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Eco-Genotoxic Effects of Certain Pesticide Mixtures to Earthworm, *Eisenia Fetida*

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Abstract In this study, Eco-Genotoxic effect of two commercially available pesticides mixtures, Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C), being used widely in agriculture were evaluated on earthworm *Eisenia fetida*. Initially LC50 for each pesticide mixture was determined by conducting acute toxicity study followed by exposing earthworms to three lowest concentrations of pesticide mixtures for 72 hours. At the end of the exposure period, the coelomocytes were isolated from the earthworm and processed for comet assay. Acute toxicity test revealed that the toxic concentration of D+T (EC) and P+C (EC) at 0.6mg/ml and 0.5mg/ml of distilled water, shown LC50 value of mortality as compare to control set of experiment. The behavior of Earthworm has been changed like body folded and less active as per the increased the dose of the toxicants. On analysis it was observed that D+T (EC) induced strand breaks at a concentration of 0.3mg/ml, whereas P+C EC induced strand breaks at 0.3 and 0.6mg/ml. From the results it is concluded that the pesticide mixtures evaluated in the study could be moderately genotoxic to earthworm *Eisenia fetida*.

Keywords: *Eco-Genotoxicity, LC50, comet assay, pesticide mixtures, Emulsifiable concentrate (EC)*

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1. Introduction

Earthworm, *Eisenia fetida* is very useful to fertilize the soil and is a natural friend of farmer. Earthworms are recognized as essential living organisms because of their important role in improvement of physical and chemical characteristics of soil and thus increasing soil fertility [1].

Due to their dynamic properties in soil and or as a result of fertilizer application in the soil, earthworm is very important for soil fertility conservation [2]. As a result of improving soil quality, earthworms were recognized as good indicator organisms by OECD in early 1980's, and for the registration of industrial fertilizers and pesticides before implementing them into the soil [3].

Prior reports demonstrate that use of pesticides as individually or in combination act danger to their lives like they are presented to pesticides polluted soil [4].

Standard toxicity tests being used now evaluate parameters such as mortality, growth and reproduction, and are widely used in toxicant effects assessment. However, at lower level, cellular and molecular mechanisms might be disturbed, without an immediate impact on the organism's physiology. Therefore, molecular biomarkers may provide complementary information regarding organism's toxicants exposure response. In particular, genotoxicity of certain pesticides may trigger damages at cellular and eventually tissue and organism level [5].

The pesticides that demonstrate non genotoxic using existing battery of tests may or may not be non genotoxic for ecologically important organisms. Thus, the analysis of DNA alterations in terrestrial organisms has shown to be an adequate technique to evaluate the genotoxic contamination of the environments with the benefit of distinguishing and enumerating the genotoxic damages deprived of the complete knowledge of the physical and chemical properties of the pollutants existing in the environment [6]. The past few years, single cell gel electrophoresis (SCGE) or comet assay has been used as a subtle, visual, consistent, swift and inexpensive technique for measuring and analyzing DNA single and double-strand breaks, alkali-labile sites, DNA cross-linking and delayed repair-site detection in eukaryotic individual cells [7,8]. In the present study eco-genotoxic effects of two pesticides mixtures were evaluated in the earthworm *Eisenia fetida*.

2. Materials and Methods

2.1 Experimental Animals

Adult earthworms (*E. fetida*), with well-developed clitellum and approximately 250-350mg weight were

procured from commercial supplier and allowed to acclimate to laboratory conditions (20 ± 2°C) for a week before the test. They were maintained in 50:50 ratio of cow manure and coco peat.

2.1.1. Pesticide Mixture

Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C), were selected as test insecticide and brought from local pesticide market of Tirupati.

2.1.2. Acute Toxicity Test

Acute toxicity test was conducted following the method described in the OECD guideline for testing of chemicals-no. 207 [3]. A plastic petri plate with a diameter of 14cm and a height of 2cm was selected as test space. Whatman No.1 filter papers were cut similarly as to the size of petri plate and placed in petri plate. Varying concentrations of test solution was prepared (0.1-1.0mg/ml) and sprayed over the filter papers of each petri plate.1ml of distilled water selected as control. For each treatment, 3 replicates were used, each consisting of one earthworm per petri plate. Earthworms were washed with distilled water, and were kept on moist filter paper for 3hours to devoid the gut content, after which it was rinsed again with distilled water, blotted on the filter paper and placed in a test petri plate. An earthworm was placed into each petri plate and surface of each petri plate was covered with a plastic film composed of small holes. Tests were done in the dark room at 28°C ±2°C for 72 hours. After 72 hours, each earthworm was monitored for mortality by a gentle mechanical stimulus to the anterior region.

2.1.3. Genotoxicity (Comet Assay)

At 72 hours, three earthworms from each lowest concentration (0.1, 0.3 and 0.6 mg/ml) of pesticide mixture were removed from the filter paper, rinsed in distilled water and slightly dried on a paper towel and coelomocytes were obtained by non-invasive extrusion method [9]. The extruded coelomocytes from the earthworm in the medium were washed in phosphate-buffered saline (PBS). The washed cells were collected, centrifuged (150 g for 10 min) and stored on ice until use. The comet assay was performed based on the modified protocol of [8]. For the basal layer, 1% normal melting agarose in PBS was prepared. To this about 25 µl of the cell suspension from each sample was mixed with 75 µl of low melting agarose (0.5% in PBS), covered with cover glass and allowed to solidify. After removal of the cover glass, the slides were immersed in 50 ml cold lysing solution and maintained dark condition at 4°C for 1 h. Slides were then transferred to a tank containing electrophoresis buffer (300 mM NaOH, 1 mM Na2 EDTA, pH >13), for 20 min to make the DNA unwinding. After electrophoresis, the slides were washed in neutralizing buffer (0.4 M Tris-HCl, pH 7.5) for 15 min and then stained with 75 µl of ethidium bromide (2µg/mL) and screened for comets using a fluorescence microscope at 400X magnification. The cells were scored by their tail intensities and the scores were categorized as 0 (undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive) based on [10]. The total amount of DNA strand breakage was expressed in total arbitrary units (AUT) defined as: AUT =N0 x0+N1 x1+N2 x2+N3 x3+ N4 x 4, where Ni is the number of nuclei scored in each category [11].

3. Results and Discussion

3.1. Acute Toxicity to Earthworm

The earthworms are farmer's natural friends and widely used for maintain the fertility of soil. The species *Eisenia fetida* is experimental animal recommended by OECD [3]. In the present experiment, filter contact test method was used for the assessment of toxic effects of pesticide mixtures, Deltamethrin 1% + Triazophos 35% EC (D+T) and Profenofos 40% EC + Cypermethrin 4% EC (P+C), on earthworm, *Eisenia fetida*. The LC50 mortality of D+T (EC) was found to be 0.6mg/ml distilled water and similarly 0.5mg/ml distilled water for P+C (EC). Therefore 0.6mg/ml of D+T (EC) and 0.5mg/ml of P+C (EC) concentration of test solution showed very toxic to earthworm. At this concentrations body of earthworm was bent, shrunken and all three replicates were died. All the earthworms were died and melt when treated with a test solution of 0.8mg/ml to 1ml of D+T (EC), and 0.7mg/ml to 1mg/ml of P+C (EC). Theses concentrations were appeared to be moderately toxic to earthworms. These results are supportive to the report of [12] where at a concentration of 5% and 10% LC50 dose induced morphological changes in earthworm. The earthworms treated with D+T (EC) at a volume of 0.1mg/ml to 0.3mg/ml, were very active and moving. Where as in the treatment of 0.4mg/ml to 0.5mg/ml the earthworms were slightly less active and moving, and in 0.6mg/ml to 0.7mg/ml, concentration of test solution earthworms was live and less active. Similarly, earthworms were active and moving when treated with a solution of 0.1mg/ml to 0.2mg/ml of P+C (EC). Where as in the treatment of 0.3mg/ml to 0.4mg/ml the earthworms were slightly less active and moving, and in 0.5ml, concentration of test solution earthworms was live and less active.

At the end of the experiment, we found that 0.6mg /ml concentration of D+T (EC) and 0.5mg/ml concentration of P+C (EC) were shown LC50 value of mortality (Table 1). These results were in consistent with the findings of the studies conducted by [1] was shown the LC50 of chloropyriphose recorded as 0.6mg/ml. In some set like 1ml, 0.9ml was very highly affected on earthworm they have been break, melt and the body was destroying. It was shown directly effect on earthworm's body and growth.

3.2. Eco-Genotoxicity of Pesticide Mixtures to Earthworm

After the 72 hours of treatment, the earthworms exposed to the three lowest concentrations of the pesticide mixtures were collected and their coelomocytes were isolated using non-invasive extrusion method [9]. Coelomocytes isolated from the earthworm were taken for comet analysis. On analysis it was observed that D+T (EC) induced strand breaks at a concentration of 0.3mg/ml (Table 2), whereas P+C (EC) induced strand breaks at 0.3

and 0.6mg/ml (Table 3). In the comet assay, the cells were scored visually based on their tail intensities and the scores were categorized as 0 (undamaged), 1 (mild), 2 (moderate), 3 (severe) and 4 (extensive) About 100 comet images were visually scored at random for each earthworm covering a total of 1000 cells per group. The percentage of damage was calculated and statistically analyzed among the control and treated groups. [13], tested genetic toxicity of pesticides in the earthworms using comet assay and did not find any significant difference in the comet length and olive tail moment at all concentrations of pesticides, either used alone or as mixture, in whole exposure period. But these findings are inconsistency with present reports. Thus, based on the results of this study, we suggest that both pesticide mixtures are safe and acceptable to earthworms if used in field at the lower dose.

Table 1. Mortality in *E. fetida* after 72 hours of exposure to various concentrations of D+T (EC) and P+C (EC)

Concentrations (mg/ml)	Mortality#	
	D+T (EC)	P+C (EC)
0.1	-	-
0.2	-	-
0.3	-	-
0.4	-	-
0.5	-	19.33 ± 1.53
0.6	4.67 ± 0.58	66.33 ± 1.53
0.7	86.67 ± 1.53	100.00 ± 0.00
0.8	100.00 ± 0.00	100.00 ± 0.00
0.9	100.00 ± 0.00	100.00 ± 0.00
1.0	100.00 ± 0.00	100.00 ± 0.00

[#] Values expressed as mean of 3 replicates; - no mortality.

Table 2. Analysis of DNA damage as measured by comet assay in coelomocytes of *E. fetida* treated with D+T (EC)

Concentration (mg/ml)	Proportion of damaged nuclei[a]					% DNA Damage (1+2+3+4)[b]	DNA Damage score (AU)[c]
	0	1	2	3	4		
Control	74.00 ±1.00	25.00 ±1.00	0.73 ±0.12	0.27 ±0.12	0.00 ±0.00	26.00 ±1.00	27.53 ±1.40
0.1	66.33 ±1.53	22.33 ±1.53	7.33 ±0.58	3.00 ±4.44	1.00 ±0.00	33.67 ±1.53	50.00 ±2.00
0.3	63.00 ±1.00	16.33 ±1.53	11.67 ±0.58	7.00 ±1.00	2.33 ±0.58	37.33 ±1.15	70.00 ±4.58
0.6	44.67 ±1.53	22.67 ±1.53	17.00 ±1.00	8.67 ±0.58	7.00 ±1.00	55.33 ±1.53	110.67 ±3.21

a: 0-4 - indicates grade of DNA damage b: percentage of damaged cells = (1+2+3+4), c: AU - Arbitrary units of DNA Damage score.

Table 3 Analysis of DNA damage as measured by comet assay in coelomocytes of *E. fetida* treated with P+C (EC)

Concentration (mg/ml)	Proportion of damaged nuclei					% DNA Damage (1+2+3+4)	DNA Damage score (AU)
	0	1	2	3	4		
Control	73.67±0.58	25.33±0.58	0.67±0.15	0.33±0.15	0.00±0.00	26.33±0.58	27.67±0.72
0.1	62.00±1.00	23.00±1.00	9.67±0.58	4.00±1.00	1.33±0.58	38.00±1.00	59.67±5.03
0.3	54.67±1.53	21.33±0.58	16.00±1.00	6.00±1.00	2.00±1.00	45.33±1.53	79.33±6.11
0.6	44.67±1.53	18.00±1.00	14.67±0.58	13.00±1.00	9.67±1.53	55.33±1.53	125.00±4.58

a: 0-4 - indicates grade of DNA damage b: percentage of damaged cells = (1+2+3+4), c: AU - Arbitrary units of DNA Damage score.

4. Conclusion

Filter paper contact test method is the best method for assessment of insecticide. By this method, we investigated LC50 mortality of pesticide mixture D+T (EC) and P+C (EC) on earthworm. We observed that 0.6mg/ml and 0.5mg/ml concentration of D+T (EC) and P+C (EC) shown LC50 mortality on earthworm *Eisenia fetida*. This species was highly affected from test solutions at a concentration of 0.5mg/ml to 1mg/ml. On analysis it was observed that D+T (EC) induced strand breaks at a concentration of 0.3mg/ml, whereas P+C (EC) induced strand breaks at 0.3 and 0.6mg/ml. From the results it is concluded that the pesticide mixtures evaluated in the study could be moderately genotoxic to earthworm *Eisenia fetida*.

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