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Research Article

In Vitro Effect of *Pelargonium sidoides* on Promastigote Forms of *Leishmania infantum* and *Leishmania tropica*

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Abstract

Leishmaniasis is recognized as a neglected disease by the World Health Organization (WHO). New treatment modalities are needed for the treatment of leishmaniasis due to the limited number of drugs that can cause toxic side effects. Therefore, studies are being carried out on herbal extracts, which can be potential candidates for the treatment. *Pelargonium sidoides* a perennial herb originating in Africa, is used to treat infectious diseases. The aim of this study was to perform *in vitro* investigation of the direct effect of *P. sidoides* commercially available root extract (EPs 7630) on promastigotes of *Leishmania infantum* and *Leishmania tropica.* For this purpose, *L. infantum* and *L. tropica* strains were grown on NNN medium and then transferred into RPMI 1640 medium supported by 10% fetal bovine serum. After mass growing, the promastigotes were placed into 96-well plates with *L. infantum* as $5x10^4$ and L. tropica as $1.5x10^5$. EPs 7630 was diluted at a concentration of 400, 200, 100 and 50 µg/mL. Afterwards, EPs 7630 was added and then counted by hemocytometry at 24, 48, 72, and 96 h. The calculations were done after the experiments repeated three times. Comparison with the control group and liposomal amphotericin B showed that EPs 7630 had no inhibitory effect on the growth of *Leishmania* promastigotes at the concentrations of 50 and 100 µg/mL, a partial inhibitory effect at 200 µg/mL, and an inhibitory effect at 400 µg/mL. It was concluded that identifying the substance(s) responsible for the antileishmanial effect of *P. sidoides* extract, conducting toxicity studies, and improving the results of these studies in *in vivo* models may be useful as steps for future clinical studies.

Keywords: *Pelargonium sidoides, Leishmaniasis, in vitro, Antileishmanial agent*

Pelargonium sidoides'in *Leishmania infantum* ve *Leishmania tropica* Promastigot Formlarına *İn Vitro* Etkisi

Öz

Leishmaniasis Dünya Sağlık Örgütü (DSÖ) tarafından ihmal edilmiş bir hastalık olarak kabul edilmektedir. Leishmaniasis tedavisinde kullanılan ilaçların sınırlı, maliyeti yüksek, toksik ve yan etkileri bulunması sebebiyle yeni tedavi yöntemleri geliştirilmeye ihtiyaç duyulmaktadır ve bu kapsamda bitkisel ekstraktlar üzerinde çalışmalar yapılmaktadır. Afrika kökenli çok yıllık bir bitki olan *Pelargonium sidoides* birçok hastalığı tedavi etmek için kullanılmaktadır. Bu çalışmada *Leishmania infantum* ve *Leishmania tropica* promastigotları üzerinde ticari olarak mevcut *P. sidoides* kök ekstraktının (EPs 7630) doğrudan etkinliğinin *in vitro* olarak araştırılması amaçlanmıştır. Bu amaçla *L. infantum* ve *L. tropica* suşları NNN besiyerinde üretildikten sonra %10 fetal sığır serumu eklenen RPMI 1640 besiyerine aktarıldı. Çoğaltıldıktan sonra promastigotlar, *L. infantum* için $5x10^4$ ve *L. tropica* için $1.5x10^5$ olacak şekilde 96 oyuklu plakalara yerleştirildi. Daha sonra, EPs 7630'un 400, 200, 100 ve 50 µg/mL konsantrasyonları ile 24, 48, 72 ve 96 saat inkübe edildi. EPs 7630'un *L. infantum* ve *L. tropica* suşlarının promastigotları üzerine etkinliği hemositometri yöntemi ile sayılarak belirlendi. Hesaplamalar, deneyler üç kez tekrarlandıktan sonra yapıldı. Kontrol grubu ve lipozomal amfoterisin B ile karşılaştırıldığında EPs 7630'un 50 ve 100 µg/mL konsantrasyonlarda *Leishmania* promastigot üremesi üzerinde inhibe edici etkisinin olmadığı, 200 µg/mL'de düşük inhibitör etkili olduğu ve 400 µg/mL'de etkili olduğu saptanmıştır. EPs 7630'un anti-leishmanial etkisinden sorumlu madde veya maddelerin incelenmesi, toksisite çalışmalarının yapılması ve bu çalışma sonuçlarının *in vivo* modellerle geliştirilmesinin klinik çalışmalara basamak olması açısından yararlı olabileceği düşünülmüştür.

Anahtar sözcükler: *Pelargonium sidoides, Leishmaniasis, in vitro, Anti-leishmanial ajan*

Introduction

Leishmaniasis is a disease caused by protozoan parasite *Leishmania* spp., which is transmitted by the bite of the insect vector female sand fly *(Phlebotomus* spp./*Lutzomyia* spp.). Leishmania is a genus in the order of Kinetoplastida and in the family of Trypanosomatidae [1,2]. Leishmaniasis is seen in 97 countries in the tropical and subtropical regions

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of America, Africa, Asia, and Europe [3]. There are four main clinical forms of leishmaniasis: Visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and Post-Kala-Azar Dermal Leishmaniasis (PKDL) [4]. It is estimated that there are seven hundred thousand to 1.5 million new cases of leishmaniasis in the world each year causing 26.000 to 65.000 deaths [5]. Leishmaniasis is accepted as a neglected tropical disease by World Health Organization (WHO) [6]. Pentavalent antimony compounds, amphotericin B and liposomal amphotericin B, miltefosine, paromomycin, and pentamidine are used in the treatment of different clinical forms of leishmaniasis [7]. Today, antimony compounds accepted as the gold standard in the treatment of leishmaniasis [8]. The very limited number of drugs and the development of resistance pose big problems in the treatment of leishmaniasis. New treatment modalities are being investigated because of the toxicity potential, high cost, and drug resistance of existing drugs [9]. Therefore, in recent years, herbal extracts have been emphasized as an alternative treatment option [10].

In this study, we aimed to conduct an *in vitro* investigation to understand the efficacy of *Pelargonium sidoides* root extract (EPs 7630) on promastigotes of *Leishmania infantum* and *Leishmania tropica*.

Material and Methods

Supply and Cultivation of Leishmania spp. Strains

In this study, *Leishmania infantum* strain (confirmed patient's isolate) was obtained from the National Parasitology Reference Laboratory of the General Directorate of Public Health belonging to Turkish Ministry of Health and *L. tropica* strain (EP 200) from Ege University Faculty of Medicine, Department of Medical Parasitology.

The NNN medium was prepared with a mixture of 3.5 g Bacteriological Agar (Oxoid, UK) and 2.5 g sodium chloride (Carlo Erba Reagents, Italy) in 230 mL distilled water in a 500 mL bottle and then heated to dissolve the agar. The solution was sterilized for 20 min at 121°C in an autoclave device (ALP, Japan) and cooled to 55°C in a hot water bath (Nüve ST30, Turkey). Then, 0.6 mL of penicillin G, 0.6 mL streptomycin sulfate, and 70 mL of defibrinated horse blood, which was aseptically collected, were added to the medium. Thus, final concentrations were penicillin G 200 IU/mL and streptomycin sulfate 200 µg/mL in the medium. The medium was then dispensed in a volume of 4.5 mL into sterilized conical centrifuge tubes (ISOLAB GmbH, Germany), and the medium was allowed to freeze in a 10° inclined position and stored at +4°C.

The *Leishmania* strains supplied were inoculated on NNN Medium and left to incubate at 24°C. After mass growing of promastigotes, they were transferred from NNN medium to cell culture flasks containing RPMI 1640 (Sigma R8755-1L, Germany) supported by 10% Fetal Bovine Serum, HEPES and 80 µg/mL gentamicin. Flasks were checked for viability of promastigotes under an inverted microscope (Leica S40/0.45, Germany). It was observed that the *L. infantum* promastigotes reached the logarithmic phase on the third day and *L. tropica* promastigotes on the second day. Accordingly, *L. infantum* promastigotes were included in the study on the third day and *L. tropica* promastigotes on the second day.

Prior to the study, the initial promastigote counting was done on a Thoma counting slide (Witeg, Germany). Thus, using the Hemocytometer Sidekick application, *L. infantum* was counted as $5x10^4$ cells and *L. tropica* as $1.5x10^5$ cells. The IC_{50} value was calculated using AAT Bioquest software *(https://www.aatbio.com/tools/ic50 calculator).*

Herbal Extract

In this study, the EPs 7630 (Dr. Willmar Schwabe, GmbH&Co. KG, Germany) standard solution was used as a root liquid extract of *P. sidoides*. The main stock solution at a concentration of 800 µg/mL was diluted into herbal extract solutions at 400, 200 and 100 and 50 µg/mL concentrations. Lipo-somal Amphotericin B (L-AmB) (Gilead Sciences, Inc., USA), known to be effective on *Leishmania* species, was used as a positive control in the present study. The dilution was made in conical centrifuge tubes to prevent *Leishmania* promastigotes from being damaged during pipetting.

In this study, 96-well flat-bottom cell culture plates (Deltalab S.L., Spain) were used. Nine study groups were designed. Firstly, 100 µL of media containing promastigotes from previously prepared cell culture flasks were placed in all wells. Different concentrations of *P. sidoides* and L-AmB were added to each well based on the study groups. Only RPMI 1640 medium was added to control-1 group and a solvent to control-2 group. The distribution and quantities among the groups are summarized in *Table 1*.

The cell culture plates were wrapped in a plastic film (Parafilm, 3M) and left to incubate in a 24°C incubator (Nüve N500, Turkey).

In this study, *P. sidoides* herbal extract was studied in triplicate for all groups on *L. infantum* and *L. tropica* strains.

Hemocytometry

Samples at a volume of 10 µL were taken from each well at 24, 48, 72, and 96 h, respectively, from cell culture plates incubated at 24°C were counted on Thoma chamber (Witeg, Germany) and calculated and recorded using the Hemo-cytometer Sidekick application. Comparison of efficiency between groups was performed using one-way analysis of variance (ANOVA).

Table 1. *Distribution of concentrations of P. sidoides, L-AmB, and solvent by groups*

Groups		*Leishmania infantum* (Initial count: 5x10⁴)	*Leishmania tropica* (Initial count: 1.5x10⁵)
1	Ps	50 µg/mL	50 µg/mL
2	Ps	100 µg/mL	100 µg/mL
3	Ps	200 µg/mL	200 µg/mL
4	Ps	400 µg/mL	400 µg/mL
5	Control 1	Promastigote medium	Promastigote medium
6	Control 2	Promastigote medium + solvent (11 µL/mL 70% ethanol)	Promastigote medium + solvent (11 µL/mL 70% ethanol)
7	L-AmB	12.5 µg/mL	12.5 µg/mL
8	L-AmB	25 µg/mL	25 µg/mL
9	L-AmB	50 µg/mL	50 µg/mL

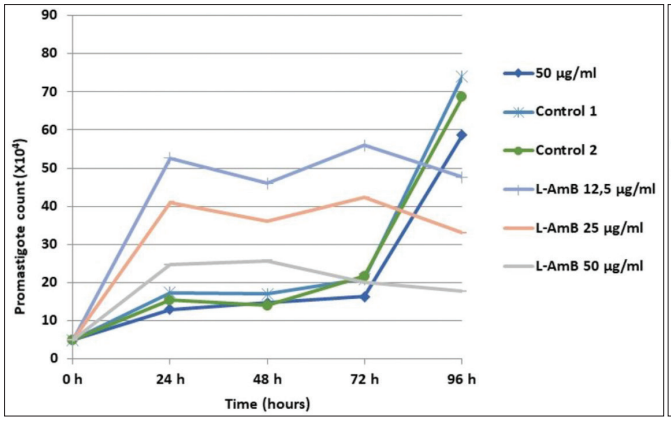

Fig 1. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 50 µg/mL

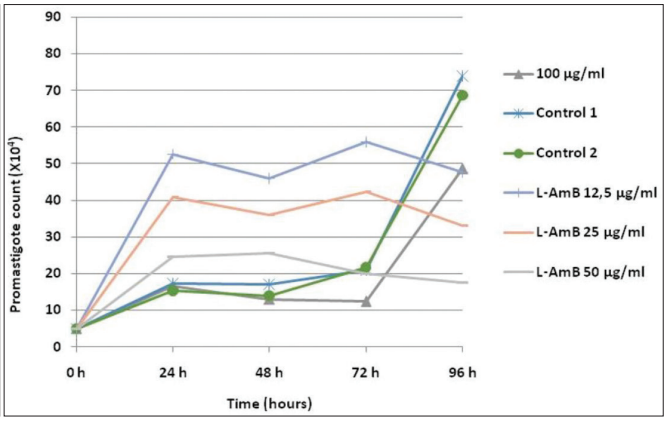

Fig 2. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 100 µg/mL

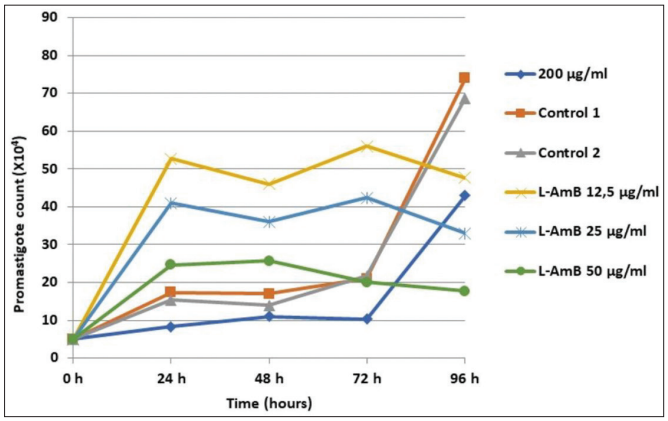

Fig 3. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 200 µg/mL

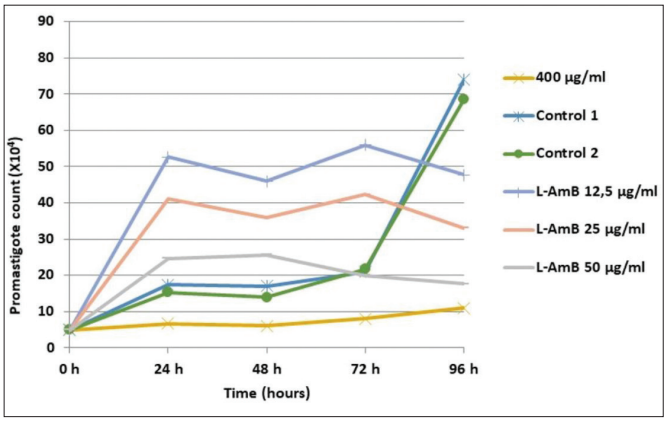

Fig 4. The effects of EPs 7630 on *Leishmania infantum* promastigotes at 400 µg/mL

Results

Samples taken from the wells at 24, 48, 72, and 96 h were counted, followed by determination of the growth rates.

At the end of 96 h of incubation at 24°C, it was found that *P. sidoides* did not have a significant inhibitory effect on the growth of *L. infantum* promastigotes at concentrations of 50 and 100 µg/mL compared to the control groups (P>0.05) *(Fig. 1* and *Fig. 2)*.

At the end of 96 h of incubation, *P. sidoides* was observed to significantly inhibit growth of *L. infantum* at a concentration of 200 µg/mL and 400 µg/mL compared to the control groups (P<0.05) *(Fig. 3* and *Fig. 4)*.

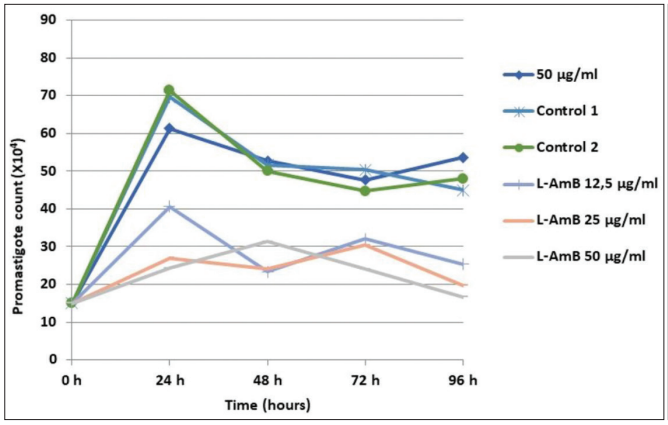

Fig 5. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 50 µg/mL

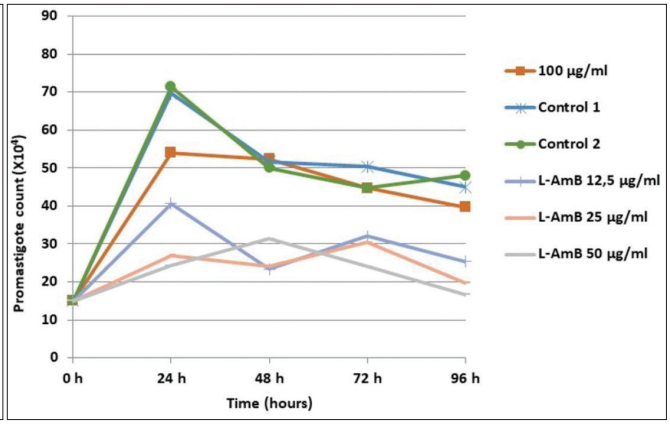

Fig 6. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 100 µg/mL

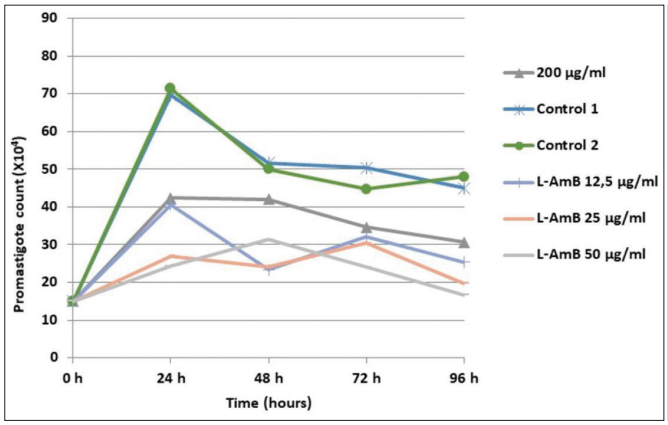

Fig 7. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 200 µg/mL

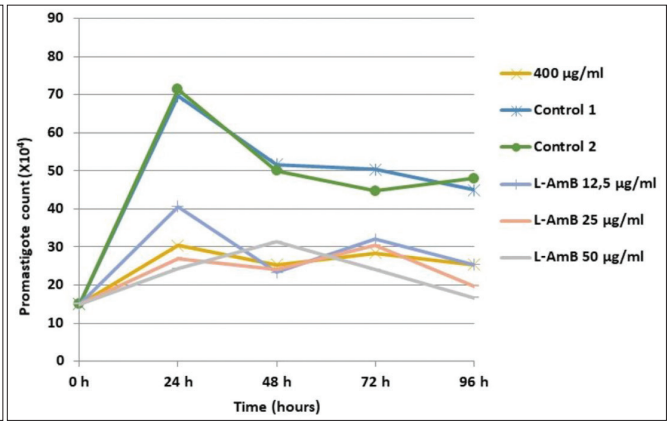

Fig 8. The effects of EPs 7630 on *Leishmania tropica* promastigotes at 400 µg/mL

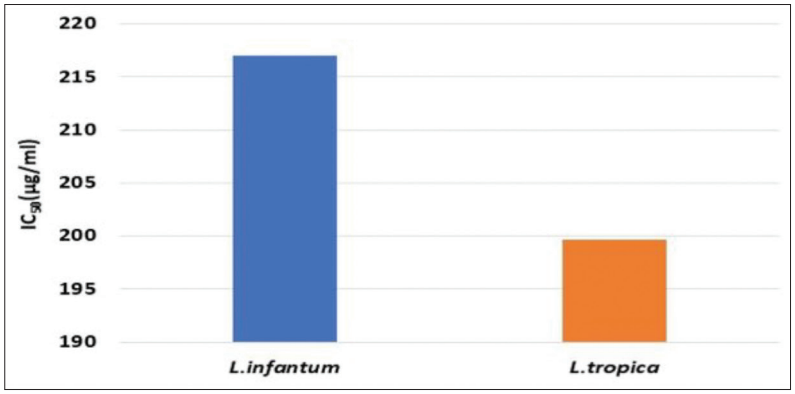

Fig 9. IC_{50} values of EPs 7630 on *L. infantum* and *L. tropica* promastigotes

At the end of 96 h of incubation at 24°C, it was found that *P. sidoides* extracts did not have a significant inhibitory effect on the growth of *L. tropica* promastigotes at concentrations of 50 and 100 µg/mL compared to the control and positive control groups (P>0.05) *(Fig. 5* and *Fig. 6)*.

It was observed that EPs 7630 significantly inhibited the growth of *L. tropica* at concentrations of 200 µg/mL and 400 µg/mL at the end of 96 h of incubation (P<0.05) *(Fig. 7* and *Fig. 8)*.

It was calculated that EPs 7630 had an IC_{50} = 217.0018 µg/mL on *L. infantum* and IC_{50} = 199.6707 µg/mL on *L. tropica* promastigotes *(Fig. 9)*.

The contribution of the solvents (ethanol) used for the dissolution of *P. sidoides* to the inhibitory effect on promastigotes was analyzed and no significant inhibitory effect was found (P>0.05).

Discussion

Leishmaniasis is a major public health problem especially in Asian, African and Latin American countries [1]. In recent years in Turkey, there has been persistent leishmaniasis cases after migration of Syrian refugees [11]. The limited number of drugs such as pentavalent antimony compounds and amphotericin B are used in the treatment of leishmaniasis. The cytotoxic effects of these drugs have led to the emergence of new drug searches.

Herbal medicines are extensively used in many countries. *P. sidoides* is a perennial herb found in the Eastern Cape Province of South Africa and the Lesotho highlands. It is used by people living in those area to treat various diseases, including diarrhea, colic, gastritis, tuberculosis, cough, liver disorders, menstrual complaints, and gonorrhea [12]. It has been reported that the antiviral effect of EPs 7630 is related to the production of interferons, which has been reported on viruses such as influenza, parainfluenza, respiratory syncytial virus (RSV), rhinovirus, coxsackie, and coronavirus [13].

Many different herbal materials have been used experimentally for the treatment of *L. donovani* and *L. tropica* [14-19].

The mechanism of anti-leishmanial action of L-AmB is thought to be drug-binding to *Leishmania* sp. ergosterol precursors causing degradation of the parasite membrane. Several *in vitro* studies have reported the effectiveness of L-AmB against *Leishmania* species. Piñero et al.[20] evaluated the *in vitro* activity of L-AmB compared to amphotericin B on different strains of *L. infantum* isolated from HIV (+) patients and noted a higher efficiency of L-AmB on promastigotes. In our study, we compared 400 µg/mL *P. sidoides* and 50 µg/mL L-AmB as a positive control on *L. infantum* and *L. tropica* promastigotes, and reported higher effectiveness of *P. sidoides* compared to L-AmB at 96 h ($P<0.05$) *(Fig. 4)*.

EPs 7630 is widely used in phytotherapy practice for therapeutic purposes. Various scientific studies have been conducted on its antibacterial and antiviral effects. In the present study, we investigated concentration-dependent *in vitro* inhibitory effect of EPs 7630 on *L. infantum* and *L. tropica* promastigotes. Ethanolic standardized extract of *P. sidoides* roots (EPs 7630) used in our experiments. When it was compared to control 1 and control 2 group (only solvent), *P. sidoides* was significantly inhibitory effect on *L. infantum* and *L. tropica* promastigotes at concentrations of 200 µg/mL and 400 µg/mL (IC_{50} = 217.0018 µg/mL and IC_{50} = 199.6707 µg/mL respectively) ($P<0.05$).

It has been reported in some studies that *P. sidoides* may not be directly effective on microorganisms. However, it has been determined that the plant is effective by disrupting the receptors and enzymes that microorganisms bind to the host cell [12]. Thäle et al.[21] investigated the effects of induced nitric oxide production on *L. major* in infected macrophages and showed that EPs 7630 alone had a lower anti-infective effect compared to its combination with IFN-gamma. In the same study, they noted that a single dose of 10 µg/mL EPs 7630 did not have any direct effect on the viability of promastigotes after 48 h. However, in our study, we found a significantly higher efficacy at higher concentrations, especially at 400 µg/mL, at 96 h. The effect of *P. sidoides* on *Leishmania* species may be mediated by the active substances, epigallocatechin and gallocatechin [22]. Also, cytotoxic effect of EPs 7630 was investigated and reported that neither EPs 7630 nor phenolic compounds, including benzoic and cinnamic acid derivatives, hydrolysed tannins and C-glycosylflavones, exhibited any cytotoxic effects [23,24]. Based on these studies, the LD_{50} value of EPs 7630 of >1000 µg/mL eliminated the expectation of any cytotoxic effect at the concentrations in our study.

As a conclusion, we detected direct antileishmanial activities of EPs 7630 against *L. infantum* and *L. tropica* promastigotes. Herbal sources gain importance in treatment applications because of their easy tolerability and fewer side effects compared to synthetic drugs. Although the screening and purification of bio-compounds from multi-molecular plant extracts requires a lot of time, planning, and cost, there is hope for further advancement in this area to effectively treat patients. According to studies, phyto-therapeutics provide a broad and promising perspective for new, safe and effective antileishmanial agents [25]. This study determined the direct efficacy of EPs 7630 solution against *Leishmania* promastigotes. It has been concluded that it may be an alternative treatment option in the future due to its lower toxic effects compared to many drugs in routine clinical practice.

Further studies are needed to identify the substance(s) responsible for the antileishmanial effect of *P. sidoides* and to perform combined with other antileishmanial compounds. Development of further *in vivo* models may be useful as a stepping stone for future clinical studies.

Acknowledgments

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Conflicts of Interest Statement

The authors declare that there is no conflict of interest.

Author Contributions

MH and EB designed the project. MH and EB provided *Leishmania infantum* and *L.tropica* strains. NG and MH carried experiments. EB, NG, FK and MH performed statistical analysis of data and wrote the article.

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