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Evaluation of anti-giardial and immunomodulatory activities of chloroform extract of *Astragalus baba-alliar* in experimental *Giardia lamblia* infection

Javad Ghasemian Yadegari[1], Seyed Bahram Mirzadeh Ahari[2], Amal Khudair Khalaf[3], Roya Darabi[1], Aram Oladi[1], Hossein Mahmoudvand[4,*]

[1]*Department of Pharmacognosy, Lorestan University of Medical Sciences, Khorramabad, Iran*
[2] *Universal Scientific Education and Research Network (USERN) Office, Lorestan University of Medical Sciences, Khorramabad, Iran* [3]*Department of Microbiology, College of Medicine, University of Thi-qar, Iraq*
[4]*Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran*

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**** Corresponding Author:***
dmahmodvand@gmail.com

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Abstract

Objective: This study evaluated the *in vitro* and *in vivo* anti-giardial effects of a chloroform extract of the aerial parts of *Astragalus baba-alliar* (ABCE) against *Giardia lamblia*.

Materials and methods: Anti-giardial activity of ABCE (100–400 µg/mL) was assessed against cysts and trophozoites. Cytotoxicity was determined in normal intestinal cells (NCM460) and SW480 cell line using MTT assay. *In vivo* efficacy was evaluated in infected mice compared with metronidazole. Intestinal expression of *TNF-α, NF-κB,* and *IL-1β* was analyzed by real-time PCR.

Results: ABCE exhibited dose-dependent in vitro activity, with the highest concentration inducing 100% eosin-positive cysts within 120 min and complete loss of trophozoite viability within 60 min ($p < 0.001$). Cytotoxicity analysis revealed selective safety, as NCM460 cells were less affected than cancerous SW480 cells. *In vivo*, ABCE markedly reduced cyst burden in a dose-dependent manner, with 40 mg/kg showing the most pronounced effect ($p<0.001$). Concomitantly, intestinal inflammatory markers decreased progressively with higher doses of ABCE, from modest reductions at 10 mg/kg to near-baseline levels at 40 mg/kg (*TNF-α* 1.17, *NF-κB* 1.08, and *IL-1β* 0.94-fold change), indicating effective modulation of the host inflammatory response.

Conclusion: ABCE demonstrated potent anti-giardial and anti-inflammatory activity with minimal toxicity to normal cells, supporting its potential as a therapeutic candidate against giardiasis. Further mechanistic and pharmacokinetic studies are warranted to confirm efficacy and safety.

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Introduction

Giardia lamblia is a flagellated protozoan that inhabits the human small intestine and has been documented globally (Adam 2001). Human infection is primarily transmitted via the consumption of contaminated food and water, as well as through direct fecal-oral contact. The resilience of the parasite's cysts to chlorination processes in water enhances its potential for transmission through aquatic

sources. While infection can manifest at any age, it is particularly prevalent among children (Laishram et al. 2012). It is estimated that there are approximately 280 million cases of human infection annually. *Giardia Lamblia* (Mahdavi et al. 2022), the etiological agent of giardiasis, is prevalent in both temperate and tropical regions and is associated with the clinical manifestations such as diarrhea (steatorrhea), particularly in pediatric populations, as well as malabsorption, abdominal cramps, and weight loss (Hooshyar et al. 2019).

The pharmacological management of this disease involves the administration of chemical agents such as metronidazole (MTZ) and furazolidone (Watkins and Eckmann 2014). However, these medications are associated with numerous adverse effects, and their efficacy remains uncertain (Vivancos et al. 2018). Some anti-giardial drugs have demonstrated mutagenic or genotoxic effects in experimental studies, while their clinical relevance in humans remains under investigation (Vivancos et al. 2018). Furthermore, there are instances of parasitic resistance to these drugs (Watkins and Eckmann 2014). Consequently, there is a growing interest in research aimed at identifying alternative compounds that exhibit fewer or no adverse effects. Recent literature has highlighted the therapeutic potential of specific herbs and their derivatives, such as *Zataria* spp., *Eucalyptus* spp., and *Allium* spp., in the treatment of *Giardia* infections (Alnomasy et al. 2021). Nevertheless, the broader application of herbal remedies for giardiasis is presently impeded by ambiguous research outcomes that often lack sufficient empirical support.

The genus *Astragalus* encompasses approximately 3,000 species of herbs, predominantly perennial, with over 250 recognized taxonomic classifications globally (Li et al. 2014). This genus exhibits a broad distribution across temperate regions, with around 800 species documented in the pastures and mountainous regions of Iran. Numerous *Astragalus* species have a longstanding history of use in traditional medicine for the treatment of various ailments including diabetes, nephritis, gastric ulcers, hypertension, and chronic bronchitis (Shahrajabian et al. 2019). Furthermore, a range of pharmacological properties associated with this genus has been established, including antioxidant activity, immune system enhancement, and antihypertensive effects, as well as antimicrobial and anti-inflammatory properties (Miraj and Kiani 2016). To the best of our knowledge, only one previous study has investigated anti-*Giardia* activity of *Astragalus* species, specifically *A. maximus* against clinical isolates of *G. lamblia*. No study has evaluated *A. baba-alliar* in either *in vitro* or *in vivo* models (Ghasemian Yadegari et al. 2022). Given the aforementioned information and the noted antimicrobial characteristics of the *Astragalus* genus, coupled with the limited research on *A. baba-alliar*, this study represents the first report evaluating the *in vitro* anti-giardial activity, *in vivo* therapeutic efficacy, and immunomodulatory effects of *A. baba-alliar* against experimental giardiasis.

Materials and Methods

Plant materials

The aerial parts of *A. baba-alliar* were collected from wild populations in the rural areas of Nurabad district, Lorestan Province, Western Iran, in May 2025. The plant material collection was carried out on privately owned property, for which no specific permissions were required. Additionally, it is affirmed that the field research did not include any endangered or protected species, in accordance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the provisions of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Plant

identity was authenticated by a qualified taxonomist at the School of Pharmacy, Lorestan University of Medical Sciences, Khorramabad, Iran. A voucher specimen has been deposited in the institutional herbarium under accession number LUMS-26354 for future reference.

Extract preparation

Air-dried aerial parts of the plant (200 g) were coarsely ground and subjected to sequential solvent extraction. Initially, defatting was carried out by maceration with n-hexane to remove non-polar lipophilic constituents. The defatted material was then extracted by maceration in 70% methanol (v/v) for 72 hr at room temperature with periodic agitation. The resulting methanolic extract was filtered through Whatman No. 1 filter paper and concentrated under reduced pressure at 50 °C using a rotary evaporator (Heidolph, Germany). The concentrated extract was subsequently partitioned against chloroform in a separatory funnel to further remove non-polar impurities. The final extract was lyophilized, weighed to determine yield percentage, and stored at −20 °C in amber vials protected from light until further analysis (Mahmoudvand et al. 2016a; Ezatpour et al. 2016). The chloroform fraction was selected because previous studies on *Astragalus* species demonstrated enrichment of bioactive lipophilic constituents with antiparasitic activity in this fraction.

Determination of total phenolic content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric assay with gallic acid as the reference standard (Singleton et al. 1999). Briefly, 0.3 mL of appropriately diluted extract or standard solution was mixed with 3 mL of 10% Folin–Ciocalteu reagent, followed by the addition of 3 mL of 7.5% sodium carbonate solution. The mixture was incubated in the dark at room temperature for 30 min to allow complete color development. Absorbance was measured at 760 nm against a reagent blank using a UV–Vis spectrophotometer (UV-2600, Shimadzu, Japan). A calibration curve was constructed using gallic acid at concentrations ranging from 10 to 500 µg/mL ($R^2 \geq 0.99$). TPC is expressed as milligrams of gallic acid equivalents per gram of dry extract weight (mg GAE/g DW). All measurements were performed in triplicate, and results are presented as mean ± standard deviation (SD). Appropriate reagent blanks were included in all assays and absorbance values were corrected accordingly. Calibration standards and quality-control samples were analyzed with each run.

Determination of total flavonoid content

Total flavonoid content (TFC) was quantified using the aluminum chloride ($AlCl_3$) colorimetric method, with quercetin as the reference standard (Phuyal et al. 2020). Briefly, 0.2 mL of extract or standard solution was combined with 0.2 mL of 2% $AlCl_3$ (w/v) and 0.1 mL of 33% acetic acid, followed by vortex mixing. The reaction volume was adjusted to 5 mL with 90% ethanol (v/v), and the mixture was allowed to equilibrate at room temperature for 30 min. Absorbance was recorded at 510 nm against a reagent blank using a UV–Vis spectrophotometer. A quercetin calibration curve was established over a concentration range of 10–250 µg/mL ($R^2 \geq 0.99$). TFC is expressed as milligrams of quercetin equivalents per gram of dry extract weight (mg QE/g DW). All assays were conducted in triplicate, and data are reported as mean ± SD. Appropriate reagent blanks were included in all assays and absorbance values were corrected accordingly. Calibration standards and quality-control samples were analyzed with each run.

In vitro anti-giardial effects of ABCE

Isolation and purification of *G. lamblia* cysts

A total of one stool sample was obtained from a male patient aged 13 years

with confirmed giardiasis referred to healthcare facilities in Khorramabad, Lorestan Province, Iran. Initial diagnosis was established by direct wet mount microscopy. For isolation of viable *Giardia* cysts intended for subsequent *in vitro* cultivation and excystation, positive stool samples were processed under sterile conditions and purified by sucrose density gradient centrifugation as described below. No formalin-containing procedures were applied to samples designated for culture in order to preserve cyst viability. All procedures involving human-derived samples were conducted in accordance with the ethical guidelines of the institutional review board, and informed consent was obtained from the patient prior to sample collection. The *Giardia* cysts used in this study were obtained from clinical isolates recovered from patients with microscopically confirmed giardiasis. Molecular genotyping of isolates (Assemblage A/B) was not performed; therefore, the findings should be interpreted as representative of clinical *Giardia* isolates rather than a specific assemblage.

Cyst purification by sucrose density gradient centrifugation

Giardia cysts were purified using sucrose density gradient centrifugation as previously described (Malekifard et al. 2020), with minor modifications. Briefly, confirmed positive stool samples were diluted in distilled water (DW) at a 12:1 ratio (v/v) and passed through a 425-µm mesh filter to remove coarse particulate matter. The filtered sediment was resuspended in 5 mL of DW, and the resulting suspension was carefully layered onto 3 mL of 0.85 M sucrose solution in a conical centrifuge tube. The preparation was centrifuged at 700 × g for 10 min at 4 °C using a refrigerated centrifuge. Following centrifugation, the cyst-enriched interface layer was carefully aspirated using a sterile Pasteur pipette, transferred to a clean tube, and washed three times with sterile normal saline (0.9% NaCl) by repeated centrifugation at 500 × g for 5 min to remove residual sucrose. The purified cysts were resuspended in sterile normal saline and stored at 4°C until use.

Cyst quantification and viability assessment

Cyst concentration was determined by direct enumeration using a Neubauer hemocytometer under light microscopy at 400× magnification. Cyst viability was assessed using the eosin exclusion assay in which, non-viable cysts with compromised membrane integrity stain red, while viable cysts remain unstained. Only preparations with viability exceeding 90% were used for subsequent experimental procedures. The final cyst suspension was adjusted to a working concentration of 1×10^5 cysts/mL in sterile normal saline prior to use.

Cultivation and collection of *G. lamblia* trophozoites

Trophozoites of *G. lamblia* were obtained via in vitro excystation of purified cysts according to previously described methodology (Azadbakht et al. 2003; Bingham and Meyer, 1979) with minor modifications. Minor modifications were introduced to improve excystation efficiency and trophozoite yield under local laboratory conditions. Briefly, cyst suspensions were exposed to aqueous hydrochloric acid (pH 1.5–2.0) at a 1:9 (v/v) ratio to simulate gastric passage and incubated at 37°C for 120 min under gentle agitation. Following incubation, suspensions were centrifuged at 600 × g for 10 min at room temperature, the supernatant discarded, and the pellet resuspended in complete TYI-S-33 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS), bovine bile (0.5 g/L), penicillin (500 IU/mL), and streptomycin (500 µg/mL), filter-sterilized through a 0.22-µm membrane prior to use. TYI-S-33 medium consisted of casein digest, yeast extract, glucose, L-cysteine,

ascorbic acid, ferric ammonium citrate, KH_2PO_4, K_2HPO_4 and bovine bile, prepared according to the original formulation of Keister (Azadbakht et al. 2003). Cultures were maintained at 37°C under microaerophilic conditions in tightly capped tubes. Culture tubes were incubated at 37°C in a slanted position (~15°) to facilitate trophozoite attachment and examined daily by inverted light microscopy for morphology, motility, and confluency. Trophozoites were harvested at mid-logarithmic growth phase (48–72 hr post-excystation) by chilling cultures on ice for 10 min to detach adherent cells, followed by centrifugation at 800 × g for 10 min at 4°C. The pellet was washed twice with sterile phosphate-buffered saline (PBS, pH 7.4) and resuspended in the appropriate experimental medium. Viability was confirmed by eosin exclusion assay, with only preparations exceeding 95% viability accepted for downstream assays, and cell density was adjusted to the required concentration using a Neubauer hemocytometer.

Anti-*Giardia* effects on cysts and trophozoites

The *in vitro* antiparasitic activity of ABCE was evaluated against *G. lamblia* cysts and trophozoites at concentrations of 1, 2, and 4 mg/mL across discrete exposure intervals of 15, 30, 60, 120, 180, 240, 300, and 360 min. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and diluted to working concentrations in sterile normal saline immediately prior to use, with the final DMSO concentration maintained at ≤0.1% (v/v) to exclude solvent-mediated cytotoxicity. Equal volumes (100 µL) of each extract dilution and parasite suspension (1×10^5 cysts or trophozoites/mL) were combined in sterile 1.5-mL microcentrifuge tubes and incubated at 37°C under static conditions for each designated interval. Metronidazole at its established minimum lethal concentration (15 mg/kg) and sterile normal saline served as positive and negative controls, respectively (Gholami et al. 2014). All assays were conducted in triplicate across three independent experiments. Following each incubation period, tubes were centrifuged at 500 × g for 5 min, the supernatant aspirated, and the pellet resuspended in 50 µL of 0.1% eosin solution (Sigma-Aldrich, Germany) for 5 min at room temperature. Thin smears were prepared on clean glass slides, cover-slipped, and examined immediately under light microscopy at 400× magnification. Parasite viability was evaluated using eosin exclusion assay as an indicator of membrane integrity. No excystation-based infectivity assay was performed. Parasites with intact membranes excluded the dye and appeared colorless, whereas non-viable organisms absorbed the stain and appeared pink to red. A minimum of 200 parasites per preparation (100 cysts and 100 trophozoites, enumerated separately) were assessed per replicate. Parasite viability was evaluated using eosin exclusion assay as an indicator of membrane integrity. No excystation-based infectivity assay was performed.

Cytotoxic effects of ABCE

Normal human intestinal epithelial cells (NCM460) and the human colorectal adenocarcinoma cell line (SW480) were obtained from the Pasteur Institute in Tehran, Iran. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum, and maintained at 37°C in a 5% CO2 atmosphere. To assess the cytotoxic effects of ABCE on both normal and cancerous human cell lines, an initial volume of 100 µL containing cell lines at a concentration of 1×10^5 cells/mL was dispensed into each well of a 96-well plate. Various concentrations of the extract were subsequently added to the wells containing the cells, followed by incubation at 37°C in a humidified 5% CO_2 atmospher for 48 hr. After removing the supernatant from the wells, 20 µL of MTT solution (0.5 mg/mL

in PBS) was introduced into each well and incubated for 4 hr in a 5% CO_2 environment at 37°C. After adding 100 µL of DMSO, the absorbance of each well was measured at 570 nm using an ELISA plate reader. The 50% cytotoxic concentration (CC50 values) was determined using the Probit analysis in SPSS software (Version 26.0). The selectivity index (SI) serves as a measure of the selective toxicity of the essential oil towards cancerous cells in comparison to normal cells, as defined by the equation CC50 of healthy cells divided by CC50 of cancer cells. An SI value exceeding 2 suggests that the extract is non-toxic to healthy cells (Mahmoudvand et al. 2016b). All cytotoxicity assays were performed in triplicate and repeated in three independent experiments.

In vivo effects of ABCE on giardiasis in mice

Animals

A total of 40 male BALB/c mice, aged 8–10 weeks and weighing 25–30 g, were housed under controlled environmental conditions (22 ± 2°C, 12-hr light/dark cycle, 50 ± 10% relative humidity) with *ad libitum* access to standard rodent chow and filtered water. All animal procedures were conducted in strict accordance with the ethical guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Ethics Committee of Lorestan University of Medical Sciences (Ethics Code: IR.LUMS.REC.1401.192).

Experimental giardiasis in mice

To enhance intestinal susceptibility to parasitic colonization, mice underwent a four-day antibiotic pretreatment regimen prior to experimental infection. Drinking water was supplemented *ad libitum* with vancomycin (0.5 mg/mL), neomycin (1 mg/mL), and ampicillin (1 mg/mL) for four consecutive days to deplete commensal microbiota and facilitate *G. lamblia* establishment. Following a 24-hr antibiotic washout period to minimize direct antiparasitic interference, each animal received a single oral inoculation of 0.2 mL of sterile normal saline containing 2×10^3 *G. lamblia* cysts via gavage. Successful infection establishment was confirmed on days 7 post-inoculation by detection of cysts in fecal samples through both direct wet mount microscopy and the formalin–ether sedimentation technique, performed on three consecutive daily samples to account for intermittent cyst shedding (Masoori et al. 2024). Only animals with confirmed, consistent cyst shedding were included in subsequent experimental groups.

Experimental groups and treatment protocol

Following confirmation of successful infection on day 7 post-inoculation, mice were randomly allocated into five experimental groups (n = 8 per group) using a random number table to minimize selection bias. All treatments were administered orally via gavage once daily for seven consecutive days according to the following regimen: (i) infected untreated control, receiving sterile normal saline; (ii) infected positive control, receiving metronidazole (MTZ) at 15 mg/kg/day (Masoori et al. 2024), the standard therapeutic dose for murine giardiasis; and (iii, iv, and v) infected treatment groups, receiving ABCE at doses of 10, 20, and 40 mg/kg/day, respectively. Selected doses (10–40 mg/kg) were based on preliminary pilot experiments and previous studies evaluating *Astragalus*-derived extracts in murine models (Nancy and Szabo, 2014). All solutions were freshly prepared daily and administered at the same time each day to minimize circadian variation. The experimental timeline for the current study was as follows:

- Day -4 to 0: antibiotic pretreatment
- Day 0: oral infection
- Day 7: infection confirmation
- Day 7–14: treatment period
- Day 15: fecal assessment and tissue collection

Post-Treatment parasitological assessment

On day 8 following the completion of treatment (day 15 post-inoculation), fresh fecal samples were collected from each animal over a 24-hr period and processed within two hours of collection to preserve cyst integrity. Cyst burden was quantified using both direct wet mount microscopy and the formalin–ether sedimentation technique, with enumeration performed using a Neubauer hemocytometer to determine the number of cysts per gram of feces. Cyst viability was subsequently assessed using the eosin exclusion assay: pellets obtained after centrifugation at 500 × g for 5 min were resuspended in 0.1% eosin solution (Sigma-Aldrich, Germany) and incubated for 5 min at room temperature. Thin smears were prepared on clean glass slides and examined immediately under light microscopy at 400× magnification. Cysts with intact membrane integrity excluded the dye and appeared colorless and were classified as viable, whereas cysts with compromised membranes absorbed the stain and appeared pink to red and were classified as non-viable. A minimum of 200 cysts per sample were enumerated to ensure statistical reliability, and results are expressed as mean percentage viability ± SD across all animals per group (Masoori et al. 2024).

Tissue harvesting and serum collection

At the experimental endpoint, mice were humanely euthanized by intraperitoneal administration of a ketamine/xylazine combination (100 mg/kg and 10 mg/kg, respectively), consistent with institutional and international guidelines for humane endpoints in rodent studies. Death was confirmed by cessation of cardiac and respiratory activity prior to tissue harvesting. The abdominal cavity was opened via midline laparotomy, and the entire duodenum was carefully excised, flushed with ice-cold sterile phosphate-buffered saline (PBS, pH 7.4) to remove luminal contents, and longitudinally incised along the antimesenteric border. Tissue processing was performed as previously described by Masoori et al. (2024). Mucosal scrapings were collected for parasitological and enzymatic analyses, while full-thickness duodenal segments were immediately snap-frozen in liquid nitrogen and stored at −80 °C until molecular and biochemical analyses. All tissue harvesting procedures were completed within 30 min of euthanasia to minimize RNA degradation and preserve tissue integrity (Masoori et al. 2024).

Expression analysis of inflammatory mediators

RNA extraction and quality assessment

Duodenal tissue samples collected from mice were processed for total RNA extraction using a commercially available isolation kit (Yekta Tajhiz Azma, Iran), strictly following the supplier's recommended protocol. Spectrophotometric quantification was carried out with a NanoDrop instrument, where A260/A280 and A260/A230 ratios were recorded to evaluate purity; only specimens falling within the 1.8–2.0 range for A260/A280 were taken forward.

Elimination of genomic DNA carry-over

Given the potential for genomic DNA to interfere with downstream expression profiling, all specimens underwent DNase I treatment (Rnase-free; Fermentas, USA) directly on the column, as outlined by the manufacturer. To confirm complete removal of residual DNA, a pre-reverse-transcription PCR was run on each RNA preparation using intron-spanning primer pairs, with the absence of any detectable band taken as evidence of successful decontamination.

Reverse transcription

One microgram of purified RNA per sample was converted into first-strand cDNA using a reverse transcription kit (Yekta Tajhiz Azma, Iran) under conditions specified by the manufacturer. Oligo(dT) primers were employed to selectively

capture polyadenylated messenger RNA populations during the conversion step. Synthesized cDNA products were kept at −20°C until required for amplification.

Primer design and specificity confirmation

Oligonucleotide primers directed at murine *TNF-α, NF-κB,* and *IL-1β* transcripts were constructed with the aid of Primer3 software and subsequently screened against the mouse reference genome via NCBI BLAST to rule out cross-reactivity with non-target sequences (Table 1) (Masoori et al. 2024). Each primer pair was deliberately positioned across exon–exon boundaries to preclude amplification of any genomic DNA that might have escaped the DNase treatment step. Full details of primer sequences, expected amplicon lengths, and optimal annealing temperatures are provided in Table 1. Amplification efficiency for all primer sets was validated and found to be within acceptable ranges (90–110%).

Table.1. Primer sequences utilized in Real-time PCR analysis

Gene	Sequence (5'–3')	Size (bp)
IL-1	F: AACCTGCTGGTGTGTGACGTTC R: CAGCACGAGGCTTTTTTGTTGT	256
TNF-α	F: TGAACTTCGGGGTGATCGGT R: GGTGGTTTGTGAGTGTGAGGG	182
NF-κB p65	F: AGGCAAGGAATAATGCTGTCCTG R: ATCATTCTCTAGTGTCTGGTTGG	209
β-actin	F: GTGACGTTGACATCCGTAAAGA R: GCCGGACTCATCGTACTCC	245

PCR Amplification Protocol

All amplification reactions were carried out on a Bio-Rad iQ5 optical platform (Bio-Rad, USA) with SYBR Green chemistry serving as the fluorescent reporter. Individual reactions were assembled in 20 µL volumes, each containing the appropriate cDNA template, paired primers, and SYBR Green master mix. The cycling program began with a 5-min hold at 96°C to achieve complete template denaturation, proceeded through 35 repetitive cycles consisting of a 30-sec denaturation phase at 96°C and a 60-sec combined annealing and extension phase at 62°C, and concluded with a 5-min final extension at 73°C. Upon completion of cycling, a continuous melt curve acquisition was performed to verify single-product amplification and rule out primer dimer formation or spurious bands.

Quantification and statistical normalization

Transcript abundance is expressed as fold change relative to the uninfected control group, derived through application of the $2^{-\Delta\Delta Ct}$ algorithm. Prior to this calculation, raw Ct values were normalized against a stably expressed housekeeping gene —β-actin whose expression consistency across all groups had been pre-verified using geNorm or NormFinder algorithms. Each target gene was amplified in independent triplicate reactions per sample, and mean Ct values were used as the basis for all subsequent calculations to minimize technical variability.

Statistical analysis

All *in vitro* experiments were performed in triplicate across three independent biological replicates to ensure both technical and biological reproducibility. Quantitative data are presented as mean ± standard deviation (SD). Normality of data distribution was assessed using the Shapiro–Wilk test prior to parametric analysis. Intergroup differences were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference

(HSD) post hoc test for multiple pairwise comparisons. For *in vivo* data, comparisons between the infected untreated control and treatment groups were additionally verified using appropriate non-parametric alternatives (Kruskal–Wallis test followed by Dunn's post hoc test) where normality assumptions were violated. All statistical analyses were performed using SPSS software (version 26.0; IBM Corp., Armonk, NY, USA). A two-tailed p-value of less than 0.05 was considered statistically significant. Dose–response relationships and LC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism (version X; GraphPad Software, San Diego, CA, USA).

Results

Total phenolic and flavonoid contents

The chloroform extract yielded 17.8 g, corresponding to a percentage of 8.9% (w/w). The analytical results indicated that the TPC and TFC were measured at 2.69 mg GAE/g DW and 0.85 mg QE/g DW, respectively.

In vitro effect on *G. lamblia* cysts

The ABCE dose-dependently demonstrated a significant increase in the mortality rate of *G. lamblia* cysts ($p<0.001$) when compared to the control group (Figure 1). The results indicated that the most pronounced effect of the ABCE occurred at a concentration of 4 mg/ml, which resulted in the absence of viable cysts based on eosin exclusion assay after 120 min ($p<0.001$). Additionally, this extract was effective at a concentration of 2 mg/ml, achieving absence of viable cysts based on eosin exclusion assay after 240 minutes ($p<0.001$). Conversely, the lowest efficacy was observed at the concentration of 1 mg/ml, which required 360 min of incubation to eliminate all *G. lamblia* cysts.

In vitro effect on *G. lamblia* trophozoites

As illustrated in Figure 2, the ABCE substantially increased the mortality rate of *G. lamblia* trophozoites compared to the control group in a dose-dependent manner ($p<0.001$). The findings indicate that *G. lamblia* trophozoites exhibited greater susceptibility to the ABCE than *G. lamblia* cysts. The most pronounced effect of the extract was noted at a concentration of 4 mg/ml, which resulted in the 100% eosin-positive trophozoites within 60 min ($p<0.001$). Furthermore, this extract also demonstrated the 100% eosin-positive trophozoites at a concentration of 2 mg/ml after 120 min ($p<0.001$). Among the concentrations examined, the lowest efficacy was observed at 1 mg/ml, where the absence of viable trophozoites based on eosin exclusion assay was observed after 240 min of incubation.

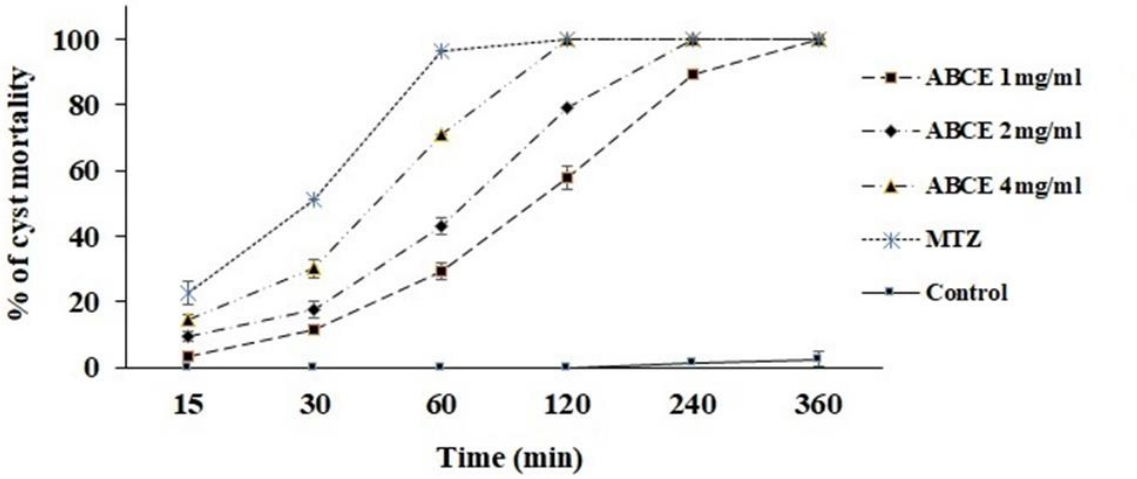

Figure 1. The impact of different concentrations of *Astragalus baba-alliar* chloroform extract (ABCE) on the cysts of *Giardia lamblia* in comparison to metronidazole (MTZ). The results are presented as mean ± standard deviation (SD) with a sample size of n=3.

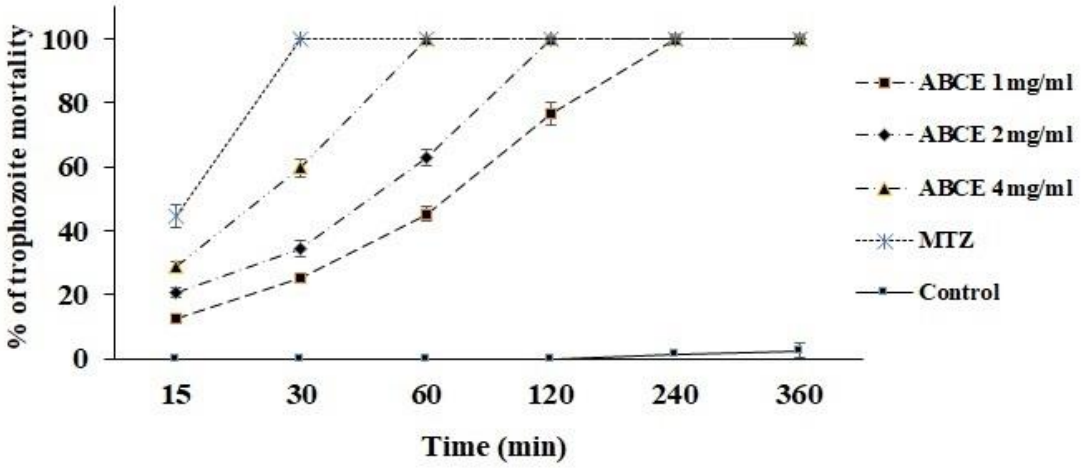

Figure 2. The impact of different concentrations of *Astragalus baba-alliar* chloroform extract (ABCE) on the trophozoites of *Giardia lamblia* in comparison to metronidazole (MTZ). The results are presented as mean ± standard deviation (SD) with a sample size of n=3.

Cytotoxic effects of ABCE

The MTT results showed that ABCE dose-dependently reduced the viability rate of normal (NCM460 cells) and cancerous (SW480 cells) human intestinal cells. The CC_{50} value of ABCE against NCM460 and SW480 cells were 2.78 and 1.26 mg/ml, respectively. The SI value was calculated 2.2 suggesting a degree of selectivity toward cancer cells

In vivo effects of extract on giardiasis in mice

As illustrated in Table 2, the application of various dosages of ABCE, particularly at 40 mg/kg over the seven-day period, resulted in a statistically significant reduction ($p<0.001$) in the mean number of *Giardia* cysts. The observed reductions were 97.7, 86.6, 95.3, and 99.0% in the groups treated with MTZ (15 mg/kg), ABCE 10 mg/kg, ABCE 20 mg/kg, and ABCE 40 mg/kg, respectively. Furthermore, Table 1 demonstrates a significant decrease ($p<0.001$) in the viability of *Giardia* cysts following treatment with various dosages of ABCE compared to normal saline group. The viability reductions recorded were 93.6, 46.1, 76.2, and 97.6% for the groups treated with MNZ (15 mg/kg), ABCE 10 mg/kg, ABCE 20 mg/kg, and ABCE 40 mg/kg, respectively.

Inflammatory markers in intestinal tissue

Compared with the healthy control group, untreated *Giardia*-infected mice exhibited significant increases in *TNF-α* (3.96-fold), *NF-κB* (4.35-fold), and *IL-1β* (3.61-fold), confirming successful induction of intestinal inflammatory responses (Figure 3). Treatment with ABCE elicited a pronounced dose-dependent attenuation of inflammatory gene expression. Administration of 10 mg/kg ABCE significantly reduced the expression levels of *TNF-α* and *IL-1β* by approximately 2.2-fold and *NF-κB* by 2.79-fold compared with the normal saline group ($p < 0.05$). Increasing the dose to 20 mg/kg further reduced the expression levels of these genes, with approximately 1.8- to 2.0-fold reductions compared with the normal saline group ($p < 0.01$) and significant reductions compared with the 10 mg/kg ABCE group ($p < 0.05$).. At the highest dose of 40 mg/kg, expression levels approached baseline, with *TNF-α* at 1.17-fold, *NF-κB* at 1.08-fold, and *IL-1β* reduced below baseline at 0.94-fold. These reductions were highly significant compared to all lower-dose groups ($p<0.001$). A statistically significant difference was detected between the 40 mg/kg ABCE treatment and metronidazole ($p<0.05$).

Table 2. *In vivo* effects of *Astragalus baba-alliar* chloroform extract (ABCE) on giardiasis in mice. Mean ± standard deviation (SD). **p<0.01 and ***p<0.001 compared to normal saline; +p<0.05 compared to MTZ.

Drug	Mean No. of *Giardia* cysts	% of cell viability
Normal saline	839.4±23.55	97.2±2.4
Metronidazole 15mg/kg	18.6±2.61***	6.22±1.15***
ABCE 10 mg/kg	112.3± 16.5***	52.44± 5.6**
ABCE 20 mg/kg	39.1±5.23***	26.6±3.66***
ABCE 40 mg/kg	8.23±2.31*** +	2.33±1.26***

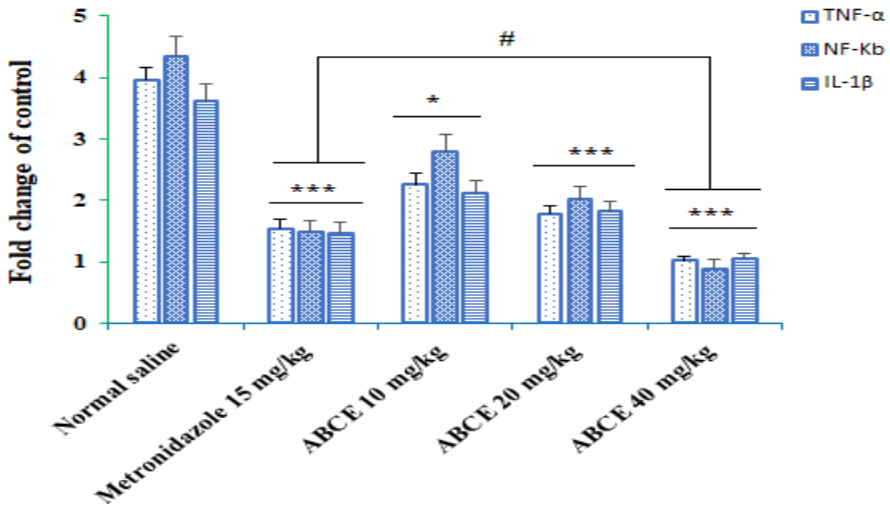

Figure 3. Comparative evaluation of the anti-inflammatory effects exerted by varying doses of *Astragalus baba-alliar* chloroform extract (ABCE) and metronidazole (METRO) on the levels of TNF-α (panel a), NF-κB (panel b), and IL-1β (panel c) in mice infected with *Giardia* (n = 8 per group). Statistical significance is indicated as follows: *p<0.05 and ***p<0.001 compared to the untreated control group (normal saline); #p<0.05 compared to metronidazole group II. Intergroup differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test.

Discussion

Synthetic agents such as metronidazole, quinacrine, and furazolidone are widely utilized in the treatment of giardiasis; however, recent reports have highlighted various adverse side effects associated with these medications (Watkins and Eckmann 2014). Consequently, there is a significant interest in identifying an effective anti-giardial agent that exhibits minimal side effects. Accordingly, the objective of this study is to examine the anti-giardial properties of the chloroform extract of *A. baba-alliar* against *G. lamblia* infection *in vitro* and *in vivo*.

The findings of our study indicated that the ABCE exhibited its most pronounced effect at a concentration of 4 mg/ml, resulting in 100% eosin-positive cysts/trophozoites after 120 and 60 min of exposure, respectively. Furthermore, this extract also achieved 100% eosin-positive cysts/trophozoites at a lower concentration of 2 mg/ml but required longer exposure times of 240 and 120 min, respectively. Conversely, the concentration of 1 mg/ml demonstrated the least efficacy, as it necessitated 360 and 240 min of incubation to completely eliminate all *G. lamblia* cysts and trophozoites, respectively. *In vivo*, we found that the application of various dosages of ABCE, particularly at 40 mg/kg over the seven-day period, resulted in a statistically significant reduction (p<0.001) in the mean number and viability of *Giardia* cysts. Because viability was determined by

eosin exclusion assay, reduced viability should not be interpreted as complete loss of infectivity. The greater susceptibility of trophozoites compared with cysts may be associated with the absence of a protective cyst wall and the higher metabolic activity of trophozoites, rendering them more vulnerable to membrane-disruptive phytochemicals such as flavonoids and terpenoids. Previous studies have shown that plant-derived polyphenols may induce oxidative imbalance, mitochondrial dysfunction, and apoptosis-like death in protozoan parasites (Ghasemian Yadegari et al. 2022).

Limited research has been conducted on the antiparasitic properties of various species within the genus *Astragalus*. Ghasemian Yadegari et al. (2022) demonstrated that the extract of *A. maximus* led to a statistically significant reduction of the viability of *G. lamblia* cysts. Specifically, at concentrations of 22.5 and 45 mg/mL, the extract was able to completely eliminate 100% of *G. lamblia* cysts after 2 and 3 hr, respectively. In an investigation by Yang et al. (2012), the extract of *A. membranaceus* exhibited effective *in vitro* anti-*Toxoplasma* activity, as evidenced by a reduction in the intracellular replication of *Toxoplasma gondii* tachyzoites after 3, 4, and 5 days of incubation. Additionally, Abdel-Tawab et al. (2020) reported that *A. membranaceus*, particularly at a concentration of 50 mg/kg, decreased the viability of *Eimeria papillata* oocysts and reduced the mean number of oocysts excreted in the feces of infected animals. Mahmoudvand et al. (2024) also demonstrated that the extract of *A. ecbatanus* evidently reduced the viability of both promastigote and amastigote forms of *Leishmania tropica* compared to the negative control. Ghasemian Yadegari et al. (2022) also reported that the extract of *A. brachycalyx* at concentrations of 225 and 450 mg/mL exhibited potent protoscolicidal effects on hydatid cyst protoscoleces. Although studies on *Astragalus* species have reported activity against a variety of parasitic organisms, including *Giardia*, *Toxoplasma*, *Eimeria*, *Leishmania*, and *Echinococcus*, direct comparisons of efficacy across these studies should be interpreted with caution. These parasites differ substantially in their taxonomy, biology, life cycle, host–parasite interactions, and susceptibility to antiparasitic agents. Therefore, the findings collectively support the general antiparasitic potential of the *Astragalus* genus rather than indicating comparable efficacy against different parasite species. In addition, variations in *Astragalus* species, extraction procedures, phytochemical composition, experimental conditions, and outcome measures may further contribute to differences observed among studies.

In the phytochemical assessment, the presence of saponins, flavonoids, terpenoids, and polysaccharides has been reported in other *Astragalus* species (Bratkov et al. 2016; Jun et al. 2012). Additionally, literature reviews indicate that prior research has established phenolic and flavonoid compounds as the primary constituents of *Astragalus* spp. (Bratkov et al. 2016; Jun et al. 2012). Concerning the impact of flavonoid compounds on microbial pathogens, earlier studies have demonstrated that these compounds exert their antimicrobial effects through mechanisms such as disruption of the cytoplasmic membrane, inhibition of nucleic acids, induction of apoptosis, interference with energy metabolism, alteration of membrane permeability, and reduction of pathogenicity (Górniak et al. 2019, Panche et al. 2016). Furthermore, polyphenolic compounds exhibit antimicrobial properties by impeding virulence factors, disrupting cytoplasmic and cell membranes, and inhibiting DNA synthesis (Miklasińska-Majdanik et al. 2018, Othman et al. 2019). Consequently, it can be posited that the observed antigiardial activity of *A. baba-alliar* extract may be partially attributed to the presence of

phenolic and flavonoid compounds within this herb.

Here, we evaluated the cytotoxic effect of ABCE on normal (NCM460 cells) and cancerous (SW480 cells) human intestinal cells by MTT method. The CC50 value of ABCE against NCM460 and SW480 cells were 2.78 and 1.26 mg/ml, respectively. The SI value was calculated 2.2 indicates that this extract is safe for healthy cells compared to cancer cells. Similarly, Mahmoudvand et al. (2022) reported that the CC50 of *A. ecbatanus* chloroform extract for normal cell lines is 4.62 mg/mL, while for cancer cell lines, it is 1.89 mg/mL.

The present investigation reveals that *Giardia* infection substantially induces intestinal pro-inflammatory pathways, as evidenced by significant elevations in *TNF-α, NF-κB,* and *IL-1β* gene expression level in untreated murine models. This pro-inflammatory response corroborates prior findings indicating that *Giardia* interacts with both epithelial and immune cells (Faria et al. 2020), thereby triggering innate immune activation and subsequent cytokine production (Cotton et al. 2015). The increased expression of *TNF-α* has been linked to disruption of the epithelial barrier, whereas *NF-κB* serves as a critical transcriptional regulator orchestrating the expression of multiple inflammatory mediators, including *IL-1β*, which collectively contribute to the amplification of mucosal inflammation (Cotton et al. 2015). The present findings indicate that ABCE exerts a dose-dependent anti-inflammatory effect in intestinal tissue of *Giardia*-infected mice, obviously downregulating *TNF-α, IL-1β,* and *NF-κB* expression. The modulation of these key pro-inflammatory mediators suggests that ABCE can effectively suppress the parasite-induced inflammatory cascade (*NF-κB* signaling pathways), which is a major contributor to intestinal mucosal damage during giardiasis.

This activity aligns with reports on other *Astragalus* species, where polysaccharides, flavonoids, and saponins have been shown to inhibit *NF-κB* activation and reduce pro-inflammatory cytokine production in both *in vitro* and *in vivo* models (Li et al. 2022). For instance, Lv et al. demonstrated that *Astragalus* polysaccharides noticeably attenuated *TNF-α* and *IL-1β* in a murine model of colitis, highlighting the role of *NF-κB* inhibition as a central mechanism (Lv et al. 2017). Similarly, Chen et al. (2023) reported that *Astragalus* flavonoids suppressed intestinal inflammation by downregulating *NF-κB* signaling and oxidative stress markers in infected or chemically induced intestinal injury models. These findings not only reinforce the therapeutic potential of ABCE as a complementary or adjunct treatment in giardiasis but also warrants further investigation in other inflammatory conditions, where NF-κB–mediated cytokine release drives pathology. Although *NF-κB* activation is predominantly regulated through phosphorylation and nuclear translocation, assessment of *NF-κB* transcript levels was used here as an indirect indicator of inflammatory pathway modulation. Future studies should incorporate protein-based approaches such as Western blotting or nuclear translocation assays.

The present study has several limitations. First, detailed phytochemical profiling of ABCE was not performed; therefore, the exact active compounds responsible for the observed biological activities remain unidentified. Second, the absence of histopathological evaluation prevented direct correlation between molecular changes and intestinal tissue recovery. Third, mechanistic investigations involving oxidative stress pathways, apoptosis induction, and parasite ultrastructural alterations were beyond the scope of this study. Another limitation is the absence of molecular genotyping of *Giardia* isolates, which precluded assessment of assemblage-specific susceptibility. In addition, systematic assessment of treatment-related toxicity,

including body weight changes, food consumption, behavioral alterations, and clinical signs, was not performed during the experimental period. Therefore, the *in vivo* safety profile of ABCE requires further investigation in dedicated toxicological studies. Finally, morphological and ultrastructural alterations of trophozoites, pharmacokinetic and long-term toxicity evaluations are required before translational application can be considered.

ABCE demonstrates potent anti-giardial and anti-inflammatory activity with minimal toxicity to normal cells, supporting its potential as a therapeutic candidate against giardiasis.

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Conflicts of interest

The authors had no competing interests.

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Ethical Considerations

The research received approval from the ethics committee at Lorestan University of Medical Sciences, located in Khorramabad, Iran, under the ethics identification number IR.LUMS.REC.1401.192.

Code of Ethics

IR.LUMS.REC.1401.192.

Authors' Contributions

HM planned and supervised; LM, and SBMA performed tests and collected data; AO and JGY were responsible for the methodology; RMK, HM, edited the manuscript. All authors have reviewed and approved the final version of the manuscript for publication.

Abbreviations

ABCE: *Astragalus bab-alliar* chloroform extract
TNF-α: Tumor Necrosis Factor-alpha
NF-κB: Nuclear Factor kappa B
IL-1β: Interleukin-1 beta

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