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ANTIPARASITIC ACTIVITY OF SILVER AND COPPER OXIDE NANOPARTICLES AGAINST *ENTAMOEBA HISTOLYTICA* AND *CRYPTOSPORIDIUM PARVUM* CYSTS

BY

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Abstract

Nanoparticles (NPs) have received more attention as antiparasitic agents. In the present study, silver and copper nanoparticles were synthesized and characterized using scanning electron microscopy (SEM), transmission electron microscope (TEM) and X-ray fluorescence (XRF). The antiparasitic activity of Ag and CuO nanoparticles were tested against two of the most environmentally spread parasites in Egypt (*Entamoeba histolytica* and *Cryptosporidium parvum*). The average sizes of synthesized Ag NPs and CuO NPs were 9 & 29 nm respectively and a significant reduction for cysts viability ($p > 0.05$) was observed for CuO NPs against *E. histolytica* cysts and Ag NPs against *C. parvum* oocysts. Moreover, LC_{50}-3h of CuO NPs for *E. histolytica* and *C. parvum* were 0.13 and 0.72 mg/l, while Ag NPs recorded 0.34 and 0.54 mg/l respectively. Accordingly, these NPs could be suggested as a new nanoform agent for safe and effective treatment of *E. histolytica* and *C. parvum* parasites.

Keywords: Antiparasitic, silver, copper, nanoparticles, *Entamoeba histolytica*, *Cryptosporidium parvum*.

Introduction

Amaebiasis, a major health problem in developing countries, is the second most common cause of death due to parasitic infection. Amaebiasis is usually transmitted by the ingestion of *Entamoeba* cysts through oral–fecal route (Shukla *et al*, 2015). Among the protozoa, *Entamoeba*, *Giardia* and *Cryptosporidia* are the major causes of waterborne diseases (Marshall *et al*, 1997; Sharma *et al*, 2003). The prevalence of *E. histolytica* among outpatients in the Dakahlia Governorate in Egypt was 9.7% (El Shazly *et al*, 2006). Besides, *Cryptosporidium parvum* infection is a leading cause of diarrhea in Egypt and its prevalence among diarrheic individuals whether outpatient or inpatient clinics was up to 49% (Youssef *et al*, 2008; Mousa *et al*, 2010; Helmy *et al*, 2013). Moreover, the commonest protozoan infection in immunosuppressed patients was *C. parvum* which reached 60.2% (Abdel-Hafeez *et al*, 2012). In Cairo and the Nile Delta between 2005 and 2007 applied ELISA technique for the detection of bacterial, viral and parasitic pathogens in 2,112 diarrheic children below 5 years of age *G. duodenalis* and *C. parvum* were the only parasites detected; 7.0 and 5.0 %, respectively (El-Mohammady *et al*, 2012).

The main motive is the expectation that nanoparticles will be able to be used in the treatment of various diseases in the future (Angeli *et al*, 2008; Debbage, 2009). It was determined that through their unique properties and large surface areas, metal oxide nanoparticles possess effective antimicrobial activities (Elechiguerra *et al*, 2005). Particularly, owing to their great chemical reactivity, nanoparticles are capable of producing reactive oxygen species (ROS), which have the ability to kill infectious agents.

The use of metal oxide nanoparticles exhibiting the antimicrobial activity offers the possibility of an efficient removal of pathogens from wastewater (Elechiguerra *et al*, 2005). The NPs may not have the pronounced antimicrobial activity when compared to the bulk formulations of the metal oxide or solutions of metal salts. But, the stability and slow release of metal ions from nanoparticles are main characteristics which

give them the advantage in use (Heinlaan *et al,* 2008).

The antimicrobial efficiency of NPs depends on the particle size (Adams *et al,* 2006). The smallest sized of NPs showed the strongest effect (Lu *et al,* 2013). Some researches indicate that silver nanoparticles, gold, chitosan, and oxidized metals have growth inhibitory or cytotoxic effect on various parasites, including *Giardia, Leishmania, Malaria, Toxoplasma* and insect larva (Elmi *et al,* 2013). Also, Ag NPs showed significant anti-leishmanial effects by inhibiting the promastigotes proliferation and meta-bolic activity (Allahverdiyev *et al,* 2011). Therefore, nanoparticles are recommended for destroying parasites (cytotoxic and inhibitory effect), because they act as more effective and less harmful drugs and also beneficial vaccines for the prevention and control of the parasites (Elmi *et al,* 2013).

The aim of the present study was to determine the efficacy of synthesized Ag and CuO nanoparticles in treatment of *Entamoeba histolytica* and *Cryptosporidium parvum.*

Subjects, Materials and Methods

Preparation of silver nanoparticles: For the preparation of silver nanoparticles two stabilizing agents, sodium dodecyl sulphate (SDS) (Sigma-Aldrich Company) and sodium citrate (Merck Company) were used. For the synthesis of silver nanoparticles, silver nitrate solution (1.0mM) and 8% (w/w) SDS were used as a metal salt precursor and a stabilizing agent. Hydrazine hydrate (Sigma-Aldrich Company) solution with a concentration 2.0mM and citrate of sodium solution (1.0mM) were used as a reducing agents. Citrate of sodium was also used as stabilizing agent at room temperature. The transparent colorless solution was converted to the characteristic pale yellow and pale red color, when citrate of sodium was used as stabilizing agent. The occurrence of color was indicated the formation of silver nanoparticles. The silver nanoparticles were purified by centrifugation. To remove excess silver ions, the silver colloids were washed at least three times with deionized water under nitrogen stream. A dried powder of the nanosize silver was obtained by freeze-drying (Guzmán *et al,* 2009).

Preparation of copper oxide NPs: Aqueous solution (250ml) of copper acetate (0.02mol) (Merck Company) is prepared in round bottom flask. 1 ml glacial acetic acid is added to above aqueous solution and heated to 100ºC with constant stirring. About 0.4gm NaOH (El-Gomhouria Chemical Co., Egypt) was added to above heated solution till the pH reached 6-7. The large amount of black precipitate was formed immediately, centrifuged, washed 3-4 times with deionized water and the precipitate was air dried in for 24 h (Lanje *et al,* 2010).

Characterization of nanoparticles: Nanoparticles prepared were characterized with the help of multiple techniques. The surface morphology of nanoparticles was characterized by a scanning electron microscopy (SEM; JEOL JSM-5600). The particles size of the resulted nanoparticles was analyzed by using transmission electron microscope (TEM) (EM 208S Philips, Netherlands) connected to a high resolution imaging system. Samples for TEM studies were prepared by placing drops of nanoparticles solutions on carbon-coated TEM copper grids. X-ray fluorescence (XRF) that was performed to detect the main chemical elemental analysis of the minerals that are present in synthesized nanoparticles. XRF measurements were carried out using the JSX-3222 element analyzer.

Collection of cysts: *E. histolytica* cysts and *C. parvum* oocysts were isolated from positive samples of farmers and their animals' feces from three different governorates Cairo, Giza and Qaluobia. All samples were used directly after arrival. A highly purified cysts suspension was achieved by combining the sucrose flotation method with a simplified sucrose gradient method (Sheffield and Bjorvatn, 1977). The feces were broken up in normal saline (0.9%) and filtered through a 300µm filter. A total of 3ml of feces sus-

pension was layered on top of 3ml of 0.85M sucrose (Sigma-Aldrich Company) and centrifuged at 2,000 for 10 min at 4°C. The cysts were aspirated with a Pasteur pipette at sucrose-water interface and washed 3 times with normal saline. The washed cysts were carefully added to the top of a discontinuous density gradient consisting of two 3ml layers of 0.85M and 0.4M sucrose. After centrifugation, the cysts concentrated at the 0.85-0.4M sucrose interface were collected and washed again. The purified cysts were suspended in normal saline and stored at 4°C for a maximum of 3 days prior to use.

Anti-parasitic activity: The nanoparticles were prepared in four concentrations (0.5, 1.0, 2.0 & 4.0mg/l). A total of 2ml of each solution was placed in test tubes, to which washed cysts were added and gently mixed. The tubes were then incubated at 37ºC for 10, 30, 60 & 180 min for each concentration. At the end of each incubation period, the upper phase was carefully removed. Untreated cysts were used as a control group in each experiment. A total of 2ml of 0.1% eosin stain was then added to the remaining settled cysts, and gently mixed. Eosin 0.1% stain was used to detect the cysts' viability for 15min. The cysts were then smeared on a glass slide, covered with a cover glass and examined under a light microscope. Dead cysts percent was determined as 100 cysts/ slide. Cysts without absorbed dye were recorded as potentially viable; otherwise recorded as dead one. The tests were repeated in triplicate (Yarahmadi *et al*, 2014).

SEM of treated *C. parvum* oocysts: Control and treated *C. parvum* oocysts by NPs were fixed by using fresh 2% glutaraldehyde for an hour, rinsed in sodium cacodylate buffer and post fixed for an hour at 4°C with 1% osmium tetraoxide in sodium cacodylate buffer solution. Suspensions were progressively dehydrated. The preparation were coated with gold salt and examined with a SEM; JEOL JSM-5600 (Brasseur *et al*, 1998).

Statistical analysis: Data were analyzed using statistical package for the social sciences (SPSS 18.0). The differences between treated and untreated samples were tested for significance using a t- test ($P < 0.05$) was considered statistically significant.

Results

Silver nanoparticles synthesis and characterization: The scanning electron microscope images showed that Ag NPs were round-shaped with a smooth surface morphology and tended to form aggregates (Fig. 1A). The particle size analysis of the nanoparticles was performed by transmission electron microscopy, where TEM images of the aqueous dispersion showed average particle size 9nm (Fig. 1B). The elemental analysis of Ag NPs was performed using XRF analysis of powder showed that 98.4% of metal composition of Ag NPs powder was Ag (Fig. 1E).

Copper oxide nanoparticles synthesis and characterization: SEM image showed spherical nanoparticles morphology (Fig. 1C). TEM photos showed that the CuO NPs are spherical and its average diameter is 29 nm (Fig. 1D). XRF recorded that 98.5% of metal composition of CuO NPs powder is Cu (Fig. 1F). Antiparasitic activity: The activity of two nanoparticles showed a significant difference between untreated groups and treated ones. Time as an effective parameter influenced mortality rate of cysts; increasing the duration of exposure also increased the mortality rate.

Entamoeba histolytica cysts recorded high mortality rates after 180 min of exposure time ranged from 73% to 100 % for CuO NPs (Fig. 3A) and from 51% to 100 % for Ag NPs (Fig. 3B). Difference between groups treated with NPs and control was significant ($P<0.05$) at concentrations 2.0 and 4.0 mg/l for Ag NPs (Fig. 3F), and at 1.0, 2.0 and 4.0 mg/l for CuO NPs (Fig. 3E). No significant difference was observed between the treated and untreated groups at 0.5 mg/l of exposure time for both nanoparticles and 1.0 mg/l for Ag NPs. The mortality rates of cysts treated with Ag and CuO nanoparticles after 180 min of exposure time by a

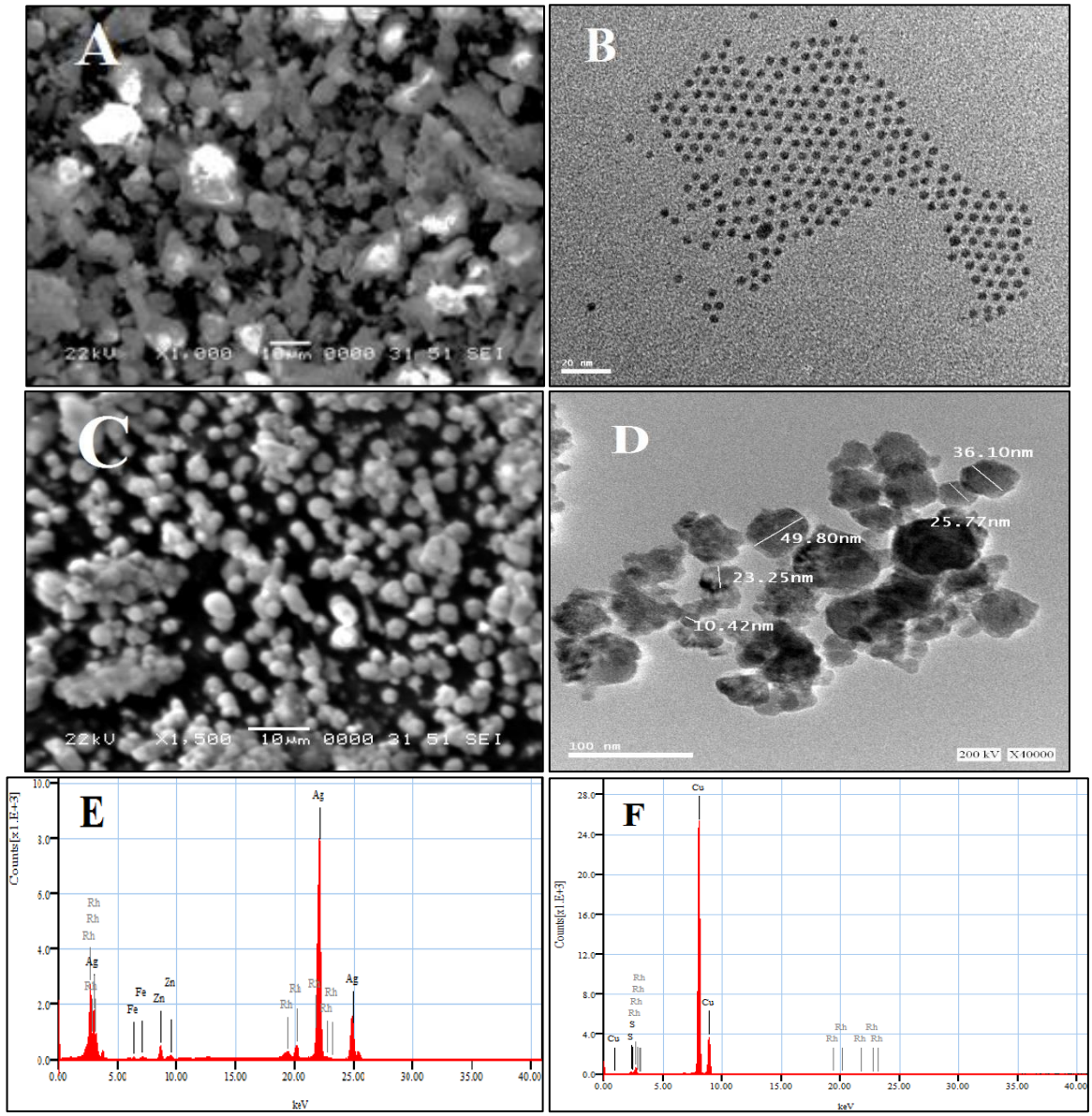

Fig. 1: A) SEM of Ag NPs, B) TEM of Ag NPs, C) SEM of CuO NPs, D) TEM of CuO NPs, E) XRF of Ag NPs, F) XRF of CuO NPs.

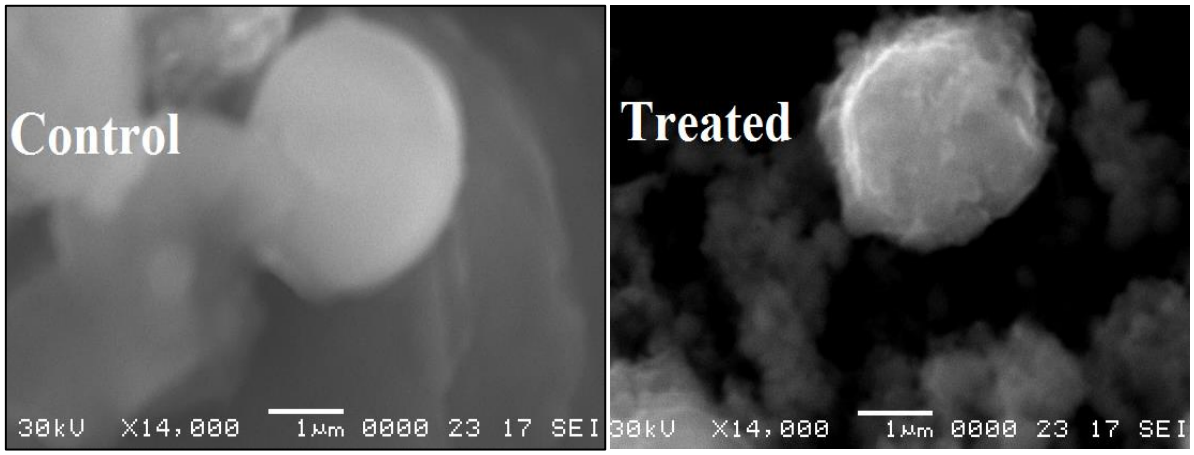

Fig. 2: SEM images of control and treated oocysts of *C. parvum* by nanoparticles.

concentration of 0.5mg/l were 51% and 63 %, by the side of 1.0 mg/l was 85 and 100% respectively, while on 2.0mg/l and 4.0mg/l mortality rate was 100% for both NPs (Fig. 3E, F). But, the mortality rate of the control group at 180 min was 8%. The present results showed that mortality rate increased gradually with the increase in Ag NPs and CuO NPs concentrations (Fig. 3A, B). Additionally, LC_{50}-3h values of NPs for *E. histolytica* cysts were 0.13 and 0.34 mg/l with CuO and Ag, respectively, which indicated that *E. histolytica* cysts more sensitive for CuO than Ag NPs. Mortality rates of *C. parvum* oocysts after 180 min of contact time mortality ranged from 39% to 100% for CuO NPs (Fig. 3C) and from 47% to 100% for Ag NPs (Fig. 3D). Difference between groups treated with Ag NPs and CuO NPs and control was significant ($P<0.05$) at concentrations 2.0 and 4.0mg/l for Ag NPs (Fig. 3F), but CuO NPs recorded significant reduction at 4.0mg/l (Fig. 3E). The mortality rate of *C. parvum* oocysts treated with Ag NPs and CuO NPs after 180 min of exposure time at a concentration of 0.5mg/l were 47% and 39%, by 1.0mg/l was 73% & 59%, by the side of 2.0mg/l was 100 and 81% respectively, while on 4.0mg/l mortality rate was 100% for both NPs (Fig. 3C, D). The mortality rate of control group at 180 min was 9%. The study results showed that the mortality rate increased gradually with the increase in Ag NPs & CuO NPs concentrations (Fig. 3E, F). Also, LC_{50}-3h values of Ag NPs for *C. parvum* were 0.54 mg/l, while CuO NPs was 0.72 mg/l, which indicated that *C. parvum* more sensitive for Ag than CuO NPs. SEM images of *C. parvum* oocysts treated with Ag & CuO nanoparticles showed changes in the oocyst wall' structure compared to control ones (Fig. 2).

Discussion

In the present study, Ag and CuO nanoparticles showed anti-parasitic activity. Whereas, *E. histolytica* and *C. parvum* cysts recorded high mortality rates after 180 min of exposure time for Ag NPs and CuO NPs. The antibacterial effects of Ag NPs were recorded (Zheng *et al*, 2008). Silver nanoparticles, gold and oxidized metals have growth inhibitory or cytotoxic effect on various parasites, including the *Giardia, Leishmania, Plasmodium, Toxoplasma* and insect larva (Elmi *et al*, 2013). Also, the Ag NPs showed significant anti-leishmanial effects by inhibition of promastigotes proliferation and metabolic activity (Allahverdiyev *et al*, 2011).

Time as an effective parameter influenced the mortality rate of parasites cysts; increasing the exposure time, improved the mortality rate. Also, in the present study, *E. histolytica* and *C. parvum* oocysts showed gradual increase in mortality rate with the increase in Ag NPs & CuO NPs concentrations. The LC_{50}-3h of nanoparticles for *E. histolytica* cysts were 0.13 and 0.34 mg/l with CuO and Ag, respectively indicating that *E. histolytica* cysts sensitive for CuO was more than Ag NPs. LC_{50}-3h values of nanoparticles for *C. parvum* oocysts were 0.54 and 0.72 mg/l with Ag and CuO, respectively indicating that *C. parvum* sensitive for Ag was more than CuO NPs. The present results agreed with Allahverdiyev *et al.* (2011) who found that Ag NPs recorded the highest suppressive effect on parasites at a concentration of 2.0mg/L, and at same concentration, the metabolic activity of *Leishmania tropica* promastigotes decreased nearly twofold, in contrast to the control group ($P<0.05$). Smith (2013) in mice studied the silver nanoparticles effects on *C. parvum* using a murine model and dielectrophoresis. They found that ionic silver released from the silver NPs has a modest but measureable effect on *C. parvum* infectivity. However, Liao *et al.* (2012) revealed that *C. parvum* oocysts monitoring method into a powerful scale due to Ag NPs treatment performed within very short time and its nontoxic agent property. So, accepted to be a good waterborne protozoan parasite determination method, rapidly diagnose and prevent the infectious outbreak. Su *et al.* (2014) used in vitro excysta-

tion and fluorogenic assays to examine disinfection capabilities of silver nanoparticles on *C. parvum* oocysts and validated their results by in *vivo* animal infectivity assays. They found that almost two orders of magnitude reduction in oocyst shedding among mice fed on silver nanoparticle treated oocysts relative to untreated ones. Thus, CuO NPs and Ag NPs could be suggested as a new nanoform agent as safe and effective treatment of *E. histolytica* and *C. parvum* besides, NPs were biologically non-toxic. However, Blinova *et al.* (2010) compared the CuO NPs toxicity with $CuSO_4$ in natural river waters samples collected in six different sites. They found that LC_{50}-48h ranged from 92.7 to >200mg/l for CuO NPs and 0.24 to 0.92mg/l to $CuSO_4$. The lower toxicity in river water samples as compared to the test media was attributed to the presence of organic matter that can strongly complex to Cu and reduce the Cu ions bioavailability.

In the present study, SEM images of *C. parvum* showed that oocysts became inactive after treatment by NPs, where changes in the structure of its wall were observed after treated with NPs compared to control, which indicated that these oocysts loss its viability. Choi and Hu (2008) consider the reason for this is the interaction of NPs with the surface of parasites. It may be posited that NPs impair the structure of lipophosphoglycan and glycoprotein molecules that are found on the surface of parasites and which are responsible for the infection. They also proposed that these molecules may be more seriously affected from ROS generated form NPs and this may lead to inhibition of parasite infection. Also, Choi and Hu (2008) reported that owing to their great chemical reactivity, nanoparticles are capable of ROS, which have the ability to kill infectious agents. The main reason for using Ag NPs in this study was their capacity to produce ROS. Moreover, Chang *et al.* (2012) discussed three different mechanisms based on oxidative stress, coordination effects, and non-homeostasis effects that potentially explain why copper nanoparticles exert toxic effects on eukaryotic cells. They reviewed that nanoparticles can diffuse into the cell directly through the pores present in cell membrane due to their small size, or they get entry through ion channels and transporter proteins present on the plasma membrane. Some nanoparticles may enter into cells via endocytosis. They found that the nanoparticles which are entered into the cell can directly interact with oxidative organelles such as mitochondria. Later, redox active proteins stimulate ROS production in cells, and ions (Cu^{2+}) produced by nanoparticles can induce ROS by various chemical reactions. ROS can induce DNA strand breaks, and affect gene expression. Schrand *et al.* (2010) hypothesized that copper nanoparticles act as effective antibacterial agent against wide range of bacterial species due to interactions with -SH groups leading to protein denaturation. Copper nanoparticles exert effect on cell membrane due to affinity towards amines and carboxyl groups on organisms' cell surface as *Bacillus subtilis* bacteria (Beveridge and Murray 1980; Ren *et al,* 2009). Once inside the cell, the nanoparticles might bind with DNA molecules and disturb the helical structure by cross-linking within & between nucleic acid strands (Stohs and Bagchi, 1995). Copper ions inside bacterial cells also disrupt biochemical processes (Kim *et al,* 2000).

The particle size analysis of prepared nanoparticles by TEM showed average particle size 9 & 29 nm for Ag NPs & CuO NPs respectively, while SEM images showed that Ag NPs were round-shaped with a smooth surface morphology and tended to form aggregates and CuO NPs were spherical shape. Allahverdiyev *et al.* (2011) revealed that Ag NPs of size ranged 10-40 nm demonstrated significant anti-leishmanial effects by inhibiting proliferation and metabolic activity of promastigotes. Nevertheless, antimicrobial activity of Cu NPs with an average size of 20 nm reported significant inhibitory activity against *Escherichia coli* followed by *Klebsi-*

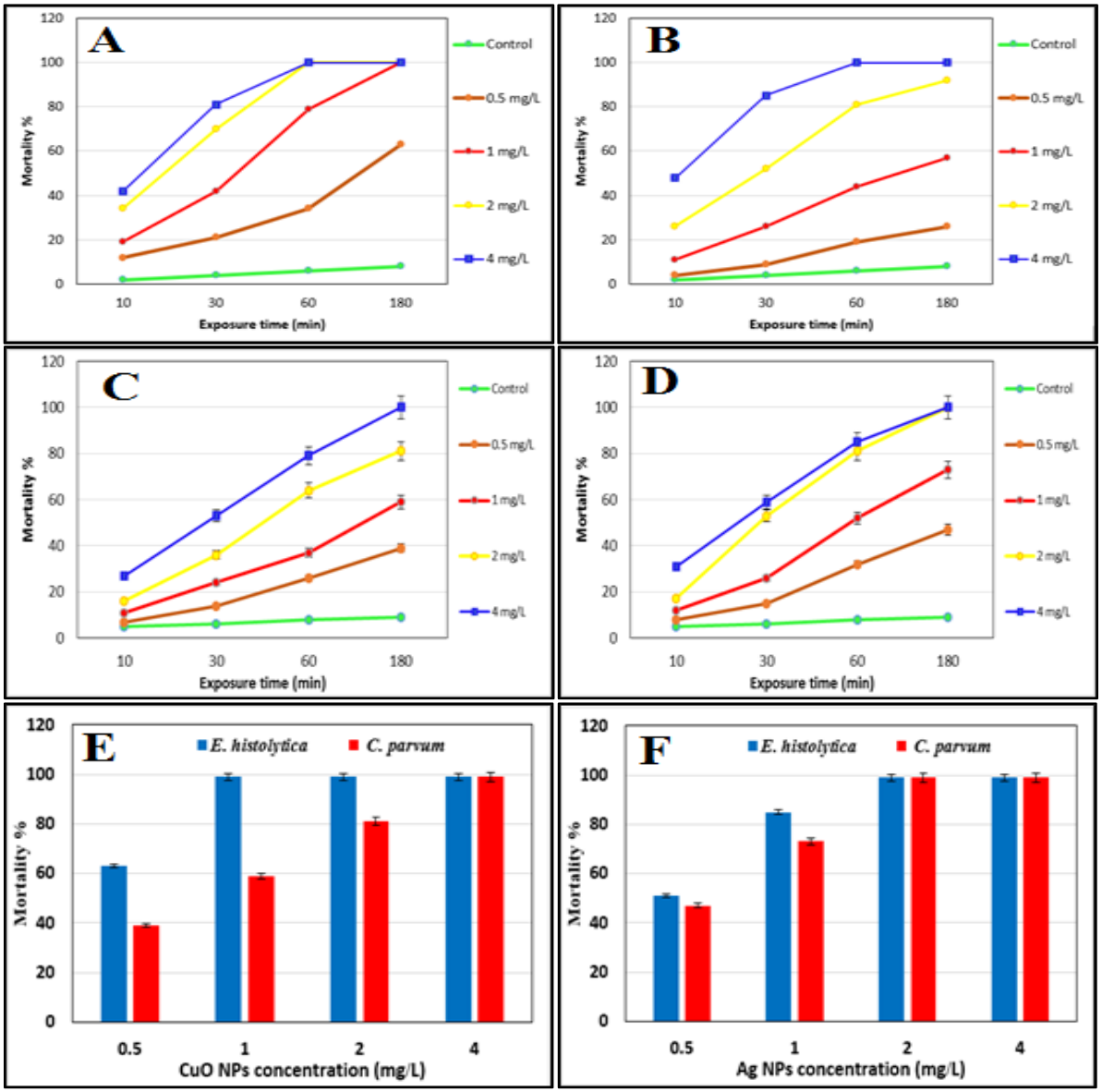

Fig. 3: Mortality rates at different exposure time of A) Treated *E. histolytica* cysts by CuO NPs, B) *E. histolytica* cysts by Ag NPs, C) Treated *C. parvum* oocysts by CuO NPs, D) Treated *C. parvum* oocysts by Ag NPs, Mortality rates after 180 min of exposure time for both parasites treated by E) CuO NPs F) Ag NPs.

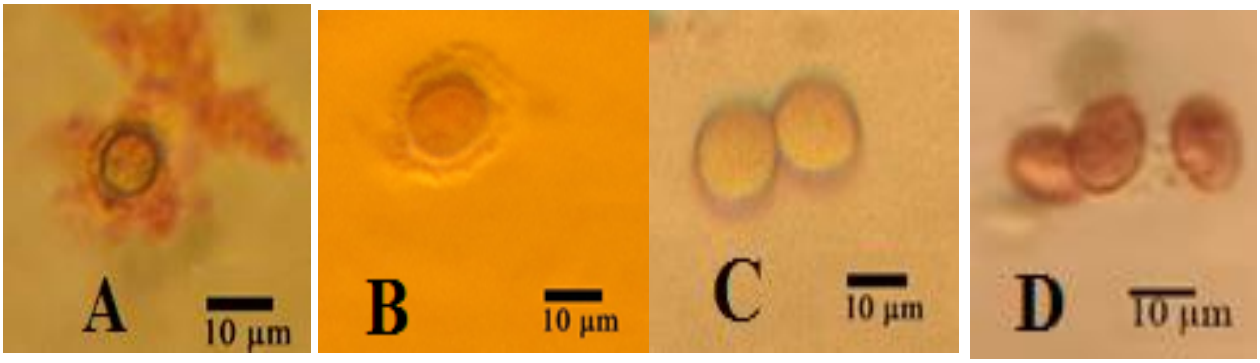

Fig. 4: Images of treated parasites by NPs and stained by 0.1% eosin A) Viable *E. histolytica* cyst B) Non-viable *E. histolytica* cyst C) Viable *C. parvum* oocyst D) Non-viable *C. parvum* oocyst.

ella pneumoniae, *Pseudomonas aeruginosa*, *Propionibacterium acnes* and *Salmonella typhi* (Shende *et al*, 2015). Generally, the antimicrobial efficiency of NPs depends on the particle size (Adams *et al*, 2006), with the activities of Ag NPs were size-dependent; the smallest one showed the strongest effect (Lu *et al*, 2013).

Generally speaking, *E. histolytica* affects anyone, and commonest in people who live in tropical areas (CDC, 2010). Besides, in Egypt *E. histolytica* was reported by many authors particularly in immunocompromized patients (El-Beshbishi *et al*, 2005; Abd-Alla *et al*, 2013; El Nadi *et al*, 2015; Foda and Singh, 2015; Banisch *et al*, 2015; El-Shazly *et al*, 2015). On the other hand, the zoonotic cryptosporidiosis was reported in nearly all the Egyptian Governorates from man particularly in the immunocompromized patients, animals and even water sources (Azab *et al*, 1985; Soliman, 1992; Youssef *et al*, 1994; El Shazly *et al*, 2007; El-Sherbini and Mohammad, 2007; Massoud *et al*, 2008; Rayan *et al*, 2009; Baiomy *et al*, 2010; Khalifa *et al*, 2014).

Conclusion

Undoubtedly, the gastrointestinal protozoan parasites particularly, *Entamoeba histolytica* and *Cryptosporidium parvum* are among the commonest causes of diarrheal illness particularly among children worldwide. Their prevalence in children with diarrhea is higher than in those without them.

The outcome results showed the capability of CuO and Ag nanoparticles to inactivate *E. histolytica* and *C. parvum* cysts. The CuO NPs and Ag NPs may represent an alternative drugs and water treatment for *E. histolytica* and *C. parvum* cysts. The treatment based on CuO NPs and Ag NPs have a very important role in overcoming amoebiasis and cryptosporidiosis. Extensive studies on this issue are ongoing and while be published in due time elsewhere.

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