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Green synthesized zinc oxide nanoparticles using neem leaf extract and their *in vitro* acaricidal activity against *Rhipicephalus (Boophilus) microplus*

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Abstract

Neem leaf extract-mediated green-synthesised zinc oxide nanoparticles (ZnO NPs) exhibited promising *in vitro* acaricidal efficacy against *Rhipicephalus* (*Boophilus*) *microplus*. The synthesized nanoparticles demonstrated dose-dependent tick mortality and significant inhibition of oviposition. UV-visible spectroscopic analysis showed a characteristic absorption peak at 370 nm, confirming the formation of ZnO nanoparticles. The surface morphology and elemental composition of the synthesized nanoparticles were characterized using scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX). SEM analysis revealed that the nanoparticles were predominantly spherical in shape, with particle sizes ranging from 53 to 92 nm. The EDX spectrum showed strong signals corresponding to zinc and oxygen, confirming the elemental composition of the synthesized nanoparticles. Elemental analysis indicated the presence of 81.58% zinc and 17.45% oxygen by weight. The calculated an LD_{50} value of green synthesized ZnO nanoparticles ranged from 4.37-6.73 mg/ml in various agro-climatic zone of Gujarat.

1. Introduction

Ticks are the most significant ectoparasites affecting livestock worldwide. They are primary arthropod vectors responsible for transmitting bacteria, viruses, protozoa, rickettsia to animals, and second only to mosquitoes as vectors of human pathogens (Zhou *et al*., 2009). In India, ticks act as major vectors for the transmission of economically important haemoprotozoan diseases such as babesiosis caused by *Babesia bovis* and *Babesia bigemina*, and anaplasmosis caused by *Anaplasma marginale* in cattle and buffaloes (Ghosh *et al*., 2006; Ghosh and Nagar, 2014; Kumar *et al*., 2019). Among different tick species, *R.* (*B.*) *microplus* is considered one of the most economically important cattle ticks in tropical and subtropical regions.

Ticks and tick-borne diseases (TTBDs) represent a major constraint to the growth and sustainability of the dairy industry (FAO, 1996). Besides transmitting pathogens, heavy tick infestation causes substantial economic losses due to blood loss, irritation, stress, reduced productivity, suppression of immune responses, damage to hides and skins, and decreased milk and meat production (Brossard and Wikel, 1997; Jongejan and Uilenberg, 2004). Globally, economic losses from tick infestations are estimated at US$ 14-18 billion annually, while the annual economic burden of tick-borne diseases (TTBDs) in cattle in India has been estimated at approximately US$ 498.7 million (Minjauw and McLeod, 2003).

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Chemical acaricides have traditionally been the primary approach for the management of tick infestations. However, the indiscriminate and prolonged application of these chemicals has contributed to the emergence of acaricide-resistant tick populations (Rodríguez-Vivas *et al*., 2006; Patel *et al*., 2019). In addition, the persistent use of synthetic acaricides has raised several concerns, including environmental pollution, accumulation of toxic residues in milk and meat, higher treatment expenses, and harmful effects on non-target organisms (Sonenshine *et al*., 2002). Resistance to widely used acaricides such as organophosphates (Ortiz-Estrada *et al*., 1995; Baxter *et al*., 1999), synthetic pyrethroids (Arantes *et al*., 1996; Davey and George, 1998; Davey *et al*., 2006; Mathivathani *et al*., 2011; Patel *et al*., 2020), and macrocyclic lactones (Benavides *et al*., 2000; Martins and Furlong, 2001; Miller *et al*., 2002) has been extensively documented in several countries, including India.

The growing problem of acaricide resistance has created an urgent need for alternative and eco-friendly tick control strategies. In recent years, ethnoveterinary medicines and nanoparticle-based approaches have attracted considerable attention as promising alternatives to conventional acaricides (Ymeti *et al*., 2007; Heng *et al*., 2013; Salam *et al*., 2012; Benelli and Lukehart, 2017). Although, plant-based ethnoveterinary practices possess acaricidal potential, their practical application is often limited due to lack of scientific validation, variability in active constituents, absence of standardized formulations, and safety concerns (Heng *et al*., 2013; Kim *et al*., 2007).

Nanotechnology has emerged as a promising field in veterinary medicine for disease diagnosis, treatment, and parasite control. Nanoparticles exhibit unique physicochemical properties such as high surface area, enhanced penetration ability, targeted delivery, improved stability, and reduced likelihood of resistance development (Nagajyothi *et al*., 2013; Benelli and Lukehart, 2017). Metallic nanoparticles such as silver, copper, nickel, titanium dioxide and zinc oxide nanoparticles have shown effective activity against a wide range of arthropod pests and parasites (Benelli and Lukehart, 2017). Among these, zinc oxide nanoparticles (ZnO NPs) have gained considerable scientific interest due to their strong antimicrobial and acaricidal properties. ZnO nanoparticles induce toxic effects by rapidly penetrating the tick body, generating oxygen species, inducing oxidative stress, disruption of cellular functions, and ultimately leading to cell death (Manna *et al*., 2013). Therefore, ZnO nanoparticles are being explored as potential alternative acaricidal agents against *R*. (*B*.) *microplus.*

Conventionally, nanoparticles are synthesized using physical and chemical methods that require expensive equipment, high energy input, elevated temperature and pressure conditions, and hazardous chemicals. These methods may also produce toxic by-products that can adversely affect the environment and human health. To address these limitations, green synthesis approaches have received growing attention in recent years. Green synthesis employs biological resources such as plant extracts, microorganisms, and biomolecules as reducing and stabilizing agents, thereby offering a cost-effective, environmentally friendly, and sustainable method for nanoparticle synthesis (Rosi *et al*., 2005; Elumalai and Velmurugan, 2015).

Among various medicinal plants, neem (*Azadirachta indica*) is well known for its antimicrobial, antiparasitic, insecticidal, and therapeutic properties, which are mainly attributed to bioactive compounds such as azadirachtin, nimbin, and salannin. Neem leaf extract can act as both a reducing and capping agent during nanoparticle synthesis, thereby facilitating the eco-friendly production of ZnO nanoparticles. Moreover, the synergistic action of neem phytochemicals and ZnO nanoparticles may enhance acaricidal efficacy against resistant tick populations.

Considering the increasing resistance of *R*. (*B*.) *microplus* to conventional acaricides and the need for sustainable tick control measures, the present study was undertaken to evaluate the *in vitro* acaricidal activity of green synthesized zinc oxide nanoparticles prepared using neem leaf extract against cattle ticks. The findings of this study may contribute to the future development of an effective, environmentally safe, and sustainable alternative strategy for tick management in livestock.

2. Materials and Methods

2.1 Plant authentication and ethical approval

Fresh leaves of *A. indica* were harvested from the campus of Navsari Agricultural University, India. Botanical identification and authentication of the plant material were carried out by Dr. B. K. Patel at the Pulses and Castor Research Station, Navsari Agricultural University, Navsari. A voucher specimen designated as *A. indica* (02) was prepared and deposited for record maintenance. The authenticated sample has been preserved in the Herbarium of the Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari. Approval to conduct the experimental work was granted by the Institutional Animal Ethics Committee (IAEC) of the same institution under Registration No. 1631/GO/Re-S/ReBi-L/12/CCSEA dated 24/12/2021, vide Project No. 135-VCN-VPA-2023.

2.2 Study area and sample collection

Adult ixodid ticks were obtained from cattle maintained under organized as well as unorganised farming systems in various agro-climatic regions of Gujarat. Tick collection was carried out using a random sampling approach, in which specimens were collected directly from cattle at the livestock owners' premises. Three representative clusters from each agro-climatic zone were included in the study, and nearly 100 fully engorged ticks were sampled for each bioassay. All experiments were conducted in triplicate to maintain consistency and reproducibility of the observations. Engorged female ticks were mainly recovered from cracks and crevices present within animal sheds. The collected specimens were transferred into individually labelled plastic containers fitted with perforated caps to allow adequate aeration during transportation. Subsequently, the samples were transported to the laboratory of the Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Navsari, for detailed investigation. Species identification of the ticks was performed according to standard morphological identification keys described by Walker *et al*. (2003).

2.3 Synthesis of zinc oxide nanoparticles

Zinc oxide nanoparticles were synthesized through a green synthesis technique employing leaf extract of *A. indica* by adopting a modified protocol described by Elumalai and Velmurugan (2015). In this environmentally sustainable approach, the bioactive phytoconstituents present in the plant extract function as natural reducing, stabilizing, and capping agents during nanoparticle formation, thereby eliminating the requirement for hazardous chemical reagents.

2.3.1 Neem leaf extract preparation

Fresh and disease-free leaves of *A. indica* were collected and cleaned thoroughly using running tap water followed by double-distilled water to eliminate adhering contaminants. The washed leaves were then shade-dried at room temperature for a period of one week prior to further processing. The dried leaves were powdered, and 20 g of leaf powder was mixed with 100 ml of double-distilled water in a conical flask. The mixture was heated at 60°C for 20 min with continuous stirring to extract bioactive compounds. After cooling, the extract was filtered using Whatman No. 1 filter paper (Figure 1). The filtrate was stored at 4°C and used as a reducing and stabilizing agent for nanoparticle synthesis.

2.3.2 Green synthesis of ZnO nanoparticles

For synthesis of ZnO nanoparticles, 5 g of zinc acetate dihydrate procured from Sigma-Aldrich was dissolved in 100 ml of double-distilled water under constant magnetic stirring. After continuous stirring for approximately 10 min, 20 ml of prepared *A. indica* leaf extract was added slowly in a dropwise manner to the reaction mixture. The pH of the solution was subsequently adjusted to 12 using 1 M sodium hydroxide, which led to the appearance of a pale white precipitate, indicating the formation of nanoparticles. The reaction mixture was further stirred for 2-3 h followed by centrifugation at 2000 rpm for 10 min. The resulting precipitate was repeatedly washed with distilled water and ethanol to remove impurities and unreacted residues. Thereafter, the purified material was dried at 60-80°C and subjected to calcination at 400°C for 2 h in a muffle furnace to obtain crystalline ZnO nanoparticles. Finally, the light yellowish-grey nanoparticle powder obtained was collected and preserved in airtight containers for subsequent experimental applications.

Figure 1: Preparation of aqueous neem leaf extract.

2.4 Characterization of green-synthesized ZnO nanoparticles

The synthesized ZnO nanoparticles were characterized to confirm their formation, structural properties, and elemental composition.

2.4.1 UV-Visible spectroscope

The nanoparticle suspension was scanned within the wavelength range of 200-800 nm. (Shimadzu UV-1900, Japan). The characteristic absorption peak observed between 350 and 380 nm indicated the formation of ZnO nanoparticles due to surface plasmon resonance.

2.4.2 Scanning electron microscopy (SEM)

Surface morphology (image), particle size and shape of particle of the synthesized nanoparticles were analysed using scanning electron microscopy (Zeiss EVO-18, Germany).

2.4.3 Energy dispersive X-ray analysis (EDX)

The elemental composition and purity of the nanoparticles were confirmed using EDX analysis (Ametek, Germany).

2.5 Bioassay test

Various concentrations of green-synthesized ZnO nanoparticles were prepared by dispersing accurately weighed quantities of fine product in distilled water. Suspensions were prepared at different double-strength (2X) concentrations of 2, 4, 8, 16 and 32 mg/ml. To obtain a homogenous dispersion and minimize nanoparticle aggregation, the prepared suspensions were first subjected to continuous magnetic stirring and subsequently ultrasonicated for approximately 15-20 min before further use. Where necessary, a minimal amount of dispersing agent was used to enhance stability. The prepared suspensions were freshly made before each experiment and used for evaluating acaricidal activity.

2.5.1 Adult immersion test (AIT)

The adult immersion test (AIT) was performed following the method described by Drummond *et al*. (1973) with slight modifications. Fresh fully engorged female ticks were used in this test. Ten randomly selected engorged ticks were used for each treatment group. Ticks of each treatment group were weighed before conducting AIT. Ticks were immersed in different concentration like 2, 4, 8, 16, and 32 mg/ml for 2 min. Same batch of ticks were treated in distilled water for 2 min as a control. Following treatment, the ticks were placed in petri dishes lined with Whatman No. 1 filter paper and maintained at room temperature for 24 h. Subsequently, the treated ticks were transferred into glass beakers covered with muslin cloth and incubated inside desiccators at 28°C with 85% relative humidity in a B.O.D. incubator. Mortality observations were recorded on the 14th day post-treatment (DPT), and the egg masses laid by surviving ticks were weighed accordingly. Tick mortality was confirmed based on the absence of motility and loss of pedal reflex upon exposure to light. The resistance status was evaluated by using following formula:

Mortality %

$$(Nt / Nw) \times 100$$

Nt = No. of dead ticks on 14th dpt,

Nw = No. of treated ticks at the start of experiment

Reproductive index

$$RI = Ew / EFw$$

Ew = Egg mass weight at 14th dpt,

EFw = Engorged female weight before treatment

Inhibition of oviposition

$$IO\ \% = (RIc - RIt) / RIc \times 100$$

RIc = Reproductive Index of control group,

RIt = Reproductive Index of treated group

2.6 Statistical analysis

Mortality percentage regression curve was plotted against working concentrations of the green-synthesized ZnO nanoparticles in microsoft office excel (Finney, 1952). The slope of regression curve and the repeatability (R^2) was noted. The median lethal concentration 50% (LC_{50}) and 95% (LC_{95}) value was calculated from following equation.

Regression equation:

Y = mx + C

Y = Mortality percentage value,

m = Slope of the curve,

C = y Intercept (correlation coefficient),

x = Concentration of ZnO nanoparticle

All experimental data were expressed as mean ± standard error (SE). Statistical evaluation of the results was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test with the help of IBM SPSS Statistics 20.00 software. Differences were considered highly significant at $p<0.01$ and significant at $p<0.05$.

3. Results

A total of 1,795 cattle were examined for tick infestation across different locations representing various agro-climatic zones of Gujarat. The collected tick specimens were identified as *R.* (*B.*) *microplus* under a stereomicroscope using standard taxonomic identification keys described by Walker *et al.* (2003). *R.* (*B.*) *microplus* had brevirostrate mouth part, oval to elongated body, hexagonal basis capitulum, bifid coxa, absence of festoons and presence of caudal process at the middle of posterior margin (Figure 2).

Dorsal view

Ventral view

Figure 2: Adult engorged female *Rhipicephalus (Boophilus) microplus.*

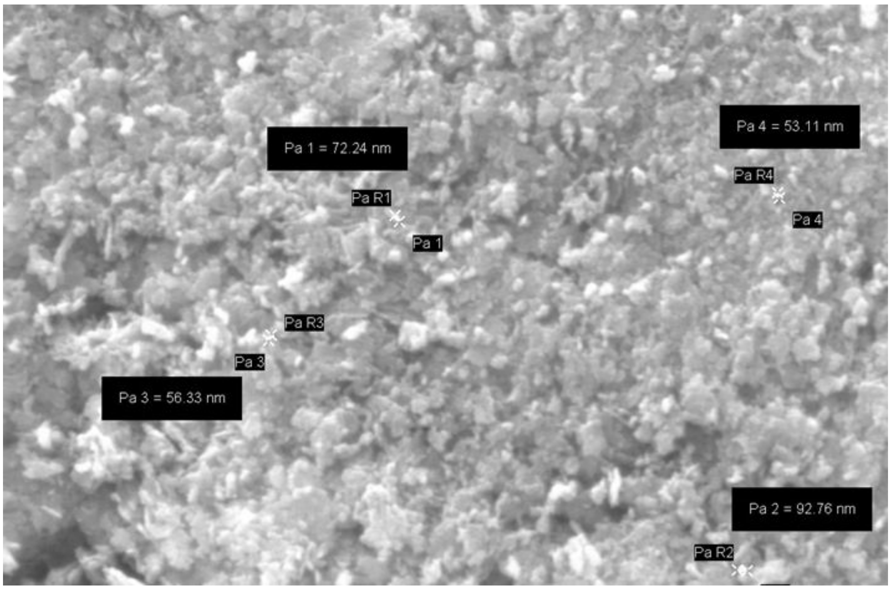


Figure 3: Topography of green synthesized ZnO by using SEM.

3.1 Synthesis of zinc oxide nanoparticles and their characterization

The pale white precipitate formed during the synthesis process was dried in a hot air oven to obtain ZnO nanopowder. The dried nanopowder was subsequently resuspended in deionized water, and characterization by UV-Visible spectroscopy.

3.1.1 UV-Visible spectroscopy

UV-Visible spectroscopy is considered one of the most commonly employed techniques for the structural and optical characterization of synthesized nanoparticles. The optical behaviour of the synthesized ZnO nanoparticles was evaluated using UV-Visible spectroscopic analysis. A prominent absorption peak detected at 370 nm confirmed the successful formation of ZnO nanoparticles.

3.1.2 Scanning electron microscopy (SEM) analysis

Scanning electron microscopy (SEM) is a sophisticated analytical technique that uses a high-energy electron beam to obtain high-resolution images of samples. The interaction between the electron beam and the sample surface provides valuable information regarding surface morphology, elemental composition, and other physical characteristics, including electrical conductivity, as reported by Reimer, 2000. In the present investigation, the morphological characteristics of the synthesized zinc oxide nanoparticles were examined using SEM in combination with energy-dispersive X-ray spectroscopy (EDX). The SEM micrographs demonstrated that the nanoparticles were mainly spherical in morphology, with particle sizes varying between 53 and 92 nm (Figure 3). In addition, the synthesized nanoparticles exhibited a tendency to aggregate, resulting in the formation of comparatively larger clusters.

3.1.3 Energy dispersive X-ray (EDX) analysis

The formation of ZnO nanoparticles was further confirmed by EDX analysis. The EDX spectrum (Figure 4) showed strong peaks corresponding to zinc and oxygen, confirming the elemental composition of nanoparticles. The analysis revealed 81.58% zinc and 17.45% oxygen by weight.

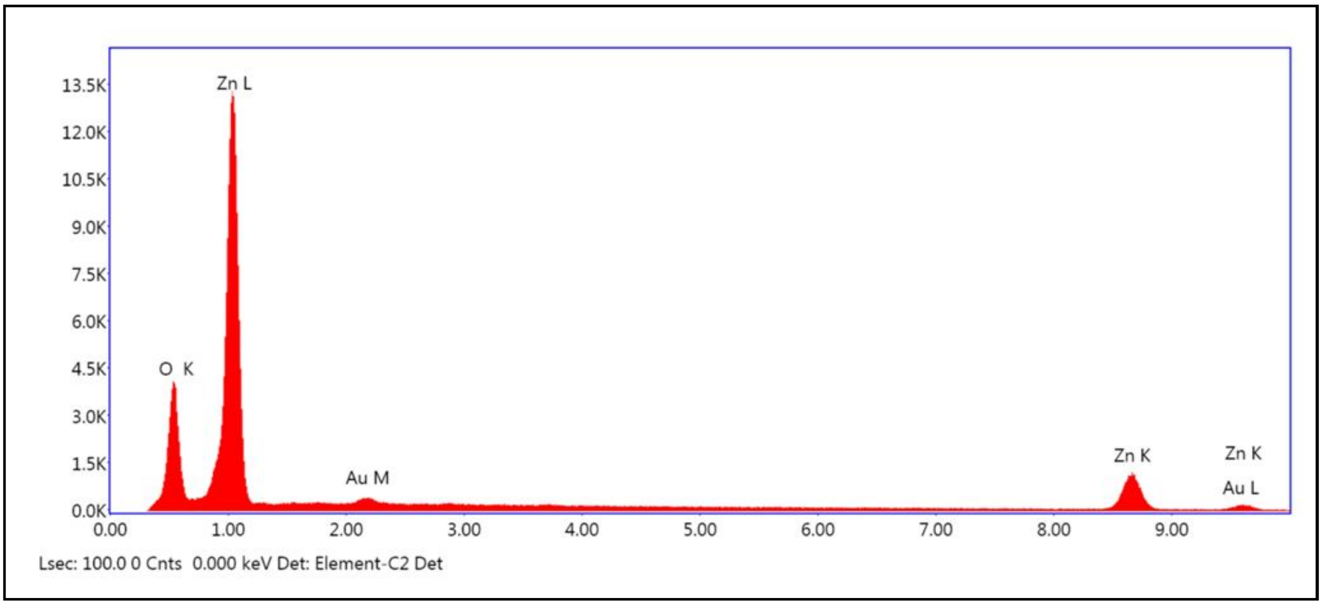


Figure 4: Strong peaks corresponding to zinc and oxygen element by using EDX analysis.

3.2 Adult immersion test (AIT) with green synthesized ZnO

The *in vitro* acaricidal efficacy of green synthesized zinc oxide nanoparticles against *R.* (*B.*) *microplus* was evaluated across seven agro-climatic zones of Gujarat by assessing reproductive index (RI), oviposition inhibition (IO%), slope of mortality curve and median lethal dose (LD_{50}) (Table 1). In zone-1 (Southern Hill), RI decreased from 0.18 ± 0.04 to 0.00 ± 0.00, while IO% increased from 66.14 ± 8.05 to 100.00 ± 0.00, with a mortality curve slope of 5.33 and an LD_{50} of 4.37 mg/ml. Similarly, zone-2 (Southern Gujarat) showed a reduction in RI from 0.45 ± 0.03 (control) to 0.29 ± 0.07 - 0.00 ± 0.00, with IO% ranging from 37.19 ± 12.20 to 100.00 ± 0.00, a slope of 2.48, and an LD_{50} of 6.63 mg/ml (Table 1, Figure 5). In zone-3 (Middle Gujarat), RI declined from 0.55 ± 0.09 to 0.28 ± 0.04 - 0.01 ± 0.01, accompanied by an IO% increase from 48.62 ± 2.57 to 99.00 ± 1.00, a slope of 5.29, and an LD_{50} of 4.37 mg/ml. Zone-4 (North Gujarat) recorded RI values ranging from 0.55 ± 0.10 (control) to 0.35 ± 0.14 - 0.02 ± 0.02, with IO% between 41.55 ± 9.51 and 96.99 ± 3.10, a slope of 4.708, and an LD_{50} of 5.58 mg/ml (Table 1, Figure 5).

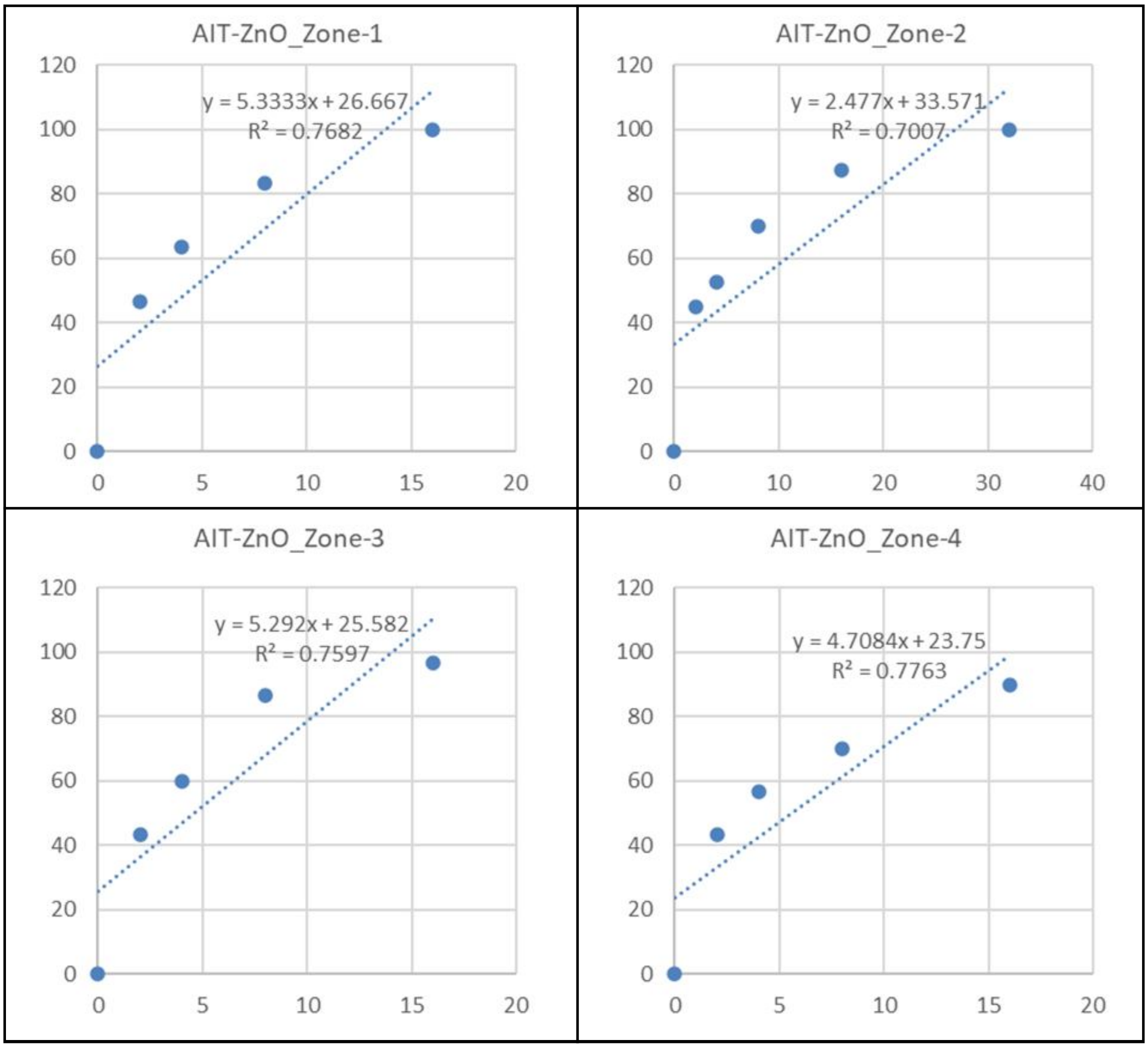
In zone-5 (North-West Arid), RI decreased from 0.55 ± 0.03 to 0.26 ± 0.09 - 0.00 ± 0.00, while IO% increased from 53.89 ± 5.34 to 100.00 ± 0.00, with a slope of 2.344 and an LD_{50} of 5.12 mg/ml. Zone-6 (North Saurashtra) exhibited RI values from 0.62 ± 0.06 to 0.53 ± 0.04 - 0.00 ± 0.00, with IO% ranging from 12.94 ± 9.03 to 100.00 ± 0.00, a slope of 4.375, and an LD_{50} of 6.00 mg/ml (Table 1, Figure 5). In zone-7 (South Saurashtra), RI declined from 0.53 ± 0.03 to 0.20 ± 0.05 - 0.00 ± 0.00, with IO% increasing from 62.13 ± 9.78 to 100.00 ± 0.00, a slope of 5.458, and an LD_{50} of 6.73 mg/ml (Table 1, Figure 5). Overall, green synthesized ZnO nanoparticles demonstrated dose-dependent acaricidal activity, significantly reducing reproductive capacity and increasing oviposition inhibition in *R.* (*B.*) *microplus* across all agro-climatic zones (Table 1, Figure 5). The lowest LD_{50} values observed in Southern Hill (zone-1) and Middle Gujarat (zone-3) indicate comparatively higher susceptibility of tick populations from these regions.

Table 1: Adult immersion test (AIT) with green synthesized ZnO nanoparticles

Zone	Concentration (mg/ml)	Mean ± S.E.					
		Tick wt. (g) before treatment	Egg mass (g) on 14th dpt	Reproductive Index	% Oviposition inhibition	% Mortality on 14th dpt	Slope, LC_{50}, R^2
Southern Hill	**0**	0.84 ± 0.03	0.44^{c} ± 0.01	0.52^{c} ± 0.02	0.00^{a} ± 0.00	0.00^{a} ± 0.00	Slope = 5.333,
	2	0.86 ± 0.07	0.15^{b} ± 0.03	0.18^{b} ± 0.04	66.14^{b} ± 8.05	46.67^{b} ± 6.67	R^2 = 0.768,
	4	0.94 ± 0.17	0.12^{b} ± 0.05	0.14^{b} ± 0.04	73.59^{b} ± 7.68	63.33^{c} ± 3.33	LD_{50} = 4.37
	8	1.00 ± 0.17	0.00^{a} ± 0.00	0.00^{a} ± 0.00	100.0^{c} ± 0.00	83.33^{d} ± 3.33	mg/ml
	16	1.01 ± 0.11	0.00^{a} ± 0.00	0.00^{a} ± 0.00	100.0^{c} ± 0.00	100.0^{e} ± 0.00	
	32	0.99 ± 0.08	0.00^{a} ± 0.00	0.00^{a} ± 0.00	100.0^{c} ± 0.00	100.0^{e} ± 0.00	
	F	0.408	92.18	64.93	73.20	132.27	
	p	0.834	0.00	0.00	0.00	0.00	
Southern Gujarat	**0**	0.83 ± 0.08	0.41^{d} ± 0.05	0.45^{c} ± 0.03	0.00^{a} ± 0.00	0.00^{a} ± 0.00	Slope = 2.477,
	2	0.68 ± 0.08	0.17^{c} ± 0.03	0.29^{bc} ± 0.07	37.19^{ab} ± 12.2	43.33^{b} ± 3.33	R^2 = 0.701,
	4	0.70 ± 0.07	0.13^{bc} ± 0.04	0.23^{abc} ± 0.08	50.92^{b} ± 14.5	53.33^{b} ± 3.33	LD_{50} = 6.63
	8	0.77 ± 0.09	0.09^{abc} ± 0.03	0.18^{ab} ± 0.09	61.25^{bc} ± 18.3	70.00^{c} ± 0.00	mg/ml
	16	0.67 ± 0.13	0.04^{ab} ± 0.02	0.14^{ab} ± 0.09	70.87^{bc} ± 18.6	86.67^{d} ± 6.67	
	32	0.80 ± 0.13	0.00^{a} ± 0.00	0.00^{a} ± 0.00	100.0^{c} ± 0.00	100.0^{e} ± 0.00	
	F	0.458	19.19	4.60	6.52	113.87	
	p	0.800	0.00	0.014	0.004	0.00	
Middle Gujarat	**0**	0.94 ± 0.03	0.51^{c} ± 0.07	0.55^{c} ± 0.09	0.00^{a} ± 0.00	0.00^{a} ± 0.00	Slope = 5.292,
	2	1.04 ± 0.18	0.30^{b} ± 0.08	0.28^{b} ± 0.04	48.62^{b} ± 2.57	43.33^{b} ± 3.33	R^2 = 0.759,
	4	1.20 ± 0.18	0.26^{b} ± 0.09	0.20^{b} ± 0.04	64.08^{c} ± 2.06	60.00^{c} ± 5.77	LD_{50} = 4.61
	8	1.10 ± 0.06	0.18^{ab} ± 0.06	0.16^{ab} ± 0.05	71.84^{d} ± 4.60	66.67^{c} ± 6.67	mg/ml
	16	1.00 ± 0.10	0.01^{a} ± 0.01	0.01^{a} ± 0.01	98.90^{e} ± 1.10	86.67^{d} ± 6.67	
	32	1.11 ± 0.13	0.01^{a} ± 0.01	0.01^{a} ± 0.01	99.00^{e} ± 1.00	96.67^{d} ± 3.33	
	F	0.507	9.07	16.65	239.45	49.60	
	p	0.766	0.001	0.00	0.00	0.00	
North Gujarat	**0**	0.96 ± 0.12	0.54^{b} ± 0.13	0.55^{c} ± 0.10	0.00^{a} ± 0.00	0.00^{a} ± 0.00	Slope = 4.708,
	2	1.11 ± 0.09	0.40^{ab} ± 0.16	0.35^{bc} ± 0.14	41.55^{b} ± 9.51	43.33^{b} ± 3.33	R^2 = 0.7763,
	4	1.20 ± 0.10	0.32^{ab} ± 0.18	0.25^{abc} ± 0.12	58.87^{bc} ± 8.31	56.67^{bc} ± 6.67	LD_{50} = 5.58
	8	1.10 ± 0.02	0.16^{ab} ± 0.01	0.15^{ab} ± 0.10	75.55^{bcd} ± 3.11	70.00^{c} ± 5.77	mg/ml
	16	1.06 ± 0.04	0.08^{a} ± 0.09	0.08^{ab} ± 0.08	89.07^{cd} ± 9.50	90.00^{d} ± 5.77	
	32	1.09 ± 0.03	0.02^{a} ± 0.02	0.02^{a} ± 0.02	96.99^{d} ± 3.10	96.67^{d} ± 3.33	
	F	1.058	2.650	3.985	11.36	56.083	
	p	0.430	0.077	0.023	0.00	0.00	
North-West Arid	**0**	0.96 ± 0.08	0.54^{d} ± 0.08	0.55^{d} ± 0.03	0.00^{a} ± 0.00	0.00^{a} ± 0.00	Slope = 2.344,
	2	0.95 ± 0.09	0.23^{c} ± 0.06	0.26^{c} ± 0.09	53.89^{b} ± 5.34	43.33^{b} ± 3.33	R^2 = 0.613,
	4	0.99 ± 0.09	0.17^{bc} ± 0.03	0.17^{bc} ± 0.03	69.68^{bc} ± 4.33	66.67^{c} ± 3.33	LD_{50} = 5.12
	8	0.99 ± 0.09	0.12^{abc} ± 0.02	0.12^{ab} ± 0.02	77.33^{c} ± 3.92	76.67^{d} ± 3.33	mg/ml
	16	0.98 ± 0.07	0.05^{ab} ± 0.01	0.05^{ab} ± 0.00	91.05^{cd} ± 0.60	86.67^{e} ± 3.33	
	32	1.02 ± 0.06	0.00^{a} ± 0.00	0.00^{a} ± 0.00	100.0^{d} ± 0.00	100.0^{f} ± 0.00	
	F	0.12	21.33	25.59	28.54	175.0	
	p	0.99	0.00	0.00	0.00	0.00	

North Saurashtra	**0**	1.09 ± 0.08	$0.69^{d} \pm 0.11$	$0.62^{d} \pm 0.06$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	Slope = 4.375,
	2	1.04 ± 0.09	$0.56^{cd} \pm 0.07$	$0.53^{d} \pm 0.04$	$12.94^{a} \pm 9.03$	$40.00^{b} \pm 5.77$	R^2 = 0.737,
	4	1.14 ± 0.11	$0.42^{bc} \pm 0.10$	$0.35^{c} \pm 0.07$	$40.61^{b} \pm 9.41$	$56.67^{c} \pm 6.67$	LD_{50} = 6.00
	8	1.08 ± 0.08	$0.23^{ab} \pm 0.10$	$0.21^{bc} \pm 0.08$	$66.23^{bc} \pm 8.25$	$70.00^{cd} \pm 5.77$	mg/ml
	16	1.09 ± 0.06	$0.07^{a} \pm 0.03$	$0.06^{ab} \pm 0.02$	$89.89^{cd} \pm 3.87$	$83.33^{d} \pm 3.33$	
	32	1.08 ± 0.05	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$100.0^{d} \pm 0.00$	$100.0^{e} \pm 0.00$	
	F	0.192	11.52	24.37	21.42	61.26	
	p	0.960	0.00	0.00	0.00	0.00	
South Saurashtra	**0**	0.99 ± 0.08	$0.53^{c} \pm 0.06$	$0.53^{d} \pm 0.03$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	Slope=5.458,
	2	0.88 ± 0.14	$0.16^{b} \pm 0.02$	$0.20^{c} \pm 0.05$	$62.13^{b} \pm 9.78$	$26.67^{b} \pm 3.33$	R^2 = 0.874,
	4	0.94 ± 0.10	$0.13^{b} \pm 0.02$	$0.14^{bc} \pm 0.03$	$72.74^{bc} \pm 6.78$	$36.67^{c} \pm 3.33$	LD_{50} = 6.73
	8	0.91 ± 0.06	$0.08^{ab} \pm 0.02$	$0.09^{ab} \pm 0.02$	$82.71^{cd} \pm 4.15$	$76.67^{d} \pm 3.33$	mg/ml
	16	0.91 ± 0.04	$0.03^{a} \pm 0.03$	$0.03^{a} \pm 0.03$	$93.63^{d} \pm 6.36$	$90.0^{e} \pm 0.00$	
	32	0.91 ± 0.04	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$100.0^{d} \pm 0.00$	$100.0^{f} \pm 0.00$	
	F	0.212	39.636	41.964	39.574	283.80	
	p	0.951	0.00	0.00	0.00	0.00	

Means with different superscript within columns are statistical different. F = Frequency, P = probability, 0ppm = control, R^2 - Goodness of fit in regression.



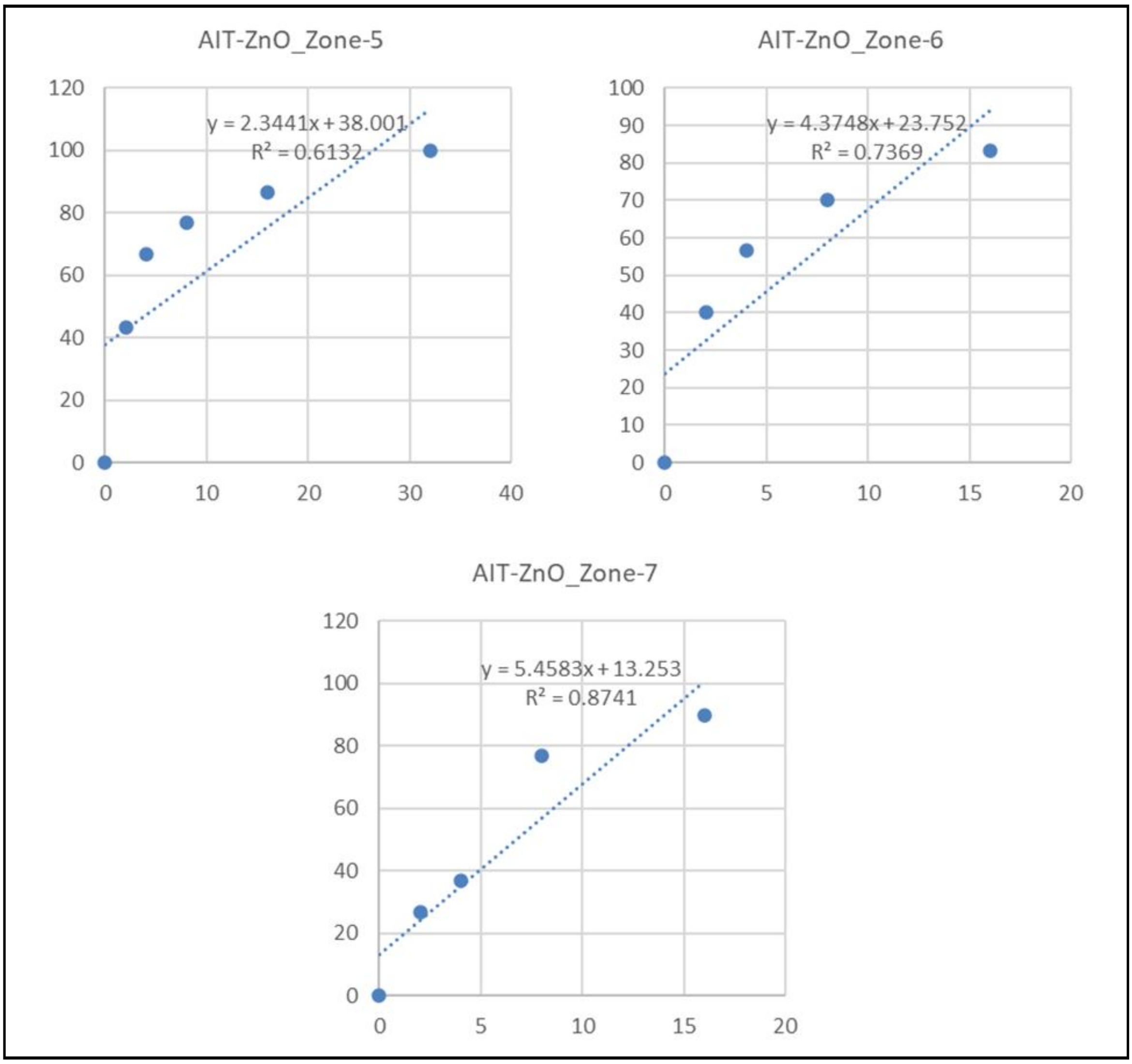


Figure 5: Regression curve showing mortality of adult tick.

4. Discussion

4.1 Synthesized zinc oxide nanoparticles and their characterization

UV-Visible spectroscopy is regarded as one of the most commonly employed analytical techniques for the structural characterization of synthesized nanoparticles. In the present study, the optical properties of the synthesized zinc oxide nanoparticles were evaluated using UV-Visible spectroscopic analysis. A distinct absorption peak was recorded at 370 nm, indicating the successful formation of ZnO nanoparticles. Similar findings have also been documented in earlier investigations conducted by Kavithaa *et al*. (2016); Banumathi *et al*. (2016); Abdelbaky *et al*. (2022).

In the present investigation, the surface morphology of the synthesized zinc oxide nanoparticles was examined using scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX). SEM micrographs demonstrated that the nanoparticles were predominantly spherical in morphology, with particle sizes ranging between 53 and 92 nm (Figure 3). The synthesized particles also exhibited aggregation, resulting in the formation of larger clusters. Such agglomeration is frequently observed in biosynthesized nanoparticles owing to the interaction of biomolecules present on the nanoparticle surface. Comparable findings have been documented by several researchers. Ojha *et al*. (2025) reported the synthesis of round-to-hexagonal ZnO nanoparticles with an average particle size of 82.39 nm, along with noticeable aggregation behaviour. Likewise, Divya *et al*. (2013) synthesized ZnO nanoparticles using leaf extract of *Hibiscus rosa-sinensis* and observed UV-Visible absorption peaks between 358 and 375 nm, with particle sizes varying from 40 to 56 nm. Similarly, Elumalai and Velmurugan (2015) employed extracts of *A. indica* for nanoparticle synthesis, whereas Parthiban and Sundaramurthy (2015) utilized *Pyrus pyrifolia* extract and confirmed the formation of crystalline ZnO nanoparticles possessing a wurtzite hexagonal structure with an average particle size of nearly 22 nm. More recent investigations have concentrated on enhancing nanoparticle formulations and assessing their efficacy under varying experimental conditions. In this regard, Ajayan and Hebsur (2020) synthesized

ZnO nanoparticles using extracts of neem and tulasi and reported particle sizes exceeding 100 nm.

In the present study, the EDX spectrum (Figure 4) exhibited prominent peaks corresponding to zinc and oxygen, thereby confirming the elemental composition of the synthesized zinc oxide nanoparticles. Elemental analysis demonstrated that the nanoparticles contained 81.58% zinc and 17.45% oxygen by weight. These observations are in close agreement with previously published findings. Jayachandran *et al*. (2021) reported zinc and oxygen contents of 78.32% and 12.78%, respectively, whereas Abdelbaky *et al*. (2022) documented 50.58% zinc and 49.42% oxygen in their synthesized nanoparticles. In contrast, Ojha *et al*. (2025) observed the presence of carbon (58.07%) along with zinc (26.34%) and oxygen (15.59%). The occurrence of carbon in biosynthesized nanoparticles is generally attributed to residual phytochemical constituents originating from the plant extracts employed during the green synthesis process.

4.2 Adult immersion test (AIT) with green synthesized ZnO nanoparticles

The calculated an LD_{50} value of green synthesized ZnO nanoparticles ranging from 4.37-6.73 mg/ml in present study. Overall, green synthesized ZnO nanoparticles demonstrated dose-dependent acaricidal activity, significantly reducing reproductive capacity and increasing oviposition inhibition in *R.* (*B.*) *microplus* across all agro-climatic zones. These findings are comparable with earlier reports. Banumathi *et al.* (2016) demonstrated a highly potent LC_{50} value of 1.7 mg/l against adult *R. (B.) microplus* using ZnO nanoparticles synthesized from *Lobelia leschenaultiana*, with mortality increasing significantly in a dose-dependent manner. Addition to Gandhi *et al.* (2017) further reported LC_{50} of 6.87 mg/l of ZnO nanoparticles synthesized from *Momordica charantia*. Naseer *et al.* (2020) and Abdel-Ghany *et al.* (2022) reported strong antibacterial activity and stage-specific toxicity against ticks, respectively. Abdel-Ghany *et al.* (2022) also observed reduced reproductive performance in *Hyalomma dromedarii*, indicating that ZnO nanoparticles can affect not only survival but also reproductive fitness. Shnawa *et al.* (2023) reported high mortality rates in *Hyalomma marginatum* using Ag-ZnO nanocomposites synthesized from *Lawsonia inermis*, demonstrating the potential of combining multiple nanomaterials. Furthermore, studies on related ectoparasites reinforce the broad-spectrum potential of green-synthesized nanoparticles. Al-Azzazy *et al.* (2024) demonstrated dose-dependent mortality and reduced egg hatchability in phytophagous mites, while Anoopkumar *et al.* (2026) reported significant mortality in *R.* (*B.*) *microplus* using ZnO nanoparticles synthesized from *Ageratum conyzoides*. These findings suggest that green nanotechnology can be effectively applied across multiple arthropod pests. Furthermore, the antimicrobial and acaricidal efficacy of *A. indica* leaf extract-mediated zinc oxide nanoparticles may be associated with the presence of diverse bioactive phytochemical constituents in neem leaves. These include carotenoids, phenolic compounds, flavonoids, triterpenoids, ketones, saponins, glycosides, steroids, and tetranortriterpenoids such as azadirachtin, as reported by Parra and Haque (2014). These phytochemicals act as both reducing and capping agents during nanoparticle synthesis and remain associated with the ZnO NP surface. The incorporation of neem-derived biomolecules within the ZnO nanoparticle matrix enhances their biological activity by providing synergistic effects, which collectively contribute to a potent defensive mechanism against a wide range of pathogens (Geetha *et al*., 2016; Babatunde *et al*., 2019).

5. Conclusion

The present study demonstrates that zinc oxide nanoparticles synthesized using neem leaf extract possess promising *in vitro* acaricidal activity against *R.* (*B.*) *microplus*. The nanoparticles exhibited dose-dependent tick mortality and inhibition of oviposition. Therefore, green synthesized ZnO nanoparticles may serve as an effective and environmentally safer alternative for tick control in livestock.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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