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Assessment of bio-efficacy and genotoxicity of commercial insecticides and their nanoemulsion counterparts against cotton leafworm, *Spodoptera littoralis*

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Abstract

The purpose of this study is to develop nanoemulsions from commercial organophosphate insecticides and evaluate their efficacy toward *Spodoptera littoralis*. The nanoemulsions with uniform particle sizes, had average sizes of 255.8, 259.9, and 206.7 nm for Dora 48% (CPF), Deleron 50% (PFF_d), and Teliton-s 72% (PFF_t), respectively. Nanoemulsion forms were more effective against *S. littoralis* larvae than their commercial formulations by 33.90 to 62.15%. Also, they inhibited acetylcholinesterase and glutathione-S-transferase more than normal formulations. As for genotoxic effects, different mutagenic endpoints were used: the chromosomal aberrations assay, mitotic index, micronucleus test, and morphological sperm abnormalities. The study highlighted the potential benefits of nanoemulsion formulations in reducing genotoxic and reproductive toxicity compared with conventional insecticides and enhancing their bio-efficacy. Developing innovative nano-formulations that could mitigate the adverse effects of traditional pesticides on ecosystem attributes and bring about a transformative revolution in the agricultural sector. However, further research is needed to fully understand the long-term effects and environmental implications of nanoemulsion insecticides.

Keywords: Bio-efficacy; Enzymatic activity; Genotoxicity; Nanoemulsions; Organophosphate insecticides.

Introduction

Cotton cultivation is one of the vital and economic crop resources in Egypt. The cotton leaf worm, *Spodoptera littoralis* Boisd (Lepidoptera: Noctuidae), is a significant, economically devastating pest of numerous crops and vegetables. It is a key pest prevalent throughout Africa and the Middle East, including Egypt [1, 2]. It represents one of the most prevalent, injurious, and destructive pests that devour valuable economic crops like cotton, clover, maize, and several vegetables. It is also recognized as the most harmful pest of over 60 other economically significant crops, ornamentals, and vegetables [1, 3]. Farmers extensively applied synthetic chemical insecticides to mitigate the threat of this pest, but this practice resulted in strains of pests with appearance impediments, its residue caused defilement to circumference, which affected all living organisms [4]. The discriminate and intensive application of traditional pesticides resulted in significant environmental issues like air pollution, groundwater, and surface water contamination, the growth of insect resistance, health hazards, eradication of natural enemies (such as parasitoids and predators), letting pest populations grow exponentially [5], and posing serious toxicological risks to humans [6]. Over the last fifty years, the development of *S. littoralis* resistance to many registered insecticides and some insect growth regulators has been attributed to the intensive and ongoing use of widespread insecticides against it [7].

To prevent these issues, evolving new efficient and environmentally friendly pesticides are necessary [8]. It is critical to look for new, safer approaches with minimal effects on the ecosystem to avoid the risks associated with chemically synthesized

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insecticides that have already been mentioned [9]. Plenty of attempts have been initiated in Egypt to evaluate the anti-insecticide efficacy of various plant products versus *S. littoralis* as an option to control such insects [10]. The use of ecologically friendly nanoparticles is currently becoming increasingly vital in the field of plant protection. Applying nanotechnology could greatly benefit agricultural pest control [11].

Nanotechnology is a promising technology causing development in the agriculture sectors such other fields [12, 13]. Nowadays, the use of this technology to design and prepare nano-pesticides has shown great potential in creating novel formulations [14]. Due to their nano-properties, like excellent adsorption, good biocompatibility, bioaccumulation, and unusual relocation, these characteristics improve the superior effects of nanomaterials but also exhibit unidentified possible bio-toxicity [15]. Several investigations have assessed the effectiveness of various nanoparticles, however, there is still limited knowledge regarding their safety in the mammalian system [16]. Therefore, in-depth research is necessary to understand how nanoparticles affect biological organs. Our study is designed to investigate a novel trial for assessing the genotoxic impact of the new formulation, nano CPF, and the conventional organophosphate insecticide chlorpyrifos (*O*, *O*-diethyl-*O*-3,5,6-trichloro-2-pyridyl phosphorothionate; CPF), to compare and assess whether one could pose more risk than the other on mammalian organs. Different mutagenic end-points were conducted in our investigation: The chromosomal aberrations (CAs) assay, mitotic index, and micronucleus test. The selection of these end-points was based on the fact that the bone marrow assay is highly sensitive in sensing chromosomal abnormalities and micronuclei development. It is also quicker, has a lower cost, and is easier to run. This assay can be applied to assess the genotoxic risks of pesticides [17]. Additionally, the assessment of sperm morphological abnormalities is investigated among the most popular diagnostics for genetic toxicology. It is possible to identify spermatogenic dysfunction-inducing chemicals and genetic modifications [18]. Nanoemulsions have been generated by different techniques, including low-energy, high-energy, and combination techniques. Recently, nanoemulsions have been widely studied as a significant delivery scheme in contradiction of several pests as an insecticide, larvicide, acaricide, repellent activity, bactericide, fungicide, and anti-parasitic actions [19, 20]. One benefit of using nanotechnology in pesticides is their high efficacy and decreased negative impacts on non-target organisms [21]. This point is crucial since it will enable us to contrast their results with the pesticides' marketing presentations [22]. Thus, this work offers a promising method for formulating water-insoluble insecticides for spray applications with a small amount of formulation and active ingredients as well as may increase the efficacy of applied insecticides. The formulated nanoemulsions were characterized using physicochemical and topographical analysis. Additionally, the cytotoxicity of formulated nanoemulsions was evaluated.

Materials and Methods

Insect rearing

The cotton leafworm, *S. littoralis* was obtained from a research laboratory, Pests and Plant Protection Department, National Research Centre,Cairo, Egypt. The culture was reared for numerous generations without any insecticidal or microbiological pressure. According to Birnbaum et al. (2021) 23], the insects (*S. littoralis*) were reared on castor bean leaves (*Ricinus communis*) in a laboratory setting with 25 ± 2 °C and 60 ± 5% relative humidity.

Chemicals and tested insecticides

Three common pesticides, Dora (CPF), Deleron (PFF_d), and Teliton-s (PFF_t) were used in this study (**Table 1**) that compared with the references of range of Resistance Action Committee Mode of Action Insecticide (IRAC MoA). They were divided into two types of formulations: commercial formulations (emulsifier concentrate) and nanoformulations (nanoemulsions). All tested insecticides belong to the same pesticide class of organophosphate pesticides (OPs) and have the same modes of action against the tested pest. Colchicine was purchased from Sigma Chemicals Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Table 1. List of insecticides with their trade names, Common name, IRAC classification, and their producers (**Guideline, I.C.H., 2023**)

Trade names	Abbreviation	Chemical group	Common name	Manufacturer	IRAC MoA
Dora 48% (EC)	CPF	Organophosphate	Chlorpyrifos	Yingde Greatchem Chemicals Co., LTD China	1B
Deleron 50% (EC)	PFF_d	Organophosphate	Profenofos	El-Nasr Co. for Intermediate Chemical, Egypt	1B
Teliton-s 72% (EC)	PFF_t	Organophosphate	Profenofos	The National Company for Agrochemicals Productions, Egypt	1B

Preparation of insecticide nanoemulsions

Two phases of the process described by Hikal et al. (2023) [24] were used to manufacture insecticide nanoemulsions (**Fig. 1**). The organic phase was prepared by dissolving 10 mL of an insecticide in a pre-mixed solution of 10 mL toluene and 1 mL butanol. The aqueous phase is made up of 70 mL of water and 9 mL of surfactant (tween 80). The organic phase was added drop-wise to the aqueous phase while agitating at 4000 rpm for 30 min to create the emulsions. After that, the nanoemulsions were formed by subjecting the mixture to a 15-minute ultrasonic treatment at 50% power, using a frequency of 20 KHz and a pulse rate of 7 cycles per second. There was just a 25 ºC temperature difference between the first coarse emulsion and the final emulsion.

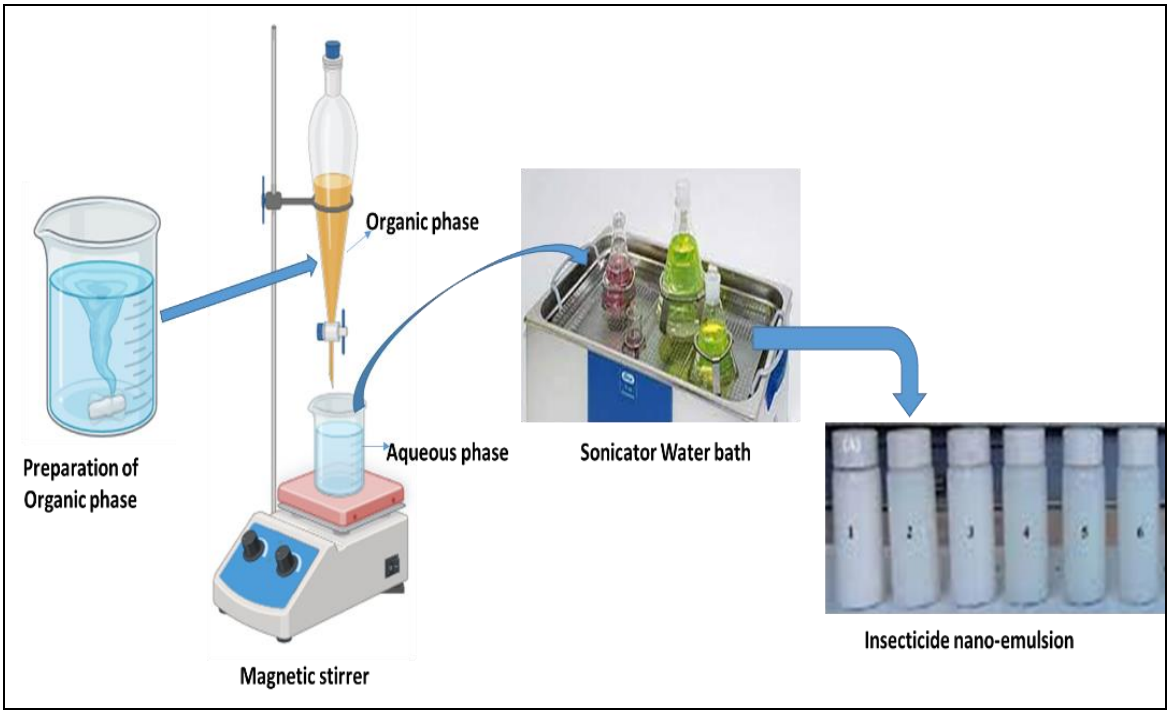

Fig. 1. Steps for preparation of insecticides nanoemulsion formulations.

Characterization of insecticide nanoemulsions size distribution

Nanoemulsion droplet size and polydispersity index (PDI) were measured using a Zetasizer Nano ZS (Malvern) at ambient temperature [25]. A PSS Dynamic Light Scattering System was used to determine the homogeneity and stability of the final nanoemulsions (Santa Barbara, California) at 23 °C. This technique employs a 632.8 nm helium-neon laser beam, scattered at 90 °C.

Transmission electron microscopy (TEM) characterization of insecticide nanoemulsions

The morphology of the stable nanoemulsions was characterized using high-resolution transmission electron microscopy (HRTEM). A small quantity of the nanoemulsion was placed on a carbon-coated copper grid, followed by staining with a phosphotungstic acid solution for one minute. After allowing the sample to dry at room temperature, it was examined and photographed using a JEOL 2100 HRTEM (Japan).

Efficacy of treated insecticides on *S. littoralis*

A leaf-dipping method was used. After dipping castor leaves in an insecticide solution for ten seconds, they were let to dry at room temperature for forty-five minutes. Five replicates of the treated leaves were offered to the larvae as food supply. Newly moulted 4th instar larvae of *S. littoralis* were used, and the mortality was recorded after 24 h of treatment.

Insect tissue preparation for biochemical studies

One gram of the treated larvae was ground in 5 mL of distilled water using a mortar for 3 min to extract enzymes. The obtained homogenate was then centrifuged under cooling at 3000 rpm for 15 min. The resulting supernatant was collected and used directly for biochemical studies. The supernatant could be stored at -20 °C for some time if necessary. The supernatant obtained from untreated larvae served as a control.

Assay of acetylcholinesterase (AChE)

The activity of acetylcholinesterase was determined in the supernatant of *S. littoralis* lysate (as an enzyme solution). Substrate specificity was evaluated through an assay involving three thioesters, following a modified process by Ali et al., (2024) [26]. In this process, a 10 µL portion of the supernatant was combined with 1.5 mL of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) dissolved in a 52 mM phosphate buffer at pH 7.2. Following thorough mixing and incubation, 50 µL of a solution containing acetyl thio-choline iodide (at a concentration of 156 mM) was introduced. The enzyme activity was measured by recording the increase in optical density resulting from the conversion of DTNB to 5-thio-2-nitrobenzoic acid, as described by Pavoni et al. (2019) [27]. Spectrophotometric monitoring at 405 nm was employed, and the appropriate corrections for spontaneous substrate hydrolysis was applied.

Assay of Glutathione-S-transferase (GST)

The supernatant of *S. littoralis* (as enzyme solution) was used to measure the activity of GST by spectrophotometr, based on the method of Darwesh et al. (2023) [28] with some alterations. An assay mixture of 1 mL (980 µL of PBS buffer pH 6.5 + 10 µL of 100 mM CDNB + 10 µL of 100 mM GSH) was made, and 900 µL of this mixture was added to a 1.5 mL cuvette with 100 µL of PBS as blank. The spectrophotometer was calibrated with 1 mL of distilled water as a blank. The absorbance was recorded every minute for three minutes at 340 nm. The initial rate of absorbance increases, directly proportional to the amount of GST in the sample.

Genotoxicity and cytotoxicity studies

Male white Swiss mice (*Mus musculus*), aged 10 weeks ±1 week, and weighing 25 g ± 2 g were used in our study. Animals were obtained from a closed random-bred colony at the National Research Centre (Cairo, Egypt) and under its ethical authority. The ambient temperature was kept at 22 °C ± 2 °C with a relative humidity of 50% ± 15% and 12 h light/dark cycle.

An important issue in the toxicity assessment is the method of administration. Although dietary supplementation may simulate human exposure to pesticide residues, the desired dose is often difficult to accurately administer in animals owing to their intake avoidance [29]. To control daily intake and simulate digestive-metabolic processes, an oral method of administration was applied in our study.

A total of 75 mice were used; 25 were used for morphological sperm abnormalities, and 50 were used for the CAs and micronucleus test examination. Mice were divided into five groups of five animals for each one. Group **I** served as the control group (untreated); Groups II and III were mice treated with CPF for 7 consecutive days at 6 and 12 mg/Kg dose levels, respectively, which were equivalent to 1/10 and 1/5 of oral LD_{50} in mice; Groups **IV** and **V** mice treated with Nano-CPF for 7 days at the same concentrations as conventional insecticide, 6 and 12 mg/Kg, respectively. The selected doses were based on previous studies by the EPA, (2000) [30]. Samples for the micronucleus test and chromosomal abnormalities were collected after 24 h. of the last treatment. In the morphological sperm abnormalities test, mice were treated for 5 consecutive days, and samples were collected 35 days after the first treatment. All experiments were approved and conducted according to the Animal Research Ethical Committee Guidelines of the National Research Centre, Egypt, and according to the guidance for the care and use of laboratory animals.

The Chromosomal aberration assay **(**CAs) was carried out as described by Fahmy et al. (2022) [31] and according to the Guideline of OECD 475 [32], for testing chemicals. Bone marrow cells were collected by flushing with hypotonic solution 0.56% KCl (pre-warmed at 37 ºC) from femur bone and incubated for 20 min at 37 ºC. The cell pellets were centrifuged at 1000 rpm for 5 min, fixed in freshly prepared aceto-methanol (acetic acid and methanol in the ratio 1:3, v/v). This step was repeated at least twice, and then the cells were suspended in a few drops of fixative, spread onto frozen slides, and run over the flame. Staining was done in 10% buffered Giemsa stain (pH 7.0) after 24 h of air drying and covering with coverslips. At least 100 well-spread metaphases were studied per animal, describing different CAs in bone marrow cells. CAs were scored under 2000× magnification with a light microscope (Olympus, Japan).

The mitotic index for cytotoxicity evaluation was calculated as a percentage by dividing cells out of the total bone marrow cells counted. The slides were examined under high power (40 X) of a compound light microscope [33].

$$Mitotic\ Index\ (MI) = \frac{Number\ of\ Dividing\ cells}{Total\ number\ of\ cells} \times 100$$

The standard test protocol and guidelines of OECD 474, [34] were followed for the micronucleus preparation from bone marrow. The bone marrow cells were separated in 3 mL of fetal bovine serum and obtained from both femurs, then centrifuged and smeared on slides. The slides were dried in the air and dipped in absolute methanol (10 ~ 20 min) for fixation. The fixed slides were stained using the May

Grünwald-Giemsa protocol. Micronuclei appeared as dark blue staining bodies in the cytoplasm of polychromatic erythrocytes (MPEs). The percentage of PCEs/100 nucleated cells (PEs + NE's) showed the ratio of erythrocytes to nucleated cells. For each animal (5 animals/group), 2000 cells (PEs + NEs) were counted. Scoring was conducted under 800× magnification with a light microscope.

The sperm abnormality assay was done following standard OECD guidelines, (2001) [35] and according to the method recommended by Wyrobek and Bruce [36] with some modifications the sampling time for this investigation was 35 days after the first injection. This is based on the principle that germ cells exposed to the chemical at a late spermatogonial stage would reach the cauda epididymis after undergoing developmental changes to give rise to sperm, which are then analyzed for shape abnormalities. Smears were stained with 1% Eosin Y. A total of 1000 sperm were counted per animal (5000 for each treatment), and different types of sperm abnormalities were scored, including head and tail abnormalities. Sperm preparations were examined by light microscopy at 800× magnification.

Statistical analyses

The data were analyzed with the computerized software SPSS (Statistical Package of Social Science, version 27, Armonk, New York: IBM Corp). Normality and homogeneity of variance were assessed using the Kolmogorov-Smirnov's test and Levene's test, respectively. One-way analysis of variance (ANOVA) followed by the Tukey HSD test was used to analyze the differences among groups with normal distribution. Results were considered significant when the *P*-value was less than or equal to 0.05.

Ethical approval

All experiments were approved and conducted according to the Animal Research Ethical Committee Guidelines of the National Research Centre, Egypt, and according to the guidance for the care and use of laboratory animals. The approval was performed according to the principles of the Ethics Committee of the National Research Centre (Approval No. 13050117, Cairo, Egypt).

Results and discussion

Preparation and characterization of insecticide nanoemulsion

Some disadvantages associated with traditional or normal forms of insecticides are poor stability, poor biological activity, coarse carrier particles, and poor dispersion. These properties increase the non-acceptance option for farmers and agricultural investors [37]. In addition, more than 90 % of insecticides run away into the environment and inhabit agricultural products in the application process. These negative impacts led scientists and researchers to introduce novel and non-traditional solutions like bioinsecticides, biologically active microbial substances, and nano-pesticides [38]. Nanoemulsion insecticide, as prepared in the current study, can be considered an option to replace the dangerous conventional one. Three insecticides, CPF, PFF_d, and Tilton commonly used to control various insects in their normal form, were converted to nanoemulsion form. The prepared nanoemulsion insecticides were characterized using high-resolution transmission electron microscopy to know the size and shape of either emulsion or insecticide particles. Also, the distribution of nanoemulsion particles in the final solution was characterized using a particle size distribution instrument. The pictures illustrated in **Fig. (2)** showed that the size of CPF-nano-emulsion particles ranged between 30 and 40 nm, containing inter-insecticide particles with size around 1 nm, and the shape of nanoparticles was spherical. Meanwhile, for PFF_d-nano-emulsion particles, the size ranged between 180 and 300 nm with a spherical shape, and the particles contained around 17 nm PFF_d particles. In the case of Tilton-nano-emulsion, the size of nanoparticles reached 100-250 nm with a uniform spherical shape, and the particles contained in their structure were insecticide particles with a size of around 20 nm (**Fig. 2**).

The particle size distribution of the prepared insecticides nanoemulsion was averaged at 255.8, 259.9, and 206.7 nm for CPF, PFF_d, and PFF_t insecticides, respectively, as illustrated in **Fig. (3)**. From the pictures (**Fig. 3**), the distribution of nanoparticles in the three insecticides was uniform. The use of nano-pesticides in agricultural and food production is currently underway [39]**.** According to an analysis by Wang et al. (2022) [40], compared to their traditional analogues, nanocides may be more effective, versatile, sustainable, and environmentally benign.

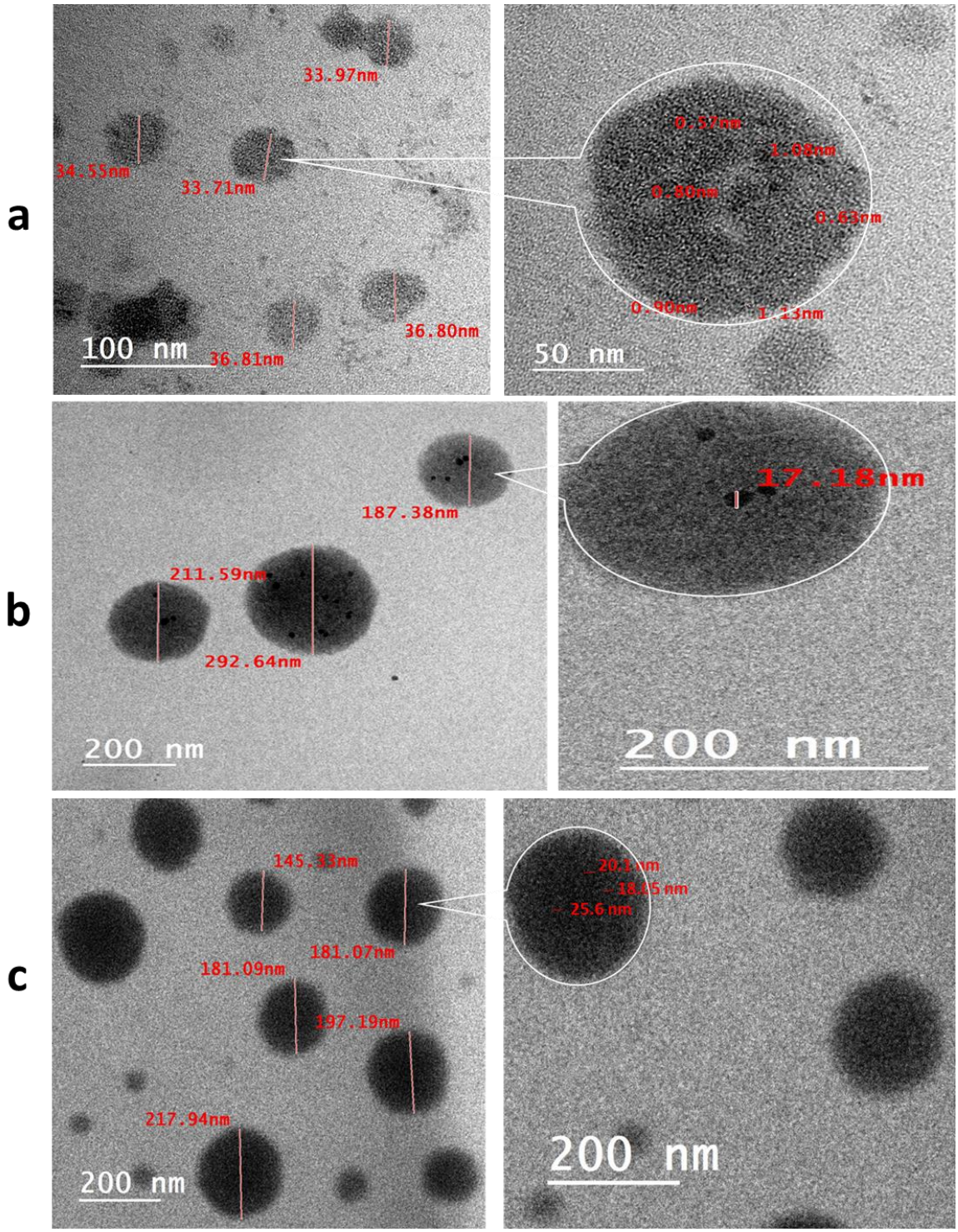

Fig. 2. HRTEM images of nanoemulsion form for CPF (a), PFF_d (b), and PFF_t (c) insecticides

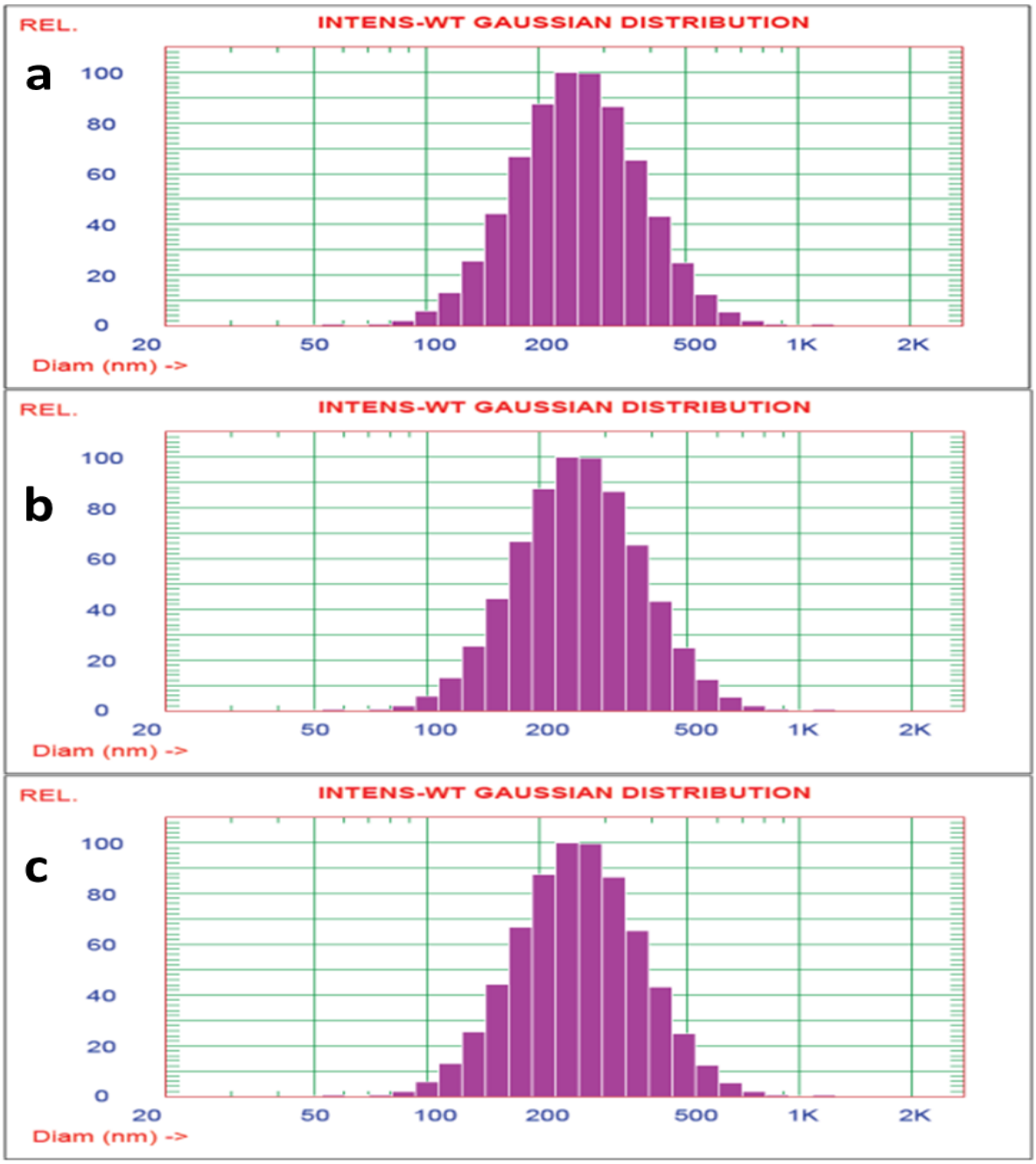

Fig. 3. Particle size distribution of insecticide nanoemulsion for CPF (a), PFF_d (b), and PFF_t (c)

Bio-efficacy of commercial and nanoemulsion insecticide formulations on *S. littoralis* larvae

All the tested insecticides (3 commons) were evaluated in conventional and nanoformulations against the 4th instar larvae of the cotton leafworm, *S. littoralis*. Data presented in **Table (2)** demonstrated the LC_{50} and LC_{90}, and slope values for the tested insecticides formulation (either EC or nanoemulsion) against *S. littoralis* under laboratory conditions using a leaf-dipping technique after 2 days of the treatment. The results in this study demonstrated that all the used formulations of insecticide exhibited noteworthy toxicity, with LC_{50} ranging from 4.96 to 9.91 mg/L for commercial formulations and 1.88 to 6.49 mg/L for nanoformulations. The compound transmutation process from the commercial formulation to the nanoformulation increased the efficiency of the CPF compound by 62.15 %, the PFF_d compound by 33.90 %, and the Tilton compound by 34.48 %. Compared to the EC and LC_{50} of nanoemulsion insecticides, all these compounds exhibited high toxicity against the targeted insect. It should be mentioned that using nanoemulsion formulations enhances the pesticides' distribution. Concerning the commercial formulations, CPF was the superior insecticide (LC_{50}= 4.962 ppm), followed by PFF_d (LC_{50} = 7.076 ppm),

while Tilton displayed the lowest toxic effects (LC_{50} = 9.909 ppm). Deteriorations in crop yield by pests and insects need the development of eco-friendly, green, safe, and novel insecticide formulations. The major problem facing the agricultural industry is using traditional agrochemicals that negatively impact crops and the environment. A nanoemulsion form of insecticides was prepared to achieve this objective by reducing the exposure of crops and the environment to harmful insecticides [41].

Table 2. Bio-efficacy of commercial and nanoemulsion formulations on *S. littoralis* larvae

Products	LC_{50} (mg/L)	95% Confidence limits (mg/L)	LC_{90} (mg/L)	95% Confidence limits (mg/L)	Slope ± SD	Intercept	X^2	df
CPF (EC)	4.962	2.963-6.937	10.283	7.238-51.027	4.049± 0.395	2.817±0.299	5.627	2
PFF_d (EC)	7.076	4.638-9.123	14.977	10.966-47.285	3.936±0.419	3.345±0.385	3.828	2
PFF_t (EC)	9.909	9.159-10.618	18.737	16.868-21.681	4.632±0.461	4.614±0.488	1.138	2
Nano-CPF	1.878	1.552-2.190	7.160	5.817-9.567	2.205±0.233	0.603±0.127	3.778	2
Nano- PFF_d	4.677	4.231-5.104	10.592	9.208-12.939	3.608±0.380	2.419±0.287	1.276	2
Nano- PFF_t	6.492	5.819-7.141	15.856	13.605-19.738	3.304±0.349	2.684±0.315	1.249	2

Mode of action of the tested insecticide formulations on the targeted insect

The enzymatic activity of *S. littoralis* larvae treated with the tested insecticides either in EC or Nano form was represented in **Table (3)**. AChE activity differed significantly among the tested insecticides and their nanoformulations. Lower AChE activities were observed in CPF (Nano) treatment (24.60 µM/mL/g tissue) than in CPF (EC) (47.31 µM/mL/g tissue), compared with control (75.7 µM/mL/g tissue). Data also cleared that PFF_d (Nano) was more effective than PFF_d (EC), with the activity of (32.17 and 41.63 µM/mL/g tissue) respectively. The highest AChE activity was observed in the Tilton treatment (53.56 and 43.34 µM/mL/g tissue) for EC and nanoformulation, respectively, compared with control (75.7). So, the inhibition percentage of the enzyme activity of the treated nano-insecticide was more effective than EC with 1.8, 1.28, and 1.46 folds for CPF, PFF_d, and Tilton, respectively.

For Glutathione-S-transferase (GST) activity, the investigated compounds' impact on GST also showed a similar action trend to AChE (**Table 3**). There was a substantial difference between each treatment and the control group. The results demonstrated that nanoformulations induced a significant decrease in GST enzyme activity than EC one, where CPF (Nano) displayed the lowest significant reduction in enzyme activity with 29.85 followed by PFF_d, and Tilton, which were 33.99 and 42.59 U/mL/g tissue, respectively. Conversely, the control sample exposed the highest enzyme activity (65.66 U/mL/g tissue). So, CPF (nano) showed the highest percent of inhibition with (54.54%).

Table 3. The activity of acetylcholine esterase (AChE) and Glutathione-S-transferase of *S. littoralis* larvae treated by the tested insecticides

Treatment	AChE		GST	
	Activity µm/mL/g	Inhibition	Activity µM/mL/g	Inhibition
Control	75.7±1.20 a	-----	65.66±1.55 a	-----
CPF (EC)	47.31±1.89 c	37.50	54.49±1.64 b	17.00
CPF (Nano)	24.60±1.89 f	67.50	29.85±0.33 e	54.54
PFF_d (EC)	41.63±1.20 d	45.01	39.68±2.35 c	39.56
PFF_d (Nano)	32.17±1.89 e	57.50	33.99±0.42 d	48.23
Tilton (EC)	53.56±0.57 b	29.25	52.19±1.13 b	20.51
Tilton (Nano)	43.34±5.87 cd	42.75	42.59±2.83 c	35.12
F-value	120.53		163.68	
***P*-value**	0.0000		0.0000	

Values that share same letter (s) within columns are not significantly different.

Genetic observations

Genotoxic studies and the evaluation of DNA damage are valuable tools for assessing the potential risks of pesticide exposure [42]**.** This study evaluated the genotoxic and clastogenic properties of a commercial pesticide CPF and its nanoform. **Table (4)** represents the frequency of CAs and MI persuaded in mice bone marrow cells after treatment with CPF and its nanoform. CAs are particularly dangerous to the cell due to the physical discontinuity of the chromosome which may lead to genome damage, loss of genetic information, and potentially cell death if a housekeeping gene is affected**.** A significant increase in the percentage of CAs, reached 18.8±0.97 and 39.8±1.53 after the treatment of animals for seven successive days with CPF at the dose levels of 6 and 12 mg/Kg, respectively. These percentages reached 8.2±0.58 and 13.4±0.87 in animals treated with the same dose levels of nano CPF, suggesting that commercial formulation has a significantly greater genotoxic impact than its nanoform.

The recorded data also confirmed that the percentage of DNA damage is strongly linked with the concentration. High-concentration treatment is more genotoxic. In this regard, exposure to acute or chronic chlorpyrifos produced reactive oxygen species that significantly damaged PC12 cells and increased DNA damage in the liver and brains of rats in a dose-dependent manner [43]**.** CPF significantly increased chromosomal abnormalities and the rate at which Chinese hamster pneumonocytes, rat bone marrow cells, and mouse spleen cells generate micronuclei [44]**.** The adverse effect of CPF was also investigated in aquatic organisms [45]**.** Regarding aberration types, metaphases including endomitosis constituted the majority of aberrant cells, followed by multiple aberrations (M.A.), which encompassed more than three forms of abnormalities within a single metaphase. Metaphases with fragments and/or breaks were also recorded **(Fig. 4).** Since gaps were observed to be correlated with DNA damage using the comet test in a study of people exposed to pesticides at work, gaps were included in our analysis as a kind of CAs**.** Gaps appear to result from single-strand breaks in the DNA, which prevent DNA polymerase from working during replication [46].

Table 4. Frequency of chromosomal aberrations & mitotic index induced in male mice after treatment with different doses of CPF and its nano-form

Experimental groups and doses	Abnormal metaphases		No. of metaphases with different types of chromosomal aberration					Mitotic index
			Structure aberrations				Numerical aberrations	
	No.	Mean± SE	Chromatid gap	Frag. and/ or break	M.A	Endomitosis	Polyploidy	Mean± SE
I-Control (non-treated)	11	2.2±0.37^{a}	5(1.0)	6(1.2)	0	0	0	6.2±0.28^{d}
II- CPF (6 mg/Kg.)	94	18.8±0.97^{d}	10(2.0)	29(5.8)	31(6.2)	22(4.4)	2(0.4)	3.5±0.24^{b}
III- CPF (12 mg/Kg.)	199	39.8±1.53^{e}	16(3.2)	42(8.4)	61(12.2)	75(15.0)	5(1.0)	2.12±0.29^{a}
IV- Nano CPF (6 mg/Kg.)	41	8.2±0.58^{b}	6(1.2)	20(4.0)	11(2.2)	4(0.8)	0	5.10±0.27^{c}
V- Nano CPF (12 mg/Kg.)	67	13.4±0.87^{c}	8(1.6)	37(7.4)	12(2.4)	9(1.8)	1(0.2)	3.04±0.32^{b}
F-value		229.86						34.45
***P*-value**		0.0000						0.0000

Values that share same letter(s) within columns are not significantly different.
Where: A total of 500 cells were analyzed for chromosomal aberrations (5 mice per group; 100 cells per mouse).
Frag. = Fragment, M.A. = More than one aberration.
Statistical analysis was performed using one-way ANOVA–Tukey's multiple comparisons test. Values with different superscript letters in each column are significantly different.

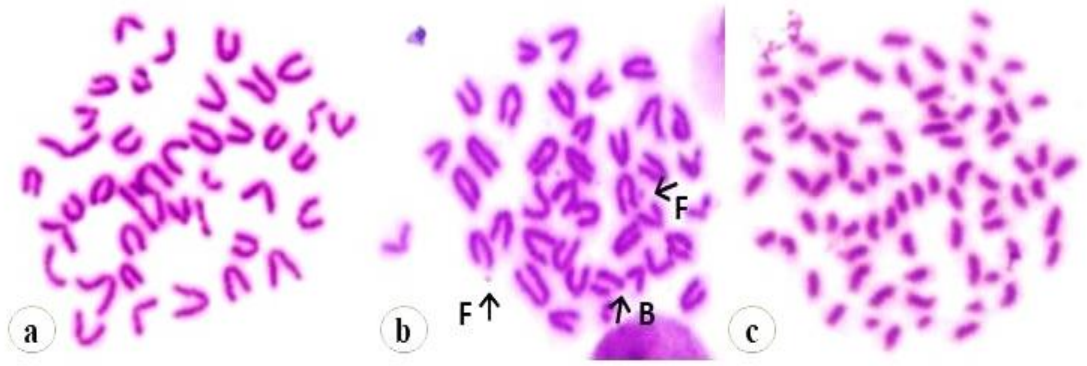

Fig. 4. Chromosomal abnormalities in mice bone-marrow cells showing normal (a), fragment and break (b), and endomitosis (c) (magnification power: 2000X).

This adverse effect of CPF may be attributed to the generation of reactive oxygen species and the induction of intracellular oxidative stress [47]. The CPF is converted to a more potent cholinesterase inhibitor, chlorpyrifos oxon (CPO), through a cytochrome P450-mediated desulfuration reaction. It is also possible that CPF-induced DNA damage may be caused by CPO alone or in combination with CPF. Moreover, most pesticides are lipophilic and can interact with living organisms through the lipid-rich biomembranes**.** Further, chlorpyrifos can cause DNA cross-links due to the presence of two methoxy groups that function as alkylating agents [43]**.** One of the most important cellular events that occurs throughout development is the cleavage of DNA into its constituent inter-nucleosomal fragments, which is essential to the generation of genotoxicity by chlorpyrifos**.** Chlorpyrifos can compete with ethidium bromide (EB), as a fluorescence probe, and binding with CT DNA instead of EB-CT DNA complex. In this way, it is capable of inducing chemical damage to the DNA of various organisms**.** The use of various mutagenic parameters in our work to support one another is a crucial factor to take into consideration. Micronucleus assay MN is a remarkable test for measuring the genotoxic potential of chemicals, DNA damage, and carcinogen screening [31]**.**

Micronuclei are extra-nuclear bodies that contain damaged chromosome fragments and/or whole chromosomes not incorporated into the nucleus after cell division**.** The results of the MN assay are shown in **Table (5) and Fig. (5).** A significant increase in the percentage of micronuclei in polychromatic cells MPEs was observed in all treated groups compared with control. This percentage reached 9.46 after treatment of animals with CPF at 6 mg/Kg. On the other hand, it recorded 5.95% in the group of animals treated with the same concentration of nano CPF, indicating the higher genotoxic potency of CPF in a common formulation than its nanoform. Our results also clearly investigated the relationship between the tested concentrations and the level of induced damage, which are important aspects to be considered. According to Eastmond (2008) [48]**,** there is a strong relationship between cytotoxicity and genotoxicity since cytotoxicity is usually a subsequent outcome of genotoxicity and frequently occurs after significant genotoxicity. The results also indicated that CPF and nano-CPF increase the number of PCEs/total counted cells, indicating severe toxicity in bone marrow cells [48]. High doses of CPF induce a significant percentage of PEs. It reached 17.25, representing about threefold more than the control **Table (5).** The mitotic index was conducted to determine whether CPF and its nanoform are cytotoxic. The recorded data were in harmony with the MN assay, which markedly indicates the cytotoxicity of CPF and its nanoform in all the treated groups **(Table 5).** The level of cytotoxicity of the tested compound can be determined by an increase or decrease in MI [41]. If the MI value is lower than that of the control, it suggests that cell division is being inhibited. This means that the chemical substance to which the cells are exposed interferes with the growth and development of the test organism.

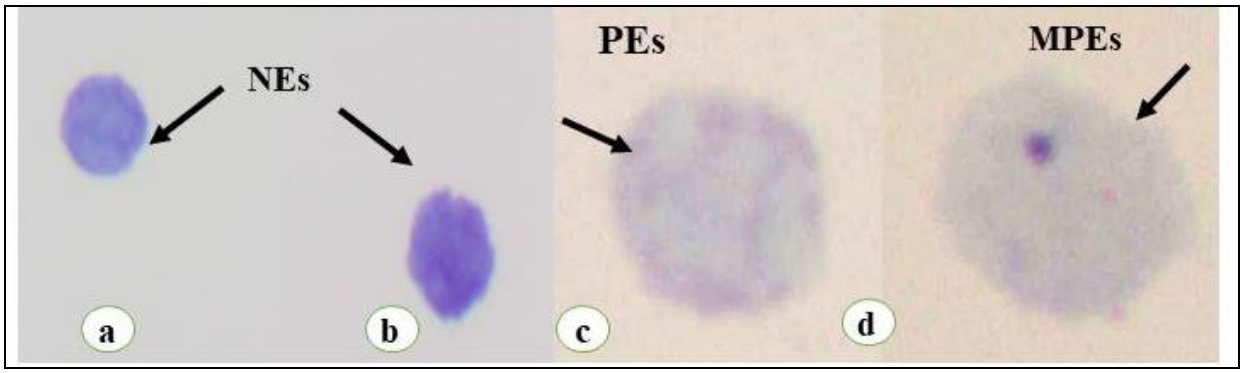

Fig. 5. Mice bone-marrow cells showing normochromic erythrocytes (a&b), polychromatic erythrocytes (c), and micronuclei in polychromatic cells (d)

Table 5. Percentage of polychromatic erythrocytes (PEs) and micronuclei in polychromatic erythrocytes (MNPEs) induced in mouse bone-marrow cells after treatment with different doses of CPF and nano CPF.

Treatment and doses	No. and percentage of PEs		No. and percentage of MNPEs	
	No.	Mean ± SE	No.	Mean ± SE
I- Control (untreated)	532	5.32 ± 0.42^{a}	9	1.70 ± 0.36^{a}
II- CPF (6 mg/Kg)	1216	12.16 ± 0.13^{c}	115	$9.46 \pm 0.59^{c,d}$
III- CPF (12 mg/Kg)	1725	17.25 ± 0.17^{d}	176	10.20 ± 0.46^{d}
IV- Nano CPF (6 mg/Kg)	824	8.24 ± 0.35^{b}	49	5.95 ± 0.43^{b}
V- Nano CPF (12 mg/Kg)	1136	11.36 ± 0.19^{c}	97	8.54 ± 0.44^{c}
F-value		263.92		55.30
***P*-value**		0.0000		0.0000

Values that share same letter(s) within columns are not significantly different.
Where: No. of examined nucleated cells = 2000 / mouse (5 mice/group). Values with different subscript letters in each column are significantly different. The data are presented as mean ± SE (n=5)

Our results confirmed that NPs have genotoxic and cytotoxic effects on mice. These effects were linked to generating reactive oxygen species (ROS), resulting in cell propagation and DNA damage**.** Once nanoparticles (NPs) come into cell contact, they cause the production of a lot of oxidative active chemicals in the cells, like superoxide, peroxides, and free radicals**.** The primary source of these compounds is the breakage of the electron transmission chain [49]**,** which may interact with internal proteins to cause the denaturation of proteins and damage to mitochondria**,** and nucleic acid [50]**.** It can also obstruct the passage of biological signals and gene information. Reactive oxygen destroys the nuclear membrane directly or interacts with DNA and related proteins to cause damage to genetic material and the production of genetic toxicity when it enters the nucleus. Additional research has shown that NPs can cause a discernible increase in the activation of stress-related genes, antioxidant defense, and genome integrity. This is always accompanied by an increase in ROS levels inside the cell. The buildup of NPs, either non-degradable or gradually degradable in the organs, is a major cause for concern. Additionally, NPs may overburden phagocytes, triggering stress responses. These responses ultimately result in inflammation and weakened defenses against additional infections**.** Although the data concerned with the genotoxicity of nano-pesticides and their mechanism of toxicity were discussed in our study, it is important to be certain that its genotoxicity is less than that induced by the conventional formula. Compared to traditional pesticides, NPs are safer for non-target creatures, according to a wealth of experimental data [40]**.** For instance, nanostructured alumina, which is being studied as an alternative to chemical insecticides, is less harmful to humans than commercially available chemical pesticides. Experimental data indicates that nanostructured alumina causes significantly less chromosomal breakage, DNA damage, and cell viability in human peripheral blood lymphocytes compared to commonly used organophosphates**.** In another study, parquet, a commonly used herbicide formulated in chitosan grafted porous carbon, exhibited significantly lower toxicity or non-toxicity towards human hepatic cells than the technical parquet. The technical parquet displayed high toxicity and resulted

in very low cell viability (44%), whereas the nano-formulated parquet showed much higher cell viability (90%). Furthermore, the administration of parquet to mice led to a 100% mortality rate within 3 days. Conversely, when mice were exposed to the nano-formulated parquet, they all managed to survive [51]**.** Consistent with our findings, other research indicates that NPs can, in comparison to conventional pesticides, reduce the toxicity of non-target organisms by 43 % [40]**.** The decrease in the toxicity of nanoformulations is due to the dispersible nature of NP, which prevents binding and allows for rapid elimination from the body. Additionally, the hydro-dispersive colloidal form of the NP prevents aggregation and enhances elimination, reducing the amount of time the NP remains in the body. Furthermore, nano-encapsulation allows for controlled release of lower, consistent amounts of NP for exposed organisms.

Measuring morphological abnormalities in sperm may be used to detect reproductive harm and perhaps hereditary mutations brought on by exposure to physical or chemical factors**.** Our data noted a significant percentage of morphological abnormalities in sperm after CPF and nano-CPF treatments with an abnormality of sperm tail and head. The maximum percentage of sperm defects reached 27.2 % after treatment with CPF at the dose level of 12 mg/Kg *vs* 2.76% for the control **(Table 6).** The amorphous heads of the sperm were the more noticeable anomalies. Sperms with coiled tails, triangle, banana, and without hook were also recorded in all treated groups **(Fig. 6).** In the highest tested dose of CPF, sperms with amorphous head and coiled tail defects represent 13.9 and 6.78 %, respectively, compared with 1.16 and 0.52 % for the control. This increase indicates its probable potential to act as germ cell mutagen, mainly due to increased ROS levels. In line with our results, Hassan et al., (2022) [52] reported that chlorpyrifos induces male reproductive damage by increasing oxidative stress, which adversely affects sperm survival. Certainly, oxidative stress is one of the main causes of DNA damage and male infertility through the loss of membrane integrality and cellular function, leading to chromatin damage. Spermatogenic arrest and male sterility have been linked to sperm head and tail abnormalities, specifically defects in nuclear chromatin integrity and insufficient sperm maturation during epididymis transport [44]. Additionally, some organophosphate compounds have been found to cause genetic damage by indirect DNA alkylation with pesticide metabolite residues**.** Consistent with our findings, prolonged exposure to CPF for 30 days at the dose levels of 5 and 10 mg/Kg resulted in reproductive dysfunction characterized by decreased levels of testosterone hormone and increased levels of follicular-stimulating hormone. This led to a decrease in sperm motility and count [48]. In vitro studies conducted with OPs have demonstrated that these pesticides have the potential to damage DNA in human sperm, causing genotoxic effects [50]**.**

Table 6. Percentage of sperm abnormalities in male mice induced after treatment with various doses of CPF and nano-CPF

Experimental groups and doses	Total abnormal sperm		No. and (%) of different types of sperm abnormalities				
			Head abnormalities				Tail abnormalities
	No.	Mean (%)±SE	Amorphous	Without hook	Triangle	Banana	Coiled tail
I- Control (untreated)	138	2.76±0.43[a]	58(1.16)	34(0.68)	12(0.24)	8(0.16)	26(0.52)
II- CPF (6 mg/Kg)	566	11.32±0.89[b]	112(2.24)	82(1.64)	33(0.66)	19(0.38)	320(6.40)
III- CPF (12 mg/Kg)	1360	27.20±1.05[c]	697(13.94)	152(3.04)	113(2.26)	59(1.18)	339(6.78)
IV- Nano CPF (6 mg/Kg)	454	9.08±0.53[b]	89(1.78)	53(1.06)	22(0.44)	17(0.34)	273(5.46)
V- Nano CPF (12 mg/Kg)	562	11.24±0.79[b]	106(2.12)	88(1.76)	31(0.62)	24(0.48)	313(6.26)
F-value		133.46					
***P*-value**		0.0000					

Values that share same letter(s) within columns are not significantly different.

Where: The total number of examined sperms is 5000 per treatment (1000 /mouse, 5 mice/group. One-way ANOVA–Tukey's multiple comparisons test was used. The values having different superscript letters in each column are significantly different from one another at $P<0.05$

It was also clear that Judo 40, a commonly used organophosphorus pesticide, could significantly impact both rat and human sperm in vitro [51]. Numerous studies confirmed the relationship between OPs and multiplicative disorders by altering genital function and reducing brain acetylcholinesterase (AChE) action, which affects the pituitary gonadotropin and leads to infertility. Males exposed to OP had a higher risk of developing abnormal semen parameters, including sperm motility reduction, decreased sperm count, and a higher incidence of sex chromosome aneuploidy in sperm [40]. Finally, our study investigated that, both CPF and its nanoform exhibited clastogenic effects. However, CPF poses a greater risk than its nanoform. Additionally, both tested compounds showed detrimental effects on reproductive organs, as evidenced by the elevation in the percentage of sperm abnormalities. Every pesticide can be toxic when the levels of exposure are high enough. Consequently, no pesticide is completely safe. It recommends that the use of NPs in environmental applications should be limited until it is established that the advantages outweigh the hazards.

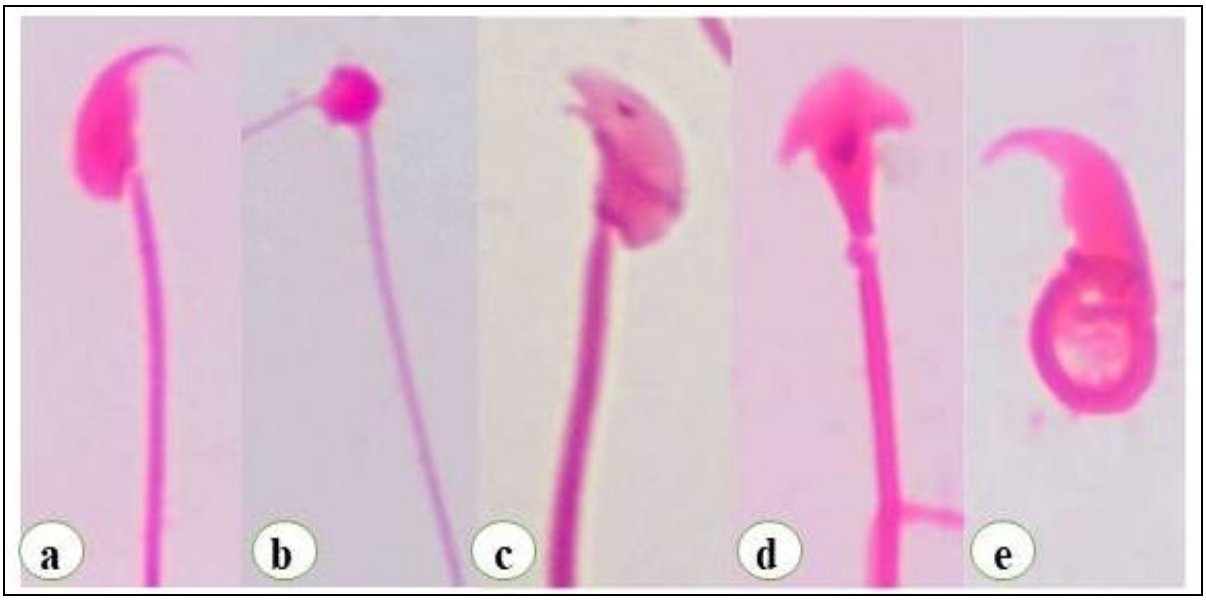

Fig. 6. Sperm abnormalities in mice showing normal (a), amorphous (b), without hook (c), triangular (d), and coiled tail (d)

Conclusions

The present study aimed to develop nanoemulsion forms of three insecticides (CPF, PFF_d, and Tilton) and compare their bio-efficacy, mode of action, and genotoxic effects with their conventional formulations against the cotton leafworm (*S. littoralis*). The results showed that the nanoemulsion insecticides had smaller and more uniform particle sizes than the commercial ones, which enhanced their distribution and penetration into the insect body. The nanoemulsion insecticides also exhibited higher toxicity and lower enzyme activity than the commercial ones, indicating a more effective inhibition of the insect's physiological processes. Moreover, the nanoemulsion insecticides caused less DNA damage, CAs, and sperm abnormalities than the commercial ones, suggesting a lower genotoxic and reproductive risk. These findings demonstrate that nanoemulsion insecticides are a promising alternative to traditional pesticides, as they can improve pest control efficiency and reduce health and environmental hazards.

Declarations

Author Contributions

N.M.A.: Visualization, Methodology, writing–original draft, Writing-review & editing, Formal analysis, Data curation. **A.M.E.B.:** Visualization, Formal analysis, Data curation, Writing– review & editing. **E.E.H.:** Animal treatments and sample collection for genetic observations, Formal analysis, Writing – review & editing, Data curation. **MSH**: Visualization, Methodology, Investigation, Formal analysis, writing–original draft, Writing-review & editing. **O.M.D.:** Visualization, Methodology, Investigation, Formal analysis, writing–original draft, Writing-review & editing.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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