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Genotoxic assessment of oxadiazon and pendimethalin herbicides on *Eisenia hortensis* by comet and micronucleus tests

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Herbicides are chemicals that are widely and unconsciously used to control unwanted weeds. Herbicides do not only damage the target animals, but they also damage the off-target soil fauna. In our study, it was aimed to determine the mutational genotoxic consequences of commonly used Oxadiazon (OD) and Pendimethalin (PM) herbicides in agriculture fields on *Eisenia hortensis* (*E. hortensis*) species by using Comet and Micronucleus tests. Consequently, the LD_{50}values for OD and PM herbicides were estimated at0.25 ppm and 0.24 ppm, respectively. Concentration series of OD and PM herbicides, ½ LD_{50}, LD_{50}, and 2 × LD_{50} were applied to *E. hortensis* for 48 h. As a negative control group, distilled water was used for both herbicides. An increase inthe concentration of damaged DNA and chromosomal aberration of *E. hortensis* coelomocytes was observed by both herbicides. Highest DNA damage (32.33 ± 2.51; 15.33 ± 1.15) was observed by the highest concentrations of OD (0.51 ppm) and PM (0.48 ppm), respectively. The negative control group had shown the least genotoxic effects. There was statistically significant ($p < 0.05$) difference among different concentration values of OD and PM herbicides.
Keywords: Genotoxicity, herbicides, micronuclei, comet assay, chromosomal aberration.

INTRODUCTION

The use of herbicides unconsciously is increasing day by day as a result of augmentation in agricultural production. Concerns about the negative effects of herbicides on nature and human health still remain important since the 1960s (Carson, 1962). Although the debate on the risks and benefits of herbicides is still ongoing. Each year, 2.5 million tons of herbicides are applied to agricultural crops worldwide. In addition to agricultural applications, herbicides are known to be used as chemical mixtures in public health, hygiene works, paper paints and swimming pool waters.

Pendimethalin (PM) and Oxidaizone (OD) are selective herbicides, applied to manage most annual grasses and several broadleaf weeds in field of cotton, potatoes, corn, soybeans, trifles, sunflowers, rice fields, soybeans and tobacco. Weed control in legumes and especially in groundnut has particularly contributed to enhance quality and to increase yield with the usage of the above-mentioned herbicides. Inspite of their better qualities, pernicious effects have been triggered by these soil micro-organisms's growth and metabolism that has also been indicated previously (Khan *et al.,* 2020). Synthetic chemicals and pesticides negatively affect non-target organisms and disrupt the balance of ecosystems (Tan, 2009). Worms found in the agricultural areas play an important role in the biodiversity of nutrients and maintaining the balance of ecosystem. Soil worms are generally considered to be beneficial for the structure and composition of the soil (Spurgeon and Hopkin, 1999; Shore and Rattner, 2001). Comet assay and MN test have been used extensively to investigate the cytogenetic effects of different mutagens, herbicides, herbal extracts and carcinogenic compounds (Ali and Cigerci, 2017; Ali and Cigerci, 2019; Drif *et al.,* 2019; Liman *et al.,* 2021a; Liman *et al.,* 2021b; Fidan *et al.,* 2022). The current study was designed to estimate the genotoxic damage of OD and PM herbicides, which are frequently used in agricultural fields, on *E. Hortensis* coelomocytes DNA by using Alkaline Comet and Micronucleus tests.

MATERIALS AND METHODS

***Collection of sample and experimental procedure*:** Collection of earthworms were conducted from polluted free area and kept in laboratory using a stock soil for acclimation. These were kept in dim light at room temperature and in moist soil. Worms (n=8) with 6-8 cm and weighing from 500-600 mg were selected from stock culture for further genotoxicty assays. Seven different concentrations of OD (0.0625 ppm, 0.125 ppm, 0.25 ppm, 0.5 ppm, 1 ppm, 2 ppm and 2.5 ppm)

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and PM (0.0625 ppm, 0.125 ppm and 0.25 ppm, 0.5 ppm, 1 ppm, 1.5 ppm and 2 ppm) were applied to selected earthworms for 48 h to find theLD_{50}. Distilled water was used as a negative control. After 48 h, each worm was touched 5 times with a light wooden stick and a live-lifeless count was made according to the reaction conditions. If the earthworms were responding and showing active movement, then those were considered live and the worms which were showing no movement upon touch were considered dead (Ciğerci, *et al.*, 2018). Counting was evaluated by Probit (EPA, version 1.5) analysis method. Then, comet assay and micronucleus test test were performed by taking ½ x$LD_{50/2}$, LD_{50} and 2xLD_{50} concentrations of both herbicides. All experimental procedures were repeated three times to validate the results.

***Comet Assay*:** Coelomocytes were collected through the extrusion buffer as described by the (Ciğerci, *et al.*, 2016). Firstly, centrifugation of coelomocytes was done for 5min at 6000 rpm. Then, supernatant was discarded; 1 ml sample was taken and mixed with 100 ul Low melting agarose (LMA, 0.8%). Microscopic slides were pre- coated with Normal melting agarose (NMA,1%). After that, Slides were coated with cell suspension mixture and covered instantly with the help of cover slips. Slides were shifted on cold ice slabs for 2 min, for gel solidification. After fixing of gel, slides were transferred in lysis buffer solution after the removal of cover slips for 60 min. Later, when the lysing procedure was completed, slides were shifted in electrophoresis chamber and chilled electrophoresis buffer was poured all over the slides; the latter were rested for 20 min for the uncoiling of DNA strands. Chamber was kept in a cold place and electrophoresis was preceded for 20 min at 25V.After the completion of electrophoresis, slides were washed with chilled deionized water and then stained with 70uL ethidium bromide (20 mg mL^{-1}) and secured with cover slips. Two slides from each concentration and sample of earthworm were examined. The level of DNA damage was measured by visualizing under fluorescent microscope. Total of100 cells per slide were counted and scored 0 to 4 depending upon the extent of DNA damage.

***Micronucleus test (MN)*:** MN was carried out, as defined earlier (Muangphra and Gooneratne, 2011b; Muangphra and Gooneratne, 2011a), with minor modifications. Rest of Coelomic fluid was utilized to proceed MN test. Coelomic fluid was treated with 1 mL Potassium chloride and was kept in the solution for five minutes. After waiting, eppendorfs were centrifuged for more another 5min at 1200 rpm. Afterwards, 1mL fixative I (50 mL fixative II, 50 mL 0.09%Nacl) was added in the fluid, the solution was kept for 5 min again and then spinned for 10 min at 1200 rpm. Subsequently, the supernatant was discarded and 1 mL fixative II (400 ml glacial acetic acid, 200 ml methanol) was added to the fluid, and waited for 5 min and then sample was centrifuged for 10 min at 1200 rpm. At last, a clean wet microscopic slide was taken; cell suspension was smeared on it. The slides were kept for drying for at least a day. After 24 h, dried slides were stained by Giemsa stain for 15 min. Entellan solution was used to fix slides permanently. About 3000 coelomocytes from 3 slides for each concentration were scored including the negative control. Compound microscope at 400 X magnification was used for rating of coelomocyte micronuclei (MNi) and binuclei (BN) cells and scoring of cell count. This procedure was performed to analyse chromosomal aberrations and cytokinesis inhibition.

***Statistical analysis*:** Data obtained from comet assay and MN studies were evaluated with the Duncan test in the SPSS package program version 23. $p < 0.05$ was considered statistically significant.

RESULTS

In our study, LD_{50}value for OD herbicide was determined as 0.25 ppm. The concentrations of OD values which were studied further for genotoxic assessment were 0.12 ppm, 0.25 ppm and 0.51 ppm. Concentration dependent genotoxic effects on *E. hortensis* were observed for both pesticides (Table 1). Highest DNA damage (32.33 ± 2.51) was found in the 0.51 ppm concentration group of OD, whereas least (6.67 ± 0.57) was observed by the negative control group. In our study, lethal concentrations applied for PM were, 0.12 ppm, 0.24 ppm and 0.48 ppm. The highest DNA damage score (15.33 ± 1.15) was observed in the highest concentration of PM. There was statistically significant ($p < 0.05$) difference among different concentrations of OD and PM herbicides.

Table 1. DNA damage scores by the Oxadiazone and Pendimethaline herbicides.

Concentrations of Oxadiazone	**DNA damage score (mean±SD)**
Control	6.67±0.57a
0.12 ppm	9.33±1.15a
0.25 ppm	18.67±1.15b
0.51 ppm	32.33±2.15c
Concentrations of Pendimethalin	
control	7.00±1.00a
0.12 ppm	7.67±0.58a
0.24 ppm	12.67±1.53b
0.48 ppm	15.33±1.15c

*Different letters in columns showing significant difference at $p<0.05$.

Micronuclei formation by the different concentrations of OD and PM are shown in graphs I and II. In the current study, OD and PM herbicides showed concentration dependent MN formation. The lowest MN frequency was observed in the control group and the highest was observed at 2xLD_{50}. There were comparatively more MNi compared to BN. There was statistically significant ($p < 0.05$) difference among different concentrations of OD and PM herbicides.

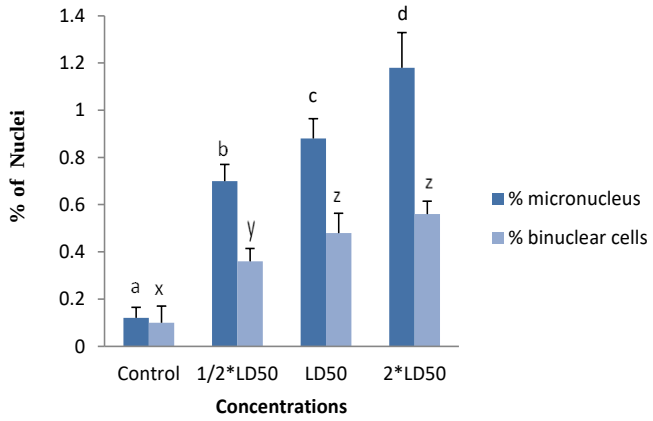

Figure 1. Percentage of Micronucleus and binuclei formation in *E. Hortensis* coelomocytes by the different concentrations of Oxadiazone herbicide.

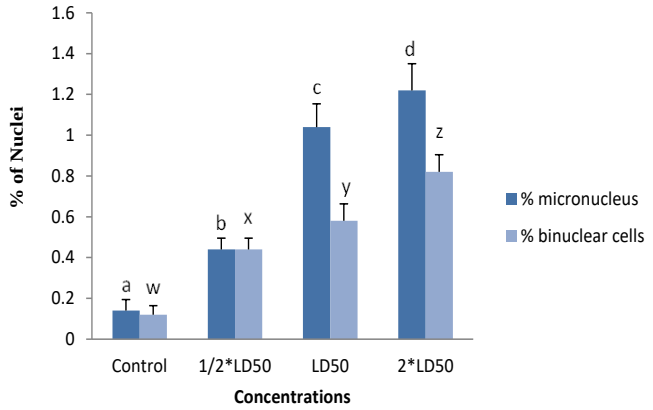

Figure 2. Percentage of Micronucleus and binuclei formation in *E. Hortensis* coelomocytes by the different concentrations of Pendimethaline herbicide.

DISCUSSION

Environmental pollution is one of the factors that negatively affect the reproduction of soil worms. Soil worms are the first to be affected by toxic soil contamination (Richert *et al.*, 1996; Savard *et al.*, 2007). Herbicides have been reported to cause damage to living creatures inside the soil. In our study, it has been shown that herbicides cause genotoxicity on *E. Hortensis* in a concentration dependent manner. Toxic effects of OD has been observed in liver of rodents, rats and mice(Reddy *et al.*, 1982; Butler *et al.*, 1988; Birchfield and Casida, 1996; Richert *et al.*, 1996). Saravanan *et al.* (2017) also demonstrated their effects on altered biochemical-hematological parameters in mammals where OD was a suspected oncogene (Mattern *et al.*, 1991), which was moderately acute toxic and caused hepatic porphyria (Birchfield and Casida, 1996). Oncogen formation by OD could be due to peroxisome proliferation (Richert *et al.*, 1996). In addition, toxic effects of PM has also been reported by the previous studies in different species(Holland *et al.*, 2002). PM contains N-nitroso compounds and nitrosamine impurities, in which N-nitroso compounds are grouped as possible carcinogen in humans. Pancreatic cancer incidents in agricultural workers who have been exposed to PM for less than seven years have been increased significantly (Andreotti *et al.*, 2009).

The comet test and MN were found to be important parameters in the determination and monitoring of genotoxic compounds in terrestrial ecosystems (Ciğerci *et al.*, 2015). In current study, increased in DNA damage was observed in *E. Hortensis* after exposure of OD and PM. İt was observed that OD and PM prodcue reactive oxygen species and induce oxidative stress in the target organism which could lead to DNA damage (Gurvinder Kaur, 2019; Sayantani Vasudeva, 2020). MN test evaluate the chromosomal aberrations due to the complete or asymmetric chromosome fragmentation during cell division. Chromosomal aberration in the current study could be due to the aneugenic and clastogenic effects of these herbicides on earthworms (Albertini *et al.*, 2000).

Different herbicides and pesticides used in the agricultural struggle in our environment cause various negative effects in the ecosystem. These effects vary according to the nature of the chemicals used, the route of administration, the dose and the target organism. As a result, these chemicals can adversely affect various species and lead to deterioration of ecosystem balance, human and environmental health. These negative effects on soil worms, which are one of the important organisms of terrestrial ecosystem, cause many living beings to be adversely affected. Therefore, this lead to the inability of the organisms to perform their basic functions, and ultimately agricultural yield is decreased (Savard *et al.*, 2007; Ciğerci, 2016; Khan, 2020).

***Conclusion*:** In our study, it was concluded that the soil worm *E. Hortensis* collected from agricultural areas and exposed to different concentrations of the herbicides (OD) and (PM) for 48h had chromosomal alteration indicated by DNA damage and Micronucleus and binuclei formation. Therefore, these results will contribute to the literature by giving a new perspective to the prevention of unconscious use of pesticides by farmers and as a model of future ecotoxicological studies.

***Conflict of Interest*:** All Authors have no Conflict of Interest.

Authors conrtibutions Statement: IHC and MMA conceived the idea. TT performed the experiments analysis. MMA drafted the article.

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