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Original article

Copper nanoparticles: Biosynthesis, characterization, and protoscolicidal effects alone and combined with albendazole against hydatid cyst protoscoleces

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ABSTRACT

Background: Surgery remains the preferred treatment option for hydatid cyst (cystic echinococcosis); however, recent studies have demonstrated that the current protoscolicidal agents used during surgery are associated with some adverse side effects such as biliary fibrosis, hepatic necrosis, and cirrhosis. The present study aims to evaluate the *in vitro* and *ex vivo* anti-parasitic effects of copper nanoparticles (CuNPs) alone and combined with albendazole on hydatid cyst protoscoleces.

Methods: CuNPs was green synthesized using C. spinosa extract. Various concentrations of CuNPs (250, 500, and 750 mg/mL) alone and combined with albendazole (ALZ, 200 mg/mL) were exposed to protoscoleces collected from the liver fertile hydatid cysts of infected sheep for 5–60 min *in vitro* and *ex vivo*. Next, the eosin exclusion test was applied to determine the viability of protoscoleces. Caspase-3 like activity of CuNPs-treated protoscoleces was then evaluated using the colorimetric protease assay Sigma Kit based on the manufacturer's instructions.

Results: Scanning electron microscopy (SEM) results showed that the particle size of CuNPs was 17 and 41 nm with the maximum peak at the wavelength of 414 nm. The maximum protoscolicidal activity of CuNPs was observed at the concentration of 750 mg/mL *in vitro,* so that 73.3 % of protoscoleces were killed after 60 min of exposure. Meanwhile, the mortality of protoscoleces was 100 % after 10 min of exposure to 750 mg/mL of CuNPs along with ALZ (200 mg/mL). Nevertheless, the findings proved that CuNPs even in combination with ALZ required a longer time to kill protoscoleces *ex vivo*. After 48 h of treating protoscoleces, CuNPs in a dose-dependent manner and at doses of 250, 500, and 750 mg/mL induced the caspase enzyme activation by 20.5 %, 32.3 %, and 36.1 %, respectively.

Conclusion: The findings of the present investigation showed potent protoscolicidal effects of CuNPs, especially combined with albendazole, which entirely eliminated the parasite after 10–20 min of exposure. The results also showed that although the possible protoscolicidal mechanisms of CuNPs are not clearly understood, the inducing apoptosis through caspases is one of the main protoscolicidal mechanisms of CuNPs. However, supplementary studies, especially in animal models and clinical settings, are needed to approve these results.

1. Introduction

Cystic echinococcosis (CE or hydatidosis) is a zoonotic infection caused by the larval stage of cestode species belonging to the genus Echinococcus [1]. According to the World Health Organization's (WHO) reports, CE is one of the 17 neglected tropical diseases (NTDs) with an important challenge both from medical and economic points of view [2]. Human and other intermediate hosts (*e.g.* sheep, goats, cattle, *etc.*) are infected once they ingest the CE eggs, which are shed in the stool of the definitive host (carnivores such as canine feline, *etc.*) [3]. After ingesting the eggs, the oncosphere hatches from the egg, penetrates the intestinal mucosa, migrates through the bloodstream to vital organs including the liver, lung, *etc.*, and forms a hydatid cyst [3].

The clinical symptoms of CE depend on various factors such as the

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involved organ, location, number of cysts in the involved organ, size of cysts, *etc.* Although CE is frequently asymptomatic, some factors including cyst rupture cause infection or anaphylactic shock, fistula in adjacent tissues (biliary tract, intestine, and bronchus), or mechanical effect on the adjacent tissues and structures [4].

The CE treatment is complex and there is a large variety of CE management around the world [4]. However, chemotherapy, surgical treatment, endoscopic interventional treatment, percutaneous methods (puncture, aspiration, injection, and re-aspiration (PAIR)), and observation without intervention (watch and wait) are considered as the main CE management modalities [5]. Overall, in small and inactive cysts, the preferred treatment is chemotherapy with benzimidazole derivatives (mebendazole and albendazole), while the first-choice treatment for large and active cysts is surgery [6]. Previous investigations have demonstrated that chemotherapy with benzimidazole compounds is related to some side effects such as methemoglobinemia, leucopenia, thrombocytopenia, hepatotoxicity, teratogenicity, and osteoporosis. According to these studies, physicians should be very careful when using these drugs [5–7].

The most important risks and complications of surgical approaches for CE treatment are the ruptures of cysts or leakage of their contents (protoscoleces), which can result in re-infection, secondary infection, anaphylaxis shock, and even death in patients [8]. To solve these problems and complications, surgeons apply various chemical protoscolicidal agents such as hypertonic saline 20 %, silver nitrate, and formalin to prevent these complications [8]. Nevertheless, reviews have shown that the existing protoscolicidal agents are associated with some adverse side effects such as biliary fibrosis, hepatic necrosis, and cirrhosis [9,10]. Consequently, the study for finding a new protoscolicidal agent is of top urgency for physicians in terms of CE treatment.

Nanomedicine is well-known as a relatively novel field of science and technology that uses nano-meter-sized materials for a wide range of medical goals including diagnostic and therapeutic applications in modern medicine, drug delivery, imaging, medical devices, vaccines, *etc.* [11]. Recently, using nanoparticles for antimicrobial purposes has been of main interest for various researchers around the world [12].

In the recent decade, various physical and chemical approaches have been investigated to synthesize the nanoparticles with a certain size and less toxicity. Among these methods, green synthesis is considered as one of the most popular, reliable, maintainable, and eco-favorable approaches for synthesizing appropriate and safe nanomaterials [13]. Generally, green synthesis of metal nanoparticles using plant products is described as a preferred choice, rather than bacteria and/or fungi mediated synthesis, because of its low cost, low toxicity, acceptable efficacy, and implementation ease [14].

From a long time ago, copper (Cu) has been considered as one of the most beneficial elements with a broad spectrum of pharmacological properties such as improving the immune system and inducing anti-inflammatory, anti-cancer, analgesic, and anti-microbial effects [15,16]. Previous investigations have shown that Cu nanoparticles (CuNPs), because of their high surface-to-volume ratio, are very reactive and merely interact with other particles. Thus, they result in various biological and therapeutic activities [17]. Recent studies have demonstrated the expansion of synergistic combinations of synthestic and medicinal herbs (*e.g. Punica granatum* L.) with some existing drugs such as albendazole (ABZ) which has led to improved efficacy as well as reduced toxicity of single drugs [18].

The present study aims to evaluate the *in vitro* and *ex vivo* antiparasitic effects of green synthesized CuNPs alone and in combination with albendazole on hydatid cyst protoscoleces.

2. Materials and methods

2.1. Green synthesis of copper nanoparticles

Fruits of *Capparis spinosa* were collected from the rural areas in Khorramabad, Lorestan, Iran. The extract was prepared by percolation method using 80 % methanol for 72 h at room temperature. The green synthesis of CuNPs was performed according to the method described elsewhere [19]. Succinctly, 75 mL of the extract mentioned earlier was added to 100 mL 0.01 M copper sulfate solution; after stirred it was kept at 60 °C for one day. Afterward, to remove all impurities it was centrifuged twice at the 12,000 rpm for 20 min. The change in color of the precursor solution from green to amber yellow with time supply evidence of CuNPs synthesis. The synthesized nanoparticles were dried in the oven at 60 °C for the more analyses.

2.1.1. UV–vis spectroscopy analysis

Surface Plasmon Resonance (SPR) of synthesized CuNPs was detected by using UV–vis spectrophotometer to confirm the Transformation of the copper ions to copper nanoparticles. Consequently, 0.3 mL of the NPS solution was diluted with 3 mL of normal saline and were evaluated by UV–vis spectrum analysis employing a spectrophotometer device (JENWAY 6405) in the range of 300–700 nm

2.1.2. Fourier transform infrared spectroscopy

FTIR (model Nicolet32) analysis was performed in the range of 400–4000 and with the resolution of 1–4 cm on the mixture of the obtained NPs along with the potassium bromide (KBr) granules with the ratio of 1–100 (1/100 ratio) after becoming tablets.

2.1.3. Scanning electron microscope (SEM)

The Specifications of synthesized nanoparticles such as size and morphology were studied by electron microscopy (Mira3, Made in Czech) with 15 kv, magnification of 10x, and resolution of 1 nm.

2.2. Collection of protoscoleces and viability

Protoscoleces of *E. granulosus* were collected from livers of the naturally infected sheep slaughtered at Khorramabad abattoir, Iran. The protocol for the preparation of protoscoleces and viability assessment is described by Moazeni et al. [20].

2.3. In vitro protoscolicidal activity

In the present study, CuNPs alone at concentrations of 250, 500, and 750 mg/mL as well as in combined with albendazole (Sigma-Aldrich, Germany) at the concentration of 200 μg/mL (the selection of this concentration was based on the primary experiments) used for 5, 10, 20, 30, and 60 min. Initially, 0.5 mL of the protoscoleces (2 × 103/mL) solution were placed in test tubes. Then 0.5 mL of various concentrations of NPs were added to each test tube. Tubes were delicately mixed and then incubated at 37 °C for 5, 10, 20, and 30 min. At the end of each incubation time, the upper phase was carefully removed and Fifty μl of 0.1 % eosin stain (Sigma-Aldrich, St. Louis, MO, USA) was then added to the remaining settled protoscoleces and mixed gently. Sedimented protoscoleces was then smeared on a glass slide, covered with a cover glass, and examined under a light microscope. The mortality rate of percentages was determined through counting 100 protoscoleces by eosin exclusion test. Besides, normal saline and Agnitrate were used as negative and positive groups [20].

2.4. Ex vivo protoscolicidal activity

To evaluate the *ex vivo* protoscolicidal activity of CuNPs alone at concentrations of 250, 500, and 750 mg/mL as well as in combined with albendazole (ALZ, Sigma-Aldrich, Germany) at the concentration of 200 μg/mL, liver fertile hydatid cysts acquired from naturally infected sheep were used. Firstly, more than half of the content of the cyst was aspirated to determine the viability of protoscoleces by eosin test. For each concentration three hydatid cysts were applied then NPs were injected into the cysts. Then some of the cyst fluid along with protoscoleces was

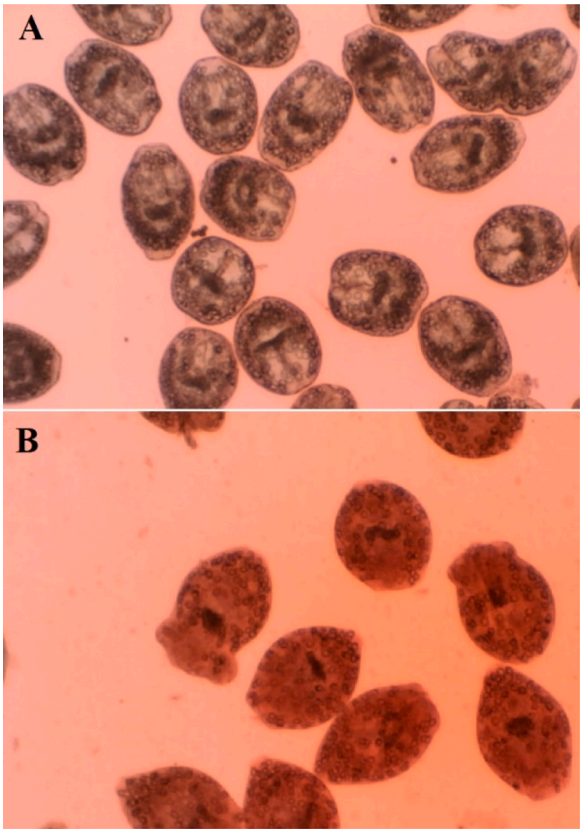

Fig. 1. Live (A) and dead (B) protoscoleces after exposure with various copper nanoparticles alone or combined with albendazole following various exposure times.

aspirated at 5, 10, 20, 30, and 60 min, and in the next step, 0.1 % eosin was placed to the precipitate. Similar to *in vitro* assay, the mortality rate of protoscoleces was calculated by an eosin exclusion test [21].

2.5. Eosin exclusion test

Eosin exclusion test for evaluation of viability of protoscoleces is based on the flame cell motility and impermeability to 0.1 % eosin solution (1 g of eosin powder in 1000 mL of distilled water); so that live protoscoleces do not absorb color and exhibited typical muscular movements and flame cell activity; but in dead protoscoleces eosin enter the cell and protoscoleces become red (Fig. 1).

2.6. Evaluating the caspase-3 like activity of CuNPs-treated protoscoleces

Caspase-3 like activity of CuNPs-treated protoscoleces was evaluated by means of the colorimetric protease assay Sigma Kit based on the manufacturer instructions. The basis of the experiment is color spectrophotometric measurement, which is caused by the release of a molecule (pNA attached to the substrate) under the activity of the enzyme caspase-3. After 48 h of treatment with CuNPs, the protoscoleces were centrifuged at 600 rpm for 5 min at 4 °C, the cell precipitate was lysed, and the cell lysate was centrifuged at 20,000 rpm for 10 min. In a final volume of 100 µl, 5 µg of supernatant was tested with 85 µl of buffer and 10 µl of caspase 3 (pNA-DEVD-Ac) substrate was incubated for 2 h at 37 °C. The light absorption of the samples was read at 405 nm with the ELISA reader.

2.7. Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used to analyze The results of this study. *in vitro* and *in vivo* experiments were carried out in triplicate. One Way ANOVA, as well as a t-test, was utilized to evaluate the variations among tested groups. $P < 0.05$ was considered statistically significant.

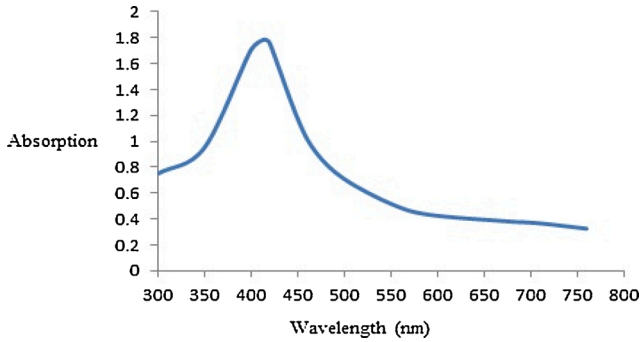

Fig. 2. The absorption spectrum of synthesized copper nanoparticles. the maximum peak of the obtained CuNPs was detected in the zone of 414 nm; where the characteristic of the resonance band of the surface plasmon happened for CuNPs.

3. Results

3.1. Characteristics of CuNPs

3.1.1. UV–vis spectrum analysis

As shown in Fig. 2, the maximum peak of the obtained CuNPs was detected in the zone of 414 nm. The presence of metallic copper was evidenced by EDX analysis. The copper nanoparticles at 1Kev revealed a sorptive peak, which is attributed to the metallic nanoparticles of copper. We found that at the wavelength of 414 nm, the characteristic of the resonance band of the surface plasmon happened for CuNPs.

3.1.2. FTIR analysis

Fig. 3 exhibits that the biomolecules in the extract decreased the copper sulfate solution; therefore, they can be used as coatings for nanoparticles. The bands at 3380, 2928, 1741, 1604, 1400, 1050, and 1271 were related to the O—H stretching of alcohol and phenol, C–H stretching of the aliphatic group, C═O stretching of ester carbonyl, C═C stretching of the aromatic ring, and C—O stretching of ester, respectively.

3.1.3. SEM analysis

According to the obtained results using SEM, the green synthesized CuNPs had spherical morphology and the size of the particles was measured between 17 and 41 nm (Fig. 4).

3.2. In vitro protoscolicidal effects of CuNPs

The *in vitro* protoscolicidal effects of different concentrations of CuNPs alone and combined with ALZ on the E. granulosus protoscoleces over 5, 10, 20, 30, and 60 min incubation are presented in Fig. 5. The findings exhibited that CuNPs, especially along with CuNPs, had considerable protoscolicidal effects compared to the control group ($p < 0.001$). The maximum protoscolicidal activity of CuNPs was observed at the concentration of 750 mg/mL, such that 73.3 % of protoscoleces were killed after 60 min of exposure. Meanwhile, the mortality of protoscoleces was 100 % after 10 min of exposure to 750 mg/mL of CuNPs along with ALZ (200 mg/mL). The mortality rate of protoscoleces in the negative and positive control group was 2.3 % and 100 % after 30 and 5 min of exposure, respectively.

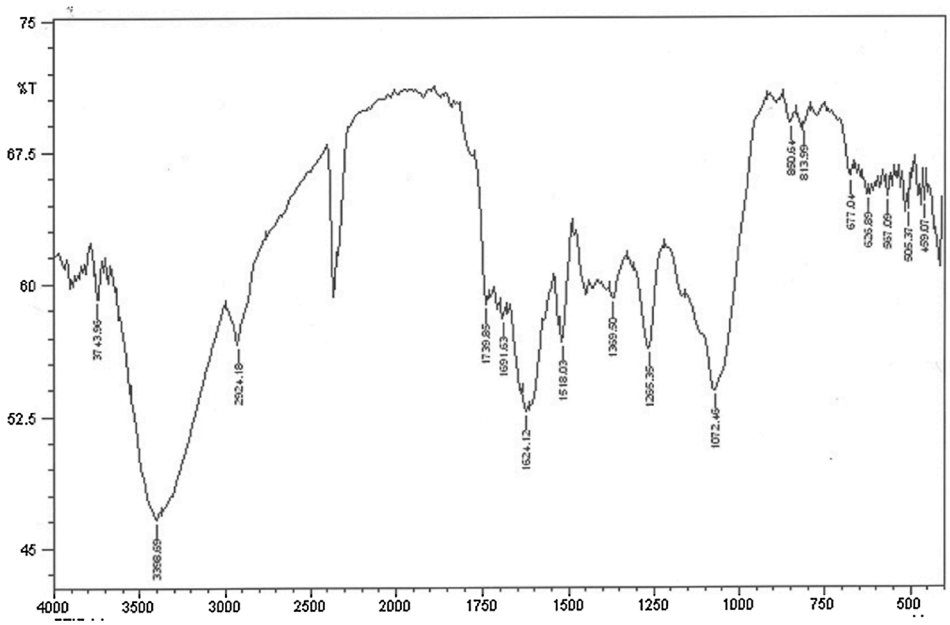

Fig. 3. The FTIR spectrum of synthesized copper nanoparticles. The bands at 3380, 2928, 1741, 1604, 1400, 1050, and 1271 were related to the O—H stretching of alcohol and phenol, C—H stretching of the aliphatic group, C═O stretching of ester carbonyl, C═C stretching of the aromatic ring, and C—O stretching of ester, respectively.

3.3. Ex vivo effect on protoscoleces

According to the obtained results, after adding CuNPs at the concentrations of 250, 500, and 750 mg/mL alone and combined with ALZ (200 mg/mL) to hydatid cysts, the CuNPs indicated considerable protoscolicidal effects alone and in combination with ALZ. Nevertheless, the findings proved that CuNPs, even in combination with ALZ, required a longer time to kill protoscoleces *ex vivo*. Fig. 6 shows the protoscolicidal effects of CuNPs alone and combined with ALZ *ex vivo* (200 mg/mL).

3.4. Evaluating caspase-3 like activity of CuNPs-treated protoscoleces

To evaluate the effect of CuNPs on the apoptosis induction in *E. granulosus* protoscoleces through the activity of caspase-3, the enzymatic activity of this enzyme was measured. For this purpose, the protoscoleces were treated with various concentrations of CuNPs for 48 h and the amount of change in the activity of the caspase-3 enzyme was measured by measuring the concentration of the released NA-p. After 48 h of treating protoscoleces, CuNPs in a dose-dependent manner and at doses of 250, 500, and 750 mg/mL induced the caspase enzyme activation by 20.5 %, 32.3 %, and 36.1 %, respectively (Fig. 7).

4. Discussion

In recent decades, nanomaterials, especially nanoparticles individually and combined with the existing/conventional antimicrobial agents, are increasingly applied to target pathogenic parasites as an alternative for current drugs [22]. It has been proven that nanoparticles because of their large surface-volume ratio and easier entry into the cell than other particles can interact with different living molecules and microbes. As a result, they can interrupt a negative activity on some microbial pathogens, especially parasites [22].

Although surgery remains the preferred treatment option for CE management, the rupture of cysts or leakage of their contents (protoscoleces) during surgery, which can result in re-infection, secondary infection, anaphylaxis shock, and even death in patients, is considered the most serious complications for this treatment approach [8]. Applying various chemical protoscolicidal agents such as hypertonic saline 20 %, silver nitrate, and formalin is one of the best strategies for preventing these complications [9]. However, previous studies have demonstrated that the current protoscolicidal agents are associated with some adverse side effects such as biliary fibrosis, hepatic necrosis, and cirrhosis [9,10]. Accordingly, we aimed to evaluate the *in vitro* and *ex vivo* anti-parasitic effects of CuNPs alone and combined with albendazole on *E. granulosus* protoscoleces.

The findings exhibited that CuNPs, especially along with CuNPs, had considerable protoscolicidal effects compared to the control group ($p < 0.001$). The maximum protoscolicidal activity of CuNPs was observed at the concentration of 750 mg/mL, such that 73.3 % of protoscoleces were killed after 60 min of exposure. Meanwhile, the mortality of protoscoleces was 100 % after 10 min of exposure to 750 mg/mL of CuNPs along with ALZ (200 mg/mL). CuNPs indicated considerable protoscolicidal effects alone and in combination with ALZ. Nevertheless, the findings proved that CuNPs, even in combination with ALZ, required a longer time to kill protoscoleces *ex vivo*. These findings promised that CuNPs specially in combination with ALZ can be used as an scolicidal agent intraperitoneally during hydatid cyst surgery.

Considering the anti-microbial effects of copper nanoparticles, previous studies have shown these NPs have considerable antimicrobial properties against a broad spectrum of microbial pathogens such as *Staphylococcus aureus*, *Salmonella enteric*, *Campylobacter jejuni*, *Escherichia coli, Listeria monocytogenes, Aspergillus niger, etc.* [23–25]. Also, Saad et al. (2015) reported that copper oxide nanoparticles with IC50 values of 0.13 mg/l for *Entamoeba histolytica* and 0.72 mg/l for *Cryptosporidium parvum* could be considered as a novel nanoform agent for treating *E. histolytica* and *C. parvum* infections [26]. In another study, Malekifard et al. (2020) showed that copper oxide nanoparticles at the concentration of 0.6 mg/mL killed 97 % of *Giardia lamblia* cysts after 180 min. This effect is similar to that of metronidazole [27].

Although the exact antimicrobial mechanisms of these nanoparticles are not yet understood, previous studies have shown that copper interacts with sulfhydryl groups (–SH) and results in the denaturation of protein in bacteria [24]. Besides, it has been proven that CuNPs can disrupt the cell membrane and multiple toxic effects such as generating reactive oxygen species, lipid peroxidation, protein oxidation, and DNA degradation in bacteria cells [28].

Inducing apoptosis or programmed cell death is considered as one of the main probable anti-microbial mechanisms of drugs. Since caspases

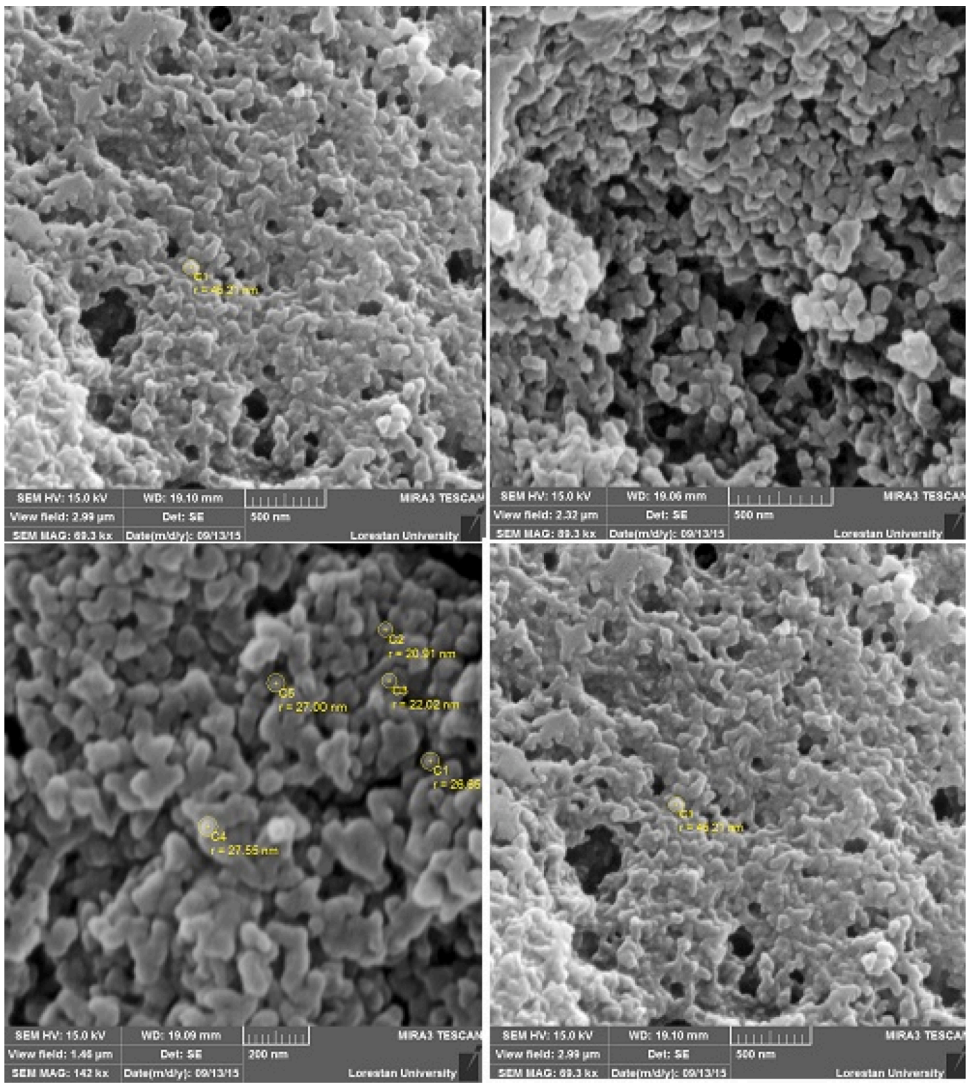

Fig. 4. Scanning electron microscope of copper nanoparticles synthesized using aqueous extract of *Capparis spinosa* fruit. The green synthesized CuNPs had spherical morphology and the size of the particles was measured between 17 and 41 nm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

are the main mechanisms of apoptosis [29], in the present study, the caspase-3 activity assay was performed to evaluate induced apoptosis in *E. granulous* protoscoleces treated with various concentrations of CuNPs. The obtained finding demonstrated that, after 48 h of treatment of protoscoleces, CuNPs in a dose-dependent manner and at doses of 250, 500, and 750 mg/mL induced the caspase enzyme activation by 20.5 %, 32.3 %, and 36.1 %, respectively. Therefore, inducing apoptosis can be suggested as one of the probable antimicrobial mechanisms of CuNPs. In line with our results, Chakraborty et al. (2017) demonstrated that CuNPs through caspase-9-mediated intrinsic pathway induced apoptosis in a human skin melanoma A-375 cell line [30]. Considering the cytotoxicity of CuNPs, Prasad et al. (2017) showed that CuNPs had no cytotoxicity at concentrations ranging from 0.5 to 1.5 μM on prostate cancer (PC-3) cell lines [31]. Ostaszewska et al. (2018) also indicated that CuNPs at the concentration of 0.15 mg/mL had no significant cytotoxicity on the rainbow trout (Oncorhynchus mykiss) hepatocytes after 4 weeks of incubation [32].

Conclusion and prospective for future work

The findings of the present investigation showed the potent protoscolicidal effects of CuNPs, especially combined with albendazole, as they entirely eliminate the parasite after 10−20 min of exposure. Based on the obtained results, although CuNPs has potent scolicidal efficacy *in vitro* and *ex vivo* as an intraperitoneal model of administration of drug to CE treatment; however, further studies are required to assess the safety and the efficiency of these NPs as a promising scolicidal agent in preclinical model (*in vivo* or animal model) and clinical setting not only in intraperitoneal administration but also from different routes such as oral route. The results also showed that although the possible protoscolicidal mechanisms of CuNPs are not clearly understood, the induction of apoptosis through caspases is one of the main mechanisms. However, the cellular and molecular mechanisms of action of these biogenic NPs against CE must be clarified. These can help provide a new vision in NPs target, and perhaps give a chance for designing a new and more effective drug for human CE in the near future.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

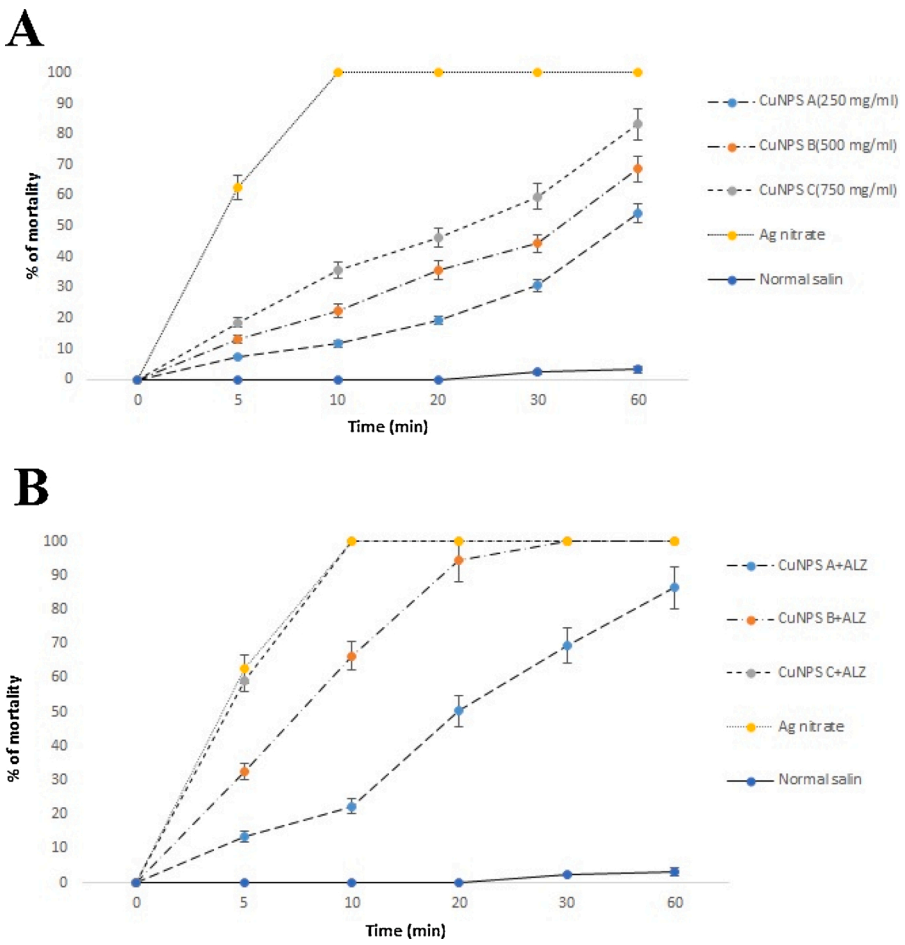

Fig. 5. *In vitro* scolicidal effects of CuNPs alone and in combined with albendazole (ALZ, 200 μg/mL) against *E. granulosus* protoscoleces at various concentrations following various exposure times. The findings exhibited that CuNPs, especially along with CuNPs, had considerable protoscolicidal effects compared to the control group ($p < 0.001$). The maximum protoscolicidal activity of CuNPs was observed at the concentration of 750 mg/mL, such that 73.3 % of protoscoleces were killed after 60 min of exposure.

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Not applicable.

Declaration of Competing Interest

The authors report no declarations of interest.

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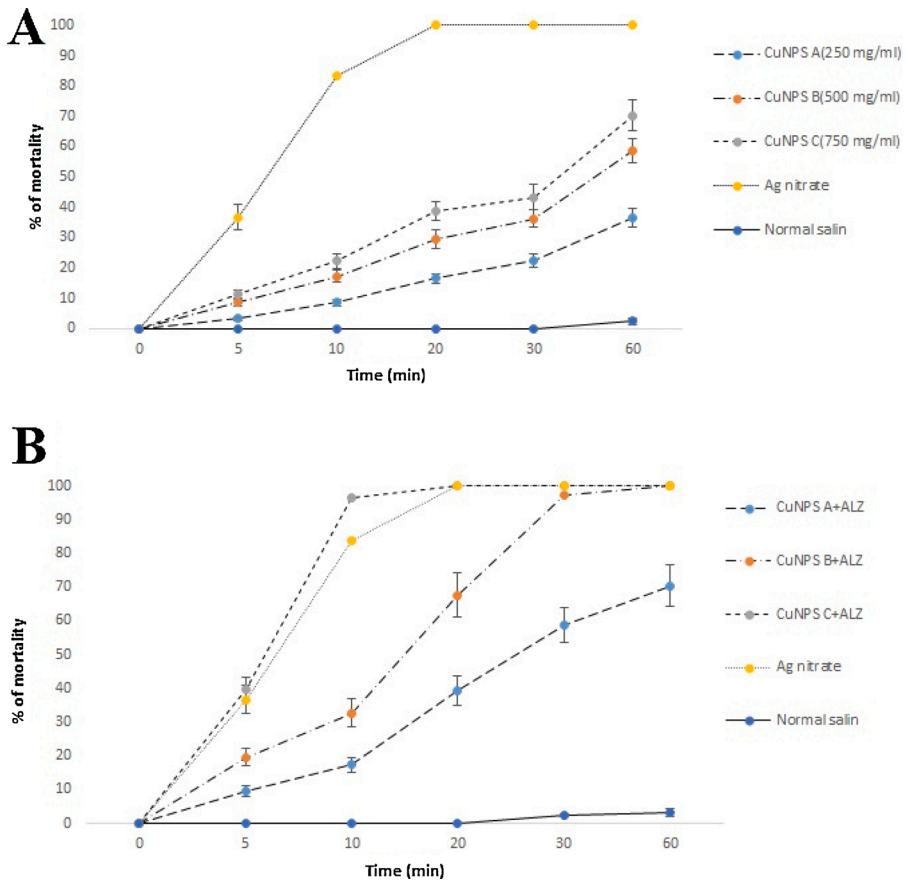

Fig. 6. Ex vivo scolicidal effects of CuNPs alone and in combined with albendazole (ALZ, 200 μg/mL) against *E. granulosus* protoscoleces at various concentrations following various exposure times. The findings proved that CuNPs, even in combination with ALZ, required a longer time to kill protoscoleces *ex vivo*.

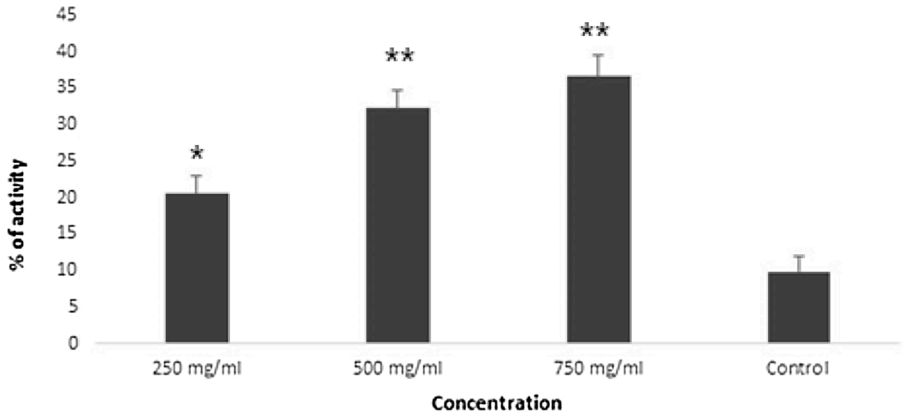

Fig. 7. Caspase-3 activity of *E. granulosus* protoscoleces at various concentrations of CuNPs. After 48 h of treating protoscoleces, CuNPs in a dose-dependent manner and at doses of 250, 500, and 750 mg/mL induced the caspase enzyme activation by 20.5 %, 32.3 %, and 36.1 %, respectively. Data show as mean ± SD from three experiments in duplicate. * $p < 0.05$, ** $p < 0.001$.

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