

Assessment of the Effects of Neem (*Azadirachta indica*) Extract on Reproductive Performance of the Earthworm, *Eisenia foetida*.

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ABSTRACT

Earthworms are important soil organisms that contribute significantly to soil structure, organic matter decomposition, nutrient cycling, and overall soil fertility. Because of their ecological importance and sensitivity to environmental contaminants, *Eisenia foetida* is widely used as a model organism in soil ecotoxicological studies. The present study was undertaken to evaluate the effects of Neem (*Azadirachta indica*) extract on the reproductive performance of *Eisenia foetida*, with particular emphasis on juvenile emergence from cocoons. Earthworms were exposed to different concentrations of Neem extract under controlled experimental conditions, and reproductive performance was assessed by recording the number of juveniles emerging from cocoons. The percentage of reproductive inhibition was determined by comparing juvenile production in treated groups with that of the untreated control. The results showed that juvenile emergence remained relatively high across the treatment groups, although an overall reduction in juvenile production was observed with increasing neem extract concentration. Statistical analysis indicated a significant effect of Neem extract concentration on juvenile emergence, while correlation analysis revealed a strong negative association between neem concentration and juvenile production. Despite this statistically significant relationship, the magnitude of reproductive inhibition remained comparatively low, indicating that the reproductive capacity of *E. foetida* was largely maintained during neem exposure. The findings suggest that Neem extract exerts a relatively mild sublethal effect on earthworm reproduction under the experimental conditions. Overall, the study indicates that neem extract have comparatively low reproductive toxicity toward *E. foetida* and may therefore represent a potentially more earthworm-compatible botanical pest-management option.

Keywords: Earthworm- *Eisenia foetida*, Neem extract, *Azadirachta indica*, reproduction, juvenile emergence, reproductive inhibition, soil ecotoxicology.

1. INTRODUCTION

Earthworms are important components of terrestrial soil ecosystems and play a fundamental role in maintaining soil quality and ecosystem functioning. Through their burrowing, feeding, and casting activities, earthworms contribute to soil aggregation, aeration, organic matter decomposition, nutrient cycling, and the formation of biologically active soil environments (Lavelle et al., 2006). Because of their close association with soil and their sensitivity to environmental contaminants, earthworms are also considered useful bioindicators for evaluating soil quality and ecological contamination. Among the species commonly used in ecotoxicological investigations, *Eisenia foetida* (Savigny, 1826), commonly known as the red wiggler earthworm, is particularly suitable because of its ease of laboratory culture, relatively short life cycle, high reproductive capacity, and sensitivity to chemical stressors (OECD, 2016).

The increasing demand for environmentally sustainable approaches to pest management has promoted the use of plant-derived pesticides and botanical products as alternatives to conventional synthetic pesticides. Neem (*Azadirachta indica* A. Juss.) is one of the most extensively investigated botanical resources used in pest management. Neem-derived products contain several biologically active compounds, among which azadirachtin is considered one of the major bioactive constituents. Neem products exhibit insecticidal, antifeedant, repellent, growth-regulating, and developmental effects against a wide range of agricultural insect pests (Schmutterer, 1990; Ascher, 1993). Consequently, neem extracts and neem-based formulations are increasingly used in integrated and sustainable pest-management practices.

Although neem-based products are generally regarded as relatively environmentally compatible compared with many conventional synthetic pesticides, their natural origin does not necessarily imply complete safety to non-target organisms. The biological activity of neem constituents raises the need for evaluating their possible effects on beneficial soil fauna. Soil organisms may be exposed to neem-derived compounds following their application to agricultural soils through direct

contact with treated soil, ingestion of contaminated organic matter, or absorption through the body surface. Previous ecotoxicological investigations have reported that neem extract and neem cake generally produced limited adverse effects on earthworm reproduction under certain experimental conditions, although effects were observed at relatively high concentrations in artificial soil (Alves et al., 2019). Similarly, studies examining azadirachtin-based insecticides have reported no significant adverse effects on earthworm survival, growth, or cocoon production at environmentally relevant exposure levels (Kreutzweiser et al., 2009). These findings indicate that the effects of neem-derived products may depend on formulation, concentration, exposure conditions, and the biological endpoint examined.

Among the different endpoints used in earthworm ecotoxicology, reproduction is considered particularly sensitive and ecologically significant for assessing chronic effects. Mortality primarily reflects relatively severe or acute toxicity, whereas reproductive impairment may occur at concentrations that do not cause immediate mortality. Consequently, changes in reproductive output can provide an early indication of sublethal stress and may have greater implications for the long-term maintenance of earthworm populations. The OECD Earthworm Reproduction Test (Test No. 222) specifically recommends reproductive output as an important endpoint for evaluating the effects of chemicals on *Eisenia fetida* and *Eisenia andrei* (OECD, 2016).

In *Eisenia fetida*, reproductive performance can be assessed using parameters such as cocoon production, cocoon viability, hatching success, and juvenile emergence. Cocoon production represents an important measure of adult reproductive activity, while juvenile emergence provides information regarding the successful completion of subsequent reproductive stages. A decrease in cocoon production or juvenile emergence in treated groups compared with untreated controls may indicate reproductive stress or impairment. Reproductive inhibition can therefore be quantified by comparing the reproductive output of exposed earthworms with that of the control group. Such measurements can reveal sublethal effects that may remain undetected when toxicity is evaluated solely on the basis of mortality or changes in body weight.

Despite the widespread use of neem-based products as botanical pesticides, information concerning their concentration-dependent effects on the reproductive functioning of *Eisenia fetida* remains comparatively limited, particularly for specific neem extracts and formulations used under local agricultural conditions. Assessment of reproductive responses is therefore important for determining whether repeated exposure to neem-derived substances may influence the reproductive capacity of this ecologically important soil organism.

Therefore, the present study was undertaken to investigate the effects of neem extract on the reproductive functioning of *Eisenia fetida* following exposure to different concentrations. The study focuses primarily on reproductive parameters particularly juvenile emergence and compares the responses of treated earthworms with those of an untreated control. The resulting data are intended to provide an assessment of the potential sublethal and chronic effects of neem extract on earthworm reproduction and to contribute to a better understanding of the ecological safety of neem-based products in agricultural soil ecosystems.

2. MATERIAL AND METHODS

A) Preparation of Neem Extract:

Fresh and healthy leaves of neem (*Azadirachta indica*) are collected and thoroughly washed first with tap water and subsequently with distilled water to remove adhering dust and impurities. The leaves are then shade-dried or dried in a hot-air oven at 40–45°C until a constant weight is attained. The dried leaves are finely powdered and passed through a 40–60 mesh sieve to obtain uniform particle size. Approximately 100 g of the powdered neem leaves is soaked in 1000 mL of distilled water, maintaining a 1:10 (w/v) ratio. The resulting mixture is filtered initially through muslin cloth and subsequently through Whatman No. 1 filter paper to obtain a clear filtrate. The filtrate is concentrated using a rotary evaporator or by gentle evaporation in a water bath at 40–45°C. The concentrated extract is further dried in a hot-air oven at the same temperature until a constant weight is obtained. The dried residue is collected as the crude neem extract, stored in airtight amber-coloured bottles under suitable conditions, and accurately weighed for the preparation of different concentrations required for the earthworm toxicity and reproductive studies.

B) Preparation of Test Soil:

Test soil was prepared as the exposure medium for evaluating the sublethal effects of Neem Extract on *Eisenia fetida*. A concentrated stock solution of Neem extract was prepared and the required treatment concentrations were subsequently prepared from the stock solution. Based on the preliminary range-finding observations and the expected relative toxicity different treatment concentrations of 0mg/Kg, 100mg/kg, 200mg/kg, 300mg/kg, 400mg/kg, 500mg/kg, and 600mg/kg, dry soil were selected for the chronic exposure experiment. For each treatment, a predetermined quantity of dry artificial soil was accurately weighed, and the required volume of the respective solution of Neem Extract was added gradually. The solution was distributed uniformly over

the soil while the soil was continuously mixed using a glass rod and mechanical blender. Thorough mixing was performed to ensure homogeneous distribution of the Neem extract throughout the soil and to minimize the formation of localized areas with excessively high or low toxicant concentrations. The control treatment was prepared in the same manner but received an equivalent volume of distilled water instead of malathion solution. Following treatment, the prepared soils were allowed to equilibrate for 24 h under controlled laboratory conditions to facilitate uniform distribution and stabilization of the insecticide within the soil matrix. After the equilibration period, the treated soils were used for the introduction of earthworm for subsequent chronic toxicity and reproductive assessment. The soil preparation and exposure procedure was conducted with reference to the principles described in OECD (1984).

C) Experimental Setup:

For each treatment, 1 kg of treated artificial soil was transferred into earth Plastic pot containers. Ten healthy, mature, clitellated earthworms were carefully introduced into each container. Three replicates were maintained for each concentration, along with an untreated control. The containers were covered with perforated cotton cloth to provide adequate aeration while reducing moisture loss. All experimental containers were maintained in an environmental chamber at $20 \pm 2^\circ\text{C}$ under continuous illumination, following the recommended conditions for earthworm chronic toxicity studies (OECD, 1984). Soil moisture was maintained close to the initial level by periodic addition of deionized water. A small quantity of finely powdered cattle manure was provided weekly as supplementary food to support normal physiological and reproductive activity during the exposure period (Edwards and Loft, 1977).

The chronic reproduction experiment was conducted for 56 days. The first 28 days represented the adult exposure and cocoon-production phase, during which cocoon production was recorded weekly. The subsequent 28 days were allowed for cocoon incubation and juvenile development. At the end of Day 56, the number of newly emerged juveniles was recorded to evaluate the reproductive effects of Neem extract.

Juvenile Emergence and Reproduction Inhibition

Following the removal of adult earthworms, the experimental containers were maintained for a further 28 days to allow adequate incubation of cocoons and development of juveniles. On Day 56, the soil from each container was carefully transferred onto sorting trays, and the emerged juveniles were separated and counted

manually. The total number of juveniles produced was considered the principal endpoint for evaluating the reproductive effects of neem extract. A decrease in juvenile production compared with the control group was interpreted as evidence of reproductive inhibition resulting from neem exposure.

The percentage reproductive inhibition was calculated using the following formula:

$$\text{Reproductive Inhibition (\%)} = \frac{\text{Number of juveniles in control} - \text{Number of juveniles in treatment}}{\text{Number of juveniles in control}} \times 100$$

Throughout the experimental duration, soil moisture and pH were monitored periodically to maintain stable exposure conditions. Moisture loss was compensated by addition of deionized water whenever necessary. At the termination of the experiment, final soil moisture and pH values were recorded (OECD, 1984)

3. OBSERVATIONS AND RESULT

The reproductive effects of Neem extract on *Eisenia foetida* were assessed by measuring juvenile emergence from cocoons following exposure to concentrations ranging from 100 to 600 mg/kg. Juvenile emergence remained relatively high across all treatment groups (Table: 1.1 and Figure: 1.1), indicating that reproductive success was largely maintained during the exposure period.

The mean number of juveniles decreased from 59.00 ± 1.00 in the control to 54.00 ± 1.00 at 600 mg/kg, while reproductive inhibition increased from 1.69% at 100 mg/kg to 8.47% at 600 mg/kg. A slight irregularity was observed at 500 mg/kg, where juvenile emergence (55.67 ± 1.53) was marginally higher than that recorded at 400 mg/kg (54.67 ± 2.08), indicating that the response was not perfectly concentration-dependent. Nevertheless, the overall trend showed a reduction in juvenile production with increasing Neem extract concentration.

TABLE 1.1: Effect of Neem extract on Juvenile emergence in *Eisenia foetida*

Sr. No.	Neem extract concentration (mg/kg)	Juvenile emergence (Mean $\pm$ SD)	Reproductive inhibition (%)
1	0 (Control)	59.00 ± 1.00	0
2	100	58.00 ± 1.00	1.69
3	200	57.33 ± 2.08	2.82
4	300	56.33 ± 2.52	4.51
5	400	54.67 ± 2.08	7.34
6	500	55.67 ± 1.53	5.64
7	600	54.00 ± 1.00	8.47

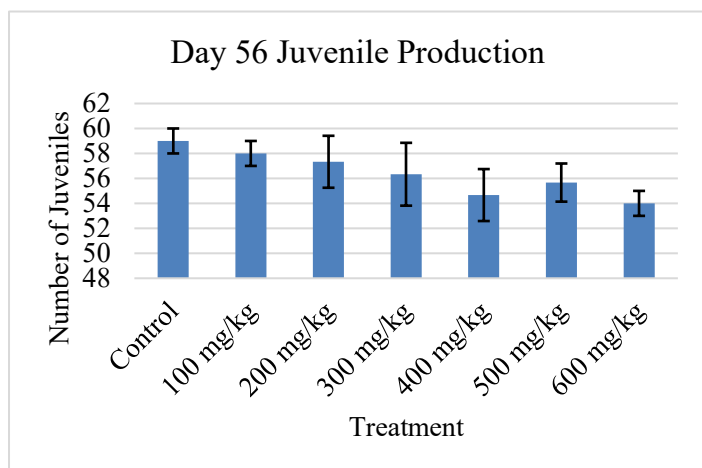


FIGURE 1.1: Effect of Neem extract on Juvenile emergence in *Eisenia foetida*

TABLE 1.2.: Pearson correlation between Neem extract concentration and juvenile emergence of *Eisenia foetida*

Reproductive parameter	Pearson's r	R ²	p-value	Correlation
Number of juveniles emerged	-0.956	0.914	<0.001	Very strong negative
Reproduction inhibition (%)	0.956	0.913	<0.001	Very strong positive

One-way ANOVA revealed a statistically significant effect of Neem extract concentration on juvenile emergence ($p = 0.029$). Pearson correlation analysis (Table:1.2) also showed a very strong negative relationship between Neem concentration and juvenile emergence, indicating that increasing Neem concentration was associated with reduced reproductive output. Overall, Neem extract produced a statistically significant but comparatively mild inhibitory effect on reproduction, with the greatest reproductive inhibition (8.47%) observed at 600 mg/kg. The consistently high juvenile emergence suggests that the reproductive capacity of *E. foetida* was largely preserved even at the highest concentration tested.

4. DISCUSSION

The present study demonstrated that exposure to neem extract produced a relatively mild but statistically significant effect on the reproductive performance of *Eisenia foetida*, as assessed by juvenile emergence from cocoons. Juvenile production remained comparatively high throughout the experimental period, decreasing from 59.00 ± 1.00 juveniles in the control group to 54.00 ± 1.00 juveniles at 600 mg/kg. Correspondingly, reproductive inhibition increased from 1.69% at 100 mg/kg to 8.47% at 600 mg/kg. This relatively small reduction indicates that neem extract had only a limited

adverse effect on the reproductive capacity of *E. foetida* under the experimental conditions.

The reduction in juvenile emergence with increasing neem concentration suggests that reproductive processes may be more sensitive to prolonged exposure than immediate survival responses. neem contains several biologically active limonoids, particularly azadirachtin, which can interfere with endocrine regulation, growth, development, feeding, and reproduction in susceptible organisms (Schmutterer, 1990; Ascher, 1993). Although these compounds are primarily associated with insecticidal activity, exposure to sufficiently high concentrations may potentially influence physiological processes in non-target soil organisms. The observed decline in juvenile production at higher concentrations may therefore reflect a mild sublethal physiological stress imposed by Neem constituents.

The present findings are broadly consistent with reports indicating comparatively low toxicity of neem-derived products toward earthworms. Alves et al. (2019) reported that neem extract and neem cake produced relatively limited effects on earthworm-related biological responses under experimental soil conditions, although responses varied according to the material and concentration used. Such findings support the view that neem-based products may be less harmful to earthworms than many conventional synthetic pesticides when applied at appropriate concentrations. However, differences in extraction method, chemical composition, formulation, soil properties, and exposure concentration can substantially influence toxicity.

The statistically significant one-way ANOVA result ($p = 0.029$) indicates that Neem extract concentration had a significant overall effect on juvenile emergence among the experimental groups. However, statistical significance should be interpreted together with the magnitude of the biological response. In the present study, the maximum reproductive inhibition was only 8.47%, indicating that although differences among treatments were statistically detectable, the actual reduction in reproductive output was relatively small. Thus, the findings suggest a mild reproductive effect rather than severe reproductive toxicity.

The Pearson correlation analysis further supported an inverse association between Neem concentration and juvenile emergence. The strong negative correlation indicates that increasing exposure concentration was generally associated with decreasing juvenile production. This relationship provides additional evidence that neem extract influenced reproductive performance. However, the response was not perfectly monotonic because juvenile emergence at 500 mg/kg (55.67 ± 1.53) was slightly higher than that recorded at 400 mg/kg (54.67 ± 2.08). Such minor fluctuations may arise from natural biological variation among earthworms, differences in cocoon viability or hatching success, and experimental variability. Therefore, the overall negative trend is more informative

than the small deviation observed at an individual concentration.

The relatively high juvenile emergence observed even at 600 mg/kg suggests that the reproductive system of *E. foetida* remained largely functional during Neem exposure. This may indicate that the concentration of biologically active compounds reaching the earthworms was insufficient to cause substantial reproductive disruption, or that earthworms possess physiological mechanisms that allow them to tolerate moderate exposure to neem-derived compounds. The findings are therefore important because reproductive endpoints can reveal subtle sublethal effects that may not be evident from mortality measurements alone.

From an ecological perspective, maintenance of relatively high juvenile production is significant because earthworm population sustainability depends strongly on successful reproduction and recruitment of juveniles. A maximum reproductive inhibition of 8.47% suggests that, under the conditions of the present experiment, Neem extract caused only a limited reduction in reproductive output. Nevertheless, repeated exposure to neem-based products in agricultural soils could potentially produce cumulative effects, particularly if high concentrations are applied frequently or if earthworms are exposed simultaneously to other environmental stressors.

Overall, the present results indicate that Neem extract exerted a statistically significant but biologically modest inhibitory effect on juvenile emergence in *Eisenia foetida*. The decrease in juvenile production and the strong negative correlation with Neem concentration suggest a concentration-related reproductive response, although the response was not completely consistent at every concentration. The comparatively low reproductive inhibition, even at the highest concentration tested, indicates that Neem extract was relatively less detrimental to earthworm reproductive performance under the experimental conditions. These findings support the potential suitability of neem-based products as comparatively earthworm-compatible botanical pest-management agents; however, further studies involving different formulations, longer exposure periods, repeated applications, cocoon viability, hatching success, and other reproductive endpoints would be valuable for a more comprehensive assessment of their ecological safety.

5. CONCLUSION

The present study demonstrated that Neem extract produced a comparatively mild inhibitory effect on the reproductive performance of *Eisenia foetida*. Juvenile emergence remained relatively high across all concentrations tested, although a general decline was observed with increasing concentration. Mean juvenile production decreased from 59.00 ± 1.00 in the control group to 54.00 ± 1.00 at 600 mg/kg, while reproductive inhibition increased from 1.69% at 100 mg/kg to 8.47% at 600 mg/kg.

The statistically significant ANOVA result ($p = 0.029$) indicates that Neem extract concentration significantly influenced juvenile emergence. Furthermore, the strong negative correlation between Neem concentration and juvenile production indicates that increasing exposure was generally associated with reduced reproductive output. However, the magnitude of inhibition remained relatively low, and the slight variation observed at 500 mg/kg indicates that the response was not strictly concentration-dependent at every exposure level.

Overall, the results indicate that Neem extract did not cause severe reproductive impairment in *E. foetida* under the conditions of the present experiment. Even at the highest concentration tested, reproductive inhibition was limited to 8.47%, suggesting that the reproductive capacity of the earthworms was largely maintained. Therefore, Neem extract appears to have relatively low reproductive toxicity toward *E. foetida* within the tested concentration range and may represent a comparatively earthworm-compatible botanical pesticide. Nevertheless, further long-term studies examining cocoon viability, hatching success, juvenile growth, repeated exposure, and field-level application conditions are recommended to establish its ecological safety more comprehensively.

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