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Assessment the Ag and ZnO Biosynthesized Nanoparticles effects on Giardia lamblia trophozoites which grown in HSP-1 culture media

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A B S T R A C T

Chemotherapy with drugs such as Metronidazole (MTZ) derivative products is currently the preferred treatment for giardiasis. However, these agents have been linked to a variety of negative side effects, varying from nausea to probable geno toxicity. Aim of this study to conduct an in vitro study on the efficiency of silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs) biosynthesized by green ecofriendly method using Pseudomonas aeruginosa and Escherichia coli against Giardia trophozoites. Methods Silver and Zinc oxide NPs formation was confirmed based on ability of selected bacteria to biosynthesized these NPs. The particle size arranges of (29.5 nm) for Ag P. aeruginosa and (32 nm) for Ag E. coli. Particle size of ZnO for P. aeruginosa was (25 nm) and (29.7nm) for E. coli. Giardia trophozoites cultivated on HSP-1 media were subjected to different concentration of biosynthesized NPs at (0.025, 0.050, 0.075 mg/ml) for (24, 48, 72 hrs.). Significant reduction ($P<0.05$) in trophozoite number was recorded by the two groups of Ag and ZnO nanoparticles for both bacteria and was 91% for Ag NPs for both *E. coli* and *P. aeruginosa* and 73% for ZnO for both bacteria at concentration of 0.075mg/ml after 72 hrs. Morphological difference appeared as destructive with the release of the cytoplasm outside. The cytotoxic effects of Ag and ZnO NPs on *Giardia lamblia* trophozoite exceeded that of metronidazole, these particles can be recommended for use, especially at higher concentrations that lead to total death rates.

Introduction

Giardia duodenalis (G. lamblia) is an enteric protozoan parasite that causes acute, watery diarrhoea or giardiasis in 280 million people each year and is a common cause of waterborne outbreaks [1]. Ingestion of infectious cysts in contaminated food or water is the most common method of parasite transmission [2]. The trophozoites can colonise as well as replicate in the host. small intestinal tract, especially the duodenum, and adhere to the human intestinal epithelium, specially the brush border, via a ventral disk as well as a specific receptor ligand.The encystation process happens since trophozoites migrate to the lower intestine, where they are released as infective cysts into the environment [3]. Metronidazole, Tinidazole, Albendazole and Nitazoxanide are the most commonly used antibiotics, but resistance to these drugs has grown in recent years [4]. More research on antibiotic resistance mechanisms and its use of drug combinations in this circumstance is critical [5]. Nanoparticles are now being considered as a substitute to antibiotics, with the potential to solve the problem of multidrug resistance in microorganisms. The biological method, which employs fungi, plants, or bacteria, is regarded as one of the most superior approaches to synthesis [6]. Use of nanotechnology for medical purposes has been named nanomedicine and is described as e applying nanomaterials for diagnosis, monitoring, control, prevention and treatment of diseases [7].

Zinc is a trace element that contains a variety of compounds with varying therapeutic activities [8]. Zinc oxide (ZnO) nanoparticles are a promising compound for use in the biomedical applications, thanks to their anticancer and antimicrobial properties [9]. Silver nanoparticles are also among the rare materials with special chemical and physical properties such as oxidation resistance and high heat resistance that are used in a variety of fields such as industry, health, and medicine. Anti-inflammatory, anti-cancer, antioxidant, anti-angiogenic and antimicrobial properties of silver nanoparticles have been linked to a variety of pharmacological properties. Prior studies have represented the antibacterial activities of silver nanoparticles on a wide spectrum of bacteria pathogens such as Escherichia coli, Candida species, and parasites such as Leishmania spp. and G. lamblia [10, 2]. Several studies in vitro have found that some inorganic nanoparticles have antibacterial property (such as metal and metal oxide). However, no report on drug resistance of microbes, particularly parasites, to nanoparticles has been published [11]. Thus the aim of this study was to find out the in vitro efficiency of Ag and ZnO nanoparticles biosynthesized by Pseudomonas aeruginosa and Escherichia coli against Giardia trophozoites.

The aim of current study examines the effectiveness of silver (Ag) and zinc oxide (ZnO) nanoparticles (NPs) biosynthesized by green ecofriendly methods using Pseudomonas aeruginosa and Escherichia coli against Giardia trophozoites in vitro.

Materials and methods:

Ethical approval:

The research was carried out in accordance with the ethical principles outlined in the Helsinki Declaration. Before taking the sample, the patient's verbal and analytical consent were obtained. To obtain this consent, a local ethics committee reviewed and approved the study protocol, subject information, and consent form using the document number including the number 3658 in 11/11/2021.

Parasite collection:

Diarrheic stool specimens were collected from Kirkuk General Pediatric Hospital from the period December 2021 to June 2022. Visual examination made before being examined under microscope, which includes its consistency, odor and color. Direct wet saline smear and light microscope was used to screen the presence of trophozoite in the specimens. Standard microbiology laboratory equipment was used, which included Microscopes, slides and cover slips. All media preparation materials, chemicals, and reagents were used to create suitable in vitro culture media as described in [12] with slightly modification, the positive samples for Giardia trophozoite were directly inoculated to culture media. Diagnosis was based on morphological characteristic under microscope by observation sucking disk, pear shape, elongated flagella and fall leaf motion [13, 14].

Preparation of Nanoparticle:

Zinc oxide Nanoparticles

The biological formation of zinc oxide nanoparticles was achieved by two types of bacteria E. coli and P. aeruginosa. The obtained nanoparticles were in particle size (25nm) and (29.7nm) from P. aeruginosa and E. coli respectively after characterization with XRD, SEM and TEM [15].

Silver Nanoparticles

Biosynthesis of silver NPs by using silver nitrate (AgNO3) solution. The silver nitrate-treated supernatant was evaporated to dryness at room temperature. The air dried biomass was investigated [16]. The obtained nanoparticle were in particle size (29.5nm) and (32nm) from P. aeruginosa and E. coli respectively after characterization with XRD, SEM and TEM. Different test concentrations of silver and Zinc oxide nanoparticles were prepared in deionized water (0.025, 0.050, 0.075 mg/ml) for each one and vortexing for five minute to demonstrate its effectiveness on Giardia trophozoite in vitro culture media [17].

In vitro assay

In order to assess the effects of Ag and ZnO nanoparticles in killing *G. lamblia* trophozoite. Three different concentrations were used (0.025, 0.050, 0.075 mg/ml) with various exposure times (24, 48, 72 hrs.). On the day of use, Culture media HSP-1 media that containing an autoclaved broth (BBL) Phytone peptone (1gm); Glucose (0.05gm); L-cysteine hydrochloride (0.15gm); Hanks balanced salt solution (85ml) and adjusted the PH at 6.7 then autoclaved 10 minute at a pressure of 15 lbs., and temperature at 121 ^{0}C, then finished by aseptically adding 15 ml of inactivated human serum (56 ^{0}C, 30 min) after filter sterilization. Culture media mediated by 50,000 units of potassium penicillin G and 0.05 g of streptomycin sulfate to 85 ml of sterile broth. In each tube (5ml) from media placed and stored at 4 ^{0}C until use for parasitic culture. Each test was run three times to ensure reliable outcomes and reduce the error rate. The volume of 50 µL (45 X 10^3 trophozoites) was transferred from the growing culture media into the wells of the micro plates. The first wells of the micro plates were set with control (untreated) as negative control and metronidazole as a positive control. 10µL of different concentration from treatment added to the rest of the wells. Eventually, the micro plates were transferred to the candle jar and incubated at 37 °C for 72 hrs. The wells were screened after (24, 48, and 72) hours, the parasite number was counted under the microscope using a Neubauer chamber. Data are reported as the number of parasites per square centimeter as determined by three counts each of three independent replicates using the following formula below, the data was collected and statistically analyzed [18, 19, 20, 21]

$$\text{Concentration (mg/ml)} = \frac{\text{Number of parasite} \times 10^4}{\text{squares counted}}$$

Statistical Analysis:

SPSS was used for statistical analysis software (ver.19, Chicago, IL, USA). ANOVA has been used to compare the results of two tests as well as control groups less than 0.05 values were deemed significant ($P< 0.05$).

Result

The samples collected were obtained during visited to Kirkuk General Pediatric Hospital and only positive sample for G. lamblia trophozoite were collected. The viability of trophozoite in examined samples for each patient was checked as appeared in figure 1. The trophozoite was used for in vitro culture in HSP-1 media. The parasite was cultivated successfully in the prepared media figure 1, 2 demonstrates the harvested trophozoites from cultures, and stained with trypan blue.

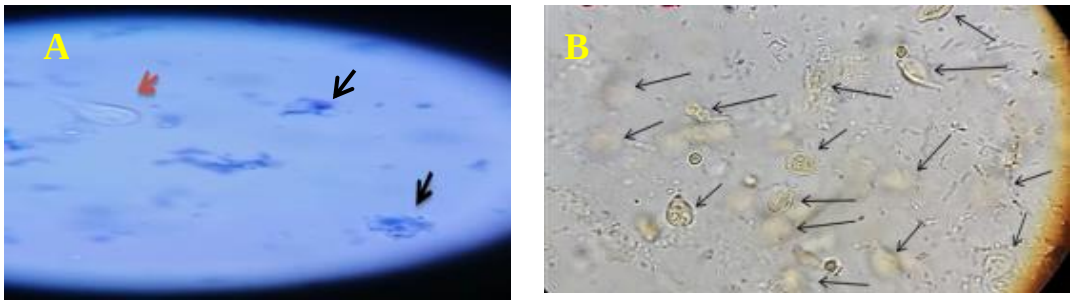

Figure 1. A: Viable trophozoite unstained with trypan blue stain (Red arrow). Disintegrated trophozoite stained with blue stain (Black arrow). B: *G. lamblia* throphozoites harvested from culture media 400X

Biosynthesized nanoparticles effect on the *Giardia* trophozoite in different concentration are illustrated in tables below.

Table 1. Direct proportion between concentration 0.025 mg/ml of Ag *E. coli* and Met. through 24, 48, 72 hours

0.025 mg/ml	Metronidazole	Ag *E. coli*	*P.* value for T. test
24 Hours	15000.0 ± 1000.0^{a}	6666.7 ± 577.3^{a}	**0.000**
48 Hours	12500.0 ± 556.8^{b}	6666.7 ± 763.7^{a}	**0.000**
72 Hours	8333.3 ± 763.7^{c}	4166.6 ± 288.6^{b}	**0.000**
***P.* value**	**0.000**	**0.003**	
LSD	**1587.2**	**1153.5**	

The table showed the significant difference between experimental times and illustrate the direct proportion in 0.025mg/ml concentration between the Metronidazole activity and time, in Ag E. coli that show a non-significant difference between 24 and 48 time hour but both significantly compared with 72 hours was scored the high activity, also a significant increase in the activity Ag E. coli compared to metronidazole at P. value < 0.05. Similar letters indicate the absence of significant differences and different letters indicate the presence of significant differences. Capital letters are to compare between treatments within a same concentration, while small letters are to compare between concentrations within the same treatment.

Table 2. Direct proportion between concentration 0.050 mg/ml of Ag E. coli and Met. through 24, 48, 72 hours

0.050 mg/ml	Metronidazole	Ag *E. coli*	*P.* value for T. test
24 Hours	11666.7 ± 763.7^{a}	5000.0 ± 300.0^{a}	0.000
48 Hours	8333.3 ± 1040.8^{b}	3333.3 ± 288.6^{b}	0.000
72 Hours	6666.7 ± 577.3^{c}	1666.7 ± 104.0^{c}	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**1631.3**	**495.0**	

In concentration of 0.050 mg/ml Metronidazole activity and Ag *E. coli* were increased significantly with increasing time through 24h at P. value <0.05. The results also showed a significant increase in the activity Ag E. coli compared to metronidazole at the same time.

Table 3. Direct proportion between concentration 0.075 mg/ml of Ag E. coli and Met. through 24, 48, 72 hours

0.075 mg/ml	Metronidazole	Ag *E. coli*	*P.* value for T. test
24 Hours	7500.0 ± 0.00^{a}	2500.0 ± 0.00^{a}	0.000
48 Hours	5833.3 ± 763.7^{a}	1666.7 ± 288.6^{b}	0.000
72 Hours	5000.0 ± 0.00^{b}	833.3 ± 144.3^{c}	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**881.0**	**372.2**	

In concentration of 0.075mg/ml there is a non-significant difference in Metronidazole activity in 24 and 48 time hours but significant with 72 time hours. In Ag E. coli which increased significantly with increasing time. The results also showed a significant increase in the activity of Ag E. coli compared to metronidazole at the same time at P. value < 0.05.

Table 4. Direct proportion between concentration 0.025 mg/ml of ZnO E. coli and Met. through 24, 48, 72 hours.

0.025 mg/ml	Metronidazole	ZnO *E. coli*	*P.* value for T test
24 Hours	15000.0 ± 1000.0[a]	6666.7 ± 763.7[a]	0.000
48 Hours	12500.0 ± 556.8[b]	6666.7 ± 288.6[a]	0.000
72 Hours	8333.3 ± 763.7[c]	5000.0 ± 0.00[b]	0.000
***P.* value**	**0.000**	**0.013**	
LSD	**1587.2**	**941.8**	

In treated group with ZnO E. coli showed a non-significant difference in between ZnO NPs treated group at 24 and 48 h but significant compared with the period at 72h, as well as the Metronidazole activity was increased significantly with increasing time. The results also showed a significant increase in the activity of ZnO E. coli compared to metronidazole at the same time at P. value < 0.05.

Table 5. Direct proportion between concentration 0.050 mg/ml of ZnO E. coli and Met. through 24, 48, 72 hours.

0.050 mg/ml	Metronidazole	ZnO *E. coli*	*P.* value for T test
24 Hours	11666.7 ± 763.7[a]	5833.3± 763.7[a]	0.000
48 Hours	8333.3 ± 1040.8[b]	3333.3 ± 288.6[b]	0.000
72 Hours	6666.7 ± 577.3[c]	1666.7 ± 104.0[c]	0.000
***P.* value**	**0.000**	**0.000**	
LSD	**1631.3**	**949.4**	

The results of the current study recorded the both Metronidazole activity and Ag E. coli were increased significantly with increasing time. The results also showed a significant increase in the activity ZnO E. coli compared to metronidazole at the same time as 24 h. at P. value < 0.05.

Table 6. Direct proportion between concentration 0.075 mg/ml of ZnO E. coli and Met. through 24, 48, 72 hours

0.075 mg/ml	Metronidazole	ZnO *E. coli*	*P.* value for T. test
24 Hours	**7500.0 ± 0.00[a]**	**2500.0 ± 0.00[a]**	**0.000**
48 Hours	**5833.3 ± 763.7[b]**	**1666.7 ± 288.6[b]**	**0.000**
72 Hours	**5000.0 ± 0.00[b]**	**1666.7 ± 144.3[b]**	**0.000**
***P.* value**	**0.015**	**0.002**	
LSD	881.0	372.2	

The results of the current study recorded a non-significant difference in both Metronidazole and ZnO E. coli activity in 48- and 72- time hours but significant with 24-time hours. The significant increase in the activity of ZnO. E. coli compared to metronidazole is at P. value < 0.05.

Table 7. Direct proportion between concentration 0.025 mg/ml of Ag P. aeruginosa and Met./ through/24,/48,/72/hours

0.025 mg/ml	Metronidazole	Ag *P. aeruginosa*	*P.* value for T. test
24 Hours	15000.0 ± 1000.0[a]	7500.0 ± 1250.0[a]	0.000
48 Hours	12500.0 ± 556.8[b]	5833.3 ± 971.2[b]	0.000
72 Hours	8333.3 ± 763.7[c]	5000.0 ± 0.00[b]	0.000
***P.* value**	**0.001**	**0.039**	
LSD	**1587.2**	**577.4**	

The results of the current study recorded the increased Metronidazole activity significantly with increasing time, in 0.025 mg/ml Ag P. aeruginosa, noted a non-significant difference between 48 and 72 time hour but both significantly compared with 24 hours. The results also showed a significant increase in the activity of Ag P. aeruginosa compared to metronidazole at the same time at P. value < 0.05.

Table 8. Direct proportion between concentration 0.050 mg/ml of Ag P. aeruginosa and Met./ through 24,/48,/72/hours

0.050 mg/ml	**Metronidazole**	**Ag *P. aeruginosa***	***P.* value for T. test**
24 Hours	11666.7 ± 763.7^{a}	5833.3 ± 971.2^{a}	0.000
48 Hours	8333.3 ± 1040.8^{b}	5000.0 ± 0.00^{a}	0.000
72 Hours	6666.7 ± 577.3^{c}	1666.7 ± 381.8^{b}	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**1631.3**	**1203.8**	

Ag P. aeruginosa in concentration 0.050 mg/ml was scored a non-significantly difference between 24 and 48 time hours but significant with 72 time hours. The results also showed a significant increase in the activity Ag P. aeruginosa compared to metronidazole.

Table 9. Direct proportion between concentration 0.075 mg/ml of Ag P. aeruginosa and Met./ through 24, 48, 72 hours.

0.075 mg/ml	**Metronidazole**	**Ag *P. aeruginosa***	***P.* value for T. test**
24 Hours	7500.0 ± 0.00^{a}	5000.0 ± 0.00^{a}	0.000
48 Hours	5833.3 ± 763.7^{b}	5000.0 ± 0.00^{a}	0.001
72 Hours	5000.0 ± 0.00^{b}	1666.7 ± 144.3^{b}	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**881.0**	**166.4**	

Table 10. Direct proportion between concentration 0.025 mg/ml of ZnO P. aeruginosa and Met./ through 24, 48, 72 hours

0.025 mg/ml	**Metronidazole**	**ZnO *P. aeruginosa***	***P.* value for T. test**
24 Hours	15000.0 ± 1000.0^{a}	10833.3 ± 1154.7^{a}	0.000
48 Hours	12500.0 ± 556.8^{b}	9166.7 ± 144.3^{a}	0.000
72 Hours	8333.3 ± 763.7^{c}	6666.7 ± 1040.8^{b}	0.000
***P.* value**	**0.001**	**0.004**	
LSD	**1587.2**	**1800.9**	

In Ag P. aeruginosa 0.075 mg/ml was scored a non-significantly difference between 24 and 48 time hours but significant with 72h was scored a high activity. The results also showed a significant increase in the activity Ag P. aeruginosa compared to metronidazole.

In ZnO P. aeruginosa concentration 0.025 mg/ml at 24 hour was scored a non-significantly difference between 24 and 48 time hours but significant with 72 h which scored a high activity. The results also showed a significant increase in activity ZnO P. aeruginosa compared to metronidazole at P. value < 0.05.

Table 11. Direct proportion between concentration 0.050 mg/ml of ZnO P. aeruginosa and Met./ through 24, 48, 72 hours

0.050 mg/ml	**Metronidazole**	**ZnO *P. aeruginosa***	***P.* value for T. test**
24 Hours	11666.7 ± 763.7^{a}	9166.6 ± 288.6^{a}	0.000
48 Hours	8333.3 ± 1040.8^{b}	6666.7 ± 763.7^{b}	0.000
72 Hours	6666.7 ± 577.3^{c}	4166.7 ± 763.7^{c}	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**1631.3**	**1289.6**	

The results of the current study recorded the activity of both Metronidazole and ZnO P. aeruginosa increased with time increasing. The results also showed a significant increase in the activity of ZnO P. aeruginosa compared to metronidazole at the same time as 24 in metronidazole with 24 in Zn P. aeruginosa at p. value < 0.05.

Table 12. Direct proportion between concentration 0.075 mg/ml of ZnO P. aeruginosa and Met./ through 24, 48, 72 hours

0.075 mg/ml	Metronidazole	ZnO *P. aeruginosa*	*P.* value for T. test
24 Hours	7500.0 ± 0.00 [a]	6666.7 ± 721.6 [a]	0.000
48 Hours	5833.3 ± 763.7 [b]	5000.0 ± 0.00 [b]	0.000
72 Hours	5000.0 ± 0.00 [b]	3333.3 ± 144.3 [c]	0.000
***P.* value**	**0.001**	**0.000**	
LSD	**881.0**	**848.9**	

A non-significant difference appeared in activity of Metronidazole in 48 and 72 time hours but significant with 24 time hours, in ZnO P. aeruginosa reveled activity was increased with increasing time. Also showed a significant increase in the activity ZnO P. aeruginosa compared to metronidazole

The number of parasite demonstrated by calculation of the inhibition rate as follow (control group – treated group/control group) ×100, calculated by hemocytometer as appeared in (figure 3A) The effect of these nanoparticles appeared in decrease trophozoite number and morphological deformity as appear in (figure 3B)

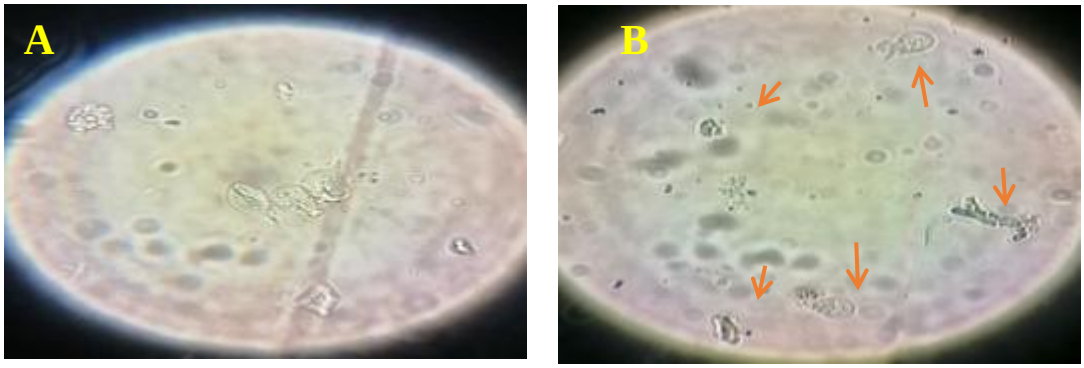

Figure 3.A: Giardia trophozoites from culture media counting on hemocytometer. B; Morphological deformities after exposure to Nanoparticle treatment

Discussion:

The flagellated intestinal parasite Giardia is a significant cause of human diarrheal disease worldwide due to ingestion of infectious cysts. Giardiasis treatment is based on the presence of trophozoites in the intestine, as well as a combination of drugs (Ag NPs) and metronidazole, the drug of choices. The current treatment has some drawbacks, such as unpleasant side effects, varying treatment efficacy, and parasite drug resistance. As a result, new anti-Giarddial drugs are being sought [22]. Recently, out of 312 papers were 4 paper (40%) including in vitro studies and 1 paper (10%) in vivo /in vitro study all were about the efficacy of nanoparticles against giardiasis [23] Nanoparticles have distinct physicochemical properties, are small in size, have a large surface area, an electrical charge, and a special shape [24]. In the recent study we discovered that the efficacy of nanoparticles was dose-dependent, higher concentration of nano-compound, the faster protozoan elimination rates were observed over time, these observations reported from daily follow up for number of trophozoites, morphological change and viability. The consequence of nanoparticles at various concentrations through variable duration results in significant reduction in trophozoites number and the considerable reduction were associated with concentration of 0.075 mg/ml through 72 hrs of time. These result reveled the ability of using NPs in higher concentration that lead to arrive total death rates. As previously mentioned about effectiveness of increase exposure time to eradicate Giardia

cyst completely by increase concentration. Use of nanoparticles in the treatment of parasitic infections revealed that nanoparticles of silver and metal oxide (ZnO) reduced the growth rates of parasites. This resulted from increased mortality in the environment where parasites live and are exposed to nano-drugs. Silver ions are thought to bind DNA, attach cell membranes, interact with the electron transport chain and adapt with the thiol group in vital enzymes. [18, 25, 26]. Several studies have found that continuous use of these chemotherapeutic agents results in pathogens that are no longer responsive to older medicine regimes. As a result, nanomedicine has paved the way [27]. NPs are recommended for parasite killing (cytotoxic and inhibitory effects) because they are more effective and less harmful drugs, as well as useful vaccines for parasite prevention and control [28]. These findings revealed the capability of using NPs at higher concentrations, which resulted in higher total death rates. Another study [33] discussed the effectiveness of increasing exposure time to completely eradicate Giardia cysts by increasing concentration. Finding the proper particle size appears to be an important step in promoting the ability to create the best delivery system for nano-drugs to penetrate the tissue. Selected and effectively released on the target cells, Morphological difference appeared as destructive with the release of the cytoplasm outside [11]. The presence of an apoptotic body in trophozoites demonstrated that Ag NPs were effective in inducing apoptosis as a reported outcome from [17]. A significant effect ($P<0.05$) of 91% was noted for Giardia trophozoite treated with Ag NPs biosynthesized by E. coli and P. aeruginosa, which was higher than the inhibition rates of 73% for the same organism treated with ZnO NPs biosynthesized by E. coli and P. aeruginosa respectively with contrast to control group (untreated). The inhibition rate reported for metronidazole 55% which was equal in its effect and not significant impact announce between two groups of NPs used and inside the same NPs group and it did not significantly differ between different concentrations of Ag NPs. These outcomes consistent with previous studies that reported that using of Ag NPs give a highest result in recovery from giardiasis [29, 30, 31 and 32]. In comparison to MTZ, ZnO NPs significantly affected Giardia group in compared to MTZ, as previously mentioned [32] when improved that ZnO have more effectiveness than MTZ in enhanced the intestinal mucosa's functional, histopathological, and immunological characteristics. We confirm that the cytotoxic effects of silver and zinc oxide nanoparticles on Giardia trophozoite exceeded those of metronidazole and that these particles can be used, particularly at higher concentrations that result in total death rates.

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