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Original paper

Evaluation of the inhibitory impact of biosynthesized silver nanoparticles using *Bacillus cereus* and *Chromobacterium violaceum* bacteria on some intestinal protozoa

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ABSTRACT. Nano materials are utilized to improve the performance of some pharmaceuticals and materials, as well as to lessen the collateral damage they cause. The purpose of this study was to look at the effect of silver nanoparticles (AgNPs) produced by bacteria on some trophozoites of intestinal parasites. The silver nanoparticles were synthesized using *Bacillus cereus* (BAgNPs) and *Chromobacterium violaceum* (ChAgNPs) bacteria. The AgNPs production was confirmed by several tests and techniques, such as electron microscopy. The results of the analysis showed that the size of these particles was within the range of 21–96.71 nm for both BAgNPs and ChAgNPs. *In vitro* and *in vivo* efficacy were tested on some trophozoites of intestinal parasites. The effect of the AgNPs on *Entamoeba histolytica* trophozoites in culture was significantly higher compared to metronidazole. The highest percentage of inhibition was 70.6% and 76.5% for the particles prepared from *B. cereus and C. violaceum* without significant differences between the two bacteria, compared to 57.6% inhibition for metronidazole. The *in vivo* effect of the AgNPs on *Giardia lamblia* exceeded that of metronidazole and led to the total disappearance of the stages from mice faeces after 3–4 days. Likewise, *Tritrichomonas muris* numbers were also reduced in infected mice treated with AgNPs, with the highest inhibition rate of 81.3%. From above can concluded these bacterially produced nanoparticles have proven strong efficacy, and it is possible to recommend their use independently after conducting studies on the extent of their effects on the body and proving their safety.

Keywords: inhibitory impact, silver nanoparticles, bacteria, intestinal protozoa

Introduction

Intestinal parasitic infection is one of the diseases that the human race has suffered from and still suffers from. Parasites cause great harm to humans and their domestic animals and lead to great deaths and huge economic damage [1]. Intestinal inflammation and dysentery diarrhea are the main symptoms caused by intestinal parasites, which may be caused by some of the most common parasites such as *Entamoeba histolytica*, *Giardia lamblia*, *Cryptosporidium parvum,* and a dozen other parasites [2]. Parasitic diseases were not excluded from the researchers' interest in trying to prevent or treat them, especially after the emergence of cases of drug resistance and its increase in recent times, which necessitated a tendency to find new treatments [3–5]. The application of nanotechnology has increased in a variety of fields, including medicine and therapy, where the focus is on drastically altering a material's qualities by altering the number of its atoms or molecules [6]. Nano materials are used to improve the performance of some materials and drugs or reduce their collateral damage. In the medical field, nanotechnology is versatile and widely used to diagnose, treat, or produce vaccines [7]. Many elements are used in their nano forms, such as gold, silver, platinum, carbon and many others, but the use of silver has captured the attention of many researchers and occupied an important place in

research paths because of its stable properties in water and air, non-oxidation, high chemical effectiveness, morphology of particles, larger surface expanse and ion release efficiency [8,9]. It is widely used in many therapeutic areas for medical parasites; it is used in the treatment of parasitic protozoa and worms. Of these, in experimentally infected animals, a combination of silver, cesium (Cs), and curcumin NPs produced the best results and a complete cure for giardiosis [10]. Also, a combination of silver and Cs for *in vivo* toxoplasmosis treatment resulted in a considerable reduction in parasite burden in the liver and spleen. Tachyzoites were found to have stopped moving and to have deformed shapes [11]. Likewise, the number of trophozoite stages following treatment with AgNPs and metronidazole was significantly reduced [12,13]. Investigation of the activity of Ag and CuO nanoparticles against *Entamoeba histolytica* and *Cryptosporidium parvum*, both nanoparticles showed a considerable reduction in cyst viability [14]. Many other studies dealt with the therapeutic aspects of silver nanoparticles against *Panagrellus redivivus* worm, trypanosomosis, malaria, leishmaniosis, echinococcosis, fasciolosis, schistosomosis [15–21]. Due to a dearth of research on the therapeutic effects of silver nanoparticles on intestinal protozoa as well as the emergence of medication resistance instances and the need for alternative or derivative therapies, we conducted this study to see how silver nanoparticles affect specific intestinal parasites.

Materials and Methods

Study design

This study was designed to assess the pharmacological activity of silver nanoparticles biosynthesized using *Bacillus cereus* and *Chromobacterium violaceum* bacteria on *E. histolytica in vivo* and *in vitro*. The study plan was altered and adjusted when the part of generating infection in laboratory mice was abortive. The objective of the study became to know the effect of the nanoparticles on some trophozoites of intestinal protozoan parasites after it was on *Entamoeba*.

Identification and isolation of the parasitic stages

The parasites were isolated from stool samples of outpatients at the General Pediatric Hospital in Kirkuk, Iraq, who had diarrhea. Microscopic inspection of stool samples based on general characteristics of each parasite revealed the existence of cysts and trophozoite stages of *Entamoeba histolytica*/*dispar*, *Entamoeba coli*, and *Giardia lamblia*, as well as the presence of RBCs in *Entamoeba histolytica* trophozoites. When the samples were positive for *E. histolytica* trophozoites containing red blood cells, the patient was instructed to give another fresh sample for transplantation. The culture was carried out by implanting 1–2 loops of the mucous component of the sample directly into the prepared medium. Using the concentration sedimentation approach, the cystic stages of all the parasites were separated from human or mouse faeces samples.

Samples collection for bacterial isolates

A set of soil samples (105) were selected in Kirkuk governorate, which included 28 samples from Laylan in regions surrounding the Khasa River and near the Industrial District, 15 samples from Laylan farm and 18 samples from Kirkuk gardens at Kirkuk University. An additional 33 water samples were collected from the Khasa river and 11 other samples from the water that is used to irrigate the Laylan farms. Samples were collected in sterile plastic bottles for the period from 1 September 2019 until 2 February 2020.

Isolation and identification of Bacillus cereus and Chromobacterium violaceum

The methods were adopted by [22,23]. One (1) g of soil or 1 ml of water samples were measured and placed in a test tube containing 9 ml of physiological salt solution. This was considered as a concentration of 10^{-1} and dilution was conducted for it up to the concentration of 10^{-6}. Taken 0.5 ml of each concentration was taken and placed in sterile culture dishes, and the nutrient agar was poured over it after cooling the medium to a temperature of 40–45°C. The dishes were left to solidify and then incubated at a temperature of 30°C for 24–48 hours. The growing bacteria were sub cultured to obtain single pure colonies. The isolated bacteria were diagnosed based on culture, microscopic characteristics, and biochemical tests mentioned in [24]. Pure bacterial colonies were taken and grown on the nutrient agar medium to determine the culturing characteristics of the bacterial isolates in terms of the shape, height, color, and texture of the growing colonies. The microscopic examination was carried out by preparing a smear from the developing colonies on

the surface of a glass slide and staining it with gram stain to identify the shape and color of the bacterial cells and their interaction with the dye. The endospore test was done using the [25] technique. Biochemical tests (Catalase, oxidase, IMViC, Protease), the ability of bacteria to decompose blood, motility tests, and the Vitek 2 compact system were used for the identification of bacteria. Since it was not possible to isolate *C. violaceum* bacteria from soil and water samples collected from different places in Kirkuk, which amounted to 105 samples, the global standard strain of *Chromobacterium violaceum* code NO. NCTC9757 obtained from the National Intercultural Association in Britain was used. And it was re-grown on Luria Brittany agar and blood agar medium. It was diagnosed using the API-20E (Biomerieux) diagnostic system, which consisted of 20 biochemical tests to ascertain the purity of the isolate [26].

Synthesis tests for silver nanoparticles
Extracellular biosynthesis of silver nanoparticles using B. cereus and C. violaceum

For the preparation of silver nanoparticles [27] route was adopted with some modifications. Principally, bacterial culture supernatant was prepared as follows: 250 ml conical flasks containing 150 ml of sterile nutrient broth medium were inoculated with bacterial isolates under aseptic conditions. The flasks were incubated in a shaking incubator at 150 rpm at 30°C for 48 hours. After the incubation period, the bacterial cultures were centrifuged at 6000 rpm for 30 minutes. The culture supernatant for each isolate was separately collected in sterile flasks for use in the biosynthesis of silver nanoparticles.

Preparation of silver nitrate solution

Taken 0.169 g of silver nitrate ($AgNO_3$) was dissolved in 1 liter of distilled water to obtain a 1 mM silver nitrate solution. The solution was sterilized with 0.22 nm microfilters, placed in dark glass bottles and kept at 4°C until use [22].

Preparation of silver nanoparticles

In 250 ml conical flasks, 10 ml of bacterial culture supernatant for each isolate was mixed separately with 90 ml of silver nitrate solution. The flasks were incubated in a shaking incubator at 120 rpm and at room temperature. The supernatant of bacterial cultures for each isolate was also incubated without adding silver nitrate solution and silver nitrate solution alone as controls. The color changes of the samples were monitored at different intervals (3, 6, 12, and 24) hours as an initial evidence for the synthesis of silver nanoparticles, which occurs as a result of the reduction of silver ions (Ag^{+}) to silver atoms (Ag) in the presence of an aqueous enzymatic extract in the supernatant of the bacterial isolates [15]. This was considered as a stock solution for silver nanoparticles (*B. cereus* BAgNPs and *C. violaceum* ChAgNPs). The dilutions were prepared with distilled water or with Locke's solution to obtain the required concentrations for therapeutic experiments.

Analysis of the properties of silver nanoparticles

Several devices were used to analyze the properties of the silver nanoparticles under study, depending on the [28,29] method. The formation of silver nanoparticles was detected by studying the optical properties of the prepared particle solutions using an UV-visible spectrophotometer with a precision of 1 nm. The device was standardized first with sterile distilled water. Two ml of silver nanoparticle solution was put into a quartz cuvette tube of the device. The absorbance was measured at 200–900 nm. This analysis was carried out in the laboratories of the Department of Chemistry/ College of Science/University of Kirkuk, using a UV-visible spectrophotometer of the type (PG Insteruments T92, British).

Fourier transmission infrared spectroscopy (*FTIR*)

This assay was conducted to identify the functional groups present in the supernatant solution of bacterial cells that are responsible for reducing silver nitrate to silver nanoparticles within the range of 450–4500 cm^{-1} and with a resolution of 4 cm^{-1}. The silver nanoparticle solutions were centrifuged at 1200 rpm for 30 minutes, the filtrate was discarded, and the precipitate was washed with distilled water three times. Then the precipitate was dried in an oven at 45°C for 24 hours. After that, a disc was made from each sample with potassium bromide (KBr) after grinding it well and placing the disc in its designated place in the apparatus. This test was carried out at Tehran University in the Islamic Republic of Iran using the FTIR apparatus.

Microscopy analysis

SEM was conducted to analyze and characterize the surfaces of silver nanoparticles and to determine their dimensions by directing a beam of electrons

onto the surface of the particles. This examination was conducted at Tehran University in the Islamic Republic of Iran, using a SEM device of the type (NOVA Nano SEM 450). TEM was used to determine the shape, size and crystal phase of the banned silver nanoparticles at a voltage of 100 kV. This examination was conducted at Tehran University in the Islamic Republic of Iran using a Philips device**.**

The inhibitory activity of silver nanoparticles against isolated parasites

In vivo inhibitory activity of silver nanoparticles

Male or female albino (Balb/c strain) mice, weighing 25–30 g, were chosen to examine the silver nanoparticle's effect *in vivo*. Mice reared under proper conditions were brought from Kirkuk University's College of Science's animal house. Mice were infected with *Giardia* or *Tritrichomonas* using a thousand cystic stages or cysts like that were given to them orally. *Tritrichomonas muris* cyst-like samples were obtained from the research laboratories of Kirkuk University. Three different concentrations (25, 50, and 75%) of silver nanoparticles were prepared. 0.1 ml of each concentration of silver nanoparticles was given to mice three times daily for five days (six mice for each concentration) [30]. The excretion patterns of the parasites in mice's faeces were checked daily to track the trophozoites and cyst excretion. The average number of cysts and trophozoite stages counted in 30–50 high magnification fields was calculated. The inhibition ratio was determined as (control group – treated group/control group x 100). Trypan blue at 0.4 percent was used to test viability. The control groups were given 0.1ml of metronidazole at a concentration of 0.8 mg/kg as a positive control and a group that was given the parasite without any therapy as a negative control.

In vitro inhibitory activity of silver nanoparticles

Culture media preparation

The Locke-Egg-Serum (LES) Medium of Boeck and Dr. Bohlav was used for cultures [31]. Initially, Locke's solution was prepared by melting (9.0 g NaCl, 0.2 g $CaCl_2$, 0.4 g KCl, 0.2 g $NaHCO_3$, and 2.5 g glucose) in 1 liter of distilled water. The solution was filtered with Whatman No. 1 paper before being autoclaved for 15 minutes at 121°C and 15 lb/in^2 pressure. To make the solid face of the culture, four cleaned eggs were rubbed with 70% alcohol. The eggs were cracked and mixed with 50 ml of Locke's solution in a sterilized container and shaken until thoroughly melted. The homogenized solution was then divided into 2.5 ml sterilized test tubes, with bubbles being released from the tubes using a disposable loop. After that, the tubes were plugged and placed in an oven at 70°C in a gradient of 1–2 cm for the solidification of the media. Finally, each tube was re-autoclaved with 4 ml of Locke's solution. The damaged medium was discarded, and the good one was stored in the refrigerator until it was needed [31]. Each tube was inoculated with 0.5 ml of inactivated human serum from a healthy individual, 0.05 ml of stock antibiotic solution (100 U per ml of penicillin and 100 g per ml of streptomycin), and a loopful of sterilized starch. All of the procedures were carried out in a sterilized environment.

Culturing of E. histolytica

Approximately ten tubes of culture media were inoculated with bloody mucoid diarrheic fresh stool samples from outpatients diagnosed with *E. histolytica* trophozoites after being warmed in an incubator at 35°C for 1 to 2 hours [32]. In each tube, two loops of mucus were inoculated. The tubes were subsequently incubated at 37°C for 48–72 hours in a candle jar under microaerophilic conditions. To remove the adhering trophozoites from the tube walls at the logarithmic phase, the cultures were chilled in ice water upright for a few minutes. The presence of trophozoites was determined by microscope screening of 0.05 ml of the liquid component from the tube bottom mixed with 0.05ml of PBS. Sub-culturing every two to three days was used to keep positive cultures going.

In vitro evaluation of the inhibitory activity of silver nanoparticles in culture media

To achieve the necessary concentrations of (25, 50, and 75%), the desired volumes of the silver nanoparticles stock solution were mixed with Locke solution. Three tubes of culture media were added with inactivated human serum, antibiotics, and starch for each concentration. After counting with a hemacytometer, approximately 5000 trophozoites of *E. histolytica* were transplanted. Incubation of the implanted tubes was performed as stated in the above step. The viability of the trophozoites was determined after (24, 48, 72) hours of exposure to 0.1 l of silver nanoparticles of each concentration by calculating alive and dead cells in 30–50 microscopic fields using 0.4 percent trypan blue

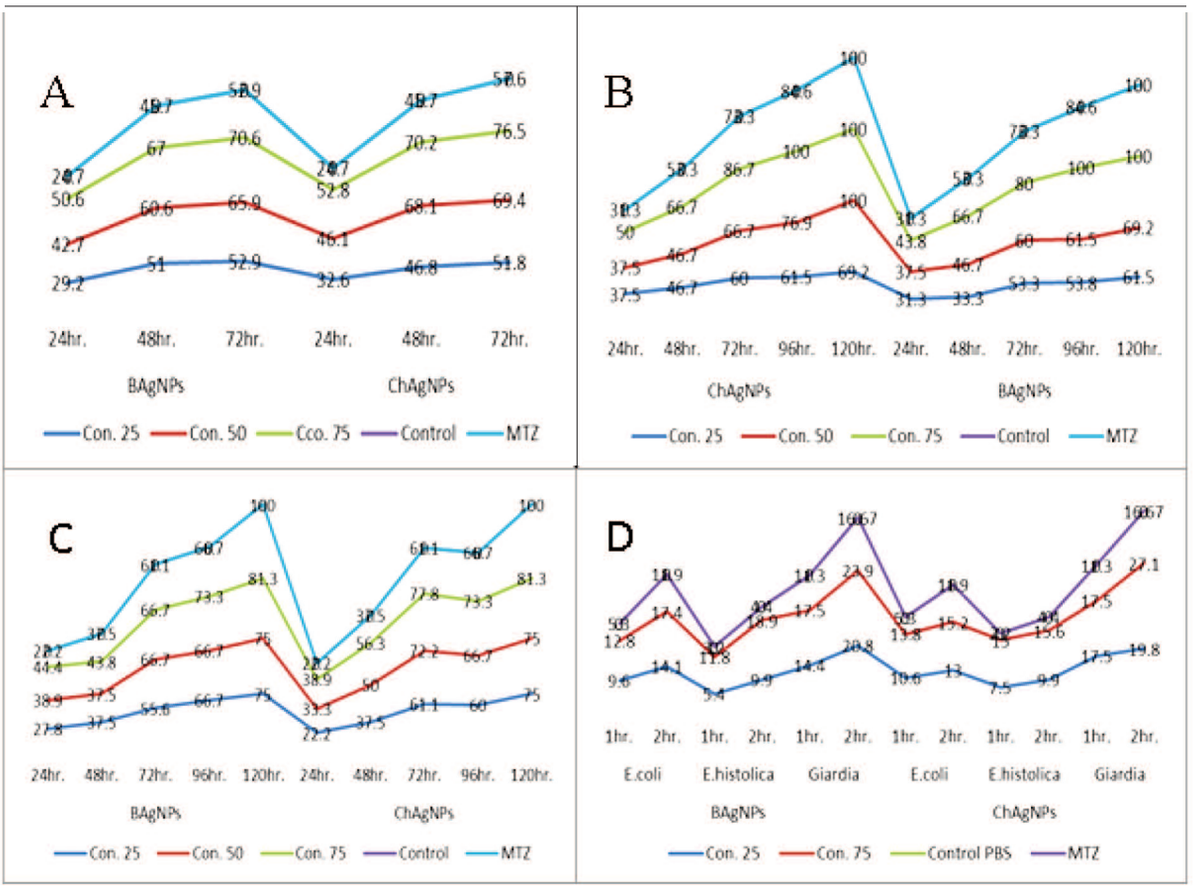

Figure 1. The inhibitory activity of silver nanoparticles against tested parasites, A (*in vitro* inhibitory effect of produced silver nanoparticles on *E. histolytica* trophozoites in culture), B (*in vivo* inhibitory effect of produced silver nanoparticles on *G. lamblia* in mice), C (*in vivo* inhibitory effect of produced silver nanoparticles on *Tritrichonas muris* in mice). The effect line of the control did not appear in the figures, as it did not include the percentage of inhibition

[33]. The percentage of alive cells (no. of alive cells/total alive and dead cells x 100) and inhibition rate (no. of alive cells in control group – no. of alive cells in treated group/no. of alive cells control group x100) of all concentrations were assessed. As negative and positive controls, 0.1 l of 0.8 g/ml metronidazole and cultures deprived of any additive were assessed. Each experiment was carried out in three replicates.

Statistical analysis

All data was analyzed using SPSS software. To find the discrepancies between the concentrations and the used times, ANOVA testes, one way and two ways without replication were used. $P \leq 0.05$ were considered significant.

Ethics approval

All research experiments and dealing with laboratory animals were carried out with the approval of the Ethics Document for Scientific Research and Dealing with Laboratory Animals with code (MUCEDLA-01) issued by Kirkuk University.

Results

Preparation of silver nanoparticles

An isolate from soil was selected to test its ability to form AgNPs using culture supernatant as a reducing agent and stabilizer in the process of synthesis of AgNPs. The positive result of AgNPs formation was inferred by observing the color changes of the reaction mixture. The results showed that the color of the supernatant solution of the bacterial culture gradually changed with the passage of time after adding silver nitrate, from pale yellow to brown. which was preliminary evidence for the formation of silver nanoparticles, and it was observed that the intensity of the color increased gradually and changed to dark brown after 24 hours. While there was no change in the color of the

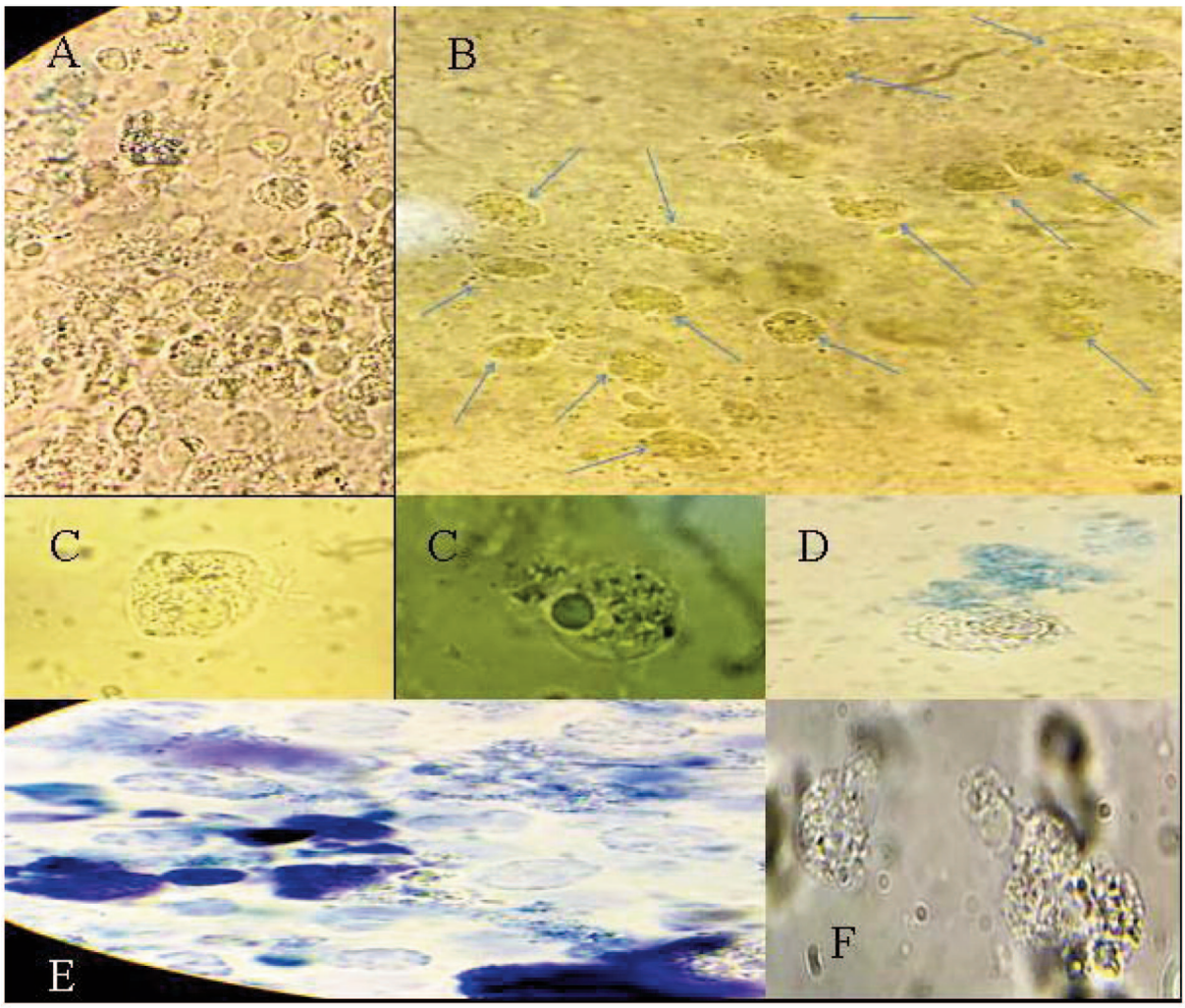

Figure 2. *E. histolytica* trophozoites: A (a mass of alive cells from culture at logarithmic phase, unstained, 400×), B (unstained alive after one day, 400×), C (unstained magnified trophozoite with obvious nucleus, 1000×), D (unstained alive trophozoite with dead one blue stained after one day, 1000×), E (unstained alive and dead stained trophozoites after two days, 400×), F (unstained dead degenerated cells after three days, 1000×)

control samples (silver nitrate solution alone and bacterial culture supernatant solution) that were incubated under the same conditions and for the same period of time.

Analysis of the properties of silver nanoparticles

In order to prove the formation of silver nanoparticles and their stability in the supernatant solution of the bacterial culture, a UV spectrometer was used to measure the absorbance spectrum, and the absorption peak was shown at 420–425 nm, which represents the absorbance peak of silver. This result indicates the biosynthesis of AgNPs particles. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to detect the surface phenotype and to determine the size and shape of B, Ch AgNPs particles. The SEM images showed the presence of multiple shapes of nanoparticles with the predominance of the spherical shape. The results of the analysis showed that the size of these particles was within the range of 21–82, 45.23–96.71 nm and a rate of 44.595, 66.30 nm for each of BAgNPs and ChAgNPs, respectively. In order to obtain more information about the shape and size of the formed silver nanoparticles, the particles were analyzed using TEM, and the results of the analysis showed the presence of different patterns of shapes with the predominance of spherical shapes, which was consistent with the results of the SEM analysis.

In vitro evaluation of the inhibitory activity of silver nanoparticles in culture media

The inhibitory result of silver nanoparticles on the parasites under experiment was significantly high, and in general, its effect increased with

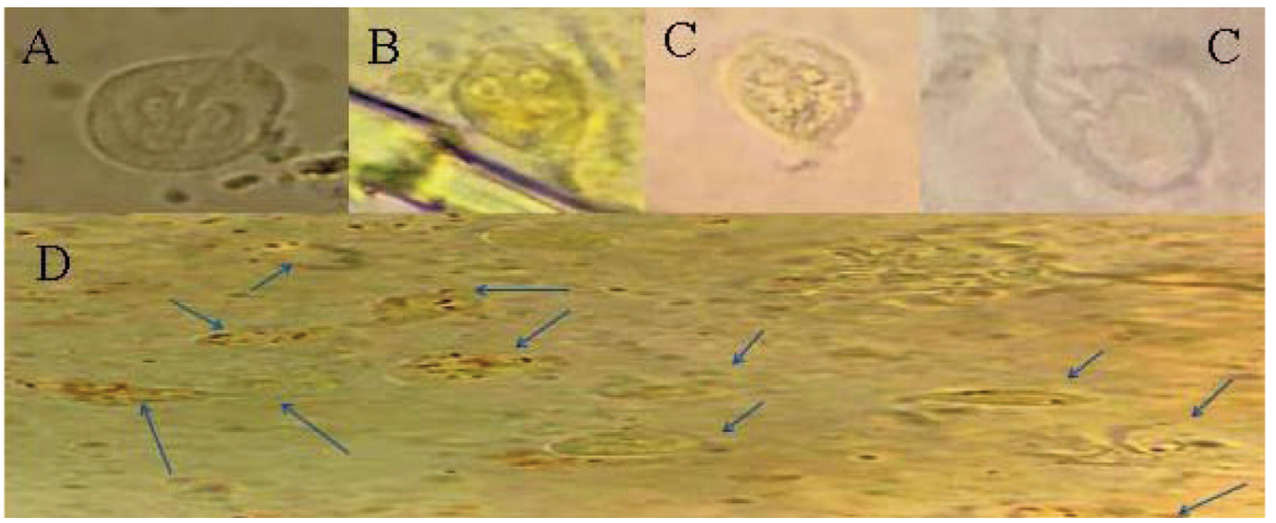

Figure 3. *Giardia* trophozoites: A (unstained alive after one day, 1000×), B (stained alive after two days, 1000×), C (stained dead after two days, 1000×), D (stained dead after three days, 400×), E (stained alive after one day, 400×)

increasing concentration and increasing time and period of exposure. The effect of the silver nanoparticles on *E. histolytica* trophozoites in culture was significantly higher compared to the positive control group represented by metronidazole and the negative control group. The highest percentage of inhibition was 70.6% and 76.5% for the particles prepared from *B. cereus* and *C. violaceum* without a significant difference between the two bacteria, compared to 57.6% inhibition for metronidazole after 72 hours of exposure (Fig. 1 and 2).

In vivo inhibitory activity of silver nanoparticles

The *in vivo* influence of the silver nanoparticles for the treatment of giardiasis exceeded that of metronidazole, with a significant difference between the bacteria under study. where the effect of particles prepared from *C. violaceum* was greater than that of *B. cereus*. And it led to the total disappearance of the parasitic stages from the faeces of infected mice after four days compared with metronidazole, which had an inhibition rate of 84.6% (Fig. 3). The effect of silver nanoparticles on *Tritrichomonas muris* in mice was less than its effect on *Giardia* and led to a significant reduction in parasites with a significant difference between the treatments and the control groups, although there was no significant difference between the particles derived from both bacteria. The percentage of inhibition ranged between 44.4 and 81.3 for a concentration of 75% for both bacteria (Fig. 4).

Discussion

The UV-visible spectrum of the formed AgNPs particles showed that the absorption peak was at 425 nm, which represents the absorption peak of silver. This result indicates the biosynthesis of AgNPs particles. These results were in agreement with a number of studies that have demonstrated the ability of *B. cereus* and other species of *Bacillus* to extracellularly synthesize silver nanoparticles using silver nitrate at a concentration of 1 mmol. The color of the reaction mixture changed first, and secondly, the absorbance peak of the surface plasmon in those studies ranged between 420 and 427 nm, which represents the absorption peak of silver nanoparticles [27,34–37]. In addition to confirming the surface morphology and determining the size and shape of AgNPs particles using SEM and TEM electron microscopy. The produced silver nanoparticles had strong action on studied parasites, with a significant difference compared to metronidazole. In general, the inhibitory effect of silver nanoparticles increased with increasing concentration, time and period of exposure. This was concordant with some other results where augmented exposure time resulted in higher fatality rates [14–16]. In our study, the effect of the silver nanoparticles on *E. histolytica* trophozoites in culture was significantly higher compared to metronidazole. The highest percentage of inhibition was 70.6% and 76.5% for the particles prepared from *B. cereus and C. violaceum* without a significant difference between the two bacteria,

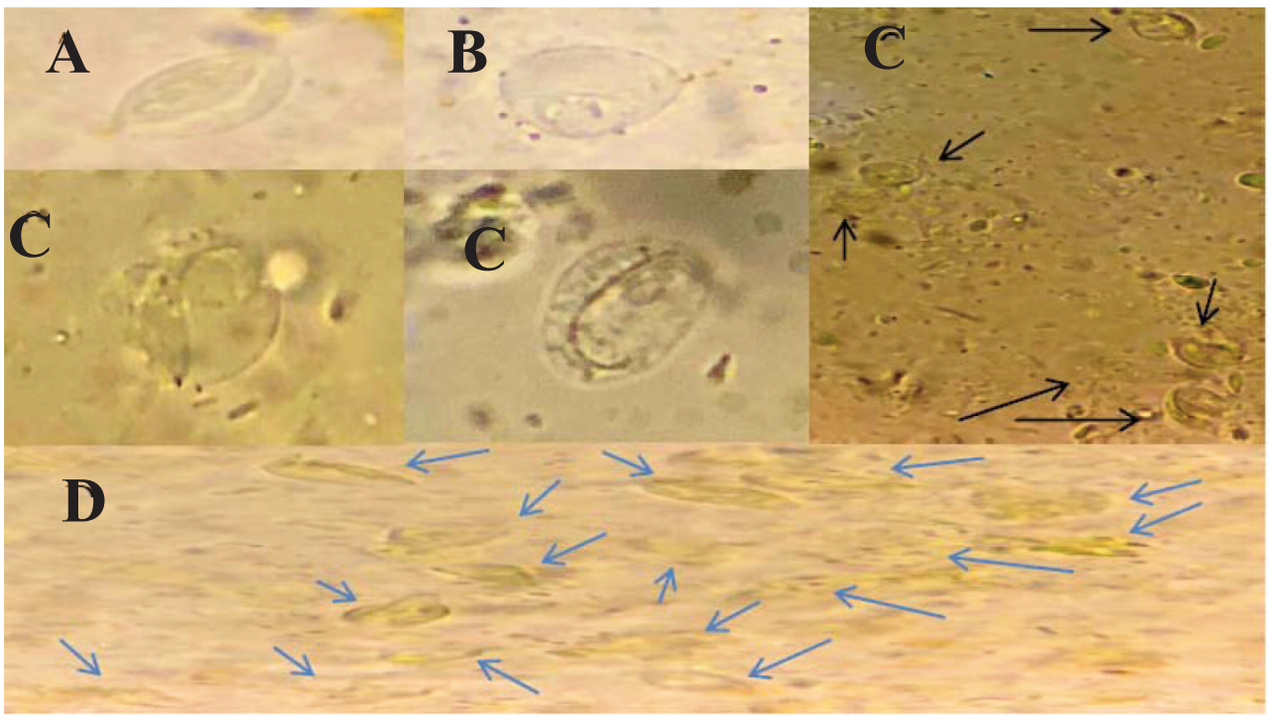

Figure 4. *Tritrichomonas muris* trophozoites: A (stained dead cell after two days, 1000×), B (stained dead cyst like stages after two days, 1000×), C (unstained alive before treatment,400×, the magnified trophozoite 1000×), D (stained alive trophozoites after one day, 400×)

compared to 57.6% inhibition for metronidazole. Moreover, the in vivo effect of the silver nanoparticles for the treatment of giardiosis exceeded that of metronidazole. And it led to the total disappearance and eradication of the parasitic stages from the faeces of infected mice after four days. Unlike *Giardia*, the effect of silver nanoparticles on *Tritrichomonas muris* in mice was less and led to a reduction in parasite numbers in infected mice. The percentage of inhibition ranged between 44.4 and 81.3, at 75% of the AgNPs produced by both bacteria. In general, the effect of the AgNPs was strong on the trophozoites of the intestinal protozoan parasite under study. Although there is no certain information about the impact mechanics of nanoparticles, according to some studies, it may be due to the ability of AgNPs to accumulate on the cell membrane, affecting its permeability and changing or destroying the internal systems of the microbe [38]. Furthermore, the dissociated silver ions from AgNPs might damage DNA or ATP [39,40]. Or with another explanation, the formed reactive oxygen species on nanoparticle surfaces may interact with respiratory enzymes or damage the plasma membrane, mitochondria and nucleic acids, causing microbes' death [41]. Silver and gold inorganic nanoparticles have been demonstrated to exhibit fascinating features such as antimicrobial activity and the potential to alter enzyme activities [15,16,40,41]. Even though during the review of the references, many studies were not actually found to show the effect of nanoparticles on intestinal parasites, only a few dealt with the effect of nanoparticles on giardiosis and amoebosis. There was a discrepancy between the results of studies on the therapeutic effects of nanoparticles, which ranged from strong total to partial or medium, and little effect. This disparity in the effect of nanoparticles between different studies may be due to the different concentrations used, the time and method of exposure, nanoparticle types, or the synthesis techniques of the nanoparticles. Among the researchers who have taken this approach, we include the following: a combination of silver, Cs, and curcumin NPs generated the best outcomes and a complete cure for giardiosis in experimentally afflicted animals [10]. *In vivo* toxoplasmosis treatment with a combination of silver and Cs also resulted in a significant reduction in parasite burden in the liver and spleen [11]. It was discovered that tachyzoites had stopped moving and had deformed shapes. The antigiardial activity of AgNPs was strong, but AgNPs and MTZ combination therapy was lower than that of the

untreated control group [12]. Similarly, after treatment with AgNPs and metronidazole, the number of trophozoite stages was dramatically reduced when compared to control [13]. The activity of Ag and CuO nanoparticles against *Entamoeba histolytica* and *Cryptosporidium parvum* revealed that both nanoparticles significantly reduced cyst survivability [14]. Several more studies focused on the medicinal potential of silver nanoparticles on parasitic diseases such as *Panagrellus redivivus* worm, trypanosomosis, malaria, leishmaniosis, echinococcosis, fasciolosis, schistosomosis [15–18]. It was concluded from the results of the current study that silver nanoparticles have a strong effect on the therapeutic aspect of protozoan intestinal parasites. Silver nanoparticles have demonstrated a high inhibitory activity against vegetative stage, and bacteria are good organisms for the biosynthesis of silver nanoparticles. We can recommend the use of synthetic silver nanoparticles for therapeutic experiments with other pathogens and also recommend conducting more extensive studies on the mechanisms of action of these nanoparticles and their toxic effects on the living body. For instance, their pharmacological properties could be an alternative or synergistic for future treatment of microorganisms.

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