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RESEARCH ARTICLE

Acaricidal, larvacidal, and repellent activity of Linalool loaded zinc oxide nanoparticles against *Hyalomma anatolicum*

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ABSTRACT

Current strategies for tick control have led to the development of resistance and environmental contamination. Consequently, there is an urgent need for research into new and effective acaricides for tick control. The aim of this study was to fabricate and characterize Linalool loaded zinc oxide nanoparticles (Lin@ZNP), and to assess the acaricidal, larvacidal, and repellent activities of Lin@ZNP against *Hyalomma anatolicum*, a prevalent tick species infesting cattle in Saudi Arabia. Lin@ZNP was synthesized using an ethanolic solution of polyvinyl alcohol. The adult immersion, the larval packet, and the assessment of vertical movement behavior of tick larvae assays were utilized to examine the acaricidal, larvicidal, and repellent activities of Lin@ZNP against *H. anatolicum*, respectively. Furthermore, the impact of Lin@ZNP on acetylcholinesterase and oxidant/antioxidant enzyme activities was investigated. Exposure of adult *H. anatolicum* to different concentrations of Lin@ZNP resulted in noticeable ($p<0.001$) reductions in the viability rate of adults and the mean number, weight, and hatchability of eggs, compared to the control group. Lin@ZNP demonstrated significant repellent effects on *H. anatolicum* larvae after 60, 120, and 180 minutes of exposure. Lin@ZNP, particularly at all concentrations, markedly suppressed the acetylcholinesterase activity of the larval stage of *H. anatolicum* ($P<0.001$); but increase in malondialdehyde (MDA) levels ($P<0.001$) and a decrease in glutathione-S-transferase (GST) levels in *H. anatolicum* larvae ($P<0.001$). Lin@ZNP exhibited considerable acaricidal, larvicidal, and repellent effects against H. dromedarii adults and larvae in a manner dependent on the dosage. Additionally, Lin@ZNP notably reduced AChE levels and antioxidant activity, while inducing oxidative stress in *H. anatolicum* larvae. Nevertheless, further research is necessary to elucidate the precise mechanisms and practical efficacy of Lin@ZNP.

Key words: Nantechnology; pesticide; tick; larvae.

INTRODUCTION

Ticks, which are part of the Ixodidae, Argasidae, and Nuttalliellidae families, are significant ectoparasites that feed on the blood of various vertebrate hosts and can transmit a wide range of pathogenic microorganisms, including parasites, bacteria, and viruses (Boulanger *et al*., 2019). The incidence of tick-borne diseases has been on the rise in recent years, posing significant health challenges for both humans and animals (Hromníková *et al*., 2022). Current strategies for tick control primarily involve the use of commercially available chemical acaricides and repellents, such as organophosphates, arsenicals, carbamates, and pyrethroids (Boulanger *et al*., 2019). However, the widespread use of these chemical agents has led to the development of resistance and environmental contamination (Sparks *et al*., 2021). Consequently, there is an urgent need for research into new and effective acaricides for tick control. Studies indicated that the primary tick species found on cattle in various regions of Saudi Arabia include *Hyalomma anatolicum*, *H. dromedarii*, *H. impeltatum*, *H. excavatum*, *Rhipicephalus annulatus*, and *R. turanicus* (Alanazi *et al*., 2021).

Bio-nanotechnology is a widely recognized field that has shown significant advancements and is extensively utilized in the realm of drug discovery (Anselmo & Mitragotri, 2019). Nanoparticles, typically ranging from 10 to 100 nm in size, exhibit a range of properties such as high bioavailability, improved pharmacokinetics, and low toxicity, making them widely applicable for pharmacological and therapeutic purposes (Formoso *et al*., 2016). Metal nanoparticles, known for their unique physical characteristics, are frequently employed as

effective vehicles for conveying both small and large drug compounds (Jamkhande *et al*., 2019). Zinc oxide nanoparticles (ZNP) have garnered significant attention as a versatile option for medical applications, demonstrating potential as an anticancer, anti-inflammatory, and antimicrobial agent (Moezzi *et al*., 2012). Linalool (Lin, 2,6-dimethyl-2,7-octadien-6-ol), also known as Lin, is a monoterpenoid compound derived from various plant sources and is known for its pharmacological properties, such as antinociceptive, anti-inflammatory, and antimicrobial effects. These attributes have been applied in both traditional and modern medicinal applications (Aprotosoaie *et al*., 2014).

Over 95% of synthetic linalool is primarily utilized for its aromatic and odor-enhancing properties in various consumer products such as cosmetics, soaps, perfumes, household cleaners, waxes, and personal care items, with only about 1% being incorporated into food and beverages for flavor and scent enhancement (Politano *et al*., 2008). Exposure to linalool is widespread among humans, stemming not only from the inclusion of this compound in processed food, beverages, cosmetics, and household goods but also from its natural occurrence in fruits and spices (Politano *et al*., 2008). Noteworthy findings from comprehensive reviews (Letizia *et al*., 2003) reveal that linalool exhibits an acute oral mammalian LD_{50} (lethal dose, 50%) of approximately 3.0 g/kg, while the acute dermal toxicity, as determined from a single rabbit study, was estimated to be 5.61 g/kg (Letizia *et al*., 2003). Linalool is not considered genotoxic or a sensitizer in humans; however, it has been shown to cause skin and eye irritation in rabbit studies when applied undiluted. Furthermore, a 32% solution of linalool in acetone has been identified as a mild dermal irritant in humans, with lower concentrations demonstrating no irritant effects (Letizia *et al*., 2003). In addintion, Politano *et al*. (2008) reported that linalool does not exhibit developmental toxicity in rats when administered at maternal doses of up to 1000 mg/kg/day.

In recent studies, the potential acaricidal properties of different metal nanoparticles, such as silver, zinc, titanium, and gold, have been investigated in relation to their efficacy against various tick species (Mahmoudvand *et al.,* 2016; Benelli *et al*., 2017; Albalawi *et al*., 2020; Alyousif *et al*., 2021; Ezzatkhah *et al*., 2021; Saadatmand *et al*., 2021). However, the results have shown variability and occasional contradictions, which may be attributed to disparities in the synthesis methods, tick species, and application techniques (Benelli *et al*., 2017; Mahmoudvand *et al*., 2017). Given the aforementioned attributes, the objective of this study was to produce and analyze the properties of zinc nanoparticles (Lin@ZNP), and to assess their effectiveness as acaricides, larvicides, and repellents against *Hyalomma anatolicum* (*H. anatolicum*), a prevalent tick species infesting cattle in Saudi Arabia.

MATERIALS AND METHODS

Synthesis of Lin@ZNP

Initially, a suspension was prepared by mixing Zn (NO3)2.4H2O and PVA (0.01%), followed by the addition of NaOH (0.5 M) with agitation on a magnetic stirrer. After 4 hours, the solution turned white, indicating the formation of ZNP. Nanoparticle formation was induced by subjecting the solution to centrifugation at 10,000 g for 10 minutes. The resulting white precipitate underwent three washes with distilled water, followed by drainage and drying in an oven at 60°C for 24 hours. To synthesize Lin@ZNP, a solution containing polyvinyl alcohol (PVA) at a concentration of 1.2%, Tween 80 at 0.1%, and Lin at 0.5% in ethanol was combined and stirred overnight at 50°C. Subsequently, 10 mL of ZNP at a concentration of 50 mg/mL was gradually added to the mixture. The combination was subsequently homogenized using sonication at 100 W and 22 kHz, employing a 5-second on/off pulse pattern at a temperature of 60°C for a duration of 60 minutes utilizing a Q700 Qsonica device, USA. Following this, the resulting nanosuspension was stored at 21°C until it was prepared for testing (Mirhosseini & Firouzabadi, 2013).

Characterization of Lin-ZNP

The nanocomposites were characterized in terms of their dimensions and visual properties using a scanning electron microscope (SEM, Mira3, Czech Republic) operating at 15 kV, with a magnification of 10x and a resolution of 1 nm. Additionally, the size distribution and zeta potential of the nanocomposites were evaluated using a Dynamic Light Scattering (DLS) instrument (Zeta sizer, Malvern, UK).

X-ray diffraction (XRD) analysis

The elemental composition of the nanosuspension was analyzed using X-ray diffraction (XRD) with a copper lamp as the radiation source emitting X beams at a wavelength of α= 1.54 A0, employing a XRD device model 2000 APD from Italy.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The functional groups present in the nanosuspension were investigated through Fourier Transform Infrared Spectroscopy (FTIR) using a Bruker Tensor 27 FTIR Spectrometer (Tensor27, Germany) operating within the range of 400-4000 cm^{-1} and a resolution of 1 cm^{-1}.

Release kinetics of Lin and ZNP

The release of Lin and Zn from the nanosuspension was evaluated using a dialysis membrane (Sigma-Aldrich, USA) in PBS at 21°C. The membrane was saturated with the nanosuspension, secured with clamps, and immersed in PBS, where it was agitated for 2 hours. At 15-minute intervals, 5 ml of the PBS solution was collected for the determination of Zn and Lin content, which was analyzed using a UV-Vis spectrophotometer (Jenway 6705 UV/Vis) and the Analyst 200 AA Spectrometer (Mundelein, USA) respectively (Erol *et al*., 2013).

Tick collection

Adult ticks were manually collected from naturally infested cattle in rural areas of Riyadh, Saudi Arabia (24.910112, 47.031268) according to the World Association for the Advancement of Veterinary Parasitology guidelines (Holdsworth *et al*., 2006). Engorged *H. anatolicum* were then identified in accordance with established protocols (Estrada Peɒa *et al*., 2004). The female ticks were rinsed twice with normal saline and subsequently dried. A total of 500 adult engorged female ticks were employed in the research. One hundred of these engorged female ticks were placed in labeled containers at a temperature of 28 ± 1 °C and a relative humidity of 85 ± 5%, with muslin cloth covering the containers to facilitate egg hatching. The eggs were allowed to hatch and progress into larvae within 18–25 days under similar incubation conditions. These larvae were subsequently employed in the larval packet test (LPT). The remaining ticks were divided into nine groups, each consisting of 10 ticks. Each group underwent three repetitions of the test, resulting in a total sample size of 270 ticks (n = 270). These tick groups were utilized to assess the acaricidal impacts of Lin@ZNP through the adult immersion test.

Adult immersion test (AIT)

The adult immersion test involved nine groups of adult ticks, which were exposed to varying concentrations of Lin@ZNP ranging from 0.312 to 200 µg/mL, as well as to normal saline (as a negative control) and deltamethrin (as a positive control) for a duration of 5 minutes at a temperature of 21°C. Subsequently, the ticks were dried and placed in a Petri dish, where they were incubated under standard conditions (at 27 ± 1°C and relative humidity of 85 ± 5%) until oviposition was complete (Gazim *et al*., 2011). After a period of two weeks, the number of ticks that laid eggs and the weight of the collected eggs were documented. To assess the hatchability rate,

the eggs were transferred to tubes and maintained under standard conditions for 21 days. Furthermore, the lethal concentration 50 (LC_{50}) and LC_{90} values of Lin@ZNP were determined using the Probit test in SPSS software version 26.0

Larvicidal activity of Lin@ZNP

The larval packet test, as described in a previous study (Matos *et al*., 2019), was utilized to evaluate the larvicidal properties of Lin@ZNP. In summary, nine sets of 100 larvae, each at 10 days old, were individually placed in the center of 7 cm×7 cm filter papers. Lin@ZNP (0.1 mL) at concentrations ranging from 0.312 to 200 µg/mL were impregnated beforehand in the paper pack and applied to the larvae. The papers were sealed to create packets, following a one-day incubation under standard conditions, the packets were examined to determine the survival rate of the larvae. Larvae exhibiting no signs of motility or movement were recorded as deceased.

Repellent activity of Lin@ZNP

The vertical larval motility behavior method was utilized to evaluate the repellent properties of Lin@ZNP (Wanzala *et al*., 2004). In this approach, a device consisting of two aluminum rods (0.7 cm×15 cm) with filter papers (7 cm×7 cm) soaked in varying concentrations of Lin@ZNP (0.625-40 µg/mL), normal saline, and 7.5% N,N-diethyl-3-methyl benzamide (DEET) was employed. Subsequently, the soaked papers were affixed to the rods. Subsequently, 10-day-old larvae (n=30) were positioned at the base of each rod and observed for a duration of 60-240 minutes. Larvae located on the upper and lower ends of the soaked filter paper were classified as not repelled and repelled, respectively.

Anti-acetylcholinesterase (AChE) activity

To examine the impact of Lin@ZNP on AchE activity, five groups of larvae (consisting of 30 larvae each) were exposed to varying concentrations of Lin@ZNP (1/3 LC_{50}, ½ LC_{50}, and LC_{50}), deltamethrin (1 mL/L, serving as the positive control), and normal saline (used as the negative control). Subsequently, the larvae were macerated for 10 minutes using a mortar and grinder in a mixture of sodium phosphate buffer (100 mM, pH 7.0), Triton X-100, and protease inhibitor, with the ratio of 1 to 5 larva weight to buffer volume, as per previous research. The degree of inhibition of the AChE enzyme was assessed following the previously described technique (Cardoso *et al*., 2020).

Oxidant/antioxidant enzyme activity

Following exposure of the larvae (five groups of larvae consisting of 30 larvae per each) to varying concentrations of Lin@ZNP (at 1/3 LC_{50}, ½ LC_{50}, and LC_{50}), the larvae homogenate was collected and the level of lipid peroxidation (malondialdehyde, MDA) and glutathione-S-transferase (GST) was determined using Lipid Peroxidation (MDA) Assay Kit (abcam, USA) and GST Assay Kit (abcam, USA), respectively (Gקrny *et al*., 2020).

Statistical analysis

The experiments were conducted three times to enhance the dependability of the findings. The data underwent analysis using one-way ANOVA through SPSS software version 26.0 to compare the groups under examination. A significance level of $P<0.05$ was deemed as indicative of a meaningful difference.

RESULTS

Charactrization of the synthesized Lin-ZNP

In the current study, the SEM analysis of the synthetic nanocomposite revealed that ZNP had a spherical shape with a solid appearance (Figure 1A), while the addition of Lin to ZNP resulted in a reduction of this characteristic (Figure 1B). EDX analysis revealed a remarkable elevation in the concentration of organic elements, especially carbon, in the Lin@ZNP nanocomposite (Figure 1C). The diffraction peaks depicted in Figure 2 were identified as the hexagonal phase (Wurtzite) of ZnO, indicating the preservation of the nanocomposite's crystalline structure. The average size of ZNP was determined to be 52.5 nm, while the size of Lin@ZNP was 105 nm. Additionally, the

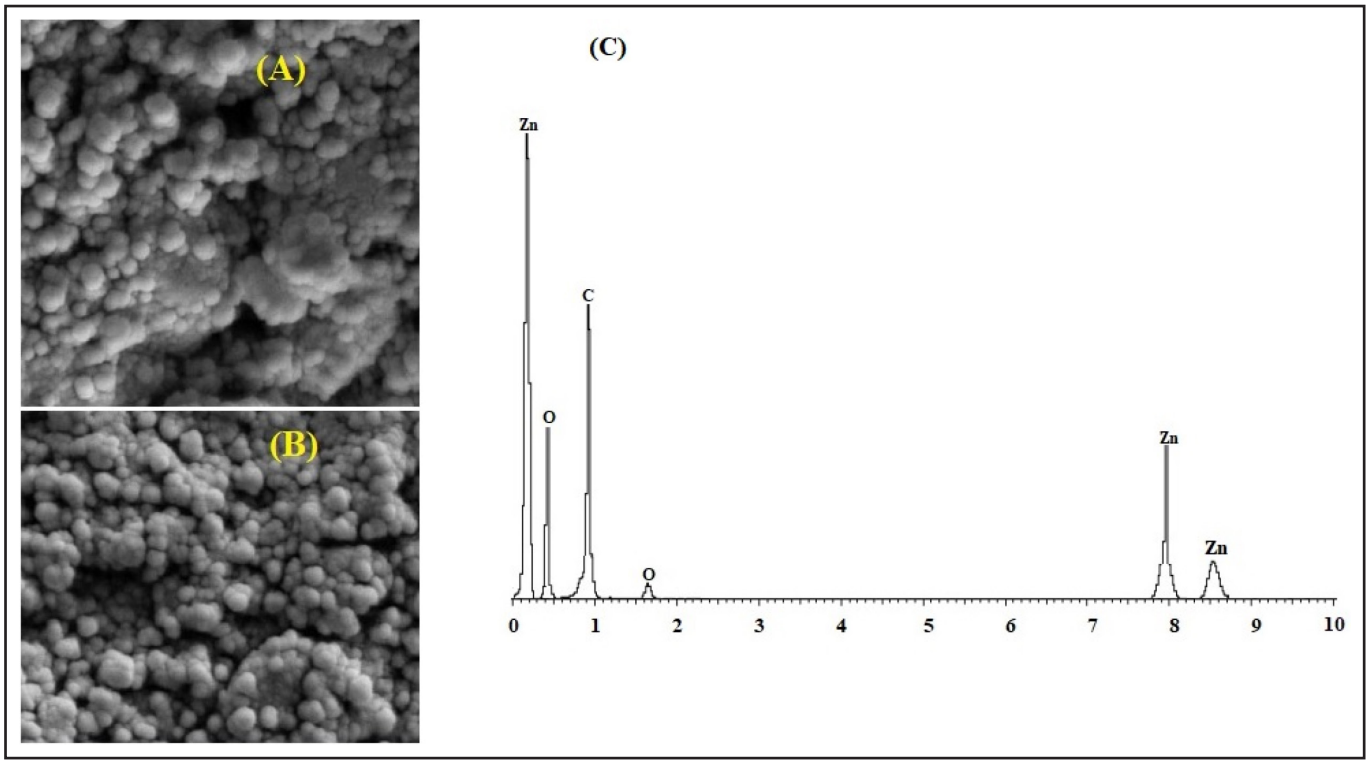

Figure 1. scanning electron microscope of zinc oxide nanoparticles (A) and linalool-zinc oxide nanoparticles (B, Lin-ZNP) as well as energy dispersive X-ray (C, EDX) analysis of Lin-ZNP.

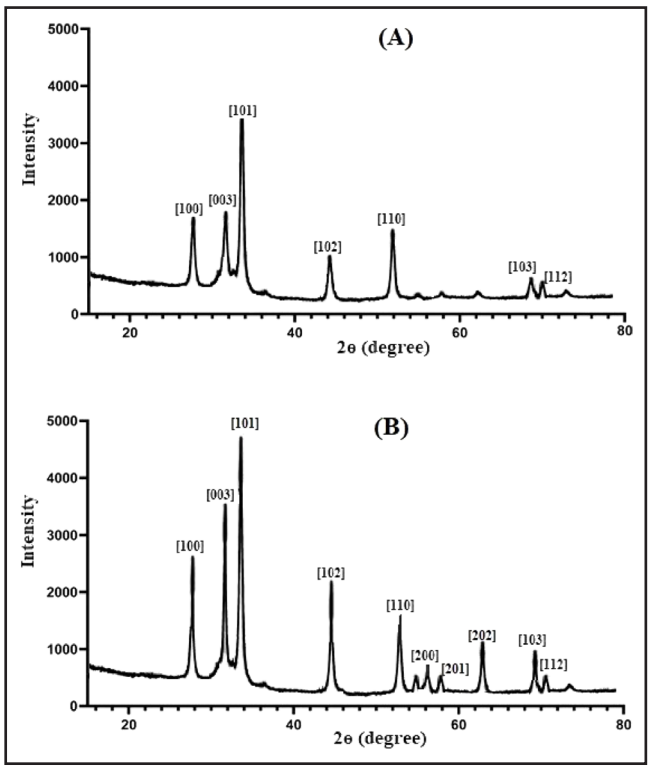

Figure 2. The X-ray diffraction analysis of the zinc oxide nanoparticles (A) and linalool-zinc oxide nanoparticles (B).

zeta potentials of ZNP and Lin@ZNP were measured to be -18.4 mV and 28.3 mV, respectively, as shown in Figure 3. As depicted in Figure 4A, the presence of absorption bands at 3312, 1523, and 712 cm^{-1} in the FTIR, are connected with the stretching of O-H bands, carbonyl group (C=O), and the existence of Zn ions, respectively. By release kinetics, we found that the release rate of ZNP and Lin was dose and time-dependently increased, peaking after 60 minutes and then stabilizing (Figure 4C).

Acaricidal effects

The results of the AIT demonstrated a significant reduction in the viability of *H. anatolicum* adults (Figure 5A) when exposed to Lin@ZNP in a manner that was dependent on the concentration (P<0.001), with the calculated LC50 and LC90 values of 89.6 and 161.3 µg/mL, respectively. Following exposure to various concentrations of Lin@ZNP, the average number, weight, and hatchability of eggs of adult *H. anatolicum* also exhibited a dose-dependent decrease (P<0.05) compared to the control group (Figure 5B-D).

Larvicidal effects

The mortality rate of larvae significantly rose (P<0.001) following exposure to varying concentrations of Lin@ZNP. All larvae exposed to Lin@ZNP at concentrations of 124 and 248 µg/mL were perished. The calculated LC50 and LC90 values for Lin@ZNP were 37.6 and 67.7 µg/mL, respectively (Figure 6).

Repellent activity

The synthesized Lin@ZNP demonstrated significant repellent effects on *H. anatolicum* larvae, with the most pronounced activity observed at concentrations of 64, 128, and 248 µg/mL, with complete repellent activity was achieved after 60, 120, and 180 minutes of exposure, respectively. These findings were statistically significant (P<0.01) and are illustrated in Figure 7.

Anti-AChE activity

According to the findings, the Lin@ZNP nanocomposite exhibited significant (p<0.001) inhibition of AChE activity in the larvae stage of *H. anatolicum* at all concentrations, as depicted in Figure 8, when compared to the control group.

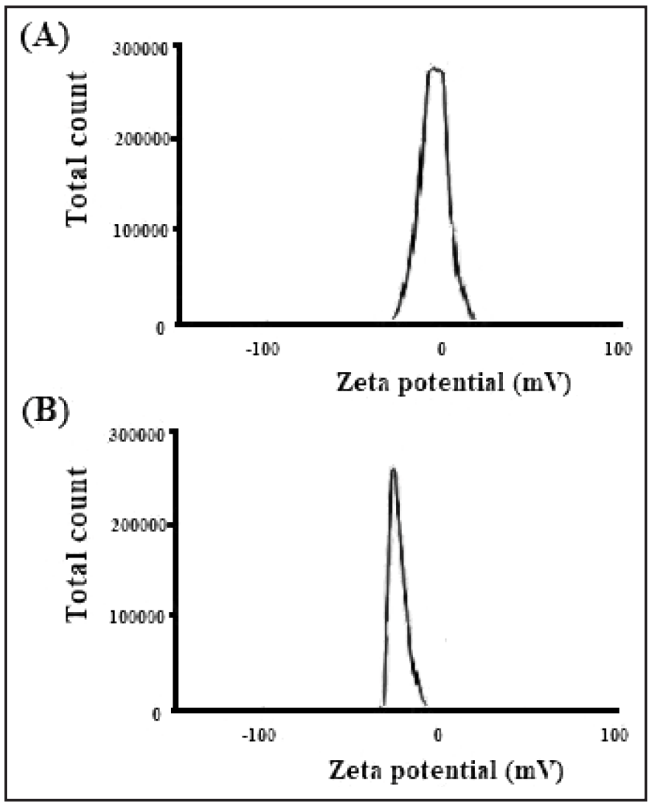

Figure 3. The results of the zeta potential of the zinc oxide nanoparticles (A) and linalool-zinc oxide nanoparticles (B).

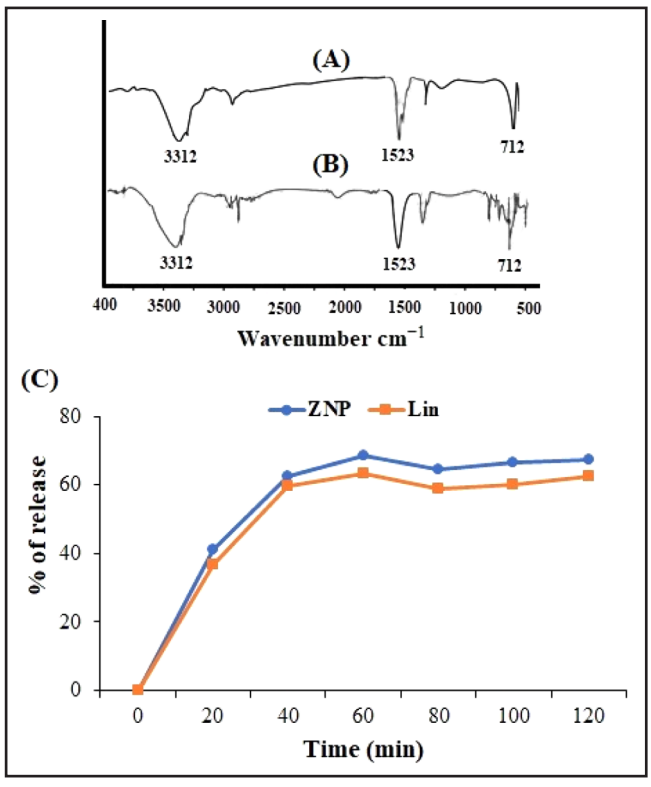

Figure 4. The Fourier transform infrared spectroscopy of the zinc oxide nanoparticles (A, ZN) and linalool-zinc oxide nanoparticles (B) and the release kinetics of linalool and ZN (C).

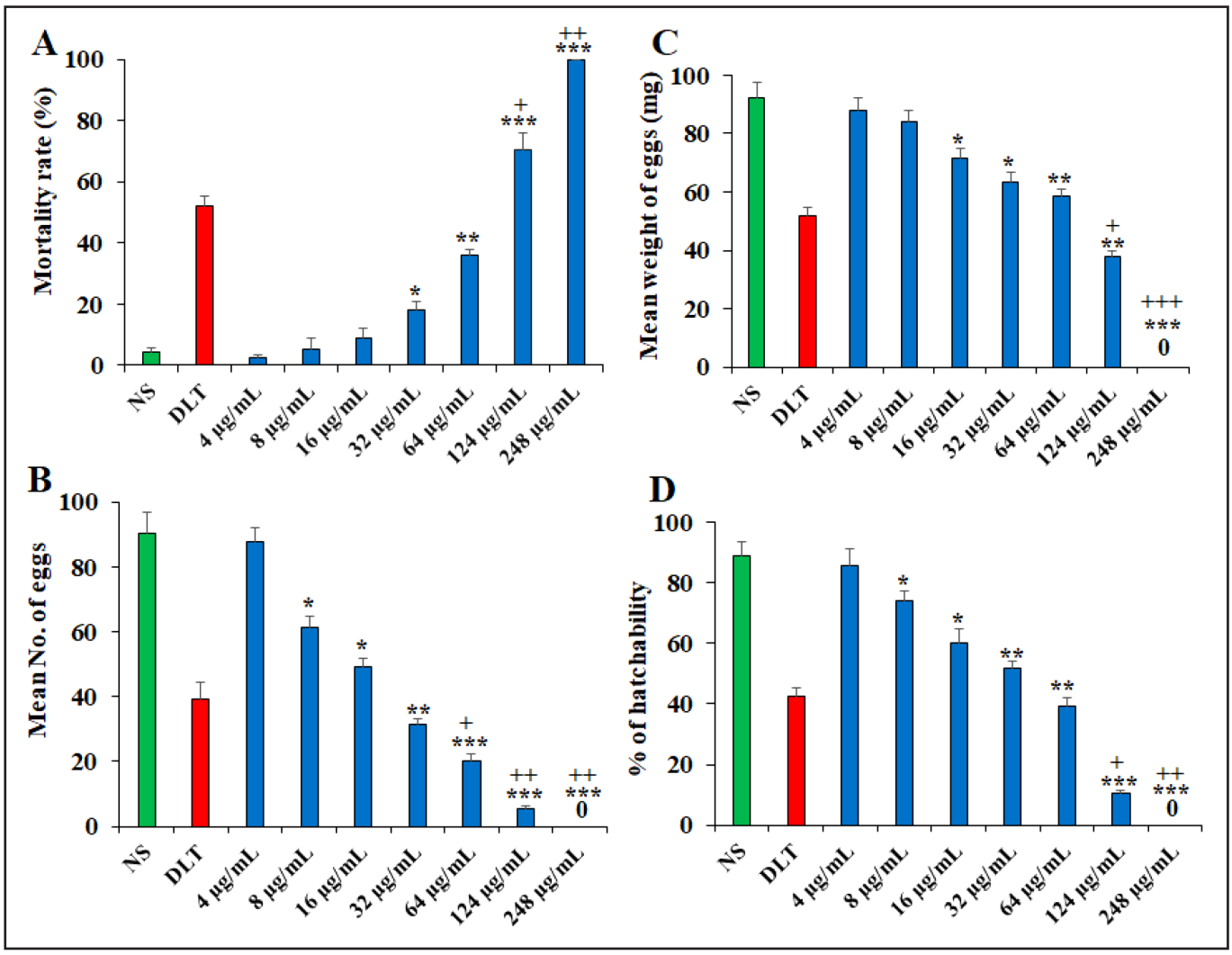

Figure 5. The acaricidal impact of Linalool loaded zinc oxide nanoparticles (Lin@ZNP) on the mortality rate of adult female *Hyalomma anatolicum* ticks (A), as well as the quantity (B), weight (C), and hatchability (D) of eggs (n = 3). * p<0.05, ** p<0.001, *** p<0.001 compared with control normal saline (NA) group. + p<0.05,++ p<0.01, and +++ p<0.001 compared to deltamethrin (DLT).

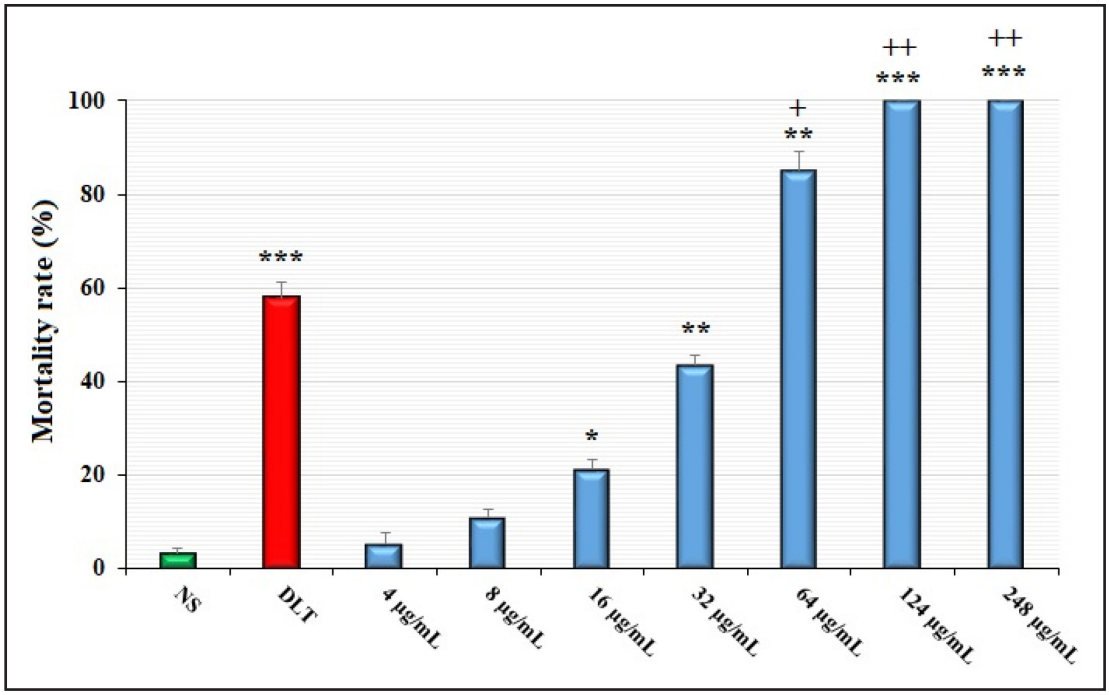

Figure 6. Larvicidal effects of Linalool loaded zinc oxide nanoparticles (Lin@ZNP) on the mortality rate of on *Hyalomma anatolicum* larvae (n = 10). * p<0.05, ** p<0.001, *** p<0.001 compared with control normal saline (NS) group. ++ p<0.01 compared to deltamethrin (DLT).

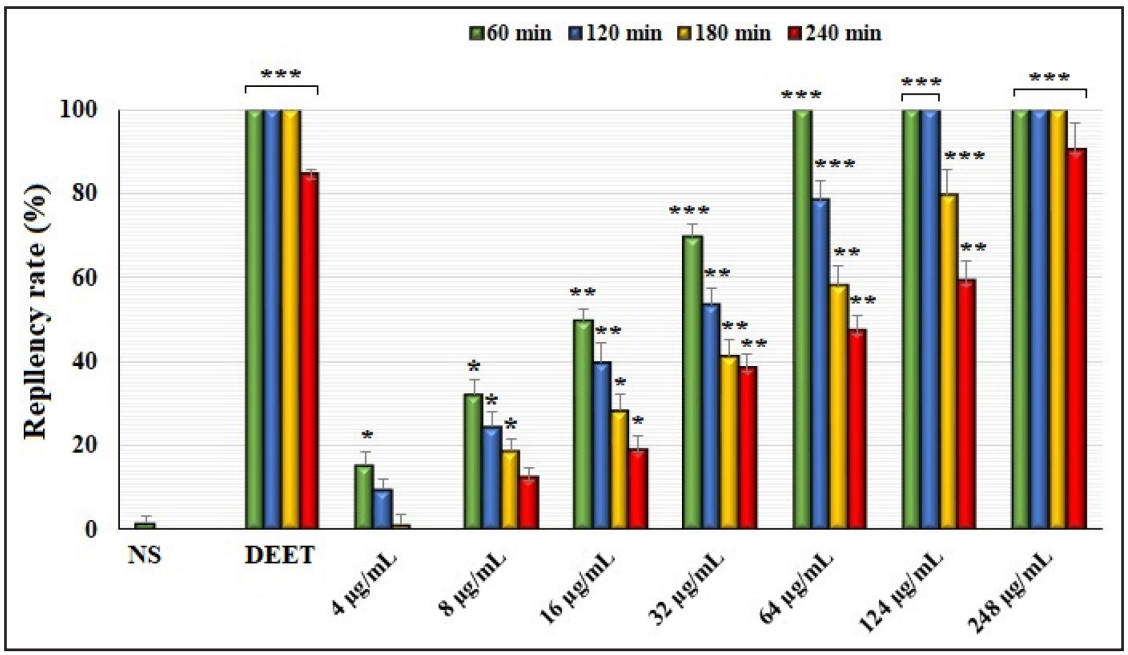

Figure 7. Repellent activity of Linalool loaded zinc oxide nanoparticles (Lin@ZNP) on the mortality rate of on *Hyalomma anatolicum* larvae (n = 10). * p<0.05, ** p<0.001, *** p<0.001 compared with control normal saline (NS) group. DEET: N,N-diethyl- 3-methyl benzamide.

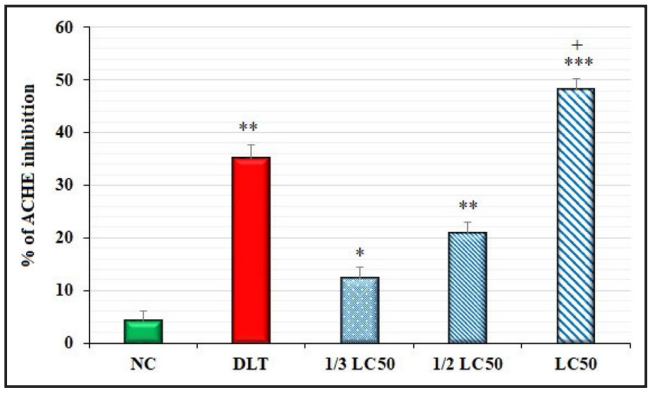

Figure 8. The effects of Linalool loaded zinc oxide nanoparticles (Lin@ZNP) on the acetylcholinesterase (AChE) activity of *Hyalomma anatolicum* larvae (n = 3). * p<0.05, ** p<0.001, *** p<0.001 compared with control normal saline (NC) group. + p<0.05 compared to deltamethrin (DLT). The lethal concentration 50 (LC_{50}) and LC_{90} values of Lin@ZNP were determined using the Probit test.

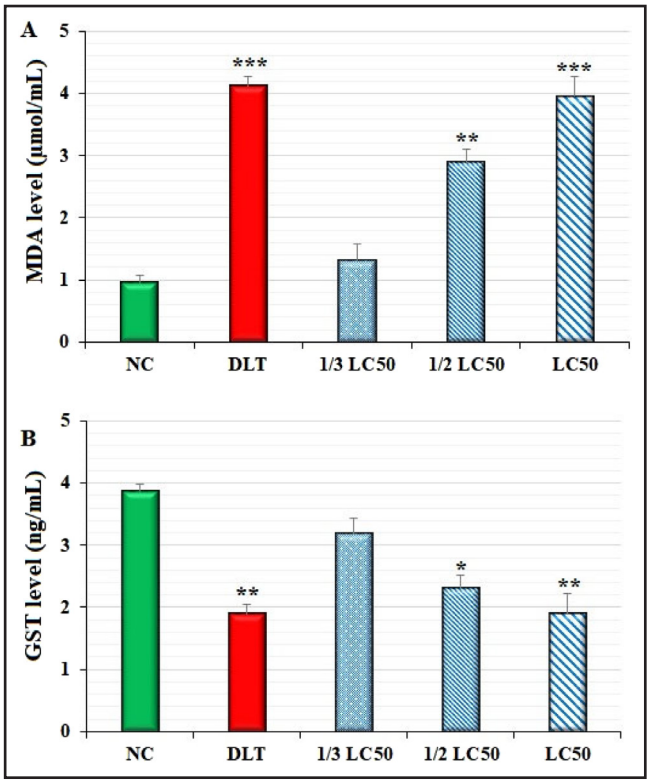

Figure 9. Effect of the Linalool loaded zinc oxide nanoparticles (Lin@ZNP) on (A) malondialdehyde (MDA) and (B) glutathione S-transferase (GST) levels in *Hyalomma anatolicum* larvae (n = 3). MDA: malondialdehyde; GST: glutathione S-transferase. *P<0.05, ** p<0.01, *** p<0.001 compared with the normal saline (NC).

Oxidant/antioxidant enzyme activity

The Lin@ZNP at ½ LC50 and LC50 resulted in significant increase in MDA levels (P<0.001) and a decrease in GST levels in *H. anatolicum* larvae (P<0.001) (Figure 9). Conversely, exposure to Lin@ZNP at 1/3 LC50 and 1/2 LC50 did not lead to any notable alterations in MDA and GST levels.

DISCUSSION

One of the important tools for measuring the shape and size of produced nanoparticles is SEM analysis, which are crucial parameters affecting their manufacturing conditions. Investigations have revealed that the stability and biological functions of nanoparticles are influenced by their size, with smaller nanoparticles typically displaying enhanced solidity and decreased aggregation (Xu *et al*., 2023). Previous studies indicated that the hydrodynamic dimensions of nanocomposites experience a notable augmentation in aqueous environments (Gupta *et al*., 2019). On the contrary, the application of particular molecules to envelop nanoparticles may lead to an increase in their dimensions, yet this coating has the potential to improve the biocompatibility and biological characteristics of the nanoparticles (Gupta *et al*., 2019). It has been proven that the surface electric charge of nanoparticles is considered as a main factor in studying

their stability, compatibility with biological systems, and catalytic activity (Mir *et al*., 2017). Bearing in mind that biogenic synthesis can elevate the ZP of nanoparticles, resulting in enhanced stability and improved control over their aggregation and agglomeration (Rivera-Rangel *et al*., 2018). Researchs also reported that some factors, e.g., size, shape, electrochemical conditions, hydrophobic interactions, and stability under different pH, temperature, and osmolarity, are associated to affect the kinetics release of nanoparticles (Herdiana *et al*., 2021). On the other hand, some investigations have revealed that degradation due to enlargement can also influence the drug release kinetics in nanoscale agents (Rakhshaei *et al*., 2019).

We found that Lin@ZNP displayed potent acaricidal, larvicidal and replent activity against *H. anatolicum*. Zaheer *et al*. (2021) demonstrated that ZNPs produced from *Azadirachta indica* extract and lemon grass displayed potent acaricidal and larvicidal effects against some *Hyalomma* spp., ticks with the LC_{50} values of 4.76-4.92 mg/L and LC_{90} values of 8.87-9.1 mg/L, respectively (Zaheer *et al*., 2021). In a study conducted by Norouzi *et al*. (2019) demonstrated that ZNPs indicated the *in vitro* acaricidal effects against *Hyalomma* spp., with the LC_{50} value of 50 mg/ml in 60 min and LC_{50} of 150 mg/ml in 30 min, respectively. Abdel-Ghany *et al*. (2022) also showed that ZNPs produced using *Melia azedarach* extract were effective against the larvae, eggs, and nymphs of *H. dromedarii* tick with the LC_{50} of 8.03, 11.6, and 3.9 mg/mL, respectively. The alteration in effects of ZNPs may be assigned to issues such as tick strain, production technique of ZNPs, assessment methodology, and concentrations used (Benelli, 2018). Detailed knowledge about the mechanisms through which nanoparticles act against insects is currently lacking. Previous studies reported that the mechanisms of action of certain nanoparticles against insects are affecting antioxidant and detoxifying enzymes, increasing the oxidative stress, increasing reactive oxygen species, lysosomal instability, increasing the membrane permeability, and DNA damage (Benelli *et al*., 2017).

Lin@ZNP nanocomposite exhibited significant ($p<0.001$) inhibition of AChE activity in the larvae stage of *H. anatolicum*. The acaricidal properties of a substance primarily involve its ability to inhibit aAChE, leading to a reduction in acetylcholine, a key neurotransmitter in the central nervous system of ticks (Mladenoviт *et al*., 2018). While the precise mechanisms by which AChE exerts its acaricidal effects are not fully elucidated, it is known that AChE inhibitors disrupt the function of autonomic ganglia and neuromuscular structures, which are essential effectors modulated by ACh (Mladenoviт *et al*., 2018). Consequently, the identification of novel acaricidal agents, particularly those with ACh-inhibitory activity, is imperative for tick control.

We found that Lin@ZNP significantly reduced antioxidant enzymes activity and increased the oxidative stress in ticks. Repeatedly, maintaining a balance between ROS production (oxidative stress) and its neutralization (antioxidant) is crucial for the survival and resilience of ticks, as it mitigates the effects of acaricidal agents. Consequently, a potent acaricidal agent that disrupts this equilibrium by reducing antioxidant activity and elevating oxidative stress can inflict severe damage on the vital physiological processes of ticks (Hernɔndez-Cruz *et al*., 2022).

CONCLUSION

The findings indicated that the Lin@ZNP exhibited considerable acaricidal, larvicidal, and repellent effects against *H. anatolicum* adults and larvae in a manner dependent on the dosage. Additionally, Lin@ZNP notably reduced AChE levels and antioxidant activity, while inducing oxidative stress in *H. anatolicum* larvae. Nevertheless, further research is necessary to elucidate the precise mechanisms and practical efficacy of Lin@ZNP.

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