Research Article

Effect of Imidacloprid and Chloropyrifos Toxicity on Earthworm *Amynthas alexandri* Under Laboratory Conditions

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ABSTRACT

Aim: To highlight the impact of commonly used insecticides on earthworms as they represent the largest fraction of soil biomass. Earthworms were exposed to different concentrations of insecticides using a contact filter paper test method. **Materials and Methods:** Acute toxicity was tested for 48 hr using a filter paper contact toxicity test (Whatman filter paper no.1). After 48 hexposure, morphological changes and DNA Damage were observed and the treated earthworms were fixed with 10% (w/v) for 24 h in formalin solution for the histological test. **Results:** Based on Median lethal concentration, the insecticides applied at the field recommended dose in the study are classified as super toxic in the contact filter toxicity test. The highest mortality was caused by Imidacloprid and found to be the most toxic. **Conclusion:** Insecticides even when used at field recommended doses cause lethal effects on various activities of earthworms including DNA damage and reduced reproductive potential leading to a poor density of earthworms including poor soil fertility. The altered morphological and histological changes were also observed when earthworms get direct or indirect exposure to insecticides. Therefore, it is important to standardize and rationalize the use of pesticides simultaneously a higher degree of awareness and education as needed at all levels including the vendors and producers of pesticides.

Keywords: Toxicity, LC_{50}, Chloropyrifos, Imidacloprid, *Amynthas alexandri.*

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INTRODUCTION

Insecticides are the widely used chemicals for pest control and integrated pest management among different categories of pesticides; the insecticides are the most extensively used compounds in terms of value. Insecticides are widely distributed in the soil ecosystem through spray runoff and leaching etc.[1,2] The use of insecticides to raise productivity is very much desired under the present scenario, but the irrational use is causing serious threats to the cropland biodiversity including farmer's health. The growth and survival of soil biota are affected by the use of pesticides.[3] Many non-target organisms including earthworms are affected by insecticides because of their intrinsic toxicity and limited species selectivity.[4-6] The toxicity of a chemical is determined by how long an organism is exposed to it, how sensitive it is, the concentration, the chemical's properties or how they work together, as well as the local environment.[7] Earthworms get exposed to different group of pesticides with different chemical nature drastically altering their physiology and morphology.[8-10] In this investigation, two categories of insecticides namely Chloropyrifos (50%) of Organophosphate class [O, O-diethyl O-3, 5, 6-trichlor- 2-pyridyl phosphorothioate; Imidacloprid (35%) of neonicotinoid [(6-chloropridin-3-yl) methyl] imidazolidin-2-ylidene] nitramide were chosen for acute toxicity testing in

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earthworms. Imidacloprid is an insecticide and is considered a possible substitute for organophosphate pesticides in many countries.[11] Imidacloprid insecticide acts as an agonist at nicotinic acetylcholine receptors. Chloropyrifos is an acetylcholinesterase inhibitor, which is a type of Organophosphorus insecticide. An excessive accumulation of acetylcholine in the synapse disrupts the normal function of the neurological system, producing toxic effects.[12] In a terrestrial ecosystem, the population decline of earthworms is considered an early warning of insecticide toxicity.

Earthworms serve a crucial role in sustaining soil fertility and provide a substantial portion of the soil's biomass and their susceptibility to insecticides makes them a model organism in determining the risk of insecticides.[13-15] This research aimed to understand the toxicity of insecticides to the dominant earthworm species *Amynthas alexandri* at the field recommended dose under laboratory conditions. The effect of Chloropyrifos and Imidacloprid, two commonly used insecticides, was calculated using the Contact filter paper toxicity method defined in the OECD[16] and ISO[17] standard test guidelines.

MATERIALS AND METHODS

Experimental organism

Earthworm (with well-developed clitellum) were collected from Grassland of Tarai Region of Kumaun Division, Kichha, U.S. Nagar, Uttarakhand. The earthworms were taken to a laboratory together with parent soil and soil material. The earthworms were acclimatized for 15 days into plastic tubs (30×12cm), containing a 5cm layer of urine free / pesticide free cow dung with moist soil (1:1). To avoid the escape a fine mesh was used to cover the plastic tubs to maintain shade and moisture thin layer of leaves and dried grass was used.[18] All the experiments were performed in the Earthworm Biotechnology and Metagenomics Laboratory, D.S.B. Campus, Kumaun University, Nainital, Uttarakhand, India.

Chemicals

Insecticides of formula grade Chloropyrifos 50% EC, (ANTH 50, 94% w/w a.i.) and Xmida (30.5 SL % Imidacloprid) were procured from local vendors and used for acute toxicity study and other chemicals were purchased from HiMedia, SRL India and Sigma Aldrich.

Determination of LC_{50}

In this study, acute toxicity was tested for 48 hr using filter paper contact toxicity test (Whatman filter paper no.1).[16] The filter paper was placed in such a way that all sides of Petri plates are covered with filter paper. The insecticides were dissolved in acetone to create varied concentrations and 1 ml of each solution was placed onto filter paper. The treated Petri plates were re-moistened after drying by adding 1ml of de-ionized water. Earthworms with well-developed clitellum were placed (one earthworm per petri-plate) and were divided randomly into ten groups. A geometric series of five concentrations i.e., Chloropyrifos (0.105, 0.157, 0.236, 0.354 and 0.532 μg/cm^2) and Imidaclorpid (0.010, 0.015, 0.022, 0.034 and 0.051 μg/cm^2) were applied to respective Petri plates for 48 hr test. Control vial was run in parallel throughout the test. The dead earthworm per group recorded, 48 hLC_{50} value and confidence interval were calculated. The morphological changes were also observed when earthworms were in direct exposure to insecticides.

Histological Analysis

According to the findings of acute toxicity tests, two different concentrations of insecticides Imidacloprid were selected to study their effect on Gastro intestinal tract (GIT). After 48 hr exposure, the treated earthworms were fixed with 10% (w/v) for 24 hr in formalin solution for histological test. The preserved tissues were washed overnight in running tap water, dehydrated in acetone, clarified in benzene, embedded in paraffin wax (melting point 60-62°C) and 4 μm cross-sections were formed immediately behind the clitellum region. Finally, Hematoxylin–Eosin (H&E) and a combination of Periodic acid–Schiff–Alcian blue were used to stain the tissue sections. The samples were examined for histology using a light microscope (OLYMPUS CX 31). Each earthworm's histology of the midgut and epidermis was qualitatively described, and histological changes were assessed.[19]

DNA damage

DNA damage was examined according with minor modifications.[20] DNA was isolated from control and treated worms. 500 μL lysis buffers were added to each tissue-containing tube and kept at room temperature for 10 min incubation and then centrifuged; cell lysate was harvested as a pellet and was further incubated at 65°C for 5 min. After incubation, the cell-containing tubes were allowed to cool down for 5 min at room temp.700 μL chloroform-isoamyl alcohols were added and the mixture was centrifuged at 12000 rpm for 5 min. The aqueous phase was transferred into fresh 2ml Eppendorf tubes and the tubes were subjected to gentle mixing by inversion after the addition of an equal amount of chilled isopropanol then the tubes were again centrifuged at 12000 rpm for 5 min. The supernatant was removed, and the pellet was air-dried for 30 min before being dissolved in 50 µL Milli-Q water. The quantification of DNA was done by using a double beam spectrophotometer (EI-3375); the harvested DNA was electrophoresed in the 1.5% Agarose gel containing 1µL/100mL (EtBr) Ethidium Bromide (HiMedia).

The gel was visualized and examined with the help of a gel documentation system (Bio-Print, VILBER, Eppendorf.[20,21]

Statistical Analysis

The data were examined using SPSS version 25 software. The LC_{50} values and 95% confidence limits were calculated followed by the standard procedures using probit analysis.[22] Data represented as mean ± SEM (n=10) and analyzed using a one-way analysis of variance (one-way ANOVA). Statistical significance of the differences among the different concentrations was compared using one-way ANOVA followed by Duncan's Multiple Post Hoc Range Test (DMRT). The results were considered significant when the $p < 0.05$ values and confidence levels were 95%.[23] Based on median lethal concentration for filter paper contact bioassays the LC_{50} was calculated based on mortality data and classified as relatively nontoxic >1000 µg cm^{-2}, moderately toxic 100-1000 µg cm^{-2}, very toxic 10-100 µg cm^{-2}, extremely toxic 1-10 µgcm^{-2}, and super toxic at <1 µg cm^{-2}.[24]

RESULTS

Acute toxicity determination (LC_{50})

The earthworms exposed to Chloropyrifos under laboratory conditions, the mortality recorded was after 12 hr of exposure. The percent mortality was recorded at 10%, 20%, 50%, 70% and 100% at 0.105µg/cm^2, 0.157 µg/cm^2, 0.236 µg/cm^2, 0.354µg/cm^2, 0.532 µg/cm^2 concentrations respectively (Figure 1). The LC_{50} of Chloropyrifos for *Amynthas alexandri* after 48 hexposure was found to be 0.234 µg/cm^2 (Confidence interval: 0.205-0.267) (Table 1). Similarly, Earthworm *Amyanthas alexandri* when exposed to Imidacloprid for 48 h the mortality was recorded after 7 hexposure and the percent mortality for the same was recorded as 30%, 50%, 70%, 80% and 100% at concentration 0.010 µg/cm^2, 0.015 µg/cm^2, 0.022 µg/cm^2,0.034 µg/cm^2,0.051 µg/cm^2 (Figure 2). The highest mortality was observed in 0.051µg/cm^2 concentration of Imidacloprid than the rest of the concentration The LC_{50} of Imidacloprid for 48 hr found to be 0.015 µg/cm^2 (confidence interval: 0.012-0.018) (Table 2).

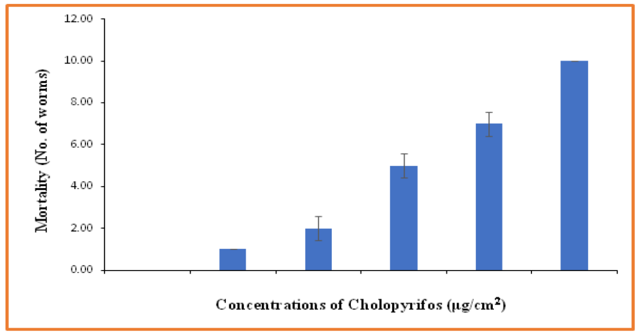

Figure 1: Mean Mortality rate of earthworm *Amynthas alexandri* after 48 hr exposure to Chloropyrifos (*n*=10). Each value is the mean ± SE of three individual observations. Values are statistically significant at *P*<0.05.

Table 1: Lethal concentrations (LC_{1-99}) of Chloropyrifos for earthworm Amynthas alexandri (*n* = 10) at 48 hr of exposure.

Lethal Concentration	Chloropyrifos (µg/cm^2)	95% Confidence Limits for Concentration: Lower Bound	Upper Bound
LC_1	0.069	0.043	0.091
LC_5	0.098	0.070	0.122
LC_{10}	0.119	0.090	0.143
LC_{15}	0.135	0.106	0.159
LC_{20}	0.150	0.121	0.174
LC_{40}	0.205	0.177	0.232
LC_{50}	0.234	0.205	0.267
LC_{80}	0.364	0.313	0.455
LC_{90}	0.459	0.382	0.615
LC_{95}	0.556	0.447	0.792
LC_{99}	0.797	0.599	1.282
Slope ± SEM	4.370±0.596	3.202	5.537
Intercept ± SEM	2.758±0.399	2.359	3.157
Chi-Square Value	7.032		
P value	<0.05		

*Control group (theoretical spontaneous rate) = 0.000.

Morphological study

Morphological changes like mucus release, coiling and curling started appearing after 12 hr of exposure when treated with Chloropyrifos (0.532 µg/cm^2- 0.105 µg/cm^2) at the highest and lowest concentration whereas Imidacloprid-treated earthworms exhibited symptoms like clitellar shrinkage and body coiling at highest concentration and tissue shrinkage at the lowest concentration within 7 hof exposure (0.051 µg/cm^2 - 0.051 µg/cm^2) (Figure 3). During the experiment, no morphological changes were observed in the control group of earthworms.

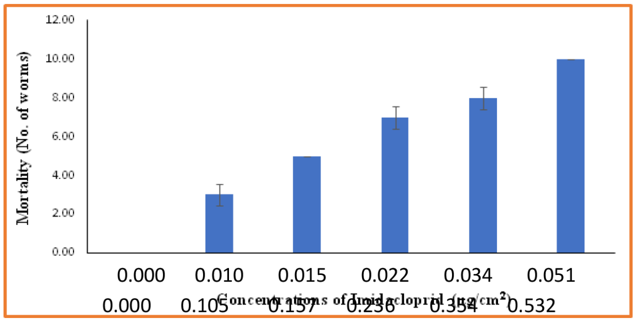

Figure 2: Mean Mortality rate of earthworm *Amynthas alexandri* after 48 h exposure to Imidacloprid (*n*=10). Each value is the mean ± SE of three individual observations. Values are statistically significant at *P*<0.05.

Table 2: Lethal concentrations (LC_{1-99}) of Imidacloprid for earthworm *Amynthas alexandri* (*n* = 10) at 48 hr of exposure.

		95% Confidence Limits for Concentration	
Lethal Concentration	Imidacloprid (µg/cm2)	Lower Bound	Upper Bound
LC_1	0.003	0.001	0.005
LC_5	0.005	0.002	0.007
LC_{10}	0.006	0.003	0.008
LC_{15}	0.007	0.004	0.010
LC_{20}	0.008	0.005	0.011
LC_{40}	0.013	0.009	0.015
LC_{50}	0.015	0.012	0.018
LC_{80}	0.028	0.023	0.036
LC_{90}	0.038	0.030	0.056
LC_{95}	0.049	0.037	0.082
LC_{99}	0.080	0.054	0.168
Slope ± SEM	3.212±0.543	2.148	4.275
Intercept ± SEM	5.849±.942	4.907	6.791
Chi-Square Value	6.530		
P value	<0.05		

*Control group (theoretical spontaneous rate) = 0.000.

Histological changes in GIT of earthworm exposed to insecticide Imidacloprid

In the present investigation, the epithelial tissue of *Amynthas alexandri* was exposed to Imidacloprid at two extreme concentrations i.e., the highest concentration 0.051 µg/cm^2 and the lowest concentration 0.010 µg/cm^2 and the worms were severely affected in both the concentrations. The histological alteration in the Gastrointestinal Tract of earthworm was observed in both concentrations and the results indicated that Imidacloprid has high toxicity even at the cellular level.

The GIT (Gastrointestinal Tract) of the unexposed worm showed an epithelial layer that is normal, an intermediate layer of longitudinal and circular muscles and blood vessels (Figure 4). With increasing insecticide concentration i.e., 0.051 µg/cm^2 the upper GIT and middle GIT showed degeneration of gut epithelium while lower GIT showed degeneration, desquamation of typhlosolar epithelium (Figure 5).

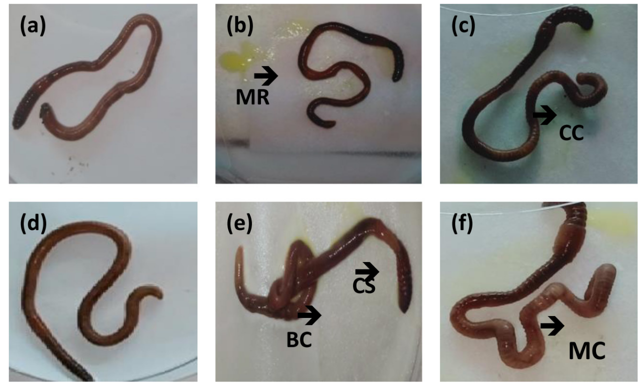

Figure 3: Morphological abnormalities observed in earthworms *Amynthas alexandri* exposed to different concentrations of Chloropyrifos and Imidacloprid, (a–f) (Control-Highest Concentration-Lowest concentration).

a: Control, b: 0.532 µg/cm2, c: 0.105 µg/cm2, concentrations of Chloropyrifos; d: Control, e: 0.051 µg/cm2, f: 0.010 µg/cm2, concentrations of Imidacloprid. BC: Body Coiling, MR: Mucus Release, CS: Clitellar Shrinkage, CC: Curling and Coiling, MC: Muscle Contraction.

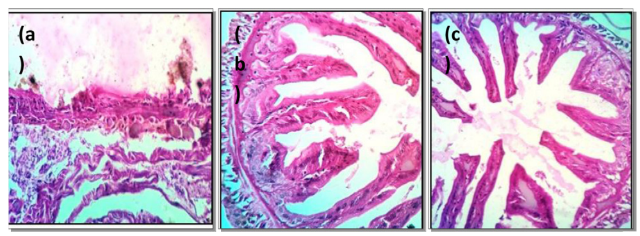

Figure 4: Effect of neonicotinoid insecticide on *Amynthas alexandri* after 48 hr exposure showing normal gut epithelium at upper, middle and lower gastrointestinal tract when treated with Imidacloprid (IM), Upper GIT (a); Middle GIT (b); Lower GIT (c) and H&E stain was used. The magnification of images is X400.

While at a lower concentration i.e., 0.010 µg/cm^2, it was observed that the upper GIT got degeneration and desquamation of gut epithelium also observed, whereas the middle GIT showed degeneration of gut epithelium. Though the concentration of the insecticide Imidacloprid was lowest, the lower GIT showed the cell debris which could have originated due to necrosis of gut epithelium (Figure 6).

DNA fragmentation assay

Pesticide-treated earthworm DNA showed fragmentation in the form of DNA shearing the degree of DNA damage induced by Chloropyrifos

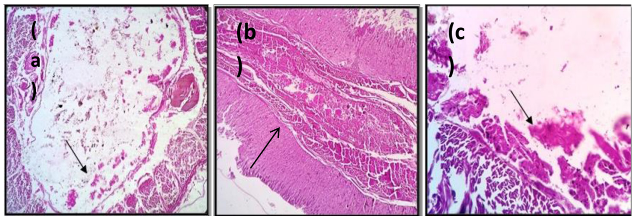

Figure 5: Effect of neonicotinoid insecticide on *Amynthas alexndrii* after 48 hr showing (a, b) degeneration of gut epithelium (Upper and middle) (c) degeneration and desquamation of typhlosolar epithelium (Lower GIT) at highest concentration of Imidacloprid. Upper GIT (a); Middle GIT (b); Lower GIT (c) and H&E stain was used. The magnification of images is X400.

Figure 6: Effect of neonicotinoid insecticide on *Amynthas alexndrii* after 48 hr showing (a) degeneration and desquamation of gut epithelium (Upper GIT) (b) degeneration of Gut epithelium (Lower GIT) (c) necrosis of gut epithelium at lower concentration of Imidacloprid and H&E stain was used. The magnification of images is X400.

and Imidacloprid was observed when the samples were subjected to electrophoresis and documented. Earthworms exposed to various concentrations of Chlorpyrifos and Imidacloprid exhibited significantly higher levels of DNA damage than did the corresponding controls (Figure 7). A higher concentration of Imidacloprid resulted in increased fragmentation in the cells followed by Chloropyrifos. These results suggest that both the insecticides at higher concentrations cause DNA fragmentation when are in direct exposure to insecticides.

DISCUSSION

Earthworms are known as biological indicator and are extensively used as model organisms in studies of ecotoxicology.[25] Insecticides affect earthworms by dermal contact and by ingesting the contaminated soil; to screen the toxicity contact filter paper test is popularly used as per OECD guidelines to determine the relative toxicity of chemicals toward earthworms. In the filter paper test, insecticides are absorbed mostly through the skin, but it cannot adequately forecast the toxicity of the soil ecosystem as a whole.[26-28]

The present findings demonstrated the toxicity of the insecticide as it significantly increases in the earthworm

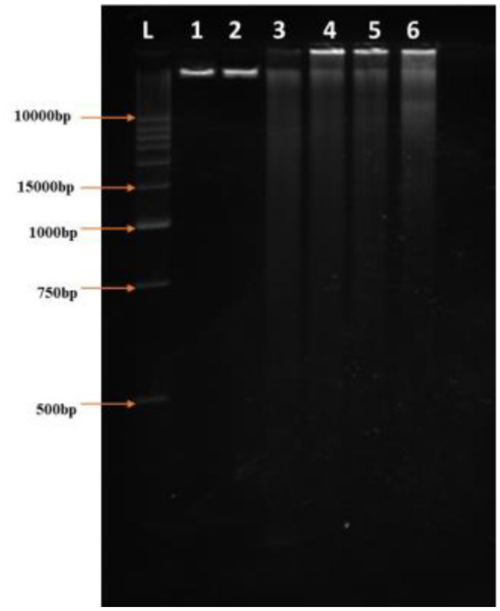

Figure 7: DNA shearing in gel electrophoresis after insecticide treatment.

Gel loading, L: 1Kb DNA Ladder, 1 and 2: Controlled DNA 3: Highest Concentration of Imidacloprid 4: Lowest Concentration of Imidacloprid 5: Lowest Concentration of Chloropyrifos 6: Highest concentration of Chloropyrifos.

Amynthas alexandri with the increase of concentration. To determine the relative toxicity of various insecticides to earthworms, contact filter paper test have been performed by many researchers and substantiate the present study findings.[4,15,29,27] The Earthworm *Eudrilus euginae* when exposed to Chloropyrifos the LC_{50} value recorded was 0.165 μg/cm^2 and caused 100% mortality at the highest concentration,[30] whereas in similar studies it observed that Imidacloprid is more toxic than Chloropyrifos to the earthworm *Eisenia foetida*.[31]

Different earthworm species have a different range of toxicity depending upon the concentration of the dose applied in the fields. Imidacloprid and Chloropyrifos are classified as super toxic and very toxic as per toxicity grade with the LC_{50} value 0.027 μg/cm^2 and 14.19 μg/cm^2.[27] Variation in morphological changes such as curling, coiling, mucus release and muscle contraction and histological changes in gastrointestinal were observed at the highest and lowest concentration. In *Lumbricus terrestris* like bleeding, muscle contraction, coiling was observed when exposed for a longer duration.[32] Similarly, *Aporrectodea nocturna and Allolobophora icterica* when exposed to Imidacloprid showed sub-lethal and lethal effects with body constriction, swelling and fragmentation.[33] Imidacloprid was found to be toxic to earthworm *Amynthas alexandri* even at the tissue level. It indicates that most of the earthworm species across the ecosystems are vulnerable to these synthetic chemicals.

The GIT (Gastrointestinal Tract) of earthworms gets highly affected when exposed to different concentrations of insecticides. The effect of neonicotinoids (Imidacloprid) when exposed at 0.2 mg/kg concentration, the midgut lining got severely damaged.[34] There are studies on other insecticides but as per the records available, no study has been made concerning the insecticide Chloropyrifos.

There are certain investigations made on the damage caused by insecticides at the genomic level i.e., Chloropyrifos causes DNA damage and also induces cell apoptosis in different types of cells.[21] Chloropyrifos are among the strong genotoxic agents, the dose or concentration with time-dependent exposure causes DNA fragmentation. In the earthworm *Eisenia fetida,* sub-chronic Imidacloprid treatment produced DNA damage.[35] The findings of Chen *et al*. 2018 demonstrated that pesticides are very harmful to this important macro fauna working as biological engineers and indicators.[36] When earthworms were treated with neonicotinoid, severe DNA damage and adverse reproductive findings were observed.[37] This study's objective is to illustrate the impact of insecticides on earthworms in the fields due to direct or indirect exposure during agriculture practices and pest management.

CONCLUSION

Insecticides even when used at field recommended doses cause lethal effects on various activities of earthworms including DNA damage and reduced reproductive potential leading to a poor density of earthworms including poor soil fertility. The altered morphological and histological changes were also observed when earthworms get direct or indirect exposure to insecticides. The toxicity of insecticides also varies with their chemical class and type of exposure. The irrational and improper application of insecticides is causing a sizable reduction in earthworm density and diversity. The current study addresses the knowledge of the farmers regarding pesticide use in general which is not at a desirable level. Therefore, it is important to standardize and rationalize the use of pesticides simultaneously a higher degree of awareness and education as needed at all levels including the vendors and producers of pesticides.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

DNA: Deoxyribonucleic Acid; **EC:** Emulsifiable Concentration; **EtBr:** Ethidium bromide; **LC:** Lethal Concentration; **OECD:** Organization for Economic Co-operation and Development; **ISO:** International Organization for Standardization; **GIT:** Gastro-Intestinal Tract; **SL:** Soluble Liquid.

Authors' contributions

This present work was carried out by all the authors. Author RM performed the experiments and major contributor to manuscript writing. NP reviewed and helped in manuscript writing. AK helped in sample collection and manuscript writing. NS performed the statistical analysis. SD helped in the overall editing and reviewing of the draft. SPS guided other authors in their work and contributed to manuscript correction. All authors read and approved the final manuscript.

SUMMARY

The findings of the present study open many vistas on the effect of insecticides on earthworms. Worldwide many insecticides have been strictly banned but it was observed that a sizeable number of insecticides are still being produced, marketed and used in India including in this study. It is concluded that when earthworms are in direct or indirect contact with insecticides face a huge of its diversity. Many histological changes were also observed in earthworms when exposed to different concentrations of insecticides. These findings will help to understand the harmful effect of insecticides on earthworms and other non-target organism.

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