Parasite Epidemiology and Control 17 (2022) e00239

Contents lists available at ScienceDirect

Parasite Epidemiology and Control

journal homepage: www.elsevier.com/locate/parepi

Anti-leishmanial activity of *Avicennia marina* (*Avicenniaceae* family) leaves hydroalcoholic extract and its possible cellular mechanisms

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ARTICLE INFO

Keywords:
Leishmania tropica
Avicennia marina
MTT
ROS
Arginase activity

ABSTRACT

Natural products are the main source of potent antioxidants and anti-leishmanial agents. This study was aimed to evaluate *Avicennia marina* (*Avicenniaceae* family) extract inhibitory effect against *Leishmania tropica* by accessing apoptotic markers and arginase activity. The *A. marina* were extracted and phytochemical analysis conducted. The inhibitory effect of *A. marina* was evaluated on *L. tropica* promastigote and amastigote forms, compared to meglumine antimoniate (Glucantime, MA) as standard drug. The level of apoptosis, Reactive Oxygen Species (ROS) production and arginase activity was assessed in *A. marina*-treated cells compared to control group. Phytochemical screening of *A. marina* extract showed strong presence of tannins and saponins. We demonstrated the inhibitory effect of *A. marina* on promastigote stages in a dose dependent manner. Also, lower 50% inhibitory concentration (IC_{50}) value of amastigotes was indicated in *A. marina* group compared with the standard group of Glucantime (60.57 ± 1.46 vs. 73.19 ± 10.12 μg/mL, respectively, $P < 0.05$). Besides, *A. marina* represented no cytotoxicity as the selectivity index (SI) was 10.7. Also, it showed the potential to induce early apoptosis of 46.5% in promastigotes at 125 μg/mL concentration. Significant reduction of arginase level was observed in both *A. marina*-treated cells and promastigotes. The promising results indicated higher effectiveness of *A. marina* in decreasing parasite growth, inducing apoptosis in promastigotes, increasing ROS production and decreasing arginase level. So, *A. marina* can be a native plant candidate for anti-leishmanial drug in tropical regions with cutaneous leishmaniasis due to *L. tropica*.

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https://doi.org/10.1016/j.parepi.2022.e00239
Received 24 September 2021; Received in revised form 8 January 2022; Accepted 14 January 2022
Available online 19 January 2022

1. Introduction

Leishmaniasis is an endemic disease caused by an obligate intracellular parasite of the genus *Leishmania*, which is known as the main health problem in areas of the Middle East, Africa, Asia, Europe and Central and South America. It affects 98 tropical and subtropical countries as well as three territories in over one billion at-risk people globally. It is estimated that between 600,000 to 1 million new cases occur worldwide annually for cutaneous leishmaniasis (CL) in 2018 (Alvar et al., 2012). Over 85% of new CL cases occurred in ten countries of Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran (Islamic Republic of), Iraq, Pakistan, the Syrian Arab Republic and Tunisia (Ruiz-Postigo et al., 2020).

Chemotherapy is a current strategy for treatment of human leishmaniasis since there is no vaccine available (Diro et al., 2014; Jabini et al., 2015). The main therapeutic approach is using of pentavalent antimonial compounds (SbV) such as sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime, MA); Although, hematopoietic (anemia or agranulocytosis), acute renal failure, cardiac arrhythmias (bradycardia, prolongation of the QT interval, flattening or inversion of the T wave), neurological (polyneuropathy) are some of the reported side effects of the SbV compounds beside painful administration (Diro et al., 2014; Chakravarty and Sundar, 2010; Lage et al., 2013). Also, second-line drugs of pentamidine, amphotericin B, paromomycin and azoles were considered as effective agents, but their usage was limited because of their high cost and long-term treatment (Diro et al., 2014). Furthermore, the reports of patients with resistant cutaneous leishmaniasis have been recently increased (Croft et al., 2006; Oliaee et al., 2018). Consequently, in recent years, much attention has been paid to plant-derived natural compounds as bioactive agents (Saedi Dezaki et al., 2016; Mahmoudvand et al., 2016; Chouhan et al., 2014). These low-toxicity as well as safe compounds can easily be made available to low-income populations everywhere (Iwu et al., 1999).

The anti-leishmanial property of main herbs has been attributed to the presence of some compounds such as alkaloids, triterpenoids, naphthoquinones, quinones, terpenes, steroids, lignans and flavonoids derivatives (Seidi, 2014; Parvizi et al., 2020; de Oliveira et al., 2017; Sifaoui et al., 2014). Mangroves are shrubs or small trees which grow in salt marshes. The term is used for tropical coastal vegetation with such species as well (Hogarth, 2015). Distribution of Mangroves is worldwide mainly in the tropic and subtropic regions, between latitudes 25° N and 25° S. The total mangrove forest region of the world was 137,800 km^2 (53,200 sq. mi) (Saenger, 2013).

A. marina (*Avicenniaceae* family) is one of the mangrove species that grows along the coast of Persian Gulf in Iran. A few chemical analyses have been conducted on *A. marina* leaf, which identified different compounds of iridoid glucosides, naphthoquinone derivatives, hydrocarbons and triterpenes (König and Rimpler, 1985; Sharaf et al., 2000; Orhan et al., 2012). This unique plant has been used far and wide in traditional medicine for the treatment of ulcers, skin diseases, smallpox and rheumatism (Namazi et al., 2013; Vinoth et al., 2019). Also, the inhibitory effect of *A. marina* extracts on breast and liver cancer cell lines has been proved (Behbahani and Sadeghi-aliabadi, 2010; Huang et al., 2016). Additionally, different studies were carried out on antiviral (Namazi et al., 2013), antimalarial (Ravikumar et al., 2011), antibacterial (Gnanadesigan et al., 2012) and antifungal (Rastegar and Gozari, 2017) activities of *A. marina*. Although the inhibitory effect of *A. marina* on different pathogens has been reported, there is no findings about anti-leishmanial effects of *A. marina* on *L. tropica*. The main goal of our study was to determine the potential inhibitory effect of *A. marina* leaf extracts on *L. tropica* promastigotes as well as amastigotes in macrophage model; the other was to identify the anti-leishmanial mechanism of *A. marina* extract by evaluating reactive oxygen species (ROS) level and arginase activity on treated cells.

2. Materials and methods

2.1. Plant material and preparation of extract

A. marina was collected from Bushehr coast, Bushehr province, South of Iran, from October 2019 to January 2020. Species identification was performed by botanist, Dr. Mirtajadini at Department of Botany, and the voucher specimen (number RB 5114) was deposited in the herbarium of the Pharmacy Faculty of Kerman University of Medical Sciences. Seventy-five gr dried and powdered leaves of *A. marina* were thoroughly extracted using both static maceration and sonication with 500 mL methanol 70% for 72 h at 25 °C. Extracts were evaporated for dryness under reduced pressure (Büchi Rotavapor R-200), then kept in −20 °C for performing bioactivity reactions.

2.2. Phytochemical screening assay

The phytochemical analysis of the extract was performed by qualitative and semi-quantitative methods to specify the presence of tannins, saponins, alkaloids, terpenoids, flavonoids and antraquinones as described elsewhere (Evans, 1998).

2.3. Cultivation of macrophage cell lines and L. tropica parasite

The *L. tropica* (MHOM/IR/2002/Mash2) promastigotes strain was cultured in RPMI-1640 medium consisted of 100 mg/mL penicillin-streptomycin (Biosera, France), and 10% heat inactivated fetal bovine serum (FBS, Gibco, Germany) then incubated at 25 ± 1 °C in 25 cm^2 culture flasks. Also, murine macrophage cell lines J774-A1 (ATCC number TIB-67, Pasteur Institute, Iran) were cultured and maintained in 50% Dulbecco's Modified Eagle Medium (DMEM) and 50% RPMI-1640 medium, enriched as above and incubated at 37 °C with 5% CO_2.

2.4. DPPH assay as an antioxidant activity

Antioxidant assay was carried out by using the method described by Benzie and Strain (Benzie and Strain, 1999). This method measures the plant extract ability to scavenge 2,2-diphenyl-1,1-picrylhydrazyl (DPPH) free radicals. Briefly, 0.1 mM of DPPH radical solution was prepared in methanol and then 1 mL of this solution mixed with the sample (3 mL) in methanol: water (8:2, *v*/v) at the concentrations of 25, 50, 100, 200 and 400 µg/mL. Following 30 min, the absorbance was measured at 517 nm. The decrease in the DPPH solution absorbance indicates the increased level in the DPPH radical scavenging activity, which was represented as % DPPH radical scavenging, and calculated by the following equation:

%DPPH radical scavenging = (control absorbance- sample absorbance)/control absorbance × 100.

The butylated hydroxyanisole (BHA) and DPPH solution without sample were used as standard control and negative control, respectively (Chuen et al., 2016).

2.5. Assessment of anti-promastigote activity and cytotoxic effects

The effects of *A. marina* extract and Glucantime (Sanofi-Aventis, Paris, France), as positive control drug, directly on *L. tropica* promastigotes and J774-A1 cell line were evaluated. Briefly, logarithmic phase promastigotes (1×10^6 cells/mL) and cell lines (5×10^5) were seeded in a 96-well flat bottom microtiter plate. Then, 10 µl of different concentrations of the extract and Glucantime (25, 50, 100, 200 and 400 µg/mL) were added to each well and incubated at 24 ± 1 °C for 72 h. The untreated control as well as treated cells with Glucantime considered as the negative and positive controls, respectively. After incubation, anti-leishmanial activity of the extract was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) solution in each well and incubated at 24 ± 1 °C for 4 h. Then, 100 µl of HCL- isopropanol was added in order to stop the reaction. The relative amount of formazan produced by viable cells was measured photometrically at 450 nm using a plate reader (BioTek-ELX800, USA). The IC_{50} and 50% cytotoxicity concentration (CC_{50}) values were determined by the probit test of SPSS software version 20 (Oliaee et al., 2020).

2.6. Anti-amastigote activity assay

J774-A1 cell line (1×10^5 /mL) were cultured in RPMI-1640 along with 50% DMEM medium on glass slides in sterile plates and incubated for 3 h at 37 °C in 5% CO_2. All cultures were performed as duplicates. Adhered macrophages were infected with *L. tropica* promastigotes (stationary growth phase) at a ratio of 10:1 parasite/macrophage and kept overnight at 37 °C in 5% CO_2. Free parasites were washed out with PBS and infected macrophages were treated with different concentrations of extract (25, 50, 100, 200 and 400 µg/mL) and maintained for 72 h at 37 °C with 5% CO_2. Amastigote burden was compared between both test and control groups. Finally the IC_{50} value was calculated by the probit test (Oliaee et al., 2020).

2.7. Detection of apoptosis induction in promastigotes

The percentage of apoptotic and necrotic cells of drug-exposed promastigotes were carried out by flow cytometric analysis using Annexin V-7AAD Apoptosis Detection Kit (B D, Biosciences, Germany). Briefly, 1×10^6 promastigotes were treated with 125 µg/mL concentration of *A. marina* extract (as the IC_{50} dose) in Eppendorf tube and incubated at 24 °C for 72 h. Then, five µl annexin-V, 5 µl 7AAD and 100 µl binding buffer (1×) were added to the sample and incubated for 15 min in the dark at room temperature. Finally, all specimens were analyzed by FACS caliber system (Mostafavi et al., 2019).

2.8. Measurement of reactive oxygen species (ROS) levels

In order to assess the level of ROS production, *L. tropica*-infected macrophages were treated with the IC_{50} dose and resuspended with PBS (pH 7.4) and labelled with 10 µM H2DCFDA (Invitrogen, USA) following incubation for 15 min at dark room. Fluorometric analysis was conducted at 492–495 nm excitation and 517–527 nm emission wavelengths using FACS Caliber system.

2.9. Measurement of arginase activity

Arginase enzymatic activity was measured in promastigotes and infected macrophages exposed with various concentrations of extract (25, 50, 100, 200 and 400 µg/mL) by using Arginase Activity Assay Kit (cat. No. MAK112, Sigma-Aldrich, USA). Briefly, 1×10^6 treated promastigotes and J774-A1 treated cells were harvested in culture medium and the cells pellet washed with PBS. The cells were lysed for 30 min in 100 µl of 50 mM Tris- HCL (pH 7.4) containing 0.4% (*w/v*) Triton x-100, followed by centrifugation at 13000 ×*g* for 10 min. 40 µl of the sample's supernatant was added to each well of a 96-well plate. Urea standard solution and substrate buffer was added to wells according to the manufacturer protocol and finally, urea reagent was used to stop the reaction. The absorbance was measured by spectrophotometer at 430 nm (Oliaee et al., 2019).

$$\text{Enzyme Activity} = \frac{\text{A 430 sample} - \text{A430 Blank}}{\text{A 430 standard} - \text{A430 Water}} \times \frac{1\text{mM} \times 50 \times 10^3}{\text{V} \times \text{T}}$$

T = Reaction time in minutes, V = Sample volume (µl) added to well (1–40 µl), 1 mM = Concentration of Urea Standard and 50 =

Reaction volume (μl).

2.10. Statistical analysis

Statistical differences between groups were determined by using ANOVA and student *t*-test. The $P < 0.05$ was considered to be significant. The statistical analyses were performed using the Prism 7.01 software (GraphPad Software, San Diego, USA) and experiments were performed duplicates.

3. Results

3.1. Phytochemical analysis

The phytochemical screening of *A. marina* hydroalcoholic extract represented strong presence of tannins and saponins, and moderate presence of terpenoids, flavonoids and alkaloids (Table 1) (Saedi Dezaki et al., 2016; Kujur et al., 2010).

3.2. DPPH assay

High radical inhibition observed by increasing the *A. marina* concentration (Fig. 1). The sample with the IC_{50} value less than 50 μg/mL has been proved as active ones (Reynertson et al., 2005). Our extract represented high scavenging effect against DPPH compared to the synthetic antioxidant BHA (IC_{50} 26.77 ± 22.6 vs. 91.05 ± 36.80 μg/mL, respectively) (Table. 2).

3.3. Effects of A. marina extract on L. tropica promastigotes

The anti-leishmanial effects of our extract and Glucantime against *L. tropica* promastigotes were assessed by MTT assay. As shown in Table 3, *A. marina* represented lower IC_{50} compared to Glucantime (125 ± 1.4 vs. 150.2 ± 11 μg/mL, respectively, $P < 0.01$). Also, significant difference was observed in promastigotes viability between *A. marina* and Glucantime by elevating concentrations ($P < 0.001$). Our results showed the viability percent of 28% in 400 μg/mL after 72 h exposure of crude extract (Fig. 2).

3.4. Anti-amastigote evaluation and cytotoxicity analysis

The IC_{50} value of *A. marina* extract against the intracellular amastigotes was calculated as 60.57 μg/mL, which showed significant decrease compared to Glucantime standard group (Table 3). Besides, the CC_{50} of *A. marina* extract was higher than Glucantime drug (650 ± 1.1 vs. 375 ± 1.2 μg/mL, respectively) (Table 3). Also, the selectivity index (SI), as the ratio of extract CC_{50} to the IC_{50} of amastigotes, represented higher level in *A. marina* compared to Glucantime (10.7 vs. 5.1, respectively), which indicated the lower toxicity of this extract. As shown in Fig. 3, the percentage of J774-A1 cell viability was significantly decreased in Glucantime-treated cells compared to extract ones, especially at 200 and 400 μg/mL concentrations ($P < 0.001$).

3.5. Apoptosis assessment

In order to assess the apoptotic or necrotic mode of *L. tropica* promastigotes, Annexin-V and 7-AAD were used, respectively. In the analysis of Annexin V-FITC/7AAD staining, four quadrants of upper right (UR), lower right (LR), upper left (UL) and lower left (LL) representing the late apoptotic cells, early apoptotic cells, primary necrotic cells and live cells, respectively. In Glucantime group, the percentage of viable cells, early apoptosis, late apoptosis and necrotic cells were 50.6%, 21.56%, 10.30% and 17.55%, respectively. In *A. marina*- exposed group, these percentages were 31.32%, 46.50%, 12.24% and 9.94%, respectively. It is obvious that the level of apoptotic cells in the early stage of apoptosis were increased in extract-exposed group compared to control group (46.5 vs. 21.5,

Table 1
Phytochemical screening of *A. marina* extract.

Secondary metabolites	Amount of presence
Tannins	
Ferric chloride test	++
Formaldehyde test	++
Phlobatannins test	++
Antraquinones	
Borntrager's test	−
Modified Borntrager's test	−
Saponins	++
Terpenoids	+
Flavonoids	+
Alkaloids	+

(−): negative reaction, (+): moderate reaction, (++): strong reaction.

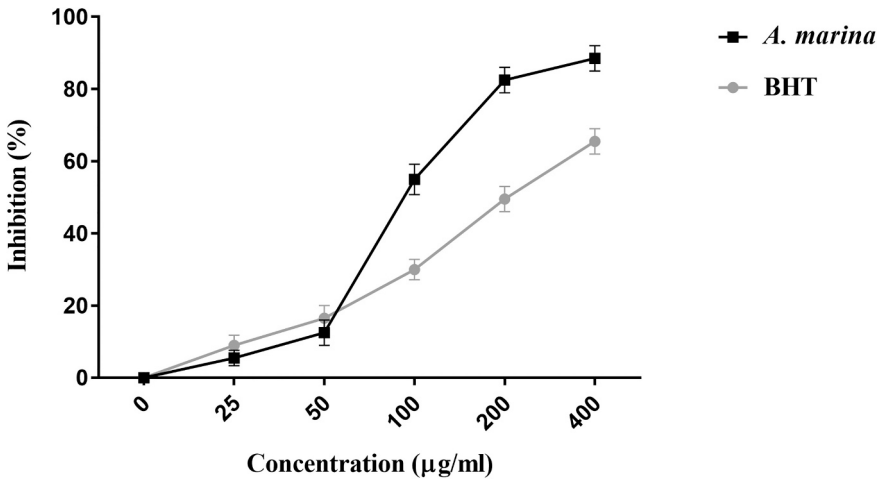

Fig. 1. Antioxidant activity of *A. marina* extract by using the free radical scavenging activity of DPPH (%) compared to BHA as a standard control. Data are means ± SD.

Table 2
The IC_{50} values of DPPH radical scavenging activity (%) of *A. marina* extract compared to BHA.

Compounds	IC_{50}[a] ± SD (µg/mL)	*P* value
A. marina	26.77 ± 22.6	$P < 0.05$
Butylated hydroxyanisole (BHA)	91.05 ± 36.80	NR

NR: Not related.
[a] IC50: 50% Inhibitory concentration of drug.

Table 3
Comparison of the IC_{50} values of *A. marina* extract on *L. tropica* amastigotes, promastigotes compared with Glucantime as positive control and the CC_{50} values of the extract on macrophage after 72 h using the SI index.

Drug	Amastigote [a]IC_{50} ± SD (µg/mL)	*P*-value	Promastigote IC_{50} ± SD (µg/mL)	*P*-value	Macrophage [b]CC50 (µg/mL)	[c]SI (selectivity index)
Glucantime	73.19 ± 10.12	NR	150.2 ± 11	NR	375 ± 1.2	5.1
A. marina	60.57 ± 1.46	$P \leq 0.05$	125 ± 1.4	$P \leq 0.01$	650 ± 1.1	10.7

NR: Not related.
[a] IC_{50}, Concentration of drug that inhibited 50% of growth in promastigotes and amastigotes.
[b] CC_{50}, Concentration of drug that inhibited 50% of growth in macrophages.
[c] SI, Selectivity index (CC_{50} of macrophage/IC_{50} of amastigote).

respectively) (Fig. 4).

3.6. ROS generation in J774-A1 cell lines

Results revealed that ROS production was significantly induced by *A. marina* extract in *L. tropica*- infected cell lines after 24 h (Fig. 5a). The ROS production in *A. marina*-treated cells represented significant increased level compared to untreated cells (7 ± 1.2 vs. 3.2 ± 0.3, $P < 0.01$, respectively) (Fig. 5b).

3.7. Arginase activity in extract-treated macrophages and promastigotes

Our findings represented decreased levels in arginase activity in two concentrations of 200 and 400 µg/mL when compared with untreated control. In treated macrophages, significant decrease was observed among control and each concentration of 200 and 400 µg/mL with 1.7-fold and 7-fold, respectively ($P < 0.001$) (Fig. 6a). Also, *A. marina*-treated promastigotes showed a significant decrease in arginase activity by increasing the extract concentrations, as compared with control group. As shown in Fig. 6b, the results showed significant reduction in enzyme activity at the last three concentrations of 100, 200 and 400 µg/mL ($P < 0.001$).

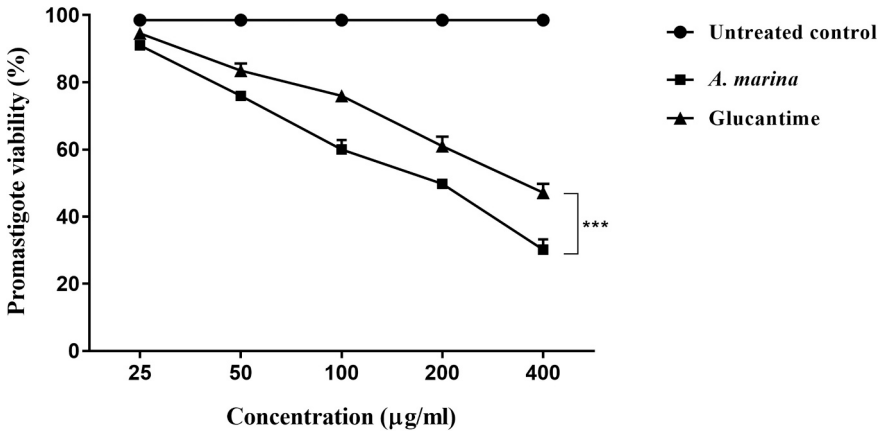

Fig. 2. Effect of *A. marina* on *L. tropica* promastigotes compared to Glucantime at different concentrations of 25–400 µg/mL. Each concentration was performed as a duplicate (***$P < 0.001$).

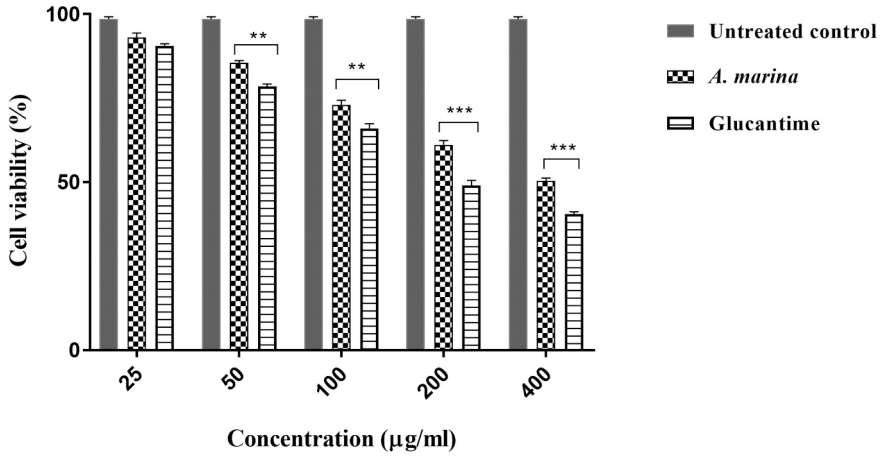

Fig. 3. Effects of *A. marina* on J774-A1 cell viability compared to Glucantime at different concentrations. Data are expressed as % of viable cells in relation to control (**$P < 0.01$ and ***P < 0.001).

4. Discussion

Leishmaniasis is a fundamental threat and has a main role in causing social and medical burden worldwide (Bailey et al., 2017). The ongoing conventional therapeutic protocol for leishmaniasis has side effects, severe pain, as well as causing parasite resistance in most species (Gervazoni et al., 2018).

Therefore, the use of effective alternative compounds is crucial, as they are less expensive, less toxic and more effective, with different action mechanisms. (Mahmoudvand et al., 2014; Ilaghi et al., 2021; Saduqi et al., 2019). From the past to date, natural products and plant-associated components have been valuable by developing novel therapeutic agents against different diseases (Parvizi et al., 2020).

In this study we examined the anti-leishmanial potency of hydro-alcoholic extract of grey mangrove on *L. tropica* parasite by accessing inhibitory effect, the level of apoptosis, ROS and arginase enzymatic activity.

In this research, *A. marina* represented higher radical inhibition than the BHA standard control; it is inconsistent with the results of previous studies (LAO et al., 2018; Kheirandish et al., 2016). In our findings, *A. marina* represented significant lower IC_{50} compared to Glucantime standard drug in promastigote and amastigote forms of *L. tropica*. Moreover, this extract had the potential to induce early apoptosis in 46.5% of the promastigotes in the IC_{50} dose (125 µg/mL) which is comparable to apoptotic level of Glucantime as the standard drug (21.56%); this is in accordance with the result of other plant-based medicines (Saduqi et al., 2019).

Additionally, the promastigotes and macrophages treated with different concentrations of *A. marina* showed the significant reduction in arginase activity compared to the non-treated group. Arginase and L-arginine play fundamental role in the biosynthesis of polyamine which is essential for *Leishmania* growth and protecting the parasite against oxidative stress agents and ROS produced by the host's immune system (Muxel et al., 2018). Importance of L-arginine in the regulation of survival and apoptosis of some eukaryote cells have been reported (Landfear, 2011; Gong et al., 2000).

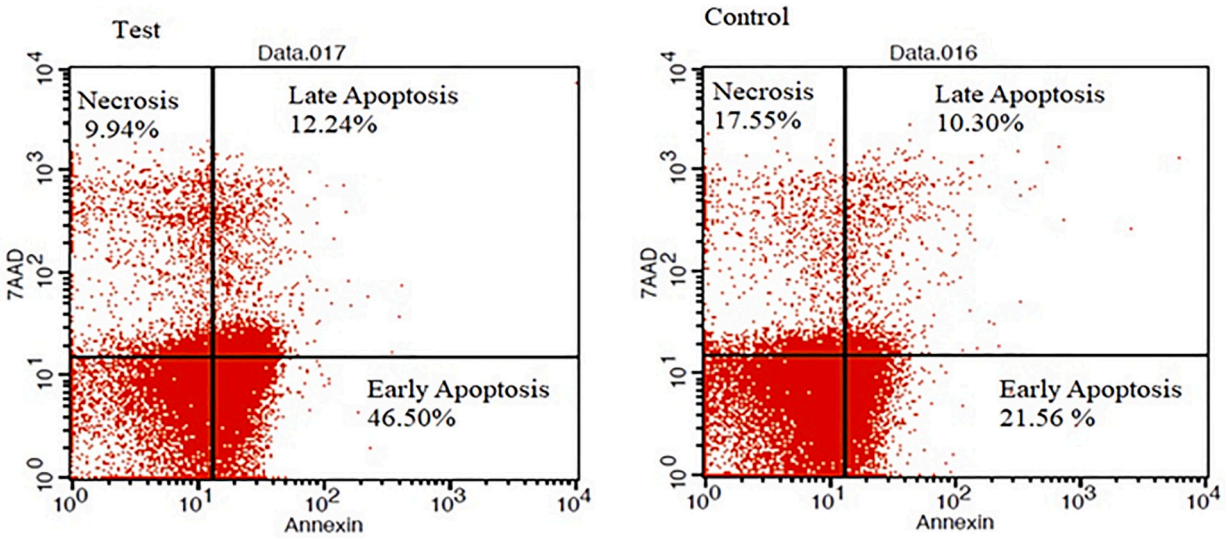

Fig. 4. The apoptotic profiles of *L. tropica* promastigotes treated with *A. marina* in 125 µg/mL concentration (IC_{50} dose) compared with the control group.

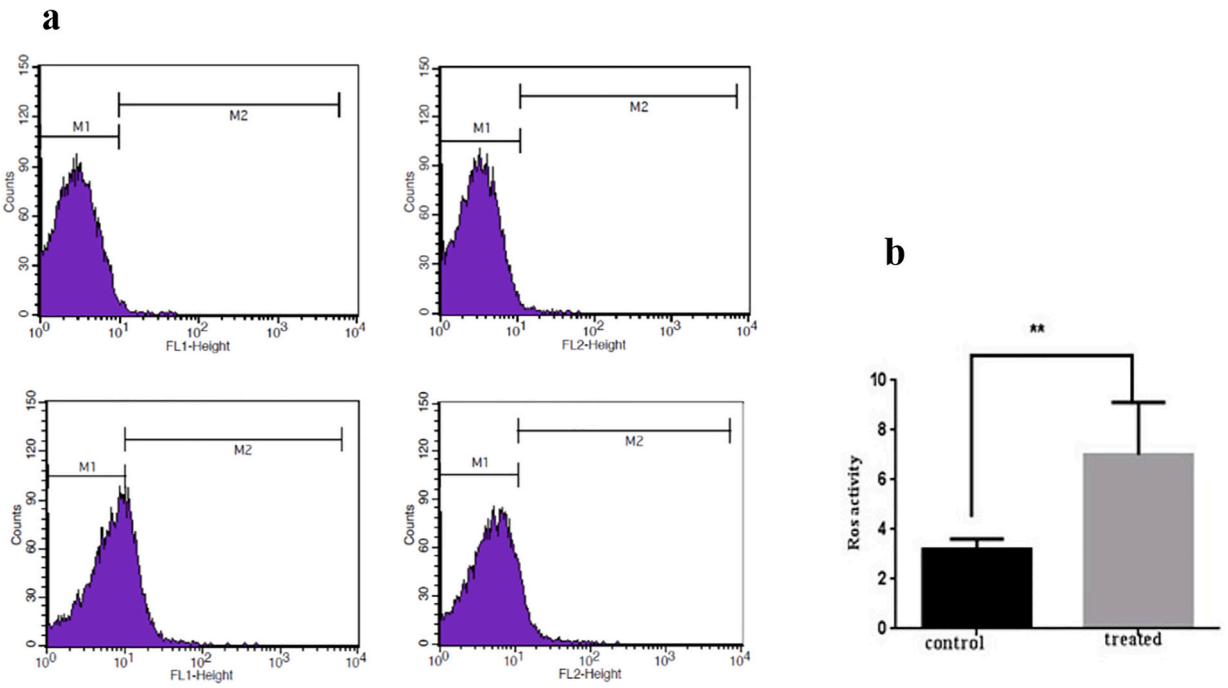

Fig. 5. The histogram represented ROS level produced by treated cells in flow cytometry analysis (a). The bar graph showed significant increase of ROS production in *A. marina*-treated cells compared to untreated cells (b). Control represents infected but non-treated cells (** $P < 0.01$).

Arginase enzyme has crucial influence in the progress of *Leishmania* infection. L-arginine can be metabolized to nitric oxide (NO) by inducible NO synthase (iNOS) or to L-ornithine by arginase. NO, as a microbicidal agent, is responsible for intracellular parasite removal. L-ornithine is a main intermediate substrate for glutamine and polyamines biosynthesis, which are both crucial for growth and reproduction of *Leishmania* parasite. Survival or death of parasite depends on the balancing between these two main enzymes (Badirzadeh et al., 2017; Roberts et al., 2004). Although Boitz et al. proved that arginase is essential for proliferation of promastigotes but not intracellular amastigotes; because *Leishmania donovani* amastigotes can readily salvage ornithine and have the access to spermidine pools of the host (Boitz et al., 2017). In the present study, decreased parasite growth in *A. marina*-exposed promastigotes or macrophages may be due to the reduced level of arginase and increased production of ROS and apoptotic markers.

As mentioned above, the effect of herbal on microorganisms has been related to substances including alkaloids, quinones, chalcones

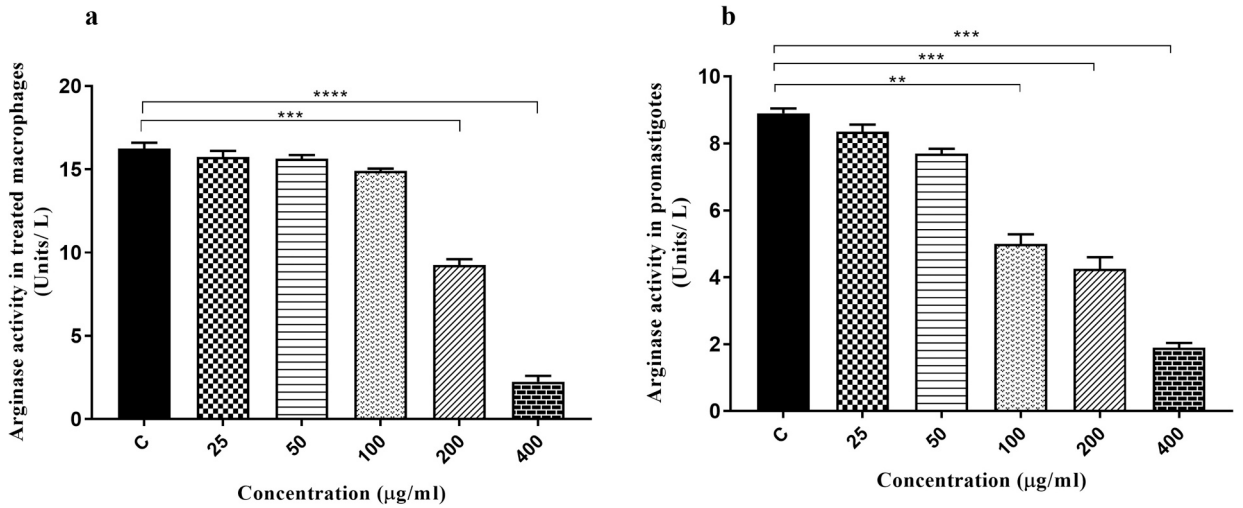

Fig. 6. Comparative levels of arginase activity in infected J774 cells (a) and *L. tropica* promastigotes (b) treated with different concentrations of *A. marina*. Significant reduction was observed in 200 and 400 μg/mL concentration compared with the control group. Error bars are SD. Each test was conducted in duplicates (**P < 0.01, ***P < 0.001 and ****$p < 0.0001$).

and triterpenoids (Lage et al., 2013; Oryan, 2015). According to our phytochemical screening, *A. marina* extract represented strong presence of both substances of tannins and saponins, and moderate presence of terpenoids, flavonoids and alkaloids. Based on the other studies, tannins induced nitric oxide synthase and cytokines gene expressions in *L. major*-infected macrophages (Kolodziej et al., 2005). Also, saponin showed a strong protease inhibitory activity in *L. braziliensis* (Zapata et al., 2020). Alkaloids disrupt respiratory chain mediators and inhibit respiration of amastigotes. Terpenoids increase the production of NO, induce apoptosis via inhibition of parasite DNA topoisomerase and inhibit the parasitic growth (Sen and Chatterjee, 2011). It has been shown that some flavonoids such as fisetin (as the most potent arginase inhibitor), quercetin, luteolin and 7, 8-hydroxyflavone with low cytotoxicity characteristics are able to inhibit arginase enzyme from *L. amazonensis* (Manjolin et al., 2013).

5. Conclusion

In the present study, we have shown that *A. marina* extract inhibited the proliferation of *Leishmania* parasite by increasing ROS production and apoptotic markers as well as decreasing arginase in parasite and extract-treated cells. The promising results demonstrated the importance of *A. marina* as a native plant in tropical region and it is potentially useful for the treatment of leishmaniasis in endemic countries.

Funding

The present study was financially supported by Vice-Chancellor for Research, Kerman University of Medical Sciences, Kerman, Iran (Grant number 95000112). The funder had no role in the study design, data collection, data analysis and manuscript preparation.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Acknowledgements

We would like to thank the staff of Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran for their assistance in performing this study.

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