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Parasiticidal Efficacy of a New Formulation of Silver Nanoparticles on *Trichinella spiralis In vitro*

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Abstract

This research illustrates the development of a new sliver nanoparticle (Ag-NPs) formulation. Its shape, size, solubility, and stability were characterized using Scanning Electron Microscope (SEM 3D), Transmission Electron Microscope (TEM 2D), Atomic Force Microscope (AFM), and Zeta size and Zeta potential. Exposure of *Trichinella spiralis* adult worms to 3, 6, 9 and 12 ppm of Ag-NPs each for 3,6,12 and 24 h. *In vitro* revealed a direct relation between mortalities and the tested drug concentration and exposure time. Anti- *T. spiralis* effect of Ag-NPs was evaluated by assessing mortality rate and damage in DNA by comet assay and by SEM analysis. Mean mortalities increased from 6.66% after exposure to 3.0 ppm/1 h to 100% after exposure to 12.0 ppm/12 h. The calculated LC50 was 3.0 ppm/10 h, 6 ppm/6 h, 9.0 ppm/4 h and 12.0 ppm/ 3.30 h, while LC100 was 9.0 ppm/24 h and 12.0 ppm/12 h. DNA genotoxic damage of dead worms was directly related to Ag-NPs concentrations for 12h using comet assay as expressed by variations in the percentage of DNA in the tail segment, tail length (μm), tail moment (μm), and olive tail moment. No significant difference ($p \leq .05$) between the recorded mortalities and DNA damage between that obtained using the Ag-NPs LC100 and that recorded using Albendazole (50 mg/kg B.W.) for 12 h. SEM images on dead worms revealed clear morphological alteration, multiple vesicles, and blebs, detachment of the epidermis and the sub-epidermal layer with partial sloughing of the cuticle, and loss of normal creases, ridges, and annulations. These morphological alterations were directly related to the concentration of the tested Ag-NPs. The tested new formulation of Ag-NPs appears to be effective in the control of Trichinellosis as an alternative to other resistant drugs.

KEYWORDS
Trichinella spiralis, Nano-Silver, DNA damage, Electron microscopy.

INTRODUCTION

Trichinellosis is an emerging and re-emerging foodborne zoonotic disease all over the world. It is caused by the aphasmid nematode worm *Trichinella* spp. It can infect a wide range of mammals, reptiles, and birds. The adult worm lives in the intestine while its laid larvae migrate to form cysts in different active body muscles especially those of the diaphragm (Taher *et al.*, 2017). Humans acquire infection through ingestion of improperly cooked pork, horses, or bear meat containing the active encysted larvae (Rostami *et al.*, 2017). Trichinellosis carries a worldwide threat to human and animal health. The estimated number of infected patients with trichinellosis reached up to 11 million all over the world (Zhang *et al.*, 2018).

Unfortunately, the currently available drugs that are commonly used for the treatment of trichinellosis have limited bioavailability, little ability to kill encysted larvae, and numerous adverse effects. Thus, the need for safer and more effective drug becomes mandatory (Fahmy *et al.*, 2020).

Silver nanoparticles (Ag-NPs) have unique physical, biological and chemical features. They have small-sized particles and a large surface area, so they are found to be potent antimicrobial against a wide range of microorganisms (Klaine *et al.*, 2008). Ag-NPs show antiparasitic activity against larvae of mosquitoes, *Rhipicephalus* ticks (Marimuthu *et al.*, 2010), and *Giardia* (Said *et al.*, 2012). Ag-NPs were investigated on *T. spiralis* larvae. Different concentrations of Ag-NPs showed marked larvicidal effects in the form of degeneration of the larvae cuticle with inhibited their ability to infect animal models (Abd-Elrahman *et al.*, 2021).

In vitro studies are considered the first step to evaluating any drug material before in vivo application. *In vitro* experiments have many benefits being low cost and simple. Also, they are considered a rapid tool for exploring the properties of the product and facilitate screening of a variety of doses till the determination of one or more lethal doses; providing a core for in vivo studies. (Fahmy *et al.*, 2020).

For this reason, the current study was carried out to assess the parasiticidal effects of a new formulation of Ag-NPs against *T.*

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spiralis adult worms *In vitro*. Mortalities were estimated depending on depression in motility rates, damage in DNA after comet assay as well as destruction in the exposed worms using Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) in comparison with control non-exposed worms.

MATERIALS AND METHODS

Ethical approval

The Declaration of Helsinki's principles guided the conduct of this study. Procedures for handling and sample collection from mice were approved by the institutional review board of the Institutional Animal Care and Use Ethical Committee (CU-IACUC) of Cairo University. The study was conducted during the period from February to June 2022 in the Department of Parasitology, Faculty of Veterinary Medicine, Cairo University, Egypt.

Tested Drugs

New formulation of Ag-NPs

Synthesis of Ag-NPs

Synthesis of Ag-NPs was carried out at Egypt's national research institution as previously described by Youssef *et al*. (2020). Silver nitrate and trisodium citrate (lab grade, Sigma, USA) were used as precursor materials. The silver colloid was synthesized by using the co-precipitation method as follows; 50 ml of 0.001 M AgNO3 were boiled then 5 mL of 1 % trisodium citrate solution were added drop by drop with vigorous agitation and heating until a change of their color to pale yellow according to the following equation ($4Ag+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4AgO + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2\uparrow$). The product was then removed from the heating device, stirred, and left to cool at room temperature then collected in dark bottles.

Characterization of the produced Ag-NPs

Characterization of silver nanoparticles was performed at Nawah Scientific and included the aspects of index, identification, and morphology, which was done to determine its physio-chemical qualities and bioactivity. The measurement conditions were 40 KV and 40 AM (1600W) at a speed scan of 0.02 and a 2-theta range of 5 to 80. To avoid burning the material, a 532 nm He-Cd edge laser line with grating 1800 (450-850 nm) and a 3.2% ND filter were utilized. The acquisition period was 10 seconds. Examination using TEM (model EM-2100 High-Resolution-Japan) with a magnification of 20X and a voltage of 200 kV to confirm 2D shape and size was used. In addition, SEM was used to check 2D shape using the Jol 2000, Japan. The suspension was applied to a mica slide under measurement conditions (size 500 X 500 nm, in contact mode, speed is 0.5 inch/sec). The Malvern, UK-made Nano Sight NS500 was used to detect zeta potential (to assess whether a sample is colloidal or not) and DLS carried out the size sector with the help of Malvern Instruments Ltd. Finally, the produced Ag-NPs were prepared as a water-soluble material containing 100 ppm/ml for experimental use.

Antioxidant Activity of the produced Ag-NPs

According to the procedure described by Bhakya *et al*. (2016), and with the aid of the stable radical of 2, 2-diphenyl-1-picrylhydrazyl (DPPH), the free radical scavenging activity of Ag-NPs and conventional vitamin C were measured. Briefly, 1.0 ml of Ag-NPs at variable concentrations (10, 20, 30, 40, 50, 75, and 100 mg/ml) were centrifuged with 1.0 ml of DPPH (1.0 ml.M in methanol), then, after 30 min incubation at room temperature in dark, A UV-Vis spectrophotometer was used to measure absorbance at 517 nm (Systronics, AU-2701). As a control, DPPH (all reagents except the sample) were utilized, and methanol was used as a blank solution. The free radical scavenging activity was calculated as a percentage of inhibition using the following formula:

$$\text{Percentage of scavenging} = \frac{Pc - Ps}{Pc} * 100$$

Where Pc = control absorbance and Ps = Ag-NPs/Vitamin C absorption.

Reference drug

Albendazole 2.5 % solution: (Pharma Swede). An oral solution was used (each 1.0 ml contain 25 mg). The dose of 50 µg/ kg.B.W. was adjusted to the required volume of the used RPMI media (Fahmy *et al*., 2020).

Parasiticidal efficacy of the produced Ag-NPs

Tested Worms

T. spiralis encysted larvae (Fig. 1-a) were collected from highly naturally infected diaphragmatic muscles of pigs that were freshly slaughtered in Cairo abattoir, Egypt. Muscles were examined in the Department of Parasitology, Faculty of Veterinary Medicine Cairo University, the infected muscles were dissected into small pieces and re-inspected using a trichinoscope. The examined larvae were extracted by digestion in pepsin-HCL as described by Taher *et al*., (2017). After removal of the digestive solution by

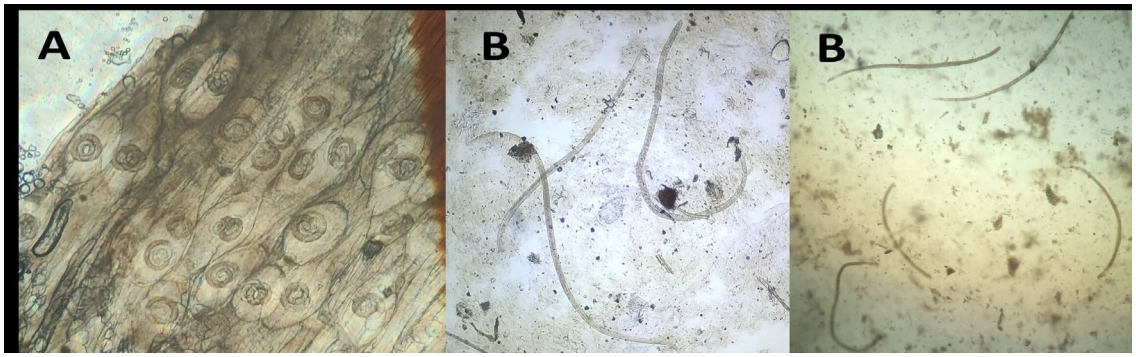

Fig. 1. (a) Fresh *T. spiralis* muscle cysts in naturally infected slaughtered pig's muscle (under trichinoscope glass slides). (b): Living 7 days old *T. spiralis* worms directly extracted from experimentally infected mice.

washing in PBS, the obtained larvae were identified according to Walden (2013) and then counted using McMaster counting chambers according to IQACA (2012). The number of larvae/ml solutions were adjusted. *T. spiralis* larvae (n.=200) were orally inoculated to 6–8 weeks old male Swiss albino mice. The animals were kept in proper cages supplied by commercial rodent feed and water ad-libitum, following the national guidelines. After 7 days post-inoculation (dpi), mice were sacrificed, their small intestines were dissected, and the remaining intestinal contents were removed by washing with warm sterile phosphate-buffered saline (PBS, pH 7.4). After that, the small intestines were cut into small pieces and kept in clean sterile suitable petri-dish immersed in warm (37 °C) saline. The dish was incubated for 3 h at 37 °C so that all worms migrated to the warm buffer solution (Fig.1-b). The worms were collected using suitable pipettes under a stereomicroscope and transferred directly to warm (37°C) incubation media (Fahmy *et al*., 2020).

In vitro experiment

The effect of the newly formulated Ag-NPs was evaluated versus adult *T. spiralis* worms. The previously extracted worms were kept in a sterile RPMI-1640 medium (Gibco Laboratories, Grand Island, NY, USA) (supplemented by 200 µg/ml streptomycin, 200 U/ml penicillin, and 20% fetal calf serum) (Huanga *et al*., 2020) at 37°C. Fifteen active worms were transferred into 6 cm diameter sterile Petri-dishes. Concentrations of 3.0 ppm, 6.0 ppm, 9.0 ppm, and 12.0 ppm of the tested Ag-NPs were adjusted in a suitable amount of the previous RPMI-1640 medium (5.0 ml/petri-dish). The worms were still under exposure each for 1h, 3 h, 6 h, 12 h and 24 h in an incubator at 37°C. At the end of each exposure time, the media containing the drug were removed, washed with warm PBS, then kept in media without drugs to evaluate the mortality percentage. Worms in blank controls RPMI media and Albendazole (50 mg/kg) as reference drugs were set simultaneously. Each concentration was tested in triplicate. The mortalities in the summation of the three trials were calculated. The mortality in each case was calculated using the following equation:

$$\text{Mortality \%} = \frac{\text{Survival\% of the control- Survival\% in exposed}}{\text{Survival\% of the control}} \times 100$$

Lethal concentrations of 50% (LC50) and 100% (LC100) were calculated after this according to Salama *et al*. (2012). The viability of the exposed worms was assessed under the microscope from the aspect of their shape and mobility. The dead worms appeared un-motile with fixed C-shaped or linear bodies. The destructive effect of the tested Ag-NPs on the DNA of the exposed worms was measured using comet assay according to Singh *et al*. (1988). Moreover, samples of dead and living worms were processed for SEM.

Evaluation of DNA Damage by Comet assay

Comet assay was adopted to analyze the damage in DNA of the worms according to Singh *et al*. (1988). DNA damage was visualized in the exposed and control *T. spiralis* worms in Ethidium bromide-stained DNA by fluorescent microscope (40x objective) using comet 5 image analysis software (Kinetic Imaging, Ltd. Liverpool, UK). Connected with a CCD camera as it can assess the quantitative and qualitative DNA damage in the cells by measuring the length and the percentage of DNA migration. The program calculated the damage to DNA as it was measured by the percentage of DNA in a tail segment (% DNA in the tail), variations in the tail length (µm), tail moment (µm), and Olive tail moment. Analysis was done on a number of 100 randomly selected cells per treatment. The Comet score was calculated according to the formula of Singh *et al*. (1988) In the range of 0- 400 arbitrary units.

SEM Examination

Destructive effects of Ag-NPs on the ultrastructure of the exposed worms and controls were investigated using SEM. At the end of the exposure time, the adherent nanoparticles were removed from the dead worms by gentle washing several times with PBS (pH 7.2). The worm samples were then fixed in 2.5% glutaraldehyde solution at 4°C for 24 h. After washing by PBS for 5 min, the fixed worms were re-washed again in PBS for 5 min, post-fixed with a solution of 2% osmium tetroxide in sodium cacodylate buffer for 1 h. The specimens were then dehydrated in ascending concentrations of alcohol and dried in the air then mounted on sputter-coated with gold and scanned by SEM (Hitachi SU8040, Japan). Photos were recorded on electron images.

Statistical analysis

The illustrated data are expressed as mean ± standard deviation (mean ± SD). Graph drawings were performed using Excel Software 2013, The data were statistically analyzed by using analysis of variance (ANOVA), The difference was considered significant when $P < 0.05$) using SPSS 27 (IBM. NY, USA) (Petrie and Watson, 1999).

RESULTS

Morphological characterization of the produced Ag-NPs

SEM and TEM data of the prepared Ag-NPs were depicted in TEM 2D and SEM 3D images (Fig. 2). The spherical structure of the produced Ag-NPs in nano-sized form was cleared by SEM photographs, then confirmed by TEM images. The creation of nanocomposite was confirmed by SEM and TEM pictures (Fig. 2).

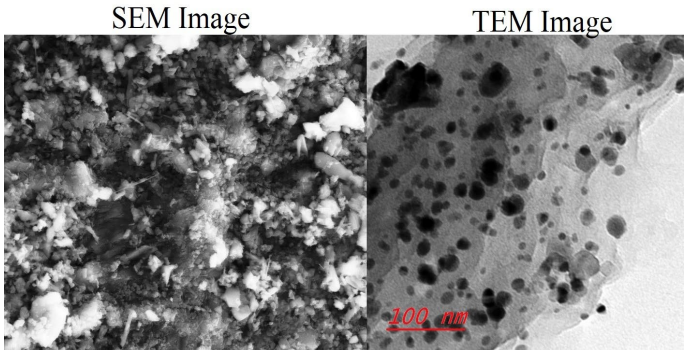

Fig. 2. Silver Nanoparticles (Ag-NPs) SEM and TEM images.

Atomic Force Microscope (AFM)

To corroborate the shape, size, concentration, and agglomeration discovered in the TEM and SEM data, Ag-NPs underwent AFM testing. Ag-NPs with a maximum thickness of less than 40 nm were shown to have a spherical structure in AFM images as shown in (Fig. 3). The image made it obvious that Ag-NPs do not typically form agglomerations in one particular place (Fig. 3).

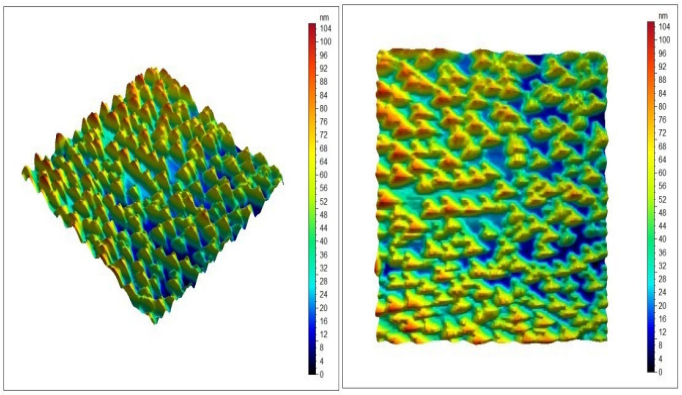

Fig 3. AFM image of the produced Ag-NPs.

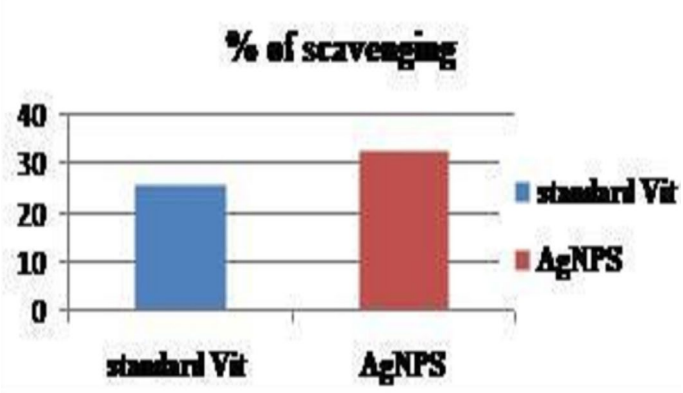

Fig. 5. Antioxidant (DPPH) efficacy of the produced Ag-NPs in comparison to Vitamin C.

Zeta size and zeta potential

Zeta size and potential were measured for the produced Ag-NPs to determine their size and stability in aqueous media. The produced Ag-NPs have a particle size of 25 nm and zeta potential of 35 mV. The obtained size was matching with the size given by TEM, SEM, and zeta potential values demonstrating that these particles have excellent stability in aqueous conditions (Fig. 4).

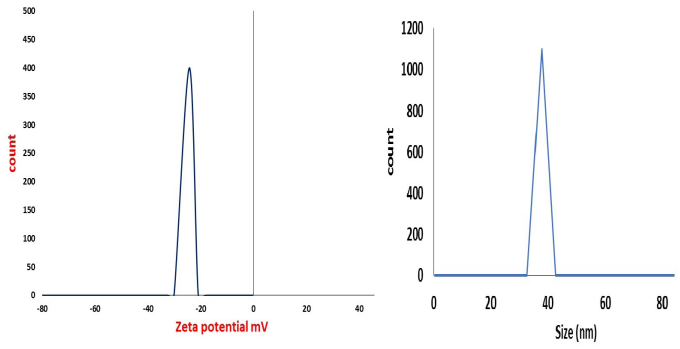

Fig. 4. Zeta size and zeta potential of the produced Ag-NPs.

Antioxidant Activity 2,2-diphenyl-1-picrylhydrazyl (DPPH method)

Evaluating the antioxidant properties of the produced Ag-NPs revealed that they possess a marked antioxidant activity reaching up to 32.45 %, which was superior to that calculated for vitamin C which has 25.36 % activity, under the same conditions. These outcomes supported the scavenging behavior of these new formulated Ag-NPs in comparison to vitamin C, as it had a strong hydrogen peroxide scavenging capacity (Fig. 5).

Parasiticidal efficacy of Ag-NPs against T. spiralis

Exposing active *T. spiralis* adult worms to 3.0, 6.0, 9.0, and 12.0 ppm of Ag-NPs in warm RPMI for 1 h, 3 h, 6 h, 12 h and 24 h as described in Table 1 and Fig. 6, revealed a direct relationship between the increase in the rate of mortality of the exposed worms and the increase in the Ag-NPs concentration and exposure time. Exposing the worms to 3.0 ppm induced 6.66% mortalities after one-hour exposure and increased gradually to 88.88% after 24h exposure time. Exposure to 6.0 ppm of the product for 1h caused mortality of 8.88% after 1 h and increased to 93.33% after 24h exposure time. Exposure to 9.0 ppm revealed 13.33% after 1h exposure time while the death of all of the exposed worms (100%) was recorded after 24 h exposure to this concentration. Increasing the concentration to 12.0 ppm caused the death of all the exposed worms after 12 h exposure. At the same time, the used reference drug killed all the exposed worms when used at a dose of 50mg/kg B.W. for 12 h, as well as three worms (6.66%) died in the control group after 24 h in media without drugs (Table 1 and Fig. 6).

Demonstration of the data present in Table 2 and in the histogram (Fig. 6) facilitate determination of the effective Ag-NPs lethal concentration (LC) LC50% and LC100 for each tested concentration. The LC50 was 3.0 ppm for 10h exposure time, 6.0 ppm/6h, 9.0 ppm / 4 h and 12.0 ppm/for 3.30 h exposure time. The LC100 was recorded as 9.0 ppm /24 h and 12.0 ppm/ after 12h exposure time (Table 2 and Fig. 6).

DNA damage in Exposed and control T. spiralis (Comet assay)

Analysis of DNA damage in dead *T. spiralis* worms exposed to different concentrations of Ag-NPs after 12 h and to the control worms in blank RPMI media were visualized using Comet assay. The data (Table 3 and Fig. 7) showed the presence of a direct relationship between the increase in the dose of Ag-NPs and the

Table 1. Number and mortality percentage in *T. spiralis* worms exposed to different concentrations of Ag-NPs

Tested conc.	Number and mortality % in 45 adult worms after being exposed to				
	1 h	3 h	6 h	12 h	24 h
3.0 ppm	3/ 6.66 ^{d}	7/15.55 ^{d}	11/24.44 ^{c}	26/57.77 ^{c}	40/88.88 ^{b}
6.0 ppm	4/8.88 ^{d}	12.26.66 ^{c}	23/51.11 ^{bc}	33/73.33 ^{b}	42/93.33 ^{a}
9.0 ppm	6/13.33 ^{c}	17/37.77 ^{c}	30/66.6 ^{b}	42/93.33 ^{a}	45/100 ^{a}
12.0 ppm	9/20.0 ^{c}	19/42.22 ^{bc}	38/84.44 ^{ab}	45/100 ^{a}	-
Albendazole (50mg/kg)	-	-	42/93.33 ^{a}	45/100 ^{a}	-
Control in blank RPMI	0	0	2/4.44 ^{d}	3/6.66 ^{d}	3/6.66 ^{d}

- Concentration before these concentrations did not induce any mortalities.
- Data represented as mean, a column with different letters are statistically significant at p-value ≤ 0.05 (One Way ANOVA)

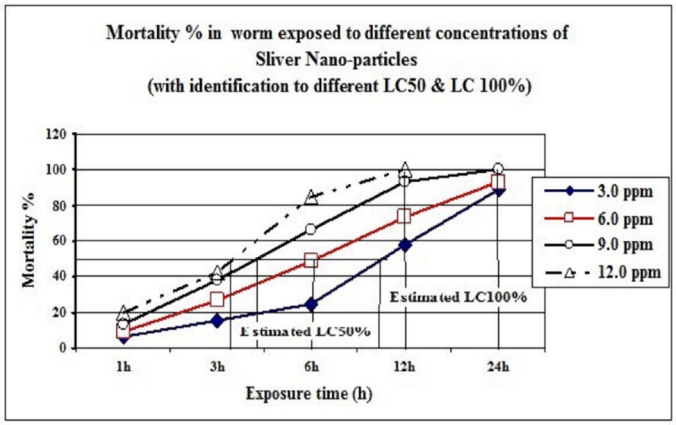

Fig. 6. Mortality % in T. spiralis adult worms exposed to different concentrations of Ag-NPs after different exposure times with determination to LC50% and LC100.

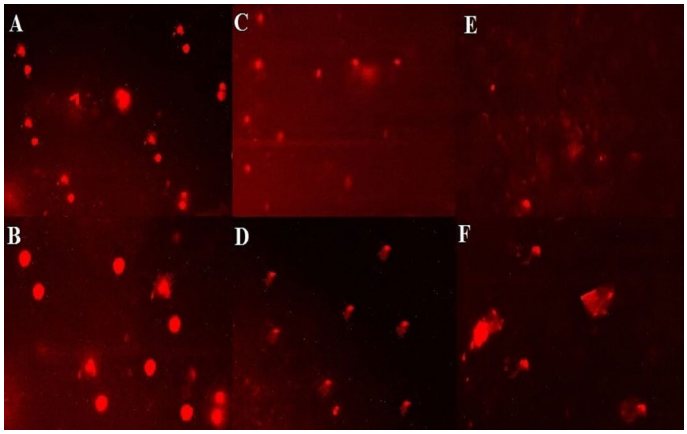

Fig. 7. Comet assay demonstrating the damage in DNA, (AandB) are the control non-exposed worms showing sound un-destructed DNA, (CandD), showing low level of damaged DNA in worms exposed to low doses (3 and 6 ppm/12 h), while (EandF) demonstrated a high level of DNA damage in worms exposed to 9 and 12 ppm/24 h

degree of DNA genotoxic damage. This damage was represented also by variations in % of DNA in the tail segment, tail length (µm), tail moment (µm), and olive tail moment by investigating the migration of DNA fragments by agarose gel electrophoresis. With the increase in the % of worm mortalities from 57.77%, 73.33%, 93.33, to 100% with increasing the Ag-NPs dose from 3.0 ppm to 12.0 ppm respectively, a significant increase ($p \leq .005$) in the % of DNA damage that increased from 10,11±0.20 to 13.30±0.28, the mean tail length (µm) increased also from 8.80 ±0.20 to 10.09±0.17, the percentage of DNA in the tail was also increased from 8.36±0.06 to 10.77±0.25, the olive tail moment differed from 1.38±0.22 to 1.81±0.19 and finally the tail moment (µm) increased also from 0.87±0.01 to 1.10±03 corresponding to the increase of the material concentration from 3.0 ppm to 12.0 ppm respectively. The data demonstrated that there is no significant difference ($p \leq .005$) between the recorded values using the high Ag-NPs concentration (12.0 ppm) with that recorded using Albendazole (50 mg/kg. B.W.). Moreover, a significant difference was recorded in comparing the obtained values with that of the living worms incubated in blank RPMI media (Table 3 and Fig. 7).

Table 2. The calculated LC50 and LC 100 of Ag-NPs versus T. spiralis adult worms

LC50	LC100
3.0 ppm/ 10 h	--
6.0 ppm/ 6 h	--
9.0 ppm/ 4 h	9.0 ppm/ 24 h
12.0 ppm /3.30 h	12.0 ppm / 12 h

SEM results

The effects of Ag-NPs on the morphology of *T. spiralis* adult worms after exposure to different concentrations of Ag-NPs for 12 h on the cuticle of the dead worms were conducted using the images obtained by SEM (Fig. 8). The results of SEM revealed marked morphological changes, multiple vesicles and blebs, sloughing and destruction of some areas of the cuticle with fissures and loss of normal annulations in the epidermal and cuticular layer of the dead worms. This morphological alteration was directly related to the concentration of the tested Ag-NPs after 12 h exposure time.

Dead worms in the group exposed to 3.0 ppm of Ag-NPs showed a mild degree of collapse and contraction without destruction in the epidermal layer of the worm cuticle (Fig. 8-B). The alteration and destruction in the morphological characters increased with the increase in the concentration to 6.0 ppm showing mild focal destruction in the epidermis of the wall with a mild degree of carrion, also, the cuticle appeared opaque and showed areas with multiple blebs, vesicles, and loss of normal creases with focal sloughing of some areas of the cuticle (Fig. 8-C). Exposure to 9.0 ppm Ag-NPs showed the disappearance of the intact annuli with corrugation and shrinking in the surface epidermal layer. The cuticle showed multiple degenerative changes, the appearance of blebs, multiple vesicles, and focal sloughing (Fig. 8-D). Serious alterations were observed in the epidermis and cuticular layer of the dead worms exposed to 12.0 ppm. The cuticle of the dead worms showed marked swellings, with numerous large blebs, fissures, and vesicles associated with loss of the normal creases, ridges, and annulations. Detachment of the epidermis and the sub-epidermal layer which appeared irregularly arranged with partial sloughing of the cuticle was observed (Fig. 8-E and F). At the same time, the control worms in blank RPMI still saved the normal morphology of the epidermis and cuticle layer as it appears intact, smooth, and non-wrinkled (Fig. 8-A and B).

Table 3. Comet parameters and level of damage in DNA of T. spiralis exposed for 12 h to different concentrations of Ag-NPs and control non-exposed worms

Tested concentration	Mortalities (%)	DNA damage (%)	Tail length (µm)	DNA in tail (%)	Tail moment (µm)	Olive tail moment
3.0 ppm/12h	57.77 ^{c}	10.11± 0.20^{b}	8.80 ± 0.20^{c}	8.36 ± 0.06^{c}	0.87 ± 0.01^{b}	1.38 ± 0.22^{b}
6.0 ppm/12h	73.33 ^{b}	11.22 ± 0.15^{b}	9.20 ± 0.32^{b}	9.72 ± 0.24^{b}	0.92 ± 0.41^{b}	1.47 ± 0.18^{b}
9.0 ppm/12h	93.33 ^{a}	12.28 ± 0.14^{a}	9.17 ± 0.21 ^{b}	10.12 ± 0.36^{a}	0.98 ± 0.38^{ab}	1.77 ± 0.22^{a}
12.0 ppm/12h	100 ^{a}	13.30 ± 0.28^{a}	10.09 ± 0.17 ^{a}	10.77 ± 0.25^{a}	1.10 ±.03^{a}	1.81 ± 0.19^{a}
Albendazole 50mg/kg/12h	100 ^{a}	12.20 ± 0.20^{a}	9.45 ± 0.21 ^{a}	10.11 ± 0.25 ^{a}	1.02 ± 0.02^{a}	1.75 ± 0.12^{a}
Control in blank RPMI	0	5.4 ± 18 **c**	7.82 ± 0.28 ^{d}	8.25 ± 0.18 ^{c}	0.65 ± 0.33^{c}	1.17 ± 0.12^{c}

Data represented by mean ± SD, a column with different letters is statistically significant at $P \leq 0.05$ (One Way ANOVA).

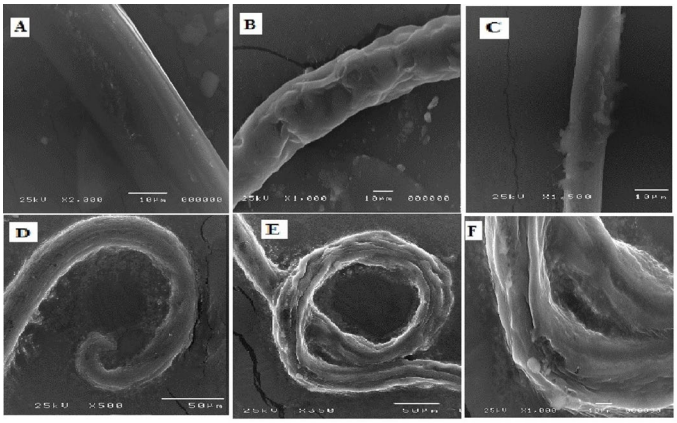

Fig. 8. SEM image to investigate the effect of Ag-NPs on T. spiralis adults. (A) Control in blank RPMI showing worm body with intact smooth non-wrinkled epidermis. (B) 3.0ppm Ag-NPs treatment group showing adult skin with a mild degree of collapse and contraction without destruction. (C) 6.0ppm exposed worm showing mild focal destructions in the epidermis of the wall with a mild degree of carrion. (D) group exposed to 9.0ppm Ag-NPs showing the disappearance of the intact annuli, with corrugation and shrinking in the surface epidermal layer. (E and F) group exposed to 12.0ppm showing serious alterations in the worm cuticle that was severely damaged and detachment of the epidermis and the sub-epidermal layer which appeared irregularly arranged.

DISCUSSION

Trichinellosis is a worldwide foodborne zoonotic disease caused by *T. spiralis* affecting different mammals including humans causing serious public health problems (Malak and Abdel-Radi 2021). Treatment of the infection with commercially available drugs has not been satisfactory due to the rapid development of drug resistance. For this reason, there is an increasing need to discover and develop alternative anti-helminthic agents (Youssef *et al*., 2019). Since 1974, nanotechnology has been regarded as a new promising field for developing effective novel materials with dimensions ranging from 1.0 to 100 nanometers (Elgadir *et al*., 2015).

In the present study, an original new formulation of highly purified effective Ag-NPs was developed in the Department of Pharmacology, Faculty of Veterinary Medicine Cairo University, by an easy, simple, and cheap sonochemical method. Characterization of the product using SEM and TEM images, confirmed its spherical shape and suitable size of Ag-NPs while zeta size and potential evaluated its size as 25 nm. and its zeta potential as -35 mV. This is agreed with previous work in the same field done by one of the authors of this work (Youssef *et al*., 2020). Moreover, the product has a considerable antioxidant activity that confirmed the potential antioxidant activity of Ag-NPs with high scavenging % (32.45%) in comparison with those of Vit. C as a reference. These results agree with Keshari *et al*. (2020), who previously characterized the antioxidant activity of Ag-NPs and reported that Ag-NPs at nano-size 20 nm showed potential antioxidant activities with high scavenging % (29.55). Nanomaterial proved by several others that it is more potent and effective in comparison with other mass materials. In the author's opinion, this was related to its special innovative physicochemical qualities such as their enormous surface to volume extent, higher reactivity, steadiness, controlled molecule size, bioactivity, bioavailability, and controlled arrival of stacked medications to the special target site (Youssef *et al*., 2020; Youssef *et al*., 2021). The proven high antioxidant efficacy of the obtained Ag-NPs add more advantages for the efficacy of this material, using antioxidant in association with parasiticidal drugs enhance the immunity of the animal as its deficiency may affect Reactive Oxygen pieces (ROS) production which are essential factors of cell division, and growth. Moreover, combinations of antioxidants and specific drugs are usually more effective than applying larger quantities of the drug alone (Abdel-Rahman and Abdel-Radi, 2022; Abou-Okada *et al*., 2021).

According to Fahmy *et al*. (2020), *In vitro* studies are essential for the primary evaluation of any new formulation before testing a similar product in vivo. *In vitro* experiments are useful easy, have low cost, simple and rapid tools for exploring the effective dose level for application of the drug in vivo. For this reason, the present study screened different upgrading doses of the locally produced Ag-NPs. Exposing active *T. spiralis* adult worms to 3.0, 6.0, 9.0, and 12.0 ppm for 1 h, 3 h, 6 h, 12 h, and 24 h revealed that the effect of the product appears of dose- and exposure time-dependent as there is a direct relationship between the increase in the rate of mortality of the exposed worms and the increase in the Ag-NPs concentration and exposure time. These facts agreed with El-Banna *et al*. (2005) and Fahmy *et al*. (2020). Moreover, the relation is indirect between the increase in the concentration of the tested dose and the exposure times concerning the values of LC50% and LC100% of the exposed worms (Salama *et al*., 2012) as it increased from 3.0 ppm/for 10h exposure time, to 12.0 ppm/for 3.30 h for LC50% and it was 9.0 ppm /24h and 12.0 ppm/ 12h for LC100%. These facts support the value of Ag-NPs as an effective anti-*T. spiralis* drug. These results have no significant difference ($P < 0.05$) from that obtained using Albendazole 50mg/kg B.W. as a reference drug. In the author's opinion and agreement with Saini *et al*. (2016) Ag-NPs are considered a potent parasiticide, they affected the worm through a reduction in ATP content of the cell, causing damage to mitochondria and increased production of ROS. Moreover, nanoparticles have potentially useful biomedical effects when applied in vivo or *In vitro* (Youssef *et al*., 2020; Youssef *et al*., 2021). Also, EL-Melegy *et al*. (2019) worked on other Ag-NPs in combination with Mebendazole on *T. spiralis* encysted larvae and concluded that Ag-NPs improved the therapeutic effect of Mebendazole treatment during the muscular phase of experimental trichinellosis.

Analysis of DNA damage in dead *T. spiralis* exposed to Ag-NPs at different concentrations for 12 h and the control worms was visualized using Comet assay. The assay demonstrated a direct relationship between the increase in the dose of Ag-NPs and the degree of DNA genotoxic damage. The damage was represented in the form of variations in % of DNA in the tail segment, tail length (µm), tail moment (µm), and olive tail moment by investigating the migration of DNA fragments by agarose gel electrophoresis. There was a significant increase ($p \leq 0.05$) in the % of DNA damage with an increase in the % of worm mortalities. At the same time, there was no significant difference ($p \leq 0.05$) between the recorded values using the high Ag-NPs concentration with that recorded using Albendazole (50 mg/kg. B.W.). Moreover, a significant difference was recorded in comparing the obtained values with that of the living worms incubated in blank RPMI media. The value of comet assay to estimate damage in DNA in the exposed worms was previously described by Kumar *et al*. (2015) and Attaullah *et al*. (2020) as they demonstrated that this degree of variations in different measured DNA parameters was reflecting the damage in DNA and this damage was increased by the increase in the concentration of tested materials.

SEM examination of dead *T. spiralis* worms after exposure to different concentrations of Ag-NPs for 12 h, revealed variable irreversible destructive effects on the worm epidermal and cuticular layer. There were marked morphological changes, multiple vesicles, and blebs, sloughing and destruction of some areas of the cuticle with fissures, and loss of normal annulations in the epidermal and cuticular layer of the dead worms. This morphological alteration was directly related to the concentration of the tested Ag-NPs after 12h exposure time. Without no marked difference between the recorded changes that occurred in worms exposed to lethal dose levels and those dead after exposure to the reference drug. These recorded changes were previously mentioned by several authors on the same parasites such as Huanga *et al*. (2020) and by Fahmy *et al*. (2020) after exposing them to different concentrations of clove oil. Moreover, the same morphological changes were recorded on the *T. spiralis* muscle larvae exposed to - by Abd-Elrahman *et al*. (2021).

CONCLUSION

The tested Ag-NPs was proved to be promising *In vitro* an-

ti-Nematodal agent that can be accepted as effective and safe alternative drug against adult *T. spiralis* worms. The newly formulated Ag-NPs possesses other advantages as it is antioxidant and able to induce irreversible damage in the parasite morphology and cause severe genotoxic damage on the DNA level. An in vivo study is running now depending on the data obtained from this *In vitro* study to evaluate the efficacy of this product as a promising parasiticidal material.

CONFLICT OF INTEREST

There are no conflicts of interest to declare.

REFERENCES

Abdel-Rahman, G.H., Abdel-Radi, S., 2022. Synergistic action of Viteselen with anti-Fasciola drug as a tool for improving fertility and hemato-biochemical biomarkers in Fasciola infected sheep. Journal of Parasitic Diseases 46, 103-112.

Abd-Elrahman, S.M., Dyab, A.K., Mahmoud, A.E.S., Elossily, N.A., Alsharif, F.M., Mohamed, S.M., Abomughaid, M.M., Elossily, N.A., 2021. Influence of chemically and biosynthesized silver nanoparticles on *In vitro* viability and infectivity of *Trichinella spiralis* muscle larvae. Annals of parasitology 67, 591-602.

Abou-Okada, M., AbuBakr, H.O., Hassan, A., Abdel-Radi, S., Aljuaydi, S.H., Abdelsalam, M., Taha, E., Younis, N.A., Abdel-Moneam, D.A., 2021 Efficacy of Acriflavine for controlling parasitic diseases in farmed Nile tilapia with emphasis on fish health, gene expression analysis, oxidative stress, and histopathological alterations. Aquaculture 541, 1-11.

Attaullah, M.K.Z., Zahoor, M.A., Mubarik, M.S., Rizvi, H., Majeed, H.N., Zulhussnain, M., Ranian, K., Sultana, K., Imran, M., Qamer, S., 2020. Insecticidal, biological and biochemical response of Musca domestica (Diptera: Muscidae) to some indigenous weed plant extracts. Saudi Journal of Biological Sciences 27, 106-116.

Bhakya, S., Muthukrishnan, S., Sukumaran, M., Muthukumar, M., 2016. Biogenic synthesis of silver nanoparticles and their antioxidant and antibacterial activity. Applied Nanoscience 6, 755-766.

El-Banna, H.A., El-Bahy, M.M., El-Zorba, H.Y., Hady, M., 2005. Anticocidial Efficacy of Drinking Water Soluble Diclazuril on Experimental and Field Coccidiosis in Broiler Chickens. Journal of Veterinary Medicine 52, 287-291.

Elgadir, M.A., Uddin, M.S., Ferdosh, S., Adam, A., Chowdhury, A.J.K., Sarker, M.Z.I., 2015. Impact of chitosan composites and chitosan nanoparticle composites on various drug delivery systems: A review". Journal of Food and Drug Analysis 23, 619-629.

EL-Melegy, M.A., Ghoneim, N.S., Noor EL-dien, N.M., Rizk, M.S., 2019. Silver Nano Particles Improve the Therapeutic Effect of Mebendazole Treatment during the Muscular Phase of Experimental Trichinellosis. Journal of American Science 15, 34-46.

Fahmy, A., Rabab Zalat, A., Rabei, A., 2020. *In vitro* evaluation of the antiparasitic activity of Syzygium aromaticum against adult and larval stages of *Trichinella spiralis*. Scientia Parasitologica 21, 94-101.

Huanga, H., Yao, J., Liua, K., Yanga, W., Wanga, G., Shia, C., Jianga, Y., Wanga, J., Kanga, K., Wanga, D., Wanga, C., Yanga, G., 2020. Sanguinarine has anthelmintic activity against the enteral and parenteral phases of *Trichinella* infection in experimentally infected mice. Acta Tropica 201, 1-10.

IQACA, 2012. Essential quality assurance standards for *Trichinella* digestion assays. ICT Qual. Assur. Comm. Append. 1). Available form: http://www.trichinellosis.org/Guidelines.html.

Keshari, A. K., Srivastava, R., Singh, P., Yadav, V. B., Nath, G., 2020. Antioxidant and antibacterial activity of silver nanoparticles synthesized by Cestrum nocturnum. Journal of Ayurveda and Integrative Medicine 11, 37-44.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. Environmental Toxicology and Chemistry 27, 1825-1851.

Kumar, A., Sharma, S., Verma, G., 2015. Insecticidal and genotoxic potential of Acorus calamus rhizome extract against Drosophila melanogaster. Asian Journal of Pharmaceutical and Clinical Research 8, 113-116.

Malak, N.M.L, Abdel-Radi, S., 2021. The Role of Ducks (Muscovy) and Catfish (Clarias Lazera) Meat in Transmitting *Trichinella spiralis* Infection. World's Veterinary Journal 11, 594-601.

Marimuthu, S., Elango, G., Kirthim, A.V., Jayaseelan, C., Rajakumar, G., Santhoshkumar, T., 2010. Evaluation of green synthesized silver nanoparticles against parasites. Parasitology Research 108, 1541-1549.

Petrie, A., Watson, P. 1999. "Statistics for Veterinary and Animal Science." 1st Ed. The Black well Science Ltd, United Kingdom, 90-115.

Rostami, A., Gamble, H.R., Dupouy-Camet, J., Khazan, H., Bruschi, F., 2017. Meat sources of infection for outbreaks of human trichinellosis. Food Microbiology 64, 65-71.

Said, D.E., ElSamad, L.M., Gohar, Y.M., 2012. Validity of silver, chitosan, and curcumin nanoparticles as anti-Giardia agents. Parasitology Research 111, 545-54.

Saini, P., Saha, S.K., Roy, P., Chowdhury, P., SinhaBabu, S.P., 2016. Evidence of reactive oxygen species (ROS): mediated apoptosis in Setariacervi induced by green silver nanoparticles from Acacia auriculiformis at a very low dose. Experimental Parasitology 160, 39-48.

Salama, M.M., Taher, E.E., El-Bahy, M.M., 2012. Molluscicidal and Mosquitocidal Activities of the Essential oils of Thymus capitatus L. and Marrubium vulgare L. American Journal of Drug Discovery and Development 2, 204-211.

Singh N.P., McCoy M.T., Tice R.R., Schneider E.L., 1988. A simple technique for quantitation of low-levels of DNA damage in Individual cells. Experimental cell Research 175, 184-191.

Taher, E.E., Meabed, E. M., El Akkad, D. M., Kamel, N.O., and Sabry, M.A., 2017. Modified dot-ELISA for diagnosis of human trichinellosis. Experimental Parasitology 177, 40-46.

Walden, H.D.S., 2013. Food born infections and intoxications, 4th edition. Food Science and Technology, 343-351.

Youssef, F., Mohamed, G., Ismail, S., Elzorba, H., Galal, A., Elbanna, H., 2021. Synthesis, Characterization and *In vitro* Antimicrobial Activity of Florfenicol-Chitosan Nano-composite. Egyptian Journal of Chemistry 64, 941-948.

Youssef, F.S., Elbanna, H.A., Elzorba, H.Y., Galal, A.M., Mohamed, G.G., Ismail S.H., 2020. Synthesis and characterization of florfenicol-silver nano-composite and its antibacterial activity against some gram positive and gram-negative bacteria. International Journal of Veterinary Science and Medicine 9, 324-330.

Youssef, F.S., Elbanna, H.A., Elzorba, H.Y., Galal, A.M., 2019. Application of some nanoparticles in the field of veterinary medicine. International Journal of Veterinary Science and Medicine. 7, 78-93.

Zhang, N., Li, W., Fu, B., 2018. Vaccines against *Trichinella spiralis*: progress, challenges and future prospects. Transboundary and Emerging Diseases 65, 1447-1458.