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In Vitro assessment of biosynthesized silver nanoparticles effect on some intestinal protozoan cystic stages

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Abstract
Silver nanoparticles has received a lot of attention recently due to its certain features, and has been used in a variety of medical fields and treatments, therefore, the purpose of this work was to, ascertain how some cystic stages of intestinal protozoan parasites are affected by bacterial biosynthesized silver nanoparticles (AgNPs). The AgNPs were synthesized using *Bacillus cereus* and *Chromobacterium violaceum* bacteria. The AgNPs production was confirmed by several tests and techniques such as a UV-visible spectrophotometer, a fourier transmission infrared spectrophotometer (FTIR), and scanning electron microscopy (SEM), cysts of *Entamoeba histolytica/dispar*, *Entamoeba coli*, *Giardia lamblia* were obtained from human stool samples. The cystic stages were purified by density gradient sucrose solution and incubated with produced silver nanoparticles at (25, 75 µl/ml). With a significant difference between the two studied bacterial species, the biosynthesized silver nanoparticles significantly inhibited all cysts belonging to the study parasites. The effect of the silver nanoparticles produced by *C. violaceum* was less than the effect of the nanoparticles produced by *B. cereus*, especially on *G. lamblia* cysts. A significant effect of 23.9 % was noted for *Giardia* cysts, which was higher than the inhibition rates of 18.9 and 17.4 % for each of the cysts of *E. hitolytica* and *E. coli*, respectively. The inhibition rate was 16.67%, which was equal to the effect of metronidazole and higher than the effect seen with cysts of *E. coli* and *E. histolytica*. Based on the findings of the current study that silver nanoparticles have an inhibitory aspect on intestinal protozoan cysts, especially with larger doses than those used in the current experiment, silver nanoparticles use in treatment or decontamination procedures may be recommended.

Keywords: silver nanoparticles, biosynthesis, bacteria, protozoa cysts

Introduction
Intestinal protozoan parasites constitute a large proportion of human diseases and are accountable for high rates of illness and death, especially in certain groups of populations and countries [1]. The trophozoites and the cysts are the two stages of intestinal protozoa development. Cysts are the infective stage and people contract the infection via ingestion these cystic stages with contaminated food or drinks. Cysts are passed outside of the host's body, they are resistant to unfavorable environmental conditions and can remain viable for long period of time in water sources and soil [2]. Integrated treatment is one way to regulate and reduce the transmission of disease throughout communities. The majority of accessible therapies are antibiotics or chemicals, over the last years, nanoparticles have been introduced into therapeutic regions such as silver and other nanoparticles [3, 4]. Silver was used in ancient civilizations in many medical fields, and it has again attracted interest after it was converted to its molecular form recently [5]. Silver nanoparticles was used in many practical domains, including industrial, medical, diagnostic, and therapeutic, due to its distinctive and unusual properties [6, 7]. There are a number of ways to make nanoparticles, including physical, chemical, and biological processes, but it seems that the biological method is the one that researchers are most interested in. Numerous varieties of bacteria, fungi, and even parts and byproducts of various plants were used as sources of living creatures to reduce the ions of silver and produce nanoparticles [6, 8, 9]. The biological approach is a risk-free, non-toxic, and sustainable one. In addition to being straightforward and uncomplicated and not requiring complicated equipment, it is regarded as a green method [10]. Furthermore, as a positive aspect of the biosynthesized nanoparticles, the biologically manufactured silver particles have a higher solubility and stability than those manufactured by other methods [11]. The emergence of drug resistance in many pathogens has prompted researchers to think about possible alternatives to existing treatments, thus in this investigation we aimed to find out the effect of biosynthesized silver nanoparticles on some intestinal protozoan cystic stages.

Materials and Methods
Study design
This work was designed in Kirkuk city, Iraq. The purpose of this work was to biosynthesize silver nanoparticles using *Bacillus cereus* and *Chromobacterium violaceum* and to assess their *in vitro* pharmacological activity on some cystic stages of intestinal protozoan parasites.

Stool samples collection
As part of standard parasitological testing, stool samples were taken in a tidy plastic cup with a tide indicator. The samples came from outpatients at Kirkuk's General Pediatric Hospital who complained of diarrhea and gastrointestinal problems. The patients were given instructions on how to take the samples in accordance with the proper procedures for taking stools samples.

Identification of the parasitic stages
Microscopic examination using normal saline or iodine was done for all stool samples. Cysts existence of *Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Giardia lamblia* was confirmed based on general characteristics of each parasite such as size, shape, nucleus numbers and other special internal inclusions.

Isolation of the parasitic stages
The positive samples for *Entamoeba histolytica*/*dispar*, *Entamoeba coli*, *Giardia lamblia* cystic stages were processed for concentration method. All samples were first filtered using gauze layers to remove big artifacts then washed several times with normal saline. Finally the sediment was re-suspended in three ml of water and was added to 3ml of 0.85M sucrose sugar solution [12] and centrifuged for 10 min. at 2000 rpm. By using micro pipette the middle layer contains cysts at water- sugar contact was removed and washed thoroughly by centrifugation. The isolated cysts were added to a tube containing 3ml of intermittent density gradient of 0.85M and 0.4M sucrose and centrifuged, the middle layer cysts were removed, washed. The purified cysts were re-suspended in normal saline and processed for viability test [12].

Isolation and identification of *Bacillus cereus* and *Chromobacterium violaceum*
Bacillus cereus bacteria was isolates from soil samples. Based on culture, microscopic features, and the biochemical tests indicated in, the isolated bacteria were identified [13, 14]. While *Chromobacterium violaceum* was obtained from the National Intercultural Association in Britain, global standard strain with code NO. NCTC9757. The purity of the isolate was ascertained using the API-20E (Biomerieux) diagnostic system [15].

Biosynthesis and characterization of silver nanoparticles
90 ml of silver solution was combined with 10 ml of each bacterial culture's supernatant after silver nitrate (AgNO3) had been dissolved in distilled water and sterilized. As per [16] procedures, the mixture was incubated. At regular intervals, the mixtures' color changes were observed (silver nitrate and bacterial supernatant solutions in separated containers were revealed the controls). The created solution was used as a stock solution for silver nanoparticle solutions. To get the appropriate working concentrations, dilutions with regular saline were performed. Using a UV-visible spectrophotometer, a fourier transmission infrared spectrophotometer (FTIR), and scanning electron microscopy (SEM), the properties of the generated silver nanoparticles were examined in accordance with the procedures described in [16, 17].

The effect of the silver nanoparticles on *the cystic stages*
With major adjustments, the test was carried out utilizing the [2] technique. Initially, 1 ml of silver nanoparticles at (25, 75µl/ml) concentrations were incubated with roughly 1000 cysts of *E. histolytica*, *E. coli* and *G. lamblia* that had been isolated as in mentioned previous step. The cysts were washed three times with PBS after incubation for 1-2 hours, and then 0.1 ml of trypan blue was mixed with 0.1 ml of cysts sediment for 15 minutes. The alive and dead cysts were counted in 30-50 microscopic fields. The inhibition rate (control group – treated group/control group *100) and percentage of viable cysts (number of alive cells/total alive and dead cells *100) were computed. Controls included an incubation with metronidazole 0.8 mg/ml and PBS. Each experiment was carried out three times.

Statistical analysis
SPSS software was used to analyze all of the data. ANOVA tests, one-way and two-way without replication, were employed to identify any differences between the concentrations and the used times. P values under 0.05 were regarded as meaningful.

Results
Biosynthesis and characterization of silver nanoparticles
By observing the color changes in the reaction solution, the formation of AgNPs is evaluated. Over time, the color transitioned from light yellow to brown. This was the main indicator that silver nanoparticles had formed. despite the fact that the hue of the control solutions remained unchanged. The absorbance peak of silver is shown by the range from 420 to 425 nm. The SEM pictures revealed the presence of several nanoparticle forms, with the spherical shape predominating. The analysis's findings revealed that these particles had sizes between 21 and 82, 45.23 and 96.71 nm, and rates of 44.595 and 66.30 nm for BAgNPs and ChAgNPs, respectively.

***In vitro* evaluation of the activity of the silver nanoparticles on cysts viability**
With a significant difference between the two studied bacterial species, the biosynthesized silver nanoparticles significantly inhibited all cysts belonging to the study parasites (table 1), with a significant effect of 23.9 percent

for *Giardia* cysts, which was higher than the inhibition rates of 18.9 percent and 17.4 percent for each of the cysts of *E. hitolytica* and *E. coli*, respectively. Figures 1, 2, 3 indicates dead and alive cysts.

Table 1: *In vitro* evaluation of the biosynthesized silver nanoparticles by *B. cereus* on cysts viability

Parasite type						
Concentration µl/ml	**No. of viable *E. Coli* cyst with inhibition rate (%In)**		**No. of viable *E. histolytica* cyst with inhibition rate (%In)**		**No. of viable *G. lamblia* cyst with inhibition rate (%In)**	
	1h %In	2h %In	1h %In	2h %In	1h %In	2h %In
25	85 9.6	79 14.1	88 5.4	81 10	83 14.4	76 20.8
75	82 12.8	76 17.4	82 11.8	73 18.9	80 17.5	73 23.9
Metronidazole	89 5.3	81 11.9	90 3.2	86 4.4	86 11.3	80 16.67
Control saline	94	92	93	90	97	96

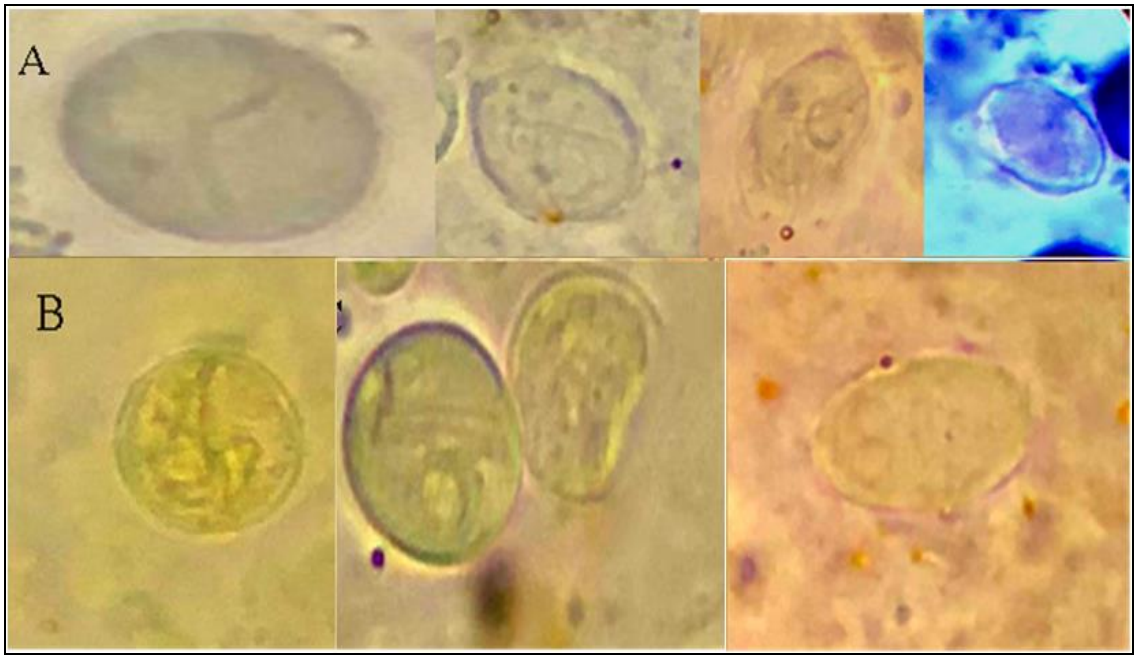

Fig 1: Samples of dead and alive *Giardia* cysts treated with silver nanoparticles s: A (dead cysts) , B (alive cyst), 1000X.

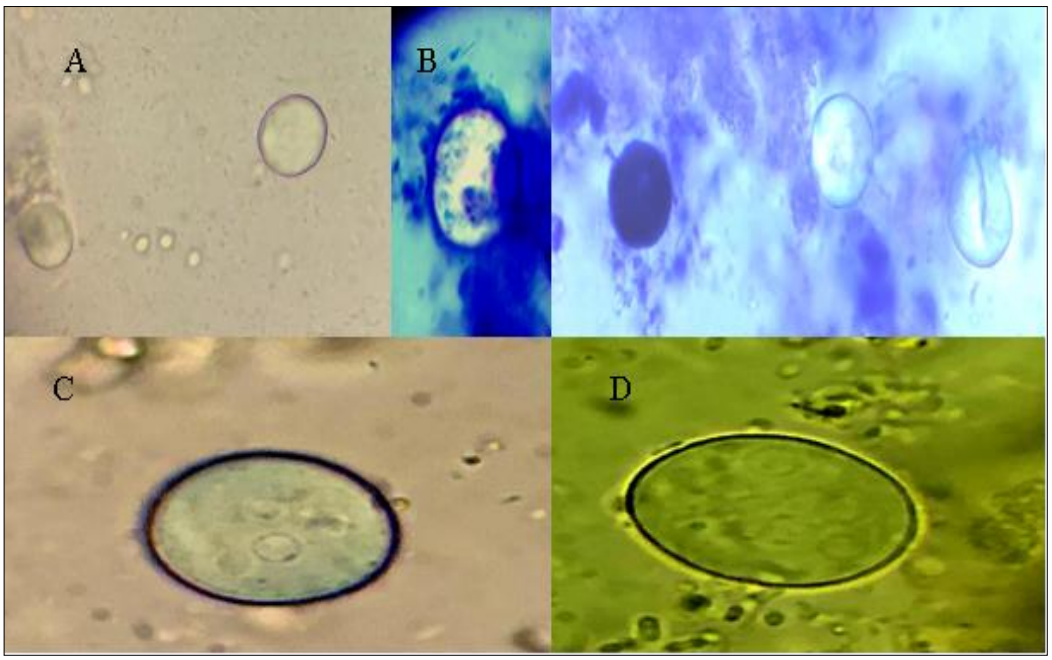

Fig 2: Samples of dead and alive *E. coli* cysts treated with silver nanoparticles : A (stained alive cysts after one hour, 400X), B (stained alive and dead cyst after two hours, 400X), C (stained alive cysts with obvious nuclei, 1000X), D (unstained alive cysts with obvious nuclei, 1000X).

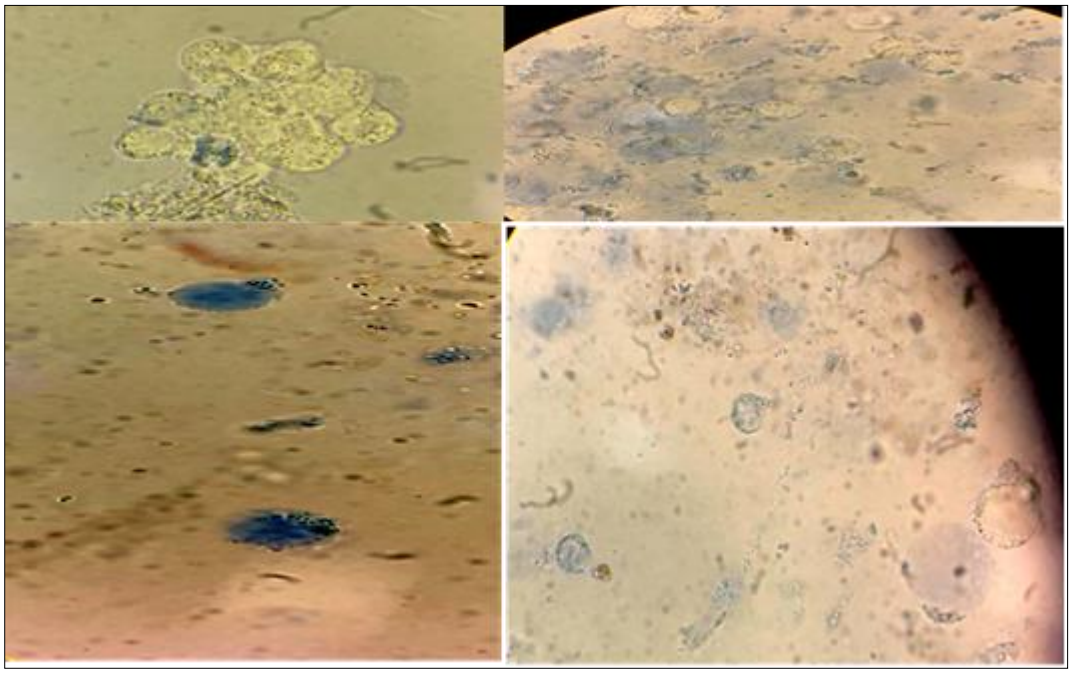

Fig 3: Samples of dead and alive *E. histolytica* cysts treated with silver nanoparticles: (stained alive and dead cysts 400X, blue stained refers to dead cysts and white stained refers to alive cysts).

According to Table 2, the inhibition rate was 16.67 at 75 µl/ml, which was equal to the effect of metronidazole and higher than the effect seen with cysts of *E. coli* and *E. histolytica*. The effect of the silver nanoparticles produced by *C. violaceum* was less than the effect of the nanoparticles produced by *B. cereus*, especially on *G. lamblia* cysts. Figures 1, 2, 3 indicates dead and alive cysts.

Table 2: *In vitro* evaluation of the biosynthesized silver nanoparticles by *C. violaceum* on cysts viability

Concentration µl/ml	**Parasite type**									
	No. of viable *E. coli* cyst with inhibition rate (%In)				**No. of viable *E. histolytica* cyst with inhibition rate (%In)**				**No. of viable *G. lamblia* cyst with inhibition rate (%In)**			
	1h	%In	2h	%In	1h	%In	2h	%In	1h	%In	2h	%In
25	84	10.6	80	13	86	7.5	81	10	80	17.5	77	19.8
75	81	13.8	78	15.2	79	15	76	15.6	80	17.5	70	16.67
Metronidazole	89	5.3	81	11.9	91	2.2	86	4.4	86	11.3	80	16.67
Control saline	94		92		93		90		97		96	

Discussion

The produced AgNPs particles' UV-visible spectra revealed that the absorption peak was at 425 nm, which corresponds to the absorption peak of silver. The biogenesis of AgNPs particles is demonstrated by this result were in line with several investigations that showed *B. cereus* and other Bacillus species could produce silver nanoparticles extracellularly. The surface plasmon's absorbance peak, which corresponds to the absorption peak of silver nanoparticles, ranged from 420 to 427 nm [16, 18, 19]. SEM and TEM electron microscopy were also used to quantify AgNPs particle size and shape in addition to confirming the surface morphology. When compared to metronidazole, the generated silver nanoparticles showed significantly stronger antiparasitic activity. With increasing concentration, exposure time, and exposure duration, silver nanoparticles' overall inhibitory effect grew stronger. This was consistent with some other findings [12, 20] where longer exposure times led to higher death rates. All of the parasite cysts included in this study were affected by the produced silver particles, with *Giardia* cysts experiencing a significant effect of 23.9 percent. This was higher than the inhibition rates for *E. coli* and *E. hitolytica* cysts, which were 18.9 percent and 17.4 percent, respectively. Even though the exact impact mechanics of nanoparticles are unknown, some studies suggest that the reactive oxygen species that develop on nanoparticle surfaces may interfere with respiratory enzymes or harm the mitochondria, nucleic acids, and plasma membrane, killing microorganism [21]. According to a different theory, DNA or ATP damage could result from dissociated silver ions from silver nanoparticles [22, 23]. They may be related to silver nanoparticles capacity to aggregate on cell membranes, which may modify their permeability and alter or destroy the microbe's internal systems [39]. Inorganic nanoparticles made of silver and gold have been shown to possess intriguing properties like antibacterial activity and the capacity to change enzyme activities [20-23]. Similar to our results, both Ag and CuO nanoparticles greatly decreased cyst survival when tested against *Entamoeba histolytica* and *Cryptosporidium parvum* [12]. Also the number of *E. hitolytica* was significantly decreased after

treatment with AgNPs and metronidazole [24]. We list the following researchers as having used this strategy: In experimentally affected mice, a mixture of silver, CS, and curcumin NPs produced the highest results and a full recovery from giardiasis [25]. Although AgNPs had a robust antigiardial effect, the combined therapy of AgNPs and MTZ had less of an impact than the untreated control group [26]. We may recommend using synthetic silver nanoparticles for their cysticidal properties in the sterilization of water and other environmental samples by assembling to chlorine particles to improve the sterilization process, especially for chlorine-resistant stages, based on the findings of the current study that silver nanoparticles have an inhibitory aspect on intestinal protozoan cysts.

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