Original Article

In Vitro and *In Vivo* Effects of *Astragalus Ecbatanus* Extract against Cutaneous Leishmaniasis

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ABSTRACT

Given the distinctive characteristics of *Astragalus* in the treatment of diseases and the strengthening of the immune system, for the first time, this study represents the first attempt to study the *in vitro* and *in vivo* leishmanicidal effects of chloroform extract of *A. ecbatanus* (AECE) on *Leishmania major*. The *in vitro* activity was determined against *L. major* (MHOM/AF/88/KK27). In addition, the effect of AECE on the generation of nitric oxide (NO) and the rate of macrophage infectivity was evaluated. The, antileishmanial effects of topical administration of AECE at 10 and 20 mg/kg were evaluated *In vivo* on cutaneous leishmaniasis in mice. The 50% inhibitory concentration (IC_{50}) index of AECE and amphotericin B for promastigotes was found to be 76.3 and 2.78 µg/mL, respectively. The number of amastigotes exhibited a dose-dependent decline following treatment with AECE. The IC_{50} and 50% cytotoxic concentration (CC50) for AECE were 39.4 and 408.3 µg/ml, respectively. The extract was observed to induce the creation of NO while simultaneously reducing the level of macrophage infection. Following a four-week course of AECE therapy, the lesions of CL were observed to have healed in the infected mice. Additionally, the number of the amastigote forms of *Leishmania* in the CL lesions was significantly reduced followingAECE therapy in infected mice ($p<0.05$). These findings demonstrate the considerable inhibitory and eliminatory effects of AECE on *Leishmania in vivo* and *in vitro*. Even though we have identified some cellular mechanisms of action for AECE, e.g. reducing the infectivity rate and the induction of NO production against *Leishmania* parasites, further experiments are essential to identify the specific mechanisms of action, assess safety, and determine its ability in animals and human subjects.

Keywords: Medicinal plants, Cutaneous Leishmaniasis, Promastigote, Amastigote

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1. Introduction

Leishmaniasis, a disease caused by *Leishmania* spp., is a prevalent global health concern, with cases reported in diverse geographical regions (1). The disease is reported to manifest in four forms: cutaneous, muco-cutaneous, diffuse and visceral forms (1). The cutaneous leishmaniasis is the most frequently reported form of the disease in many countries such as Iran (2). The principal chemical drugs employed for the control and the treatment of leishmaniasis are pentavalent antimony agents, e.g., glucantime (meglumine antimoniate, MA). Other drugs used include Amphotericin B, metronidazole, diamidine, pentamidine, allopurinol, ketoconazole, astroconazole, dapsone, and paramomycin (3). However, these agents are not free of side effects, and the recent studies have indicated that they may belinked to some complications such as gastrointestinal, liver and kidney disorders as well as drug resistance to these agents (4). In recent years, additional attempts have been made to identify and develpo alternative drugs and to enhance the efficacy of existing common drugs. *Astragalus* is a medicinal plants that has been demonstrated to possess excellent healing properties (5).Approximately 3,200 species of this plant are known to exist in the world, with approximately 500 species distributed across the Americas and another 2,700 species distributed across other regions of the world. *The Astragalus plant is particularly well-suited to* tropical climates, exhibiting robust growth in regions with limited water access and high humidity (5). Iran is home to approximately800 different species of herbaceous plants, shrubs and bushes in Iran. This plant is of significant importance due to its rapid growth and perennial durability (6). The most important species for therapeutic use are *A. kotschyanus, A. ecbatanus, A. chaborasicus, A. teheranicus, A. gummifer labill, A. ecbatanus.* The plants of this genus have have been demonstrated to possess therapeutic properties such as strengthening the immune system, relieving pain in the kidneys and bladder, preventing diabetes, antimicrobial and anti-cancer ones (6, 7). Given the unique properties of *Astragalus* in the treatment of diseases and strengthening the immune system, study initially sought to assess the *in vitro* and *in vivo* anti-leishmanial effects of chloroform extract of *A. ecbatanus* (AECE) on *Leishmania major*.

2. Materials and Methods

2.1. Plant Materials

Initially, herbarium specimens from *A. ecbatanus* aerial parts were obtained in July 2022 from the mountainous areas of Nurabad city in western Iran. Following the identification of the herb by a plant scientist, an archive specimen was reserved in the herbarium of Razi Herbal Medicines Research Center in Khorramabad, Iran (No. LUMS-22564).

2.2. Extraction Preparation

Two hundred grams of plant materials were ground and degreased with n –hexane, and extraction was performed using the maceration technique. Subsequently, the collected extract was subjected to a rotary evaporator at 52°C and 100 rpm under vacuum conditions. Subsequently ,the solution was poured into a decantation funnel containing chloroform and evaporated under the aforementioned conditions (8).

2.2.1. Phytochemical Screening

The principal phytochemical investigation of the *A. ecbatanus* root chloroformic extract was also conducted to assess the existence of phytochemicals, including flavonoids, tannins, and alkaloids according to the methods described in previous study (9).

2.2.2. Total Phenolics and Flavonoids Content

In this study the level of phenolics and flavonoids substance was determined by two colorimetric approaches: Folin-Ciocalteau's reagent colorimetric and aluminum chloride.

2.3. Cell and Parasite

TheJ774-A1 cell lines, sourced from thePasteur Institute in Iran, were maintained in DMEM with 10% FBS at 37 °C with 5% CO2 (10). The *L. major* (MHOM/AF/88/KK27) promastigotes were cultured in 1640-RPMI with 20% FBS, penicillin/streptomycin (100 ml/IU) at 23±1°C.

2.4. Effect of Extract On Promastigotes

Initially, 100 µl of promastigotes (1×10^6/cells) were exposed to the extract at concentrations of 6.25-100 µg/mL in a 96-well plate and incubated at 24°C for 48 h. Subsequently, MTT suspension (20 µL, 5 mg/mL) was added to the wells and the plates were incubated under 5% CO_2 at 37°C for a further 4 h. Subsequently, the stopping solution (DMSO, 100 µl) was added, and the absorbance of the wells was recorded at 570 nm obtained using an ELISA reader (11). Amphotericin B (AmB) was utilized as the positive control.

2.5. Effect of Extract on Amastigotes

Macrophage cells (0.1 ml, 1×10^5/mL) were maintained in 24-well plates with coverslips on the base at 37°C in 5% CO_2 in order to facilitate observation. Subsequently, stationary phase promastigotes, at a concentration ten times greater than that of the macrophage cells (1×10^6/mL), were introduced to the wells and incubated at 37°C in 5% CO_2 for one day. Subsequently, the extract (6.25-100 µg/mL) and MA were combined with to the infected macrophages and incubated for an additional 48 hours. At the end, the smears were arranged and stained with by Giemsa, and examined under a light microscopy (12).

2.6. Effect on the Level of Macrophage Infection

The promastigotes (1×10^6/mL) were firstly exposed to the chloroform extract of *A. ecbatanus* at the IC50 concentration for 120 minutes at 21°C. Subsequently, the, pre-treated parasites were exposed to macrophages cells for 24 hours. At the end, the smears were organized and stained with Giemsa, and examined under a light microscopy (13).

2.7. Efficacy on Nitric Oxide (NO) Release

The macrophage cells (1×10^5/mL) were initially exposed to the extract at a concentration of 1/4 to ½ IC_5, normal saline, and LPS+IFN-γ (10 U/ml) for 48 hours. After obtaining the upper phase of the combinations (100 µl from each) was

obtained and the efficacy of the extract on NO release was determined using the Nitric Oxide Assay commercial kit (Sigma-Aldrich, Germany) (13).

2.8. Cytotoxic Activity on Macrophage Cells

The assay was conducted in accordance with the method employed forthe assessment of extract effects on promastigotes, as delineated in the MTT assay. Subsequently, the 50% cytotoxic concentration (CC50) was then estimated using the Probit test in SPSS software. The selectivity index (SI) was calculated using the following formula: CC_{50} /IC_{50}. This was done for amastigotes (14).

2.9. In Vivo Effects on CL

2.9.1. Animal

A total of 32 male BALB/c mice, weighing between 25 to 30 grams ,were maintained in accordance with optimal standard conditions. To observe the CL, 0.1 mL of promastigotes (10^6 cells/mL) were subcutaneously injected into the base of the animal tail (13).

2.9.2. Study Design

Once the lesions of *Leishmania* were observed (after the sixth week), the 6th weeks), the infected mice were divided into four groups. The first group received normal saline, the second group received MA at 25 mg/kg (10), the third group received AECE at 10 mg/kg/day, and the fourth group received AECE at 20 mg/kg/day. Each group was treated for 28 days.

2.9.3. Effects of AECE on Cutaneous Leishmaniasis

Subsequently, the dimensions of lesions were gauged with the aid of a Vernier caliper Additionally, the number of parasites was assessed in the impression smears stained with Giemsa, which were obtained from the lesions under study using a light microscope (13).

2.10. Statistical Analysis

All in vitro examinations were repetitive three times. The data were analyzed using SPSS software version 26.0 with one-way analysis of variance (ANOVA). The level of significance was set at $p<0.05$.

3. Results

3.1. Phytochemical Assessment of AECE

The yield of the extract was 17.2 grams (9.6%, w/v). Phytochemical assessment revealed the existence of saponins, flavonoids, terpenoids, and polysaccharides, as documented in Table 1.

3.2. *In vitro* Leishmanicidal and Cytotoxicity of AECE

The AECE exhibited a marked ($P<0.001$) reduction in promastigote viability compared to the control drug, with the IC_{50} of 76.3 µg/mL (Table 2). The findings of the macrophage model indicated that AECE exhibited favorable leishmanicidal activity on amastigotes as evidenced by a dose-dependent response (Figure 1), with the IC_{50} of 39.4 µg/mL (Table 2). With regard to the cytotoxicity effects, the CC_{50} of the AECE was determined to be 408.3 with a selectivity index of 11.2 (Table 2).

Table 1. The main phytochemical analysis results of the chloroformic extract from A. ecbatanus.

Phytochemical	Presence
Flavonoids	++
Polysaccharides	+
Saponins	+
Terpenoids	+

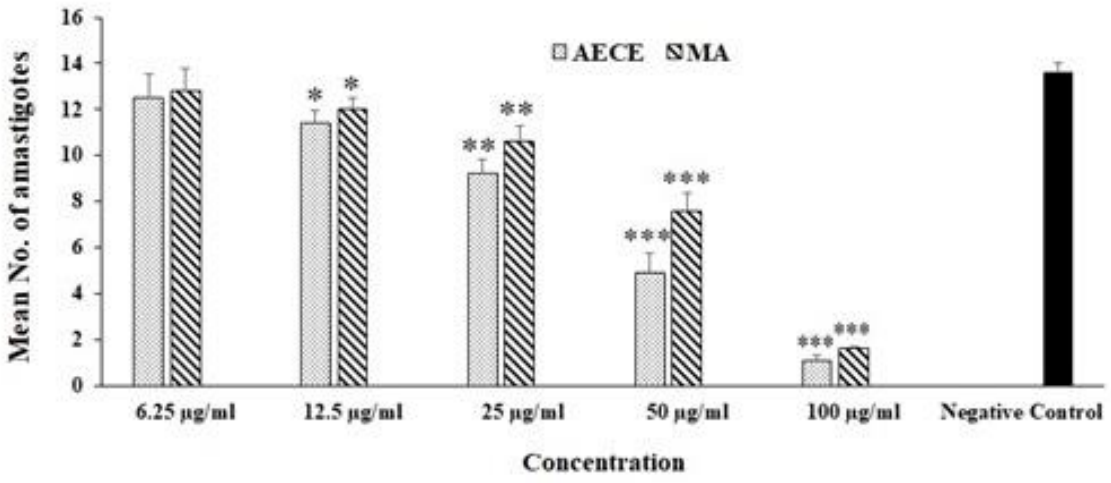

Figure 1. Effect of *A. ecbatanus* chloroform extract (AECE) and glucantime (MA) on mean numbers of amastigote forms of *Leishmania major*. Mean ± standard deviation. (n=3). *p<0.05, ** p<0.01, and *** p<0.001.

Table 2. Efficacy and cytotoxicity of *A. ecbatanus* chloroform extract against *Leishmania* amastigote and promastigote forms. Mean ± standard deviation. (n=3)

Drug	Promastigote IC_{50} (µg/mL)	Amastigote IC_{50} (µg/mL)	CC_{50} (µg/mL)	SI
A. ecbatanus	76.3 ± 5.12	39.4 ± 2.32	408.3± 5.66	11.2
Glucantime	-	56.9 ± 5.12	1165.3± 13.2	20.47
Amphotericin B	2.31± 0.087	-	-	-

3.4. Effect of AECE on NO Release

Table 3 illustrates that the *A. ecbatanus* chloroformic extract stimulated the production of NO, with the greatest effect of AECE on NO release ($p < 0.001$) observed at ½ IC_{50}.

3.5. *In vivo* antileishmanial effects of AECE

As illustrated in Figure 2, following four weeks of AECE therapy, lesions of CL were healed in the infected mice. Additionally, the number of the amastigote forms of *Leishmania* in the CL lesions was significantly reduced after AECE therapy of the infected mice ($p<0.05$).

Table 3. The impact of A. ecbatanus chloroformic extract on the production of nitric oxide (NO).

Concentration (µg/mL)	NO release (nM)
¼ IC_{50}	4.95 ± 0.28
1/3 IC_{50}	10.2 ± 1.12*
1/2 IC_{50}	19.1 ± 1.42*
Non-treated	3.14 ± 0.31
IFN-γ+LPS	40.21± 2.65

* $p < 0.001$

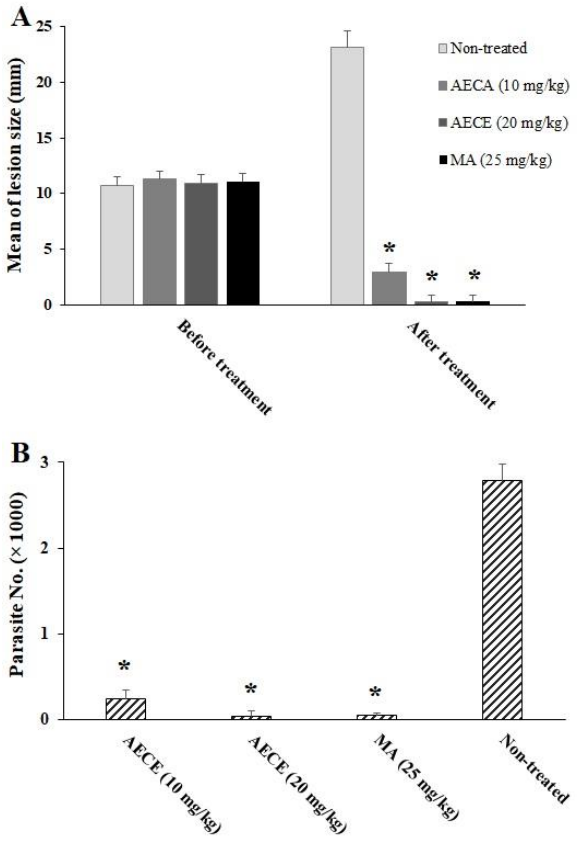

Figure 2. *In vivo* antileishmanial effects of *A. ecbatanus* chloroformic extract *(*AECE) on cutaneous leishmaniasis in mice through assessing the lesion size (A) and the number of parasites (B). * P<0.001

4. Discussion

Recently, there has been a notable increase in scientific interest in the use of medicinal herbs for the treatment of a broad range of diseases (15). The primary motivations for the use of natural products are their extensive accessibility, cost-effectiveness, minimal complications, high efficiency, and antimicrobial properties (16). For example,*Astragalus* spp., have been demonstrated to have therapeutic effects, including the strengthening the immune system, the alleviation of pain in the kidneys and bladder, the prevention of diabetes, antimicrobial ,and anti-cancer ones (10). Given the unique properties of *Astragalus* in the treatment of diseases and strengthening the immune systemthis study initially sought to assess the *in vitro* and *in vivo* anti-leishmanial properties of AECE on *L. major*. The MTT test results demonstrated that the AECE significantly ($P < 0.001$) reduced the viability of promastigotes. Additionally,the findings of the macrophage model indicated thatAECE demonstrated a favorable leishmanicidal effect on amastigotes, exhibiting a dose-dependent response. In the animal model, the lesions of CL were healed in the infected mice ollowing four weeks of AECE therapy. Additionally, the number of the amastigote forms of *Leishmania* in the CL lesions was significantly reduced following AECE therapy in the infected mice. A number of recent studies have demonstrated the antileishmanial effects of various herbs and their derivatives. However, the findings of these surveys are not entirely consistent due to the lack of accurate mechanisms and the diverse effectiveness of the surveys themselves. Accordingly, more comprehensive and precise surveys are required (15, 16). In several research projects, the ability of *Astragalus* spp. to combat a range of off various harmful microorganisms, including bacteria, fungi (*Candida* spp), viruses, and parasites (such as *Eimeria papillata* and *Toxoplasma gondii*), has been investigated. (17-19). However, to the best of our knowledge, no study has documented the antileishmanial effects of *A. ecbatanus.* A phytochemical assessment of AECE revealed the presence of saponins, flavonoids, terpenoids, and polysaccharides were approved in AECE. Phenolic and flavonoid compounds are widely recognized as the most prevalent phytochemicals found in the *Astragalus* genus. (20). It has been demonstrated that flavonoids and phenolic compounds can inhibit the synthesis of nucleic acids, disrupt the cytoplasmic and cell membranes, prompt apoptosis, change the membrane permeability, inhibit the pathogenicity, and exert synergistic effects with current agents, thereby preventing and eliminating the pathogens (21-24).Therefore, it can be suggested that the promising antileishmanial effects of *A. ecbatanus* are associated with the presence of these phytochemicals. Macrophage cells are the primary immune cells responsible for the removal of *Leishmania* parasites. These cells, through the synthesis and subsequent emission of nitric oxide, have been demonstrated to inhibit and eliminate the *Leishmania* parasite (25). The results demonstrated that the AECE stimulated the production of NO, with the greatest impact on NO release ($p<0.001$) observed ½ IC50. The CC50 of the AECE was 408.3, with a selectivity index of 11.2; indicating that the extract is highly specific for intracellular parasites and has low cytotoxicity to host cells. These findings indicated that AECE has a considerable impact on the inhibition and elimination of *Leishmania* parasites *in vitro*. While, some cellular mechanisms of AECE have been identified, such as a reduction in the infectivity rate and the induction of NO production against *Leishmania* parasites, however, further studies are essential to identify the specific mechanisms of action, safety, and its ability to be used in animal and human subjects.

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Authors' Contribution

SNT designed and conceptualized the survey; HM, JGY, and SP performed the experiments and data analysis; LM supervised and critically reviewed the manuscript.

Ethics

The research was approved by the ethics committee of Lorestan University of Medical Sciences, Khorramabad, Iran (IR.LUMS.REC.1400.204).

Conflict of Interest

The authors declare that there are no conflicts of interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

References

1. Monzote L. Current treatment of leishmaniasis: a review. *Open Antimicrob Age J*. 2009;1(1):9-19.
2. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: a review. *F1000Research*. 2017;6: 750.
3. Albalawi AE, Alanazi AD, Sharif I, Ezzatkhah F. A systematic review of curcumin and its derivatives as valuable sources of antileishmanial agents. *Acta Parasitol.* 2021; 66: 797-811.
4. Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, Andrade CA. Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Acta Trop. 2011;118(2):87-96.

5. Rios JL, Waterman PG. A review of the pharmacology and toxicology of Astragalus. *Phytother Res*. 1997;11(6):411-418.
6. Li X, Qu L, Dong Y, Han L, Liu E, Fang S, Zhang Y, Wang T. A review of recent research progress on the Astragalus genus. *Molecules*. 2014;19(11):18850-18880.
7. Pouramini A, Sh J, Shayan P, Ebrahimzadeh E, Namavari M, Shirian S. Molecular and serological detection of Neospora caninum in multiple tissues and CSF in asymptomatic infected stray dogs in Tehran, Iran. Iranian Journal of Veterinary Medicine. 2017;11(2):105-12.
8. Niazi M, Saki M, Sepahvand M, Jahanbakhsh S, Khatami M, Beyranvand M. In vitro and ex vivo scolicidal effects of Olea europaea L. to inactivate the protoscolecs during hydatid cyst surgery. *Annals Med Surg*. 2019; 42:7-10.
9. Ezatpour B, Saedi Dezaki E, Mahmoudvand H, Azadpour M, Ezzatkhah F. In Vitro and In Vivo Antileishmanial Effects of Pistacia khinjuk against Leishmania tropica and Leishmania major. *Evid Based Complement Alternat Med*. 2015; 2015: 149707.
10. Albalawi AE, Khalaf AK, Alyousif MS, Alanazi AD, Baharvand P, Shakibaie M, Mahmoudvand H. Fe3O4@ piroctone olamine magnetic nanoparticles: Synthesize and therapeutic potential in cutaneous leishmaniasis. Biomed Pharmacother. 2021;139: 111566.
11. Hesami D, Ghaffarifar F, Dalimi Asl A, Nasiri V, Ghasemi E, Jorjani O. A study on the effects of Pistacia atlantica Desf. essential oil on Leishmania major in vitro and in vivo. Iranian J Med Arom Plants Res. 2019;35(1):44-53.
12. Mahmoudvand H, Ezzatkhah F, Sharififar F, Sharifi I, Dezaki ES. Antileishmanial and cytotoxic effects of essential oil and methanolic extract of Myrtus communis L. The Korean J Parasitol. 2015;53(1):21.
13. Albalawi AE, Abdel-Shafy S, Khudair Khalaf A, Alanazi AD, Baharvand P, Ebrahimi K, Mahmoudvand H. Therapeutic potential of green synthesized copper nanoparticles alone or combined with meglumine antimoniate (glucantime®) in cutaneous leishmaniasis. Nanomaterials. 2021; 11: 891.
14. Mahmoudvand H, Kheirandish F, Mirbadie SR, Kayedi MH, Riabi TR, Ghasemi AA, Bamorovat M, Sharifi I. The potential use of methotrexate in the treatment of cutaneous leishmaniasis: in vitro assays against sensitive and meglumine antimoniate-resistant strains of Leishmania tropica. Iranian J Parasitol. 2017;12(3):339.
15. Tajbakhsh E, Khamesipour A, Hosseini SR, Kosari N, Shantiae S, Khamesipour F. The effects of medicinal herbs and marine natural products on wound healing of cutaneous leishmaniasis: A systematic review. Microbial Pathogen. 2021;161:105235.
16. Gharirvand Eskandari E, Setorki M, Doudi M. Medicinal plants with antileishmanial properties: A review study. Pharm Biomed Res. 2020;6(1):1-6.
17. Mikaeili A, Karimi I, Shamspur T, Gholamine B, Modaresi M, Khanlari A. Anti-candidal activity of Astragalus verus in the in vitro and in vivo guinea pig models of cutaneous and systemic candidiasis. Rev Bras Farmacogn. 2012;22(5):1035-1043.
18. Kanaan H et al. Screening for antibacterial and antibiofilm activities in Astragalus angulosus. J Intercul Ethnopharmacol. 2016;6(1):50-57.
19. Khan MN, Ahmed M, Khan MW, et al. In vitro Pharmacological Effects of Astragalus eremophilus and Melilotus parviflora. Acta Biol Hung. 2018;69(4):411-422.
20. Bratkov VM, Shkondrov AM, Zdraveva PK, Krasteva IN. Flavonoids from the genus Astragalus: phytochemistry and biological activity. *Pharmacogn Rev*. 2016;10(19):11-32.
21. Górniak I, Bartoszewski R, Króliczewski J. Comprehensive review of antimicrobial activities of plant flavonoids. Phytochem Rev. 2019;18(1):241-272.
22. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016;5:e47.
23. Miklasińska-Majdanik M, Kępa M, Wojtyczka RD, Idzik D, Wąsik TJ. Phenolic Compounds Diminish Antibiotic Resistance of Staphylococcus Aureus Clinical Strains. Int J Environ Res Public Health. 2018;15(10):2321.
24. Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front Microbiol. 2019;10:911.
25. Karampetsou K, Koutsoni OS, Dotsika E. Quantification of Nitric Oxide and Reactive Oxygen Species in Leishmania-infected J774A.1 Macrophages as a Response to the in vitro treatment with a Natural Product Compound. Bio Protoc. 2019;9(23):e3442.