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Research Article

Synthesis, Characterization and Toxicity Assessment of Chlorantraniliprole Nanosuspension and Its Environmental Risk

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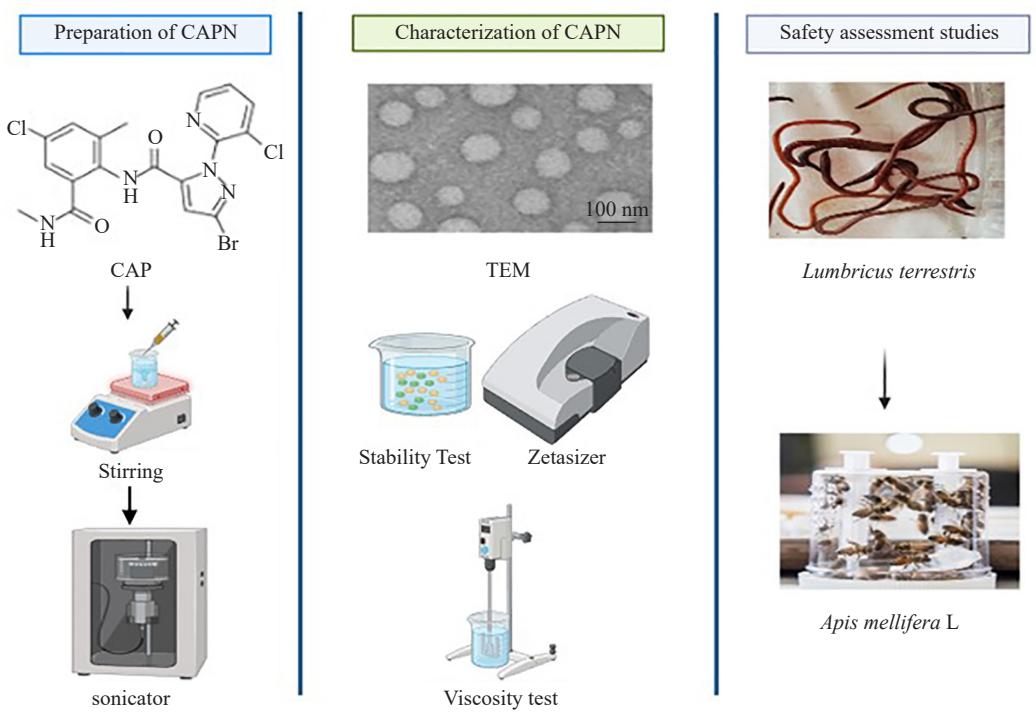
Graphical Abstract:



Note: Chlorantraniliprole (CAP); Chlorantraniliprole Nanosuspension (CAPN); Transmission Electron Microscopy (TEM)

Abstract: Chlorantraniliprole is an anthranilic diamide-class insecticide that functions as a ryanodine receptor agonist. It is recognized for its high efficacy, low mammalian toxicity, prolonged persistence, and minimal residual presence. This study developed a stable Oil-in-Water (O/W) nanosuspension of chlorantraniliprole using a combination of a solvent and the non-ionic surfactant Tween-80. A high-energy emulsification process, namely ultrasonication, was employed to achieve a nanometer-scale particle size. The successful formation of the chlorantraniliprole nanosuspension was verified using dynamic light scattering and transmission electron microscopy. Its stability was assessed through tests

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for viscosity, centrifugation, and resilience to heating-cooling and freeze-thaw cycles. The environmental safety of both the conventional Chlorantraniliprole (CAP) and Chlorantraniliprole Nanosuspension (CAPN) insecticides was evaluated using earthworms (*Lumbricus terrestris*) and honeybees (*Apis mellifera* Linnaeus) as model organisms. Earthworms were subjected to concentrations ranging from 3.75 to 60.0 mg/L for 30 days. Concurrently, the fate and transport of CAP within the soil-earthworm system were monitored. After the 30-day exposure, treatment with 60.0 mg/kg of CAP and CAPN resulted in earthworm mortality not exceeding 20%. Furthermore, a significant reduction in earthworm body weight to 7.59 and 8.09 gm was recorded following application of CAP and CAPN, respectively. In a parallel bioassay, honeybees were exposed to the same range of concentrations (3.75-60.0 mg/L), with mortality evaluated at 12, 24, 36, and 48-hour intervals. Mortality of honeybees reached 100% after application of CAP and CAPN for 48 h. Based on recent studies on CAPN, which highlight its enhanced solubility, stability, and bioefficacy compared to conventional formulations of CAP, there is a clear need for expanded field application research to measure its real-world efficiency, especially since laboratory trials have shown promising results.

***Keywords*:** chlorantraniliprole, nanosuspension, toxicity assessment, *Lumbricus terrestris*, *Apis mellifera* Linnaeus

1. Introduction

Chlorantraniliprole is an anthranilic diamide insecticide that is highly effective against Lepidoptera.[1] It is a stomach poison as well as a contact poison.[2] Chlorantraniliprole's insecticidal action occurs by activating the ryanodine receptor in insect smooth and striated muscle cells. As a result, intracellular calcium is released, which leads to muscular weakness and paralysis, ultimately killing the pest.[3] It is difficult to prepare formulations of chlorantraniliprole that are efficient and environmentally safe due to its low solubility in water (1.023 mg/L) as well as various organic solvents.[4] Chlorantraniliprole is available mostly as suspension concentrates or Water-Dispersible Granules (WDG).[5] It was specifically developed for the control of lepidopteran pests (order Lepidoptera), such as moths and butterflies, which are major defoliators of crops worldwide. Key target pests include economically damaging species like the diamondback moth (*Plutella xylostella*), cotton bollworm (*Helicoverpa armigera*), fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), black cutworm (*Agrotis ipsilon*), and obliquebanded leafroller (*Choristoneura rosaceana*).[6] Nanoformulations address these issues by reducing particle size to the nanoscale (typically 20-75 nm), enhancing surface area, improving solubility through increased wettability and dispersibility, and enabling targeted delivery. Consequently, lower doses can be used than with the conventional formulation to achieve the same results.

Nanoscale formulation (20-75 nm) enhances pesticide performance by increasing surface area, solubility, and foliar adhesion. This enables targeted delivery with lower doses, reducing environmental runoff and residue while prolonging efficacy.[7,8] Nanotechnology has demonstrated that sparingly soluble pesticides can be made more soluble, dispersible, precise, bioavailable, and safe with nanotechnology, which has led to considerable interest in the model and structure of nanopesticides.[4,9] Nanopesticides have been developed through nanotechnology to achieve high-efficiency and sustainable agriculture, and are becoming the primary method for developing pesticides.[10] To overcome the poor solubility of the pesticide chlorantraniliprole in both water and organic solvents, a stable nanosuspension was fabricated using wet media milling. This process successfully generated a nano-delivery system characterized by an average particle size of 56 nm.[8] The nanosuspension is more dispersible, foliar-wettable, and retains better on foliage than a Suspension Concentrate (SC) formulation.[8] Nanoformulations of chlorantraniliprole demonstrate superior efficiency in eliminating target pests compared to traditional suspensions, primarily due to enhanced bioavailability, faster uptake, and increased toxicity at lower concentrations. This leads to quicker feeding cessation, higher larval mortality, and reduced sublethal effects like prolonged development or reduced reproduction in survivors.[11,12]

Pesticides can affect earthworms, either directly or indirectly, through the skin of their outer skin or through the consumption of polluted soil particles.[13] Earthworms are adversely affected at various stages of their lives by chemicals entering the soil.[14] Similarly,[15] *L. terrestris* has been designated a non-target organism for the environmental risk assessment of genetically modified crops. A well-established association exists where reduced pesticide application correlates with increased earthworm densities, a phenomenon consistently observed in organic farming systems. However, attributing this effect solely to pesticides is complex, as it is complicated by a network of co-occurring abiotic and biotic factors.[16]

The honeybee is one of the most important pollinators and contributes significantly to crop yields and plant biodiversity. Around the world, pollination services provided by *Apis mellifera* are crucial for crop production.[17] The widespread phenomenon of Colony Collapse Disorder (CCD) has been linked to the global decline of honeybee populations, resulting in the loss of millions of colonies and significant economic damage to the beekeeping industry. Honeybee exposure to pesticides is primarily categorized as either unintentional or intentional. Unintentional exposure occurs during foraging, as bees come into contact with or consume contaminated nectar, pollen, and water.[18]

The objective of this research was two-fold: first, to formulate a nanosuspension of chlorantraniliprole, and second, to evaluate the risk assessment of Chlorantraniliprole (CAP) and Chlorantraniliprole Nanosuspension (CAPN) to non-target species, specifically honeybees (*Apis mellifera*) and earthworms (*Lumbricus terrestris*). We conducted controlled laboratory exposures for durations of 12, 24, 36 and 48 hours to compare the effects of varying exposure lengths and to determine the potential for recovery in honeybees following acute exposure.

2. Materials and methods

2.1 *Chemicals*

Chlorantraniliprole Technical Material (5-bromo-*N*-[4-chloro-2-methyl-6-(methylcarbamoyl)phenyl]-2-(3-chloropyridin-2-yl)pyrazole-3-carboxamide) (TC, 95%, w/w) and Coragen (20% SC) were obtained from Agrimatco Co (22R3+R9V, First Sheikh Zayed, Giza Governorate 3244211, Egypt). Dimethyl Sulfoxide 99% (DMSO) was obtained from Alpha Chemicals Co. in India. Tween 80 was bought from Sigma-Aldrich Co. (Spruce Street, St. Louis, MO, USA). Xanthine (99.5%) was obtained from Otto Chemie Co. (No. 603, Tardeo, Mumbai, Maharashtra 400034, India). Propylene Glycol was bought from Henan Jinhe Industry Co. (Zhengzhou, Henan Province, China). Throughout the study, all experiments were conducted using deionized water.

2.2 *Preparation of chlorantraniliprole nanosuspension*

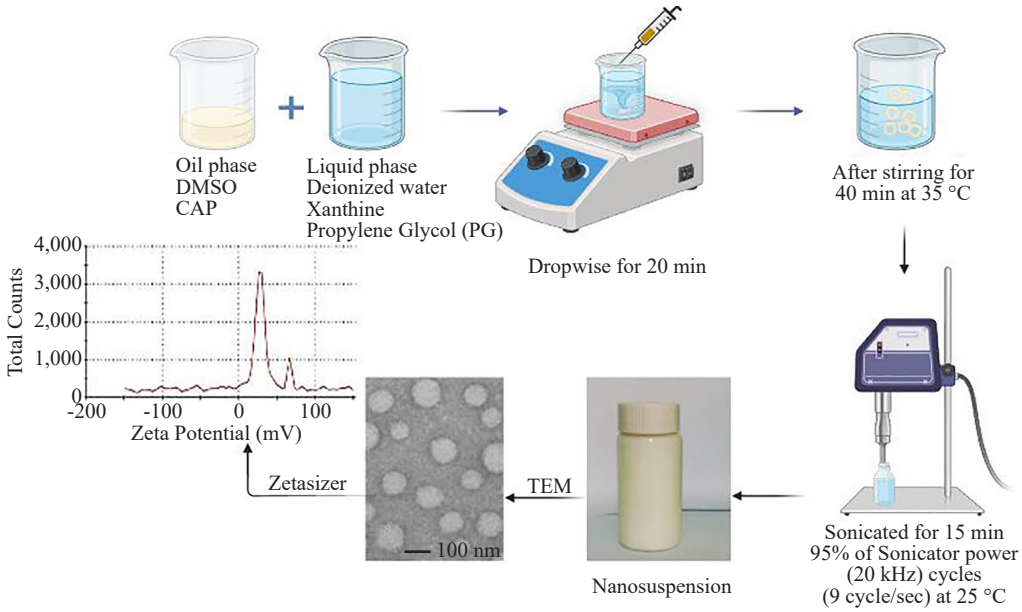

Figure 1. A schematic diagram showing the preparation of chlorantraniliprole nanosuspension

A nanosuspension of chlorantraniliprole (0.5% w/v) was synthesized by first creating an oil phase with the insecticide dissolved in DMSO (oil phase: 10 mL). An aqueous phase was prepared by mixing deionized water with

Tween 80 (5 mL), xanthine (0.1 g), and propylene glycol (2 g), and bringing it to a final volume of 100 mL with deionized water under continuous stirring. The oil phase was then slowly introduced into the aqueous phase and stirred at 4,000 rpm for 40 min at 35 °C to form a coarse nanosuspension. The nanosuspension was then sonicated using an ultrasonic probe (Ultrasonic Homogenizers HD2070 with High-Frequency (HF) generator (GM2070), ultrasonic converter UW2070, booster horn (SH213G), and probe microtip MS73, Ø 3 mm).[19] For final particle size reduction, the mixture was subjected to an ultrasonic probe for 15 min at 95% power with a pulse rate of 9 cycles/s (Figure 1).

2.3 *Characterization of CAPN*

2.3.1 *Transmission Electron Microscopy (TEM) observation of droplets*

The morphological characteristics and size distribution of the CAPN were characterized using TEM. Bright-field imaging was employed, supplemented by increased magnification and selected-area electron diffraction, to comprehensively evaluate the nanoparticle morphology and dimensions. Sample preparation involved diluting a small aliquot of the nanosuspension with deionized water, followed by deposition onto carbon-coated copper TEM grids. Imaging was performed using a JEOL JSM-1200EX II transmission electron microscope operating at an accelerating voltage of 80 kV with a 20 μm aperture.

2.3.2 *Zeta potential and particle size measurements*

For characterization, the chlorantraniliprole nanosuspension was diluted tenfold with deionized water to minimize artifacts from multiple scattering effects. The diluted sample was subsequently analyzed using a Zetasizer Nano ZS 90 instrument to determine key physicochemical parameters, including hydrodynamic particle size, polydispersity index, and zeta potential. All measurements were performed in triplicate at controlled room temperature to ensure methodological reproducibility and data reliability.

2.3.3 *Stability test*

The physical stability of the nanosuspension was assessed under accelerated storage conditions at sub-ambient (-21 °C), ambient (25 °C), and elevated (54 °C) temperatures. Each stability test was conducted for 14 days. All analyses were performed in triplicate to guarantee the reproducibility and consistency of the obtained results.[20]

2.3.4 *Viscosity measurement*

The dynamic viscosity of the neat nanosuspension was determined using a digital viscometer maintained at 25 °C with a rotational velocity of 200 rpm. Prior to each measurement, the sample was allowed to equilibrate thermally for 120 s. Triplicate measurements were conducted to ensure reproducibility, and results are reported in millipascal-seconds (mPa·s).

2.4 *Safety assessment studies of CAP and CAPN on honeybees (A. mellifera L.)*

The experimental model utilized adult worker honeybees (*Apis mellifera*) of a Carniolan-Egyptian hybrid strain. Colonies were maintained at the El-Sabahia Research Station in Alexandria, under the auspices of the Agricultural Research Center, Ministry of Agriculture. The study specifically targeted foraging-age bees, which represent the terminal stage of the adult life cycle and are typically older than 20 days.[21] Prior to collection, healthy colonies were pacified by brief puffing of smoke for 30-60 s. Foraging-age bees were subsequently dislodged from top frames into a sterile plastic container, which was immediately sealed for secure transport to the laboratory facility.[22] During transport, bees were maintained under controlled thermal conditions at 25 ± 2 °C. Following arrival, specimens were systematically allocated into experimental cohorts of 50 individuals and transferred to specialized rearing cages. These groups were provisioned ad libitum with a 10% sucrose solution and maintained under standardized environmental conditions (25 ± 2 °C; 65 ± 5% relative humidity) throughout the experimental period.

This study evaluated the acute toxicity of CAP and its CAPN on honeybees through oral exposure.[15] Test solutions were prepared at five concentrations ranging from 3.75 to 60.0 mg/L in 10% (w/v) sucrose, where 60.0 mg/L is the field

application rate from CAP to control Fall armyworm; each concentration involves 30 bees and 3 replicates. Groups of thirty bees were housed in plastic containers and briefly anesthetized with carbon dioxide prior to treatment. Each group received a cotton wick saturated with the corresponding treatment solution for 24 hours. A control group was provided with an untreated sucrose solution only. All experiments were conducted under standardized environmental conditions of temperature, humidity, and light. Mortality was assessed at 12, 24, 36, and 72-hour intervals, with death defined as the inability to walk or fly when stimulated.[22]

2.5 *Safety assessment studies of CAP and CAPN on earthworms (Lumbricus terrestris)*

Adult specimens of *Lumbricus terrestris* were collected from agricultural soils in the Bahara Governorate region. Following transport to the laboratory, the earthworms were maintained in moist soil and acclimatized for seven days under controlled feeding conditions. In accordance with Heimbach›s methodology,[23] the organisms were housed in large plastic containers (38 × 60 × 10 cm) fitted with muslin cloth covers and containing an artificial soil substrate maintained at 22 °C. Only sexually mature earthworms exhibiting well-developed clitellae were selected for the experiment. Due to the hermaphroditic nature of earthworms, no sexual differentiation was applied. Twenty-four hours prior to experimentation, the adults were transferred to damp filter paper and maintained at 23 °C in complete darkness for 48 hours to allow gut evacuation. The artificial soil substrate was formulated according to established protocols,[24] consisting of 70% industrial sand, 20% kaolin clay, and 10% sphagnum peat moss, with pH adjusted using calcium carbonate.

To assess growth inhibition, earthworms were selected, rinsed with tap water, and surface-dried on filter paper before initial weighing. Longitudinal mass measurements were conducted for each specimen following 1, 2, 3, and 4 weeks of exposure. Triplicate independent replicates were performed for all mass determinations. Growth inhibition was quantified based on relative weight changes using the formula:

$$\text{Growth inhibition (\%)} = \left(\frac{C_{\text{w}} - T_{\text{w}}}{C_{\text{w}}} \right) \times 100$$

where C_{w} represents the mean weight change in control groups and T_{w} the mean weight change in treatment groups.

An acute toxicity assay was performed by incorporating CAPN and CAP into artificial soil at concentrations of 3.75, 7.5, 15.0, 30.0, and 60.0 mg (active ingredient)/kg soil. Each concentration involves 30 earthworms and 3 replicates. Control groups received artificial soil amended with deionized water only. Soil moisture was maintained at 35% of total weight and monitored at biweekly intervals. All exposures were conducted under continuous illumination at 23 ± 2 °C. The experimental design included four exposure periods (1, 2, 3, and 4 weeks), with three replicates per concentration and duration, each containing ten earthworms. Mortality was assessed weekly by carefully rinsing earthworms from the soil matrix. Death was confirmed by the absence of response to gentle tactile prodding, consistent with Organization for Economic Co-operation and Development (OECD) guideline 207.[25] Probit regression analysis[26] was applied to mortality data to determine Median Lethal Concentration (LC_{50}) values.

2.6 *Statistical analysis*

Statistical analysis was performed using one-way Analysis of Variance (ANOVA) with a significance level of $p < 0.05$. Post-hoc comparisons of means were conducted using the Student-Newman-Keuls (SNK) test. Mortality data for both honeybees and earthworms were adjusted using Abbott's formula to normalize for control mortality.[27] Corrected mortality values were plotted against concentration, and dose-response curves were generated using probit analysis in SPSS software.[26,28] Additionally, least-squares regression analysis was applied to evaluate the correlation between compound concentration and earthworm relative growth rate.

3. Results and discussion

3.1 *Characterization of chlorantraniliprole nanosuspension*

Figure 2a presents TEM images characterizing the morphological features of the chlorantraniliprole nanosuspension. The analysis revealed well-defined nanometric droplets with diameters ranging from 32.16 to 51.81 nm. These TEM-derived measurements demonstrated strong agreement with particle size distributions obtained using dynamic light scattering (Zetasizer Nano ZS), thereby validating the accuracy of the size characterization. It should be noted that the presence of larger particulate aggregates may influence light scattering measurements, potentially leading to overestimation of hydrodynamic diameter in polydisperse systems.[29]

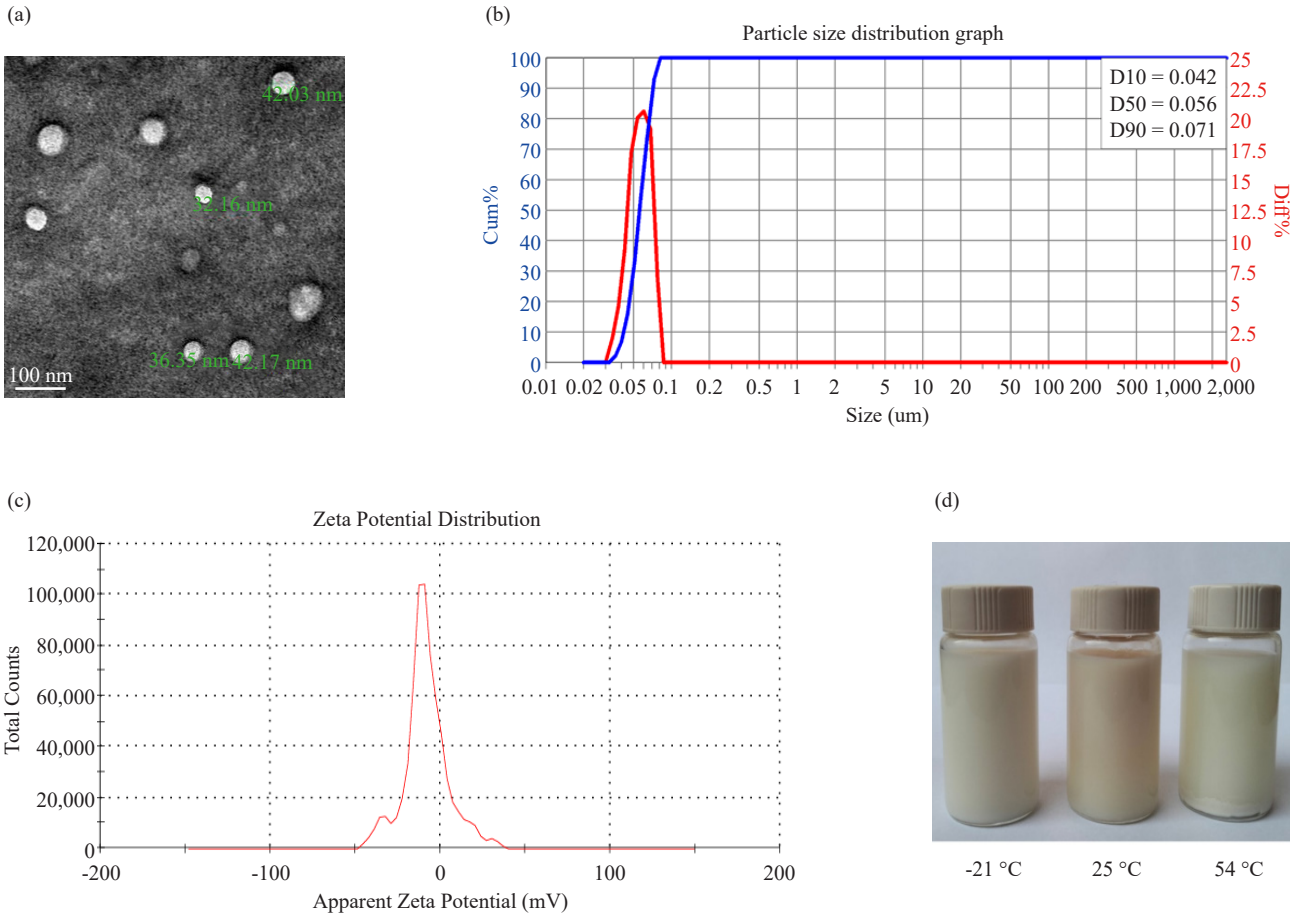

Figure 2. Transmission electron micrograph (a); particle size distribution (b); Zeta potential (c) and storage stability at -21 °C, 25 °C and 54 °C (d) of chlorantraniliprole nanosuspension

The colloidal stability of the aqueous chlorantraniliprole nanosuspension was characterized by monitoring key physicochemical parameters, including zeta potential, particle size distribution, and population homogeneity. Dynamic light scattering measurements indicated an average particle size of 32-52 ± 0.2 nm with a Polydispersity Index (PDI) of 0.272 ± 0.010 (Figure 2b). The narrow size range and low PDI suggest effective particle size reduction and homogeneity, which are critical for enhancing dispersibility, foliar wetting, and bioavailability against target pests, such as those in rice fields, while minimizing environmental release.[30] The current PDI of 0.272 falls within this optimal range, suggesting reduced aggregation risks compared to less stable formulations where PDI > 0.4 leads to phase separation and reduced efficacy.[31] A PDI value below 0.3 is generally considered indicative of a narrow, monodisperse size distribution.[32] The observed PDI, being well under this threshold, suggests a highly homogeneous system with

substantial resistance to common destabilization processes such as Ostwald ripening and particle sedimentation.[33]

The stability of the nanosuspension was confirmed by its zeta potential, a key indicator of the electrical charge at the particle interface. The nanomaterial was found to have a zeta potential of -3.29 mV (Figure 2c), indicating a negative charge. This charge is attributed to the absorption of OH^- ions onto the particle surface, which creates sufficient electrostatic repulsion to prevent aggregation and maintain a homogeneous distribution.[34,35] The strongly negative zeta potential also suggests that anionic surfactants have molecularly bonded to the pesticide, providing the necessary electrostatic force to ensure the suspension's long-term stability.[36]

As demonstrated in Figure 2d, the CAPN formulation was engineered for exceptional physical stability. This was validated through accelerated stability testing under controlled thermal stress conditions, including storage at -21 °C, 25 °C, and 54 °C for a minimum of 24 hours. All experiments were conducted in triplicate to ensure reproducibility. The observed stability is attributed to synergistic stabilization mechanisms, combining electrostatic repulsion from surface charges and steric hindrance provided by adsorbed polymers, which collectively prevent particle aggregation.[20,37]

The rheological properties of the chlorantraniliprole nanosuspension were characterized, revealing a dynamic viscosity of 16 mPa·s of a value selected to optimize delivery performance. Analysis indicated that viscosity was governed by a combination of factors, including the volume fraction of the dispersed phase, the nature of interparticle interactions, droplet size distribution, and the physicochemical properties of the formulation components. Additionally, the acidic characteristics of the system suggest potential enhancement of its biological activity.[38]

3.2 *Toxicity of CAPN and CAP compounds on non-target organisms*

3.2.1 *Honeybee (A. mellifera)*

In this regard, Table 1 shows the mortality (%) of honeybees after application of CAP and CAPN for 12, 24, 36, and 48 hours. The CAP showed an increase in honeybee mortality with higher concentrations over time. CAP at 60.0 mg/L, mortality rates reached 94% after 48 hours. Compared with the control group, the CAPN also showed a higher mortality in honeybees at any of the tested concentrations within 48 hours, with mortality rates remaining at 13.33% at 12 hours. At the highest concentration tested (60.0 mg/L), mortality rates reached 90% after 48 hours. Compared with CAP, CAPN showed lower toxicity at a concentration of 15.0 mg/L, with mortality 40.33, 55.33, 57.67, and 65.33% at 12, 24, 36, and 48 h, respectively. CAP against *A. mellifera* reported 24 h Median Lethal Dose (LD_{50}) values of ~ 0.5-2 μg/bee (equivalent to ~ 10-50 mg/L topical), with nanosuspensions reducing acute toxicity by ~ 5% due to controlled release, mirroring CAPN's lower mortality here (e.g., 55.33% vs. CAP's 59.67% at 15.0 mg/L, 24 h). Honeybees possess detoxification mechanisms (involving enzymes like cytochrome P450s and glutathione S-transferases), but their genetic repertoire for these enzymes is limited compared to other insects, potentially making them more vulnerable to synergistic effects from multiple toxins.[39]

The control group exhibited low mortality (3.33% after 48 hours), confirming the reliability of the experimental conditions and indicating that observed mortality was attributable primarily to compound exposure rather than extraneous variables. Overall, the Chlorantraniliprole Nanosuspension (CAPN) exhibited moderate toxicity to honeybees across the tested concentrations and exposure durations. Due to the limited affinity of chlorantraniliprole for honeybee ryanodine receptors, the insecticide was anticipated to demonstrate low acute toxicity, with no significant adverse effects expected on locomotor performance in treated individuals.[40] While chlorantraniliprole is relatively safe for many organisms, it is highly toxic to springtails. However, elevated concentrations of both the nanosuspension and conventional formulation resulted in increased honeybee mortality. These findings highlight the necessity of evaluating agrochemical impacts on non-target organisms such as pollinators and reinforce the need for sustainable agricultural approaches to protect pollinator health and biodiversity.

The toxicity of CAP and its CAPN toward honeybees was evaluated via oral exposure using spiked sucrose syrup. As summarized in Table 2, for both CAP and CAPN, toxicity increases significantly with exposure time (from 12 h to 48 h). CAP: LC_{50} decreases from 19.71 mg/L (12 h) to 7.86 mg/L (48 h). CAPN: LC_{50} decreases from 22.39 mg/L (12 h) to 8.62 mg/L (48 h). At every time point, CAPN has a slightly higher LC_{50} than CAP, meaning the nanosuspension appears slightly less acutely toxic than the conventional formulation in this lab test. At 48 h: CAP LC_{50} = 7.86 mg/L vs. CAPN LC_{50} = 8.62 mg/L. These results align with sublethal and lethal exposure paradigms in honeybee toxicology, where even low doses can trigger cascading failures in individual and colony health. The similarity in potency between CAP and CAPN implies that structural modifications (if CAPN represents a variant) do not substantially alter acute

lethality, though subtle differences emerge at mid-concentrations (e.g., CAPN's lower initial rise at 7.5 mg/L). These results are ecologically significant given honeybees' vital role as pollinators in maintaining biodiversity and supporting agricultural systems.[41] The potential impact of pesticides on non-target organisms such as bees underscores the necessity of comprehensive toxicological assessments to minimize adverse effects and support the development of safer agrochemical practices.

Table 1. Effects of the CAP and CAPN on the percentage of mortality of the honeybee after 12, 24, 36, and 48 hours of application

Compound	Conc. (mg/L)	Mortality (%) ± SE			
		12 hours	24 hours	36 hours	48 hours
Control	-	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$3.33^{a} \pm 3.34$
CAP	3.75	$15.67^{a} \pm 3.34$	$18.00^{b} \pm 0.00$	$20.00^{b} \pm 0.00$	$30.33^{c} \pm 3.34$
	7.5	$25.00^{ab} \pm 0.00$	$32.67^{bc} \pm 3.34$	$35.67^{c} \pm 3.34$	$48.00^{d} \pm 0.00$
	15.0	$46.33^{bc} \pm 3.34$	$59.67^{c} \pm 3.34$	$60.67^{cd} \pm 3.34$	$70.67^{e} \pm 3.34$
	30.0	$55.33^{c} \pm 3.34$	$68.67^{cd} \pm 3.34$	$70.00^{e} \pm 0.00$	$78.00^{e} \pm 0.00$
	60.0	$78.67^{c} \pm 3.34$	$88.00^{d} \pm 0.00$	$90.00^{e} \pm 0.00$	$94.00^{e} \pm 0.00$
CAPN	3.75	$13.33^{a} \pm 3.34$	$16.00^{ab} \pm 0.00$	$18.00^{b} \pm 0.00$	$30.00^{c} \pm 0.00$
	7.5	$20.33^{ab} \pm 3.34$	$30.00^{b} \pm 0.00$	$37.00^{cd} \pm 0.00$	$45.33^{d} \pm 3.34$
	15.0	$40.33^{b} \pm 3.34$	$55.33^{c} \pm 3.34$	$57.67^{cd} \pm 3.34$	$65.33^{d} \pm 3.34$
	30.0	$55.33^{bc} \pm 3.34$	$66.67^{c} \pm 3.34$	$67.33^{d} \pm 3.34$	$77.67^{de} \pm 3.34$
	60.0	$76.67^{c} \pm 3.34$	$86.67^{cd} \pm 3.34$	$88.00^{e} \pm 0.00$	$90.00^{e} \pm 0.00$

A one-way Analysis of Variance (ANOVA), performed with a significance level of $p \leq 0.05$, confirmed that the values within each column were not uniform. To specify which means differed significantly, a subsequent Student-Newman-Keuls (SNK) test was conducted. As a result, values within a column bearing the same superscript are not significantly different ($p < 0.05$) according to Student-Newman-Keuls (SNK) test.

Table 2. Acute toxicity of CAP and CAPN against bees (*A. mellifera L.*)

Insecticide	LC_{50}[a] (mg/L)	Confidence limits		Slope[b] ± SE	Intercept[c] ± SE	(X^2)[d]	p
		Lower	Upper				
CAP 12 h	19.71	16.37	24.15	1.46 ± 0.19	-1.89 ± 0.15	2.22	0.528
CAP 24 h	12.95	10.91	15.26	1.69 ± 0.16	-1.89 ± 0.19	2.44	0.486
CAP 36 h	11.92	10.01	14.06	1.68 ± 0.17	-1.81 ± 0.19	2.37	0.499
CAP 48 h	7.86	6.33	9.44	1.61 ± 0.16	-1.44 ± 0.16	2.36	0.500
CAPN 12 h	22.39	18.73	27.39	1.55 ± 0.15	-2.09 ± 0.19	1.28	0.734
CAPN 24 h	14.41	12.21	16.99	1.71 ± 0.16	-1.98 ± 0.19	1.66	0.645
CAPN 36 h	12.11	10.71	15.17	1.62 ± 0.15	-1.79 ± 0.19	2.32	0.509
CAPN 48 h	8.62	6.88	10.43	1.49 ± 0.15	-1.39 ± 0.18	0.27	0.966

[a] Lethal concentration producing 50% mortality
[b] Slope ± Standard Error of the concentration-death regression line
[c] Intercept ± Standard Error of the Regression Line
[d] Chi square

3.2.2 *Earthworms L. terrestris*

Table 3 summarizes the mortality rates (%) of the earthworm *Lumbricus terrestris* following exposure to CAP and CAPN over periods of 1 to 4 weeks. Evaluating the toxicity of these compounds on earthworm survival is essential for determining their potential risks to soil ecosystems and for advancing sustainable agricultural practices that protect soil biodiversity and ecological functions.

Contemporary ecotoxicology is increasingly adopting a refined framework for assessing contaminant impacts on living organisms. Beyond recording overt lethal outcomes, researchers now routinely employ a range of biomarkers—such as measures of growth, reproduction, genotoxicity (e.g., DNA damage), and cellular stress responses (e.g., antioxidant enzyme activity)—to detect subtler, sublethal effects. These biomarkers provide a deeper understanding of the latent impairments caused by environmental pollutants.[42]

Low mortality in earthworms was only evident at the highest dose (60 mg/kg), resulting in mortality rates ranging from 0% to 15% for CAPN after four weeks. The conventional chlorantraniliprole formulation similarly exhibited concentration-dependent toxicity, producing mortality rates between 4.44% and 20% under identical conditions. These findings highlight the compound's time- and dose-dependent ecotoxicological profile.

The environmental impact of chlorantraniliprole is characterized by notable taxonomic selectivity. While demonstrating low affinity for ryanodine receptors in mammals, birds, and fish, resulting in favorable safety profiles for these taxa, it exhibits high potency against numerous aquatic invertebrates due to their structurally susceptible receptor variants. This differential activity underscores the compound's selective mechanism of action and emphasizes the importance of taxon-specific risk assessment in ecological toxicology.[43]

Table 3. Effects of the CAP and CAPN on the percentage of mortality of the earthworm *L. terrestris* after 1, 2, 3, and 4 weeks of application

Compound	Conc. (mg/kg)	Mortality (%) ± SE			
		1 week	2 weeks	3 weeks	4 weeks
Control	-	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
CAP	3.75	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	7.5	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	15.0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	30.0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	60.0	$4.44^{a} \pm 2.94$	$10.00^{b} \pm 0.00$	$13.33^{c} \pm 3.34$	$20.00^{c} \pm 0.00$
CAPN	3.75	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	7.5	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	15.0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	30.0	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
	60.0	$0.00^{a} \pm 0.00$	$3.33^{a} \pm 3.33$	$10.00^{b} \pm 0.00$	$15.00^{c} \pm 0.00$

A one-way Analysis of Variance (ANOVA), performed with a significance level of $p \leq 0.05$, confirmed that the values within each column were not uniform. To specify which means differed significantly, a subsequent Student-Newman-Keuls (SNK) test was conducted. As a result, values within a column bearing the same superscript are not significantly different ($p < 0.05$) according to Student-Newman-Keuls (SNK) test.

Table 4 presents the effects of chlorantraniliprole nanosuspension and formulation on the weight of *L. terrestris* in artificial soil throughout 1, 2, 3, and 4 weeks after application. By examining the weight change of earthworms over time, insights can be gained into how these treatments might affect soil-dwelling organisms and, in turn, how they

may affect terrestrial ecosystems at large. It is essential to understand these effects so as to maintain the biodiversity of soils and ecosystem services in natural environments. The weights of *L. terrestris* exhibit diverse responses to chlorantraniliprole nanosuspension and formulation treatments. Over varying concentrations and time intervals, the earthworm weights display fluctuations, suggesting distinct reactions to the CAP and CAPN treatments.

Table 4. Effects of the CAP and CAPN on the weight of the earthworm *L. terrestris* after 1, 2, 3, and 4 weeks of application

Compound	Conc. (mg/kg)	Weight (g) ± SE			
		1 week	2 weeks	3 weeks	4 weeks
Control	-	$9.32^{cd} \pm 0.12$	$9.68^{cd} \pm 0.12$	$10.02^{cd} \pm 0.02$	$10.31^{cd} \pm 0.12$
CAP	3.75	$9.52^{c} \pm 0.16$	$8.84^{bc} \pm 0.07$	$8.98^{bc} \pm 0.06$	$8.74^{bc} \pm 0.07$
	7.5	$9.72^{c} \pm 0.06$	$8.86^{bc} \pm 0.06$	$8.89^{bc} \pm 0.04$	$8.74^{bc} \pm 0.05$
	15.0	$8.30^{b} \pm 0.07$	$6.96^{a} \pm 0.38$	$7.22^{a} \pm 0.38$	$6.94^{a} \pm 0.41$
	30.0	$7.73^{ab} \pm 0.03$	$7.44^{ab} \pm 0.16$	$7.61^{ab} \pm 0.12$	$7.34^{ab} \pm 0.41$
	60.0	$10.92^{d} \pm 0.03$	$7.59^{b} \pm 0.19$	$7.59^{c} \pm 0.19$	$7.59^{c} \pm 0.19$
CAPN	3.75	$9.05^{c} \pm 0.02$	$8.40^{b} \pm 0.05$	$8.40^{b} \pm 0.03$	$8.44^{b} \pm 0.05$
	7.5	$9.52^{c} \pm 0.05$	$8.59^{bc} \pm 0.09$	$8.52^{bc} \pm 0.02$	$8.04^{b} \pm 0.03$
	15.0	$8.07^{b} \pm 0.04$	$6.37^{a} \pm 0.17$	$6.91^{a} \pm 0.04$	$6.55^{a} \pm 0.05$
	30.0	$7.16^{a} \pm 0.05$	$7.19^{a} \pm 0.04$	$9.40^{d} \pm 0.09$	$10.22^{d} \pm 0.04$
	60.0	$10.72^{d} \pm 0.12$	$7.48^{a} \pm 0.02$	$7.91^{b} \pm 0.03$	$8.09^{b} \pm 0.02$

A one-way Analysis of Variance (ANOVA), performed with a significance level of $p \leq 0.05$, confirmed that the values within each column were not uniform. To specify which means differed significantly, a subsequent Student-Newman-Keuls (SNK) test was conducted. As a result, values within a column bearing the same superscript are not significantly different ($p < 0.05$) according to Student-Newman-Keuls (SNK) test.

The control group's weight values were relatively stable over the 4 weeks, providing a basis for assessing the effects of experimental substances. It is important to consider the data from control and treated groups in order to gain a clearer understanding of how each substance influences earthworm weights under artificial soil conditions. Weight is impacted over time by both formulation and chlorantraniliprole nanosuspension, especially at higher concentrations. The most reliable weight loss seems to occur around 15.0 mg/kg.

It was confirmed that the treatments inhibited normal growth because the control group gained the most weight. A weight increase at 30.0 mg/kg was one of the nanosuspension's variable effects, which could indicate varying metabolism or bioavailability. The amount of chlorantraniliprole residues in soil dropped below 20% after 42 days for all concentrations tested. While no adverse effects were observed on earthworms at 0.1 and 1.0 mg/kg, significant weight loss occurred at 10.0 mg/kg after 42 days, and chlorantraniliprole accumulation in earthworms increased with concentration.[24] There is a clear difference in sensitivity between bees and earthworms because chlorantraniliprole specifically targets and strongly activates insect Ryanodine Receptor (RyR) calcium channels. Bee RyRs are highly sensitive, causing fatal calcium release. Earthworm RyRs have very low affinity, so the insecticide has little effect. Furthermore, bees are exposed orally, and earthworms have limited dermal absorption and ingest the compound in soil, where it is often tightly bound, reducing its availability.

4. Conclusions

In this study, CAPN had a mean particle size of approximately 50 nm and excellent stability. Thorough evaluations of CAPN's efficacy and safety are essential, as the results are crucial for the development and application of future nanopesticides. CAPN showed moderate to high mortality in bees at all concentrations tested within 48 hours, which is not significantly different from the mortality exhibited by CAP under the same conditions. Mortality rates remained at 90% and 94% for doses of 60.0 mg/L of CAP and CAPN, respectively, at 48 h of application. Mortality rates ranged from 0% to 15% after 4 weeks of exposure in earthworms, indicating a moderate impact that was dependent on concentration. Higher concentrations and longer exposure periods led to increased mortality. The weights of *L. terrestris* exhibited diverse responses to CAP and CAPN treatments.

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Conflict of interest

The authors declare no competing financial interest.

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