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Original article

Antiparasitic Effects and Cellular Mechanism of *Astragalus maximus* Chloroform Extract Against Clinical Isolates of *Giardia lamblia*

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Abstract

Background and objectives: Chemical drugs such as metronidazole, quinacrine and furazolidone are used to treat giardiasis. Although these drugs are useful in most cases, they are associated with some side effects. The present investigation was designed to evaluate the antiparasitic effects and cellular mechanisms of *Astragalus maximus* chloroformic extract against both trophozoites and cysts of *Giardia*. **Methods**: The extraction was done based on the maceration method with 70% methanol. The in vitro anti-*Giardia* effects of various concentrations of *A. maximus* extract (11.25, 22.5, and 450 mg/mL) were evaluated on cysts and trophozoites of *G. lamblia* for 15-360 min. In addition, the effects on the plasma membrane permeability and the induction of apoptosis in the trophozoites of *G. lamblia* were studied. **Results**: *Astragalus maximus* extract significantly ($p<0.0001$) declined the viability of cysts of *G. lamblia*; in both concentrations of 22.5 mg/mL and 45 mg/mL, the extract killed 100% of *G. lamblia* cysts after 240 and 360 min of incubation. The results also showed that the extract in both concentrations of 22.5 mg/mL and 45 mg/mL, killed 100% of *G. lamblia* trophozoites after 120 and 240 min incubation The findings revealed that treatment of *G. lamblia* trophozoites with *A. maximus* extract increased the permeability of the plasma membrane in a dose dependent response. The extract, especially at the concentration of 10 mg/mL, significantly ($p<0.001$) induced caspase-3 activation. **Conclusion**: The present study showed the promising in vitro antiparasitic effects of *A. maximus* extract against both trophozoites and cysts of *G. lamblia* by affecting the permeability of the plasma membrane and induction of apoptosis. Further investigations especially in animal models and clinical setting are required to clarify the accurate efficacy and mechanisms against *G. lamblia* infection.

Keywords: apoptosis; *Astragalus maximus*; cysts; giardiasis; trophozoites

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Introduction

Giardia lamblia, also known as *Giardia intestinalis*, is one of the most common pathogens of the gastrointestinal tract which infects a wide range of vertebrates, including humans [1]. The infection caused by this protozoan is called "Giardiasis" and its prevalence in developing countries varies from 10 to 50% [2]; whereas nearly 280 million people in the world are infected with this parasite every year [3]. Humans become infected through the

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consumption of contaminated food and water and direct fecal-oral contact with the cyst of this parasite. Due to the resistance of cysts of this parasite to chlorination of water, its spread through human water is common [4]. Giardiasis can cause acute or chronic infection with various clinical manifestations such as acute watery diarrhea, steatorrhea, prolonged diarrhea, abdominal cramps, malabsorption and weight loss syndrome in infants and children [5]. At present, chemical drugs such as metronidazole, quinacrine and furazolidone are used to treat giardiasis. Although these drugs are useful in most cases, they are associated with some side effects such as unpleasant taste in the mouth, gastrointestinal upset, nausea, headache, leukopenia, neurotoxicity, restlessness, seizures and dizziness and disrupt in the treatment process. On the other hand, carcinogenic and mutagenic effects of some of the above drugs in laboratory animals have been proven [6,7]; therefore, it is necessary to find a potent anti-giardial drug that has few side effects and can be effective against both trophozoites and cysts of *Giardia*.

In all historical periods, human beings have always used plants and their derivatives to relieve their physical and mental illnesses, and the use of medicinal plants has always been an effective method of treatment [8]. Today, the use of medicinal plants in the treatment of diseases is expanding and the extraction and study of the properties of plant compounds and the presentation of the mechanisms of action of these compounds in the treatment of various diseases have been considered. *Astragalus* is one of the most important plants of the Fabaceae family with more than 2000 different herbaceous and shrub species [10]. Various *Astragalus* spp. have been broadly used in traditional medicine to treat several diseases including blood disorders, pneumonia, gastrointestinal and renal disorders, allergies, edema and urinary incontinence, anorexia, diabetes, hypertension as well as various infections [11]. Furthermore, studies have shown that *Astragalus* spp. displayed various pharmacological properties in the modern medicine such as antioxidant, neuroprotective, anti-cancer, antiaging, hepatoprotective, and antimicrobial properties [12,13]. *Astragalus maximus* is one of the *Astragalus* species which mostly grows in middle east countries such as Iran, Iraq, and Turkey [14,15]. To the best of our knowledge, there is no study on anti-parasitic effects of *A. maximus*; meanwhile, previous studies showed various biological and pharmacological properties of this plant. The present investigation was designed to evaluate the antiparasitic effects of *A. maximus* chloroformic extract against both trophozoites and cysts of *Giardia*.

Materials and Methods

Ethical considerations

This study was approved by the ethics committee of Lorestan University of Medical Sciences, Khorramabad, Iran, with the ethics number of IR.LUMS.REC.1400.197.

Chemicals

Sodium carbonate (7%), aluminum chloride ($AlCl_3$), bovine bile, and 20% heat-inactivated fetal calf serum were provided from Merck, Germany. Folin-Ciocalteau's reagent, eosin powder, Triton X-100 (2.5%,) and protease caspase-3 kit were purchased from Sigma-Aldrich, Germany.

Plant materials

Astragalus maximus aerial parts were collected from rural regions of Noorabad district, Lorestan Province, Iran, in June 2021. The obtained materials were then identified by a botanist and a voucher sample (No. 1400197) was archived at the Department of Pharmacognozy, College of Pharmacy, Khorramabad, Iran.

Extraction

The air dried aerial parts of the plant (250 g) were ground and then after defattingg with -n hexane, extraction was done by maceration method with 70% methanol. The methanol extract was then concentrated by a rotary evaporator at a temperature of 50 °C, under vacuum. The remaining extract was decanted with chloroform in the decantation funnel and kept at −20 °C until testing [14].

Phytochemical analysis

The primary phytochemical analysis of the extract was performed to confirm the presence of tannins, saponins, alkaloids, flavonoids, and glycosides, based on previous investigations [17].

Total phenolics content

By Folin-Ciocalteau's reagent colorimetric process we determined the total phenolics content

using gallic acid as the standard [18]. Folin-Ciocalteu solution (3 mL) along with the extract (0.3 mL) were added to 3 mL of sodium carbonate. After 30 min of incubation in the darkness, the absorbance of suspension was measured at 760 nm using spectrophotometer. The total phenolics content was reported as mg gallic acid equivalents (GAE) per gram of sample in dry weigh (mg GEA/g DW).

Total flavonoids content

Total flavonoid content was measured by aluminum chloride colorimetric method using quercetin as the standard [19]. To perform this method, 0.2 mL of the extract or standard solution was added to 0.2 mL of 2% AlCl3 and 0.1 mL of aqueous 33%acetic acid and stirred well. Finally, the reaction mixture was made with 90% ethanol to a volume of 5 mL. The tubes were kept at room temperature for 30 min. Absorption was measured by spectrophotometer at 510 nm as mg quercetin equivalent per gram dray weight (mg QE/ g DW).

***Giardia lamblia* cyst collection and isolation**

Giardia lamblia cysts were collected from fecal samples of infected patients with giardiasis who were referred to general hospitals in Khorramabad, Lorestan province. Stool samples were then placed in sterile plastic containers containing normal saline and transferred to the Parasitology Laboratory, Department of Clinical Laboratory Sciences, Lorestan University of Medical Sciences, Khorramabad, Iran. Regardless of the positive test result, stool samples were evaluated by two methods, direct and formalin-ether with a light microscope [20]. The cysts were then concentrated by sucrose 0.85 M gradient method [21]. The stool samples were diluted in a ratio of 1: 12 with distilled water and transferred to a container containing glass pearls and were shaken for 5 minutes. After passing the suspension through a strainer, 5 mL of distilled water was added to the precipitate and the above solution was slowly added to 3 mL of 0.85 M sucrose solution and centrifuged at 600 g for 10 min at 4 °C. The cysts were collected in the middle layer using a Pasteur pipette and washed three times with normal saline kept at 4 °C until testing. The standard method of vital staining of cysts with 0.1% eosin was used to determine the percentage of cyst viability. Finally, the number of the cysts was adjusted by a hemocytometer to 1×10^5 cysts/mL in distilled water.

***Giardia lamblia* trophozoites collection**

The trophozoites were obtained through the excystation of *G. lamblia* cysts according to the method described by Bingham and Meyer [22]. Aqueous hydrochloric acid (pH 2) at a ratio of 1:9 as the induction suspension was added to the cyst suspension kept at 37 °C for 2h. After centrifuging the combination at 600 rpm for 10 min, the supernatant was discarded and the sediment was added to the medium containing filter-sterilized TYI-S-33 culture complemented with bovine bile, 20% heat-inactivated fetal calf serum, streptomycin (500 µg/mL) and penicillin (500 IU/mL) and kept at 37 °C in a slant.

In vitro anti-*Giardia* effects

In order to determine the in vitro anti-*Giardia* effects of the extract, 0.1 mL of various concentration (11.25, 22.5, and 45 mg/mL) were added to 100 µL of cysts and trophozoites suspension (1×10^5 parasite/mL) in the test micro tubes and were kept warm at 37 °C for 15-360 min [22]. After discarding the upper phase of the solution, 50 µL of 0.1% eosin stain was added to the remaining settled cysts and trophozoites. Finally, smears were prepared on a glass slide and the viability rate of the cyst and trophozoites were recorded by counting 100 trophozoite and cysts with a light microscope at a 400x magnification, whereas the live and dead parasites looked colorless and pink, respectively [23]. The cysts and trophozoites treated with normal saline and those treated with metronidazole were considered as negative and positive controls, respectively.

Plasma membrane permeability of *Giardia* trophozoites

The effect of the extract on permeability of plasma membrane of *Giardia* trophozoites were studied by Sytox green stain test. In this study, parasites exposed with Triton X-100 and normal saline were considered as the positive and negative controls, respectively. The plasma membrane permeability was determined using a microplate reader (BMG, CLARIOStar, Germany) for 4 h [24].

Induction of the caspase-3 activity

The effect of the extract on the induction of caspase-3 activity of trophozoites was studied based on the colorimetric protease assay. The method is based on the formation of dye

produced by a molecule (pNA combined to the substrate) followed by the enzyme caspase-3 activity. After treating the trophozoites with different concentrations of the extract, the combination was centrifuged at 700 rpm for 5 min at 4 °C. In the next step, the remaining cells were lysed, and centrifuged at 20,000 rpm for 10 min. Lastly, the supernatant (5 µL) was added to 85 µL buffer and 10 µL caspase 3 (pNA-DEVD-Ac) solution and was incubated for 120 min at 37 °C. The absorption of the solution was determined by the ELISA reader (Biotek Elx800 Microplate Reader, USA) at 410 nm [25].

Statistical analysis

All tests were performed at least three times. The results were analyzed by the SPSS statistical package, version 25.0 (SPSS, Inc, USA.). One-way analysis of variance (ANOVA) was used to compare the intergroup differences. $P< 0.05$ was considered statistically significant.

Results and Discussion

The yield for chloroform extract was 24.2 g (9.6%, w/v). By phytochemical examination, the presence of saponins, flavonoids, terpenoids, and polysaccharides were confirmed in the extract (Table 1). The results of analysis and measurement of the contents of secondary metabolites showed that total phenolic and flavonoid contents were 0.94 (mg GEA/g DW) and 0.67 (mg QE/g DW), respectively.

Figures 1 and 2 exhibit the in vitro anti-*Giardia* effects of different concentrations of the extract on cysts and trophozoites of *G. lamblia* at 37 °C for 15-360 min. Based on the obtained results, *Astragalus maximus* extract significantly declined the viability of cysts of *G. lamblia* ($p<0.0001$); whereas in both concentrations of 22.5 mg/mL and 45 mg/mL, killed 100% of the *G. lamblia* cysts after 240 and 360 min incubation. Similarly, the results also showed that *A. maximus* extract in both concentrations of 22.5 mg/mL and 45 mg/mL, killed 100% of the *G. lamblia* trophozoites after 240 and 120 min incubation indicating that trophozoites were more susceptible to the extract.

Table 1. The primary phytochemical analysis of the *Astragalus maximus* chloroform extract

Phytochemical	Test	Presence
Flavonoids	Ammonia test, Alkaline reagent test	+
Polysaccharides	Nitroprusside test	+
Saponins	Frothing test	+
Terpenoids	Salkowski test	+

The findings revealed that treatment of *G. lamblia* trophozoites with *A. maximus* extract increased the permeability of plasma membrane in a dose dependent response through increasing the detected fluorescence (Figure 3).

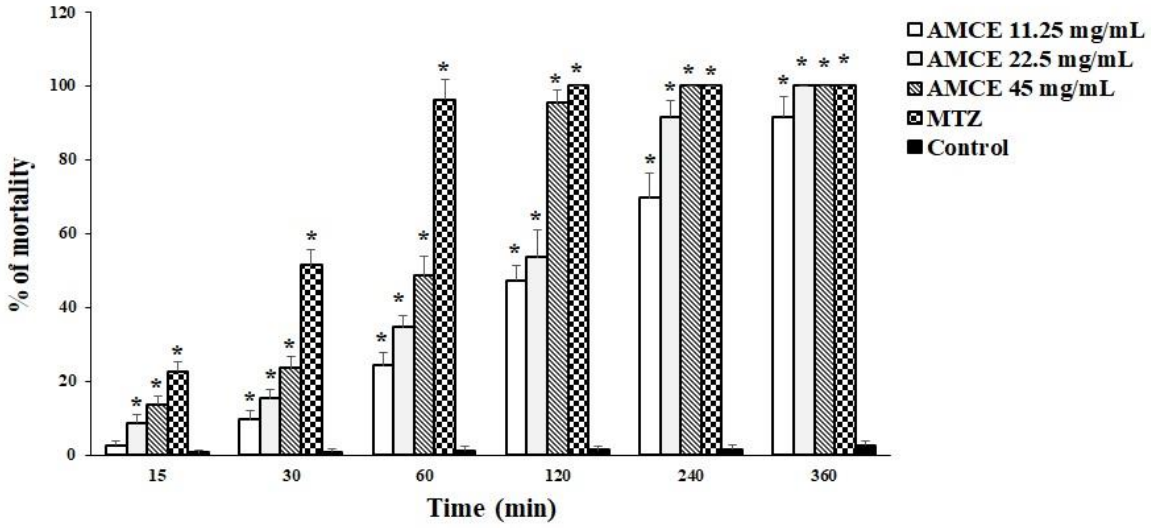

Figure 1. The in vitro anti-*Giardia* effects of different concentrations of *Astragalus maximus* chloroform extract (AMCE) on cysts of *Giardia lamblia* at 37 °C for 15-360 min in comparison with metronidazole (MTZ); Data are expressed as mean ± SD. * $p<0.05$ statistically different compared with the control group (n=3)

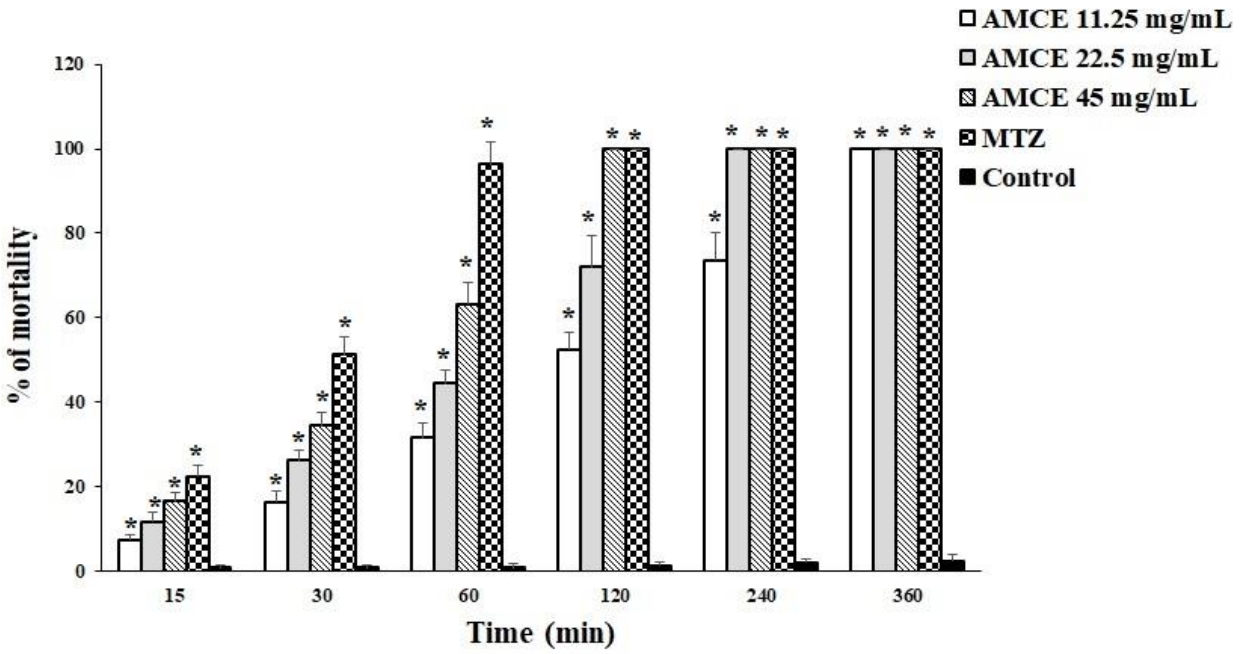

Figure 2. The in vitro anti-*Giardia* effects of different concentrations of *Astragalus maximus* chloroform extract (AMCE) on trophozoites of *Giardia lamblia* at 37 °C for 15-360 min in comparison with the metronidazole (MTZ); Data are expressed as mean ± SD (n=3). * $p<0.05$ statistically different compared with the control group

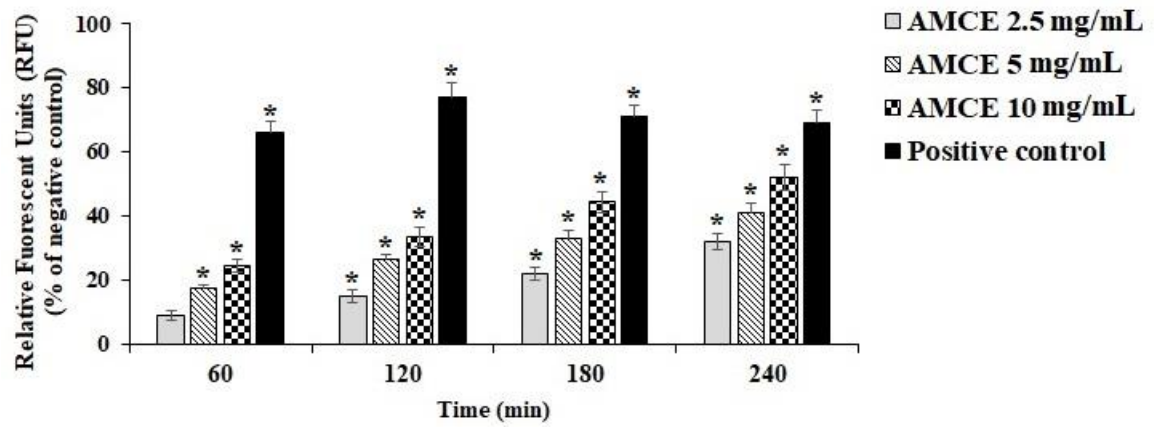

Figure 3. The plasma membrane permeability of *Giardia lamblia* trophozoites exposed to various concentrations of *Astragalus maximus* chloroform extract (AMCE) when compared with the positive (Triton X-100) and negative (normal saline) controls; Data are expressed as mean ± SD (n=3). * $p<0.05$ statistically different compared with the negative control group.

Figure 4 shows the effects of various concentrations of *A. maximus* extract on the caspase-3 activity in *G. lamblia* trophozoites. The extract at the concentrations of 2.5, 5, and 10 mg/mL induced caspase-3 activation by 7.3%, 16.4%, and 25.7%, respectively ($p<0.001$).

In recent decades, the discovery, development and high production of chemically synthesized agents have improved health care around the world. Nevertheless, large proportions of the people in developing countries still rely on traditional remedies and medicinal herbs for their primary care [26]. Today, chemical drugs such as metronidazole, quinacrine and furazolidone are used to treat giardiasis; although these drugs are useful in most cases, but they are associated with some side effects [7]; therefore, it is necessary to find potent anti-giardial drugs that show few side

effects and can be effective against both trophozoites and cysts of *Giardia*. Based on the obtained results, *Astragalus maximus* extract significantly declined the viability of cysts of *G. lamblia* ($p<0.0001$); whereas at the concentrations of 22.5 and 45 mg/mL, completely eliminated the of *G. lamblia* cysts after 240 and 360 min. for *G. lamblia* trophozoites, the results also showed that the extract at the concentrations of 22.5 and 45 mg/mL completely eliminated the of *G. lamblia* trophozoites after 240 and 120 min; indicating that they were more susceptible to the extract.

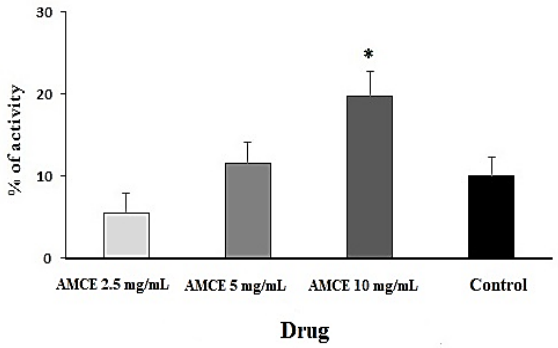

Figure 4. The effects of various concentrations of *Astragalus maximus* chloroform extract (AMCE) on the caspase-3 activity in *Giardia lamblia* trophozoites; Data are expressed as mean ± SD (n=3). * $p<0.05$ statistically different compared with the control group

Yang et al. have investigated the antiparasitic effects of aqueous extract of *A. membranaceus* against tachyzoites of *Toxoplasma gondii* RH strain in HeLa cell culture medium. They have reported *A. membranaceus* extract after 72, 96 and 120 h incubation significantly reduced the intracellular replication of tachyzoites [27]. Abdel-Tawab et al have demonstrated that *A. membranaceus* extract significantly reduced the viability of *Eimeria papillata* (coccidia) oocysts in a dose-dependent manner. Their results also showed that the *A. membranaceus* extract at the dose of 50 mg/kg significantly reduced the number of oocytes exerted in feces of infected rats (about 57%), along with a significant reduction in the number of parasitic stages in the jejunal segments [28]. In addition, antimicrobial effects of *Astragalus* spp. on some pathogenic bacteria (e.g., *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium,* and *Klebsiella pneumoniae* as Gram-negative bacteria and *Staphylococcus aureus* and *Enterococcus faecalis* as Gram-positive bacteria), fungi (e.g., *Aspergillus flavor, A. niger, A. fumigatus* and *Candida albicans*) and pathogenic viruses (e.g., influenza virus) have been reported in various studies [29-31].

Nowadays, one of the principle cellular mechanisms in the inhibition of pathogenic microorganisms is the rupture and effect on permeability of plasma membrane [32]; here we found that treatment of *G. lamblia* trophozoites with *Astragalus maximus* extract increased the permeability of plasma membrane in a dose dependent response. Another mechanism involved in the inhibition and survival of pathogenic microorganisms is the prompt of cell death or apoptosis [33]. Based on the obtained results, *A. maximus* exract at the concentrations of 2.5, 5, and 10 mg/mL significantly induced caspase-3 activation, one of the most mediators involved in apoptosis, by 7.3%, 16.4%, and 25.7%, respectively ($p<0.001$).

In the present study, by phytochemical examination, the presence of saponins, flavonoids, terpenoids, and polysaccharides were confirmed in the extract. Moreover, the results of analysis and measurement of the contents of secondary metabolites showed that total flavonoid content were 1.61 mg GEA/ g DW. Previous studies have showed that phenolic and flavonoids compounds are considered as the main components of *Astragalus* spp. Various subclasses of flavonoids have been reported from the genus *Astragalus* including flavones, flavonols, flavanones, flavanonols, chalcones, aurones, isoflavones, isoflavanes, and pterocarpans [34,35]. Considering the antimicrobial activity of flavonoids, studies have proposed that these compounds reveal their antimicrobial mechanisms through disruption of cytoplasmic membrane function, inhibition of nucleic acid synthesis, cell-membrane damage, induction of apoptosis, disrupting energy metabolism, inhibiting the biofilm formation, changing the membrane permeability, and reduction of the pathogenicity [36,37]. Reviews also revealed that polyphenolic compounds such as flavanols, flavonols and phenolic acids exhibit their antimicrobial activity through inhibiting the virulence factors, interacting and disruption of cytoplasmic membrane, inhibiting the formation of biofilm formation, cell membrane disruption, inhibition of DNA synthesis and synergistic effect with antibiotics [38,39]. Hence, it can be

suggested that the promising antigiardial effects of *Astragalus maximus* extract is related to the presence of phenolic and flavonoid compounds in this plant.

Conclusion

The findings of the present study showed the promising in vitro antiparasitic effects of *A. maximus* chloroform extract against both trophozoites and cysts of *G. lamblia* with the highest effect at the concentrations of 22.5 and 45 mg/mL. Our findings also exhibited that effect of permeability of plasma membrane as well as induction of apoptosis are the probably cellular mechanisms of this extract against *G. lamblia*; however, further investigations especially in animal models and clinical setting are required to clarify the accurate efficacy and mechanisms of *Astragalus maximus* against *G. lamblia* infection.

Acknowledgments

None

Author contributions

Javad Ghasemian Yadegari was involved in conception and design of the study; Amal Khudair Khalaf performed data analysis and critical revision of the manuscript; Roya Darabi was responsible for data collection and critical revision; Hossein Mahmoudvand supervised the study and prepared the draft of the manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

GAE: gallic acid equivalents; $AlCl_{3:}$ aluminum chloride; QE/g DW: quercetin equivalent per gram dry weight; AMCE: *Astragalus maximus* chloroform extract; GEA/g DW: mg gallic acid equivalents per gram of sample in dry weight