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ORIGINAL ARTICLE

In vitro/in vivo Assessment and Cellular Mechanisms of *Astragalus spinosus* Extract Against *Leishmania Major*

Hamdan I. Almohammed, Abdullah D. Alanazi[1]

Department of Basic Medical Sciences, College of Medicine, Almaarefa University, Riyadh, [1]Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Ad-Dawadimi, Saudi Arabia

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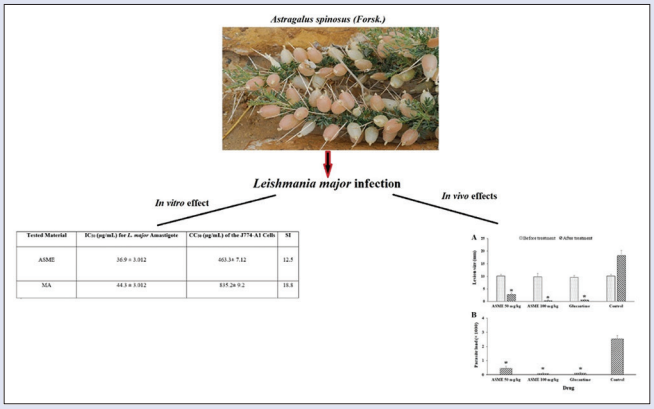
ABSTRACT

Background: In recent years, increasing resistance to synthetic agents, their long-term treatment, and lasting side effects, has faced many problems in treatment of leishmaniasis; so that finding a new high-efficacy antileishmanial drug with minimal side effects seems very necessary. This experimental study was aimed to evaluate the *in vitro* and *in vivo* leishmanicidal activity and cellular mechanisms of *Astraglus spinosus* methanolic extract (ASME) against *Leishmania major* infection. **Materials and Methods:** *In vitro* antileishmanial effect of ASME was evaluated on intracellular amastigotes of *L. major* in macrophage model. The effect of ASME on the NO production of macrophage cells was determined based on the Griess reaction for nitrites. Effect of ASME on the caspase-3-like activity of *L. major* promastigotes was performed according to the measuring the rate of color spectrophotometry. The 50% cytotoxic concentrations (CC_{50}) of the ASME on macrophages were measured to assess the cytotoxicity of ASME. In addition, *in vivo* effects of ASME were evaluated in infected BALB/c mice by measuring of the diameter of CL lesions and parasite load in the tested mice before and after 28 days of therapy. **Results:** The mean number of intracellular amastigotes of *L. major* significantly ($P < 0.001$) decreased with the IC_{50} value of 36.9 ± 3.012 µg/mL and 44.3 ± 3.012 µg/mL for ASME and MA, respectively. Although more NO was produced by increasing the concentrations of the ASME, but, a notable rise was detected at the IC_{50} ($P < 0.001$). ASME especially at the concentrations of ½ IC_{50} and IC_{50} significantly provoked the caspase-3 activation, by 10.3%, 25.6%, and 29.8%, respectively. The measured CC_{50} value of ASME and MA was 463.3 µg/mL and 835 µg/mL, respectively. Treatment of the infected mice with various doses of ASME (50 and 100 mg/kg for 28 days), markedly declined the mean diameter of the CL lesions and parasite load in tested mice. **Conclusion:** Based on the obtained results, ASME can be considered as a promising herbal drug candidate for the isolation and production of a new alternative agent for CL treatment. As a result, this survey presented adequate results in the parasite eliminating in both *in vitro* and *in vivo* assay. Nevertheless, additional studies are needed to elucidate the accurate mechanisms of action of ASME and its effectiveness in clinical subjects.

Key words: Amastigote, antileishmanial, cytotoxicity, leishmanicidal, macrophage, promastigote

SUMMARY

- We evaluated the antileishmanial effects of *Astraglus spinosus* methanolic extract (ASME).
- This survey presented adequate results in the parasite eliminating in both *in vitro* and *in vivo* assay.
- ASME provoked the caspase-3 like activity and nitric oxide production
- ASME can be considered as a new alternative agent for treatment of CL caused by *L. major.*



Abbreviations used:
CL: cutaneous leishmaniasis; *WHO:* World Health Organization; *ASME: Astragalus spinosus* methanolic extract; *mg QE/g DW:* mg quercetin equivalent per gram dray weight; *GAE:* mg gallic acid equivalents; *mg CE/g DW:* mg Catechin Equivalent per gram dray weight; *MA:* Meglumine antimoniate; *NO:* nitric oxide; CC_{50}: 50% cytotoxic concentrations; *MTT*: (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); *SI:* Selectivity index; IC_{50}: Half-maximal (50%) inhibitory concentration; *ANOVA:* One-way analysis of variance.

Correspondence:

Prof. Abdullah D. Alanazi,
Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Ad-Dawadimi, Saudi Arabia.
E-mail: aalanazi@su.edu.sa
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INTRODUCTION

Leishmaniasis is a group of protozoan diseases that can be transmitted to humans and animals in most parts of the world.[1] This disease has always been considered as an important health problem and causes great financial and human losses. The World Health Organization (2020) reports that more than 300 million people in nearly 90 countries are at risk of leishmaniasis. The number of people living with this disease is currently 12 million and it is estimated with nearly 2 million new patient observe every year.[2] Leishmaniasis can be clinically divided into four categories: cutaneous, cutaneous-mucosal, diffuse, and visceral

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leishmaniasis, the cutaneous form of which is more common and is found in abundance in some countries, such as Saudi Arabia.[3] Saudi Arabia, mainly Al-Hassa Oasis, Al-Qassim province and the rural areas around Riyadh city, is endemic foci of Cutaneous Leishmaniasis (CL),[4,5] where, the number of leishmaniasis infection among patients was more than 26,300 cases in last decade.[6]

Inefficiency of reservoir and carrier control methods, treatment costs, side effects of treatment with antimicrobial compounds, long duration of treatment and nonresponse to them, justifies the search for an effective vaccine against leishmaniasis.[7] However, no effective and reliable vaccine has been developed for this disease, and the fight against this disease has always been considered in the national planning of countries.[8] The preferred drugs for CL treatment are pentavalent compounds such as meglumine antimoniate (glucantime) and sodium stibogluconate (pentostam), which have been used as the drug of choice for decades.[3] In recent years, new anti-leishmaniasis agents, e.g., miltefosine, amphotericin B, ketoconazole, and paromomycin have been introduced to treat the clinical forms of leishmaniasis.[9] However, increasing resistance to these drugs (due to inhibition of polyamine biosynthesis, resistance to arsenite, declined biological decrease of pentavalent compounds, elevated levels of trypanothione, binding of DNA minor groove, etc.),[10,11] their long-term treatment, and lasting side effects (e.g. accumulation of drug in liver and spleen tissues, cardiac arrhythmia, muscle pain, pancreatitis, and hepatitis), has faced many problems in treatment of leishmaniasis; so that finding a new high-efficacy anti-leishmanial drug with minimal side effects seems very necessary.[12]

Given that herbal medicines in many cases do not have any significant side effects and on the other hand are available and cheap, this issue emphasizes the need to use native plants of each region for this purpose.[13] Plant extracts and their derivatives are expected to provide a rich resource of new medicinal agents. Indigenous plants are commonly used in endemic countries to treat many infectious agents, including leishmaniasis.[14]

Astragalus spinosus from family Fabaceae is one of the most prevalent *Astragalus* spp. in the Middle-Eastern and African countries.[15] Previous investigations have reported a number of phytoconstituents in *A. spinosus*, e.g., trigonoside, formonetin, quercitin, spino coumarin, astraseiversianin trigonoside, kaempferol, cycloastragenol, luteolin, tragalloside, coumaric, gallic, and cinnamic acids.[16,17] Traditionally, *A. spinosus* has been broadly applied for treating a several diseases and illness, for example, wound healing, leukemia, allergic responses, insect bites, and inflammatory reactions.[18-21] In addition, a number of studies have reports various pharmacological properties of this plant (e.g., immunostimulant, antianxiety, antidepressant, hepatoprotective, cardioprotective, antibacterial, antifungal) in modern medicine.[22,23]

Today, the comprehensive approval of the usage herbs and their derivatives for treating CL is suspended due to some ambiguities in their performance and even their toxicity. On the other hand, the high biological and pharmacological activities of *A. spinosus* and considering that in recent years it has been recommended to use native herbs of each region to treat diseases, we aimed to evaluate the *in vitro* and *in vivo* antileishmanial effects and cellular mechanisms of *A. spinosus* methanolic extract (ASME) against *Leishmania major* infection.

MATERIALS AND METHODS

Plant materials

The *A. spinosus* materials (aerial parts) were collected in May 2021 from meadows of Eastern-Riyadh province. Central part of Saudi Arabia. The plant materials were taxonomically by a botanist (Dr. Misfer AlQhatani) identified at the Department of Biological Sciences, Faculty of Science and Humanities, Shaqra University, Saudi Arabia. Finally, a voucher specimen was also archived at College of Medicine, Almaarefa University, Riyadh, Saudi Arabia (TUMA-2021--33).

Preparing of methanolic extract

Two hundred g of air-dried and powdered plant material were subjected by percolation method with 70% methanol at 24°C for 72 hr. The obtained extracts were filtered by a filter paper (Whatman No. 3, Sigma, Germany). and concentrated in vacuum at 55°C by a rotary evaporator (Heidolph, Germany) and crude extracts were kept at 4°C.[17] The extract was yielded was 17.3 g (8.65%, w/v).

Phytochemical analysis

The existence of flavonoids, tannins, glycosides, alkaloids, and saponins as the primary phytochemical compounds was determined based on the methods described elsewhere.[24]

Total phenol content

We used the Folin-Ciocalteau's reagent colorimetric assay to measure total phenol content of ASME using gallic acid as standard.[25] At first, ASME (300 µL) was added to Folin-Ciocalteu reagent (300 µL) and sodium carbonate 7% (300 µL). The absorbance of mixture was read at 760 nm using spectrophotometer and results was presented as mg gallic acid equivalents (GAE)/g dry weight.

Total flavonoid content

In this study, the aluminum chloride (AlCl3 2%) colorimetric assay with quercetin as standard was used to determine the total flavonoid content of ASME based on the techniques defined by Phuyal *et al.*[26] To do this, ASME or standard solution (300 µL) along with aluminum chloride (300 µL) were mixed with aqueous acetic acid (200 µL). in the next step, the volume of mixture was reached to 5 mL with 90% ethanol. Lastly, the optical density of mixture was read at 430 nm and the total flavonoid was exhibited as mg quercetin equivalent per gram dray weight (mg QE/g DW).

The tannin-condensed contents

The tannin-condensed contents were determined based on the technique defined by Broadhurst and Jones.[27] In this method, ASME and Catechin as the control were mixed along with 5 mL vanillin-HCl. Finally, the optical density of mixture was read at 510 nm and the tannin content was presented as mg Catechin Equivalent per gram dray weight (mg CE/g DW).

Parasite cultures

Strain *L. major* (MHOM/TM/82/Lev) and murine macrophage cells (J774-A1) which were prepared from the cell bank of the Department of Biological Sciences, Faculty of Science and Humanities (Saudi Arabia) were cultured in Schneider's medium (SIGMA, St. Louis, MO, USA) improved 10% heat-inactivated fetal bovine serum (SIGMA, St. Louis, MO, USA) and supplemented antibiotics (100 µg of streptomycin/mL, 100 U penicillin/mL) at 37°C in 5% CO_2.

Antiamastigote assay

At first, macrophages cells (10^5/mL) were plated in 24-Well Lab-Tek (containing 1 cm^2 cover slips put on their floor) at 37°C in 5% CO_2. The cells were then exposed with promastigotes in the stationary phase at 10:0:1 parascell ratio and incubated for 24 hr at the same conditions. Next, 1000 µL of medium contains various concentrations

of ASME (2.5, 5, 10, 25, 50, and 100 µg/mL) and meglumine antimoniate (MA) were separately added to each well for 48 hr. Finally, slides were fixed in absolute methanol, stained with Giemsa dye, and studied under light microscopy. Number of amastigotes inside 100 macrophages were recorded. The 50% inhibitory concentrations (IC_{50}) were determined by the Probit test in SPSS software. The analyses were done in triplicate and the findings were indicated as mean ± standard deviation.[28]

Effect of nitric oxide (NO) production

The effect of ASME on the NO production of macrophage cells was determined based on the Griess reaction for nitrites. To do this, after exposing the macrophage cells with ASME at 1/4 IC_{50}, ½ IC_{50}, IC_{50} for 48 hr, 100 µL of supernatants of mixture were moved into a 96-well microplate and then 50 µL of Griess reagent (Sigma-Aldrich, Germany) A and B (each of 40 mg/mL) were added to each well. The level of NO production of macrophage cells was studied through analysis the plates at 540 nm in an ELISA reader (BioTek-ELX800).[6]

Effect on the caspase-3 like activity

This method was performed based on the release of a molecule (substrate-bound pNA) under the activity of caspase-3 enzyme and subsequently measuring the rate of color spectrophotometry. Promastigotes (10^6) were incubated with ASME at 1/4 IC_{50}, ½ IC_{50}, IC_{50} for 48 hr. After centrifuging the mixture at 800 rpm, the sedimentary cells were lysed. Then 5 µl of the reaction supernatant was mixed to 85 µL of buffer and 10 µL of caspase 3 solution (pNA-DEVD-Ac, 15 mg/mL) and incubated for 2 hours at 37°C. The light absorption of mixture was read at 405 nm with an ELISA reader.[6]

Cytotoxic effects

The 50% cytotoxic concentrations (CC_{50}) of the ASME on macrophage cells were determined to assess the cytotoxicity of ASME. The macrophages cells (10^5 cells/mL) were treated with ASME at 25, 50, 100, 250, 500, and 1000 µg/mL for 48 hr in 96-well microplates at 37°C with 5% CO_2. The colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was subsequently utilized to measure the viability of macrophage cells t. In addition, the selectivity index (SI) ratio was measured from the following division: CC_{50} for macrophage/IC_{50} for intracellular amastigotes.[25]

In vivo effect on CL in BALB/c mice

A total of 32 male BALB/c mice weighing between 20 and 25 grams and aged 6 to 8 weeks were provided from animal breeding of Shaqra University, Saudi Arabia. Animals were kept in a colony room under a 12:12 hr light/dark cycle at (21 ± 2)°C with *ad libitum* access to food and water. To induce the CL in the tested mice, 100 µl of *L. major* promastigotes in stationary phase (2 × 106 parasites/mL) of were subcutaneously inoculated into the tail of mice.[27] The study was approved by Ethical Committee of College of Medicine, AlMaarefa University, Saudi Arabia on 25/01/2022 with the code of IRB06-25012022-09.

CL treatment in mice

Forty days after induction of CL, as soon as CL lesions were detected, the mice were accidentally allocated into five groups containing 8 mice per each group:

(i) topically cured with ASME 50 mg/kg/day for 28 days;
(ii) topically cured with ASME 100 mg/kg/day for 28 days;
(iii) cured with the intralesional injection MA (30 mg/kg/day);
(iv) cured the normal saline.

The selection of the tested doses and duration of treatment was based on the primary experiments and previous studies.[20,28,29] The size of CL lesions in the tested mice before and after 28 days of therapy, was measured through a Vernier caliper. In addition, to measure the load of parasites in the treated mice, the smears obtained from the lesions were fixed in absolute methanol, stained with Giemsa dye, and studied under light microscopy.[6,28]

Statistical analysis

The SPSS Statistics for Windows, Version 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp) was used to data analysis. Unpaired samples *t*-test and one-way analysis of variance (ANOVA) was used to compare the obtained findings between groups. $P < 0.05$ was measured statistically significant.

RESULTS

Phytochemical analysis

Flavonoids, terpenoids, tannin, saponins, glycosides and polysaccharides were present in ASME based on the primary phytochemical analysis. By the secondary metabolites of ASME we found that total flavonoid, phenolic, and tannin content was 32.3 ± 0.61 mg QE/g DW, 18.51 ± 0.41 mg GEA/g DW, and 0.94 ± 0.14 (mg CE/g DW), respectively [Table 1].

Effect of intracellular amastigotes

Based on the results, the mean number of intracellular amastigotes of *L. major* significantly ($P < 0.001$) decreased after treatment of infected macrophages with various concentration of ASME in a dose-dependent response. The calculated IC_{50} value for ASME and MA was 36.9 ± 3.012 µg/mL and 44.3 ± 3.012 µg/mL, respectively [Table 2].

Effect on the NO production

As presented in Table 3, although more NO was produced by increasing the concentrations of the ASME, but, a notable ($P < 0.001$) increase was observed at IC_{50} ($P < 0.001$) in comparison to the control group.

Caspase-3-like activity

The Caspase-3-like activity of promastigotes exposed with ASME was determined by the colorimetric protease systems. Based on the obtained results, ASME especially at the concentrations of ½ IC_{50} and IC_{50} significantly ($P < 0.001$) provoked the caspase-3 activation, by 10.3%, 25.6%, and 29.8%, respectively [Figure 1].

Cytotoxicity on the macrophage cells

Based on the results of MTT assay, ASME displayed no significant ($P > 0.05$) cytotoxicity against macrophage cells. The measured CC_{50} value of ASME and MA was 463.3 µg/mL and 835 µg/mL, respectively. Moreover, the calculated SI of >10 for ASME and MA indicates their safety to macrophages and their specificity to parasites [Table 3].

Table 1: The primary phytochemical examination of *Astragalus spinosus* methanolic extract

Phytochemical	Test	Attendance
Flavonoids	Ammonia	+
Tannins	$FeCl_3$ substrate	+
Saponins	Frothing	+
Alkaloids	Mayer and Dragendorff's reagents	+
Glycosides	Nitroprusside	+
Terpenoids	Salkowski	+

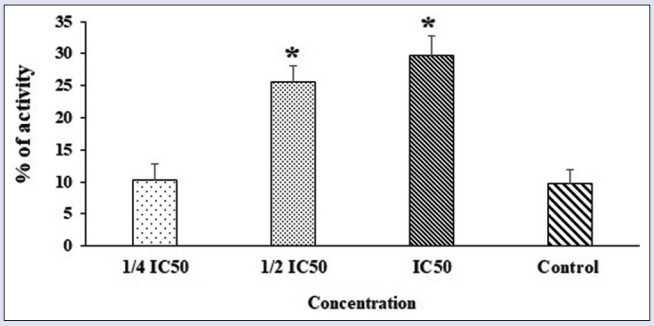

Figure 1: The effect of *A. spinosus* methanolic extract (ASME) on Caspase-3-like activity of *L. major* promastigotes by the colorimetric protease methods. The findings are indicated as mean ± standard deviation (n = 3). * $P < 0.05$ significant difference in comparison with control

In vivo antileishmanial effects

Based on the obtained *in vivo* assay, followed by treatment with ASME at 50 and 100 mg/kg for 28 days, the mean diameter of the CL lesions markedly ($P < 0.001$) reduced by 7.3, and 9.6, respectively. Whereas, in infected mice of treated with MA as control group, the mean diameter of the CL lesions meaningfully ($P < 0.001$) declined by 9.1 mm. Conversely in mice receiving normal saline, the size of the CL lesions expanded by 8.1 mm in the. By term of parasite load, after topical treatment with ASME at 50 and 100 mg/kg for 28 days, the mean number of parasites was markedly ($P < 0.001$) declined by 0.46×10^3 and 0.081×10^3, respectively; whereas this value for MA was 0.094×10^3 [Figure 2].

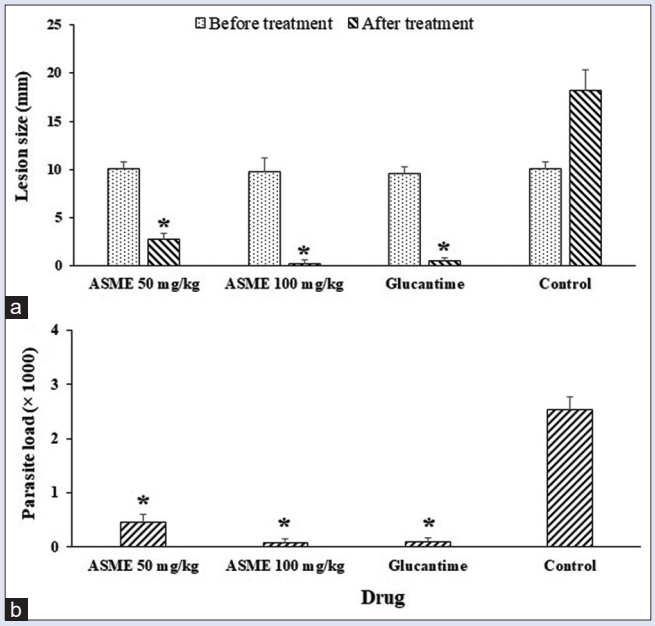

Figure 2: Effect of various concentrations of *A. spinosus* methanolic extract (ASME) on (a) the lesions size and (b) the mean number of parasites (parasite load) in BALB/c mice infected by *L. major*. The findings are indicated as mean ± standard deviation (n = 3). * $P < 0.05$ significant difference in comparison with control

Table 2: Anti-amastigote and cytotoxicity of *A. spinosus* methanolic extract (ASME) glucantime. The findings were indicated as mean±standard deviation. (n=3)

Tested Material	IC_{50} (µg/mL) for Amastigote	CC_{50} (µg/mL) of the J774-A1 Cells	Selectivity index
ASME	36.9±3.012	463.3±7.12	12.5
Glucantime	44.3±3.012	835.2±9.2	18.8

Table 3: The effect of *A. spinosus* methanolic extract (ASME) on production of nitric oxide (NO) in J774-A1 macrophage cells. The findings are indicated as mean±standard deviation (n=3)

Concentration (µg/mL)	Production of NO (nM)
¼ IC_{50}	7.2±0.58
½ IC_{50}	14.3±0.74*
IC_{50}	17.6±1.51 *
Non-treated	5.2±0.84

IC_{50}: The 50% inhibitory concentrations; * $P<0.001$

DISCUSSION

Cutaneous leishmaniasis is considered as an important endemic disease in some countries of tropical and subtropical regions, including Saudi Arabia.[30] According to recent reports, this disease has increased in some parts of the world, especially in the Middle East. The usual treatment for leishmaniasis is the use of pentavalent antimony compounds, which are expensive, scarce, and have severe side effects.[31] In addition, treatment with them is time consuming, and several cases of drug resistance to these compounds have been reported in recent seals. Which has called into question their therapeutic efficacy.[32,33] Therefore, the use of herbal compounds and products that are available and low cost and also have fewer side effects is necessary as a new treatment strategy, especially in native areas. This experimental study was aimed to evaluate the *in vitro* antileishmanial effects and cellular mechanisms of ASME against *L. major* intracellular amastigotes as well as its *in vivo* effect on infected mice with CL lesions. The results revealed that the mean number of intracellular amastigotes of *L. major* significantly ($P < 0.001$) decreased after treatment of infected macrophages with various concentration of ASME in a dose-dependent response. The calculated IC_{50} value for ASME and MA was 36.9 ± 3.012 µg/mL and 44.3 ± 3.012 µg/mL, respectively. Although, there is few documented study regarding the antimicrobial effects of *A. spinosus*; however, in various studies, the antimicrobial effects of *Astragalus* plants have been studied. Ashour *et al.* have reported the antimicrobial effects of three Saudi *A. sieberi, A. armatus,* and *A. spinosus* against some fungal strains and Gram-positive and Gram-negative bacteria through the diffusion agar method.[17] Previous studies also reported the promising *in vitro* antifungal, antibacterial and antibiofilm effects of *A. angulosus, A. hamosus, A. gummifer, A. microcephalus, A. talasseus, A. acmophyllus,* and *A. membranaceus* extracts against some fungal pathogenic strains, Gram-positive and Gram-negative bacteria in.[34-38]

By phytochemical analysis, we found that ASME is contains flavonoids, terpenoids, tannin, saponins, glycosides, and polysaccharides; in addition, the total flavonoid, phenolic, and tannin content was 32.3 ± 0.61 mg QE/g DW, 18.51 ± 0.41 mg GEA/g DW, and 0.94 ± 0.14 (mg CE/g DW), respectively. In consistent with present results, Ashour *et al.* have shown that the presence of carbohydrates, glycosides, sterols, triterpenes, tannins, flavonoids, phenolic compounds, and saponins in *A. sieberi, A. armatus,* and A. *spinosus*. They also reported the total flavonoid was 19.21, 17.8, and 37.91 mg QE/g DW for *A. sieberi, A. armatus, and A. spinosus*, respectively; whereas these values were 21.13, 21.72, and 49.12 mg GEA/g DW for total phenolic of *A. sieberi, A. armatus,* and *A. spinosus*, respectively.[17] Reviews showed that the antimicrobial effects of herbal extracts have been associated to the attendance of some secondary metabolites and bioactive compounds.[39-41] Considering the antimicrobial mechanisms of flavonoids and phenolic compounds, previous studies showed

the antifungal (e.g., *Candida* spp, *Aspergillus* spp., *Penicillium* spp.), antiviral (e.g., respiratory syncytial virus, poliovirus and Sindbis virus), antibacterial (e.g., Gram-positive and Gram-negative bacteria), and antiparasitic (e.g., *Cryptosporidium parvum* and *Encepha-litozoon intestinalis, Plasmodium falciparum, Leishmania* spp.).[40,42-46] By the antimicrobial mechanisms of these secondary metabolites, studies revealed that the flavonoids, and phenolic compounds displayed their antimicrobial mechanisms of action via the suppression of nucleic acid creation, blockage of cytoplasmic membrane function, suppression of energy metabolism, prevent bacterial virulence factors, display a synergistic effect with current synthetic drugs, etc.[47]

Today, NO-dependent cytostatic and cytotoxic effects induced by triggered macrophages against a number of parasites especially intracellular ones have been proven.[48-52] Studies showed that CL, NO-producing agents which used topically to treat lesions demonstrated a promising efficacy in mice models.[53] Furthermore, animal investigations have revealed that effective chemotherapies for visceral leishmaniasis are associated with the activation of the NO pathway. Our findings demonstrated that, although more NO in macrophage cells was produced by increasing the concentrations of the ASME, but, a notable [$P < 0.001$] increase was observed at IC_{50} ($P < 0.001$) in comparison to the control group.

Apoptosis is a main pathway in removing abnormal cells which are no longer required.[54] Caspase-3 is one of the key factors of apoptosis which involves in induction of death protease and consequently induces cell death in *Leishmania* parasites.[55] In this study, the Caspase-3-like activity of promastigotes exposed with ASME was determined by the colorimetric protease systems. ASME at ½ IC_{50} and IC_{50} markedly provoked the caspase-3 motivation, by 10.3%, 25.6%, and 29.8%, respectively.

Based on the results of MTT assay, the measured CC_{50} value of ASME and MA was 463.3 µg/mL and 835 µg/mL, respectively; where the SIs higher than 10 for ASME and MA indicates their safety to macrophages and their specificity to parasites. Ashour *et al.* have revealed the cytotoxic effects of *A. spinosus* extract against tumor cell lines, HCT-116, HepG-2, and A-549 with CC_{50} values of 22.6, 50.2, and 29.1 µg/mL, respectively; whereas these values were 28.8, 39.8, and 47.2 µg/mL for *A. sieberi* against HCT-116, HepG-2, and A-549, respectively.[17]

CONCLUSION

Based on the obtained results, ASME can be considered as a promising herbal drug candidate for the isolation and production of a new alternative agent for CL treatment. As a result, this survey presented adequate results in the parasite eliminating in both *in vitro* and *in vivo* assay. Nevertheless, additional studies are required to elucidate the accurate mechanisms of action of ASME and its effectiveness in clinical subjects. Concerning the limitations of the present study, we can point to issues such as the lack of tissue toxicity evaluation and failure to characterize and identify the phytochemicals present in the ASME using cutting-edge analytical techniques like mass spectroscopy and high-performance liquid chromatography.

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Authors contribution

H.I.A, was involved in conception, study design and data collection; **A.D.A** supervised the study and was responsible for data analysis and writing the manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Conflicts of interest

There are no conflicts of interest.

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