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# Green synthesis, characterization, acaricidal, larvacidal, and repellent activities of copper nanoparticles of *Astragalus sinicus* against *Hyalomma anatolicum*

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## ABSTRACT

**Objective:** To green synthesize and characterize copper nanoparticles (CuNPs) using *Astragalus sinicus*, as well as evaluate the acaricidal, larvacidal, and repellent activities of CuNPs against *Hyalomma anatolicum* (*H. anatolicum*), one of the most prevalent ticks infesting cattle in Saudi Arabia.

**Methods:** CuNPs were green synthesized by adding the *Astragalus sinicus* extract to a copper sulfate solution. The acaricidal, larvicidal, and repellent activities of CuNPs against *H. anatolicum* were assessed *via* the adult immersion test, the larval packet test, and the vertical movement behavior of tick larvae, respectively. The effects of CuNPs on acetylcholinesterase as well as oxidative enzyme activities were examined.

**Results:** The green synthesized CuNPs displayed a spherical form with a size range of 15-75 nm. After exposure of adult *H. anatolicum* to different concentrations of CuNPs, the viability rate of adult *H. anatolicum* and the mean number, weight, and hatchability of eggs were noticeably reduced, in comparison to the control group ($P$<0.001). In addition, the viability rate of larvae considerably declined ($P$<0.001) with the $LC_{50}$ and $LC_{90}$ values of 11.30 and 20.34 μg/mL, respectively. The maximum repellent activity of CuNPs was observed at 50, 100, and 200 μg/mL with complete repellent activity after 60, 120, and 180 min of exposure, respectively. CuNPs, mainly at ½$LC_{50}$ and $LC_{50}$ concentrations, markedly suppressed the acetylcholinesterase activity of the larval stage of *H. anatolicum* ($P$<0.001). Moreover, CuNPs, mainly at $LC_{50}$ dose, significantly elevated malondialdehyde level while declining glutathione-S-transferase level in *H. anatolicum* larvae ($P$<0.001).

**Conclusions:** CuNPs show potent acaricidal, larvicidal, and repellent activities against adults and larvae of *H. anatolicum*. However, further studies must be performed to clarify the precise mechanisms and the efficacy of CuNPs in practical use.

**KEYWORDS:** Insecticide; Tick; Pesticide; *Astragalus sinicus*; Nanomedicine; *Hyalomma anatolicum*; Acaricidal activity; Larvacidal activity

### Significance

The use of chemical acaricides has led to the emergence of resistance and coordinated ecological contamination. In this study, CuNPs green synthesized by *Astragalus sinicus* extract showed potent acaricidal, larvicidal, and repellent activities against adults and larvae of *Hyalomma anatolicum* by inhibiting the level of acetylcholinesterase and declining antioxidant activity. Further studies must be performed to clarify the precise mechanisms and the toxicity of CuNPs against non-target organisms.

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## 1. Introduction

Ticks (belonging to the families of Ixodidae, Argasidae, and Nuttalliellidae) are the main bloodsucker ectoparasites and can infest various vertebrate hosts and transmit a broad spectrum of pathogenic microbial agents, *e.g.*, parasites, bacteria, and viruses[1]. In recent decades, tick-borne diseases have been increasing, rising health constraints for both humans and domestic animals[2]. In domestic animals, several tick-borne infections, *e.g.*, babesiosis, anaplasmosis, result in severe problems in the production of milk, meat, and leather[3,4]. Nowadays, tick control approaches generally focus on applying commercially available chemical acaricides and repellent agents, *e.g.*, organophosphates, arsenicals, carbamates, as well as pyrethroids[5]. In recent years, the use of chemical agents has resulted in the emergence of resistance as well as coordinated ecological contamination[6,7]. Therefore, studies on novel and reliable acaricides to be applied for the control of ticks are urgently required.

Bio-nanotechnology is well-known as a science displaying new and remarkable advances and broadly utilized for drug discovery[8]. Nanoparticles (10 to 100 nm) displayed various properties, *e.g.*, high bioavailability, improved pharmacokinetics of components, and low toxicity, which are broadly applied for pharmacological and therapeutic goals[9]. It has been proven that the present chemical and physical processes for nanoparticle synthesis are often expensive, toxic, and harmful to the environment[10]. Green synthesis is recognized as a consistent, cost-effective, non-toxic method in the synthesis of nanoparticles using herbs and their derivatives, whereas their secondary metabolites cause prompt ion bioremediation and subsequently the synthesis of metal nanoparticles[11].

Recently, the acaricidal effects of various metal nanoparticles (*e.g.*, silver, zinc, titanium, and gold) have been reported against various ticks[12]. Copper (Cu) is an essential mineral for living organisms; because it has a key role in the construction of the respiratory enzyme cytochrome oxidase C[13]; in addition, Cu is used in agriculture for pest control and providing soil nutrients[14]. Among metal nanoparticles, copper nanoparticles (CuNPs) revealed different pharmacological effects such as anticancer, antioxidant, antinociceptive, and anti-inflammatory[15,16]. Moreover, CuNPs showed potent antimicrobial, antiviral, and antiparasitic properties against some bacteria (*e.g.*, *Bacillus* spp., *Salmonella* spp., and *Staphylococcus* spp.), pathogenic fungi (*e.g.*, *Aspergillus* spp., and *Fusarium* spp.), virus (*e.g.*, human influenza A and avian influenza), and parasites (*e.g.*, *Leishmania* spp., *Toxoplasma gondii*, and *Echinococcus* spp.)[15,17–21]. Considering the abovementioned properties, this work aimed to synthesize and characterize the CuNPs, as well as evaluate their acaricidal, larvacidal, and repellent activities against *Hyalomma anatolicum* (*H. anatolicum*), one of the most prevalent ticks infesting cattle in Saudi Arabia.

## 2. Materials and methods

### 2.1. Green synthesis of CuNPs

*Astragalus sinicus* aerial parts were gathered in June 2022 from the rural district of Riyadh, Saudi Arabia and identified by a botanist at Shaqra University, Saudi Arabia. A voucher sample was deposited at herbarium of Shaqra University, Saudi Arabia with No. 2022.14.1654. For extraction, 250 g of dried and powdered materials were used by percolation with water for 3 d at 21 ℃. After filtering, the extract was evaporated in a vacuum at −55 ℃, and reserved at −4 ℃[22,23]. To obtain nanoparticles, 50 mL of the prepared aqueous extract was poured into a beaker, and 100 mL of copper sulfate solution (Sigma-Aldrich, Germany) at a concentration of 1 mM was added to the extract and then stirred for 10 min. Finally, the reaction was at room temperature after 12 h. The change in color of the extract from blue to dark yellow with the formation of turbidity indicated the production of nanoparticles.

### 2.2. Characterization of green synthesized CuNPs

#### 2.2.1. UV–Vis spectroscopic analysis

The decrease of copper ions to nanoparticles was confirmed by determining the surface plasmon resonance (SPR) of CuNPs through Vis-UV spectrum analysis by a spectrophotometer (Shimadzu UV2550, Japan) in the range of 300-700 nm.

#### 2.2.2. Physical characterization of CuNPs

The physical characterization of CuNPs, *e.g.*, size and shape, was estimated by a scanning electron microscope (SEM, Mira3, Czech) with 15 kV, a magnification of 10×, and a resolution of 1 nm. The size of nanoparticles was also assessed using a dynamic light scattering device (Zeta sizer, UK, Malvern).

#### 2.2.3. X–ray diffraction (XRD) analysis

The Cu presence in CuNPs and the crystal configuration were studied by evaluating the Ka-ray source using an XRD device model 2000 APD, Italy.

#### 2.2.4. Fourier transform infrared spectroscopy (FTIR) analysis

FTIR analysis is a powerful tool for chemical and biochemical studies that is used to detect various functional groups. To do this, CuNP powder was mixed with potassium bromide to yield tablets and studied using a device (Tensor27, Germany).

### 2.3. Ticks collection

Adult female *H. anatolicum* (500 mg) were obtained from naturally infested cattle in rural regions of Riyadh, Saudi Arabia (24.910112,

47.031268) and they were identified according to the standard guidelines[24]. Ticks were washed with normal saline and dried. Several ticks were then used for adult immersion analysis, and the remaining were kept at standard conditions to get eggs and consequently larvae.

### 2.4. Adult immersion test

Briefly, nine groups of adult ticks (10 ticks per each) were separately immersed with CuNPs at 0.312-200 μg/mL, normal saline (negative control), and deltamethrin (positive control) for 5 min at 21 ℃. Adult immersion test was performed in triplicate. After drying the ticks, they were put in a Petri dish and incubated at standard conditions until oviposition was complete[25]. After two weeks, the number of ticks leaving eggs and the weight of the collected eggs were recorded. To determine the hatchability rate, eggs were moved to tubes and kept for 21 d at standard conditions. Additionally, the lethal concentration 50% ($LC_{50}$) and 90% ($LC_{90}$) values of CuNPs were determined using the Probit test.

### 2.5. Larvicidal activity of CuNPs

The larval packet test was used to assess the larvicidal effects of CuNPs as explained elsewhere[26]. Briefly, nine groups of larvae (15 larvae per each) at 10 days old were separately put in the center of filter papers (7 cm×7 cm) and CuNPs at 0.312-200 μg/mL were added to them and closed to make packets. After one-day incubation in standard conditions, packets were tested to determine the rate of larvae viability. The larvae with no motility or movement were recorded as dead larvae.

### 2.6. Repellent activity of CuNPs

The vertical larval motility behavior approach was used to assess the repellent effects of CuNPs as described by Wanzala *et al*[27]. Briefly, a device with two aluminum rods (0.7 cm×15 cm) with filter papers (7 cm×7 cm) was soaked with CuNPs at 0.312-200 μg/mL, normal saline, and 7.5% N,N-diethyl-3-methyl benzamide. Then, the soaked papers were cut on the rod. Next, 10-day larvae ($n$=30) were placed at the base of each rod and monitored for 60-240 min. Larvae found on the upper and lower end of the soaked filter paper were measured as not repelled and repelled, respectively.

### 2.7. Anti-acetylcholinesterase (AChE) activity

In order to investigate the effects of CuNPs on AChE activity, nine groups of larvae (15 larvae per each) were separately exposed to CuNPs (⅓$LC_{50}$, ½$LC_{50}$, and $LC_{50}$), deltamethrin (1 mL/L, positive control), and normal saline (negative control), and were macerated using a mortar and a grinder for 10 min in a mixture of sodium phosphate buffer (100 mM, pH 7.0), Triton X-100, and protease inhibitor (at a ratio of 1 to 5 larva weight:buffer volume) based on the previous studies[28,29]. The level of inhibition of the AChE enzyme was determined according to the technique explained previously[28,29].

### 2.8. Oxidative enzyme activity

Briefly, after exposure of the larvae to CuNPs (at ⅓$LC_{50}$, ½$LC_{50}$, and $LC_{50}$), the larvae homogenate was obtained and the level of lipid peroxidation (malondialdehyde, MDA) was determined colorimetrically as previously described elsewhere[30]. The absorbance of the combination was read by a spectrophotometer at 530 nm. The level of glutathione-S-transferase (GST) as an indication of antioxidant activity was determined on the larvae homogenate acquired, followed by exposure of the larvae to CuNPs (at ⅓$LC_{50}$, ½$LC_{50}$, and $LC_{50}$) based on the reaction of 5,5′-dithiobis (2-nitrobenzoic acid, Sigma-Aldrich, St. Louis, MO, USA) with GST. Then the absorbance of the combination was read by a spectrophotometer (Shimadzu UV2550, Japan) at 340 nm[31].

### 2.9. Protein determination

The protein level in the larvae homogenate was determined by adding Bio-Rad reagent (0.3 mL) to 10 μL of supernatant of the larvae homogenate. After incubation for 5 min at 21 ℃, the absorbance was read at 570 nm and the level of protein concentration was obtained using a bovine serum albumin standard curve.

### 2.10. Statistical analysis

All tests were performed in triplicate to increase the reliability of the results. The data were analyzed by one-way ANOVA using SPSS software ver. 26.0 to compare the tested groups. $P$<0.05 was considered significantly different.

### 2.11. Ethical statement

This work was permitted by the Ethics Committee at Almaarefa University, Saudi Arabia (IRB23-030).

## 3. Results

### 3.1. UV-vis analysis and physical characterization of CuNPs

The peak of the Vis-UV device was around 505 nm, which indicated the presence of zinc nanoparticles. SEM analysis revealed that the green synthesized CuNPs displayed a spherical form. For size distribution, the green synthesized CuNPs were in the range of

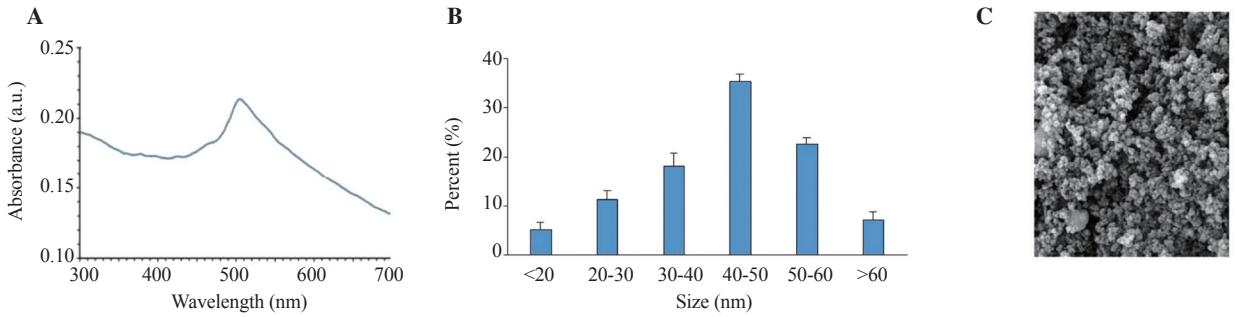


**Figure 1.** UV-Vis spectroscopic analysis (A), size distribution (B), and scanning electron microscope (C) of the green synthesized copper nanoparticles at a magnification of 10×, and a resolution of 1 nm.

15-75 nm, the maximum distribution of particle size was observed at 40-50 nm (Figure 1).

The results showed that the synthesized nanoparticles had a crystalline structure. The presence of a diffraction peak at 31.5º, 44.9º, 55.3º, 63.8º, and 74.9º, corresponding to planes 108, 111, 201, 218, and 003, respectively, indicated the presence of CuNPs with a monoclinic crystalline phase (Figure 2).

FTIR analysis showed that the bands at 3 478, 3 235, 2 895, 2 735, 1 703, 1 512, 1 467, and 1 185 $cm^{-1}$ were attributed 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 (Figure 3).

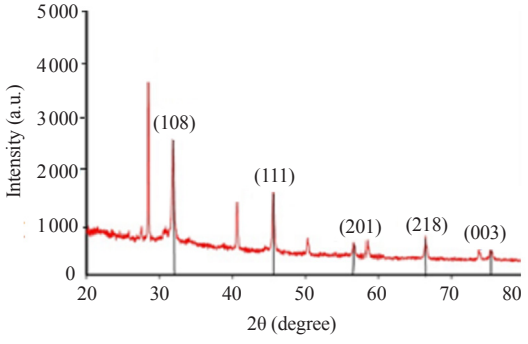


**Figure 2.** X-ray diffraction analysis of the green synthesized copper nanoparticles.

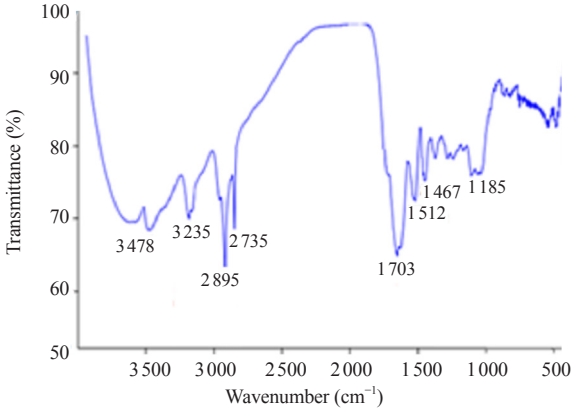


**Figure 3.** Fourier transform infrared spectroscopy analysis of the green synthesized copper nanoparticles.

### *3.2. Acaricidal effects of CuNPs*

The adult immersion test showed that CuNPs considerably reduced the viability rate of *H. anatolicum* adult in a dose-dependent manner ($P<0.001$). In particular, CuNPs at 50, 100, and 200 µg/mL resulted in 100% mortality of adult *H. anatolicum* (Figure 4A). The obtained $LC_{50}$ and $LC_{90}$ values for CuNPs were 23.03 and 41.40 µg/mL, respectively. After exposure of adult *H. anatolicum* to different concentrations of CuNPs, the mean number, weight, and hatchability of eggs were dose-dependently declined ($P<0.05$), in comparison to the control group (Figure 4B-D).

### *3.3. Larvicidal effects of CuNPs*

The mortality rate of larvae was considerably increased ($P<0.001$) after exposure to different concentrations of CuNPs. CuNPs at 25, 50, 100, and 200 µg/mL killed 100% of larvae. The obtained $LC_{50}$ and $LC_{90}$ values for CuNPs were 11.30 and 20.34 µg/mL, respectively (Figure 5).

### *3.4. Repellent activity of CuNPs*

CuNPs showed promising repellent activity against *H. anatolicum* larvae ($P<0.01$). The maximum activity was reported at 50, 100, and 200 µg/mL with complete repellent activity after 60, 120, and 180 min of exposure, respectively ($P<0.001$) (Figure 5).

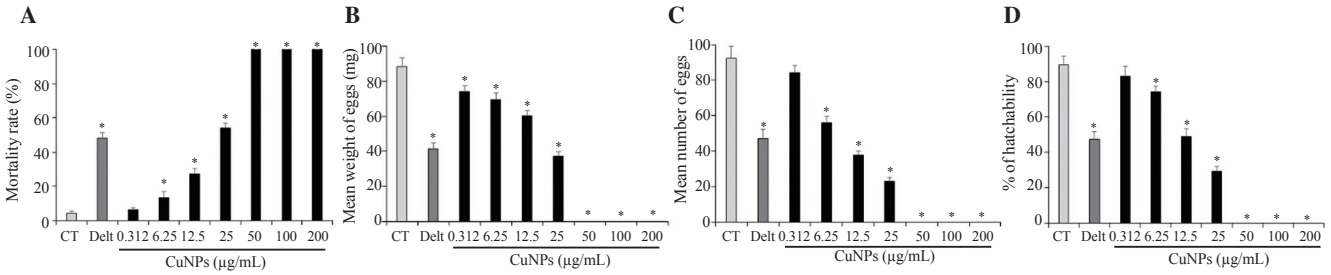


**Figure 4.** Acaricidal effects of the green synthesized copper nanoparticles on mortality rate (A) on adult females of *Hyalomma anatolicum* as well as weight (B), number (C), and hatchability (D) of eggs ($n$ = 3). $^{*}P$<0.001 compared with the control group. CT: control; CuNPs: copper nanoparticles; Delt: deltamethrin.

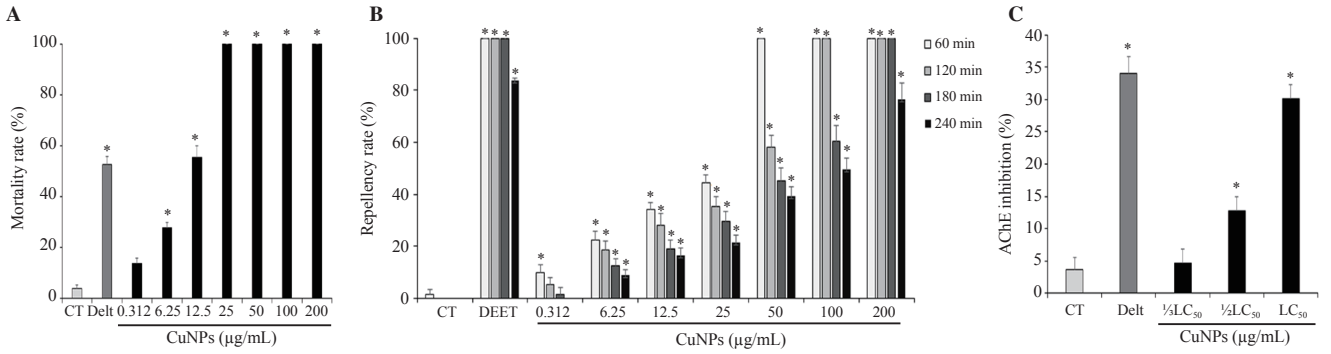


**Figure 5.** Larvicidal effect (A) and repellent (B) and anti-acetylcholinesterase (AChE) activities (C) of the green synthesized copper nanoparticles against *Hyalomma anatolicum* larvae ($n$ = 3). $^{*}P$<0.001 with the control group. DEET: N,N-diethyl- 3-methyl benzamide.

### *3.5. Anti-AChE activity of CuNPs*

The findings revealed the CuNPs mainly at ½$LC_{50}$ and $LC_{50}$ concentrations markedly suppressed the AChE activity of the larvae stage of *H. anatolicum* in comparison to the control group (Figure 5).

### *3.6. Effect of CuNPs on oxidative enzymes*

CuNPs at $LC_{50}$ significantly elevated the MDA level, while decreasing the level of GST in *H. anatolicum* larvae ($P$<0.001) (Figure 6). In contrast, the CuNPs at ⅓$LC_{50}$ and ½$LC_{50}$ caused no significant changes in MDA and GST levels. In addition, protein levels were determined in both control and extract-exposed larval homogenates. The protein level in control larval homogenates was (4.23 ± 0.02) μg/μL, while the protein level in the CuNPs exposed larval homogenates was (3.86 ± 0.04) μg/μL.

## 4. Discussion

In this study, we successfully synthesized CuNPs using *Astragalus sinicus* aqueous extract. The results confirmed that the CuNPs displayed a spherical form and had a size range of 15-75 nm with a maximum distribution size of 40-50 nm. Several herbs, *e.g.*, *Nerium oleander*, *Capparis spinosa*, *Punica granatum*, *Olea europaea*, *Postia puberula*, *Aegle marmelos*, *Zingiber officinale*, have been applied for the green synthesis of CuNPs and they produced CuNPs in a spherical form with different sizes ranging from 2-500 nm depending on extract concentration and synthesis conditions[32].

Our results showed CuNPs considerably and dose-dependently reduced the viability rate, mean number, weight, and hatchability of eggs of *H. anatolicum* adults in comparison to the control group. Additionally, we found that after exposure of the larvae stage of *H. anatolicum* to CuNPs, the viability rate of larvae considerably declined. CuNPs especially at 25, 50, 100, and 200 μg/mL killed 100% of larvae. In addition, the maximum repellent activity of CuNPs was observed at 50, 100, and 200 μg/mL after 60, 120, and 180 min of exposure, respectively.

Vivekanandhan *et al.* reported that CuNPs green synthesized by *Metarhizium robertsii* displayed potent insecticidal properties against some mosquitoes such as *Culex quinquefasciatus*, *Tenebrio molitor*, *Anopheles stephensi*, and *Aedes aegypti*[33]. Recently, Rahman *et al.* demonstrated that the chemically synthesized CuNPs had considerable larvicidal (3rd and 4th instar larvae) and antifeedant effects against *Spodoptera frugiperda*, one of the main agricultural pests[34]. Muthamil Selvan *et al.* showed that CuNPs green synthesized using *Tridax procumbens* extract had promising larvicidal effects against *Aedes aegypti* larvae with an $LC_{50}$ value of 4.2 mg/mL[35]. Previously, it has been proven that green synthesized nanoparticles, especially CuNPs displayed potent feeding, fertility

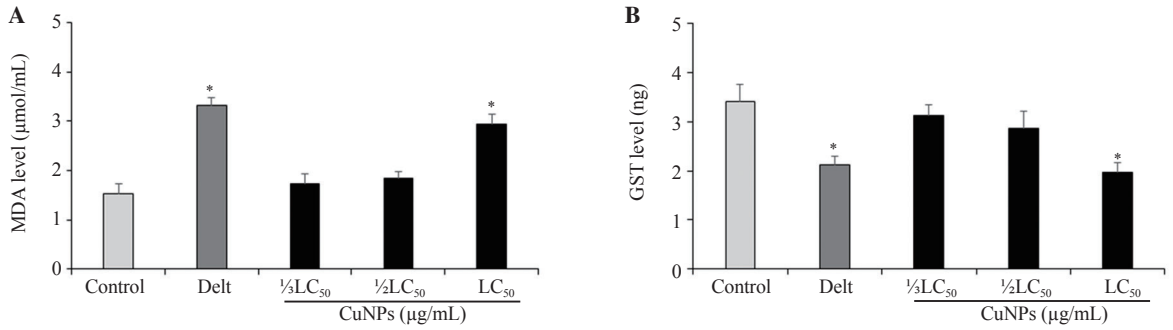


**Figure 6.** Effect of the green synthesized copper nanoparticles on MDA (A) and GST (B) levels in *Hyalomma anatolicum* larvae ($n$ = 3). MDA: malondialdehyde; GST: glutathione S-transferase. $^{*}P<0.001$ compared with the control group (normal saline).

inhibitory, and development regulatory effects against various grain insect pests[36]. Nanoparticles can penetrate the epithelial and endothelial cells through the transcytosis route and penetrate dendrites, axons, blood vessels, and lymphatic vessels, which cause oxidative stress in organisms[37]. On the other hand, studies showed that copper displayed its biocidal activity through nucleic acid degradation, alteration and inhibition of protein synthesis and inhibition of their biological activity, increased permeability of the plasma membrane, and peroxidation of membrane lipids[38].

AChE is considered one of the key enzymes in the nervous system of arthropods and participates in the balanced transduction of neuronal signals *via* the fast hydrolysis of the acetylcholine facilitator in the synaptic site, and the majority of insecticides display their efficacy by preventing AChE[39].

Our results revealed that the CuNPs mainly at $½LC_{50}$ and $LC_{50}$ concentrations markedly suppressed the AChE activity of the larvae stage of *H. anatolicum* in comparison to the control group. In line with our results, Rahman *et al.* have reported that the CuNPs markedly declined AChE activity of *Spodoptera frugiperda* larvae by 60.25% at 500 ppm through the colorimetric assay[34]. Concerning the effect of CuNPs on oxidative enzymes, we found that after exposure of *H. anatolicum* larvae to CuNPs, mainly at $LC_{50}$, the MDA level was significantly elevated while a significant reduction in the level of GST in *H. anatolicum* larvae was observed. It has been proven that GST and MDA are well-known to have a key role in the clearance of xenobiotic and endogenous drugs in arthropods and they can be considered drug-resistance factors[40,41]. The protein level in control larval homogenates was (4.23 ± 0.02) μg/μL, while the protein level in the CuNPs exposed larval homogenates was (3.86 ± 0.04) μg/μL; indicating that the decrease in total protein content can be due to the natural increase of hydrolytic and detoxifying enzymes that frequently occur shortly after treatment[42].

In conclusion, green synthesized CuNPs using *Astragalus sinicus* extract showed potent acaricidal, larvicidal, and repellent activity against adults and larvae of *H. anatolicum* in a dose-dependent manner. The green synthesized CuNPs also markedly decreased the levels of AChE and GST and increased MDA levels in *H. anatolicum* larvae. Despite these results, further studies must be performed to clarify the precise mechanisms and the efficacy of CuNPs in practical use.

## Conflict of interest statement

The authors declare that they have no competing interests.

## Acknowledgments

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## Authors' contributions

HSG and ADA designed the work and contributed to the concept. BMA, AFS, MSA, and QAHM performed experiments. ADA and HSG wrote and performed the critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

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