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THE EFFECT OF DIRECT AND INDIRECT USE OF NANOPARTICLES ON COTTON LEAF WORM, *SPODOPTERA LITTORALIS*

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ABSTRACT: Nanoparticles proved its important role to control pests even by directly use or indirectly as an additive to other pesticides. Copper and Zinc Nanoparticles showed significant effect on the 2nd instar larvae of *Spodoptera littoralis* even alone or combined with Vertimec.
ZnO, CuO+Vertimec, ZnO+Vertimec, and CuO nanoparticles and Vertimc caused mortality with 100, 100, 86.67, 73.33 and 33.33 %, resp. Some treatments showed malformation and morphological changes by nanoparticles adsorption through the integument of the 2nd larval stage of *Spodoptera littoralis*.
Most of the dead larvae malformed and underwent liquefaction. Apoptosis was showed through DNA damage response, which increased with the addition of Vertimec to nano metal particles but nano ZnO recorded the highest effect. Oxidative stress interacted effectively with induced DNA as a result of treatments.

Keywords: Spodoptera littoralis, Nanoparticles, CuO, ZnO, Vertimec, DNA, Apoptosis, Oxidative Stress.

INTRODUCTION

Spodoptera littoralis (Bosid) (Lepidoptera: Noctuidae) is an extremely serious pest. The larvae can defoliate many economically important crops cutting across over 40 families, such crop includes cotton and tomatoes (EPPO 2008).The use of insecticides in agriculture field causes biological imbalance (Yadav 2010), and some new eco-friendly formulations pesticides became the target (Bulmer *et al*. 2009, Zhang and Xiao-zhen 2010 , Cloyd and Bethke 2011).

Nanoparticles possess distinct physical, biological and chemical properties associated with their atomic strength (Leiderer and Dekorsy, 2008).Nanoparticles (which are 1-100 nm in diameter) are agglomerated atom by atom, and their size (and some-times shape) may be maintained by specific experimental procedure (Roy 2009).They can be arranged or assembled into ordered layers, or mine layers (Ulrich *et al*., 2006).

Such self-assembly is due to forces such as hydrogen bonding, dipolar forces, hydrophobic interactions, surface tension, gravity and other forces. Thus nanotechnology deals with the targeted nanoparticles as and when the particles exhibit different physical strength, chemical reactivity, electrical conductance and magnetic properties (Nykypanchuk *et al*., 2008).

Nanotechnology, a promising field of research opens up in the present decade a wide array of opportunities and is expected to give major impulses to technical innovations in a variety of industrial sectors in the future. Nowadays, nanotechnology has being embraced in the world of pesticides and pest control (Harper 2010) and has a potential to revolutionize modern day agriculture pest control, different groups of nano pesticide overcome like insecticides, fungicides, herbicides (Matsumoto *et al*. 2009) .

The new nanotechnology with materials having unique properties than their macroscopic or bulk counter parts, has promised applications in various fields.

Nano-pesticides and nano-encapsulated pesticides are expected to reduce the volume of application and slow down the fast release

kinetics (Niemeyer and Doz 2001, Leiderer and Dekorsy, 2008).

The toxic effects of NPs can be attributed to the small size and large surface area, thereby increasing chemical reactivity and penetration in the living cells (Gojova *et al.*2007, Medina *et al.*2007,Pan *et al.*2009).

Oxidative stress is a state of redox disequilibrium in which reactive oxygen species (ROS) production overwhelms the antioxidant defence capacity of a cell; thereby, it could lead to adverse biological consequences. ROS are generated during photo-activation, of some chemicals on the particle surface, or as a consequence of the interaction between particles and cellular components (Dreher 2004, Hirakawa *et al*.2004).

The *Spodoptera littoralis* as a common pest on many crops was selected in the present study as a model to assess the toxicity and eventuality of insect's body toxicity. Oxidative stress especially with Vertimec treatment, DNA damage and apoptosis had been adopted in the present study.

MATERIALS AND METHODS

Insect

Spodoptera littoralis was cultured on leaves of the castor oil plant (*Ricinus communis* L.) in Plant Protection Research Institute, Agriculture Research Center, Mansoura Branch, Egypt. Larvae were kept at 25±1 °C, 70% RH and 16L: 8D of photoperiod. The second instar larval stage of the insect was used in the insecticidal bioassay.

Pesticide

Vertimec (Abamectin 1.8 EC) was used with specific quantity in all treatments (1 ml /100 ml).

Used Nanoparticles and Characterization

Zinc Oxide and copper oxide nanoparticles were prepared at Nanotech Egypt as followed:

The Zinc oxide nano rods are prepared depending on the process involves the ultrasonic irradiation of 50 mL of aqueous solution containing 0.05 M of zinc acetate and 0.05 M of tri-ethanolamine, at 80°C for two hours. The resulting product was washed several times with de-ionized water followed by methanol. Filtered and dried at 022 ° C in air for 2 hours. The structural morphology of the particle was done by scanning electron microscope. The particles are found to be rod shaped with 100 to 250 nm dia and 1 to 2 nm lengths.

Other common method that used to prepare CuO-NPs is wet chemical precipitation method (Kida *et al*.2007). CuO nano rods with the width range of 5-15 nm can prepared using copper nitrate [Cu(NO3)2.3H2O] as precursor and water-ethanol mixture solvent and NaOH as reducing agent at 77-82 ºC. The study showed that the breadth increased with increases Cu ions concentrations.

Bioassay

Leaf discs of castor oil plant were prepared and dipped in each treatment. Every treatment had three replicates with 10 larvae/replicate. Mortality and other results were taken after 24 hours of the exposure. The main amount used of all nanoparticles in this study was 0.01 g/L and 0.1 ml/L of Vertimec.

DNA Fragmentation

S. littoralis larval haemocytes were collected at 24hours, washed with PBS (pH 7±2) buffer with 0±1% DTT and centrifuged for 1 min at 1000 g. The pellets were then incubated in lysis buffer (20 mM Tris–HCl, pH 8±0, 10 mM EDTA and 1% NP-40) for 5 min and the supernatants collected after being centrifuged at 1600 g for 5 min. SDS and RNase A was added to a final concentration of 1% and 5 µg/ml, respectively. After 2 h of incubation at 56 °C, the supernatants were digested with proteinase K at 37 °C for 2 h to a final concentration of 2±5 µg/ml. The DNAs were precipitated with ethanol, dissolved in TE and separated by electrophoresis on 1±5% agarose gels. DNA damage was visualized under fluorescence microscope (Carl Zeiss) after staining with a fluorescent DNA-binding dye ethidium bromide. Results were analyzed by Comet IV software. DNA ladder assay was

conducted according to the method of Ishizawa *et al.* (1991) with slight modification. DNA samples were analysed byhorizontal electrophoresis for 5h in 2%agarose gelpre-stained with 1 mmol / Lethidiumbromide. The DNA was visualized under UV light.

Morphological Study

The 2nd instar larvae of *Spodoptera littoralis* were compared before and after treatments. Changes in their bodies were photographed and discussed.

Antioxidant of Enzyme Activities

APX activity was measured by estimating the rate of ascorbate oxidation (extinction coefficient 2.8 mM/cm). The 3 mL reaction mixture contained 50mM phosphate buffer (pH 7.0), 0.1 mM H2O2, 0.5 mM sodium ascorbate, 0.1 mM EDTA and a suitable aliquot of enzyme extract. The change in absorbance was monitored at 290 nm and enzyme activity was expressed as unit's min/mg protein (Nakano and Asada 1981).

SOD activity was measured by the photochemical method as described by Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of nitro blue tetrazolium (NBT) reduction at 560 nm in the presence of riboflavin and light. The reaction mixture contained 45 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 13 mM methionine, 0.17 mM NBT in ethanol, 0.007 mM riboflavin and enzyme aliquot. Blanks were kept in the dark and the others were illuminated for 15 min. One unit of SOD is the amount of extract that gives 50% inhibition to the rate of NBT reduction.

Statistical Analysis

The statistical software SPSS for Windows 16.0 was used to perform T-test. Values of $p<0.05$ and $p<0.001$ were considered as statistically significant values.

RESULTS

Certain Effects of Nanoparticles on *S.littoralis*

Results showed that used nanoparticles at 0.01 g quantity, caused mortality and malformation in Fig.(1). Mortality recorded 100% and 73.33% in case of ZnO and CuO nanoparticles, resp. While, that ratio decreased to 86.67% in case of ZnO+Vertimec and increased to 100% with Vertimec+CuO , however Vertimec itself caused 33.33%.

Malformation was noticed clearly with treatments including nanoparticles + Vertimec with the same ratio gained in both treatments (33.33) % .Therefore, CuO only malformed *S.littoralis* larvae by 16.73%. All malformed larvae in all treatments died and counted in mortality % and also showed as separately ratio.

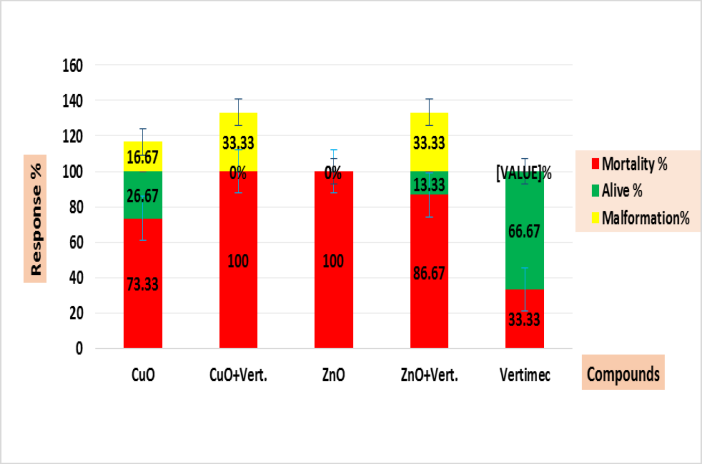


Fig. (1) Effect of nanoparticles alone and with Vertimec on the 2nd larval stage of *Spodoptera littoralis*

The statistical analysis depending on T-test showed significant differences among treatments. The highly significant treatments with all its responses were CuO , ZnO+Vertimec , CuO+Vertimec , ZnO and then less significant treatment was with Vertimec.

Morphological Effect

2nd stage of *Spodoptera littoralis* larvae affected highly after acute exposure to nanoparticles with and without Vertimec. The most effective treatments were occurred by CuO+Vertimec and ZnO+Vertimec followed by CuO.

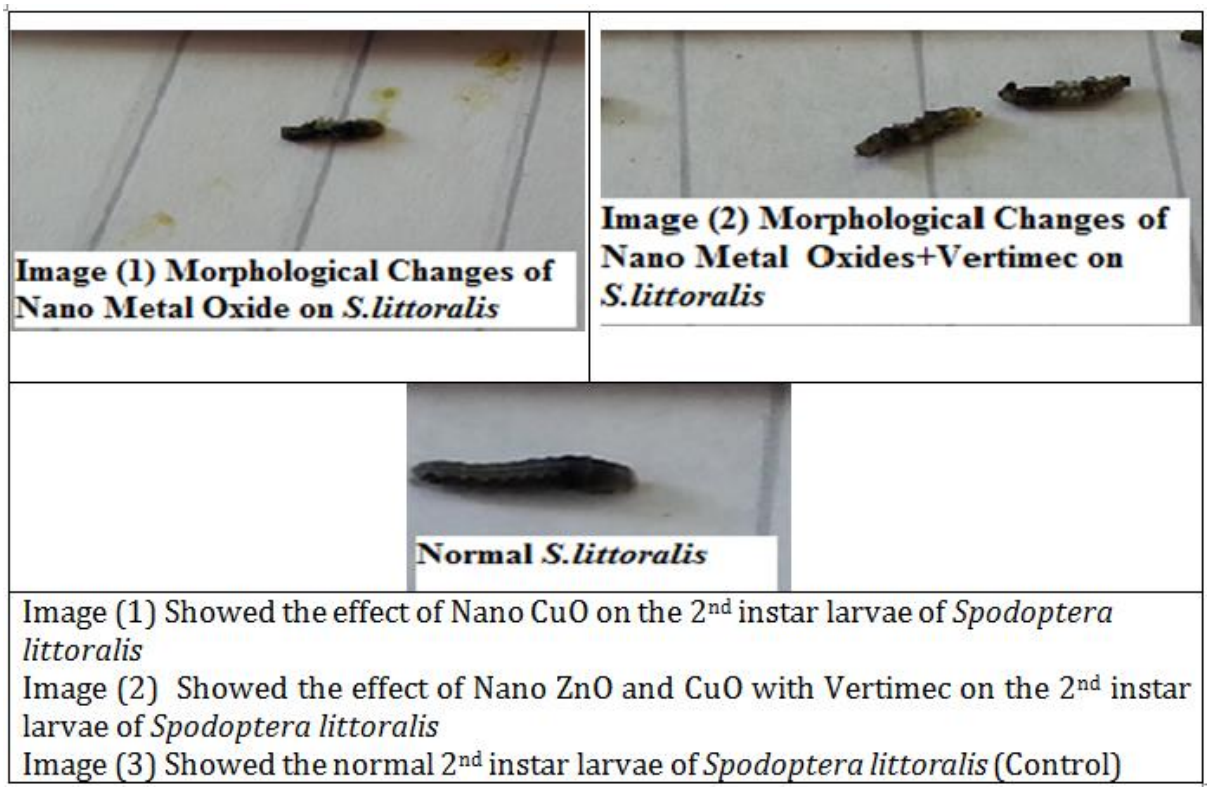


Image (1) Showed the effect of Nano CuO on the 2nd instar larvae of *Spodoptera littoralis*
Image (2) Showed the effect of Nano ZnO and CuO with Vertimec on the 2nd instar larvae of *Spodoptera littoralis*
Image (3) Showed the normal 2nd instar larvae of *Spodoptera littoralis* (Control)

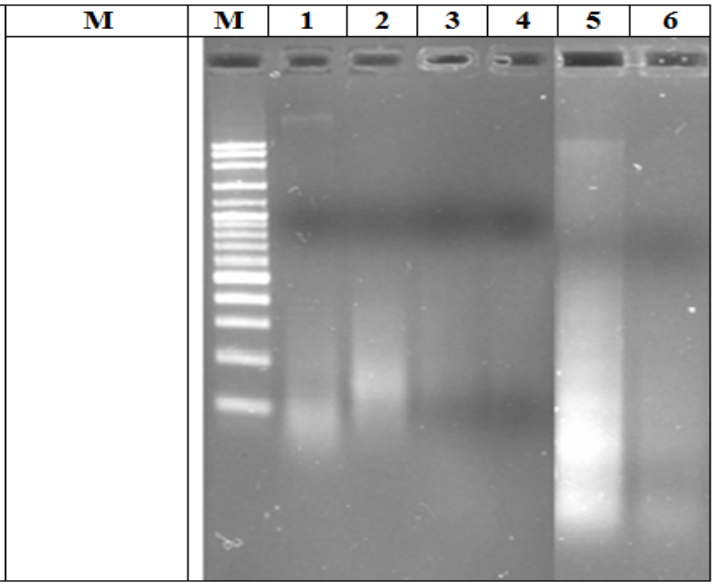
Malformation appeared during the duration of each treatment till death occurring after 24 hrs as showed at Images (1, 2 and 3). Larvae colors were turned to dark grayish. All malformed larvae were died after their bodies liquefy and melanize as usual when infected with baculoviruses which mainly occurred in case of Vertimec+Nano particles.

The integuments were exploded and body contents were out especially in case of treatments with the nano metal oxides. Even after that, the nanoparticles were able to cause their effect on other alive larvae as the same as virus particles but with faster death.

DNA Damage and Apoptosis

There were significant increase in apoptotic cases in the treated insects, for 24 hours, in comparable with the control group ($p<0.001$) Image (4).

Image (4) DNA fragmentations of *Spodoptera littoralis* larvae treated with 0.01 g/L of nanoparticles of CuO and ZnO, Vertimec and mixtures for 24 h. Lane M low molecular weight DNA ladder, lane 1 CuO+Vertimec group, lane 2 ZnO+Vertimec group, lane 3 CuO group, lane 4 Vertimec group, lane 5 ZnO group, lane 6 Control group



Cell apoptosis was confirmed by DNA fragmentation assay. A total of 0.01 g/L of nano particles, exposed group for 24 hours showed typical DNA ladder patterns of discontinuous DNA fragments while Vertimec treatment and control groups still remained as genomic DNA. That assay showed that the use of Vertimec significantly increased the apoptosis effect of nanoparticles while nano ZnO alone showed the most highly induced DNA damage. Image (4).

Reactive Oxygen Scavengers (ROS)

Reactive Oxygen Scavengers (ROS) in treatments were significantly lower decrease than control ($P < 0.05$). Table (1) showed that Ascorbate Peroxidase (APX), and Superoxide dismutase (SOD) in control are higher than treatments. Decreased ratio percentages of APX than control recorded 19.93, 13.90, 65.73, 31.09, 54.18 % for ZnO, ZnO+Vertimec, CuO, CuO+Vertimec and Vertimec, resp. In the same arrangement, SOD ratio decreased in treatments than control with 24.43, 7.17, 72.40, 29.29, and 50.27%.

Table (1) Reactive Oxygen Scavengers (ROS) Ratio during Nano-metals Treatments with Control Comparison

Treatments	[1]ROS (Reactive Oxygen Scavengers)		[2]Decreased Ratio %	
	APX	SOD	Sort 1	Sort 2
ZnO	11.11±0.76a	9.78±0.07a	19.93	24.34
ZnO+Vertimec	7.75±0.02a	2.88±0.84a	13.90	7.17
CuO	36.64±0.95b	29.09±0.74b	65.73	72.40
CuO+Vertimec	17.33±1.15b	12.02±0.84b	31.09	29.92
Vertimec	30.20±0.97b	20.20±0.65c	54.18	50.27
Control	55.74±0.81c	40.18±0.52c		

[1]ROS (Reactive Oxygen Scavengers) APX-Ascorbate peroxidase and SOD-Superoxide dismutase (unit/mg protein).
Values are expressed as the means ±SE. Mean
[2] Decreased Ratio % = ROS ratio of the tested strain / ROS ratio of the control strain*100

It is stated that Vertimec able to increase the free radicals in Verimec in the treated larvae specifically with the use of nano ZnO and CuO,resp.

DISCUSSION

Nanomaterials including polymeric nanoparticles, iron oxide nanoparticles, gold nanoparticles, and silver ions have been exploited as pesticides. Various aspects of nanoparticle formulation, characterization, effect of their characteristics, and their applications in management of plant diseases (Al-Samarrai 2012) and their potential for use in insect pest management (Bhattacharyya *et al*.2010) such as *Helicoverpa armigera* (Vinutha *et al*.2013) have been reported.So the present paper tried to test other nanoparticles and tested their effects alone and with Vertimec on *S.littoralis.*

The pediculocidal and larvicidal activity of synthesized silver nanoparticles using an aqueous leaf extract of *Tinospora cordifolia* showed maximum mortality against the head louse *Pediculus humanus* and fourth instar larvae of *Anopheles subpictus* and *Culex quinque* fasciatus. Synthesized silver nanoparticles possessed excellent antilice and mosquito larvicidal activity (Jayaseelan *et al*.2011).

Our gained results were in the same trend with Nel *et al*. (2006) .They suggested that nanomaterials may penetrate cell membranes, lodge in mitochondria and trigger injurious responses. Results of recent studies confirmed that nanomaterials probably trigger cytotoxic effects by injuring the plasma membrane (Lin *et al.*2006, Li *et al*.2008, Sayes *et al*.2005). Mode of action occur destruction of the natural water barrier, the waxy layer of the cuticle, results in the desiccation of arthropods, Desiccation follows Fick's law of diffusion. Water absorption by silica particles is not important, since there is no chemical alteration of the absorbed lipids that can be described as physisorption (Leiderer and Dekorsy, 2008).Consequently, increase of zinc dose in its normal molecule size, led to the accumulations of zinc in the larval hemolymph and fat body, and more zinc was accumulated in fat body than in hemolymph of *Spodoptera litura* Fabricius.

The apoptosis of hemocytes was significantly induced at high zinc concentration (1000 mg·kg-1) in the insect diet, and the apoptosis rate was 63.63%, which was remarkably higher than that at control and lower concentrations (50—500

mg·kg-1). That action can be explained depending on the toxic actions of metals which cause oxidative damage to DNA, proteins and lipids (Beyersmann 2012). Nanometals able to be accumulated and penetrated more than normal molecules.

The mode of action can be mentioned in specific points as a result of interaction of free radicals with DNA which may occur in different ways, including modification of sugar moieties of DNA and production of apurinic/apirymidinic (Powell *et al.* 2005). Furthermore, Reactive Oxygen Species (ROS) can also cause damage of all components of a cell (Gurr *et al*.2005). ROS-induced DNA damage is the main cause of p53 activation in the DNA damage response which explains the apoptosis clearly.

Furthermore, the morphological changes revealed that multiple numbers of proteins bind to a single or multiple NP molecules. That concept was detected through the interaction between α-lactalbumin protein and nanoparticles by tryptophan fluorescence and circular dichroism spectroscopic techniques (Ashe 2011). The tryptophan fluorescence measurement of the protein revealed the fact that the protein undergoes a havoc structural change while interacting with NP. The tryptophan fluorescence quenching revealed that tryptophan residues are possibly in the binding site. The disruption of cell membrane and mitochondrial membrane leads to the production of additional ROS (Nel *et al*.2006, Hussain *et al*.2005).

Loss of the biological functions of proteins may be due to oxidative modification that leading to the production of carbonyl groups (=C=O).These groups are stable and specific, and their appearance causes permanent changes in the structures of the proteins (Davies *et al*. 1999, Dalle-Donne *et al*. 2003).

Circular dichroism spectroscopic measurement confirmed the change of secondary structure of the protein in the presence of NP which could also explained the apoptosis occurred in DNA bands in our research.

Although the protein may retain most of its native structure after adsorption on the NP surface, in some cases the thermodynamic stability of the protein was decreased, making the protein more sensitive to chemical denaturants such as urea (Shang *et al*., 2007).

Consequently, while the exact mechanisms of the antibacterial action had not yet been clearly understood, it had been suggested that the rule of reactive oxygen species (ROS) generated on the surface of the particles, zinc ion release, membrane dysfunction, and nanoparticles internalization were the main cause of cell swelling (Nair et al.,2008).

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