Original Article

In vitro and *in vivo* effects of green-synthesized silver nanoparticles against *Giardia lamblia* infection

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How to cite this article: **Golabi Azad S, Cem Özyurt H. *In vitro* and *in vivo* effects of green-synthesized silver nanoparticles against *Giardia lamblia* infection. Archives of Razi Institute. 2024;79(1):218-225.** DOI: 10.32592/ARI.2024.79.1.218

Article Info:
Received: 8 September 2023
Accepted: 7 October 2023
Published: 29 February 2024

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ABSTRACT

The current experimental study is designed to examine the *in vitro* and *in vivo* effects of green synthesized silver nanoparticles (AgNPs) against *Giardia lamblia*, a major cause of parasitic diarrhea. The precipitation method was employed for the green synthesis of AgNPs by *Astragalus ecbatanus* aqueous extract. In the, *in vitro*, *Giardia lamblia* cysts and trophozoites were exposed to AgNPs at 10, 20, and 40 mg/mL for 10–360 min. The effects of AgNPs on trophozoite plasma membrane and their cytotoxic effects on normal and colon cancer cells were evaluated using Sytox green and MTT assay for cell viability. The *in vivo* assay included BALB/c mice, infected by *Giardia*, treated with AgNPs at 10, 15, and 20 mg/kg/day for one week. On the 8th day post-infection, stool examination was conducted to assess the presence of *Giardia* cysts and the reduction rate. The size distribution of AgNPs ranged between 5 and 80 nm, with the maximum particle size observed at 40–60 nm. AgNPs significantly ($P<0.001$) increased the mortality of *Giardia lamblia* trophozoites in a dose-dependent manner. Specifically, AgNPs at concentrations of 200 and 300 µg/mL destroyed *Giardia lamblia* cysts after 4 and 2 h, respectively. Trophozoites of *Giardia lamblia* showed more sensitivity to AgNPs compared to cysts. At concentrations of 100, 200, and 300 µg/mL, AgNPs eliminated all trophozoites after 4, 2, and 1 h of treatment, respectively. AgNPs dose-dependently reduced ($P<0.001$) the parasite load and viability of *Giardia lamblia* cysts. Exposure of *Giardia lamblia* trophozoites to AgNPs dose-dependently increased the plasma membrane permeability as indicated by rise in the exposed fluorescence. The CC_{50} value AgNPs for colon cancer and normal cell lines was 402.3 µg/mL and 819.6 µg/mL, respectively. The selectivity value greater than 2 (2.04), suggests that these AgNPs are safe for normal cells in comparison with cancer cells. This experimental study showed that AgNPs green synthesized by *Astragalus ecbatanus* exhibited significant *in vitro* and *in vivo* anti-*Giardia* activity, positioning them as potential candidates for *Giardia* infection treatment. Nevertheless, further research on the precise mechanisms of action and comprehensive exploration of all toxicity aspects associated with this type of AgNPs need to be considered.

Keywords: Giardiasis, Treatment, Cyst, Trophozoites, Mice

1. Introduction

Diarrhea, a gastrointestinal infection, precipitates a significant depletion in body fluid volume and is stands as one of the most important health concerns, particularly among children worldwide, contributing to approximately 35 million deaths annually (1). Despite notable advances in rapid diagnosis and treatment of affected individuals, diarrhea persists as a substantial health challenge (2). Various microbial pathogens, including viruses (e.g., enterovirus and rotavirus), bacteria, and parasites (e.g., *Giardia lamblia* and *Entamoeba histolytica*), are implicated in causing diarrhea (3). *Giardia lamblia*, a prevalent gastrointestinal parasite, induces giardiasis in a broad spectrum of vertebrates, including humans (4). Similar to many intestinal diseases, the prevalence of giardiasis varies based on health conditions, with higher incidence in regions with temperate and tropical climates as opposed to colder areas (5). Transmission of with this parasite is primarily occurs through the consumption of contaminated food and water, as well as direct contact with *Giradia lambia* cysts (5). Giardiasis can manifest as asymptomatic or exhibit symptoms such as watery diarrhea, malabsorption, and weight loss (6). Currently, several synthetic drugs like metronidazole, furazolidone, and tinidazole are available for giardiasis treatment (7). However, these medications entail various complications including nausea, vomiting, tongue roughness, digestive disorders, skin rashes, hives, and thick white secretions (8). Additionally, reports indicate the emergence of parasite resistance these drugs (9), and some are contraindicated during pregnancy (8). Hence, it is essential to identify drugs with lower complications and higher efficacy. Nanoparticles, emerging and extraordinary materials widely employed in medicine, the pharmaceutical industry, and disease treatment, offer promising avenues for addressing this need (10). heir nanoscale particle size and surface reactivity render nanoparticles valuable for biomedical applications (11). In recent years, certain nanoparticles, such as gold, selenium, and silver, have been used in the treatment of parasitic diseases like giardiasis (12). Nano silver products, in particular, are advantageous due to their antibacterial, antifungal, and antiviral properties at low concentrations and an extended shelf life (13). Despite substantial research on the effect of silver nanoparticles (AgNPs) on giardiasis, the results have varied, at times, been contradictory (14). This experimental study aims to investigate the *in vitro* and *in vivo* effects of green-synthesized AgNPs against *Giardia lamblia*, a prominent cause of parasitic diarrhea.

2. Materials and Methods

2.1. Green Synthesis and Characterization of AgNPs

The precipitation method was used to green synthesis of AgNPs by reducing of silver ions ($AgNO_3$) through *Astragalus ecbatanus* aqueous extract as previously studied (15). Initially, aerial parts of the plant were collected from rural regions of Kermanshah province, Western Iran, in June 2021, and subjected to maceration method with water for 48 h. The surface plasmon resonance (SPR) of AgNPs was measured employing a UV–vis spectrophotometer (Shimadzu UV2550, Japan) to assess the transformation of the Ag ions to AgNPs. Additionally, the synthesizes of nanoparticles by *A. spinosus* extract was confirmed through X-ray diffraction (XRD, 2000 APD, Italy) analysis. Various characteristics of AgNPs, including size and shape, were evaluated using a scanning electron microscope (SEM) (Mira3, Made in Czech).

2.2. Collecting the *Giardia lamblia* Cysts

Giardia lamblia cysts were collected from stool specimens of patients infected with *Giardia lamblia*, referred to health centers in Famagusta, North Cyprus. The collected specimens were confirmed by parasitological examinations, including direct and formalin-ether tests (16). The sucrose 0.85 M grade assay was used to concentrates the cysts as previously described (16). In brief, after diluted the cysts with distilled water at the ratio of 12:1, the combination was filtered, and the superior phase was gently transferred to a 0.85 M sucrose solution. Following centrifuging, the cysts from central layer were collected and adjusted to a concentration of 1×10^5 cysts/mL.

2.3. Isolation of *Giardia lamblia* Trophozoites

The excystation method was employed to obtain *Giardia lamblia* trophozoites, following established protocols (17). In summary, aqueous hydrochloric acid, serving as a triggering solution, was mixed with the cyst suspension (1:9) and kept warm at 37 °C for 120 min. After centrifugation, the superior portion was added to the TYI-S-33 medium recovered with bovine bile, 20% FCS, and strep/pen (500 IU/mL) at 37 °C.

2.4. *In vitro* Anti-*Giardia* Effects of AgNPs

One hundred µL of AgNPs at concentrations ranging from 25 to 300 mg/mL were mixed with 100 µL of cysts

and trophozoites solution in tubes, and the combination was incubated for 15-360 min at 37 ℃ (16). After removing the upper phase, eosin stain solution (0.1%) was added to the mixture, and the mortality rate of the cysts and trophozoites was estimated by counting 100 parasites using a light microscope (400x magnification). Surviving and deceased parasites were differentiated by their color, with live parasites appearing colorless and dead ones appearing pink. Metronidazole (MTZ) (50 µg/ml) (17) and untreated parasites served as positive and negative controls, respectively.

2.5. Effects of AgNPs on Parasites' Plasma Membrane

The effects of AgNPs on trophozoites' plasma membrane w determined using the Sytox green examination based on a previously reported method. Trophozoites treated with normal saline and Triton X-100 were designated as negative and positive controls, respectively. The Effects of AgNPs on plasma membrane permeability were examined using a microplate reader for 240 mins (18).

2.6. Cytotoxic Effects of AgNPs

Normal human intestine epithelial cell (NCM460, ATCC) and human colorectal adenocarcinoma cell line (SW-480, ATCC) were cultured in DMEM (Sigma-Aldrich Germany) supplemented with 10% FCS at 37 ℃ with 5% CO_2 (19). One hundred cells (1×10^5/mL) were exposed to AgNPs (25-300 mg/mL) in 96-well plate and incubated at 24 ° C for 48 h. Subsequently, 20 µL of MTT liquid was added to mixture and incubated again in 5% CO_2 at 37 ℃ for 240 min. After adding 100 µl of DMSO, the absorbance of wells was measured at 570 nm using an ELISA plate reader. Following the calculation of the 50% cytotoxic concentrations (CC50), the selectivity index (SI) was determined as CC50 healthy cells / CC50 cancer cells. An SI higher than 2 indicates that the agent is safe for normal cells (19).

2.7. Animals

Forty male NMRI mice (8-10 weeks old), weighing 25-30g, were housed in optimal conditions with food and water *ad libitum.*

2.8. Establishment of Giardiasis in Mice

Mice were infected using oral administration of 0.2 mL of cysts solution (1×10^5 cysts/mL) (16, 17). Subsequently, stool examination (SE), including direct smear and formalin-ether procedure, were performed until *Giardia* cysts were detected in their feces (20). After six days, the mice were assigned to five groups: (i) normal saline; (ii) metronidazole (15 mg/kg) (17); and AgNPs at 10, 15, and 20 mg/kg/day for one week (16, 17).

2.9. *In vivo* Effects of AgNPs Against Giardiasis in Mice

On the 8th day post-infection, SE was performed to determine the presence of *Giardia* cysts and calculate the reduction rate. Additionally, the viability rate of the collected cysts was assessed through eosin exclusion assay, where pink and colorless cysts were indicative of dead and live cysts, respectively (20).

2.10. Statistical analysis

All data were analyzed using One-way ANOVA and *t*-test in SPSS software version 22.0. A significance level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Characterization of AgNPs

The reduction of Ag^+ ions to AgNPs was evident through the transformation of the culture medium to a dark brown color. A UV-vis spectroscopy revealed a peak at 398 nm, confirming the green synthesis of AgNPs (Figure 1A). The XRD pattern showed that peaks including 112, 205, 225, and 315 at 37.23º, 42.01º, 62.11º, and 75.02º, respectively, corresponding to nanocrystals and silver cubic structures; while, non-appearance of other peaks affirmed the purity of AgNPs used in the analysis (Figure 1B). The size distribution of AgNPs ranged from 5 to 80 nm, with the maximum particles size observed in the 40-60 nm range. (Figure 1C).

3.2. *In vitro* Anti-*Giardia* Effects of AgNPs

As illustrated in Figure 2, AgNPs significantly ($p<0.0001$) increased the mortality of *Giardia lamblia* cysts in a dose-dependent manner. Specifically, AgNPs at concentrations of 200 and 300 µg/mL eradicated *Giardia lamblia* cysts after 4 and 2 h, respectively. Notably, trophozoites of *Giardia lamblia* were more sensitive to AgNPs compared to cysts. At concentrations of 100, 200, and 300 µg/mL, AgNPs eliminated all trophozoites after 4, 2, and 1 h of treatment, respectively.

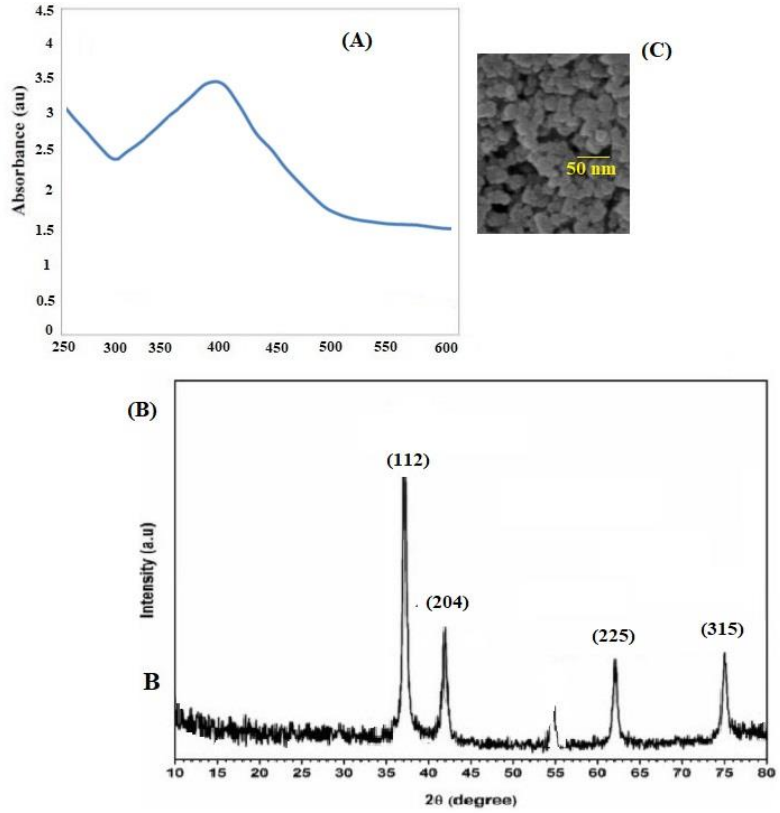

Figure 1. UV-vis spectroscopy (A), X-ray diffraction (B), and scanning electron microscope (C) of synthesized silver nanoparticles.

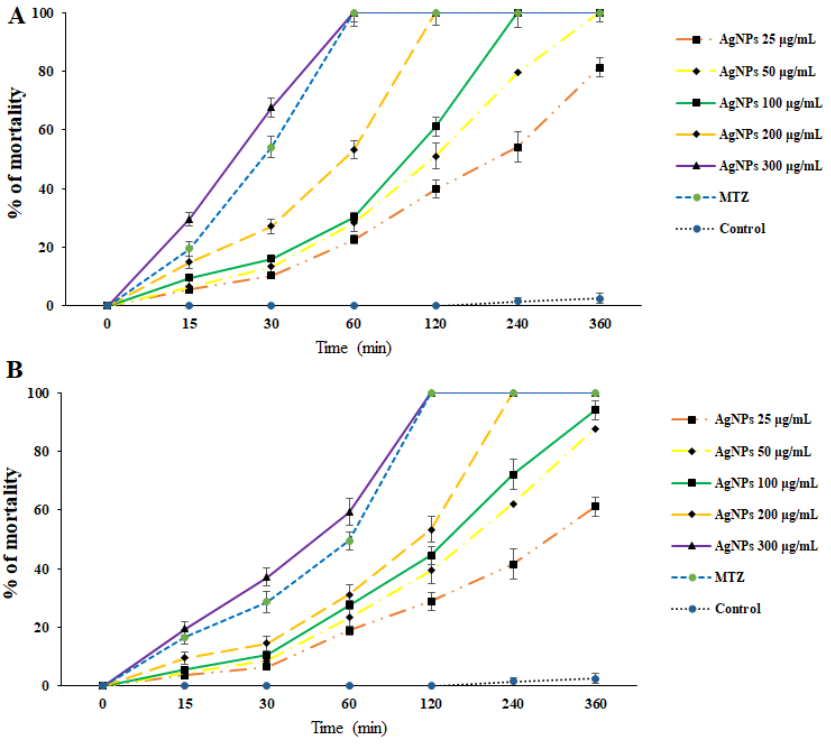

Figure 2. *In vitro* effects of silver nanoparticles (AgNPs) and metronidazole (MTZ) on the *Giardia lamblia* trophozoites (A) and cysts (B). N=3.

3.3. *In vivo* Effects of AgNPs Against Giardiasis in Mice

Figure 3 shows the *in vivo* effects of AgNPs on the load and viability of cysts defecated from mice with giardiasis. AgNPs demonstrated a dose-dependently reduction in the parasite load and viability of *Giardia lamblia* cysts. The reduction rate of cysts following treatment with AgNPs at 10, 15, 20 mg/kg were 76.9, 91.4, and 97.8%, respectively.

3.4. Effects of AgNPs on Parasites Plasma Membrane

Sytox green revealed that exposure of *Giardia lamblia* trophozoites to AgNPs dose-dependently increased the plasma membrane permeability of trophozoites , as evidenced by the elevated fluorescence (Figure 4).

3.5. Cytotoxic effects of AgNPs

Regarding cytotoxicity, AgNPs exhibited more toxic and inhibitory effects on colon cancer cells compared to normal cells (Figure 5). The CC_{50} value for AgNPs in colon cancer and normal cell lines was 402.3 µg/mL and 819.6 µg/mL, respectively. The SI value greater than 2 (2.04) indicates that these AgNPs are safer for normal cells compared to cancer cells.

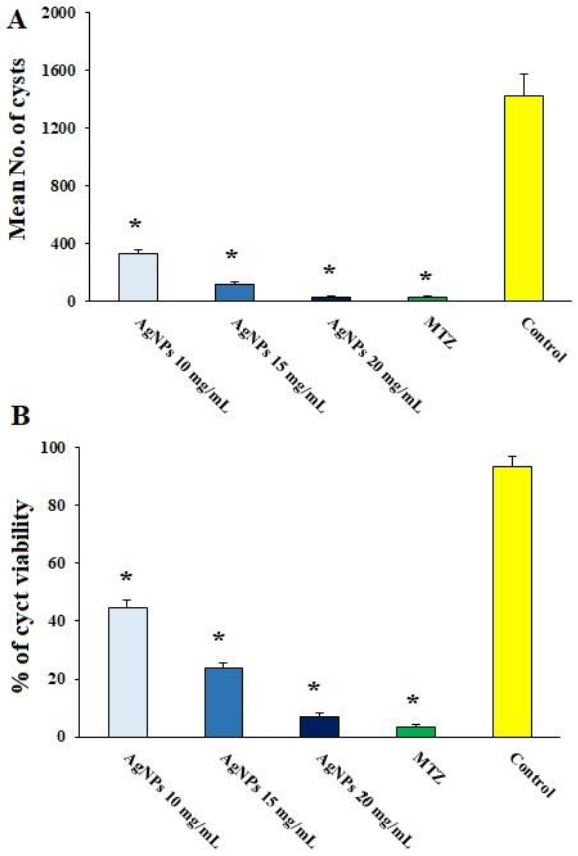

Figure 3. *In vivo* effects of silver nanoparticles (AgNPs) and metronidazole (MTZ) on the parasite load (A) and viability (B) of cysts expelled from mice with giardiasis. * P<0.001 with control (normal saline).

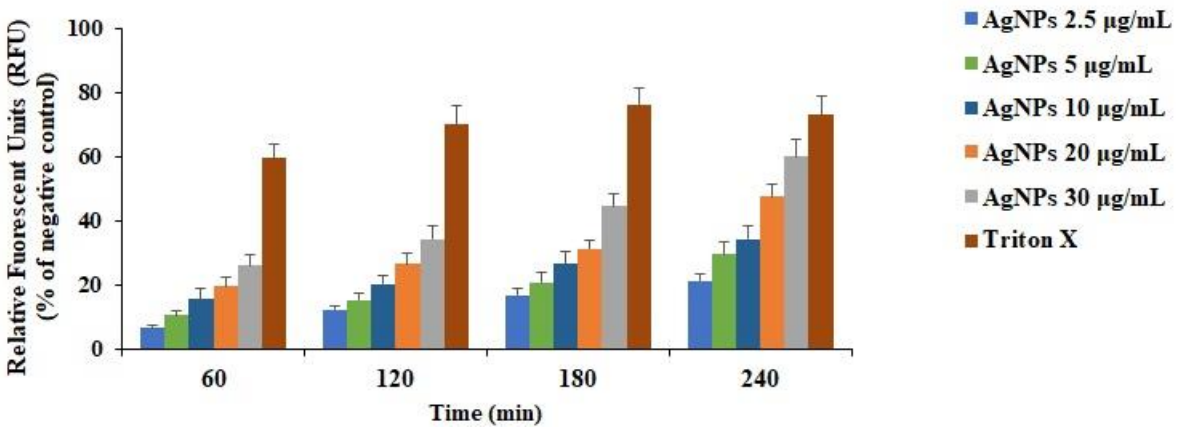

Figure 4. Effects of various concentrations of silver nanoparticles (AgNPs) on plasma membrane in the trophozoites of *Giardia lamblia*.

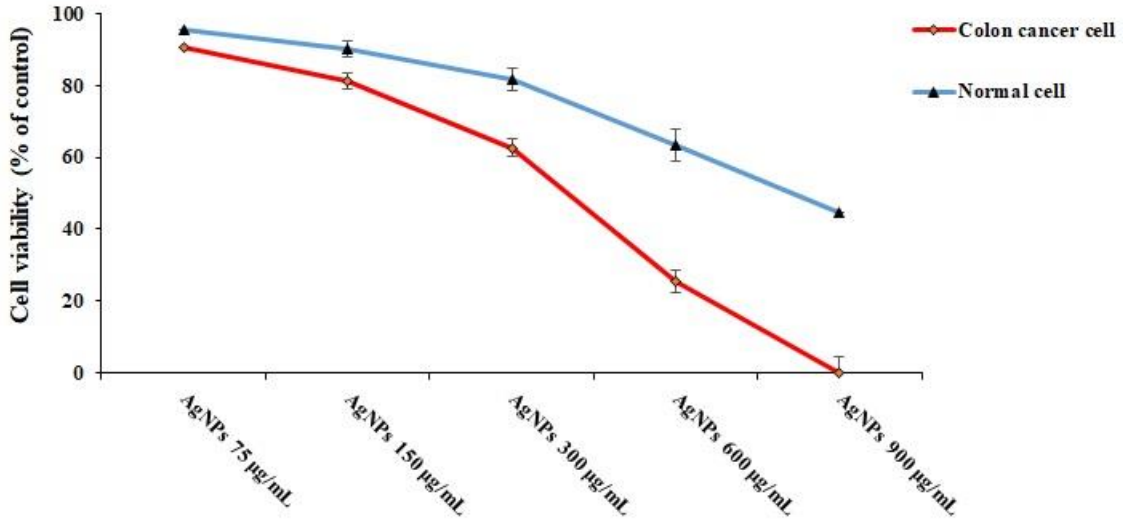

Figure 5. Cytotoxic effects of silver nanoparticles (AgNPs) on normal (NCM460, ATCC) and cancer (SW-480, ATCC) cell lines.

4. Discussion

Today, it has been etablished that the application of AgNP can yield novel agents for the treatment of parasitic diseases. Evaluating of the inhibitory properties of AgNPs on parasites and understanding their potential mechanisms can provide a hypothetical source for the further progress of AgNPs parasiticides (14). The present experimental study was designed to investigate the *in vitro* and *in vivo* effects green-synthesized AgNPs against *Giardia lamblia*, as one of the main causes of parasitic diarrhea. Our findings indicated that the AgNPs ranged from 5 to 80 nm, with the maximum particle distribution observed in the 40-60 nm. Reviews have demonstrated that synthesized AgNPs exhibits sizes with the range of 1–100 nm and display a wide variation of morphologies, such as spherical, disc-shaped, conical, and sheet-like structures. The toxicity of AgNPs are linked to their characteristics, such as shape, concentration, and chemical coating (21). Synthesis approaches using herbal extracts, with bioactive composites like flavonoids, phenolic, and terpenoids acting as reducing and stabilizing agents, have gained attention due to their cost-effective, environmentally friendliness and simplicity (22). In *Giardia lamblia* parasites, both cystic and trophozoite forms, are being considered as the infective and invasive forms, respectively (4). Trophozoites, through adhesion and lysis of the intestinal epithelial cells, invade and affect the colon and subsequently leading to gastrointestinal disorders (4). Our study showed that AgNPs significantly ($p<0.0001$) increased the mortality of *Giardia lamblia* cysts in a dose-dependent manner. Notably, trophozoites of *Giardia lamblia* were more sensitive to AgNPs than cysts. *In vivo*, AgNPs dose-dependently reduced the parasite load and viability of *Giardia lamblia* cysts. In line with our findings, Idan *et al.* (23) showed that chemically synthesized AgNPs at 100 µg/g significantly decreased the parasite load in stool of *Giardia*-infected mice, while remained trophozoites in intestine of infected mice. In recent years, the antiparasitic effects of AgNPs, synthesized by different methods, have been proven effective against various protozoan such as *Leishmania* spp., *Cryptosporidium* spp., *Plasmodium* spp., and *Toxoplasma gondii*), and helminthic parasites such as *Strongylides*, *Haemonchus*, and *Gigantocotyle explanatum* (14). The antiparasitic mechanisms of AgNPs, entry of Ag ions and the release of reactive oxygen species (ROS), leading to increased expression of apoptotic mediators, DNA damage, and inhibition of protein synthesis (14). Our study revealed that exposure of *Giardia lamblia* trophozoites to AgNPs dose-dependently increased plasma membrane permeability, indicated by rising the exposed fluorescence, showing that Ag ions in AgNPs are able to interrupt the fluidity and integrity of the parasites' cell membrane. This disruption enhances permeability, damages intracellular critical organelles, affects cell function of cells, and ultimately leads to cell death (14, 24, 25). Regarding to cytotoxicity of AgNPs, our results showed that the SI value was greater than 2 (2.04), suggesting that these AgNPs are safe for normal cells compared to cancer cells. In conclusion, this experimental study showed that AgNPs green-synthesized by *A. ecbatanus* exhibit considerable *in vitro* and *in vivo* anti-*Giardia* activity, positioning them as potential candidates for *Giardia* infection treatment. Nonetheless,

further research is warranted to elucidate the precise mechanisms of action and clarify all toxicity aspects associated with this type of AgNPs.

Acknowledgment

The authors express their gratitude to the staff of Pharmaceutics Laboratory, Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, North Cyprus.

Authors' Contribution

SGA conducted experiments and collected data. HCO supervised, directed, and managed the study. SGA wrote the initial draft, and HCO critically revised the paper. All authors approved the final version for publication.

Ethics

The procedure was authorized by the Ethical Committee of the Faculty of Pharmacy, Eastern Mediterranean University, Famagusta, North Cyprus (EMU-22-147434).

Conflict of Interest

The authors declare no conflicts of interest.

Grant Support

None.

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