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Original Article

In Vitro Antileishmanial Activity of *Achillea santolina* Essential Oil against *Leishmania infantum* Promastigote by Methylthiazole Tetrazolium (MTT) and Trypan Blue Colorimetric Methods

Ayrom, F[1], Rasouli, S[2]*, Shemshadi, B[1]

1. Department of Pathobiology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Department of Parasitology, Faculty of Veterinary, Urmia Branch, Islamic Azad University, Urmia, Iran

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Corresponding Author: sohrab_rasouli86@yahoo.com

Abstract

Leishmaniasis causes parasitic infections, especially in developing countries. The disease has not yet been controlled because of the absence of an effective vaccine and low-cost treatment. *Achillea santolina* essential oil (ASEO) might control the disease as it has antimicrobial properties. This study investigated the *in vitro* antileishmanial activity of ASEO against *Leishmania infantum* promastigote using the methylthiazole tetrazolium (MTT) and trypan blue colorimetric methods. The standard strain of *L. infantum* (MCAN/IR/96/LON49) promastigotes was prepared and cultured in a 96-well Novy-MacNeal-Nicolle (NNN) medium. The effects of different concentrations of saline, ASEO, and glucantime (10, 50, 100, 200, 500, and 1000 mg/mL) were examined in 24-, 48-, and 72-hour intervals using the MTT and trypan blue test methods.The use of ASEO reduced viability in all concentrations compared to the control group in times of 48 (p<0.05) and 72 h (p<0.05). Treatment with glucantime and ASEO had similar efficiency with the concentration of 1000 mL/mg in both methods after 72 h. The results showed that viability was significantly lower in the ASEO group with increases in time using both methods (p<0.05). Cohen's Kappa coefficient showed a significant agreement between the obtained results for the two methods (Kappa=0.856; p<0.001).In sum, the results showed *in vitro* antileishmanial activity of ASEO, but more clinical studies are needed to confirm the efficiency. ASEO can be used as an agent and/or in combination with synthetic agents for the treatment of leishmaniasis disease.

Keywords: antileishmanial activity, *Leishmania infantum*, *Achillea santolina*, methylthiazole tetrazolium, trypan blue method

Activité Antileishmanienne In vitro de l'huile Essentielle d'*Achillea santolina* contre *Leishmania infantum* Promastigote par les Méthodes Colorimétriques au Méthylthiazole Tétrazolium (MTT) et au Bleu Trypan

Résumé: La leishmaniose provoque des infections parasitaires, en particulier dans les pays en développement. La maladie n'a pas encore été maîtrisée en raison de l'absence d'un vaccin efficace et d'un traitement peu coûteux. L'huile essentielle d'*Achillea santolina* (*ASEO*) pourrait contrôler la maladie car elle possède des propriétés antimicrobiennes. Cette étude a examiné l'activité antileishmanienne in vitro de l'ASEO contre le Promastigote de *Leishmania infantum* en utilisant les méthodes colorimétriques au méthylthiazole tétrazolium (MTT) et au bleu trypan. La souche standard de promastigotes de *L. infantum* (MCAN/IR/96/LON49) a été préparée et cultivée dans un milieu 96 puits Novy-MacNeal-Nicolle (NNN). Les effets de différentes concentrations de solution saline, d'ASEO et de glucantime (10, 50, 100, 200, 500 et1000 mg/mL) ont été

examinés à des intervalles de 24, 48 et 72 heures à l'aide des méthodes de test MTT et bleu trypan. L'utilisation d'ASEO a réduit la viabilité à toutes les concentrations par rapport au groupe témoin dans des délais de 48 ($p<0.05$) et 72 h ($p<0.05$). Le traitement au glucantime et à l'ASEO a eu une efficacité similaire avec la concentration de 1000 mL/mg dans les deux méthodes après 72 h. Les résultats ont montré que la viabilité était significativement plus faible dans le groupe ASEO avec des augmentations de temps en utilisant les deux méthodes ($p<0.05$). Le coefficient Kappa de Cohen a montré une concordance significative entre les résultats obtenus pour les deux méthodes (Kappa=0.856; $p<0.001$). En somme, les résultats ont montré une activité antileishmanienne in vitro de l'ASEO, mais d'autres études cliniques sont nécessaires pour confirmer l'efficacité. ASEO peut être utilisé comme agent et/ou en combinaison avec des agents synthétiques pour le traitement de la leishmaniose.
Mots-clés: activité antileishmanienne, *Leishmania infantum*, *Achillea santolina*, méthylthiazole tétrazolium, méthode au bleu trypan

1. Introduction

The *Leishmania* genus is an intracellular parasite that causes leishmaniasis disease (1). Strains of this genus cause cutaneous, mucocutaneous, and visceral diseases (2). The bite of an infected female phlebotomine sand fly transfers this parasite to a human host. The parasite causes one million new infections and 65,000 deaths annually (3). It also causes a skin-mucosal response and systemic and fatal diseases by involving the viscera and internal organs (4). Parasitic infections are responsible for cutaneous leishmaniasis disease all over the world, though it is mainly found in developing countries (5), such as Iran, Afghanistan, and Syria (6).

The disease has not yet been controlled, because an effective vaccine and low-cost treatment are lacking (7). Several synthetic agents can be utilized for treatment of the disease, such as glucantime and pentostam. These agents block the production of adenosine triphosphate by disrupting parasitic enzymes (8). These drugs have limitations, however, such as toxicity, costliness, and drug-resistance (9). A need exists for novel drugs for the treatment of leishmaniasis disease. Natural herbal products derived from medicinal plants in the forms of oral remedies, ointments, and poultices is an efficient and cheap strategy for treating infections (10).

The *Achillea* L. (Asteraceae) genus is found mainly in Europe and Asia. In Iran, it is traditionally used as an anti-inflammatory and for the treatment of anxiety (11). *Achillea santolina* L. grows in barley and fallow fields (12). The *Achillea* oil contains terpenoids (1,8-cineole camphor, borneol, pinenes, artemisia ketone, santolina alcohol, farnesane, caryophyllene, and its oxides, cubebene, germacrenes, eudesmol, α-bisabolol and oxides, farnesene, γ-gurjunene, γ-muurolene and chamazulene) (13). *A. santolina* shows antimicrobial activity due to its compounds, flavones, and polyphenols (14). *In vitro* studies have reported the antileishmanial activity of *A. millefolium*, *A. absinthium L.*, and walnut (*Juglans regia L.*) leaves (15). To date, however, no study has investigated the *in vitro* antileishmanial activity of *Achillea santolina* essential oil. This study is the first to evaluate the *in vitro* antileishmanial activity of *Achillea santolina* essential oil (ASEO) against *L. infantum* promastigote using the methylthiazole tetrazolium (MTT) and trypan blue colorimetric methods.

2. Material and Methods

2.1. Preparation of the *A. santolina* Essential Oil

The aerial parts of the *A. santolina* plant were collected from the town of Makoo, located in West-Azarbaijan province, Iran, during the flowering period and identified by an expert botanist in the Biology Department of Islamic Azad University, Urmia Branch. The collected parts were dried at room temperature and then hydro-distilled for the production of essential oil using the Clevenger apparatus for 3 h. The extracted essential oil was dried using anhydrous sodium sulfate-

Na2SO4, then weighed and kept in dry amber vials at 4 ˚C for future analyses. The mean of production was calculated as percentage weight by weight (% w/w) of the dry plant material and was found to be 28% w/w. The compounds were analyzed by GC-MS, and major compounds were investigated as reported by Ahmed, Aburjai (16). In brief, 10 µL was diluted using 1 mL GC-grade n-hexane (Merk Company-Germany), and then 1 µL of each diluted sample was injected by automated injector into the GC/MS system for analysis by a variant chrompack CP-3800 GC/MS/MS-200 (Satum, Netherlands) equipped with a split-splitless injector and DB-5 capillary column (5 % diphenyl 9 5% dimethyl polysiloxane; 30 m x 0.25 mm ID, 0.25 µm film thickness). The injector temperature was set at 250 ˚C with a split ratio of 1:30. A linear temperature program was applied to separate compounds. The column temperature was kept at 60 °C for 1 min. The temperature was then increased to 250 ˚C at a rate of 3 ˚C/min, and then held constant at 250 ˚C for 2 min, with a total runtime of about 66 min. Helium gas was used as the standard in a flow rate of 1 mL/min. A hydrocarbon mixture of n-alkanes (C8-C20) was used. The linear retention index was used for per-compound separated by GC/MS by the value of its retention time and the retention times of the reference n-alkanes.

2.2. Cultivation of *L. infantum* Promastigote

The standard strain of *L. infantum* (MCAN/IR/96/LON49) promastigotes was prepared from Urmia University of Medical Science and cultured in a 96-well Novy-MacNeal-Nicolle (NNN) medium containing 1.4% agar (Sigma-Aldrich), 0.6% NaCl (Merck), 31% defibrinated rabbit blood, 625 units/mL penicillin, 625 units/mL streptomycin, and RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mL-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin, and 20 mM HEPES buffer (all from Lonza) as the liquid phase (17).

2.3. The MTT and Trypan Blue Tests

The promastigotes (10,000 cells) were cultured in each well and incubated for 24 h. The cells in each well were treated with the agents in the determined concentrations. The cellular environment was changed after 48 h of treatment, and MTT compound was added into the structure and incubated in darkness for 3 h. The media containing MTT was removed, 100 µL DMSO was added into each well to dissolve furmazan crystals, and the plate was shacked. An ELISA reader (Stat fax 2100, USA) was used to read optical densities (ODs) at the wavelength of 570 nm with an additional 630 nm filter. Growth of the promastigotes was considered and 100 µλ were picked up and added into each well. The most appropriate concentration of promastigotes was 10^6 parasites/mL, and a concentration of 10^6 parasites/mL was prepared, cultured in the same culture media, and diluted with liquid media of 1640 RPMI. The diluted promastigotes were transferred into plates containing media culture and investigated in smear form. The different concentrations of the ASEO and glucantime (10, 50, 100, 200, 500, and 1000 mg/mL) were used in times of 24, 48, and 72 hours. The MTT test and trypan blue test were conducted as reported by Rahiminejad, Hazrati Tappeh (8). Wells with no ASEO or drug were considered as the control and treated with saline in the same concentrations. Experimental groups included ASEO and glucantime that were treated with different concentrations of ASEO and glucantime (10, 50, 100, 200, 500, and 1000 mg/mL). Overall, there were 18 treatments with 10 replications for each treatment.

To determine viability, 0.1 mL cellular suspension was mixed with 0.1 mL Trypan blue. The stained and non-stained cells were detected as dead and live, respectively by the methods. Viability was calculated as follows;

Viability (%) = $\frac{Viable\,cells\,(n)}{Total\,number\,of\,cells}$, % cytotoxicity= 100- % viability

2.4. Data Analysis

Graph Pad Prism software (6.07 version) was used for statistical analyses, and the results were reported as

means ± SD. The ANOVA test was used for comparisons of all concentrations. The agreement between methods was investigated by Cohen's Kappa coefficient.

3. Results

3.1. Major Compounds of the ASEO

GC-MS of the ASEO identified 23 compounds (92.90%), major compounds included. The constituents attributed to ASEO were 92.90%. The major compounds included 1, 8-cineole (eucalyptol) (27.30%), β-thujone (19.60%), and bornyl acetate (10.80%). Monoterpene hydrocarbons (4.90%), sesquiterpene hydrocarbons (3.70%), oxygenated sesquiterpenes (5.40%), and nonterpenoids (6.80%) were also observed. The results are presented in Table 1.

3.2. Viability

The effects of ASEO and glucantime on viability (%) are shown in Figure 1. The results showed that treatment with glucantime in all concentrations could reduce viability compared to ASEO (p<0.05) and the control (p<0.05). The highest efficiency was observed in the concentration of 1000 mg/mL and at times of 48 h and 72 h. The results also showed that in both methods, the use of glucantime had better efficiency compared to the other groups (p<0.05). The application of ASEO reduced viability in all concentrations compared to the control group in times of 48 (p<0.05) and 72 h (p<0.05). Concentrations of 50 mg/mL and higher decreased viability compared with the control group in the time of 24 h (p<0.05). In sum, treatment with ASEO decreased viability compared to the control group.

3.3. The Comparison of the Methods

The results for the methods comparison are illustrated in Figure 2. The results for glucantime and ASEO showed that viability was significantly lower in trypan blue than in MTT methods. In other words, the MTT method detected more live cells than the trypan blue method (p<0.05). Cohen's Kappa coefficient showed good agreement between the results obtained with the MTT and the Trypan blue test methods (Kappa=0.856; p<0.001).

3.4. The Effects of ASEO During Time

The effects of ASEO on viability (%) in different methods during the tested times are shown in Figure 3. As can be seen, viability was significantly lower with increases in time in both methods (p<0.05).

Table 1. Chemical composition of ASEO

No.	AI_{exp}	AI_{lit}	Compounds	%
1	932	924	α-Thujene	1.70
2	947	946	Camphene	1.70
3	974	969	Sabinene	1.90
5	1006	1002	α-Phellandrene	1.80
6	1017	1014	α-Terpinene	1.60
7	1031	1026	1,8-cineole (eucalyptol)	27.30
8	1058	1054	γ-Terpinene	1.30
9	1092	1098	*trans*-Sabinene hydrate	0.70
10	1109	1101	α-Thujone	1.30
11	1119	1112	β-thujone	19.60
12	1147	1141	Camphor	3.30
13	1158	1156	2-acetyl-3-ethyl pyrazine	0.90
14	1203	1199	γ-Terpineol	0.90
15	1245	1238	Cumin aldehyde	1.60
16	1289	1287	Bornyl acetate	10.80
17	1296	1289	Sabinyl acetate	4.30
18	1367	1356	Eugenol	3.70
19	1384	1374	Linalol isobutanoate	0.60
20	1482	1484	Germacrene-D	1.70
21	1505	1506	(Z)-α-Bisabolene	1.90
22	1611	1613	β-Biotol	1.50
23	1670	1649	β-Eudesmol	2.80

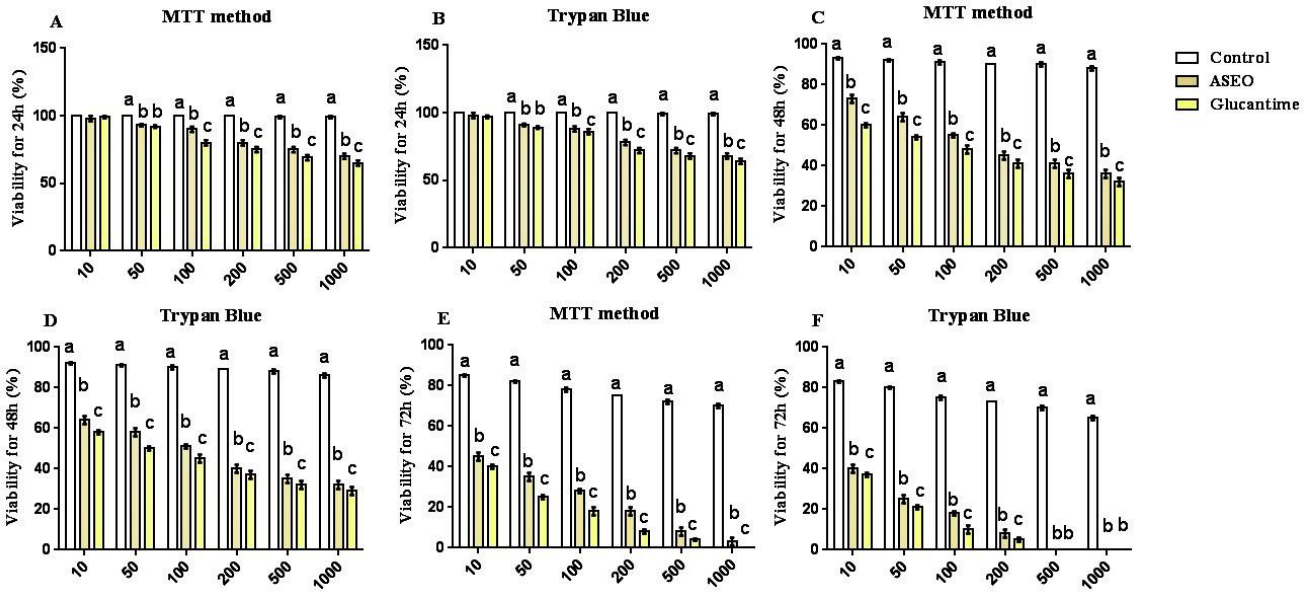

Figure 1. Effects of ASEO and glucantime on viability (%). The letters (a-c) show significant differences on the same day.

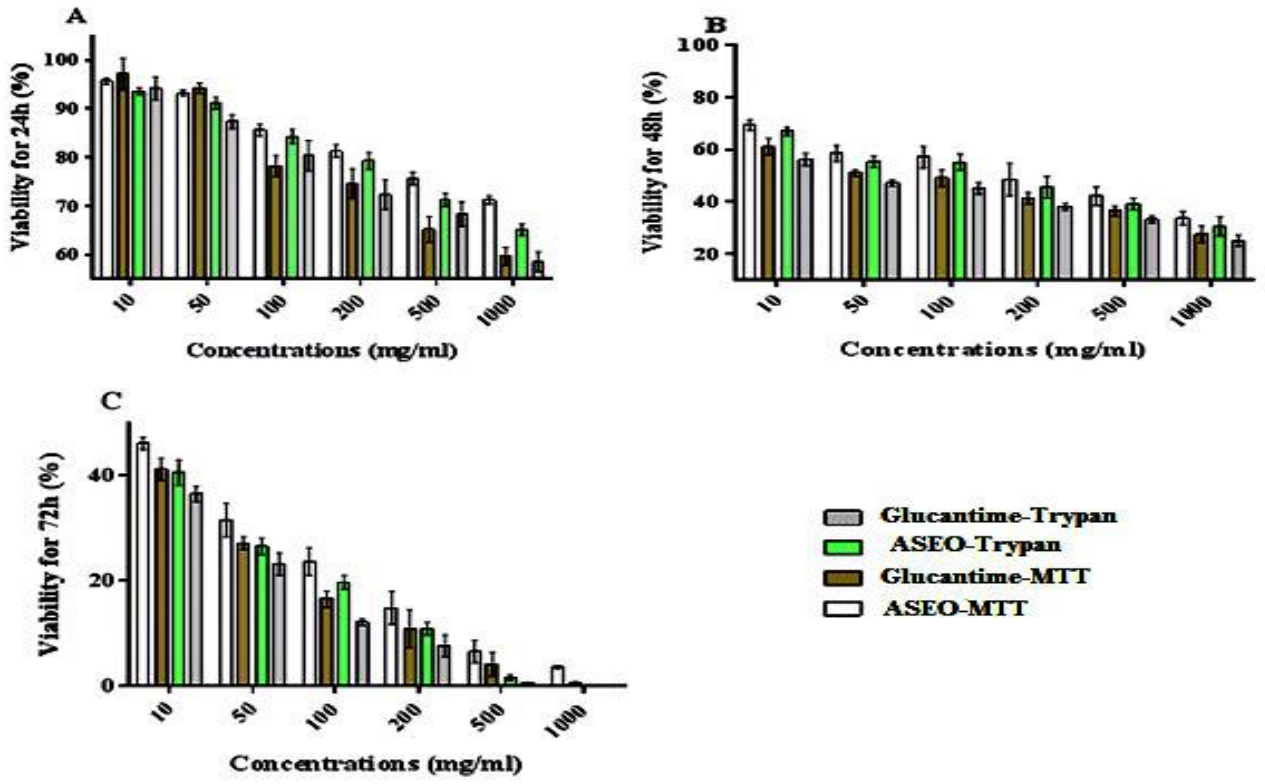

Figure 2. Effects of ASEO and glucantime on viability (%) in different methods.

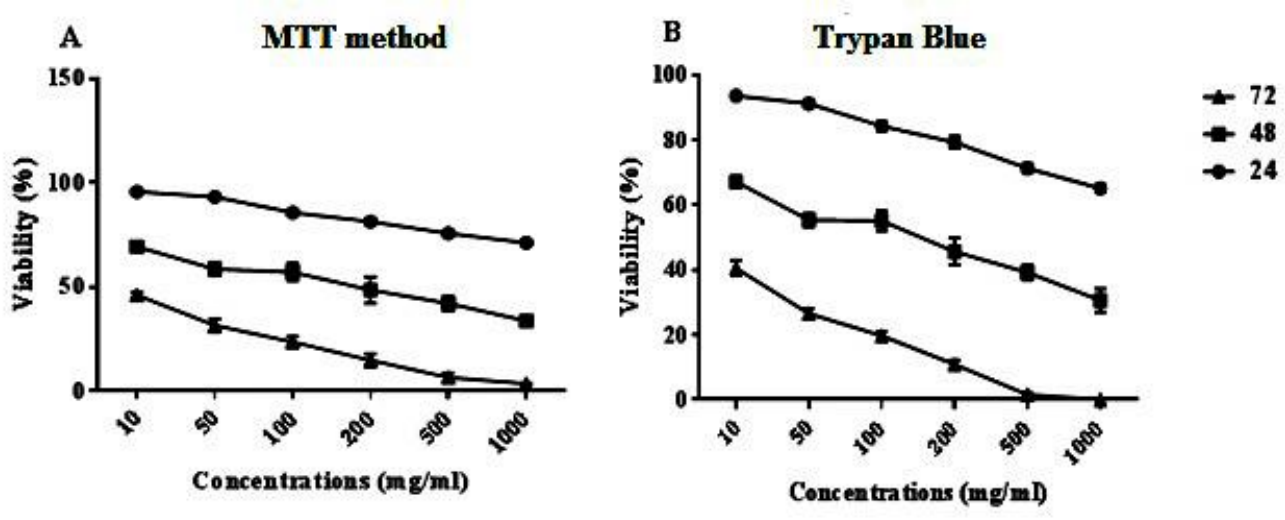

Figure 3. Effects of ASEO on viability (%) in different methods over time.

4. Discussion

In this study, the *in vitro* antileishmanial activity of ASEO against *L. infantum* promastigote was investigated by MTT and trypan blue colorimetric methods, and the results were compared with those achieved with glucantime. There is no efficient treatment for forms of leishmaniasis, because the common agents have toxic effects, require a long duration of treatment, and face the development of resistance (18). Medicinal plants are rich sources of antileishmanial drugs that are safe and used in endemic countries (19). The current results showed that ASEO had an acceptable antileishmanial activity, but the glucantime agent had comparatively better antileishmanial activity; treatment with glucantime and ASEO, however, had similar efficiency in the concentration of 1000 mL/mg with both methods after 72 h, indicating there is no difference between the groups in the highest concentrations. In other words, treatment with ASEO and glucantime had similar efficiency with increases in time. Seemingly, treatment with ASEO required more time to kill *L. infantum* promastigotes. The antileishmanial activity of ASEO could be attributed to its compounds. The results showed that the highest compound was 1,8-cineole (27.30%). Similar to the current findings, Nosratabadi, Sharifi (19) attributed the antileishmanial activity of methanolic and aqueous extracts of eucalyptus to 1,8-cineole. Terpenes including monoterpene hydrocarbons, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes comprised 14% of ASEO. Terpenes are known to have anti-malarial and antileishmanial activity. Yousefi, Eskandari (20) showed that terpenes have antileishmanial activity by changing the metabolites of various metabolic cycles, such as the galactose metabolism pathway, sphingolipid biosynthesis pathway and the biosynthesis pathways of valine, leucine, and isoleucine. ASEO might also prevent the growth of *L. infantum*. Previous studies have reported the efficiency of *A. absinthium* extract in inhibiting the growth of *L. major* (21). Similar to the current findings, Dalimi, Delavari (22) evaluated the effect of *Achillea biebersteinii afan* essential oil on *L. major* promastigote and reported the *in vitro* antileishmanial activity of *A. biebersteinii* on *L. major* promastigote. The *in vitro* antileishmanial activity of *A. absinthium* growth on *L. major* was reported by Rahiminejad, Hazrati Tappeh (8) whose results were similar to the current ones. Moreover,

other studies have demonstrated the *in vitro* antileishmanial activity of *A. millefolium*, *A. absinthium L.*, and walnut (*Juglans regia L.*) leaves (15). Parallel to the current findings, Maspi, Ghafarifar (23) assessed the anti-leishmanial effects of hydroalcoholic extract of *Calendula officinalis* on *L. major* promastigotes and reported a strong anti-leishmanial activity for the extract.

In sum, active compounds of ASEO have antileishmanial activity. The current results showed that ASEO had similar efficiency compared to synthetic agents in higher concentrations and for a longer time. In other words, ASEO needs more time and higher concentrations are required for showing antileishmanial activity. In sum, the active compounds of ASEO can compete with synthetic agents in higher concentrations and for longer times and also act as a safe agent without limitations. Both methods had similar efficiency in the time interval of 48 h, but trypan blue detected more dead cells; this difference is due to the different methods used to detect viability.

4. Conclusion

In the current study, ASEO was extracted, its major compounds were investigated, and its *in vitro* antileishmanial activity against *L. infantum* promastigote was assessed using MTT and trypan blue colorimetric methods. ASEO had similar efficiency as the synthetic agent of glucantime but required more time and higher concentrations. Its efficiency was approved by both methods. ASEO can be used for the treatment of leishmaniasis disease after confirmation by clinical studies. The present preliminary study shows that ASEO can be considered as a natural compound for antileishmanial activity and can be used in the structure of the agents for the treatment of leishmaniasis disease.

Authors' Contribution

S. R. designed the study idea, supervised, and helped prepare the manuscript. The work was financially supported by F. A.. B. Sh. helped in the preparation of the manuscript.

Ethics

All the procedures were approved by the Ethics Committee at the Islamic Azad University, Tehran, Iran under the project number of 2020-9546825-9.

Conflict of Interest

The authors declare that they have no conflict of interest.

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