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Immune-enhancing activity of *Astragalus maximus* extract for inhibiting the *Toxoplasma gondii* infection: experimental research

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Background: This study aimed to evaluate the in-vitro anti-*Toxoplasma* effects and cytotoxicity effects of *Astragalus maximus* chloroformic extract (AMCE) on the *T. gondii* Rh strain.

Methods: In-vitro effects of AMCE (2–64 μg/ml) on tachyzoites were measured by MTT assay for 48 h. The effects of AMCE on infectivity rate and intracellular parasites into macrophage cells (J774-A1) were evaluated. The Griess reaction assay and quantitative real-time PCR were used to determine the nitric oxide (NO) and the mRNA expression levels of IFN-γ and iNOS in infected J774-A1 macrophage cells.

Results: The mortality rate of the parasites significantly ($P < 0.001$) increased in a dose-dependent manner with an IC_{50} value of 9.85 μg/ml. The rate of infection and the mean number of intracellular tachyzoites in macrophage cells were significantly reduced ($P < 0.001$) after exposure of the macrophage cells to AMCE. The mRNA expression levels of IFN-γ, iNOS, and NO production in macrophage cells after treatment with the AMCE were increased, especially at the concentration of ½ IC_{50} and IC_{50} ($P < 0.001$).

Conclusion: The findings of the current *in vitro* investigation revealed favorable anti-*Toxoplasma* effects of AMCE against tachyzoites and intracellular forms of *T. gondii*. Despite the fact that the accurate anti-*Toxoplasma* mechanisms of AMCE are not clear, our results showed that triggering NO production and cellular immunity can be considered as the main mechanisms of action of AMCE for controlling and eliminating *T. gondii*. However, further surveys are mandatory to assess the efficacy and safety of AMCE in an animal model and its accurate mechanisms of action.

Keywords: isoflavone, natural products, nitric oxide, toxoplasmosis

Introduction

Toxoplasma gondii is an obligate intracellular protozoan, the causative agent of toxoplasmosis in humans and animals, and a parasite with widespread worldwide distribution. Acquired infections usually occur by eating raw and undercooked meat containing tissue cysts or by eating vegetables and water contaminated with oocysts excreted in feline feces[1]. Congenital *Toxoplasma* infections occur with placental transfer of tachyzoites in early pregnancy infections. Acquired toxoplasmosis in immunocompetent people mainly occurs as benign and self-limiting lymphadenopathy and rarely causes severe brain and eye manifestations[2]. In immunocompromised people, especially AIDS patients, toxoplasmosis is an important opportunist and the most common cause of encephalitis in these patients[3]. Also, toxoplasmosis is one of the important congenital infections that has a wide clinical spectrum and varies from the birth of a baby without obvious clinical symptoms to abortion, stillbirth, and the birth of babies with severe brain and eye complications (hydrocephaly or microcephaly, brain calcification, and

HIGHLIGHTS

- *Astragalus maximus* extract increased the mortality rate of the *Toxoplasma gondii* tachyzoites.
- Significantly reduced the mean number of intracellular tachyzoites in macrophage cells.
- Increased the mRNA expression levels of IFN-γ and iNOS in infected J774-A1 macrophage cells.
- We showed immune-enhancing activity of the extract for inhibiting the *T. gondii* infection.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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chorioretinitis)[4,5]. The diagnosis of toxoplasmosis is typically established by serologic tests (to measure immunoglobulin G and immunoglobulin M), amplification of specific nucleic acid sequences (i.e. PCR), histologic demonstration of the parasite and/or its antigens (i.e. immunoperoxidase stain), or by isolation of the organism[5].

Toxoplasmosis is commonly treated with a combination of atovaquone, pyrimethamine, and sulfadiazine. Pyrimethamine is an inhibitor of dihydrofolate reductase, a key enzyme in the folate synthesis pathway. Sulfadiazine is an inhibitor of the dihydroptyruate enzyme, which is another enzyme in this pathway. Although these drugs and other synthetic anti-*Toxoplasma* drugs have a good inhibitory effect on *Toxoplasma*, side effects are their main limitation[6,7]. For example, pyrimethamine, which is one of the main anti-*toxoplasma* drugs, causes disruption in hematopoiesis due to its suppressive effect on the bone marrow[7]. Today, obtaining an anti-*Toxoplasma* drug with optimal efficacy and minimal side effects is one of the research priorities of *Toxoplasma*, and in this regard, the products obtained from medicinal plants can be one of the options. In addition, the antiparasitic effects of many medicinal plants have been mentioned in traditional medicine[8]. In addition, in recent years, more and more studies have been conducted on the effectiveness of medicinal plants on various diseases, including parasitic diseases[8].

In recent decades, the use of herbal medicines for the treatment of giardiasis is increasing due to their high efficacy and low side effects[9]. *Astragalus* plant is an annual or perennial plant of the *Fabaceae* family, whose height reaches 75–100 cm[10]. Plants of the genus *Astragalus* include about 2000–3000 species of herbs and shrubs, mostly perennials, with more than 250 taxonomic divisions in the world[11]. This genus is widely distributed throughout the temperate regions of the world, and so far, about 800 species have been identified in the pastures and mountainous areas of Iran. Many species of *Astragalus* have long been used in traditional medicine to treat diabetes, nephritis, stomach ulcers, high blood pressure, and chronic bronchitis[12]. In addition, various pharmacological properties of this genus, such as antioxidants, boosting immunity system immune system, antihypertensive, antimicrobial, and anti-inflammatory effects have been proven[13–15]. To the best of our knowledge, there is no scientific document on the anti-*Toxoplasma* activity of *Astragalus* spp. So this study aimed to evaluate the in-vitro anti-*Toxoplasma* effects and cytotoxicity effects of *A. maximus* chloroformic extract (AMCE) on the *T. gondii* Rh strain.

Methods

Plant materials

Aerial parts of *A. maximus* were obtained from the rural regions of Nurabad district, Western Iran, in June 2021. After identifying the herbal materials by a botanist, a voucher specimen was archived in the herbarium of the Razi Herbal Medicines Research Center, Khorramabad, Iran (No. LUMS-26354).

Preparing the chloroform extract and phytochemical analysis

Air-dried plant materials (250 g) were powdered and degreasing with n-hexane. The maceration method with chloroform was used for extraction. The obtained extract was concentrated by a rotary evaporator at 50°C and 100 rpm, under vacuum situations, and was kept at −4°C until testing[15,16]. The primary phytochemical analysis of the AMCE was performed to confirm the presence of tannins, saponins, alkaloids, flavonoids, and glycosides, etc., based on the previous investigation[17].

Parasite

Tachyzoites of the *T. gondii* RH strain were kindly prepared from the Department of Medical Parasitology, Tehran University of Medical Sciences, Tehran, Iran. Tachyzoites were preserved via the serial intraperitoneal passages in BALB/c mice. After 72 h, tachyzoites were obtained and centrifuged at 200*g* for 5 min at 21°C for discarding artifacts and peritoneal cells. By removing the supernatant, the remaining parasites were re-covered with PBS and by a hemocytometer slide, and were adjusted into 1×106 parasites per each milliliter.

Macrophage cells

Macrophage cells (J774-A1) were purchased from the cell bank of the Pasteur Institute of Iran and cultured in RPMI1640 liquid culture medium containing 10% inactivated fetal bovine serum and 1% penicillin–streptomycin antibiotic and cultured in sterile flasks at 37°C in the atmosphere. They are incubated containing 5% CO_2 and 95% humidity.

In-vitro effects of AMCE on tachyzoites

To perform this assay, after adding 100 µl of *T. gondii* tachyzoites to each well of a 96-well plate, various concentrations of AMCE (2–64 µg/ml) were put into the wells, and the plate was incubated for 48 h at 37°C. Then, 20 µl of MTT solution (5 mg/ml) was added to the tested wells and were incubated under the same conditions for 4 h. Lastly, 50 µl of the dimethyl sulfoxide as the stop solution was added to the wells and their absorbance was measured read at 570 nm using an ELISA reader. Tachyzoites exposed with normal saline and pyrimethamine were considered the negative and positive controls, respectively[18].

Effect of AMCE on infectivity rate and intracellular parasites

To evaluate the effect of AMCE on infectivity rate and intracellular parasites, at first J774-A1 cells (1×105 cells/ml) were put in the 24-well plate and were incubated at 37°C for 24 h. Consequently, cells were exposed with *T. gondii* tachyzoites (1×106/ml) and plates were incubated at 37°C for 24 h. After this time the infected cells were exposed with various concentrations of AMCE (2–64 µg/ml) for 48 h. Lastly, treated cells were stained with Giemsa, and examined by light microscopy to assess the infection rate and the mean number of intracellular parasites through examination 100 cells[19].

Table 1

The oligonucleotide primers utilized for real-time PCR

Primer	Sequence
iNOS	Forward – 5′TGCCGGAAGGCGGCTCATTC-3′
	Reverse – 5′CGCAGTGCGTTGCGCATACC-3′
IFN-γ	Forward – 5′-TGCCGGAAGGCGGCTCATTC
	Reverse – 5′-CGCAGTGCGTTGCGCATACC-3′
β-actin	Forward – 5′GTGACGTTGACATCCGTAAAGA-3′
	Reverse – 5′GCCGGACTCATCGTACTCC-3′

mRNA expression of genes (iNOS and IFNγ) in infected macrophages with T. gondii

The mRNA expression levels of IFN-γ and iNOS in infected J774-A1 macrophage cells treated with AMCE (½ IC_{50} and IC_{50}) were examined by quantitative real-time PCR. At first, total RNA was extracted based on the instructions of the RNeasy tissue kit (Qiagen). Next, the complementary DNA was obtained through random primers according to the instructions of the commercial kit (Qiagen). A negative control sample with sterile water was included in the nested reactions. The extracted DNA from the RH strain of *T. gondii* was utilized as a positive control. Lastly, the obtained complementary DNA was considered for real-time PCR through SYBR Green (Qiagen). The thermal cycle for this experiment was 92°C for 6 min, 42 cycles of 92°C for 10 s and 55°C for 30 s, respectively. The iQTM5 optical system software (Bio-Rad) was applied for the $\Delta Ct - 2$. The oligonucleotide primers utilized for real-time PCR were presented in Table 1. The results were standardized by the value found from the β-actin mRNA.

Determining the nitric oxide (NO) production

J774-A1 macrophage cells (1×105/ml) were treated with various concentrations of AMCE (¼ IC_{50}, ½ IC_{50}, and IC_{50}) for 48 h, in a 96-well plate. In the next step, 20 µl of the supernatant was mixed with the nitrite assay buffer (80 µl), Griess reagent A (10 µl, Sigma-Aldrich), and B (10 µl). Lastly the absorbance of the solution was read at 540 nm in an ELISA reader (BioTek-EL × 800)[18]. The cells treated with the combination of lipopolysaccharide (LPS, 10 ng/ml) along with IFN-γ (10 U/ml) were considered as the positive control[20].

Statistical analysis

All tests were performed in triplicate to increase the reliability of the results. After measuring the normality of the data with the help of the Shapiro–Wilk test, the Analysis of Variance(ANOVA), and Tukey's *post hoc* test were used to analyze the data using SPSS version 26.0 software. $P < 0.05$ was considered as the significance level. The 50% inhibitory concentrations (IC_{50}) and the 50% cytotoxic concentrations (CC_{50}) were calculated through the Probit test. The significance level was considered $P < 0.05$. All tests were repeated three times.

Results

Phytochemical analysis and secondary metabolites

The AMCE yielded 16.8 g (6.72%, w/v). Phytochemical experiments displayed the attendance of flavonoids, saponins, terpenoids, and polysaccharides; where the evaluation of the amount of secondary metabolites showed that the total flavonoid and phenolic content was 3.89 (mg QE/g DW) and 5.12 (mg GEA/g DW), respectively.

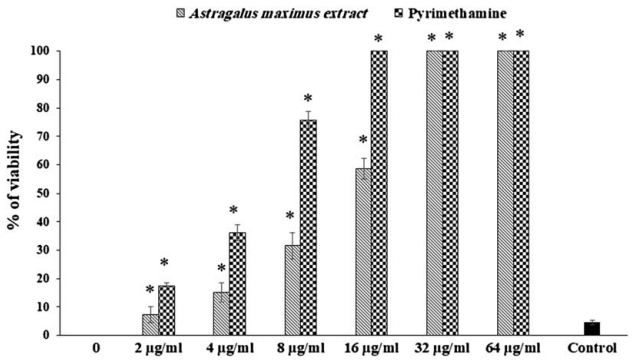

Figure 1. Comparison of the anti-*Toxoplasma* effects of different concentrations of *Astragalus maximus* chlorophormic extract and pyrimethamine on *Toxoplasma gondii* tachyzoites *in vitro*. Mean ± SD ($n = 3$); *$P < 0.001$.

In-vitro effects of AMCE on tachyzoites

The effect of different concentrations of AMCE on the viability of *T. gondii* tachyzoites IS depicted in Figure 1. After 48 h of exposure of *T. gondii* tachyzoites with different concentrations of AMCE, the mortality rate of the parasites significantly ($P < 0.001$) increased in a dose-dependent manner compared to the control group. The results also showed that the IC_{50} for AMCE and pyrimethamine drug were reported as 13.65 and 5.76 µg/ml, respectively.

Effect of AMCE on the inhibition of infection in macrophages

The obtained results showed that the maximum inhibitory activity of the AMCE was detected at 64 µg/ml, where it significantly reduced the amount of infection in macrophages ($P < 0.001$) by 92.4%. AMCE in doses of 16 and 32 µg/ml also reduced the level of contamination by 61.4 and 81.7%, respectively ($P < 0.001$) (Fig. 2).

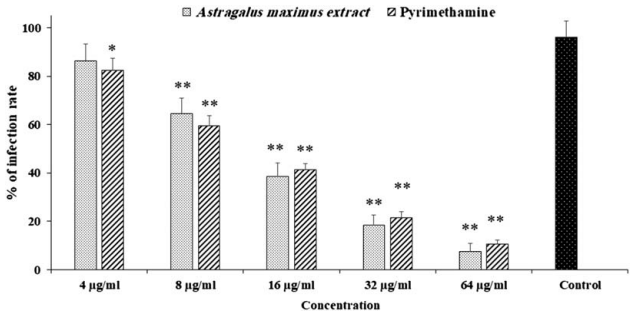

Figure 2. The effect of *Astragalus maximus* chlorophormic extract on the infection rate of J774-A1 cells after 48 h of incubation. Mean ± SD ($n = 3$); *$P < 0.01$ and **$P < 0.001$.

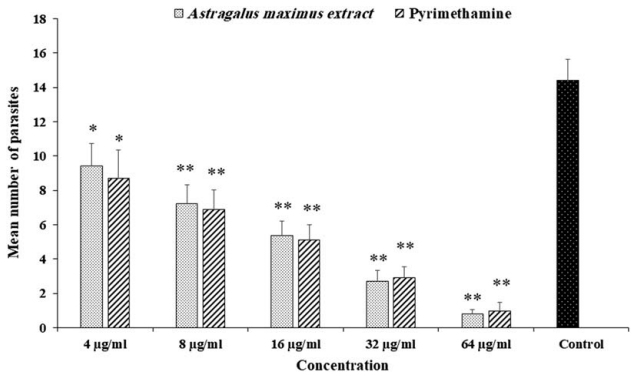

Figure 3. The effect of *Astragalus maximus* chlorophormic extract on the number of parasites in macrophages after 48 h of incubation. Mean ± SD ($n = 3$); *$P < 0.01$ and **$P < 0.001$.

Effect of AMCE on intracellular parasites

Microscopic studies revealed that the average number of intracellular tachyzoites decreased significantly ($P < 0.001$) after exposure of infected cells to different concentrations of AMCE. So that the AMCE at concentrations of 16, 32, and 64 µg/ml reduced the average number of parasites inside macrophages to 5.36, 2.68, and 0.8, respectively (Fig. 3).

mRNA expression of genes (iNOS and IFNγ) in infected macrophages with T. gondii

As shown in Figure 4, the mRNA expression levels of IFN-γ and iNOS in infected J774-A1 macrophage cells treated with AMCE, especially at IC_{50}, were significantly ($P < 0.001$) elevated in quantitative real-time PCR.

Effect of AMCE on the NO production in macrophage cells

The findings of the Griess reaction assay showed that the amount of NO production in macrophage cells after treatment with the AMCE was increased, especially at the concentration of ½ IC_{50} and IC_{50} ($P < 0.001$) in comparison with the control group (Table 2).

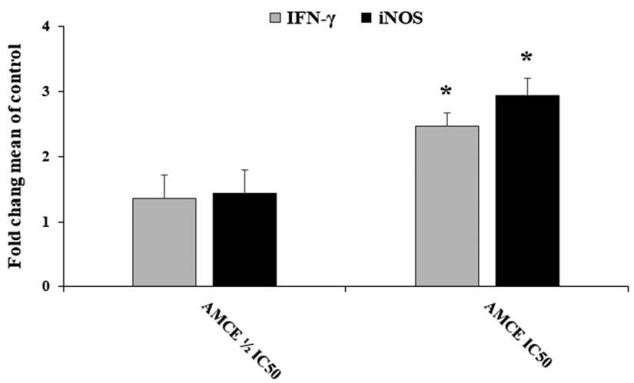

Figure 4. mRNA expression of genes (iNOS and IFNγ) in infected macrophages with *Toxoplasma gondii* treated with *Astragalus maximus* chlorophormic extract in comparison with the control group. Mean ± SD ($n = 3$); *$P < 0.001$.

Table 2

The effect of *Astragalus maximus* chloroform extract on nitric oxide (NO) production in J774-A1 macrophage cells in comparison with the lipopolysaccharide (LPS) along with IFN-γ as the positive control

Drug	Concentration (µg/ml)	NO production (µM)
AMCE	¼ IC_{50}	6.17 ± 0.72
	½ IC_{50}	7.9 ± 0.89*
	IC_{50}	12.4 ± 1.56*
Nontreated	–	4.21 ± 0.31
IFN-γ + LPS	–	44.3 ± 3.51

Mean ± SD ($n = 3$). IC_{50}: the 50% inhibitory concentrations.
*$P < 0.001$ significant difference compared with nontreated macrophage cells.

Discussion

In recent decades, the use of herbal medicines for the treatment of giardiasis has been increasing due to their high efficacy and low side effects. To the best of our knowledge, there is no scientific document on the anti-*Toxoplasma* activity of *Astragalus* spp., so this study aimed to evaluate the in-vitro anti-*Toxoplasma* effects and cytotoxicity effects of AMCE on the *T. gondii* Rh strain. We found that, after 48 h of incubation of *T. gondii* tachyzoites with different concentrations of AMCE, the mortality rate of the parasites significantly ($P < 0.001$) increased in a dose-dependent manner compared to the control group. The results showed that, according to the microscopic examination, the rate of infection and the mean number of intracellular tachyzoites in macrophage cells was significantly reduced ($P < 0.001$) after exposure of the macrophage cells to AMCE.

Considering the antiparasitic effects of *Astragalus* spp., in a study conducted by Yang *et al.*[21] *Astragalus membranaceus* extract showed effective in-vitro anti-*Toxoplasma* activity through the reduction of intracellular replication of *T. gondii* tachyzoites after 72, 96, and 120 h incubation. Another study conducted by Abdel-Tawab *et al.*[22] reported that *A. membranaceus* especially at the concentration of 50 mg/kg markedly decreased the viability of *Eimeria papillata* oocysts as well as the mean number of oocytes exerted in the stool of infected animals. Ghasemian Yadegari *et al.*[23] have reported that *A. brachycalyx* extract at concentrations of 225 and 450 mg/ml had potent protoscolicidal effects on hydatid cyst protoscoleces. Furthermore, previous studies also revealed that *Astragalus* spp. have effective antimicrobial activities against various Gram-negative (e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Klebsiella pneumonia*) and Gram-positive (*Enterococcus faecalis* and *Staphylococcus aureus*), as well as *Aspergillus* spp., *Candida* spp., and influenza virus[24–26]. Phytochemical experiments displayed the attendance of flavonoids, saponins, terpenoids, and polysaccharides; where the evaluation of the amount of secondary metabolites showed that the total flavonoid and phenolic content was 3.89 (mg QE/g DW) and 5.12 (mg GEA/ g DW), respectively. Likewise, reviews exhibited that previous studies have shown that phenolic and flavonoids compounds are measured as the main composites of *Astragalus* spp.[27,28]. Previous studies revealed that these components showed their antimicrobial mechanisms through cytoplasmic membrane disruption, nucleic acid inhibition, stimulation of apoptosis, disturbing energy metabolism, altering the permeability of the membrane, and decrease of the pathogenicity[29,30].

Polyphenols compounds also display their antimicrobial action by hindering the virulence factors, cytoplasmic membrane and cell membrane disruption, and DNA synthesis inhibition[31,32]. Therefore, it may be proposed that the favorable anti-*Toxoplasma* activity of AMCE is associated with the attendance of phenolic and flavonoid compounds in this herb.

Previous studies confirmed that macrophage cells by generating nitric oxide eliminate and control the intracellular pathogens[33]. Now, it is proven that *T. gondii* infection in macrophages inhibits NO production in these cells[34]. The mRNA expression levels of IFN-γ and iNOS in infected J774-A1 macrophage cells treated with AMCE, especially at IC_{50}, was significantly ($P < 0.001$) elevated in quantitative real-time PCR. The findings of the Griess reaction assay showed that the amount of NO production in macrophage cells after treatment with the AMCE was increased, especially at the concentration of ½ IC_{50} and IC_{50} ($P < 0.001$) in comparison with the control group, represented that AMCE's increasing of NO production can be probably considered as one of the possible mechanisms for controlling of *T. gondii* parasites. As the main limitations of the present investigation, we can mention the lack of *in vivo* studies in animal models of toxoplasmosis and the clarification of all toxicity aspects, which we will try to clear these surveys in future studies.

Conclusion

The findings of the current *in vitro* investigation as the first step to find new anti-*Toxoplasma* agents revealed the potent anti-*Toxoplasma* effects of AMCE, against tachyzoites and intracellular forms of *T. gondii*. We also found that triggering the NO production can be considered as one of the main mechanisms of action of AMCE for controlling and eliminating *T. gondii*. However, further surveys are required to assess the efficacy and safety of AMCE in an animal model and its mechanisms of action before use in the clinical phase.

Ethical approval

This study was approved by the ethics committee of Lorestan University of Medical Sciences, Khorramabad, Iran, with the ethics number of IR.LUMS.REC.1401.186.

Consent for publication

Not applicable.

Sources of funding

None.

Author's contribution

H.M.: supervisor and writing; A.K.K. and S.B.: data collection; J.G.Y.: data analysis; A.K.: study design and data collection.

Conflicts of interest disclosure

The authors declare that there are no conflicts of interest.

Guarantor

Hossein Mahmoudvand.

Provenance and peer review

Not commissioned, externally peer reviewed.

References

[1] Innes EA. A brief history and overview of *Toxoplasma gondii*. Zoonoses Public Health 2010;57:1–7.
[2] Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. Int J Parasitol 2000;30:1217–58.
[3] Hill DE, Chirukandoth S, Dubey JP. Biology and epidemiology of *Toxoplasma gondii* in man and animals. Anim Health Res Rev 2005;6:41–61.
[4] Saadatnia G, Golkar M. A review on human toxoplasmosis. Scand J Infect Dis 2012;44:805–14.
[5] Weiss LM, Dubey JP. Toxoplasmosis: a history of clinical observations. Int J Parasitol 2009;39:895–901.
[6] Goldstein EJ, Montoya JG, Remington JS. Management of *Toxoplasma gondii* infection during pregnancy. Clin Infect Dis 2008;47:554–66.
[7] Dunay IR, Gajurel K, Dhakal R, *et al*. Treatment of toxoplasmosis: historical perspective, animal models, and current clinical practice. Clin Microbiol Rev 2018;31:e00057–17.
[8] Cheraghipour K, Masoori L, Ezzatpour B, *et al*. The experimental role of medicinal plants in treatment of *Toxoplasma gondii* infection: a systematic review. Acta Parasitol 2021;66:303–28.
[9] Keyhani A, Ziaali N, Shakibaie M, *et al*. Biogenic selenium nanoparticles target chronic toxoplasmosis with minimal cytotoxicity in a mouse model. J Med Microbiol 2020;69:104–10.
[10] Rios JL, Waterman PG. A review of the pharmacology and toxicology of Astragalus. Phytother Res 1997;11:411–8.
[11] Li X, Qu L, Dong Y, *et al*. A review of recent research progress on the Astragalus genus. Molecules 2014;19:18850–80.
[12] Shahrajabian MH, Sun W, Cheng Q. A review of Astragalus species as foodstuffs, dietary supplements, a traditional Chinese medicine and a part of modern pharmaceutical science. Appl Ecol Environm Res 2019;17:13371–82.
[13] Miraj S, Kiani S. *Astragalus membranaceus*: a review study of its anticarcinoma activities. Der Pharmacia Lettre 2016;8:59–65.
[14] Ghasemian-Yadegari J, Nazemiyeh H, Asnaashari S, *et al*. Chemical characterization of the essential oil from aerial parts of *Astragalus Maximus* from northwest of Iran. Adv Biosci Clin Med 2015;3:32–9.
[15] Turgut-Kara N, Arı S. Micropropagation of *Astragalus Maximus* willd. Biotechnol Biotechnol Equip 2006;20:20–2.
[16] Niazi M, Saki M, Sepahvand M, *et al*. In vitro and ex vivo scolicidal effects of Olea europaea L. to inactivate the protoscolecs during hydatid cyst surgery. Ann Med Surg 2019;42:7–10.
[17] Evans WC. Trease and Evans' pharmacognosy. General Pharmacology. 1997;2:291.
[18] Kharazmkia A, Al-Abodi HR, Yadegari JG, *et al*. Potential effects of alpha-pinene, a monoterpene commonly found in essential oils against Toxoplasma gondii infection; an in vitro and in vivo study. J Paras Dis 2022;46:1–7.
[19] Alnomasy SF. In vitro and in vivo Anti-*Toxoplasma* effects of allium sativum essential oil against *Toxoplasma gondii* RH strain. Infect Drug Resist 2021;14:5057.
[20] Albalawi AE, Khalaf AK, Alyousif MS, *et al*. Fe3O4@ piroctone olamine magnetic nanoparticles: Synthesize and therapeutic potential in cutaneous leishmaniasis. Biomed Pharmacother 2021;139:111566.
[21] Yang X, Huang B, Chen J, *et al*. In vitro effects of aqueous extracts of *Astragalus membranaceus* and *Scutellaria baicalensis* GEORGI on *Toxoplasma gondii*. Parasitol Res 2012;110:2221–7.
[22] Abdel-Tawab H, Abdel-Baki AS, El-Mallah AM, *et al*. In vivo and in vitro anticoccidial efficacy of *Astragalus membranaceus* against Eimeria papillata infection. J King Saud Univ-Sci 2020;32:2269–75.
[23] Ghasemian Yadegari J, Khudair Khalaf A, Darabi R. Antiparasitic effects and cellular mechanism of *Astragalus Maximus* chloroform extract against clinical isolates of Giardia lamblia. Res J Pharm 2022;9:5–13.

[24] Mikaeili A, Karimi I, Shamspur T, *et al*. 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;22:1035–43.
[25] Kanaan H, ElMestrah M, Sweidan A, *et al*. Screening for antibacterial and antibiofilm activities in Astragalus angulosus. J Intercult Ethnopharmacol 2017;6:50.
[26] Khan MN, Ahmed M, Khan MW, *et al*. In vitro pharmacological effects of Astragalus eremophilus and Melilotus parviflora. Biologia Futura 2018;69:411–22.
[27] Bratkov V, Shkondrov A, Zdraveva P, *et al*. Flavonoids from the genus Astragalus: phytochemistry and biological activity. Pharmacogn Rev 2016;10:11.
[28] Jun YM, Kim EH, Lim JJ, *et al*. Variation of phenolic compounds contents in cultivated *Astragalus membranaceus*. Kor J Med Crop Sci 2012;20:447–53.
[29] Górniak I, Bartoszewski R, Króliczewski J. Comprehensive review of antimicrobial activities of plant flavonoids. Phytochem Rev 2019;18:241–72.
[30] Mahmoudvand H, Kheirandish F, Ghasemi Kia M, *et al*. Chemical composition, protoscolicidal effects and acute toxicity of Pistacia atlantica Desf. fruit extract. Nat Prod Res 2016;30:1208–11.
[31] Miklasińska-Majdanik M, Kępa M, Wojtyczka R, *et al*. Phenolic compounds diminish antibiotic resistance of Staphylococcus Aureus clinical strains. Int J Environ Res Public Health 2018;15:2321.
[32] Othman L, Sleiman A, Abdel-Massih RM. Antimicrobial activity of polyphenols and alkaloids in middle eastern plants. Front Microbiol 2019;10:911.
[33] Cabral GRA, Wang ZT, Sibley LD, *et al*. Inhibition of nitric oxide production in activated macrophages caused by *Toxoplasma Gondii* infection occurs by distinct mechanisms in different mouse macrophage cell lines. Front Microbiol 2018;9:1936.
[34] Padrão JC, Cabral GRA, da Silva MFS, *et al*. Toxoplasma gondii infection of activated J774-A1 macrophages causes inducible nitric oxide synthase degradation by the proteasome pathway. Parasitol Int 2014;63:659–63.