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Assessment of oxidative stress biomarkers in liver fluke *Dicrocoelium dendriticum* following exposure to copper oxide and zinc oxide nanoparticles

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Abstract

Dicrocoeliasis is a globally significant condition impacting both economic and public health. The lack of effective vaccines and emergence of drug-resistant flukes have prompted research into alternative treatments. Metallic nanoparticles have recently been studied for their potential as anthelmintic agents. This research examined the *in vitro* anthelmintic activity of copper oxide (CuO-NPs) and zinc oxide nanoparticles (ZnO-NPs) against *Dicrocoelium dendriticum*. Using adult motility inhibition tests and oxidative stress biomarkers, including glutathione peroxidase , glutathione S-transferase , superoxide dismutase , and malondialdehyde , this study evaluated the effects of CuO-NPs and ZnO-NPs. Flukes were treated with various concentrations of nanoparticles (1.00, 4.00, 8.00, 12.00, and 16.00 ppm) for 24 hr. The CuO-NPs and ZnO-NPs demonstrated concentration- and time-dependent anthelmintic activity. Higher concentrations (12.00 and 16.00 ppm of CuO-NPs, and 16.00 ppm of ZnO-NPs) significantly inhibited worm motility compared to the controls. The nanoparticles induced oxidative stress in the flukes, with decreased superoxide dismutase, glutathione S-transferase, and glutathione peroxidase activities and increased malondialdehyde levels. Based on these findings, CuO-NPs and ZnO-NPs exhibit potential as therapeutic agents for controlling and treating *D. dendriticum*. However, further studies are necessary to assess their safety and efficacy *in vivo* for managing parasitic infections.

Introduction

Dicrocoeliasis is a common disease caused by a hepato-biliary duct trematode affecting both humans and a variety of grazing herbivores. It is considered one of the greatest threats to livestock production in endemic areas.[1,2] *Dicrocoelium* species, along with *Fasciola* species, belong to a group known as food-borne trematodes.[3,4] The liver fluke *Dicrocoelium dendriticum* is widespread in Europe, Asia, North Africa, and North America and is considered the main cause of this disease. This infection is now classified as a neglected tropical disease and contributes to significant health problems and economic impacts. Most *D. dendriticum* infections are asymptomatic or cause only mild symptoms, resulting in many cases going undetected. While mild cases usually do not cause serious problems, severe infections can cause serious health problems in affected animals.[5,6]

The clinical and economic impact of dicrocoeliasis is significant, mainly due to the losses caused by the destruction of infected livers and digestive disorders due to the hepato-biliary changes. These problems result in reduced live weight, slower growth rates, and reduced milk production. In addition, the costs associated with anthelmintic treatments must also be taken into account.[7]

There is currently no vaccine to prevent dicrocoeliasis, making chemotherapy one of the most widely used control strategies.[4] Chemical anthelmintics, including those from the benzimidazole and probezimidazole families, including albendazole, have been widely used. However, these drugs are not easily accessible in remote rural areas and have significant drawbacks. These include the possible development of drug resistance, adverse drug reactions, residual effects, toxicity issues, and high veterinary costs. Additionally, although albendazole is an option for treating dicrocoeliasis, it has been reported to be toxic to camelids.[8] There is a growing concern about the increasing incidence of drug resistance in flukes, highlighting the need to explore alternative treatments.[4]

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Due to their nano-scale size and significant properties, nanoparticles are used in various scientific fields, including cancer therapy, drug delivery, and medicine.[9] Their ability to produce reactive oxygen species (ROS) makes them an effective agent for eliminating infectious pathogens.[10] Furthermore, due to their small size, nanoparticles can easily pass through membrane barriers, resulting in higher reactivity.[11] Many environmentally friendly and effective nanoparticles have been successfully developed to eliminate intestinal parasites and liver flukes.[12-17]

Copper is a widely used element in various industries, especially electrical applications, due to its affordability. Recently, copper oxide nanoparticles (CuO-NPs) and other metal nanoparticles have been used to prevent and control parasites, such as mosquito larvae, *Fasciola hepatica,* and *Giardia duodenalis*, due to their powerful effects.[17-19]

Zinc is an essential element for human health; however, it can also be toxic to micro-organisms.[20] Zinc oxide (ZnO) is a mineral contained in zincite that is non-toxic and is often used to treat skin conditions in humans.[21] Zinc oxide nanoparticles (ZnO-NPs) have attracted increasing attention due to their safety for humans and animals, as well as their stability under various conditions.[14] These nanoparticles exhibit diverse physicochemical properties, making them highly effective as anti-bacterial and anti-parasitic agents.[14,16,17,22]

The current study proposes that CuO-NPs and ZnO-NPs can act as anthelmintics by inducing DNA damage and oxidative stress. This is attributed to the ability of metallic nanoparticles to generate oxidative stress and produce free radicals in biological systems.[17,23] Therefore, this research aimed to evaluate the anthelmintic effects of CuO-NPs and ZnO-NPs by measuring various parameters, such as the motility of adult worms. In addition, the study investigates the influence of CuO-NPs and ZnO-NPs on the development of oxidative stress by evaluating several biomarkers, including superoxide dismutase (SOD), glutathione peroxidase (GSH), malondialdehyde (MDA), and glutathione S-transferase (GST), by *in vitro* methods.

Materials and Methods

Nanoparticles. Copper oxide nanoparticles (size: 10.00 - 40.00 nm) and ZnO-NPs (size: 10.00 - 30.00 nm) were obtained from Iranian Nanomaterials Pioneers Company (Mashhad, Iran). These nanoparticles were originally manufactured by the US Research Nanomaterials Inc. (Houston, USA). To create a homogeneous suspension, the nanoparticles were dispersed in highly pure water and sonicated at 100 W and 40.00 kHz for 40 min. The ZnO-NPs and CuO-NPs were then serially diluted in sterile ultra-pure water and sonicated for an additional 40 min. During the dilution process, magnetic rods were added to the suspensions to prevent particle aggregation or sedimentation.[23]

Preparation of CuO-NPs and ZnO-NPs suspensions. To prepare nanoparticle suspensions with different concentrations, the previously described procedures were followed.[13] Stock suspensions of CuO-NPs and ZnO-NPs were prepared in phosphate-buffered saline at the pH of 7.40. An ultra-sonic probe (Branson Ultrasonics Corp., Brookfield, USA) was used intermittently for 10 min at 30.00 W to sonicate the solution, which helped to prevent agglomeration and ensure uniform dissolution. Different concentrations of CuO-NPs (1.00, 4.00, 8.00, 12.00, and 16.00 ppm) and ZnO-NPs (1.00, 4.00, 8.00, 12.00, and 16.00 ppm),[14,17,23] were prepared in RPMI 1640 medium (Sigma-Aldrich, St. Louis, USA) by diluting the stock solution (Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany), supplemented with 5.00% (v/v) fetal bovine serum (Sigma-Aldrich) and 10.00 mL L^{-1} penicillin-streptomycin (Yakhteh Fanavar Company, Isfahan, Iran) solution.[14]

Parasite collection. Adult flukes of *D. dendriticum* were collected from the bile ducts and gallbladders of cattle slaughtered at the local slaughterhouse in Urmia, Iran. The flukes were thoroughly washed in Hank's balanced salt solution (Corning Inc., New York, USA) and subsequently incubated separately in RPMI 1640 medium containing different concentrations of CuO-NPs and ZnO-NPs.[24] Only completely intact and actively motile worms were selected for the study.

***In vitro* treatment of parasites.** To examine the *in vitro* effects of nanoparticles on adult *D. dendriticum* worms, a total of 10 worms were cultured in triplicate in 5.00 mL of RPMI medium supplemented with 5.00% (v/v) fetal bovine serum, contained various concentrations of nanoparticles. The cultures were incubated for 24 hr at 37.00 ± 1.00 ˚C. Albendazole (50.00 µg mL^{-1}; Daroupakhsh Pharmaceutical Company, Tehran, Iran) and phosphate-buffered saline were included in the assays as positive and negative controls, respectively. After incubation, the adult *D. dendriticum* worms were thoroughly rinsed with phosphate-buffered saline. The parasites were then homogenized in 100 mM Tris-HCl buffer (pH: 7.40; Yekta Tajhiz Azma, Tehran, Iran) and centrifuged at 10,000 *g* for 30 min at 4.00 ˚ C, and the supernatant was collected and stored at – 80.00 ˚ C until use.[24]

Observation of parasite mortality and mobility. In this study, parasite mortality and mobility were monitored every 4 hr for up to 24 hr after the worms were incubated in various concentrations of nanoparticles. The mobility of control worms (without nanoparticles) was also recorded. Using a dissecting microscope (Nikon, Tokyo, Japan), set at 2.00 × magnification, number of live and dead worms was counted separately for each concentration. Viability was determined by the absence of motility, with dead worms showing no movement under microscopic observation.

A 5-point qualitative scale was employed to assess parasite mobility.[15,23] The experiment was repeated three times, and the results were presented as a percentage of mortality. The percentage of mortality was calculated for each concentration using the following formula:[16]

$$Mortality\ (\%) = \frac{number\ of\ dead\ worms}{total\ number\ of\ worms\ per\ test} \times 100$$

Glutathione peroxidase assay. To assess GSH activity, the GSH detection kit from RanDox Laboratories Ltd. (Dublin, Ireland) was used. The manufacturer's recommended measurement method was strictly followed, and the absorption reduction was measured spectrophotometrically (AA-6800; Shimadzu Corporation, Kyoto, Japan) at 340 nm using a blank sample as a reference.[25] The protein content of the supernatant was determined through the Lowry colorimetric method, using bovine serum albumin (EMD Millipore, Kankakee, USA) as a standard. The results were expressed based on the protein content of the parasite homogenate.[23]

Glutathione S-transferase assay. The GST assay was performed according to the method described by Habig *et al.*, where the established protocol was carried out.[26] In this assay, 10.00 mM GSH (Kyowa Hakko Bio Co., Ltd., Tokyo, Japan) and 1.00 mmol 1-chloro-2,4-dinitrobenzene (Tokyo Kasei Kogyo Company, Ltd., Tokyo, Japan) were used as substrates. To start the assay, 50.00 µL of the protein sample was added to 100 mM potassium phosphate buffer (pH: 6.50; Karoon Phosphate Products Complex, Abadan, Iran). Enzyme activity was calculated as nmol of 1-chloro-2,4-dinitrobenzene conjugate produced *per* min *per* mg of protein using a molar extinction coefficient of 9.60×10^3 Mol *per* cm.

Estimation of superoxide dismutase (SOD) activity. The SOD activity was calculated using a standard commercial kit (Randox Laboratories Ltd.) using the xanthine-xanthine oxidase assay method.[27] The SOD activity was measured at a wavelength of 505 nm using a standard curve as a reference.

Assessment of malondialdehyde (MDA) level. To evaluate MDA level as a biomarker of lipid peroxidation, the method described by Buege and Aust was applied.[28] Specifically, one volume of homogenate was mixed with two volumes of a stock solution containing 15.00% v/v trichloroacetic acid (Caesar & Loretz GmbH, Hilden, Germany), 0.375% v/v thiobarbituric acid (Sigma-Aldrich, St. Louis, USA), and 0.25 mol L^{-1} hydrochloric acid (Nirou Chlor Co., Isfahan, Iran). After the mixture was heated and cooled, it was centrifuged at 1,000 rpm for 10 min to give a clear solution. The absorption was then measured at 535 nm, and the MDA content was calculated using a molar absorption coefficient of 1.56×10^5 Mol *per* cm. The final MDA content was recorded in nmol *per* mg of protein.

Statistical analysis. Statistical analysis was performed using SPSS Software (version 26.0; IBM Corp., Armonk USA). The homogeneity of variances was assessed using the Levene test. To compare the parameters between the control and treatment groups, both one-factor and two-factor ANOVA were used in addition to the Bonferroni *post hoc* test. Data are presented as mean ± standard deviation, and a p value ≤ 0.05 was considered statistically significant.

Results

Physicochemical characterization of CuO-NPs and ZnO-NPs. The crystalline structure of CuO-NPs was analyzed using X-ray diffraction patterns. The X-ray diffraction pattern was obtained at room temperature with a PANalytical X'Pert Pro™ X-ray diffractometer equipped with a nickel filter, using Cu Kα radiation (λ = 1.54056 Å) as an X-ray source. Transmission electron microscopy analysis revealed that the average diameter of the CuO-NPs was about 20.00 nm. Furthermore, transmission electron microscopy characterization of ZnO-NPs revealed that the highest diffraction peaks corresponded to the hexagonal phase of ZnO-NPs, which had a crystallite size of 212 Å. The spherical structure of the ZnO-NPs was visualized by transmission electron microscopy, and the diameters ranged from 20.00 to 30.00 nm.[23]

Adult worm motility test. In a motility test for adult worms, 24 hr exposure to different concentrations of CuO-NPs and ZnO-NPs (1.00, 4.00, 8.00, 12.00, and 16.00 ppm) resulted in a significant decrease in the motility of the adult worms. The inhibition rate was significantly higher in the treated worms compared to the negative control group (Table 1). Notably, exposure to 16.00 ppm of both nanoparticles completely inhibited the motility of the adult worms during the first 12 hr of observation (Table 1).

Adult worm mortality test. In this test, increasing the concentrations of CuO-NPs and ZnO-NPs, as well as the duration of exposure led to the destruction of adult worms. It was found that the highest concentration (16.00 ppm) of both nanoparticles caused 100% mortality within the first 12 hr of observation. In this study, the positive controls showed a mortality rate of 100% within 16 hr of observation, while the negative control showed a mortality rate of about 8.56% after 24 hr (Table 2).

Superoxide dismutase activity. The activity of SOD, the main anti-oxidant enzyme in *D. dendriticum* worms, was significantly reduced. The highest concentrations (16.00 ppm) of CuO-NPs and ZnO-NPs produced the maximum inhibitory effect (Table 3).

Glutathione peroxidase activity. The concentration of GSH significantly decreased upon treatment with CuO-NPs and ZnO-NPs. As shown in Table 3, GSH activity significantly decreased ($p \leq 0.05$) after exposure to different concentrations of the nanoparticles.

Table 1. The effect of various concentrations (ppm) and incubation time of copper- and zinc oxide nanoparticles on the motility of *Dicrocoelium Dendriticum*.

Time (hr)	Control	Albendazole	CuO-NPs (ppm)					ZnO-NPs (ppm)				
			1.00	4.00	8.00	12.00	16.00	1.00	4.00	8.00	12.00	16.00
0	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
4	++++	+++	++++	++++	++++	++++	+++	++++	++++	++++	+++	+++
8	++++	++	++++	++++	+++	+++	+	++++	++++	+++	++	+
12	++++	+	++++	+++	+++	+	-	++++	+++	++	+	-
16	++++	-	+++	+++	+	-	-	+++	+++	++	-	-
20	++++	-	+++	++	-	-	-	+++	++	+	-	-
24	+++	-	++	+	-	-	-	++	-	-	-	-

++++: High; +++: Moderate; ++: Low; +: Very low; -: No motility.

Assessment of lipid peroxidation. It was found that the content of MDA, the main end product of lipid peroxidation process occurring under oxidative stress, increased in a concentration-dependent manner. A significant increase in MDA values was observed at higher concentrations of CuO-NPs and ZnO-NPs compared to the control worms (Table 3).

Glutathione S-transferase activity. The specific activity of GST was significantly reduced when the worms were treated with higher concentrations of CuO-NPs and ZnO-NPs (Table 3).

Discussion

Given the increasing drug resistance to current formulations, it is important to explore new methods to address the problem of helminthic parasites, which pose significant medical challenges. Dicrocoeliasis is a food-borne parasitic disease of the human biliary tract caused by *D. dendriticum*. The disease causes significant financial losses and serious health problems as it reduces animal production and leads to condemnation of liver of ruminants. Given the limitations of existing drugs, drug delivery represents a crucial area for improving the treatment options for these diseases.[29] This study aimed to investigate the anthelmintic properties of CuO-NPs and ZnO-NPs in the treatment of dicrocoeliasis using *in vitro* tests.

Nanoparticles are effectively used to combat various parasitic diseases. However, it should be borne in mind that they can also lead to harmful biological effects at the cellular level. Therefore, after establishing non-cytotoxicity and conducting clinical trials, nanoparticles may find extensive applications as anti-parasitic agents among consumers.[30] They target parasite viability, reduce worm burden, inhibit egg production, and alter the concentration of anti-oxidant enzymes in the worms while simultaneously inducing apoptosis.[16,23,31]

Conducting *in vitro* analysis of anthelmintics before testing them *in vivo* is a smart strategy that can save time and money while reducing the number of animals needed to develop new therapeutics to treat and control the parasites.[23]

Worm motility has long been considered a crucial factor in testing the effectiveness of anthelmintics because worms must move to find suitable micro-habitats and obtain food from the host.[32] In this study, 12 hr exposure to the highest concentration (16.00 ppm) of both nanoparticles had a significant effect on adult *D. dendriticum* motility. It is important to note that the inhibition rate varies depending on exposure time and nanoparticle dose. Similarly, other studies have shown that different doses of silver nanoparticles strongly influence the motility of *D. dendriticum,* in a time- and concentration-dependent manner.[6] A study by Ravvaz *et al.*[17] showed similar results, indicating that the motility of *F. hepatica* stopped after 12 hr of exposure to 16.00 ppm CuO-NPs and ZnO-NPs.

Furthermore, bioengineered silver nanoparticles demonstrated anthelmintic effects on *Haemonchus contortus* at various stages of its life cycle, including eggs and adult parasites.[33]

Oxidative stress is harmful to worms because it can disrupt the normal function of important enzymes and proteins, alter cellular macro-molecules, and promote cell death.[34] Under normal circumstances, ROS concentrations remain relatively constant; however, factors, such as medications, stress, and illness, can lead to increased ROS levels. The ROS mainly target DNA, proteins, and lipids.[35,36] A variety of drugs and nanoparticle products have been found to stimulate ROS production and induce apoptosis.[23,31,37]

Recently, ROS-mediated apoptosis has emerged as an effective strategy to combat parasitic infections, including helminthic parasites.[34,38] Among various drug and vaccine targets, the glutathione-dependent detoxification system, including GSH and GST, has emerged as a promising candidate.

Key enzymes play a crucial role in conjugating reduced GSH to xenobiotics, which increases their water solubility and ultimately facilitates their excretion from flukes.[39,40] These molecules can be utilized to validate the effectiveness of new compounds or drugs. Enzymatic and non-enzymatic molecules in the glutathione family are essential for the survival of flukes because they are involved in anti-oxidative and detoxification processes.[24]

Table 2. Effects of various concentrations and incubation time (hr) of copper- and zinc oxide nanoparticles on the mortality of *Dicrocoelium dendriticum*.

Time	Control	Albendazole	CuO-NPs (ppm)					ZnO-NPs (ppm)				
			1.00	4.00	8.00	12.00	16.00	1.00	4.00	8.00	12.00	16.00
0	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Aa}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ac}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ae}	0.00 ± 0.00^{Ad}	0.00 ± 0.00^{Ac}
4	0.00 ± 0.00^{Ea}	23.76 ± 1.36^{Cc}	0.00 ± 0.00^{Ed}	1.22 ± 0.04^{Ed}	10.87 ± 1.03^{Dd}	35.67 ± 1.23^{Bc}	58.09 ± 1.56^{Ab}	0.00 ± 0.00^{Ed}	0.00 ± 0.00^{Ed}	13.40 ± 0.87^{Dd}	41.77 ± 0.48^{Bc}	61.56 ± 1.87^{Ab}
8	0.00 ± 0.00^{Da}	67.87 ± 0.78^{Bb}	0.00 ± 0.00^{Dd}	9.47 ± 1.98^{Dd}	20.32 ± 0.78^{Cc}	60.40 ± 0.78^{Bb}	93.05 ± 1.24^{Aa}	0.00 ± 0.00^{Dd}	7.33 ± 0.21^{Dd}	26.97 ± 0.56^{Cc}	70.04 ± 1.22^{Bb}	97.05 ± 1.65^{Aa}
12	0.00 ± 0.00^{Ea}	98.67 ± 0.98^{Aa}	8.69 ± 0.28^{Ecd}	30.16 ± 1.07^{CDc}	42.67 ± 0.22^{BCb}	89.43 ± 0.43^{Aa}	100.00 ± 0.00^{Aa}	9.43 ± 1.07^{Ecd}	20.35 ± 0.67^{Dc}	56.47 ± 1.78^{Bb}	92.08 ± 1.56^{Aa}	100.00 ± 0.00^{Aa}
16	3.87 ± 0.57^{Da}	100.00 ± 0.00^{Aa}	15.04 ± 1.03^{Dc}	57.32 ± 0.23^{Bb}	90.08 ± 0.45^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	18.67 ± 0.11^{Dbc}	61.54 ± 1.08^{Bb}	97.43 ± 1.08^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}
20	6.04 ± 0.42^{Ca}	100.00 ± 0.00^{Aa}	18.21 ± 0.6 ^{Bb}	89.66 ± 1.54^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	20.78 ± 1.32^{Bb}	90.76 ± 0.24^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}
24	8.56 ± 0.98^{Ca}	100.00 ± 0.00^{Aa}	31.79 ± 1.05^{Ba}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	35.06 ± 0.85^{Ba}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}	100.00 ± 0.00^{Aa}

$^{a\text{-}e}$ Different superscripts within the same column indicate a significant toxic effect of each concentration of nanoparticles within different exposure times ($p < 0.001$).
$^{A\text{-}E}$ Different superscripts within the same row indicate a significant toxic effect of various concentrations of nanoparticles during each exposure time ($p < 0.001$).

Table 3. Alteration in the level of oxidative stress markers in *Dicrocoelium dendriticum* treated with various concentrations of copper- and zinc oxide nanoparticles after 24 hr.

Parameters	Control	CuO-NPs (ppm)					ZnO-NPs (ppm)				
		1.00	4.00	8.00	12.00	16.00	1.00	4.00	8.00	12.00	16.00
SOD	1.78 ± 0.87^{c}	2.08 ± 1.66^{a}	1.99 ± 1.43^{ab}	1.47±0.28^{d}	1.32 ± 0.65^{d}	1.11 ± 1.13^{e}	2.15 ± 1.65^{a}	1.91 ± 1.43^{b}	1.71 ± 1.58^{c}	1.45 ± 0.39^{d}	1.03 ± 1.05^{e}
GSH	31.66 ± 0.78^{a}	27.32 ± 0.32^{a}	20.32 ± 0.16^{b}	18.65 ± 0.32^{bc}	14.23 ± 0.49^{cd}	10.34 ± 0.43^{d}	29.98 ± 1.08^{a}	23.25 ± 1.87^{b}	15.32 ± 0.12^{cd}	11.77 ± 1.76^{d}	9.08 ± 1.65^{d}
MDA	0.57 ± 0.49^{d}	1.04 ± 0.43^{d}	1.04± 0.51^{d}	0.77 ± 0.49^{c}	1.87 ± 0.86^{b}	1.76 ± 0.97^{a}	1.86 ± 0.40^{d}	1.08 ± 0.54^{d}	1.54 ± 0.79^{c}	1.32 ± 0.90^{b}	1.21 ± 0.99^{a}
GST	41.07 ± 1.65^{a}	36.78 ± 1.66^{ab}	32.40 ± 0.04^{bc}	27.80 ± 1.45^{c}	25.90 ± 1.66^{cd}	20.40 ± 1.53^{d}	35.76 ± 1.09^{ab}	31.09 ± 2.75^{bc}	29.40 ± 1.74^{c}	21.05 ± 1.76^{d}	19.54 ± 0.87^{d}

SOD: Superoxide dismutase (U mg^{-1} protein); GSH: Glutathione peroxidase (U mg^{-1} protein); MDA: Malondialdehyde (nmol mg^{-1} protein); and GST: Glutathione S-transferase (U mg^{-1} protein).
$^{a\text{-}e}$ Different superscripts within the same row indicate a significant effect ($p < 0.001$).

The GSH serves multiple functions in the cellular anti-oxidant defense mechanism, including maintaining redox status by scavenging ROS. A decrease in GSH levels can lead to an imbalance in the redox processes within parasites.[41] This phenomenon has been observed in flukes treated with CuO-NPs and ZnO-NPs, as noted in previous studies.[24] Such a decrease ultimately disrupts intra-cellular redox homeostasis, impairing the worms ability to scavenge free radicals and electrophilic xenobiotics. The reduction in GSH activity after exposure to varying concentrations of CuO-NPs and ZnO-NPs may occur due to the destruction of anti-oxidant enzymes or degradation of essential minerals or vitamins.[15] Research has indicated that during oxidative stress, GSH-related enzymes utilize glutathione to detoxify peroxides generated by excessive ROS production, leading to a depletion of the substrate.[42]

The GST, along with its phase II detoxification function, has been reported to help parasites develop resistance to commonly used anthelmintics by catalyzing the conjugation of reduced glutathione *via* a sulfhydryl group to electrophilic sites on various substrates.[24] This finding prompted us to investigate the effects of CuO-NPs and ZnO-NPs on the GST molecule. In contrast to the dose-dependent decrease in GSH levels, we observed a significant reduction in GST activity in worms treated with CuO-NPs and ZnO-NPs. This is consistent with previous reports about the liver flukes *Fasciola gigantica* and *F. hepatica.*[17,24] Our study demonstrated that the activities of both GST and GPX decreased in worms incubated with CuO-NPs and ZnO-NPs. Other studies have indicated that CuO-NPs and ZnO-NPs can cause oxidative and nitrosative damages to biomolecules.[14,15,23]

We found that treating *D. dendriticum* with varying concentrations of CuO-NPs and ZnO-NPs resulted in different effects. The use of these nanoparticles appears to induce oxidative stress in the parasites through the production of ROS. In response, the flukes increased the activity of anti-oxidant enzymes, such as SOD, to scavenge the ROS generated by nanoparticle treatment. The SOD enzyme helps convert superoxide radicals into hydrogen peroxide,[27] along with other anti-oxidants that work collectively to combat ROS. However, this protective system was disrupted when the worms were treated with the highest concentrations (12.00 and 16.00 ppm) of CuO-NPs and ZnO-NPs. We observed a significant inhibition of SOD activity in *D. dendriticum* at these high nanoparticle concentrations. This inhibition could be due to the enzyme saturation resulting from an over-production of hydroxyl ions and ROS, rendering the detoxification mechanism ineffective in *D. dendriticum*, as previously reported in other liver flukes, such as *F. hepatica* and *Gigantocotyle explanatum.*[13,17]

In conclusion, CuO-NPs and ZnO-NPs demonstrated promising *in vitro* efficacy against adult flukes of *D. dendriticum*. The study indicates that both compounds

exert anthelmintic effects by causing oxidative damage to biomolecules. Notably, these effects are concentration-dependent, with higher concentrations of CuO-NPs and ZnO-NPs impairing the anti-oxidant systems of *D. dendriticum* and damaging lipids and proteins. Therefore, the study suggests that both compounds could be further investigated for the development of innovative drug formulations aimed at controlling helminthic infections. However, it is crucial to note that nanoparticles can have harmful biological effects at the cellular level. Establishing non-cytotoxicity and conducting clinical studies are essential before considering their use as anti-parasitic agents in consumers. Also, further research is needed to gain a deeper understanding of the functional significance of these compounds and determine their effects on parasites under *in vivo* conditions. This knowledge will be vital for achieving sustainable control of liver fluke infections.

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Conflict of interest

The authors declared no conflict of interest.

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