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Research Article

Protective role of melatonin against allethrin-induced oxidative and histopathological alterations in the medicinal leech *Hirudo troctina Johnson, 1816 (Annelida, Clitellata, Hirudinida)*

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Abstract: Pesticides such as allethrin, a synthetic pyrethroid widely used in agriculture and domestic pest control, can enter aquatic environments and exert toxic effects on non-target organisms. This study aimed to evaluate the impact of allethrin on the medicinal leech *Hirudo troctina*, focusing on histopathological and biochemical alterations in the body wall and botryoidal tissue, and to investigate the potential protective effect of melatonin. Leeches were divided into four groups: a control, two allethrin-exposed groups (0.4 and 0.8 µg/L), and one co-treated with allethrin (0.8 µg/L) and melatonin (50 µg/L) for 21 days.

Histological observations revealed dose-dependent tissue alterations in the body wall and botryoidal tissue, including cuticle detachment, vacuolization, cellular disorganization, and muscle fiber fragmentation. Co-treatment with melatonin markedly improved tissue integrity and reduced degenerative changes. Biochemical assays demonstrated a significant increase in lipid peroxidation (MDA) and a decrease in antioxidant enzyme activities (SOD, CAT, GPx) following allethrin exposure, confirming oxidative stress induction. Melatonin supplementation restored antioxidant activities and reduced MDA levels, indicating strong cytoprotective and antioxidative effects.

The combined histological and biochemical findings highlight that allethrin induces oxidative and structural damage in *H. troctina*, while melatonin effectively mitigates these effects. These results underscore the value of *H. troctina* as a sensitive bioindicator for aquatic pollution and suggest melatonin as a promising agent for counteracting pyrethroid-induced toxicity.

Keywords: Pyrethroid insecticide; medicinal leech; antioxidant defense; tissue damage; redox imbalance; enzymatic activity; environmental biomarker.

1. Introduction

Pesticides, crucial for modern agriculture, are commonly utilized to combat pest infestations and enhance crop yields (Kaur et al., 2024). Among them, allethrin, a synthetic pyrethroid insecticide, is extensively employed in household pest control products due to its efficacy against a variety of pests (Gupta et al., 2013).

In aquatic ecosystems, allethrin can enter water bodies through various pathways, including runoff from agricultural fields, residential areas, and wastewater discharges (Caliskan, 2017; Farag et al., 2021). Agricultural runoff containing allethrin residues from treated crops can contaminate surface waters, while runoff from urban areas can introduce allethrin from household use (Zubairi et al., 2021). Additionally, allethrin can persist in the environment due to its resistance to degradation, leading to long-term accumulation in sediments and aquatic habitats (Rahman et al., 2020).

Once introduced into water bodies, allethrin can undergo photolysis, hydrolysis, and microbial degradation, but these processes may be slow, allowing allethrin to persist and exert its toxic effects on aquatic organisms (Mauck et al., 1976). The indiscriminate use of allethrin raises significant environmental concerns due to its potential toxicity to non-target organisms (Mužinić & Želježić, 2018). Studies have demonstrated that allethrin exposure can have detrimental effects on aquatic species, disrupting ecological balance and posing risks to biodiversity (Kodidasu et al., 2022; Werner & Moran, 2008). Non-target organisms, including beneficial insects, pollinators, and aquatic organisms, are particularly vulnerable to the toxic effects of allethrin (Antwi & Reddy, 2015).

In aquatic ecosystems, allethrin can accumulate in water bodies and sediment, posing risks to fish, amphibians, and invertebrates (Mujahid et al., 2021). Allethrin disrupts the nervous system of aquatic organisms, leading to paralysis and mortality (Werner & Moran, 2008). Additionally, allethrin can interfere with reproductive and developmental processes in aquatic species, potentially leading to population declines and ecological imbalances (Madhubabu & Yenugu, 2014).

Chronic exposure to sublethal concentrations of allethrin may impair growth, reproduction, and behavior in aquatic organisms, compromising their long-term survival (Diao et al., 2011; Hasenbein et al., 2015; Khaled & Saidi, 2023). One such non-target organism of interest is the medicinal leech species *Hirudo troctina*. Medicinal leeches, renowned for their therapeutic properties, have a long history of use in traditional medicine (Lemke & Vilcinskas, 2020). However, recent concerns have emerged regarding the potential impact of pesticide contamination on medicinal leech populations (Saglam, 2018).

The body wall of *Hirudo troctina*, which serves as a critical interface between the leech and its environment, may be particularly susceptible to pesticide toxicity due to its direct exposure to waterborne pollutants (Saglam, 2018). The importance of leeches in toxicity monitoring stems from their sensitivity to environmental contaminants and their role as bioindicators of aquatic ecosystem health (Khaled et al., 2023).

Leeches are known to accumulate pollutants from their surrounding environment, making them valuable tools for assessing the presence and toxicity of contaminants in aquatic habitats (Khaled et al., 2023). Their ability to bioaccumulate toxicants, including pesticides like allethrin, highlights their potential for monitoring pollutant levels and assessing ecological risks. Moreover, leeches serve as indicators of ecosystem integrity, reflecting

changes in water quality and habitat degradation over time (Elliott & Kutschera, 2011). Research into the effects of allethrin on the body wall of *Hirudo troctina* is essential for understanding the ecological consequences of pesticide contamination in aquatic ecosystems.

By investigating the physiological and histological changes induced by allethrin exposure, researchers can elucidate the mechanisms underlying pesticide toxicity in medicinal leeches. Furthermore, exploring potential mitigation strategies is crucial for preserving the therapeutic potential of medicinal leeches and safeguarding their ecological role in aquatic environments (Elliott & Kutschera, 2011).

This study aimed to evaluate the toxic effects of the pyrethroid insecticide allethrin on the medicinal leech *Hirudo troctina*, with particular emphasis on histopathological and biochemical alterations in the body wall and botryoidal tissue. Additionally, the study evaluated the potential protective role of melatonin in mitigating allethrin-induced toxicity.

2. Material and Methods

2.1. Collection of Leeches

Samples of *Hirudotroctina* Johnson, 1816 (Figure 1 A, B) were procured from the Chiba dam (36° 41′ 52″ N, 10° 46′ 17″ E) located on the river Chiba in northeast Tunisia during July 2022. The leeches were housed in laboratory conditions in aerated glass containers at 20°C and were fed calf blood on a weekly basis.

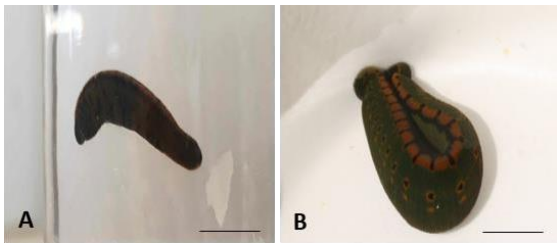

Figure 1. Adult specimen of the medicinal leech *Hirudo troctina* Johnson, 1816. Scale bar in A: 1.5cm; in B: 1cm

2.2. Experimental Design

The leeches were randomly divided into four groups; each replicated three times with 15 leeches per replication. The first group served as the control and consisted of untreated leeches. The remaining groups were subjected to different treatments: allethrin (0.4 µg/L), allethrin (0.8 µg/L), and a combination of allethrin (0.8 µg/L) with melatonin (50 µg/L) over a period of 21 days.

The allethrin and melatonin concentrations used in this study were inspired by previous studies (Leake, 1977; WHO, 1989) as well as preliminary assays in our laboratory. The LC50 of allethrin in aquatic invertebrates is 2.1 ppb (WHO, 1989). All experimental procedures complied with ethical standards for invertebrate handling.

2.3. Histological Assessment

Five specimens of *H. troctina* were randomly selected from each aquarium for histological evaluation. Following dissection, the body wall and botryoidal tissue were excised. The latter is located within the loose connective tissue between the musculocutaneous layer and the digestive tract. All tissues were fixed in 4% neutral buffered formalin for 24 hours.

The samples were then dehydrated, embedded in paraffin wax, sectioned to a thickness of 6 µm, and stained with eosin and hematoxylin for examination under a Leica Dm 500 light microscope.

For semi-quantitative assessment of tissue damage, three to five representative microscopic fields were examined per specimen. Structural alterations were graded according to severity as follows: 0 = no alteration; 1 = mild alteration (<25% of tissue affected); 2 = moderate alteration (25–50% affected); 3 = severe alteration (>50% affected). In the body wall, the following lesions were evaluated: epidermal vacuolization, cuticle detachment, muscle fiber fragmentation, and cellular disorganization.

In the botryoidal tissue, loss of cord cohesion, cytoplasmic vacuolization, lumen formation, and nuclear pyknosis were assessed. The total lesion score per individual was calculated, and the mean score per aquarium was used as the experimental unit for statistical analysis.

2.4. Oxidative Stress Evaluation

For oxidative stress assessment, five specimens of *H. troctina* were randomly selected from each aquarium, and their body wall and botryoidal tissue were homogenized in 2 ml ice-cold tris-buffered saline. After centrifugation, the obtained supernatants were used for biochemical assays.

2.4.1. Measurement of Lipid Peroxidation

Lipid peroxidation was measured using the method described by (Buege & Aust, 1978), based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA). The absorbance was evaluated at 530 nm.

2.4.2. Superoxide Dismutase (SOD) Activity

SOD activity was determined according to the method of (Beyer & Fridovich, 1987), where one unit of SOD was defined as the amount of enzyme that caused a 50% inhibition of NBT at 25°C. The absorbance was measured spectrophotometrically at 560 nm.

2.4.3. Catalase (CAT) Activity

CAT activity was assessed following the protocol outlined by(Aebi, 1984), by measuring the dismutation of H_2O_2 at 240 nm.

2.4.4. Glutathione Peroxidase (GPx) Activity

GPx activity was determined using the method described by (Flohé & Günzler, 1984), based on the oxidation of NADPH at 340 nm.

2.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9. For biochemical and histological analyses, five leeches were randomly sampled from each aquarium. Data were averaged per aquarium, and statistical analyses were performed using the aquarium as the experimental unit (n = 3 per treatment). Prior to analysis, data were tested for normality of distribution and homogeneity of variance.

Differences between the control and treated groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparisons. Statistical significance was considered at $P < 0.05$.

3. Results and discussion

3.1. Histological study

3.1.1. Body wall alterations

Histological examination of *Hirudo troctina* body wall sections revealed significant structural differences between the control and exposed groups. In the control leeches (Figure 2 A-C), the body wall exhibited a normal organization consisting of a thin cuticle covering a well-defined epidermis composed of a single layer of columnar epithelial cells. Beneath the epidermis, circular and longitudinal muscle layers were clearly distinguishable, with tightly packed fibers and intact nuclei.

The epidermal cells appeared homogeneous, with no signs of degeneration or vacuolization, and the overall structure maintained a compact and continuous appearance.

In leeches exposed to 0.4 µg/L of allethrin (Figure 2 D-F), many histological alterations were observed compared to the control. The epidermis exhibited slight vacuolization, and some epithelial cells appeared swollen or irregular in shape. Detachment of the cuticle from the underlying epithelial layer was occasionally

noted, suggesting early structural disruption. The muscular layers, however, remained relatively preserved with only minor spacing between muscle fibers, indicating an initial stress response without extensive tissue damage.

Exposure to a higher concentration of 0.8 µg/L allethrin (Figure 2 G-I) caused more pronounced degenerative changes in the body wall. The epidermal layer showed severe disorganization and cytoplasmic vacuolization, accompanied by partial detachment of the cuticle and loss of cell integrity.

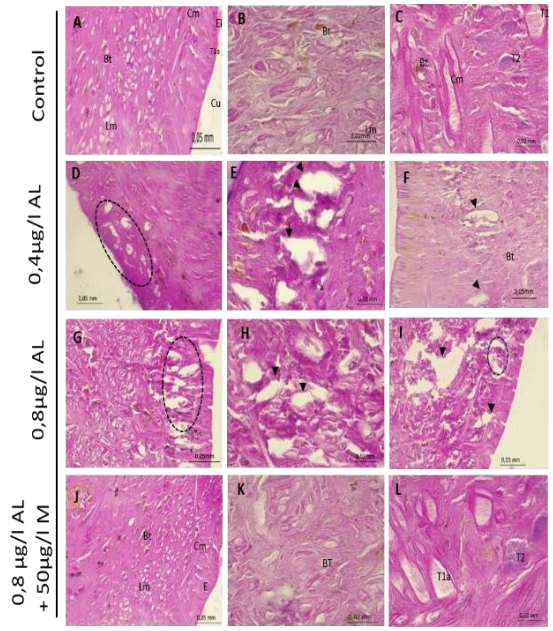

Figure 2. Histological effects of allethrin and melatonin on *H. troctina* body wall (H&E). (A-C) Control: normal cuticle (Cu), epidermis (E), muscle layers (Lm, Cm), and secretory cells (T1a, T2). (D-F) 0.4 µg/L allethrin: mild vacuolization and cuticle detachment. (I-K) 0.8 µg/L allethrin: severe epidermal degeneration, epithelial rupture, and muscle fragmentation. (L-N) 0.8 µg/L allethrin + 50 µg/L melatonin: improved tissue integrity and muscle reorganization. Scale bars: 40×, 100×.

In some areas, the epithelium appeared ruptured, and the underlying connective tissue displayed increased intercellular spaces and early signs of inflammatory cell infiltration. The muscular layers exhibited fragmentation and irregular orientation of muscle fibers, indicating deep structural impairment.

These alterations were clearly dose-dependent and reflected the toxic impact of allethrin on the epithelial and muscular architecture of the leech body wall. Co-exposure to 0.8 µg/L allethrin and 50 µg/L melatonin (Figure 2 J-L) resulted in a marked improvement in the histological organization of the body wall compared to allethrin exposure alone.

The epidermis appeared more regular, with reduced vacuolization and better preservation of cell boundaries. The cuticle was mostly attached to the underlying epithelium, and the muscle layers regained their compact arrangement with minimal fiber disruption.

This partial restoration of tissue integrity indicates a potential protective effect of melatonin against allethrin-induced histopathological damage, particularly in maintaining epidermal cohesion and muscle fiber organization.

3.1.2. Botryoidal Tissue alterations

Histological observations of the botryoidal tissue of *Hirudo troctina* revealed distinct morphological differences among the control and treated groups. In the control leeches (Figure 3 A-B), the botryoidal tissue appeared as compact cords of large, rounded granular cells surrounded by a thin connective layer.

The cells were densely arranged, forming uniform masses without any visible intercellular spaces or signs of degeneration. This organization reflected a normal, inactive state of the botryoidal tissue under physiological conditions.

In leeches exposed to 0.4 µg/L of allethrin (Figure 3 C-D), moderate histological changes were observed.

The previously compact cords of the botryoidal tissue began to lose their cohesion, with some cells showing irregular outlines and reduced cytoplasmic density.

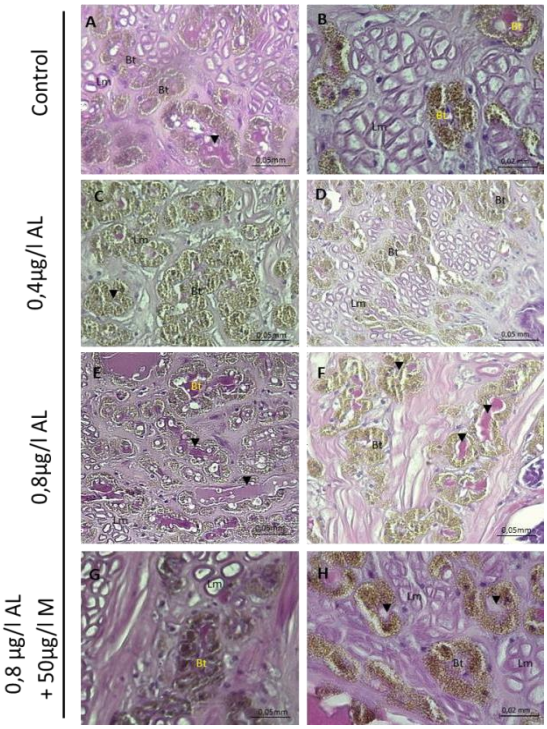

Figure 3. Botryoidal tissue morphology in *H. troctina* (H&E). (A-B) Control: compact granular cords (Bt). (C-D) 0.4 µg/L allethrin: initial disorganization. (E-F) 0.8 µg/L allethrin: activated/flattened cells and vessel formation (arrowheads). (G-H) 0.8 µg/L allethrin + 50 µg/L melatonin: attenuated damage/restored cords. Mag: 40×, 100×.

Intercellular spaces became slightly more pronounced, and a few cells appeared elongated or flattened, suggesting the onset of structural reorganization. Small vacuoles were occasionally observed in the cytoplasm, indicating early cellular stress and mild degenerative processes. However, the overall architecture of the botryoidal cords remained discernible, reflecting a partial but reversible alteration.

At the higher concentration of 0.8 µg/L allethrin (Figure 3 E-F), the degenerative changes in the botryoidal tissue were markedly increased. The cords of granular cells were disorganized and fragmented, and the tissue adopted a loose, tubular-like architecture with the formation of luminal spaces, indicating their activation.

Numerous cells appeared shrunken, with darkly stained, pyknotic nuclei and irregular cytoplasmic boundaries, indicative of advanced degeneration.

The presence of cell debris and empty spaces suggested lytic processes (Arif et al., 2021).

Furthermore, the surrounding connective tissue showed signs of infiltration by hemopoietic and inflammatory cells these alterations clearly demonstrate the dose-dependent damaging effect of allethrin on the botryoidal tissue (Singh et al., 2024).

Co-treatment with 0.8 µg/L allethrin and 50 µg/L melatonin (Figure 3 G-H) led to significant improvement in the histological appearance of the botryoidal tissue compared to allethrin exposure alone.

The cords of granular cells partially regained their compact organization, and most cells appeared rounded with well-defined nuclei and moderate cytoplasmic density.

The lumen-like structures observed in the allethrin-only group were less frequent or absent, and the overall tissue organization resembled that of the control. The reduced cellular degeneration and the restoration of cord-like arrangement indicate a clear protective effect of melatonin, likely related to its antioxidant and cytoprotective properties.

As shown in (Table 1) semi-quantitative scoring confirmed the descriptive histological observations. Lesion severity increased significantly in a dose-dependent manner in both the body wall and botryoidal tissues ($p < 0.05$), with the highest alteration index recorded in the 0.8 µg/L allethrin group. Co-treatment with melatonin significantly reduced lesion scores compared to the allethrin-only group, suggesting partial preservation of tissue structure.

Table 1. Semi-quantitative histopathological scoring of tissue alterations in leeches exposed to allethrin and melatonin. Values are expressed as mean ± SD (n = 3 independent aquaria per group). Different superscript letters indicate significant differences among groups ($p < 0.05$). Scoring scale: 0 = no alteration; 1 = mild (<25% affected); 2 = moderate (25–50% affected); 3 = severe (>50% affected).

Group	Body wall score (Mean ± SD)	Botryoidal score (Mean ± SD)
Control	0.50 ± 0.25^{a}	0.40 ± 0.30^{a}
0.4 µg/L	4.20 ± 0.80^{b}	3.80 ± 0.60^{b}
0.8 µg/L	9.10 ± 1.00^{c}	8.50 ± 0.90^{c}
0.8 µg/L + Melatonin	5.00 ± 0.70^{b}	4.20 ± 0.50^{b}

3.1.3. Oxidative Stress Biomarkers

Exposure of *Hirudo troctina* to allethrin caused significant biochemical alterations indicating oxidative stress (Figure 4).

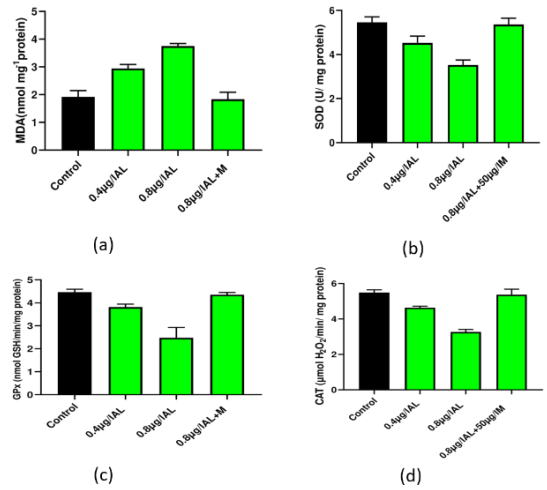

Figure 4. Effects of allethrin and melatonin on oxidative stress biomarkers in *Hirudo troctina*. Allethrin exposure significantly increased MDA levels and decreased SOD, CAT, and GPx activities compared to the control group ($P < 0.05$). Melatonin co-treatment significantly decreased MDA content and restored antioxidant enzyme activities toward control levels, demonstrating its role in mitigating allethrin-induced oxidative stress. Values are expressed as mean ± SD. Statistically significant differences were considered at $P < 0.05$.

The level of malondialdehyde (MDA), a marker of lipid peroxidation, showed a significant increase ($P < 0.05$) in both allethrin-treated groups compared to the control. The rise was dose-dependent, with the highest MDA concentration recorded in the 0.8 µg/L allethrin group, reflecting enhanced membrane lipid damage (Figure 4 a).

The activities of the main antioxidant enzymes superoxide dismutase (SOD) (Figure 4b), catalase (CAT) (Figure 4d), and glutathione peroxidase (GPx) (Figure 4c) were markedly affected by allethrin exposure. SOD, CAT and GPx activities significantly decreased ($P < 0.05$) in allethrin-exposed leeches compared to controls, indicating inhibition of the primary antioxidant defense system (Figure 4 b, c, d). Co-treatment with melatonin (50 µg/L) notably improved the oxidative balance.

MDA levels were significantly reduced, while SOD, CAT, and GPx activities increased compared to the allethrin-only groups, approaching near-control values. These results demonstrate that melatonin effectively mitigated allethrin-induced oxidative stress and restored antioxidant capacity in *H. troctina*.

Exposure of *Hirudo troctina* to allethrin resulted in both histopathological and biochemical disturbances, showing the compound's potent oxidative and cytotoxic effects on aquatic invertebrates. In this study, the body wall and botryoidal tissues exhibited dose-dependent degeneration, including vacuolization, cuticle detachment, cellular disorganization, and muscle fiber fragmentation, while the biochemical parameters confirmed the occurrence of oxidative stress.

The significant increase in malondialdehyde (MDA) levels in allethrin-exposed groups indicates elevated lipid peroxidation, reflecting the overproduction of reactive oxygen species (ROS) and subsequent membrane damage. Similar elevations in MDA have been observed in aquatic organisms exposed to pyrethroids such as deltamethrin and cypermethrin (Alnoaimi et al., 2021; Jiang et al., 2021). In parallel, the activities of antioxidant enzymes superoxide dismutase (SOD),

catalase (CAT), and glutathione peroxidase (GPx) showed significant alterations.

Such enzymatic fluctuations are typical of organisms under pyrethroid stress and signify an imbalance between ROS generation and antioxidant defense. Indeed, our findings correlates well with (Hong et al., 2018) how showed that deltamethrin exposure on the chinese mitten crab, *Eriocheir sinensis* induced genotoxicity and oxidative stress. Also, exposure of *Bufotes viridis L.* to different concentrations of deltamethrin induced oxidative stress and disruption of the antioxidant potential (Radovanović et al., 2017).

These biochemical changes correlate with the histological evidence of cellular degeneration observed in the epidermal and muscular layers. ROS-induced lipid peroxidation compromises cellular membranes, leading to cytoplasmic vacuolization, cell rupture, and disorganization of connective and muscular tissues. The pronounced tissue damage observed in the high-dose allethrin group therefore reflects the cumulative oxidative stress exceeding the leech's antioxidant capacity.

The observed lesions, including cuticle detachment, vacuolization, cellular disorganization, and muscle fiber fragmentation, are consistent with previous findings in aquatic invertebrates exposed to pyrethroids such as deltamethrin and cypermethrin and other toxicants (Jiang et al., 2021; Khaled et al., 2023; Pandya et al., 2025). At the cellular level, allethrin is known to target voltage-gated sodium channels, causing prolonged depolarization of nerve and muscle membranes, ultimately leading to paralysis and tissue degeneration(Wakeling et al., 2012).

The epidermal and muscular damage observed in this study could thus result from impaired ion homeostasis and secondary oxidative stress. Indeed, pyrethroid toxicity has been strongly linked to excessive production of reactive oxygen species (ROS), leading to lipid peroxidation, mitochondrial dysfunction, and cell death (Guven et al., 2018; Romero et al., 2017). This mechanism may contribute to the extensive vacuolization and nuclear condensation observed, which are morphological features often associated with oxidative cellular injury.

The botryoidal tissue, which plays a central role in detoxification, storage, and hemopoiesis in leeches, was also severely affected by allethrin. The disorganization of botryoidal cords, cytoplasmic vacuolization, and cell lysis suggest that allethrin compromises metabolic and immune functions of this organ. Similar degenerative effects were reported in the botryoidal tissue of *Erpobdella testacea* exposed to phosphate-processing effluents and heavy metals, confirming its vulnerability to oxidative insults (Ben Ahmed et al., 2025).

The infiltration of hemopoietic and inflammatory cells in the allethrin-treated groups may represent a defense response to tissue injury and xenobiotic stress (Mujahid et al., 2021).

Co-exposure with melatonin markedly alleviated allethrin-induced histological alterations. Leeches treated with melatonin showed better preservation of epidermal structure, reduced vacuolization, and improved muscle and botryoidal organization.

These findings corroborate the well-established antioxidant role of melatonin, which scavenges ROS, enhances the activity of endogenous antioxidant enzymes such as SOD, CAT, and GPx, and stabilizes cellular membranes (Marinho et al., 2019; Karthi & Subramanian, 2015). In aquatic organisms, melatonin supplementation has been shown to mitigate pesticide-induced oxidative stress and restore tissue integrity in gills, liver,

and muscle (Acharyya et al., 2024; Moniruzzaman et al., 2020). The observed protective effect of melatonin in *H. troctina* therefore highlights its potential as a cytoprotective agent in invertebrate models exposed to environmental contaminants.

The results of this study provide strong evidence that allethrin exerts a dose-dependent cytotoxic effect on the structural and functional components of the leech body wall and botryoidal tissue, primarily mediated through oxidative stress mechanisms.

Conversely, melatonin confers partial protection by counteracting ROS-induced damage and maintaining tissue integrity. Given the ecological importance of medicinal leeches as bioindicators, these findings also underscore the broader environmental implications of allethrin pollution in freshwater habitats. Persistent exposure to pyrethroids could disrupt the population dynamics of benthic invertebrates and, consequently, affect the trophic structure and ecological stability of aquatic ecosystems.

4. Conclusion

In summary, allethrin exposure leads to significant histopathological and oxidative alterations in the body wall and botryoidal tissue of *Hirudo troctina*. Melatonin demonstrates a notable protective role, likely via its antioxidant and anti-apoptotic properties. This study reinforces the potential of *H. troctina* as a sensitive bioindicator for monitoring pyrethroid contamination and highlights melatonin's promise as a mitigative molecule against pesticide-induced toxicity.

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