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Oxidative and Histological Effects of Herbicide Glufosinate-Ammonium and Cyanobacteria Extracted Anatoxin-a on Land Snails *Monacha cartusiana*

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Abstract

Pesticides are widely used in a variety of applications, resulting in significant discharge into the terrestrial environment. Hence, the present study was performed to evaluate the effect of two pesticides (Glufosinate-ammonium and Anatoxin- a) on biochemical parameters, oxidative stress, nuclear factor kappa B (NF-KB) activity, and histopathology of the digestive gland of the land snail *Monacha cartusiana*, which is often employed as a bioindicator of terrestrial pollution. There are no further studies on the effect of *Anabaena flos-aquae* extract on land snails. The results showed that LC_{50} of Glufosinate-ammonium and Anatoxin-a after exposure to land snail *Monacha cartusiana* for 96 hrs were 66.6 and 5.3 mg L^{-1}, respectively. LC_{50} values reveal the potential activity of Anatoxin-a than Glufosinate-ammonium against the tested snails. Within four days of exposure to glufosinate-ammonium, there were no deaths among treated snails. After the second day of exposure to Anatoxin-a, mortality percentages appeared and gradually rose with increasing concentrations and exposure time. After 28 days of exposure to LC_{25} of tested pesticides, AST, ALT, ALP, MDA, CAT, GPX, and GST levels increased while TAC levels were decreased. NF-KB immunopositivity was much more severe in the cells of tested snails treated with Anatoxin-a than Glufosinate-ammonium. Histopathological examination of the digestive gland showed that sublethal concentration of Glufosinate-ammonium produced excessive secretion and vacuolation. Anatoxin-a induced severe cellular damage in digestive tubules. In conclusion, two pesticides caused alterations in oxidative stress, NF-KB activity, and histological nature of *M. Cartusiana* that confirmed the toxicity of both pesticides for the living species in the terrestrial environment.

Keywords: *Monacha cartusiana*; Glufosinate-ammonium; Anatoxin-a; Oxidative stress; NF-KB activity; Histopathological changes.

1. Introduction

Environmental contamination is a recurring issue as well as an undesirable byproduct of human activity (Desouky *et al.,* 2013). An increasing human population necessitates increased agricultural productivity, which may need the usage of industrially generated chemicals, such as pesticides. These compounds are beneficial in certain ways, but they frequently have negative effects on the environment and, as a result, endanger human health (Stara *et al.,* 2018). Pesticides are undeniably a source of public concern due to their propensity for movement from one environmental compartment to another, as well as their effects on non-target biota. In Egypt, the widespread usage of pesticides in agricultural areas is commonplace. The application of these contaminants may cause hazards to non-target organisms (Klassen, 1986).

Herbicides, which are used to control weeds, comprise a diverse group of chemical products. One of the most often utilized herbicides in natural areas is Glufosinate-ammonium. Due to its high crop safety, ability to inhibit the synthesis of amino acids required for protein formation in vulnerable plants, and potential to be rapidly degraded in the soil, Glufosinate-ammonium is a highly effective herbicide used to control weeds **(**Hack *et al*., 1994; David *et al*., 2010).

The most studied classes of cyanotoxins are microcystins, nodularin, saxitoxins, cylindrospermopsin, and anatoxins. The literature on their environmental prevalence, biosynthesis, characteristics, and health importance evaluated was published after 2000 (Svircev *et al*., 2019). Cyanobacterial neurotoxins are divided into three classes (anatoxin-a, homoanatoxin, and saxitoxins) based on their structure: alkaloids. Neurotoxicity mechanism includes: anti acetylcholinesterase activity, anti-phosphatase activity, postsynaptic cholinergic agonist action (Metcalf and Codd, 2004), and protein kinase C activators are some of the mechanisms of neurotoxicity (Fujiki *et al*., 1990). Moreover, Cyanotoxins like organophosphorus insecticides cause toxicity by irreversibly inhibiting acetylcholinesterases (Metcalf and Bruno, 2017). Anatoxin-a is derived from cyanobacteria *Anabaena flos-aquae* and may play an important role in insect control (Saber *et al*., 2018). In addition to toxigenic cyanobacteria or particular cyanotoxins, there are some indications of relevant exposure to environmental health problems in animal poisoning occurrence (Krienitz *et al*., 2005). Anatoxin-a showed promising larvicidal

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activity against some insects such as mosquitoes (Costa *et al*., 2019).

Terrestrial snails are helpful models for assessing the risk of metals and pesticides in soil (Scheifler *et al*., 2003; Laguerre *et al*., 2009). They may be exposed to three sources of pollution: soil, plants, and air. Land snails are a suitable model organism for ecotoxicological investigations of pollutant-induced alterations and are frequently utilized as a bioindicator of soil contamination because they can accumulate and respond to both organic and inorganic substances of various origins at the organism and cellular levels (Regoli *et al*., 2005). *Monacha cartusiana* (Phylum: Mollusca, Class: Gastropoda, Muller 1774) is a land snail found throughout a wide geographical range in the Mediterranean and Southeastern Europe (Pieńkowska *et al*., 2019). In Egypt, *M. cartusiana* snails infest various economic crops with a high occurrence and population density (Lokma, 2013).

Glufosinate-ammonium and Anatoxin-a in animal tissues have been studied in only a few research studies. Therefore, the goal of the current study was to scrutinize the impacts of Glufosinate-ammonium and Anatoxin-a on the biochemical parameters, oxidative stress, NF-KB activity, and histopathology of the digestive gland of the land snail *M. cartusiana* and to censoriously assess its utility as a bioindicator for Glufosinate-ammonium and Anatoxin-a toxicity in the terrestrial environment.

2. Materials and Methods

2.1. Chemicals

Glufosinate ammonium was obtained from Sigma-Aldrich (Germany). Different concentrations of Glufosinate ammonium ($C_5H_{15}N_2O_4P$) were freshly-prepared daily. It is a liquid form with the commercial name ‘‘Basta’’. Percentage of Glufosinate ammonium equals 40% w/v, and inert ingredients equal 60 %w/v. One liter of Glufosinate ammonium equals 400 g of active ingredient (400 g/l). The stock solution was prepared by adding 2.5 ml (=1 gram) of Glufosinate ammonium and was completed to1L by adding distilled water to give following concentrations (30, 60, 90 and 120 $mg.L^{-1}$)

2.2. Preparation of Cyanobacterial extract

The alga *Anabaena flos-aquae* was obtained from Prof. Dr. Yassin El-ayouty at Zagazig University's Phycology Lab. *Anabaena flos-aquae* was grown on BG-11 medium (Stanier *et al.,* 1971). The algal culture was maintained at 25°C ± 2°C, under white fluorescent illumination of 120 $\mu Em^{-2}s^{-1}$ provided by fluorescent tubes. The culture was aerated with air current and subjected to photoperiod of light: dark (16:8hrs). A pH meter was used to adjust the pH of the medium to 7.4 using 0.1N HCl and NaOH. At the mid-logarithmic phase, algal cells were harvested by centrifugation at 10,000 rpm (4°C) for 15 minutes using Multi-tube under cooling centrifuge (Vision SCIENTIFIC CO., LTD., South Korea), washed three times with sterile distilled water, and air-dried. Anatoxin-a was extracted from *Anabaena flos-aquae* using the modified method (Harada *et al.,* 1993).

Approximately 10 grams of dried algal cells were extensively grounded in 5 ml methanol and 50 mM acetic acid, agitated for 24 hours at 150 rpm in dark, and the supernatant was collected by centrifuging at 8000 rpm. The extraction was carried out three times. The solution's pH was raised to 7.7 by adding 7% ammonium hydroxide. The extract was dried on a vacuum evaporator and recorded as the yield of crude extract. The sample was dissolved in water to finally achieve stock concentration of 2 $g.L^{-1}$ and stored in darkness at 4°C. Extraction with 50 mM acetic acid–methanol was determined to be the most successful since it yielded better recovery and allowed less pigment to be extracted from cell pellets (Harada *et al.,* 1989). The sample was determined using a T80, UV/VIS Spectrophotometer within the wavelength range of 200-400 nm. The extract had a single absorption peak at 225 nm (Figure 1) which may give indication for the presence of *Anabaena* toxin. A single absorption peak of anatoxin-a at 225 nm was confirmed by AL-Sultan and Aubaed (2017) and Gugger *et al.* (2005).

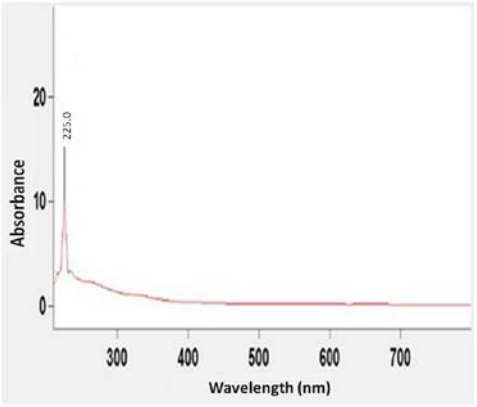

Figure 1. Ultraviolet spectrum of *Anabaena* extract

2.3. Collection and acclimatization of snails

Adult *M. cartusiana* snails (Muller, 1774) (8 ± 0.4 mm lengths, 10.3 ± 0.6 mm width, and 1.6 ± 0.1 g weights) were gathered by hand from fields cultured with Egyptian clover at Alashraf village, Zagazig District, Sharkia Governorate, Egypt, during September 2020. The gathered snails were taken to the laboratory and maintained in glass boxes (40 x 30 x 30 cm) filled with moist soil of about 10 cm height and covered with a nylon-mesh covering. They nourished on fresh lettuce leaves every day for a week. The tested snails were housed at 22°C± 3°C and a humidity of 80-90%**.**

2.4. Toxicity test and Experimental design

The tested snails were categorized into three groups. The first group is the control group (n=10), whereas the second group was divided into two subgroups (each subgroup is pooled of 40 snails, 10 snails for each concentration of two pesticides) the first subgroup treated with four concentrations of Anatoxin-a (2, 4, 6, 8 mg/l) and the 2nd subgroup exposed to Glufosinate-ammonium concentrations (30, 60, 90, 120 mg/l) for five days for the toxicity test. The toxicity test occurred using the poisonous bait technique. Each concentration was combined with 5 grams of sugarcane syrup. The mixture was then completed with wheat bran to 100 grams and humidified with little quantities of water. The LC_{50} was calculated using three replicates per concentration. Tested snails were investigated by a stainless steel needle (WHO, 1965), and mortality percentages were counted after 24, 48, 72 and 96 days treatments. The third group was fed baits comprising LC_{25} of the used pesticides for 28 days for biochemical,

oxidative stress, immune-histochemical and histological investigations. The ethical guidelines for animal experiments stated in the Declaration of Helsinki were followed, approval number 55.

2.5. Biochemical and oxidative stress biomarkers

Snail shell was detached, and the foot muscle was isolated under ice (4^0C). 0.5gm of the digestive gland of unexposed and LC_{25}-exposed snails for 28 days were dissected out and homogenized in 10mL of ice-cold 0.05M Phosphate buffer saline. The homogenates were centrifuged at 8000 rpm at 5 °C, and the supernatants were kept at - 80 °C. Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were analyzed according to Breuer (1996). Assay of alkaline phosphatase (ALP) was carried out according to Moss (1982). Malondialdehyde (MDA), total antioxidant capacity (TAC), Catalase (CAT), Glutathione peroxidase (GPX), and glutathione s-transferase (GST) were analyzed according to the corresponding assay kit protocol (Sat 450, AMS, Italy).

2.6. Immunohistochemical studies

Paraffin sections (5 µ) were mounted on positively charged glass slides (Biogenex, USA). The slides were placed in an open plastic container with enough antigen retrieval solution to completely cover them (Citrate buffer solution, pH 6). Slides were put in a microwave oven (Samsung 800 Watts) at power 10 for 5 minutes. The slides were then washed several times in deionized water before being immersed in PBS for 5 minutes. Endogenous peroxidase blocking reagent comprising hydrogen peroxide and sodium azide was used to incubate tissue sections (DAKO peroxidase blocking reagent, Cat. No. S 2001). Except for the tissue portion, the excess buffer was blotted off and the slides were dried. The sections were then treated with one to two drops of a supersensitive primary monoclonal antibody [against Nuclear Factor Kappa B (NF-B)]. For 60 minutes, slides were incubated horizontally in a humid chamber at room temperature. The slides were washed for 5 minutes after the excess reagent was thrown away. In two successive phosphates buffer saline jars (PBS). 1-2 drops of the ready-to-use DAKO EnVision + system were applied for 20 minutes at room temperature after blotting excess buffer. The sections were rinsed with PBS as before and blotted. DAB (diaminobenzidine) was used as a chromogen and counterstained with Mayer's hematoxylin. NFkB immunoreactivity was evaluated under light microscopy in terms of severity (Eissa and Shoman, 1998).

2.7. Histological examinations

Both control and treated digestive glands of the snail were dissected out and fixed in Formalin. Specimens were dehydrated in ascending series of ethanol concentrations, and then cleaned in Xylene for 20 minutes before being embedded in paraffin wax. Sections (4–5 m thick) were cut, mounted, and stained with Eosin and Hematoxylin.

2.8. Statistical analysis

All results were executed using SPSS version 20 (SPSS, Richmond, VA, USA). One-way ANOVA followed by Duncan's multiple range tests as a post-hoc test was used to compare the significant differences among treatments at $P < 0.05$. Probit analysis was used to determine LC_{50} by the graphic way of the curve dose-effect according to Finney (1971).

3. Results

3.1. Toxicity test

Table 1 showed that glufosinate-ammonium exposure resulted in no deaths among exposed snails within four days. After the second day of Anatoxin-a exposure, mortality percentages occurred. With increasing concentrations of tested pesticides and exposure periods, mortality percentages increased. The LC_{50} values of Anatoxin-a and Glufosinate-ammonium were 5.3 and 66.6 mg/l, respectively, whereas LC_{25} were 1.33 and 16.65 mg/l, respectively. Anatoxin-a had a toxicity index of 100%, whereas Glufosinate-ammonium had a toxicity index of 7.9% (Table 2).

Table 1. Effect of different concentrations of Glufosinate-ammonium and Anatoxin- a on mortality percentages of *M. cartusiana* at different exposure periods.

Tested pesticides	Time / Conc.	Percentage mortality (%)			
		24 hr.	48 hr.	72 hr.	96 hr.
Glufosinate-ammonium ($mg.L^{-1}$)	30	0±0.00	0±0.00	0±0.00	0±0.00
	60	20±5.8	20±5.8	26.7±3.3	40±0.00
	90	56.7±3.3	70±0.00	70±0.00	76.7±3.3
	120	66.7±3.3	86.7±3.3	100±0.00	100±0.00
Anatoxin- a ($mg.L^{-1}$)	4	0±0.00	23.3±3.3	30±0.00	36.7±3.3
	6	40±0.00	46.7±6.7	46.7±6.7	50±0.00
	8	50±0.00	63.3±3.3	66.7±3.3	70±0.00
	10	83.3±3.3	100±0.00	100±0.00	100±0.00

- Data are represented as means of samples ±SE. Concentration (Conc.)

Table 2. Lethal toxicity values, sublethal concentrations, and toxicity index of Glufosinate ammonium and Anatoxin-a against *M. cartusiana* under laboratory conditions.

Tested pesticide	LC_{50} ($mg.L^{-1}$)	LC_{25} ($mg.L^{-1}$)	Toxicity index (%)	Slope
Glufosinate-ammonium	66.6	16.65	7.9	7.81±0.73
Anatoxin-a	5.3	1.33	100	4.54±0.49

3.2. Biochemical biomarker in clover snail M. cartusiana exposed to LC_{25} of two tested pesticides.

Table 3 shows that after 28 days of exposure, ALT, AST, and ALP levels in clover snails treated with Glufosinate-ammonium were significantly increased ($P < 0.05$) by 20.7%, 19.7%, and 144.5% while the exposure of clover snails to Anatoxin-a caused a significant increase by 56.9%, 91.2%, and 192.6% as compared with the control groups.

Table 3. Physiological parameters of *M. cartusiana* treated with LC_{25} of Glufosinate-ammonium and Anatoxin-a for 28 days.

Pesticides	ALT (U/l)	AST (U/l)	ALP (U/L)
Control	23.2 ± 0.6^{a}	28.4 ± 0.50^{a}	136.0 ± 6.0^{a}
Glufosinate-ammonium	28.0 ± 0.9^{b}	34.0 ± 0.95^{b}	332.5 ± 2.5^{b}
Anatoxin-a	36.4 ± 0.55^{c}	54.3 ± 1.25^{c}	398 ± 20^{c}
F-value	91.44	200.18	126.11
P-value	0.002	0.001	0.001

Each value is mean of 3 samples ± SE -Means with different alphabetical superscripts at each column among tested pesticides are significantly different at $P < 0.05$ (one way ANOVA and subsequent post hoc multiple comparisons with Duncan's Multiple Range Test).

3.3. Oxidative stress and antioxidant biomarkers in clover snail M. cartusiana exposed to LC25 of two tested pesticides.

Table 4 shows that after 28 days of exposure, MDA, CAT, GPx and GST levels in clover snails treated with Glufosinate-ammonium were significantly increased ($P<0.05$) by 32.3%, 77.1% ,14.5% and 49.4% while the exposure of clover snails to Anatoxin-a caused a significant increase by 310.1%, 95.8%, 78.9% and 182.4% compared by the control groups. TAC levels in clover snails treated with Glufosinate-ammonium were significantly decreased ($P<0.05$) by -23.5 % while the exposure of clover snails to Anatoxin-a caused a significant decrease by -80.9% compared by the control groups**.**

Table 4. Oxidative stress and antioxidant biomarkers of *M. cartusiana* treated with LC_{25} of tested pesticides for 28 days.

Pesticides	MDA (nmol)	TAC (µmol/l)	CAT (U/g)	GPX (U/g)	GST(U/g)
Control	14.4 ± 1.2^{a}	2.30 ± 0.34^{a}	0.48 ± 0.02^{a}	0.76 ± 0.04^{b}	1.76 ± 0.35^{a}
Glufosinate-ammonium	19.1 ± 2.1^{a}	1.76 ± 0.22^{b}	0.85 ± 0.11^{b}	0.87 ± 0.03^{b}	2.63 ± 0.67^{a}
Anatoxin-a	59.05 ± 9.55^{b}	0.44 ± 0.11^{b}	0.94 ± 0.05^{b}	1.36 ± 0.16^{b}	4.97 ± 0.93^{b}
F-value	18.61	16.63	27.42	10.86	14.16
P-value	0.02	0.02	0.01	0.04	0.03

Each value is mean of 3 samples ± SE -Means with different alphabetical superscripts at each column among tested pesticides are significantly different at $P < 0.05$ (one way ANOVA and subsequent post hoc multiple comparisons with Duncan's Multiple Range Test).

3.4. NF-kB immunoreactivity

NF-kB immunopositivity was observed in the digestive gland cells of clover snail *M. cartusiana*. NF-kB positivity was detected in the perinuclear areas of cells of digestive tubules (Plate 1 B, C, and D) of exposed snails compared to the control groups (plate 1 A). Immunopositivity was much more severe in snails treated with Anatoxin-a than Glufosinate-ammonium.

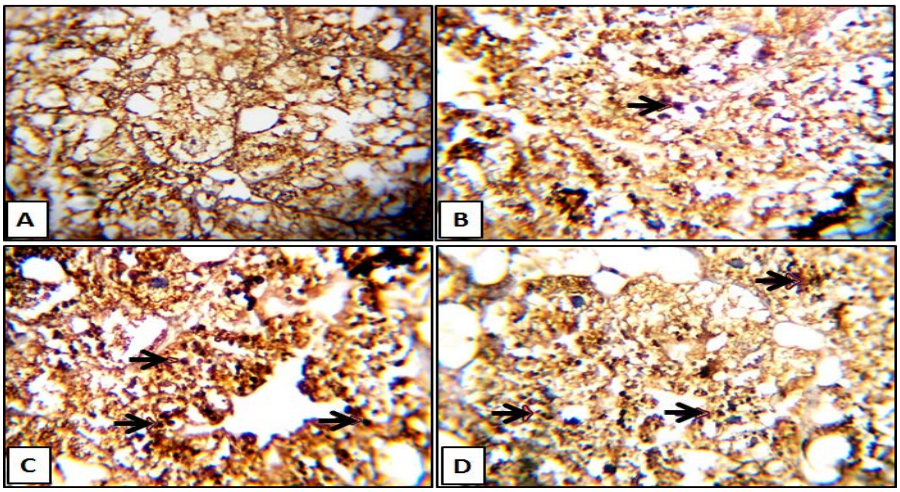

Plate 1. Nuclear factor kappa B staining of snail digestive gland. (A) NF-kB staining of digestive gland of control snails. (B) NF-kB staining of digestive gland of snail exposed to LC_{25} Glufosinate-ammonium after 28 days of exposure (arrows refer to NF-kB immunopositivity). (C and D) NF-kB staining of digestive gland of snail exposed to LC_{25} Anatoxin-a after 28 days of exposure (arrows refer to NF-kB immunopositivity).Magnifiction power (400X).

3.5. Histological changes of the digestive gland of M. cartusiana

The digestive glands comprise branched blindly ending follicles circumscribed by connective tissue (Plate 2A). Each follicle of the digestive gland is encircled by a thin layer of circular muscle fiber. The cells constituting the wall of each tubule are predisposed around an irregular lumen. Investigation of such cells demonstrated the presence of three types of cells digestive, excretory, and calcium cells (plate 2B). The digestive cells are the most plentiful cell type and fill most of the volume of each tubule. The excretory cells are less abundant and more significant than digestive cells, and cytoplasm of these cells is occupied by large vacuole having one or more yellow-brownish secretory granules. The calcium cells are the least abundant compared to the digestive and excretory cells, and their nuclei are situated at the basal half of the cell and are spherical ovoid in shape (plate 2A). After exposure to Glufosinate-ammonium, mild histological abnormalities such as mild tubular disruptions, excessive luminal secretion, and vacuolation (plate 2C), excretory cells showed an increase in the number and size of brownish-yellow granules (plate 2 D).

Anatoxin-a caused an increase in the number of vacuoles in the tubular epithelial cells of the digestive gland of snails and resulted in a widening of the lumen. The digestive and secretory cells are swollen and vacuolated with a thin cytoplasmic layer surrounding the large vacuole (plate 2E). Excessive tubular disruption is observed, including epithelial cell lysis, extensive vacuolation, and lumen reduction (plate 2F).Discussion

Investigating the impacts of pesticides discharged into the terrestrial environment on the terrestrial ecosystem becomes a topic of interest. Several studies were conducted to investigate the release of these pesticides into the terrestrial environment in response to these concerns. The current study focuses on the impact of two pesticides (Glufosinate-ammonium and Anatoxin-a) on the land snail *M. cartusiana*, which is frequently used as a bioindicator of terrestrial pollution.

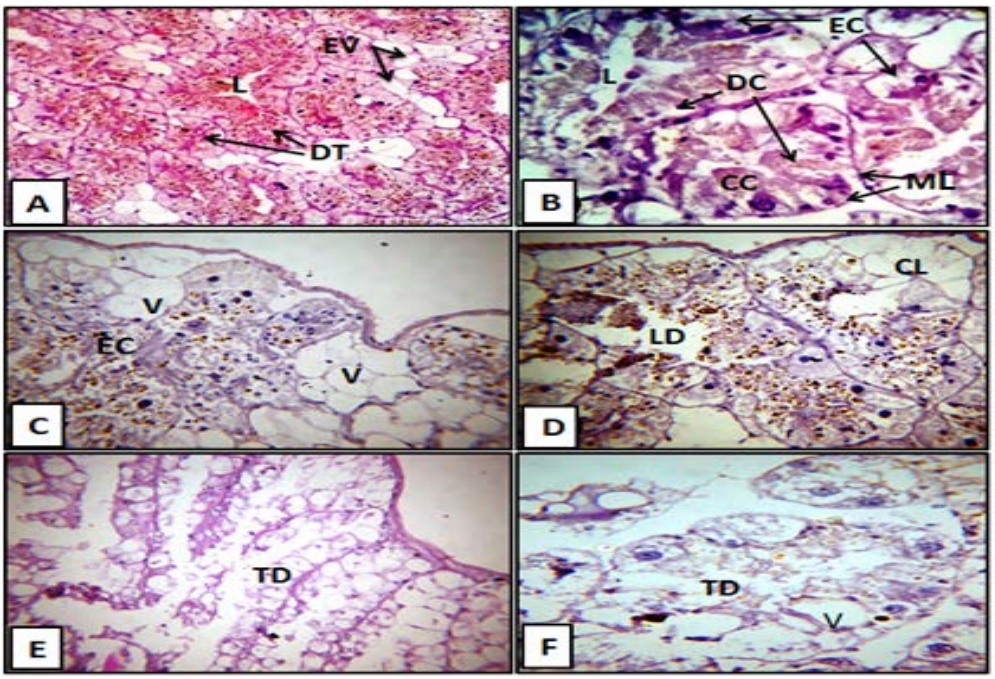

Plate 2. Light micrograph of the digestive gland of *M. cartusiana* snails. (A): The digestive gland of control group displaying the usual cellular structure (X100). (B): The digestive gland of control group displaying different types of cells forming the digestive tubules (X 400). (C&D): T.S. of digestive tubules after exposure to $LC_{25}$of Glufosinate-ammonium for 28 days showing mild tubular disruptions such as excessive secretion and Vacuolation (X100 & 200). (E&F): T.S. of digestive tubules after exposure to $LC_{25}$of Anatoxin-a for 28 days showing tubular degeneration(X100 & 400). **CC:** Calcium cell; **CL:** Cell lysis; **DC:** Digestive cell; DT**:** Digestive tubules; **EC:** Excretory cell; **EV:** Excretory vacuoles; **L:** Lumen; **LD:** Lumen dilatation; **ML:** Muscle layer; **TD:** Tubular disruption; **V:** Vacuolation.

3.6. Toxicity test

According to the results, Anatoxin-a was more toxic to *M. cartusiana* than Glufosinate-ammonium. These findings are compatible with those of Druart *et al.* (2011), who reported that Glufosinate-ammonium has a diminished toxic effect on land snail *Helix aspersa*. Silva dos Santos *et al.* (2019) recorded that Anatoxin-a has a neurotoxic effect on *Nauphoeta cinerea* cockroaches. The differences in toxicity between two pesticides may be accredited to the differences in susceptibility and tolerance associated with its accumulation, biotransformation, and excretion (Reddy *et al.*, 2012). Furthermore, the susceptibility of adult snails to pesticides varies depending on the mode of action, biological agent against the target organism, and the chemical component produced by the microorganisms. Two mechanisms mediate the toxic action of Anatoxin-a; first, it stimulates muscle contraction by acting as an ACh agonist, but it is continuous, unlike Ach; second, by inhibiting acetylcholinesterase, it increases the neurotransmitter within the synaptic cleft. The result of these two mechanisms is paralysis which could lead to the death of snails (Osterbauer and Dobbs, 2009). On the contrary, Glufosinate-ammonium does not inhibit neurotransmitter receptors or affect the catecholamine neurotransmitter tissue concentrations. This reason may be due to the weak toxic effect of Glufosinate-ammonium.

3.7. Biochemical biomarker in clover snail M. cartusiana exposed to LC_{25} of two tested pesticides.

Biochemical investigations have been used for evaluating the health status, impacts of stressors, and the adaptive capability of organisms to the external environment (El-Sayed *et al.*, 2019). Amino-transferase enzymes (ALT&AST) catalyze the inter-conversion of α-ketoacids and amino acids, and their echelons indicate heart dysfunction and liver impairment (Ghouri *et al.*, 2010). The current study found that ALT and AST increased in the digestive gland of treated snail with two pesticides. This increase may result from toxin exposure or destruction of the hepatic cell (Farkas *et al.*, 2004). These findings are in agreement with Abdel-Halim *et al.* (2020) and Gaber *et al.* (2021) who stated that ZnONPs and Methomyl (Copter 90%) caused an increase in the activity of AST and ALT in treated *M. cartusiana*, respectively. Also, Abd El-Atti *et al.* (2020) stated that Biozed (fungal) and Biogard (Bacterial) biocide caused an increase in the levels of ALT and AST in *M. cartusiana*. ALP is an enzyme responsible for removing phosphate groups from several kinds of molecules such as proteins and nucleotides by hydrolysis and is used to detect liver damage (Thomas, 2006). The present study revealed that Anatoxin-a and Glufosinate-ammonium significantly increased ALP levels in the digestive gland of treated *M. cartusiana*. Such increments are in harmony with the results of Sharaf *et al.* (2015) who stated that Diazinon, Lambda-cyhalothrin, and Methomyl exposure increased ALP levels in *M. cartusiana*. This increase may be due to destruction of the hepatic cell.

3.8. Oxidative stress and antioxidant biomarkers in clover snail M. cartusiana exposed to LC_{25} of two tested pesticides.

The reactive oxygen species (ROS) has a substantial role in the biological system's physiological control of cell function. Enzymatic and non-enzymatic antioxidant mechanisms are adequate in biological systems to cope with the continuous generation of ROS. When the generation of ROS exceeds their neutralization by antioxidant mechanisms within an organism, oxidative stress (OS) occurs (Regoli and Giuliani, 2014). Malondialdehyde (MDA) used as a biomarker of oxidative stress is one of the final products of polyunsaturated fatty acids peroxidation in the cells (Davey *et al.*, 2005). The current study displayed that MDA levels increased significantly in clover snail *M. cartusiana* after treatment with two pesticides. Likewise, Sharaf *et al.*, (2015) illustrated that MDA levels increased in the clover snail, *M. cartusiana*, after exposure to Diazinon, Lambda-cyhalothrin, and Methomyl. The increment may induce this elevation in free radicals, which causes the overproduction of MDA. Total antioxidant capacity (TAC) measures an organism's antioxidant state by assessing the antioxidant response against the free radicals accumulated in a tissue sample (Richetti *et al.*, 2011). In addition, the current study revealed that the TAC level was decreased in pesticides exposed to the snail. In agreement with the current study, Morad *et al*., 2022 found that TAC levels decreased in *Biomphlaria alexandrina* snails, after exposure to myco-synthesized nano-selenium. This reduction can be attributed to its defensive role against damages induced by free radicals. Catalase (CAT) and Glutathione peroxidase (GPx) are antioxidant enzymes and are considered the first line of defense against ROS (Van der Oost *et al*., 2003). In the current study, CAT and GPX activity was increased in the digestive gland of *M. cartusiana* upon exposure to two pesticides. Such increment in CAT and GPX activities may be an adaptive mechanism to prevent the accumulation of toxic reactive oxygen (Regoli *et al.*, 2006). In the same regard, Sharaf *et al.* (2015) found that CAT activity increased in the digestive gland of the clover snail, *M. cartusiana*, after treatment with Diazinon, Lambda-cyhalothrin, and Methomyl. Glutathione-s-transferase (GST) plays a vital role in defending cells and tissues from oxidative stress (Havelková *et al.*, 2008). GST activity was found to be significantly increased in the digestive gland of clover snail *M. cartusiana* following exposure to the tested pesticides. This increase may be attributed to its defensive role against damages induced by oxyradical. On the contrary, Abdel-Halim *et al.* (2020) reported that ZnONPs caused a decrease in the activity of GPX and GST in treated *M. cartusiana*.

3.9. NF-kB immunoreactivity

Nuclear factor Kappa B (NF-KB) is present in an inactive form in the cytosol linked to inhibitory regulatory proteins called IKB. There are different inducers to NF-KB activity, comprising oxidative stress resulted from reactive oxygen species and hypoxia, leading to NF-KB immunopositivity in tissues (Topal *et al.*, 2015). Another inducer is the lipid peroxidation intermediates (Campo *et al.*, 2008). Furthermore, the treatment of cells with Anatoxin-a and Glufosinate-ammonium increases the activity of NF-KB in the digestive gland of clover snail *M. cartusiana*. Immunopositivity was much more severe in snails treated with Anatoxin-a than Glufosinate-ammonium. The increment in the activity of NF-KB may be due to this elevation in reactive oxygen species.

3.10. Histological changes of the digestive gland of M. cartusiana

Histological investigations are significant biomarkers in toxicological studies. They are utilized as indicators of environmental stress since they deliver a particular biological end-point of historical exposure and divulge the direct effect of the toxicant in the organs (Ramesh *et al.*, 2018). In the current study, many histopathological alterations were estimated in the digestive gland, which is the vital organ that performs the detoxification mechanism (Henry *et al.*, 1991). The digestive gland of untreated snail is composed of three types of cells (digestive, excretory, and calcium cells). This finding agrees with Ismail *et al.* (2013), who detected the presence of three types of cells in the wall of tubules of the digestive gland. Based on the Histological examination of the digestive gland, the sub-lethal concentrations of Glufosinate-ammonium caused mild tubular disruptions such as excessive luminal secretion and vacuolation. Sub-lethal concentrations of Anatoxin-a resulted in severe tubular disruption, including cellular destruction. Similar changes were detected in the digestive gland of clover snail, *M. cartusiana* treated with Biozed (fungal), and Biogard (Bacterial) biocide (Abd El-Atti *et al.*, 2020).

In conclusion, this study has revealed that two pesticides (Glufosinate-ammonium and Anatoxin-a) have toxic effects against *M. cartusiana*. However, Anatoxin-a

was more toxic to treated snails than Glufosinate-ammonium. Anatoxin-a and Glufosinate-ammonium caused alterations in biochemical, oxidative stress biomarkers, NF-KB activity, and severe histopathological changes of *M. Cartusiana*. Therefore, these pesticides must be used prudently, and their release into the terrestrial environment should be closely monitored and regulated. Furthermore, the current study demonstrated the efficacy of land snails, *M. cartusiana*, as a bioindicator for Anatoxin-a and Glufosinate-ammonium toxicity. Furthermore, it suggested that long-term treatment could reveal the dangers of pesticide addiction on macroinvertebrates and human life.

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Author contribution

R.M. Said conceived and designed the experiment. A H. Al-Badwy conceived and designed the experiment. A. A. Mohamed conceived and performed the experiment. All authors contributed to writing, reading, and approving the final manuscript.

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Data included in the article.

Declarations

Conflict of interest

The authors declare no competing interests.

Additional information

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