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

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ABSTRACT
Leishmaniasis causes parasitic infections, especially in developing countries. The disease has not still been controlled, due to the absence of an effective vaccine and low-cost treatment. Achillea santolina essential oil (ASEO) might control the disease due to its antimicrobial properties. This study was conducted to investigate the In vitro antileishmanial activity of the ASEO against Leishmania infantum promastigote by Methyl Thiazole 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 the different concentrations of saline, the ASEO and Glucantime (10, 50, 100, 200, 500, and 1000 mg/mL) were used in times of 24, 48, and 72 hours by the MTT test, and Trypan blue test methods.

The use of the ASEO could reduce viability in all the concentrations compared to control group in times of 48 (P<0.05) and 72 h (P<0.05). The treatment with Glucantime and the ASEO had similar efficiency in the concentration of 1000 mL/mg for both methods after 72 h. The results showed that viability was significantly lower in the ASEO group with the increase in time in both methods (P<0.05). Cohen’s Kappa coefficient showed a significant agreement between the obtained results for methods (Kappa=0.856 and P<0.001).
In sum, the results showed *In vitro* antileishmanial activity of the ASEO, but the confirmation of the efficiency needs more clinical studies. It 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*, Methyl Thiazole Tetrazolium, Trypan blue method

**INTRODUCTION**

The *Leishmania* genus is an intracellular parasite that causes leishmaniasis disease (Boon Ong et al., 2020). The strains of the genus cause cutaneous, mucocutaneous, and visceral diseases (Alexandre et al., 2020). The bite of an infected female phlebotomine sand fly transfers it to the human host. The parasite annually causes one million new infections and 65,000 deaths (WHO, 2019). It also causes the skin-mucosal part and causes systemic and fatal diseases by the involvement in the viscera and internal organs (Rezaei et al., 2020). Cutaneous leishmaniasis disease is responsible for parasitic infections all over the world and is mainly found in developing countries (Sosa et al., 2019), such as Iran, Afghanistan, Syria, etc. (Badirzadeh et al., 2020). The disease has not still been controlled, due to the absence of an effective vaccine and low-cost treatment (Kedziersk, 2010). Several synthetic agents are utilized for the treatment of the disease, such as Glucantime and Pentostam. The agents block the production of adenosine triphosphate by disrupting parasitic enzymes (Rahiminejad et al., 2018). These drugs have limitations, such as toxicity, costliness, and drug-resistance (Croft and Coombs, 2003). It needs to prepare novel drugs for the treatment of leishmaniasis disease. The use of natural herbal products, such as medicinal plants and their derivations is an efficient and cheap strategy for the treatment of infections in forms of oral remedies, ointment, and poultice (Gharirvand Eskandari et al., 2020).

The *Achillea* L. (Asteraceae) genus is mainly found in Europe and Asia. It is traditionally used as an anti-inflammatory and for the treatment of anxiety among Iranian population (Golalipour et al., 2004). *Achillea santolina* L. grows in barley and fallow fields (Darier and Tammam, 2012). The *Achillea* oil is contained 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) (Nemeth and
Bernath, 2008). The *A. santolina* showed antimicrobial activity due to its compounds, such as flavones, and polyphenols (Eddouks et al., 2003). Studies have reported *In vitro* antileishmanial activity of the *A. millefolium*, *A. absinthium* L., and walnut (*Juglans regia* L.) leave (Yektaeian et al., 2012). So far, any study has not been investigated the *In vitro* antileishmanial activity of *Achillea santolina* essential oil. This study was for the first time evaluates the *In vitro* antileishmanial activity of *Achillea santolina* essential oil (ASEO) against *L. infantum* promastigote by Methyl Thiazole Tetrazolium (MTT) and Trypan blue colorimetric methods.

**MATERIALS AND METHODS**

**The preparation of the *A. santolina* essential oil**

The aerial parts of the *A. santolina* were collected from Makoo town placed in the West-Azarbayjan province of Iran during the flowering period and identified by an expert botanist in Biology Department in Islamic Azad University, Urmia Branch. The collected parts were dried at room temperature and then hydro-distilled for the production of the 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 it was 28% w/w. The compounds were analyzed by GC-MS and major compounds were investigated as reported by Ahmed et al. (2020). Summary, 10 µL was diluted by using 1 mL GC-grade n-hexane (Merk Company-Germany) and then 1 µL of each diluted sample was injected using a automated injector into the GC/MS system for analysis by a variant chrompack CP-3800 GC/MS/MS-200 (Satum, Netherlands) equipped with split-splitless injector and DB-5 capillary column (5 % diphenyl 95% 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. For separating compounds, a linear temperature program was applied. 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 was held constant at 250°C for 2 min, with a total runtime of about 66 min. Helium gas was used as a standard in a flow rate of 1 mL/min. A hydrocarbon mixture of n-alkanes (C8-C20) was used. Finally, linear retention index was used for per compound separated by a GC/MS by the value of its retention time and the retention times of the reference n-alkanes.
**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 liquid phase (Saki et al. 2009).

**The MTT and Trypan blue tests**

The promastigotes (10000 cells) were cultured in each well and incubated for 24h. The cells per well were treated with the agents in the determined concentrations. The cellular environment was changed after 48h treatment, MTT compound was added into the structure and incubated in darkness for 3h. The media containing MTT was removed, 100 µL DMSO was added into per well for dissolving furmazan crystals and the plate was shacked. ELISA reader (Stat fax 2100, USA) was used for reading optical densities (ODs) at the wavelength of 570 nm with an additional 630 nm filter. The promastigotes growth was considered and 100 µλ were picked up and added into per well. The most appropriate concentration of promastigotes is $10^6$ 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 et al., (2018). The wells lack of the ASEO and drug were considered as control and treated with saline in the same concentrations. Experimental groups included ASEO, Glucantime and control that were treated with different concentrations of the ASEO, glucantime and saline (10, 50, 100, 200, 500 and 1000 mg/mL). In sum, we had 18 treatments with 10 replications for per 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;

$$\text{Viability (\%)} = \frac{\text{Viable cells (n)}}{\text{Total number of cells}}, \% \text{ cytotoxicity} = 100 - \% \text{ viability}$$
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 the comparison all the concentrations. The agreement between methods was investigated by Cohen’s Kappa coefficient.

RESULTS
Major compounds of the ASEO
The results for GC-MS of the ASEO identified 23 compounds (92.90%) and major compounds included. The constituents amounted to ASEO were to be 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.

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

The comparison of the methods
The results for the comparison of the methods are illustrated in Figure 2. The results for Glucantime and ASEO showed that viability was significantly lower in Trypan blue compared to MTT methods. It means that MTT method detected more live cells compared to Trypan blue method (P<0.05). The results for Cohen’s Kappa coefficient showed a good agreement between the obtained results for the MTT test, and Trypan blue test methods (Kappa=0.856 and P<0.001).
The effects of the ASEO during time

The effects of the ASEO on viability (%) in different methods during the time are shown in Figure 3. The results showed that viability was significantly lower with the increase in time in both methods (P<0.05).

DISCUSSION

In this study, we investigated In vitro antileishmanial activity of the ASEO against *L. infantum* promastigote by MTT and Trypan blue colorimetric methods and compared the results with Glucantime. It is no efficient treatment for forms of leishmaniasis, because the common agents have toxic effects, long duration of the treatment and development of resistance (Hadighi et al., 2007). Medicinal plants are rich sources of antileishmanial drugs that are safe and used in endemic countries (Nosratabadi et al., 2015). The results showed that ASEO showed an acceptable antileishmanial activity. The results showed that the Glucantime agent had better antileishmanial activity compared to the ASEO, but the treatment with Glucantime and the ASEO had similar efficiency in the concentration of 1000 mL/mg for both methods after 72 h. It means non-difference between groups in the highest concentrations. In other words, the treatment with ASEO and Glucantime has similar efficiency with the increase in the time. Seemingly, the treatment with the ASEO needs more time for killing *L. infantum* promastigotes. Antileishmanial activity of the ASEO could be attributed to its compounds. The results showed that the highest compound was 1,8-cineole (27.30%). Similar to our findings, Nosratabadi et al. (2015) attributed the antileishmanial activity of methanolic and aqueous extracts of eucalyptus to 1,8-cineole. Terpenes including Monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygenated sesquiterpenes comprised 14% of the ASEO. Terpenes are known to have anti-malarial and antileishmanial activity. Yousefi et al. (2014) showed that terpenes have antileishmanial activity by changing the metabolites of various metabolic cycles, such as galactose metabolism pathway, sphingolipid biosynthesis pathway as well as the biosynthesis pathways of valine, leucine, and isoleucine. The ASEO might also prevent the growth of *L. infantum*. Previous studies have reported the efficiency of *A. absinthium* extract for inhibiting the growth of *L. major* (Soosaraei et al., 2017). Similar to our findings, Dalimi et al. (2015) evaluated the effect of *Achillea biebersteinii afan* essential oil
on *L. major* promastigote and reported *In vitro* antileishmanial activity of *A. biebersteinii* on *L. major* promastigote. *In vitro* antileishmanial activity of *A. absinthium* growth of *L. major* was reported by Rahiminehad et al. (2018) that was similar to our results. Similarly, other study has shown *In vitro* antileishmanial activity of the *A. millefolium, A. absinthium L.*, and walnut (*Juglans regia L.*) leave (Yektaeian et al., 2012). Parallel to our findings, Maspi et al. (2010) assessed the anti-leishmanial effect 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 the ASEO have antileishmanial activity. The results showed that the ASEO had similar efficiency compared to synthetic agent in higher concentrations and a longer time. It means that the ASEO needs more time and higher concentrations are required for showing antileishmanial activity. In sum, active compounds of the ASEO can compete with synthetic agents in higher concentrations and longer time and also act as safe agents without limitations. Both methods had similar efficiency in a time of 48h, but Trypan Blue detected more dead cells that their differences are due to different methods for detection of viability.

**CONCLUSION**

In the current study, we extracted the ASEO, investigated the major compounds, and assessed its *In vitro* antileishmanial activity of the ASEO against *L. infantum* promastigote by MTT and trypan blue colorimetric methods. The ASEO had similar efficiency with a synthetic agent of Glucantime in more times and higher concentrations. The efficiency of the ASEO was approved by both methods. The ASEO can be used for the treatment of the leishmaniasis disease after confirming by clinical studies. The present preliminary study shows that the 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 the leishmaniasis disease.

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**ETHICS**

All used procedures were approved by Ethical Committee of Islamic Azad University, Science and Research Branch.
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AUTHOR CONTRIBUTIONS
Dr Rasouli designed the idea and supervised it and helped to prepare the manuscript. The work was supported by Fatemeh Ayrom. Dr Shemshadi helped in the preparation of the manuscript.

DISCLOSURE OF INTEREST
None.

REFERENCES


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Figure 1 The effects of the ASEO and Glucantime on viability (%). The letters (a-c) show significant difference for same day.
Figure 2 The effects of the ASEO and Glucantime on viability (%) in different methods.

Figure 3 The effects of the ASEO on viability (%) in different methods during time.