniasis. The conventional drugs are toxic and expensive. In addition, patients receive a long treatment with these drugs which have adverse effects and unfortunately there are some limitations during the treatment. The aim of this study is to investigate the *in vitro* anti-leishmanial activities of four different extracts of *Sarcopoterium spinosum* against *Leishmania tropica*. Initially, different concentrations of ethanol, methanol, n-hexane, and water extracts of *S. spinosum* were incubated with *L. tropica* promastigotes. After 72 hours of incubation, the growth of *L. tropica* promastigotes was significantly inhibited and the percentage of inhibition ranged between 42.8 and 100 %. Among these extracts, the most efficient growth inhibition (100 %) was obtained with methanol extract (at a dose of 50 µg/ml). In conclusion, *S. spinosum* may be a potential source for the development of novel therapeutic agents to treat *L. tropica* infection.

Keywords: Anti-leishmanial activity, *in vitro*, *Leishmania tropica*, *Sarcopoterium spinosum*.

### ***Sarcopoterium spinosum*'un *Leishmania tropica*'ya karşı *in vitro* anti-leishmanial etkisi**

**Özet:** Leishmaniasis'de ülseratif cilt lezyonları, yıkıcı mukozal inflamasyon ve dissemine visceral enfeksiyon gibi kompleks klinik semptomlar ortaya çıkabilmektedir. Konvansiyonel ilaçlar toksik ve pahalıdır. Bunun yanında, hastalar yan etkilere sahip bu ilaçlar ile uzun süre tedavi edilmekte ve maalesef tedavide bazı kısıtlılıklar bulunmaktadır. Bu nedenle, bu çalışmada *Sarcopoterium spinosum*'un dört farklı ekstresinin *L. tropica*'ya karşı *in vitro* anti-leishmanial etkilerinin araştırılması amaçlanmıştır. İlk olarak, farklı konsantrasyonlardaki *S. spinosum*'un etanol, metanol, n-hekzan ve su ekstraktları *L. tropica* promastigotları ile inkübe edilmiştir. İnkübasyonun 72 saat sonrasında, tüm ekstraktlar *L. tropica* gelişimini %42.8 ve %100 arasındaki değişen oranlarda önemli derecede inhibe etmiştir. Ekstreler arasında, en etkili gelişim inhibisyonu (%100) metanol ekstresi (50 µg/ml) ile elde edilmiştir. Sonuç olarak, *S. spinosum*, *L. tropica* enfeksiyonunu tedavi etmek için yeni teröpatik ajanların geliştirilmesinde potansiyel bir kaynak olabilecektir.

Anahtar sözcükler: Anti-leishmanial etki, *in vitro*, *Leishmania tropica*, *Sarcopoterium spinosum*.

### **Introduction**

More than 20 species of genus *Leishmania* which cause leishmaniasis in human and other mammals are transmitted through the bite of infected female phlebotomine sand flies. Leishmaniasis is endemic in 102 countries/regions and approximately 350 million people living in this geography are under the risk of leishmaniasis (26).

In Turkey, prevalent *Leishmania* species are *Leishmania infantum* and *Leishmania tropica*. *L. infantum* causes human visceral leishmaniasis (HVL) and canine leishmaniasis (CanL) (12, 15). On the other hand, anthroponotic cutaneous leishmaniasis (ACL) is mainly caused by *L. tropica* and lately, it has been showed that *L. infantum* is also responsible for ACL (22, 18, 8).

Complex clinical symptoms such as ulcerative skin lesions, destructive mucosal inflammation, and disseminated visceral infection can appear in leishmaniasis (1). The first recommended treatment for all forms of leishmaniasis is pentavalent antimony but it has some adverse effects such as anorexia, myalgia, arthralgia, chemical pancreatitis, leucopenia, and cardiotoxicity (11). In addition to side effects, it is reported that in some cases, this drug gives rise to drug-resistant parasites when used in high doses (2-3). The second option is the use of amphotericin B and pentamidine although they have considerable degree of toxicity (16).

Overall, the conventional drugs are toxic, expensive and availability is low in developing countries. Besides, patients receive a long treatment with these drugs which

have adverse effects (3). These reasons are sufficient to investigate the efficiency of new natural therapeutic agents having less toxicity (4, 9). Moreover, the World Health Organization (WHO) underlines that plants used in traditional medicine should primarily be investigated against leishmaniasis (25).

Different parts of *Sarcopoterium spinosum* have traditionally been used as a medicinal plant for the treatment of diabetes, digestive problems, and cancer or to relieve pain among the people (20). In Turkey, *S. spinosum* spreads naturally in Çanakkale, İstanbul, Sinop, İzmir, Aydın, and Adana regions. Phytochemically, the aerial and underground parts of *S. spinosum* were reported to contain triterpenoids such as ursolic acid, tormentic acid, and sitosterol while the leaves were reported to have carotenoids, flavonoids, and its derivatives such as catechin, epicatechin, quercitrin, quercetin, and hyperoside (17, 19, 24).

The present study aimed to investigate the *in vitro* anti-leishmanial activities of four different extracts of *S. spinosum* against *L. tropica* in comparison with glucantime which is the reference drug for the treatment of leishmaniasis. Due to wide variations in chemical structure and polarities of compounds present in *S. spinosum*, in the present work, solvents having different polarities were chosen for the extraction of secondary metabolites.

### Material and Methods

*Plant material and preparation of extracts:* *Sarcopoterium spinosum* was collected from Seferihisar town of İzmir located in western Turkey. The plant was taxonomically identified and deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, İzmir, Turkey.

Aerial parts of the plant material were cut into small pieces, dried at room temperature, and powdered in a grinder. The powdered material was then used in extraction. Ethanol, methanol, n-hexane, and water were used for the preparation of plant extracts. Briefly, ethanol, methanol, and n-hexane extracts were prepared by maceration technique in which 5 g of the powdered plant material was dissolved in 50 ml solvent at room temperature. Water extracts were prepared by 2% infusion and all extraction solvents were filtered and evaporated under reduced pressure in a rotary evaporator (Buchi, Germany). The extracts were lyophilised and stored at +4 °C until use (14).

*Cultivation of parasite:* *Leishmania tropica* promastigotes (MHOM/TR/2004/EP95) isolated from Turkey were grown in NNN medium and then cultured in RPMI-1640 medium (Biochrom, Germany) supplemented with 10% fetal calf serum (FCS, Sigma-Aldrich, Germany) as described (14).

*Anti-leishmanial assays:* For *in vitro* testing, 25 µg/ml; 50 µg/ml; 100 µg/ml; 200 µg/ml; 400 µg/ml concentrations of ethanol, methanol, n-hexane, and water extracts of *S. spinosum* were prepared. All extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) at a final concentration of 0.5% (v/v) that does not affect parasite growth rate, mobility or morphology, and diluted in RPMI medium supplemented with 10% FCS (27).

*Leishmania tropica* promastigotes in the logarithmic growth phase were transferred to 24-well plate ( $10^6$  parasites/well) containing RPMI medium supplemented with 10% FBS. Thereafter, different concentrations of *S. spinosum* extracts were added to each well and incubated at 25°C. As a reference drug, glucantime prepared in DMSO (25 µg/well) was used. For untreated groups, two wells were used. One of them contained DMSO (25 µg/well) in RPMI medium with 10% FBS and promastigote ( $10^6$  parasites/well) while the other contained only RPMI medium with 10% FBS and promastigote ( $10^6$  parasites/well). The number of parasites was counted with a hemocytometer under a light microscope at 12<sup>th</sup>, 24<sup>th</sup>, and 72<sup>th</sup> hours. All *in vitro* experiments were run in triplicate and the results were expressed as mean percent of inhibition in the number of parasites (13).

*Phytochemical analysis:* Standard screening tests were performed on each extracts. The extracts were analysed for the presence of flavonoids, tannins, triterpenoids and alkaloids using routine protocols (23).

*Statistical analysis:* Data obtained from this study were processed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). IC<sub>50</sub> was calculated by nonlinear regression method with 95% confidence interval. A two-tailed unpaired t test and analysis of variance (ANOVA) with 95% confidence interval were used to determine the significance between the results of assays. All *P-values* <0.05 were considered statistically significant.

### Results

*Phytochemical analysis:* N-hexane, methanol, ethanol, and water extracts exhibited positive results for flavonoids, tannins, and triterpenoids. No extracts showed positive result for alkaloids.

*In vitro anti-leishmanial activity:* The results obtained at 12<sup>th</sup> and 24<sup>th</sup> hours were not statistically significant compared to control group. Also, there was no difference between the amounts of promastigotes in untreated groups. All plant extracts had inhibitory activity against *L. tropica* promastigotes after 72 hours compared to negative control even at concentration of 25 µg/ml (*P*=0.0078). Percent inhibition values among different extracts were compared to each other and statistically

significant difference was observed between extract concentrations ( $P < 0.05$ ). Accordingly, when we compare the extract groups with each other, statistically significant inhibition was detected in the groups of promastigotes treated with ethanol (25  $\mu\text{g/ml}$ ,  $P = 0.04$ ; 200  $\mu\text{g/ml}$ ,

$P = 0.006$ ), methanol (50  $\mu\text{g/ml}$ ,  $P = 0.006$ ; 100  $\mu\text{g/ml}$ ,  $P = 0.005$ ; 200  $\mu\text{g/ml}$ ,  $P = 0.006$ ), and n-hexane (200  $\mu\text{g/ml}$ ,  $P = 0.006$ ) extracts. The inhibition percentages of all extracts and  $\text{IC}_{50}$  values at 72<sup>th</sup> hour are comparatively given in Table 1 and Figure 1.

Table 1. *In vitro* efficacy of *Sarcopoterium spinosum* extracts at 72<sup>th</sup> hour.

Tablo 1. *Sarcopoterium spinosum* ekstrelerinin 72. saatteki *in vitro* etkinliği.

Sample	$\text{IC}_{50}$ ( $\mu\text{g/ml}$ )	Doses ( $\mu\text{g/ml}$ )	% Inhibition	*P-value
Ethanol extract	10.2	25	71.5	0.002
		50	71.5	
		100	71.5	
		200	100	
		400	100	
Water extract	28.4	25	42.8	0.025
		50	71.5	
		100	71.5	
		200	71.5	
		400	100	
Methanol extract	25.2	25	42.8	0.008
		50	100	
		100	100	
		200	100	
		400	100	
N-hexane extract	80.2	25	42.8	0.020
		50	42.8	
		100	71.5	
		200	100	
		400	100	
Glucantime		25	100	
**Untreated control groups		-	0	

\*P-values were obtained by comparing the inhibitory effects of different *S. spinosum* extracts against *L. tropica* promastigotes with negative control groups.

\*\*There was no difference between the amounts of promastigotes in untreated control groups.

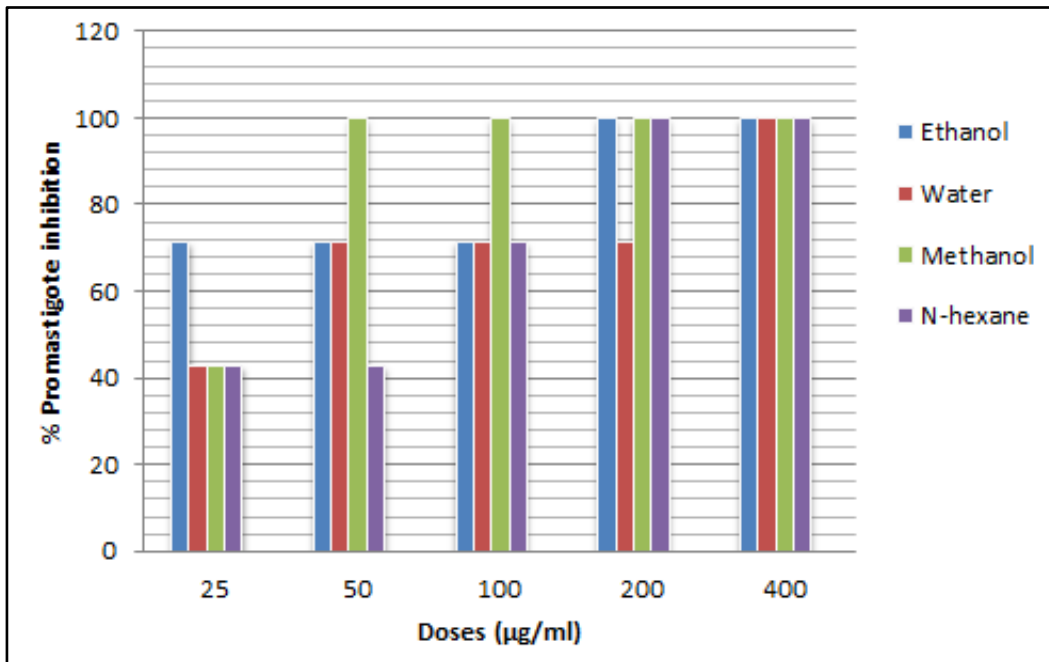


Figure 1. Comparing percent inhibition values of four different extract obtained from *S. spinosum*.

Şekil 1. *S. spinosum*'dan elde edilen dört farklı ekstreinin yüzde inhibisyon değerlerinin karşılaştırılması.

Among extracts having a concentration of 25 µg/ml, the highest percent inhibition (71.5%) on parasite growth was observed in ethanol extract. Also, methanol extract at a concentration of 50 µg/ml showed 100 % inhibition on parasite growth. When we analyzed the results of all extracts obtained at a concentration of 100 µg/ml, the percent inhibition didn't change except the n-hexane where the percent inhibition increased to 71.5%. Ethanol, methanol and n-hexane extracts at concentrations of 200 and 400 µg/ml showed 100% inhibition while water extract had only 100% inhibition at concentrations of 400 µg/ml. The reference drug glucantime had 100 % inhibition on parasite growth after 24-48 hours of incubation.

IC<sub>50</sub> values for ethanol, water, methanol, and n-hexane extracts were 10.2, 28.4, 25.2, and 80.2, respectively (Table 1).

### Discussion and Conclusion

The plants can be defined as an enormous source of compounds which can be used as drugs in treating various diseases. *Sarcopoterium spinosum* is traditionally used to treat various diseases and is so abundant in the Mediterranean region including Turkey (21). The root part of *S. spinosum* is reported to be used as an anti-diabetic drug in Arab folk medicine. Also, it is used in digestive problems and to relieve pain (20).

In this study, we investigated the *in vitro* anti-leishmanial activities of four different extracts of *S. spinosum*. The results showed that after 72 hours of incubation, all extracts significantly ( $P < 0.0001$ ) inhibited the growth of *L. tropica* promastigotes with a range between 42.8 - 100% compared to negative control group. Among all extracts, 100% parasite growth inhibition was first observed for the methanol extract at a concentration of 50 µg/ml with an IC<sub>50</sub> value of 25.2 µg/ml. The ethanol extract, having an IC<sub>50</sub> value of 10.2 µg/ml, was found to be the most effective extract with 71.5% inhibition even at a concentration of 25 µg/ml. Likewise, *S. spinosum* was previously reported to have an inhibitory effect on promastigotes of *Leishmania donovani*, the causative agent of human visceral leishmaniasis. Moreover, anti-malarial activity of *S. spinosum* against *Plasmodium falciparum* was also reported in the same study. Another significant outcome was the non-cytotoxicity of methanol and dichloromethane extracts of *S. spinosum* in mammalian cell culture (6).

A lower anti-leishmanial activity of methanol extract of *S. spinosum* at a concentration of 200 µg/ml against promastigotes of *Leishmania major*, the causative agent of zoonotic cutaneous leishmaniasis was also reported. This extract was evaluated as inactive against *L. major* (5). Contrary to this, methanol extract of *S. spinosum* at a concentration of 50 µg/ml showed 100% inhibitory

activity against *L. tropica* promastigotes in the present study. The lack of consistency among studies may be due to the use of different *Leishmania* species. Also, it is known that *L. tropica* and *L. major* can show different clinical courses. Thus, depending on *Leishmania* species the efficiency of drugs can also change.

Extracts prepared from different plants such as *Casearia sylvestris*, *Piptocarpha macropoda*, *Trembleya parviflora*, *Samanea tubulosa*, and *Plectranthus neochilus* with IC<sub>50</sub> values below 20 µg/mL were found to have promising anti-leishmanial activity against different *Leishmania* species (1). Based on this finding, ethanol extract of *S. spinosum* having an IC<sub>50</sub> value below 20 µg/mL has a potential to be used as an alternative and less toxic drug against promastigotes of *L. tropica*.

Preliminary phytochemical analysis revealed that secondary metabolites such as flavonoids, tannins, and triterpenoids were present in the extracts. Among them, it was previously reported that flavonoids and triterpenoids had anti-leishmanial activity without significant toxicity to mammalian cells (7). In addition, flavonoids have been shown to have anti-protozoal effects by inhibiting synthesis of heat shock proteins (Hsp90, Hsp70 and Hsp27) (7). On the other hand, it was shown that tannins had inhibitory effect against *L. donovani* promastigotes (10). Thus, the inhibitory effect of the extracts used in this study could be due to presence of wide range of secondary metabolites.

In conclusion, the results obtained in this study showed that the ethanol extract of *S. spinosum* with lowest IC<sub>50</sub> value, may be a potential source for the development of novel, natural, and less toxic therapeutic agent for the treatment of *L. tropica* infection. Further analysis focusing on testing the efficiency of *S. spinosum* in animal models and conducting bioactivity guided assays to identify the active constituents and mode of action needs to be performed.

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- Address for correspondence:**  
 Assoc. Prof. Dr. Hüseyin CAN  
 Ege University, Faculty of Science, Department of Biology,  
 Molecular Biology Section, 35040-Bornova/İzmir, Turkey.  
 e-mail: huseyin.can@ege.edu.tr