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Original Article

In Vitro Assessment of Anthelmintic Activities of AgO Nanoparticle against Liver Fluke *Dicrocoelium dendriticum*

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Abstract

Background: Dicrocoeliasis is an important helminthic zoonosis reported from many parts of the world. Due to low-performance medications, drug delivery is a great challenge in improving the treatment of this liver fluke infection. We aimed to determine the anthelmintic properties of Nanosilver oxide (AgO) against *Dicrocoelium dendriticum* infection.

Methods: The impacts of various concentrations of AgO nanoparticles (50-200 μg/ml) for 12-24 hours were compared with closantel, a chemical drug. The anthelmintic efficacy was evaluated using the scanning electron microscopy (SEM) technique. The synthesized nanoparticles were analyzed for structural assessment using XRD, UV–VIS spectroscopy, and SEM. The XRD pattern shows the formation of AgO nanoparticles.

Results: The UV-VIS spectra showed the broad peak, corresponding to Ag nanoparticles. SEM images of treated parasites by AgO (200 μg/ml) showed severe damage, which includes complete loss of sensory papillae and destruction of prominent network structures and tegument vesicles. The mortality rate increases with the increase in the concentration and exposure time of the parasite to nanoparticles. Besides the MTT assay, the toxicity of AgO, at concentrations of 800 μg/ml was 8.7%.

Conclusion: AgO NPs have potent anthelmintic effects on liver fluke *D. dendriticum*. This is the first research that assessed the effect of AgO NP on liver fluke *D. dendriticum*. Hence, the present study provides a basis for future research on the control of this common trematode.

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Introduction

Dicrocoeliasis is a worldwide hepatic bile duct trematode disease, which parasites both humans and a wide range of grazing mammals and counts as one of the major threats to livestock production in endemic areas (1). *Dicrocoelium* spp. is an important helminthic food-borne zoonosis that is reported in some country of the world. Most true human infections are caused by *D. dendriticum*, which occurs in some parts of the world (2, 3).The importance of dicrocoeliasis is often forgotten in the food safety and hygiene community due to the rarity of human clinical cases.

However, the World Health Organization Foodborne Disease Burden Epidemiologic Reference Group notes that *D. dendriticum* was included on its list of causative agents for which disease burden estimates could be made (4).

It is now recognized that dicrocoeliasis is a neglected parasitic disease, causing major public health problems and significant economic impacts, including a reduction in the production of ruminants (4-6). Dicrocoeliasis does not show any symptoms in the majority of cases or causes only minor sings, so most cases remain undiagnosed. Infection usually causes mild clinical symptoms, but serious health problems may be seen in severe animal infections (6, 7).

The severe pathological changes including distension, hardened liver, pallor, abscesses, granulomas, and fibrosis found in the liver and biliary system of animals with sever *D. dendriticum* infection. In the advanced stage of infection, hyperplasia of bile duct epithelium, periodical inflammation, and cholangitis with thickened bile ducts are associated with infection. In the terminal stage and chronic disease, it leads to the development of biliary fibrosis and liver cirrhosis (8, 9). The abdominal pain, flatulence, dyspepsia, increase in bilirubin and albumin and watery diarrhea have been reported during dicrocoeliasis (7). There are narrow therapeutic choices for the treatment of disease in animals and drugs need to be used as an unapproved indication. It is troublesome to regulate whether anthelmintic drugs applied at dose rates and routes endorsed for grazing herbivores are able to eradicate parasite in definitive host as well. The possible hazard, of either inefficient levels or the danger of expansion of anthelmintic resistance or leading to toxic levels, is accordingly high (4, 10, 11). Nowadays, chemotherapy widely used as one of the strategies to dicrocoeliasis control. However, other alternative treatment may be used due to cost of small ruminants treating and drug resistance emergence (3).

At present, chemical anthelmintic drugs, including Benzimidazole, pro-benzimIdazole families, and Albendazole have been widely used. However, these drugs are not easily available in distant rural areas and also have some serious disadvantages. Toxicity of albendazole in chemotherapy of camelids dicrocoeliasis is reported (10). Further studies are needed concerning the safety of other benzimidazoles in animals, due to consumption higher dosage rate against *D. dendriticum* compare to other tapeworms (11).

In the recent decade, nanoparticles (NPs) due to their defined properties, have considerable interest, which makes them a favorable candidate for anthelmintic application. Among a wide variety of nanoparticles, silver nanoparticles (AgNPs) are one of the most vital and fascinating nanomaterials that are involved in biomedical applications (3, 12). One of the most vital and unique applications of AgNPs that makes them ideal in the field of medicine is using them as antimicrobial agents, as well as for use in nanotoxicology studies (13). One of the most investigated nanostructures is AGNPs which have promising, and interesting characteristic benefits for various enhanced biomedical applications in recent years (14).

AgNPs are convinced to have legitimate dramatic capabilities for the improvement of antimicrobial agents, drug-delivery formulations, identification and diagnosis platforms, and performance-enhanced therapeutic alternatives (15). The exact mechanism of the action of silver nanoparticles on cells is not yet fully understood. The effect of AgNP on cells has several mechanisms, including adhesion to the surface of the cell wall and membrane, penetration into cells and destruction of intracellular organelles and biomolecules, induction of oxidative stress, and regulation of signal transduction pathways (16). It is widely believed that AgNPs, which can be easily ionized, can affect cells through Trojan horse mechanisms(17). The phagocytosis of AgNPs stimulates inflammatory signaling through the production of reactive oxygen species (ROS) in macrophage cells, and activated macrophage cells induce TNFα secretion. Elevated TNFα levels cause cell membrane damage and apoptosis (18). A number of studies have shown the toxicity of AgNPs on hepatocytes and neuronal cells of rats, mouse stem cells, and human lung epithelial cells in relation to cells, and healthy mammalian cells (19–24).

Bio-nanoparticles of silver are proper to subsequent scientific studies and practical medical use due to low toxicity, economic cost production and diverse potential to solving some biological problems.

We aimed to investigate the anthelmintic activities of AgONPa in comparison to the chemical drug, closantel, against *D. dendriticum*.

Methods

Ethical Approval

The study protocol was approved by the Ethics Committee of Kashan University of Medical Sciences, Iran, (Approval ID is IR.KAUMS.MEDNT.REC.2018.23).

Synthesis of AgO nanoparticle

One gram of silver nitrate, under ultrasonic irradiation (180W), dissolved in 20 ml deionized water and stirred to archive a clear solution. Then, 1.5 g of potassium persulfate (K2S2O8) was added as a powerful oxidant under 180 W ultrasonic irradiation and stirred for 15 min. Finally, the gray sediment was filtered and washed by distilled water three times. The precipitate dried at 60 °C for 24 hours.

Collection of D. dendriticum

Adult fresh *D. dendriticum* were isolated from the liver of slaughtered goats and sheep from the Kashan slaughterhouse. The collected parasites were identified based on the morphological and morphometric characteristics of the isolated parasites.

MTT viability assay

The MTT assay was used to measure the viability of the Hela cells to find the optimum concentrations of AgO. Some 10^5Hela cells per well were placed in 96-well plates along with introducing different concentrations of AgO to each well and keeping at 37 °C, 5% CO2 for 24 h. Afterward, the MTT solution (20 µl, 5 mg/ml in PBS) was added to each well and further incubated for 3 h. After 3 hours, the supernatant of each well was removed and 100 µl of DMSO was added to each well. After 15 min incubation with DMSO, the ELISA plate reader was used for reading the absorbance of each well at 570 nm (14) (25).

Cell Viability (%) = 100 * OD sample/OD control

In vitro assays

The investigation was carried out in three groups: experimental groups NPs: 50, 100, 150, and 200 µg/ml), positive control (closantel: 50, 100, 150, and 200 µg/ml) and negative control (only RPMI1640 media culture). Under sterile conditions, liver flukes were transferred into the 24 well, each well contained 1ml of RPMI1640 (50IU/ml of penicillin, 50IU/ml of streptomycin, 50% V/V of FBS, and 2% of sheep red blood cells). Afterward, 1ml of AgONPs were added individually to

each well and incubated for 12, 18, and 24h at 37 °C in an atmosphere of 5% CO_2. The number of live worm was checked during these times.

Motility examination

The motility time (12, 18, and 24 h) of worms after incubation in different concentrations of the treatments and control groups was calculated and the viability of experiments was measured based on the motility criteria (the whole body moving high, parts of the body moving, less movement of the whole body and complete loss of motility). The motility assay in control groups was noted, according to the absence of the experimental compounds. All worms in each experiment were observed individually at hour intervals according to the motility criteria established.

Scanning electron microscopy (SEM) sample preparation

For the determination of the ultrastructural alteration in the tegumental of worms, we used electron microscopy (SEM). In the first step, the treated and control flukes were fixed with sodium cacodylate buffer (pH: 7.4, 0.2 M) and glutaraldehyde in phosphate buffer (2.5% v/v) for 4 h at 4 °C, and then the parasites were washed 3 times in phosphate buffer (pH: 7.4). In the second step, they were dehydrated in ascending ethanol concentrations (70%, 80%, 95%, and 100%) for 30 min, final dehydration was achieved in hexamethyldisilazane and consequently dried in the vacuum oven. Finally, mounted on stubs, sputter-coated with gold, and they were photographed by SEM (ZEISS-DSM 960A, Germany) at the central laboratory of the Institute for Color Science and Technology, Tehran, Iran.

Results

Structural study

Crystal structure and phase purity of the synthesized AgONPs were measured by X-ray powder diffraction (XRD). Based on the XRD pattern of Silver Oxide NPs (Fig. 1), the observed diffraction peaks can be indexed to the pure Monoclinic phase of AgO with space group P21/c (JCPDS no. 80-1269).

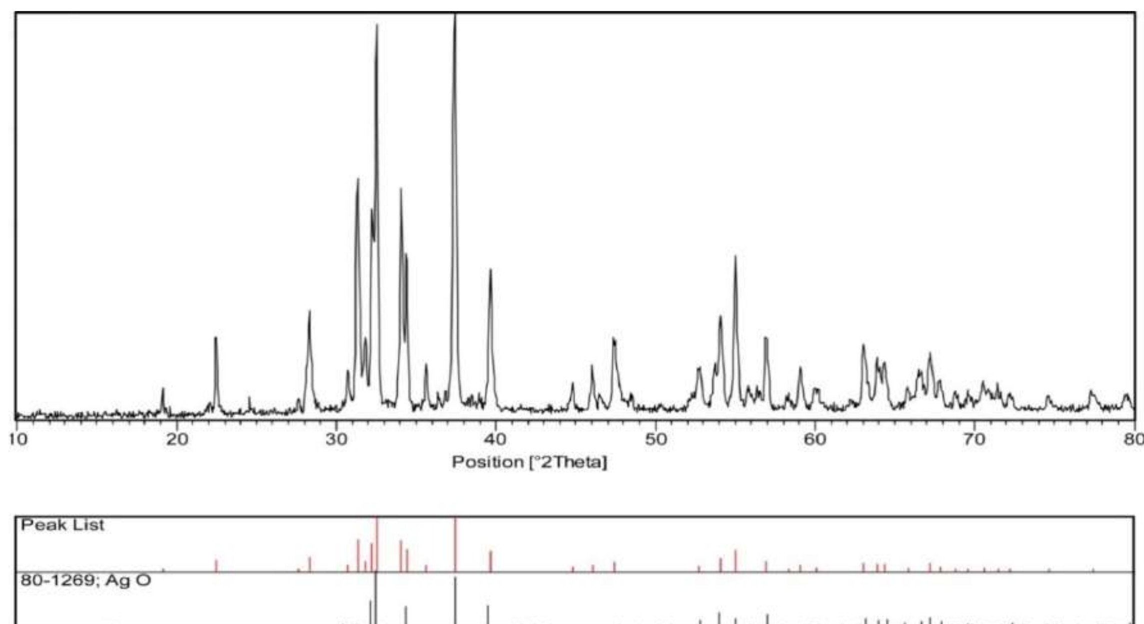

Fig. 1: XRD pattern of Silver Oxide nanoparticle

The crystallite diameter (Dc) of AgONPs based on the Scherrer equation ($Dc= K\lambda/\beta\cos\theta$) was calculated to be 19.59 nm. Where β is the breadth of the observed diffraction line at its half intensity maximum, K is the so-called shape factor, which usually takes a value of about 0.9, and λ is the wavelength of the X-ray source used in XRD. Further prove the composition of AgO was performed by energy dispersive spectroscopy (EDS), as shown in Fig. 2a. EDS spectrum reveals that NPs composed of only Ag and O atoms. The SEM images demonstrated silver Oxide NPsmainly consists of rice-shape NPs with an average size of 20-40 nm (Fig. 2b).

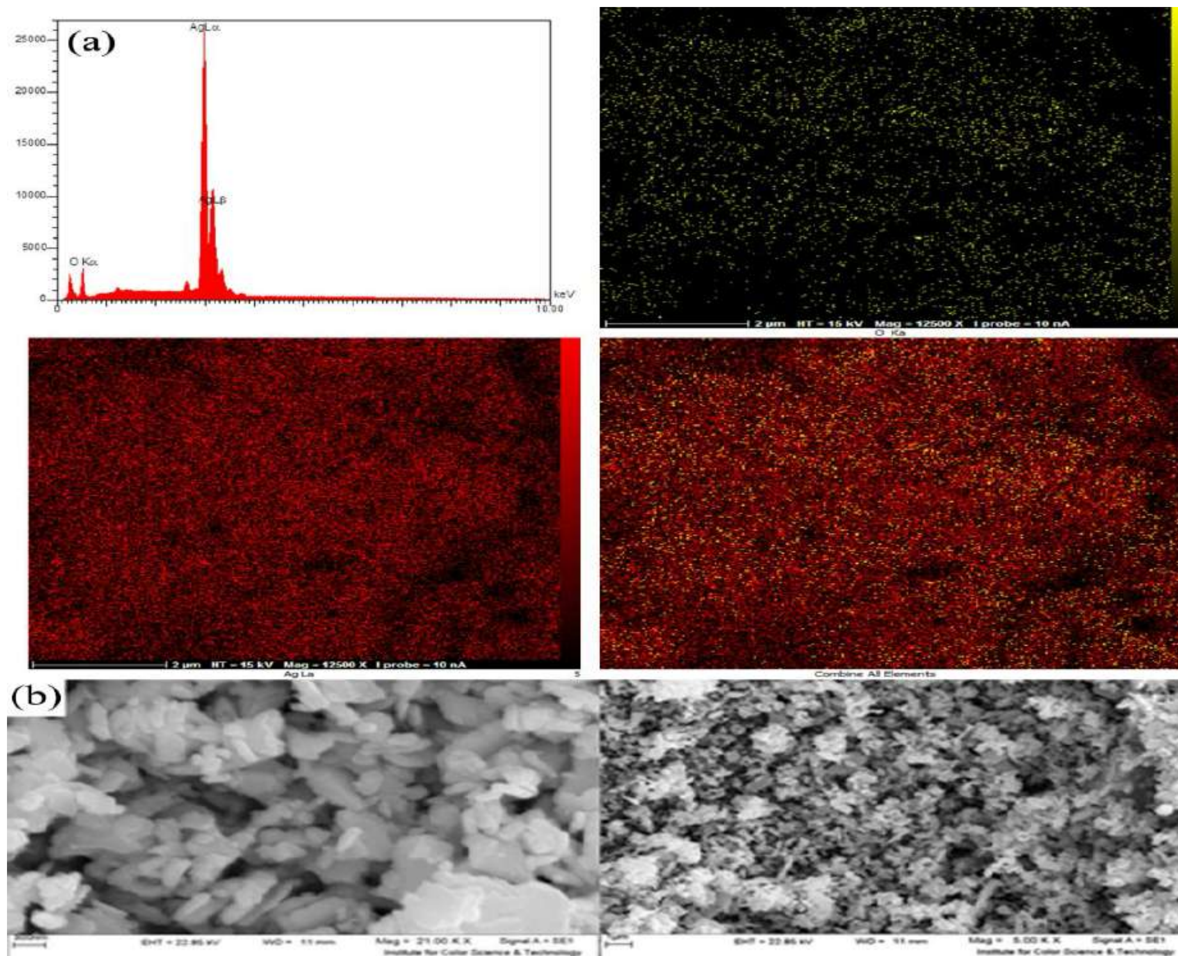

Fig. 2: (a) Energy dispersive spectroscopy (b) SEM images of Silver Oxide nanoparticle nanoparticles with diameters of 20 nm

Besides, Furrier transform infrared spectroscopy (FT-IR) was also carried out to check the presence of certain functional groups in AgONPs, as depicted in Fig. 3a.

FT-IR spectrum shows three absorption bands at 3182, 1059, 705 cm-1 which can be attributed to the stretching and bending vibrations of H2O molecules (3182 and 1059 cm-1) and the Ag-O bond (705 cm-1).The optical property of as-synthesized AgONPs was determined using UV-vis spectroscopy. Fig.3b depicts (αhν) 1/2 vshν curve of AgONPs calculated based on the Wood and Taucequation. αhυ = (hv-Eg) n where α is the absorbance, h

the Planck constant, ν the photon frequency, Eg the energy gap, and n the pure numbers associated with the different types of electronic transitions.

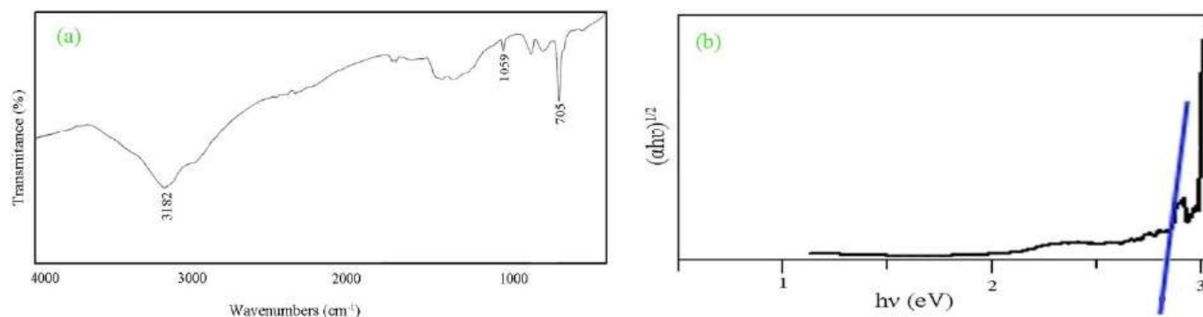

Fig. 3: (a) FT-IR pattern (b) UV-Vis pattern of Silver Oxide nanoparticle

For n=1/2, 2, 3/2, and 3, the transitions are directly allowed, indirectly allowed, directly forbidden, and indirectly forbidden, respectively. Each energy gap was determined by the extrapolation of each linear portion of the curves to a= 0. Hence, the energy gap of AgONPs was calculated to be2.9 eV.

In vitro toxicity assay

Toxicity values (TC50) of HeLa cells were measured after 24 h incubation and were compared with positive and negative control groups (Table 1 and Fig. 4).

Table 1: Toxic values (TC50) of Hela cell exposed to the AgO nanoparticles

Concentration	50(*μg/ml*)	100(*μg/ml*)	150(*μg/ml*)	200(*μg/ml*)
Survival %	100	99.3	80.2	79.3
Toxicity%	0	0.7	19.8	20.7

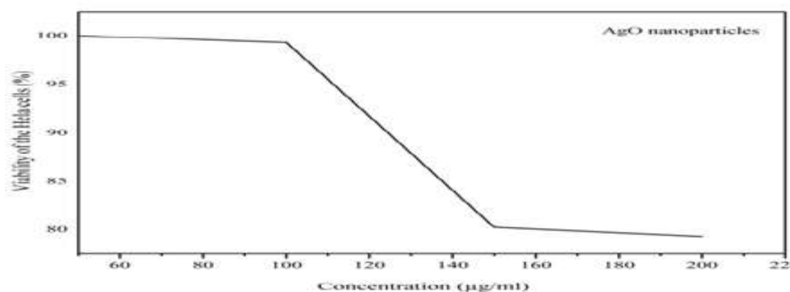

Fig. 4: TC50 values of Hela cell after 24 h incubation with AgO nanoparticle

Worm motility assays

After 12 h incubation at a concentration of 150μg/ml of AgONPs as well as the highest concentration of 200 μg/ml all worms were dead. Furthermore, after 12 h incubation at 200μg/ml of closantel, all worms died. The decrease in the motility rate of flukes treated was both time and concentration-dependent (Table 2).

Table 2: Comparison Lethal concentration (LC) values of AgOwith control group (closantel) during 12, 18, and 24 h

Groups	LC (Hour)	LC10	LC25	LC50	LC75	LC90
AgO nanoparticle	12	87.9	95.03	102.9	110.9	120.1
	18	39.7	60.6	83.8	101.2	115.1
	24	39.1	45.8	52.5	59	62.5
Closantel	12	89.5	106.3	125	143.6	160.4
	18	89.5	106.3	125	143.6	160.4
	24	87.8	95	102.9	110.9	118
Comparison between groups		P<0.001				

Scanning electron microscopy (SEM)

By the SEM technique, the control worms seem normal with unchanged tegument around suckers, and their oral and ventral suckers are round and smooth. Besides, sensory papillae at the edges and inside the oral sucker, tegumental ridges network, and vesicles look unaltered. Besides, ridge walls and valley floors cover are densely by tegumental vesicles in the entire body, which seem to be like spherical structures (Fig. 5, a-d).

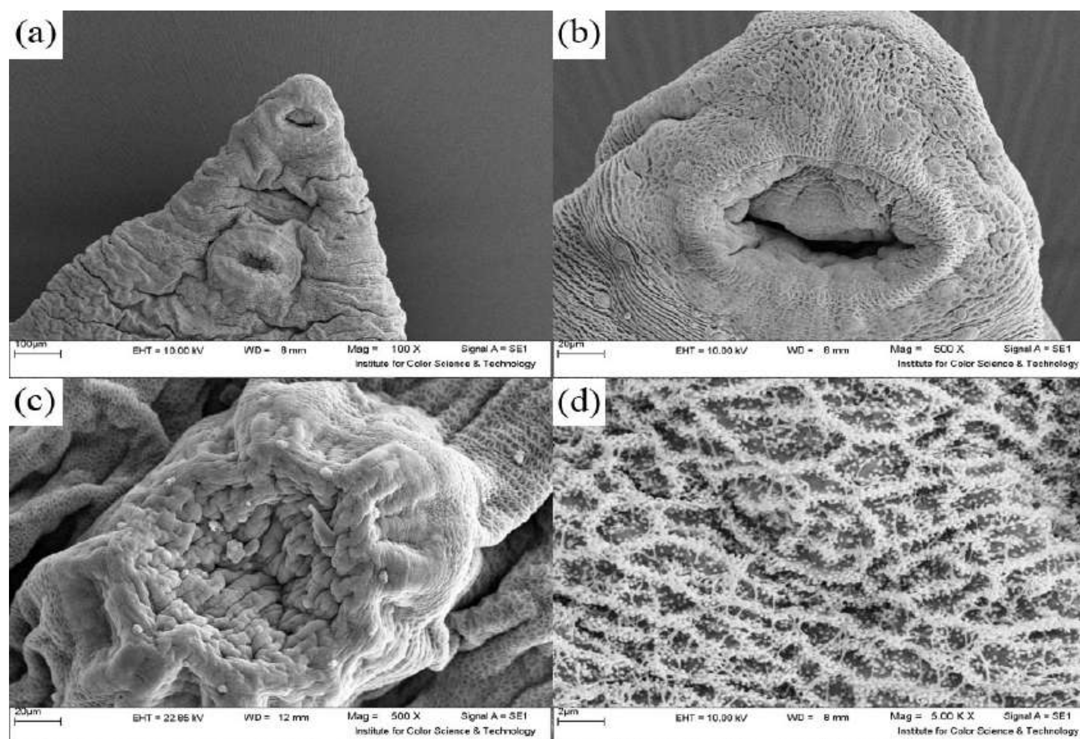

Fig. 5: SEM images of adult *D. dendriticum*(Control) worms incubated in RPMI showing: (a, b) round and smooth oral sucker (OS) and ventral sucker (VS), larger than OS, (c) normal and intact tegumental enfolding's around sucker, sensory papillae at the edges and inside OS, (d) tegumental ridges and vesicles covering the valley floors

SEM images of treated worms with AgONPs (100 µg/ml) demonstrated that their tegumental region endured a variety of changes, including appearing severe swelling, swollen and blistering (Fig. 6a), destroying sensory papillae (Fig. 6b), and also destroying the network structure and tegument vesicles (Fig. 6, c, d).

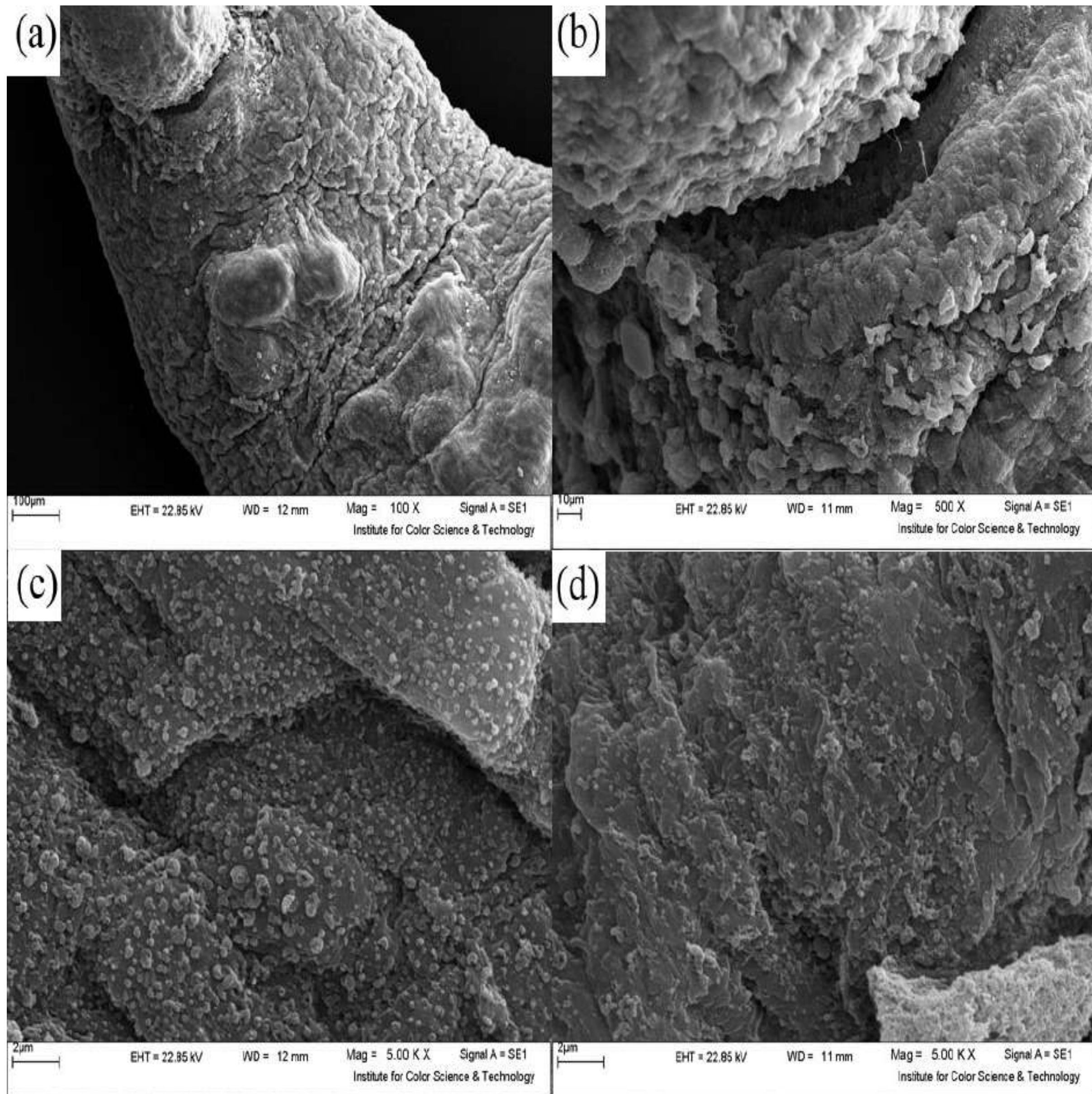

Fig. 6: SEM showing the effect of 100 µg/ml AgO NPs on the tegumental surface of adult *D. dendriticum*: (a) swollen and blister on the surface of tegument, (b) loss of sensory papillae and severe tegumental damage, (c,d) complete destruction of prominent network structure and tegument vesicles

The tegumental changes in treated worms with closantel (200 µg/ml) are shown in Fig. 7a-f. Based on Fig. 7a, swelling, erosions, and blebs appeared on the surface tegumental. Besides, cirri were damaged and lost their natural appearance (Fig. 7b). Also, sensory papillae disappeared completely, and oral and ventral suckers are flaky completely insofar as a little recognizable structure remained (Fig. 7c-f).

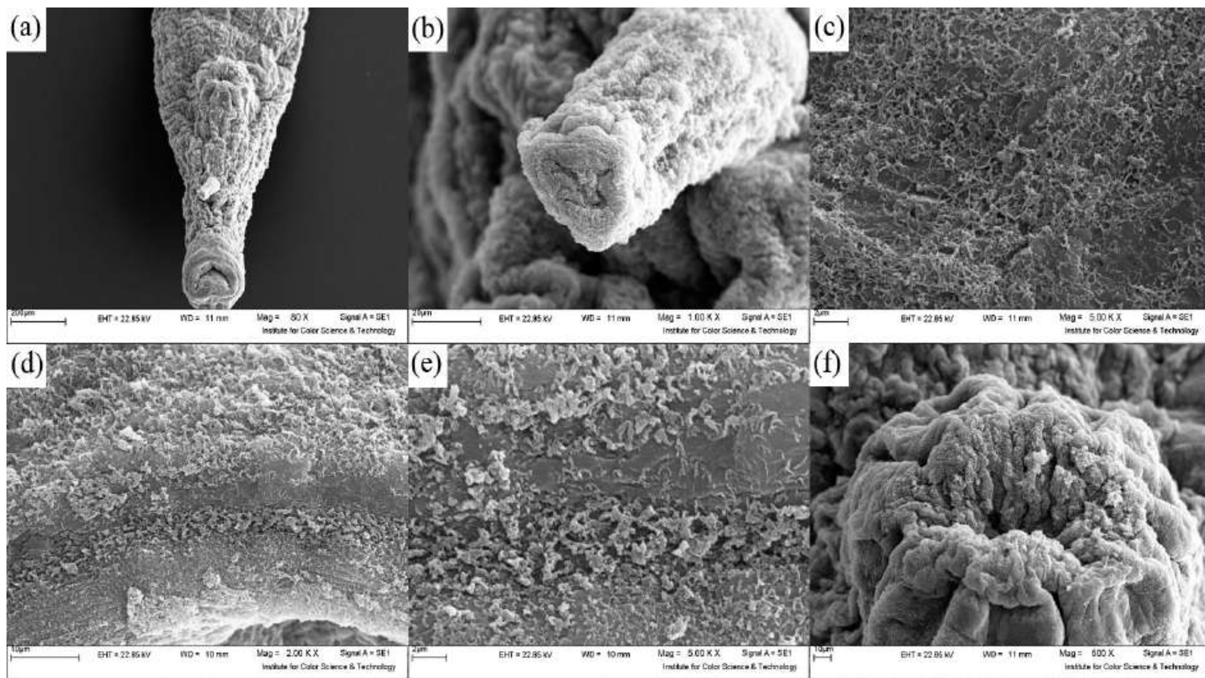

Fig. 7: SEM showing the effect of 200 µg/ml closantel the tegumental surface of adult *D. dendriticum*: (a) swelling on the surface of tegument, (b) loss of natural morphology of cirrus and severe damage (c-e) complete destruction of prominent network structure and tegument vesicles, (f) complete loss of sensory papillae

Discussion

Dicrocoeliasis is currently treated with available chemotherapeutic anti-helminthic drugs, but overuse has led to the development of resistance to these drugs over time (3). The past decade has seen remarkable progress in the field of nanomedicine for parasite control. One of the most demanded of nanoparticles in different groups of metal nanoparticles, is silver nanoparticles (AgNPs) due to a number of activities in exponentially various precincts.

These nanoparticles have demonstrated remarkable effects such as damaging parasite membranes, destroying DNA (deoxyribonucleic acid), inhibiting protein synthesis, and generating free radicals. Increased use of AgNPs-enriched products may lead to increased levels of toxicity affecting living organisms such as parasites(25-28).Given the widespread use of AgNPs, it is important to assess the risks of these nanoparticles. Previous study has demonstrated the ability of AgNPs to induce harmful biological and cellular effects (26). These nanoparticles adhere to the cell walls and membranes of microorganisms and can enter the cell. They damage cell organelles, cause the production of reactive oxygen species, and impair signal transduction (13). Several studies report applications where good results have been obtained when using silver nanoparticles for the control of pathogenic microorganisms in the medical and public health fields (18, 30). Despite the widespread use of silver nanoparticles, few studies have been performed on AgNPs against Platyhelminth parasitic infections (26, 31–33).

Besides, several investigations have been performed on the anti-parasitic impact of the AgNPs on the *Gigantocotyle explanatum*, *Haemonchus contortus*, *Ancylostoma caninum*, and *Fasciola hepatica* (33–36). AgO have a high attitude to create reactive oxygen species (ROS) and free radicals, which are responsible for causing oxidative stress and apoptosis leading to cell death, which ends up with acceptable antibac-

terial, antifungal, antioxidants and anti-parasite (37). Indeed, the enormous creation of ROS in cells by direct interaction with particles is at present accepted as one of the major mechanisms of cellular toxicity of nanoparticles (38–40). ROS have many signaling and information functions; however, excessive ROS can collapse the antioxidant defense system, leading to the damage of DNAs, lipids, and proteins (40, 41). AgNPs with larger surface areas provide better contact with organisms and easily rupture the cell wall. It has also been shown that smaller nanoparticles are more toxic than bigger ones. The toxicity of Ag NPs is dependent on the concentration, pH of the medium, and exposure time to pathogens (42). Yin et al. study showed that nano scale size and large ratio of surface area to volume may be cause of potency of AgNPs. These character increased permeability of membrane cell, produce reactive oxygen species and releasing silver ions that resulting to interrupt the replication of DNA (43).

Also, antioxidant enzymes have been recognized as important modulators in AgNPs-induced oxidative stress. Two of them, catalase (CAT) and superoxide dismutase (SOD), are prominent for maintaining the level of ROS in organisms and are used as bio-indicators of increased ROS production (25). Prior studies showed that AgNPs induce oxidative stress due to altering the activity of CAT and SOD enzymes *in vivo* and in vitro assays. The alteration of the enzyme activity may be associated with either the regulation of gene or due to the direct surface interaction of the enzymes with AgNPs (44). The molecular mechanisms of the interaction between enzymes and nanoparticles were also reported (45).

In vivo studies are needed to complement the results of the present study.

Conclusion

AgONPs can be used to treat *D. dendriticum* infection. SEM demonstrated that AgONPs have dose-dependent anthelmintic efficiency. Nonetheless, additional research is necessary to evaluate the *in vivo* efficacy of this treatment as well as its toxicity on a definitive host.

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Conflict of interest

The authors declare that they have no competing interests.

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