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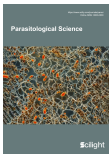

Article

Green Tea Anti-Acanthamoeba Activity Regulated by Silver Nanoparticles

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Abstract: Efforts to utilise naturally derived substances in parasitology are gaining increasing interest. Among these are nanoparticles, whose antiparasitic properties may be enhanced when combined with plant extracts rich in bioactive compounds, beneficial to the host but toxic to parasites. This study evaluated whether silver nanoparticles coated with green tea (GT(AgNPs)), enhance the antiparasitic effects of silver nanoparticles (AgNPs) or green tea extract (GT) alone. Transmission electron microscopy revealed phagocytosis of nanoparticles, which accumulate in the vacuoles of amoebae. Notably, amoebae exposed to GT(AgNPs) exhibit cytoplasmic vacuolization and advanced cytoplasmic organelle disintegration, indicating strong toxicity even at lower levels of nanoparticle accumulation. Green tea extract alone is also toxic to amoebae, but without induction of cytosolic vacuolization. GT extract alone induced high expression of cysteine proteinase, paralleled by active autophagy. GT(AgNPs) did not have such effect on cysteine proteinase gene expression in amoebae. The mechanisms underlying the toxicity of silver nanoparticles and green tea appear to differ and are probably influenced by the condition of vacuole formation in trophozoites of *Acanthamoeba castellanii.*

Keywords: *Acanthamoeba castellanii*; silver nanoparticles; green tea; antiparasitic activity; amoeba cysteine proteinase; gene expression

1. Introduction

Both life stages (the trophozoite and the cyst) of free-living facultative parasites of the *Acanthamoeba* genus can be infectious to humans, leading to soil- and water-borne opportunistic *acanthamoebiasis* [1–4]. The wide distribution in different natural and anthropogenic environmental of Acanthamoeba complicates efforts to develop effective prevention strategies for infections Antiparasitic treatment can be challenging and may fail due to various factors, including drug resistance, formation of resistant cyst stages, and resistance to disinfection treatments [5].

In the search for new preventive strategies and parasite drug treatments, there has been growing interest in silver nanoparticles (AgNPs). These nanoparticles are a significant advance in nanotechnology, noted for their exceptional physicochemical properties. Their excellent antimicrobial properties, stability, and minimal chemical reactivity make them useful in various biological and medical applications, including antiparasitic treatments [6]. Silver nanoparticles (AgNPs) are effective against many parasites, including the unicellular organisms such as *Cryptosporidium parvum* [7], *Toxoplasma gondii* [8], *Plasmodium* genus [9] and *Leishmania major* [10] as well as helminths such as Monogenea [11], cystic echinococcosis [12], *Toxocara vitulorum* [13], and *Haemonchus contortus* [14,15]. Different parasites are susceptible to silver nanoparticle treatment, and the mechanism of action may be nonspecific metabolically. Compared to traditional antiparasitic drugs, AgNPs could provide an alternative or complementary therapeutic option, particularly in cases of drug resistance [16]. Due to their high surface area

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and unique physicochemical characteristics, AgNPs can effectively destroy many pathogens through oxidative stress induction, cell membrane damage, and metabolic inhibition [17]. They also exhibit antioxidative effects [18]. Further research is needed on the toxicological mechanisms induced by silver nanoparticles at the subcellular level [19,20].

Naturally derived compounds, including plant extracts that contain bioactive substances, are being investigated as a potential treatment for *Acanthamoeba* infections. These compounds are known for their therapeutic properties, such as antibacterial, antiparasitic, and anti-inflammatory effects, making them valuable in medicine and veterinary science [21]. For instance, *Camellia sinensis*, commonly consumed as tea, is recommended for its health benefits [22]. The extracts from this plant have shown amoebicidal activity, as they inhibit the growth of trophozoites and reduce cyst formation of *Acanthamoeba in vitro*. The primary action of the herb is believed to affect cell membrane integrity through compounds like epigallocatechin-3-gallate (EGCG). Nevertheless, transient exposure of pathogens to *C. sinensis* does not significantly hinder trophozoite growth [23,24], which raises questions about the green tea stability treatment. Combining silver nanoparticles with herbal extracts can enhance their efficacy and safety by reducing toxicity. Compounds found in these herbs may work synergistically to boost the antiparasitic effects of the nanoparticles. To reduce the likelihood of potential side effects, a combined approach is recommended [25]. The interaction between silver nanoparticles and herbal extracts leads to the parasite's membrane integrity disruption, induction of apoptosis, and interference with cellular metabolism. Herbal extracts can also serve as natural stabilizers and carriers, enhancing the bioavailability of nanoparticles [26]; The plant extract used in producing silver nanoparticles not only acts as a reducing agent for silver ions but also functions as a coating agent [27,28].

Amoebae are often used as biological models for studying the toxicity of nanomaterials. Analyzing the response of amoebae to silver nanoparticles may help better understand mechanisms of uptake, accumulation, induction of oxidative stress, and DNA damage [29–32].

Rab1/RabD family small GTPase (GTPase), vacuolar proton ATPase (ATPase), and cysteine proteinase II are proteins involved in phagocytosis in *A. castellani*. Previous studies have shown that silencing the GTPase gene disabled phagosome formation in the ingestion of *E. coli*. In contrast, ATPase gene silencing allowed phagosome development but inhibited phagolysosome formation. Silencing the cysteine proteinase gene inhibited *E. coli* digestion and amoeba cell rupture [33]. Other studies report that some cysteine proteases in *Acanthamoeba* sp. are involved in both the encystment process and trophozoite reactivation [34,35].

The effects of green tea extract on amoebae are crucial for developing new therapies and understanding their mechanisms of action. Given the distinct activities of silver nanoparticles and green tea, we examined their individual and combined effects. Evaluating the antiparasitic activity of nanoparticles combined with green tea extract presents a novel approach for more effective and targeted drug delivery. Specifically, we examined the susceptibility of the *Acanthamoeba castellanii* genotype T4 axenic strain 309 cultures to these compounds. Additionally, we analyzed the expression of genes involved in the phagocytosis activation pathway to determine whether silver nanoparticles could modulate the amoeba's response to treatment with silver nanoparticles and green tea extract.

2. Materials and Methods

2.1. Preparation of Silver Nanoparticles, Green Tea Extract, and GT(AgNPs)

Green tea leaves (Loyd, Mokate, Poland) were frozen within liquid nitrogen and ground into a fine powder using a laboratory mortar. The extract was prepared by brewing 10 g of tea leaves' powder in 100 mL of MiliQ water (60 °C, 15 min). The debris was removed by two 10-min centrifugations (at 9000 rpm and 15,000 rpm) followed by filtration with a 0.22 µm syringe filter.

For the tea extract-based assays, the extract was first dried using the rotary evaporator and then redissolved to reach the required concentrations.

To obtain the green tea silver nanoparticles (GT(AgNPs)), we modified the method described by [36]. Briefly, 750 µL of $AgNO_3$ solution (10 mM) was added dropwise to a solution containing 12 mL of the green tea extract and 13 mL of MiliQ water, with continuous stirring, for 2 h. The nanoparticles were then purified by six consecutive cycles of centrifugation (10,000 rpm, 10 min) and resuspension in distilled water. They were then stored at 4 °C.

Silver nanoparticles without extract were prepared as a control. NPs were synthesized using a modified version of the method described by Agnihotri et al., (2014) [37]. 48 mL of an aqueous solution containing 2 mM $NaBH_4$ and 2 mM trisodium citrate (TSC) was first heated to 60 °C and stirred for 30 min. Subsequently, 2 mL of 11.7 mM $AgNO_3$ solution was added dropwise. The reaction mixture was then brought to 90 °C, and the pH was

adjusted to 10.5 with 0.1 M NaOH. Stirring was continued at 90 °C for an additional 20 min until a distinct color change indicated nanoparticle formation.

Depending on the batch of tea used for the synthesis, the G-Tea NPs were around 60 nm in diameter, usually containing a small proportion of particles around 30 nm in diameter. AgNPs were slightly smaller with a diameter of around 30 nm. Each batch of particles was examined using FEI Nova NanoSEM 450.

2.2. Acanthamoeba Culture

Acanthamoeba castellani genotype T4, free-living strain 309, pathogenic to mice, was cultured axenically in Bacto-Casitone liquid medium [38]. Amoebae were cultured in glass tubes at 28 ± 2 °C [39] and kept in humid and dark conditions in the incubator (UniEquipGmbH). To synchronise the growth of the cyst-free culture, the medium was replaced with fresh medium 15 h before the protozoa were collected for testing.

2.3. Antiproliferative Activity of Nanoparticles and Green Tea Extract

Amoebae were cultured in 2 mL of medium per well [40]. Antiproliferative activity of silver nanoparticles, green tea extract, and GT(AgNPs) was evaluated in vitro in 24-well polystyrene cell culture plates (NEST). A suspension containing 5×10^4 cells/mL was added to each well. Control wells contained amoebae without reagents.

To determine the kinetics of culture growth inhibition by amoebicidal AgNPs, trophozoites and cysts were counted in a Bürker hemocytometer on days 1, 3, 5, and 7. The percentage of inhibition was calculated from the number of amoebae in the control wells (CTRL), which was taken as 100%. The percentage of inhibition was calculated by using the following formula:

$$\text{Inhibition (\%)} = 100 - N_{(T)}/N_{(CTRL)} \times 100$$

where $N_{(T)}$ is the number of treated amoebae and $N_{(CTRL)}$ is the number of amoebae in the control group (CTRL).

Cultures were also monitored for 5000, 1000, 500, and 100 µg/mL of AgNPs, GT, and GT(AgNPs) to determine the effect of different reagent concentrations on amoeba proliferation. Tests were performed in triplicate. Cultures were maintained for 5 and 7 days at 28 °C in a humid dark chamber. The growth of amoeba cultures was examined using a modified light microscope at 20× or 40× objective magnification. Photographs were taken with an M16 microscope camera (Opta-Tech MW 50, Warsaw, Poland).

2.4. Evaluation of Acanthamoeba castellanii Sensitivity to Amphotericin

To compare the magnitude of the nanoparticles' activity, the cytotoxicity of amphotericin against amoebae was assessed. Amphotericin B aqueous solution, 250 µM (Sigma-Aldrich Inc., St. Louis, MI, USA) was diluted with sterile deionized water to obtain the final concentrations (125^{-1} µM) used for in vitro studies. The cytotoxic assay was performed on 96-well plastic plates (NUNC) filled with 200 µL of 5×10^4 /mL amoeba suspension in culture medium and then supplemented with an aliquot of diluted amphotericin B to obtain the required drug concentrations. Control wells were filled with the same 20 mL volume of deionized water. Samples were monitored 1, 3, and 5 days after culture treatment with each concentration tested in triplicate. The number of live trophozoites was counted in a Bürker hemocytometer using 0.04% toluidine blue.

2.5. Transmission Electron Microscope (TEM)

A pellet of amoebae was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, overnight at room temperature. Samples were washed in cacodylate buffer thrice and stained with 1% osmium tetroxide in ddH_2O overnight at room temperature. Samples were washed in ddH_2O and dehydrated through a graded series of ethanol (30%, 50%, 70%, 80%, 96%, absolute ethanol, and acetone). Samples were embedded in epon resin (SERVA) and polymerized for 24 h at 60 °C in the incubator (Agar Scientific, London, England), then 70 nm sections were cut with a diamond knife on RMC MTXL ultramicrotome (RMC Boeckeler Instruments, Tucson, AZ, USA). The sections on copper grids were not contrasted. Samples were analysed in a LIBRA 120 transmission electron microscope produced by Carl Zeiss (Oberkochen, Germany), at 120 keV. Photographs were made with a Slow-Scan CCD camera (ProScan, Germany), using the EsiVision Pro 3.2 software.

2.6. RNA Purification and RT-qPCR

Total RNA was isolated with the Maxwell® RSC instrument using the Maxwell® RSC simplyRNA Tissue Kit (Promega). RNA concentrations and purity were assessed using the NanoDrop One (Thermo Fisher, Waltham, MA, USA).

Reverse transcription was performed on 100 ng of RNA from each sample, using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA, USA), according to the manufacturer's recommendations. The following program was used for reverse transcription: 46 °C for 20 min (reverse transcription), followed by reverse transcriptase inactivation at 95 °C for 1 min. The resulting cDNA was stored at −20 °C until further use. The volume of each reaction was 20 µL.

Real-time PCR was performed with the Biorad CFX96 thermal cycler (Biorad). The RT-qPCR program consisted of preincubation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 30 s. A melt curve analysis was conducted from 65 °C to 95 °C with 0.5 °C increments.

Primer sequences and characteristics are provided in Table 1S. 18S and HPRT were used as reference genes, as their expression is among the most stable under variable conditions [41]. All reactions were performed using the iTaq Universal SYBR Green Supermix (Biorad). The volume of each reaction was 20 µL. Each cDNA sample was analyzed in two technical replicates per gene. RT-qPCR data analysis was conducted using CFX Maestro Software (v. 1.0, Biorad). ANOVA followed by Tukey's post-hoc test was used to determine the significance of differences in gene expression between groups.

2.7. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 10.4.1, San Diego, CA, USA). A -value ≤ 0.05 was considered statistically significant. IC_{50} was calculated with QuestGraph IC_{50} calculator (AAT Bioquest, Inc., Pleasanton, CA, USA, https://www.aatbio.com/tools/ic50-calculator, accessed on 27 April 2025).

3. Results

3.1. Acanthamoeba Growth in Culture

The amoeba cultures are shown in Figure 1. In the control group (CTRL), the amoebae were actively moving and dividing, with numerous acanthopodia protruding from their surfaces and possessing well-defined vacuoles (Figure 1A). A few cysts were also observed in the control culture. After exposure to AgNPs, GT extract, and GT(AgNPs), amoebae reduced their mitogenic activity, changed shape, altered phagocytic behaviour, and declined in number. In cultures treated with AgNPs, the amoebae became flattened and adhered tightly to the plate surface, producing numerous vacuoles filled with silver nanoparticles (Figure 1B). When exposed to GT extract, the amoebae assumed an oval shape. Compared to the CTRL culture, no adherent forms on the bottom of the plate were reduced in size. They resembled the pseudocyst stage and induced autophagy. No dividing forms were observed (Figure 1C). The number of amoebae decreased when treated with GT(AgNPs). The cells in this culture appeared small, irregularly shaped, motionless, and filled with irregular vacuoles containing few nanoparticles (Figure 1D). Treatment of *Acanthamoeba* culture with AgNPs, GT extract, and GT(AgNPs) caused changes in the morphology of trophozoites.

3.2. TEM Acanthamoeba castellanii Ultrastructure Analysis

TEM analysis examined the subcellular morphology of the amoebae to confirm the lesions and the general condition of the culture, visualised by light microscopy. This study emphasised the impact of silver nanoparticles and green tea extract on amoebae over a 10-day culture period. Representative images were selected upon exposure to a concentration of AgNPs that exhibited anti-amoebic activity (i.e., 5000 µg/mL). GT extract and GT(AgNPs) were used at the same concentration as AgNPs to compare their toxicity levels. The ultrastructural analysis was performed on days 3 and 10 of the axenic culture. In all examined groups, the ultrastructure of the trophozoites exhibited notable changes compared to the CTRL (Figure 2A,B). Large vacuoles containing nanoparticles were surrounded by mitochondria in amoebae cultured with AgNPs (Figure 2C–F). In cultures treated with GT extract and GT(AgNPs), the amoeba showed a size reduction, acanthopodia depletion, and cell surface smoothing. After treatment with GT, at day 3, reduced cytoplasm around the nucleus was accompanied by fragmented organelles and the formation of cytoplasmic compartments (Figure 2G,H). By day 10, the cytoplasm became thinner, and granule numbers diminished. Along with the amoeba's oval shape and less dense nucleus, all these changes indicated the progression of autophagy (Figure 2I,J). Amoebae treated with GT extract showed a change in size and defragmentation, with lysis of intracellular material increased with time. When exposed to GT(AgNPs), amoeba also decreased in size, with fewer acanthopodia, adopting oval morphology (Figure 2K,L). Nanoparticles adhered to the cell membrane, but phagocytic vacuoles did not form, in contrast to amoebae treated with AgNPs (Figure 2D). The particles were also observed in the cytoplasm outside vacuoles (Figure2I,J), suggesting an

alternative entry pathway into cellular compartments. As shown in Figure 2M,N, amoebas treated with GT(AgNPs) exhibited a delayed and reduced phagocytosis of these particles compared to those treated with silver nanoparticles alone. These changes were accompanied by extensive cytoplasmic vacuolization resembling advanced autophagy, as illustrated in Figure 2K–N, leading to the disintegration of the amoebae. Green tea extract and green tea delivered into the cell as GT(AgNPs) induced autophagy in *Acanthamoeba* trophozoites.

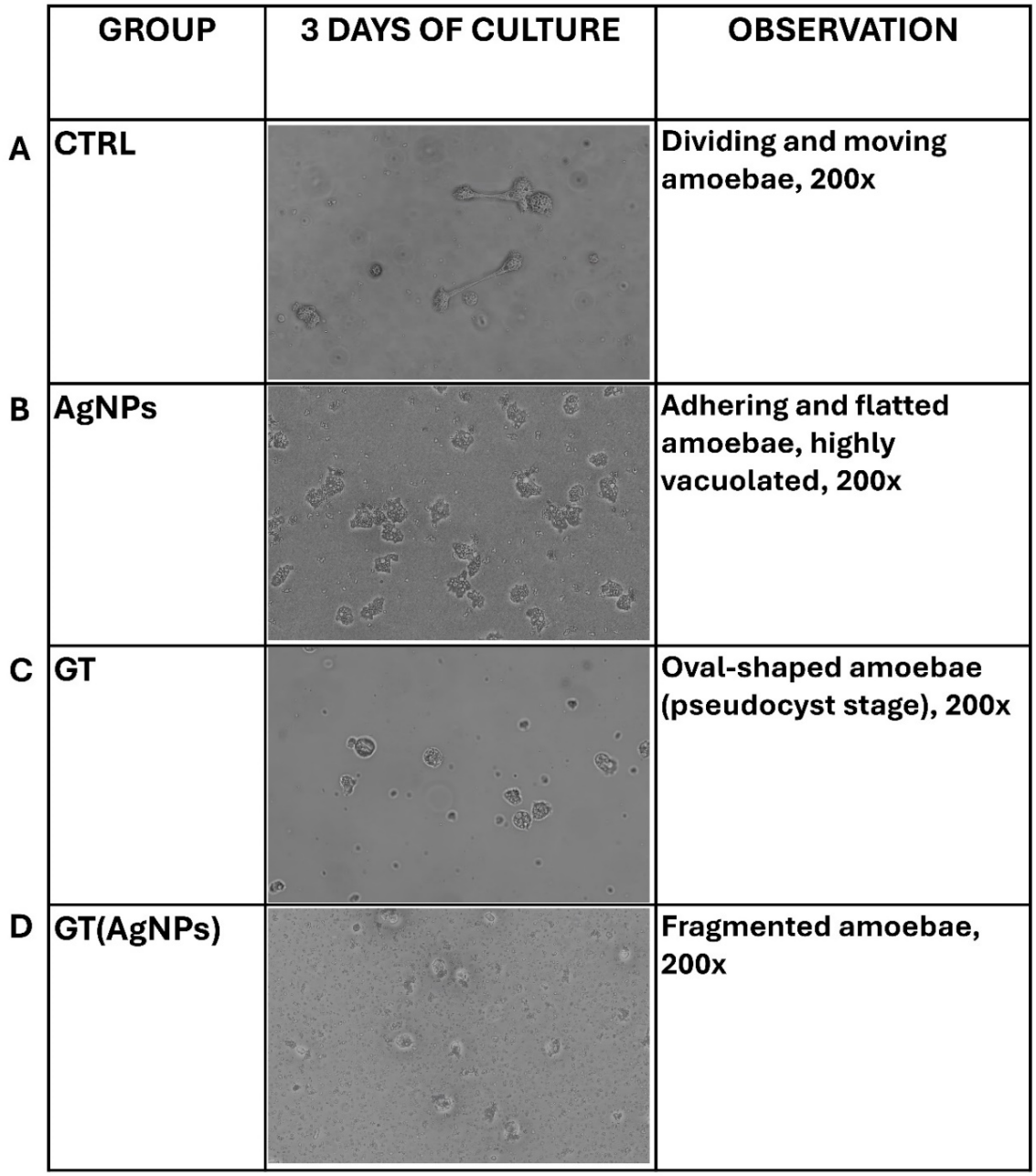

Figure 1. Trophozoite morphological changes in the 3-day culture of *Acanthamoeba castellanii*: (**A**) control (CTRL); (**B**) after exposure to silver nanoparticles (AgNPs); (**C**) green tea extract (GT); and (**D**) GT(AgNPs). All compounds were administered to the axenic culture at a 5000 µg/mL concentration.

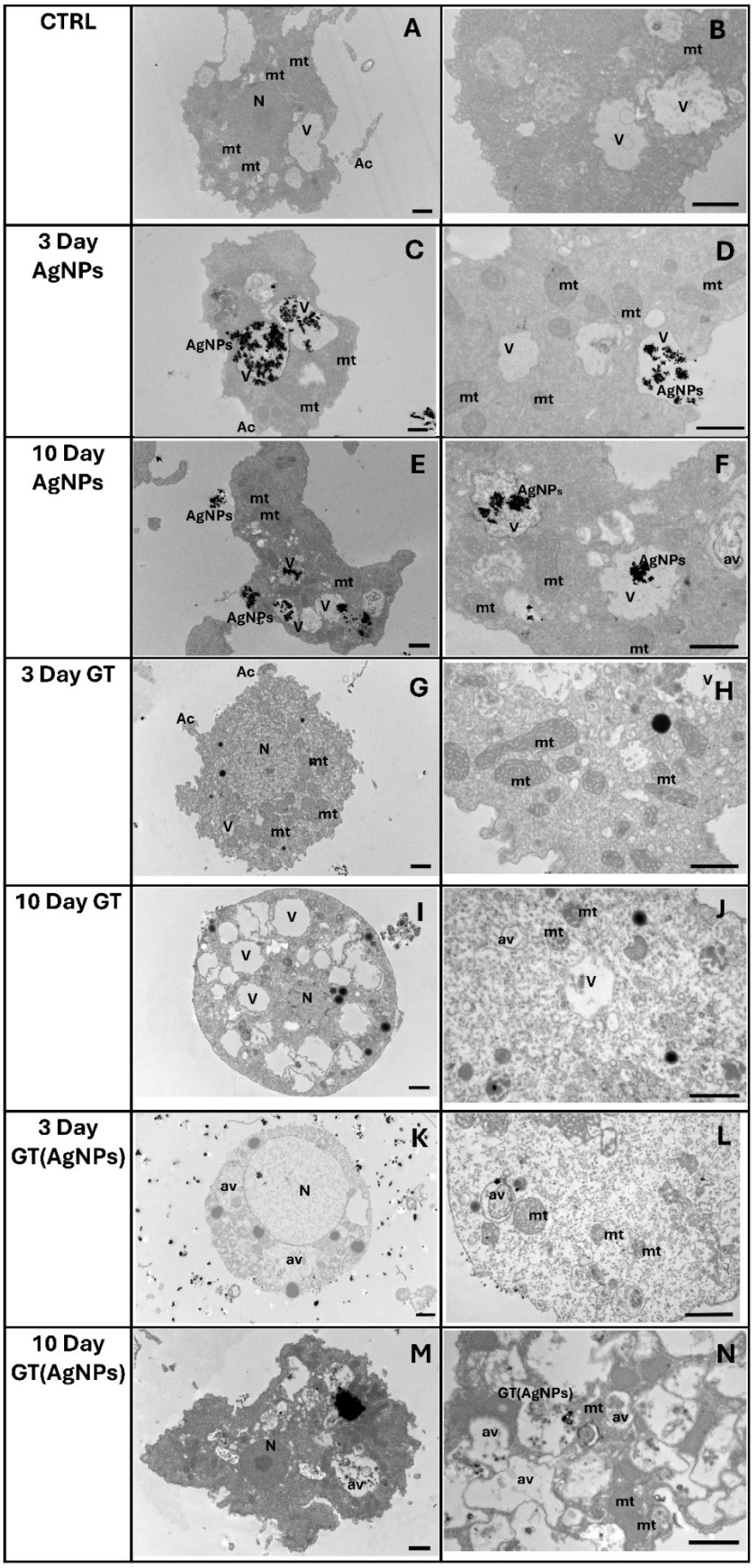

Figure 2. TEM pictures of *Acanthamoeba castellanii* trophozoites collected at day 3 and 10 of exposure to AgNPs, GT extract, and GT(AgNPs). Images were collected from different cells at the following magnifications: 12,000× (**A**,**C**,**E**,**G**,**I**,**K**,**M**); 25,000× (**F**); # 30,000× (**B**,**D**,**H**,**J**,**L**,**N**). Scale bar = 1 μm. Picture captions: acanthapodia (Ac); autophagic vacuole (av); mitochondrion (mt); nucleus (N); vacuole (V).

3.3. Growth Inhibition of Acanthamoeba in Cultures

The sensitivity of amoebae to AgNPs, GT extract, and GT(AgNPs) was assessed at concentrations of 10,000, 5000, 1000, 500, and 100 µg/mL for each compound. In the cultures treated with 10 mg/mL, growth was significantly inhibited by AgNPs (20.2%, $p = 0.014$), GT extract (45.8%, $p = 0.040$), and GT(AgNPs) (61.65%, $p < 0.026$) after 24 h. Subsequently, the amoebicidal effect increased for AgNPs (53%, $p < 0.0001$), GT (85%, $p < 0.0001$), and GT(AgNPs) (91%, $p < 0.0001$) on day 3. The inhibition remained temporary at a lower level on day 5, but later on day 7 the greatest effect was observed between CTRL and GT–amounting to 95.5% inhibition at $p < 0.0001$; 91.3% inhibition between CTRL and GT(AgNPs) at $p < 0.0001$, and 58.8% inhibition between CTRL and AgNPs at $p = 0.035$ (Figure 3, Table S2). Green tea extract strongly affected the biological condition of *Acanthamoeba* trophozoites. No increase in cyst numbers was seen in any culture.

The IC_{50} (concentration of drug inhibiting 50% of amoebae growth compared to the control culture) for AgNPs was identified for 10 mg/mL. GT extracts achieved such an effect 24 h after treatment. Silver nanoparticles could maintain stable anti-amoebic activity at lower concentrations than 10 mg/mL, and the effectiveness of the treatment was not strongly dose-dependent (Figure 3). GT extracts and GT(AgNPs) at a concentration of 10,000 mg/mL showed very high toxicity, which was statistically confirmed despite slight differences between both groups.

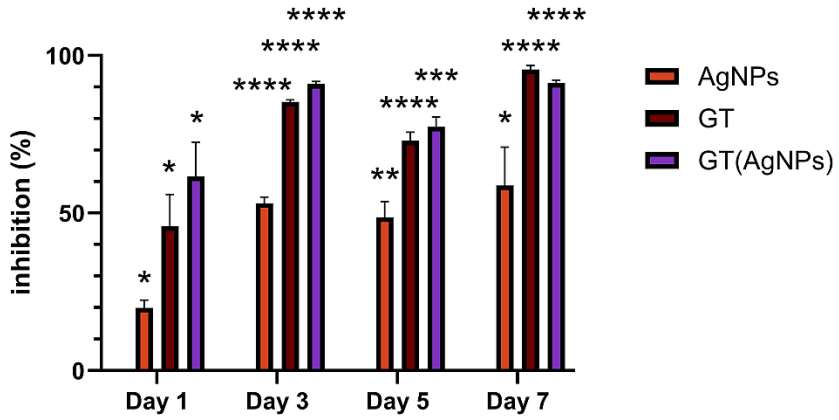

Figure 3. Growth inhibition of *Acanthamoeba castellanii* cultures after exposure to 10,000 µg/mL silver nanoparticles (AgNPs), green tea extract (GT) and silver nanoparticles coated with green tea extract (GT(AgNPs)). ANOVA and post-hoc-Tukey statistical analysis are shown in Supplementary Materials (Table S2). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.0001$.

In subsequent tests, reagents were used at lower concentrations. The effect of decreasing concentrations of AgNPs, GT, and GT(AgNPs) on the growth of *Acanthamoeba* cultures was monitored using compounds at 5000, 1000, 500, and 100 µg/mL on days 5 and 7 (Figure 4A,B). For AgNPs, GT, and GT(AgNPs), equal anti-amoebae activity was observed at 5000 µg/mL concentration. AgNPs demonstrated more stable activity against *Acanthamoeba* than GT and GT(AgNPs). The 5000 µg/mL concentration was the most effective in inhibiting culture growth ($p < 0.0001$). GT extract and GT(AgNPs) exhibited more pronounced toxic effects on day 5 than on day 7 (Figure 4A). The culture seemed to regenerate as trophozoite abundance increased in samples with compound concentrations lower than 5000 µg/mL on day 7 (Figure 4, Table S3). For concentrations of 1000 µg/mL and lower, GT and GT(AgNP) extracts exhibited comparable toxicity. This toxicity was significantly weaker than that of AgNPs. Additionally, in samples with GT(AgNP), there was even a recovery of amoeba growth in the culture compared to cultures with only AgNPs.

Green tea at a concentration of 10 mg/mL exerted strong and stable amoebicidal activity till day 7 of culture (Table S4). The inhibition of amoeba growth culture exposed to AgNPs achieved not more than 60% growth inhibition (Figures 3 and 4A,B, Tables S2 and S3). AgNPs maintained the amoeba count at the lowest level across all tested concentrations compared to GT and GT(AgNPs), where the number of amoeba returned to the control levels at 100 and 500 µg/mL after seven days of culture. A 5000 µg/mL concentration was selected for further molecular-level analysis of amoebic changes. That concentration gave comparable IC_{50} on day 7th for all compounds.

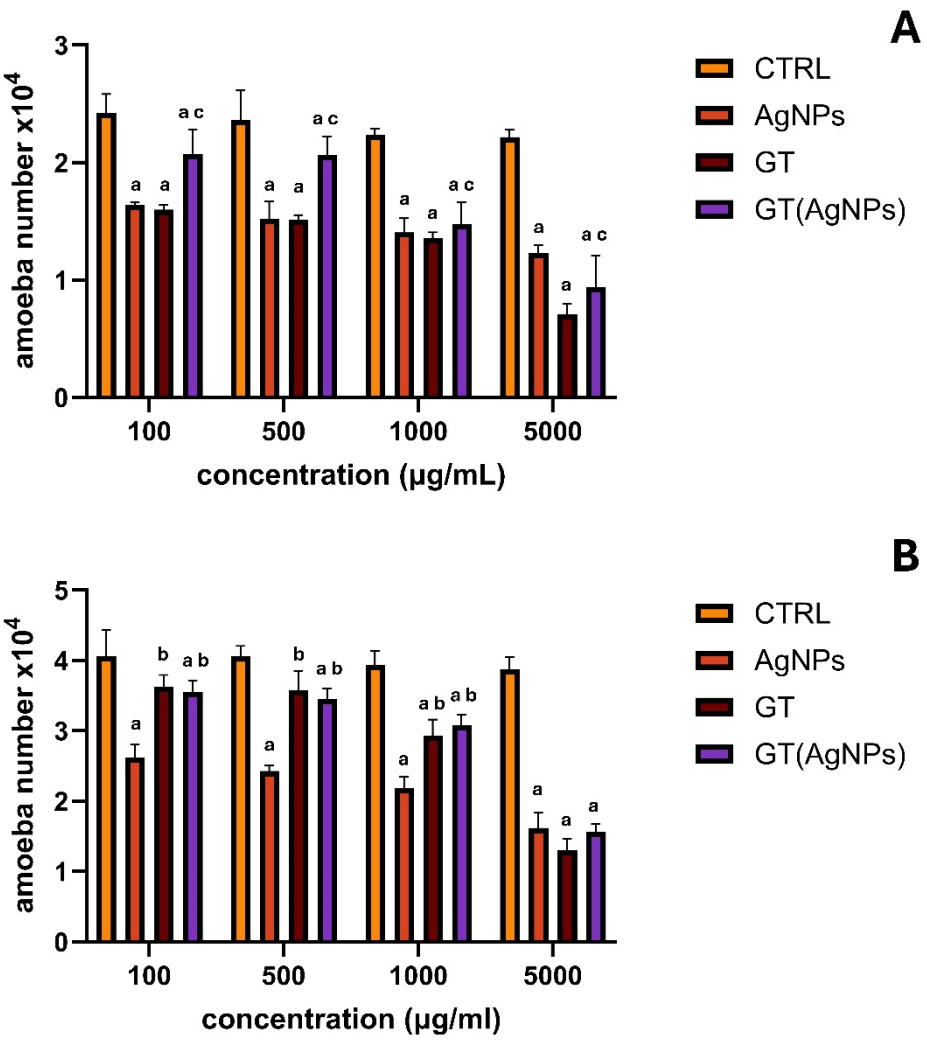

Figure 4. The concentration-dependent growth of *Acanthamoeba castellanii* culture in control (CTRL) and after exposure to silver nanoparticles (AgNPs), green tea extract (GT) and silver nanoparticles coated with green tea extract (GT(AgNPs)). (**A**) 5 days after exposure; (**B**) 7 days after exposure. Differences that are statistically significant in relation to CTRL are marked as "a", in relation to AgNPs as "b" and in relation to GT as "c". $p < 0.05$; ANOVA and post-hoc-Tukey statistical analysis with statistical significancies between the groups are shown in Supplementary Materials (Table S3).

3.4. Cytotoxicity of Amphotericin B to Acanthamoeba castellanii

The effect of Amphotericin B treatment on *Acanthamoeba castellanii* is presented in the Supplementary Materials (Table S4). The results were generated by counting amoebae in three independent samples under a light microscope using a Bürker hemocytometer. Amphotericin B increased IC_{50} with treatment duration; on day 3 of culture, IC_{50} was 3.39 µM, and after 5 days of culture, IC_{50} increased to 23.94 µM.

In contrast, AgNPs, GT extract, and GT(AgNPs) exhibited lower cytotoxicity than Amphotericin B and achieved this activity at an IC_{50} of 5000 µg/mL. However, unlike to Amphotericin B [42], Acanthamoeba cultures did not exhibit increasing resistance to the nanoparticles or GT extract over time. Growth inhibition of Acanthamoeba castellanii cultures was maintained throughout the experiment after exposure.

3.5. Cysteine Proteinase, GTPase, and ATPase Gene Expression in Acanthamoeba Trophozoites

In the GT-treated amoeba group, cysteine proteinase gene expression was significantly higher than in all other groups ($p < 0.005$). There were no significant statistical differences between the groups in the expression of GTPase and ATPase genes (Figure 5).

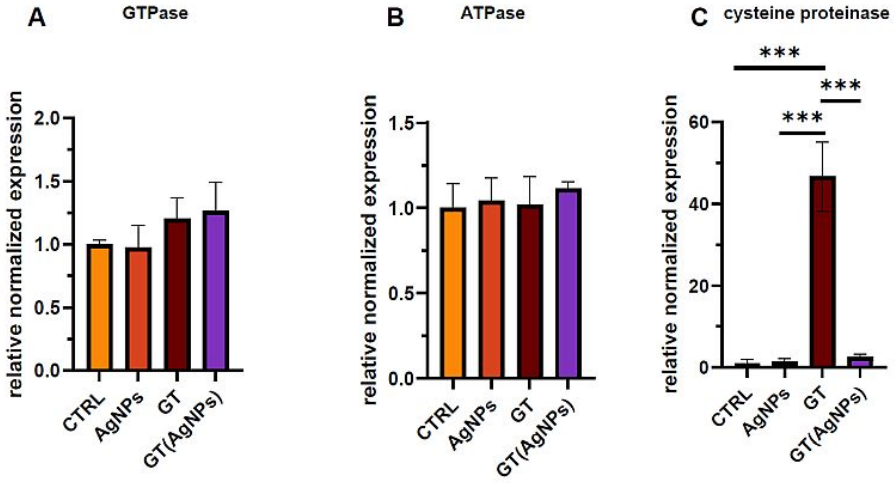

Figure 5. The relative normalized expression of GTPase (**A**), ATPase (**B**), and cysteine proteinase (**C**) in trophozoites of *Acanthamoeba castellanii* after 5 days of culture being exposed to silver nanoparticles (AgNPs), green tea extract (GT), and silver nanoparticles coated with green tea extract GT(AgNPs)). ANOVA and post-hoc-Tukey statistical analysis are shown in Supplementary Materials (Table S5). *** $p < 0.005$.

4. Discussion

Previous studies have demonstrated the antiparasitic properties of silver nanoparticles [43–45], as well as green tea extract [46]. Despite their different modes of action, these agents produce similar results: reduced parasite survival. The manner in which these compounds are delivered into cells may be crucial for enhancing their target activity. Due to their ability to penetrate cell membranes and also by phagocytosis, nanoparticles can deliver active substances precisely into the intracellular environment, thereby increasing the effectiveness of treatment [17,47,48].

This study examined the potential toxic effects of silver nanoparticles (AgNPs), green tea extract (GT), and green tea-coated silver nanoparticles (GT-AgNPs) on *Acanthamoeba castellanii*. Treatment with these substances reduced amoeba numbers and caused distinct changes in trophozoite morphology. These differences in subcellular structure suggested that the toxicity mechanisms might be distinct for nanoparticles and green tea and nanoparticles coated with green tea. For the first time we show that silver nanoparticles coated with green tea extract did not impact the expression of phagocytosis-related genes when compared to green tea extract alone.

Several characteristics of in vitro amoeba culture have been described [31,42,49,50]. Other studies [51,52] have shown that *Acanthamoeba* transfers foreign particles by phagocytosis, engulfing them into vacuoles. Amoeba also takes up silver nanoparticles, including those conjugated with other substances. We measured the number of trophozoites, their ultrastructure, and their sensitivity to various nanoparticle concentrations during the early, late log, and stationary phases of amoeba growth. Intensive AgNPs accumulation was found to cause vacuole formation and changes in mitochondrial structure but with minor changes in cytosol density. Consequently, the number of trophozoites was reduced in the late and stationary phases of culture growth. It has been suggested that, once incorporated into the cytosol or other intracellular organelles, silver nanoparticles induce oxidative stress, disrupt enzymatic pathways, and cause the production of free radicals, ultimately leading to trophozoite death [53,54]. This explains the reduced number of trophozoites in culture. Silver nanoparticles can physically damage the lipid bilayer and inhibit DNA synthesis in cells in a concentration-dependent manner [55,56]. Damage to the cytoskeleton and DNA leads to autophagy and the activation of p53-dependent apoptosis pathways. Meanwhile, damage to mitochondria induces apoptosis demonstrating a cytotoxic effect [57]. In our study, we observed changes consistent with autophagy and ongoing apoptosis at the subcellular level. These changes included vacuoles engulfing cytosolic organelles, but were not so intensive as exposed to green tea amoeba.

The efficacy of silver nanoparticles depends on their size. Greater membrane damage and higher apoptosis rates in bacteriahave been observed for AgNPs with a diameter of 10 nm compared to larger particles [58]. In our study, the amoebae's response was not strongly dose dependent, as their response appeared later, which could be due to the different diameters of silver nanoparticles. The diameter was 30 nm for AgNPs, which has confirmed antimicrobial activity, and 60 nm for GT(AgNPs) [28,59]. The size of nanoparticles for antiparasitic treatment appears essential and requires further investigation

From the culture data we noticed that AgNPs exhibited anti-amoebic activity already at lower concentrations. In the long and stationary phases of culture, the amoebicidal activity of GT extract was lower than that of AgNPs at concentrations below 5000 μg/mL. GT(AgNPs) also did not increase the efficacy of the anti-amoeba treatment compared to the GT extract alone. Despite these evident destructive changes, the cytosol of the amoebae exposed to the GT extract and the GT(AgNPs) became less dense and was filled with many vacuoles, which are typical of intensive autophagy in *Acanthamoeba* trophozoites. The amoebicidal effect of green tea is evident and supported by subcellular analysis of *Acanthamoeba*. In contrast to AgNPs, green tea treatment reduced trophozoite size, inhibited acanthopodia formation, reducing the amoebae's adherence ability, and generation of osmotic vacuole caused by autophagy-like, or pseudocyst-like changes observed in earlier studies [60]. TEM analysis shows that mitochondria lost their tubular ultrastructure and became spherical. Although the function of mitochondria might decreased, the abundance of vacuoles containing ingested cellular particles increased. Similar changes were observed in studies investigating the cytotoxic effects of amphotericin on amoeba cell cultures [24,42]. We also found, that GT affected cellular ultrastructure in trophozoites, depleting cytoplasmic contents, and decreasing cytoplasmic density. Additionally, we observed cell swelling accompanied by severe plasma membrane alterations and membrane blebbing. Green tea may target cell membranes, thereby affecting the integrity of parasites. GT(AgNPs) induced the most pronounced changes, and as with previous treatments, no cyst formation was observed, which was also provided by Dickson et al., 2020 [46] for green tea extracts. Trophozoites ingested only few nanoparticles, but most were preferentially deposited in the cytoplasm during the early and late phases of culture growth. In the stationary phase, large vacuoles filled with granular cellular material appeared. Mitochondria were swollen and lost their tubular structure, and reduced in size. These findings suggested that the GT(AgNPs) were initially adsorbed onto the external trophozoite membrane during the early phase of culture, and were subsequently absorbed or transferred into the cytoplasm, inducing a strong toxic effect. This toxicity produced a high inhibitory effect on amoeba cultures at a greater concentration of green tea. The damage of cell membranes by lipid peroxidation may disrupt membrane permeability and compromises cell integrity [61,62]. High toxicity is associated with induced autophagy and destabilization of mitochondrial structures [16,63]. In our studies these effects were particularly notable for treatments involving GT extract or GT(AgNPs) where formation of phagocytic vacuole was not observed. However, nanoparticles were still absorbed into the cell cytoplasm, triggering toxic effects. Two compounds derived from the tea plant (*Camellia sinensis), i.e.*, epigallocatechin-3-gallate and caffeine, significantly inhibited trophozoite replication and encystation [23,24].

The antioxidant catechins in green tea may affect the stability and bioactivity of silver nanoparticles [64]. What is interesting in our study, GT extract appeared to promote regeneration of the amoeba population during the stationary phase of amoeba growth in culture. AgNPs may also induce lipid peroxidation [61,62], and therefore, damage to many subcellular structures was evident in our study. However, GT in some extent attenuates this effect [65]. In comparison to GT extract alone, lower toxicity was observed in the low concentrations of GT(AgNPs), where phagosomes contained only a small number of nanoparticles coated with green tea. GT coating the AgNPs may attenuate the cytotoxic effect of silver ions [66]. These observations require confirmation at the molecular level, including evaluation of phagocytic enzyme activity.

Due to *Acanthamoeba castellanii* unique life cycle, amoebae exhibit resistance to several clinical drugs [67]. Given the amoebicidal activity of GT(AgNPs), it was valuable to compare their activity with that of amphotericin B. Interestingly, amphotericin B is a compound that shows an increase in IC_{50} over time with prolonged treatment, which also was observed with GT treatment. The antibiotic exhibits some activity against *Acanthamoeba* by binding to ergosterol—the primary sterol in the amoeba plasma membrane—resulting in pore formation and subsequent cell death [42]. The impact of green tea constituents on cell membrane properties during anti-amoebic treatment also seems to be critical and requires further investigation. These findings highlight the multiple mechanisms through which green tea-coated nanoparticles affect cell membrane integrity, leading to cytotoxic effects.

Our study suggests that the cytotoxic effects of nanoparticles and green tea extract operate through different mechanisms. Three genes involved in the phagocytosis process in *Acanthamoeba* were monitored for expression: vacuolar-type H+-ATPase, Rab1/RabD family small GTPase, and cysteine proteinase II [33,68,69]. Only the cysteine proteinase II gene expression was significantly increased in amoeba trophozoites treated with green tea extract, while the expression of other genes tested was not changed. AgNPs, either alone or coated with green tea, did not change the expression of any of the investigated genes. The function of the cysteine proteinase II we studied are not well understood, apart from its described role in digesting the contents of phagolysosomes [33]. Another study showed that other Acanthamoeba cysteinę protease is present in large organelles and is crucial in the early phase of encystment [69]. This can suggest that upregulation of protein cysteinase II following the treatment with GT extract could be a defense mechanism and could be related to encystment initiation. However, it would be

necessary to investigate whether the specific cysteine proteinase II we studied also participates in encystation. In GT(AgNPs)-treated amoebae the cysteine proteinase II gene was not upregulated. Possible explanation, supported by microscopic images is that the cellular damage was too severe to initiate the encystment process. Determining whether a particular cysteine proteinase also plays a role in susceptibility to silver nanoparticles and green tea compound requires further research.

The results highlight the complex interactions and effects of silver nanoparticles and green tea extract. Both treatments as the bioactive compounds in green tea and silver nanoparticles, leading to increased cellular damage and unchanged gene expression, except cysteine proteinase II, which expression was elevated by GT extract alone.

5. Conclusions

Silver nanoparticles were phagocytosed by *Acanthamoeba* trophozoites and exerted toxic effect on the parasite. Nanoparticles were engulfed and kept in amoeba vacuoles raised subcellular destruction probably by over loading with silver nanoparticles which resulted in dying of amoeba. Green tea may affect cell membrane stability, leading to amoeba death. Destructive changes were preferentially identified as affecting mitochondria structure and autophagy before amoeba death. As the most destructive over a prolonged period of culture, the treatment with GT(AgNPs) resulted in strong vacuolization, decreased cytoplasmic density, autophagosome formation, swelling of mitochondria, and disorganization of nuclear structure with chromatin dissolution. Based on ultrastructure TEM analysis we established that GT(AgNPs) exhibited distinct toxic effects compared to AgNPs alone. Moreover, GT extract was also as active as GT(AgNPs) and additionally caused a significant increase in expression of the cysteine proteinase II gene, which is also involved in the digestion of phagolysosome content.

Because of the evident cytotoxic effect exerted by 5mg/mL concentration of all compounds, it seems, that all treatments may be used topically or in vitro. Although the IC50 values observed in this study (5–10 mg/mL) are relatively high and unlikely to be achievable through systemic administration, the intended use of GT(AgNPs) and related formulations is primarily for topical or localized applications. For instance, due to the environmental persistence of *Acanthamoeba*, such formulations may be useful in surface disinfectants, contact lens solutions, or water treatment systems, where high local concentrations can be safely deployed. Additionally, the demonstrated antimicrobial and antiparasitic properties of GT(AgNPs) suggest potential for inclusion in hydrogel dressings or topical sprays, enabling effective local delivery to wounds or skin surfaces. These application modes mitigate the need for systemic exposure while leveraging the high local efficacy observed in vitro.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/25081509 31047511/ParSci-1209-revised-1-SI.zip.

Author Contributions

Conceptualization: M.D., L.S.; methodology: M.D., L.S., J.N., M.W., J.P.; validation: M.W., M.G., M.D., L.S., J.N.; formal analysis: M.W., J.P., M.D.; investigation: M.W., M.G., J.N., L.S., M.D.; resources: J.P., M.D.; data curation: M.W., L.S., J.N., M.D.; writing—original draft preparation: J.P., M.W., M.D., L.S.; writing—review and editing: M.W., J.P., L.S., M.D.; project administration, J.P.; funding acquisition: J.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The raw data required to reproduce these findings are available from the Supplementary Materials.

Conflicts of Interest

The authors have declared no competing interests.

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