

Anti-parasitic Effects of Herbal Extract-Based Silver Nanoparticles on the Trophozoite and Cystic Forms of *Acanthamoeba* Protozoa



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Abstract

Background: *Acanthamoeba* is a globally dispersed protozoan that can cause different clinical manifestations in infected individuals. Various drugs have been proposed against its drug-resistant forms.

Objective: The present study examined silver nanoparticles (NPs) with a good anti-parasitic background. More precisely, the study focused on evaluating the anti-parasitic effect of silver nano-scale particles on protozoan trophozoite and cysts by microscopic counting and flow cytometry after exposure to different concentrations.

Methods: To this end, MTT assay and IC50 were used to assess the macrophage toxicity and cysts/trophozoites, respectively.

Results: Based on the results, 100 ppm silver NPs had better anti-parasitic effects than 80 ppm concentration and even the standard treatment of *Acanthamoeba* on both trophozoite and cystic phases. Macrophages toxicity at 100 ppm concentration was similar to the control group.

Conclusion: In general, further studies should be conducted to confirm the present results given the significant effects of silver NPs against trophozoite and parasite cysts.

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Background

Acanthamoebiasis occurs due to infection by a globally distributed protozoan group, which belongs to the *Acanthamoebidae* family. The most well-known species of this genus is *Entamoeba castellanii*, which is capable of causing diseases such as keratitis and encephalitis in humans. In the former, unilateral eye involvement occurs in healthy individuals, and the latter is more frequently observed in immunocompromised individuals.^{1,2} In the case of *Acanthamoeba* keratitis, contact lenses are extremely involved and most infections are related to this case.³ *Acanthamoeba*'s disease is usually painful and can be difficult to treat due to the sensitivity of the surrounding tissue. To date, various treatments have been suggested for *Acanthamoeba* infections although challenges such as resistance to treatment and high toxicity of some drugs are still ahead.

Recently, with the advancement of technology, especially in the field of medical biotechnology, related sciences have been affected as well. The advent of revolutionary nano-materials in basic medical sciences has brought about applications such as treatment, drug delivery, extraction of nucleic acid content, and the like.⁴⁻

⁶ Nano-sized particles, because of their small size and

thus greater penetration, can be a suitable candidate for alternative treatments regarding diseases that are difficult to access.

Silver nanoparticles (NPs) are among the popular particles which have been widely used against pathogenic organisms such as bacteria and viruses. This is because the mechanism of action of these NPs causes them to die by affecting the respiratory chain of the bacteria and binding to surface glycoproteins. The importance of this NP becomes clear when knowing that there is no resistance, and every time organisms succumb to exposure to the NP.

The use of silver NPs against parasites, which are also among pathogenic organisms, can be somewhat promising. In this regard, the antiparasitic effects of this NP on leishmania and malaria parasites have been tested, the findings of which are highly satisfactory.^{7,8} However, it should be noted that the use of this material in high amounts is toxic to host cells and is not permitted, thus low concentrations of the NP should be tested and used to resolve this issue.

Given the above-mentioned explanations, this study aimed to investigate the effect of silver NPs with different concentrations on the *Acanthamoeba* forms whether cysts or trophozoites in vitro.

Methods and Materials

Preparation of Parasites

Acanthamoeba protozoa were isolated from the soil source and cultured to isolate the parasite cyst and trophozoite, cultured in a medium, along with *Escherichia coli* bacteria at the Medical Parasitology Department of Tarbiat Modares University. The parasitic trophozoite and cystic forms were obtained after three days and three weeks in the mentioned medium at about 26°C, respectively. Both forms of parasites were washed with sterile saline at their stated time according to the study by Pazoki et al.

Preparation of Silver Nanoparticles

Nano-size silver particles were synthesized in the Pharmaceutical Biotechnology and Pharmaceutical Sciences Research Centre, Pharmacy Faculty, Tehran University of Medical Sciences. Previous studies have suggested that the incorporation of NPs with synthetic or organic materials could have a synergistic effect on NP activities. Accordingly, 100 ppm and 80 ppm concentrations of silver NP were combined with *Zingiber officinale* and *Thymus* extracts, respectively.

Evaluation of Cell Viability

The viability evaluation of trophozoites and cysts with different concentrations of silver NPs was performed by Trypan blue staining and the flow cytometry method.

Microscopic Test (Trypan Blue)

It is a type of staining that is caused by damage to the cell membrane (trophozoite or cyst) as dye penetrates the cells, thus the nucleus, cytoplasm, and other organelles appear to be colored microscopically. Conversely, when none of the forms of the protozoan are damaged, the cell membrane inhibits the Trypan blue penetration into the cell, which means that the cell is alive. After picking up a certain amount of *Acanthamoeba* trophozoites and cysts ($\approx 15 \times 10^4$), the effects of 80 and 100 ppm concentrations of silver NPs were monitored at 24, 48, and 72 hours after exposure. The Trypan blue reagent was added at 0.4% concentration and cells were counted after 10 minutes by Neubauer slide under 40X and 100X microscopic magnifications. In the preparation of negative and positive controls, the parasites were mixed with equal ratios of distilled water and 0.01% polyhexanide, respectively.

Flow Cytometry to Cell Death Evaluation

Flow cytometry was used to measure cell death. This method is based on the relationship between the size, the number of cells, and the amount of light diffraction. The cell death (apoptosis) rate was detected by using the Annexin-V-FLUOS staining kit (Roche, Germany). The procedure was carried out according to the manufacture's protocol, and the results were analyzed by FlowJo software. The half-maximal inhibitory concentration (IC50) was measured for the concentrations of 80 ppm and 100 ppm

for both trophozoite and cystic forms, followed by MTT assay for macrophages.

Results

Based on the findings of the trophozoite form by Trypan blue staining with the 80 ppm concentration of the silver NP, 4, 3, and 2 parasites were observed in each microscopic field after 24, 48, and 72 hours, respectively. Conversely, these values for the control group were 5, 4, and 3 parasites, respectively (Figure 1). Similarly, 5, 4, and 3 alive trophozoites were found at 100 ppm concentration of silver NPs after 24, 48, and 72 hours. In contrast, the number of live trophozoites per microscopic field was 10, 5, and 4 in the control group, respectively (Figure 2).

Similar results were observed for the *Acanthamoeba* cyst at the same concentrations and times when exposed to silver NPs (Figures 3 and 4). In the graphs, the vertical

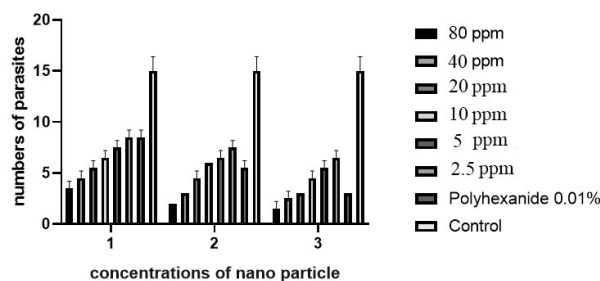


Figure 1. *Acanthamoeba* Trophozoites in the Presence of Multiple Concentrations (80 ppm) of Silver Nanoparticles After 24, 48, and 72 Hours of Microscopic Counting.

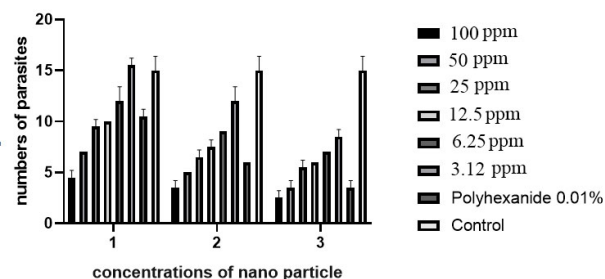


Figure 2. *Acanthamoeba* Trophozoites in the Presence of Multiple Concentrations (100 ppm) of Silver Nanoparticles After 24, 48, and 72 Hours of Microscopic Counting.

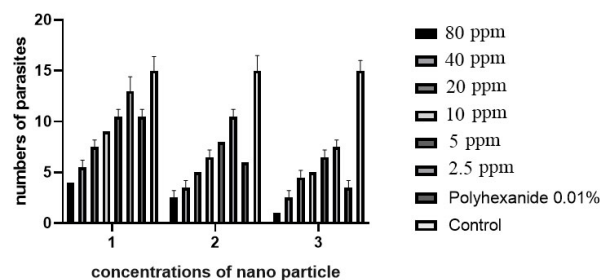


Figure 3. *Acanthamoeba* Cysts in the Presence of Multiple Concentrations (80 ppm) of Silver Nanoparticles After 24, 48, and 72 Hours of Microscopic Counting.

and horizontal axes represent the number of live parasites (the survival rate), as well as the concentration and time, respectively. Each experiment is repeated three times.

The findings indicated that silver NPs were more effective in both concentrations compared to the control group and showed a better effect over time.

The findings of flow cytometry also confirmed those obtained from the microscopic stage. It should be noted that flow cytometry was only performed for the cystic phase of the parasite (Figure 5).

Macrophage MTT Assay and IC50

The MTT test findings demonstrated that high concentrations of NPs, especially 2 ppm, have a similar effect on macrophages compared with the control group (Figure 6).

The IC50 was evaluated for both trophozoite and cystic phases after 72 hours according to Table 1.

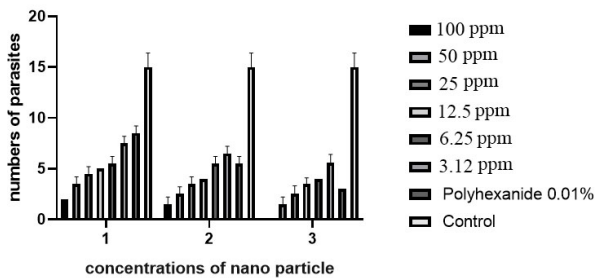


Figure 4. *Acanthamoeba* Cysts in the Presence of Multiple Concentrations (100 ppm) of Silver Nanoparticles After 24, 48, and 72 Hours of Microscopic Counting.

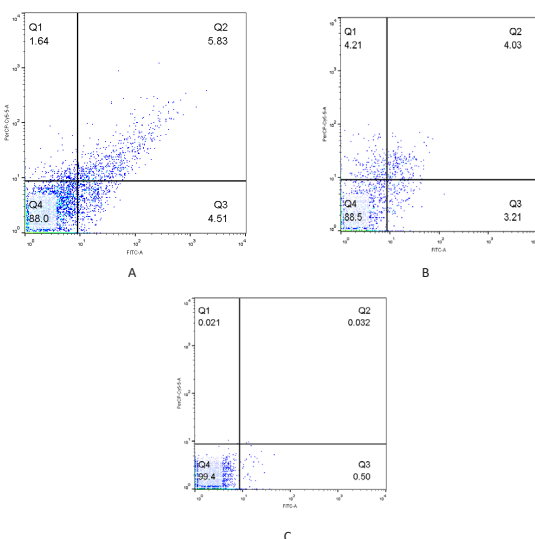


Figure 5. *Acanthamoeba* Cyst Apoptosis in the Presence of Silver NPs by Flow Cytometry: (A) Under 80 ppm of Silver NPs, (B) Under 100 ppm of Silver NPs, and (C) Viability of Most Protozoan Trophozoite and Cystic Forms in the Control Group. Note. NP: Nanoparticle.

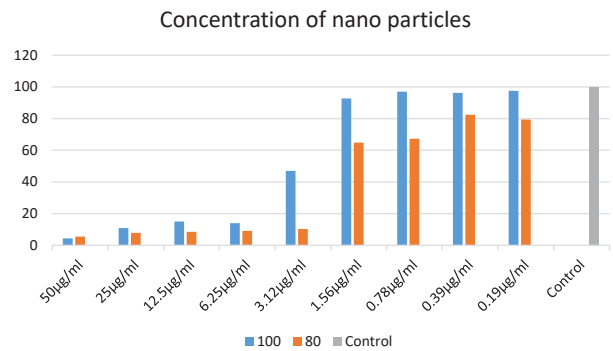


Figure 6. Macrophage MTT Test Results at Different Concentrations of Silver Nanoparticles. Note. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

Discussion

The findings showed that silver NPs expectedly provided promising results. The high lethality for both trophozoite and cystic forms while the low lethality for macrophages made this NP a suitable candidate for the treatment of *Acanthamoeba* infection. The anti-parasitic background of this NP has been strengthened by comparing our findings with those of other studies, and this material may rank the first among anti-parasitic substances for at least *Acanthamoeba*.

Acanthamoeba is one of the few free-living protozoans with rare but severe infections for humans. It is a ubiquitous protozoan and can be transmitted from the water and soil, thus many people around the world are exposed to it although this does not mean that all of these people are at risk of the disease.⁹ The infection can involve specific organs such as the eyes, skin, central nervous system, and lungs or many of them manifested as a disseminated infection. Three forms of the disease are caused by *Acanthamoeba* infection.¹⁰

In the disseminated infection, multiple organs are affected by the infection alone or in combination. This widespread infection often occurs in people with immune deficiencies.¹¹ Another form of infection that can occur in healthy people is manifested as *Acanthamoeba* keratitis that engulfs the eye and results in visual impairments or blindness if treated inadequately. The disease is mostly associated with eye contact lenses.¹² The third and the most dangerous form of the disease that can be fatal is the development of granulomas amebic encephalitis in the infected central nervous system.^{13,14}

Up to now, various studies have been performed to improve the clinical and laboratory treatment of parasitic disease by chemical or plant derivatives.^{15,16} Pazoki et al have recently evaluated the plant extract (*Artemisia aucheri*) effect on trophozoite and cyst forms in an in vitro condition.⁹ Although similar approaches were applied in the above-mentioned study and the present study and the results were somewhat similar, the difference relies on the amount of the applied material for killing the parasite, indicating that silver NPs could have a much greater effect on killing trophozoites and parasitic cysts while being less

Table 1. IC50 Values for the Two Concentrations and Both Parasite Phases

	Ag 80 ppm	Ag 100 ppm
Trophozoite	2.2 µg/mL	1.5 µg/mL
Cyst	4.6 µg/mL	10 µg/mL

Note. IC50: Half-maximal inhibitory concentration.

toxic to macrophages.

Previous studies have emphasized the anti-parasitic property of silver NPs on several important protozoans such as *Leishmania* spp., *Entamoeba histolytica*, *Toxoplasma gondii*, and *Cryptosporidium parvum*.¹⁷⁻¹⁹

In their study, Ismail et al investigated the anti-leishmaniasis effect of silver oxide NPs, which resulted in a more favorable effect than the standard *Leishmania* drugs.⁷ Accordingly, our findings are in line with those of the above-mentioned studies representing that silver NPs have even greater anti-parasitic activity compared to the *Acanthamoeba* choice drug.

Gaafar et al concluded that silver NPs, alone or in combination with chitosan, have a high anti-toxoplasmic effect in addition to having a greater impact on biocompatibility.²⁰ It should be noted although chitosan was not used with NPs in the current study, it was suggested that bio-elements could be combined with NPs.

The present study has some limitations including the difficulty in cultivating and isolating protozoa because of opportunistic fungi from soil sources with growth parasites. The lack of a suitable animal model for *Acanthamoeba* also overshadows the findings of this study. However, the obtained results cannot be generalized to in vivo conditions.

In contrast, the tested antiparasitic agent was modified to be nano-scale, permeable, and effective compared to its raw form, and the required steps for evaluating an antiparasitic agent in vitro (e.g., colorimetry, flow cytometry, and the like) were performed for herbal extract-based silver NPs.

Conclusion

According to the findings and their comparison with previous observations, the anti-parasitic activity of silver NPs in combination with the herbal extract on the *Acanthamoeba* protozoa was highly significant. However, further studies in different dimensions are needed to confirm the results.

Authors' Contributions

FG and SB designed the study. AKS, PT, YKS, and LZ contributed to study implementation and collaborated in the analysis and interpretation of data. AK and SB collaborated in the manuscript writing and revision. FG contributed in reviewing and editing the manuscript. All the authors commented on the drafts of the manuscript and approved the final version of the article.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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