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Toxicity assessment of novel *Bacillus thuringiensis* -based bioinsecticides formulation isolated from desert locust

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Abstract

Biopesticides formulations based on *Bacillus thuringiensis* strains were successfully used in biological control against different insects such as desert locusts and grasshoppers. This study throw light on the impact of two biopesticides formulations (MR1 and MR2) prepared from *Bacillus thuringiensis* isolated from desert locust individuals collected from southeast of Egypt on male of albino rats. The rats were divided randomly into five groups, control (P1) was given distilled water, groups (P2) and (P3) were orally intubated with $2X10^7$, $8X10^7$ cfu's /kg as low and high doses of MR1 formulation, respectively. Groups (P4) and (P5) were orally intubated with $2.6X10^7$, $1X10^8$ cfu's/ kg as low and high doses of MR2 formulation, respectively. The results of this study indicated an increase in Hb, RBCs, MCV, TP and AST and a decrease in WBC, MCHC and ALT. Histopathological examination showed mild vacuolar degeneration of hepatocytes and pyknotic nuclei of hepatocytes. However, Histopathological examination of kidney showed engorged dilated renal blood vessels and mild to moderate necrotic tubular epithelium. Results of this investigation showed the safety of both MR1 and MR2 formulations derived from *Bacillus thuringiensis* isolates for mammals. These formulations exhibit promising potential as a safe bio-pesticide that can be used against locusts and grasshoppers

Keywords: Biochemical parameters; Biopesticides formulation; *Bacillus thuringiensis*; Hepatic toxicity; Renal toxicity; Histopathological impacts.

1. Introduction

The relentless surge of insect pests poses a significant threat to global food security and public health. Conventional insecticides, while effective, raise concerns regarding environmental contamination, non-target organism toxicity, and the emergence of resistant pest populations. In this context, the search for sustainable and environmentally friendly pest control solutions has become paramount. The bacterium *Bacillus thuringiensis* (*Bt*) occupies a prominent position in this arena, boasting potent insecticidal properties through the production of Cry toxins [1]. However, limitations such as narrow host specificity and susceptibility to environmental degradation hinder the comprehensive utilization of *Bt*-based bioinsecticides. Intriguingly, the desert locust (*Schistocerca gregaria*), a notorious migratory pest, harbors within its gut a diverse consortium of microorganisms, including novel *Bt* strains with potentially unique insecticidal attributes. Exploiting these indigenous *Bt* strains for bioinsecticides development presents a compelling opportunity to overcome the limitations of existing formulations. Grasshoppers and Locusts (Orthoptera: Acrididae) are considered among the most devastating agricultural pests. It is very critical to control these pests for food security. These control measures often require cooperation between international organizations. Locusts and Grasshoppers outbreaks can be triggered by climate changes [2]. Most grasshoppers and locusts control methods used neurotoxic chemical pesticides [3, 4]. In Kenya and Ethiopia, 1.6 million hectares of cultivated regions were treated with broad-spectrum pyrethroid and organophosphate Conventional pesticides to combat the swarms of desert locust upsurge in 2019 to 2021 [5]. Though chemical pesticides are considered

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effective means of control because of their prolonged activity, but increased concerns have been raised about their environmental damage and safety to humans [6]. These concerns became a stimulus to find alternative means of control that are safe for the environment and sustainable [7]. Recently two bacterial isolates from desert locust cadavers showed potentiality to be used as a bio pesticide against locusts and grasshoppers, these isolates were introduced as wettable powder formulation. Formulation is the final product resulting from mixing the microorganism with different inert and adjuvants that provide protection from environmental conditions and also improve the viability of these microbes [8]. Any novel bio pesticides must be evaluated on their benefits against their environmental risk [9, 10]. Egypt boasts a diverse range of registered biopesticides for various plants and pests, including *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus thuringiensis* subsp. kurstaki, and *Beauveria bassiana*. Despite their widespread use, limited research explores the biosafety of these formulations on mammals and ecosystems. Notably, few studies like El-Saadany et al. [11] have highlighted potential side effects of isolated *Bacillus thuringiensis* on mammals. This study investigated the toxicity assessment of novel *Bt*-based bioinsecticides formulations isolated from desert locusts. The primary objective is to evaluate the insecticidal potency and non-target organism toxicity of these formulations. This study aims to evaluate the toxicity of the bacterial formulations based on novel *Bacillus thuringiensis* isolates (MR1and MR2) on male Albino rats. We hypothesize that:

1- The selected *Bt* formulations demonstrate minimal toxicity towards mammals.
2- Contribute to the ongoing research on *Bt*-based bioinsecticides and pave the way for their practical implementation in desert locust management programs.
3- The successful development of safe and effective *Bt*-based bio insecticides from desert locusts can provide a valuable tool for sustainable locust control, contributing to food security and environmental protection in vulnerable regions. Hence, this study evaluates the oral toxicity of *Bt*-based bioinsecticides in rats through hematological, histopathological, and biochemical analyses. The findings will contribute valuable insights into potential exposure risks associated with *Bt*-based bioinsecticides, ultimately informing safe and sustainable pest management practices.

2. Materials and methods

2.1. Bacillus thuringiensis formulation :

In this study, *Bacillus thuringiensis* formulations as Wettable powder (WP) were prepared from two novel strains of *Bacillus thuringiensis* isolated from desert locust (*S. gregaria*) at locust and grasshoppers research department, Plant Protection Research Institute, Agricultural Research Centre, Egypt. According to the identification by 16S rRNA gene sequencing, both isolates were identified as *Bt* MR1 (OM666062) and *Bt* MR2 (OM666063).

2.2. Animals and housing:

Male albino rats were purchased from the Egyptian Holding Company for Biological Products and Vaccines, Helwan Farm, Cairo, Egypt. All test animals were examined for clinical signs for 7 days as an acclimatization period. Rats were kept in plastic cages with an open mesh of stainless steel and allowed access to food and water during the whole experiment. Albino rats were kept in an environmentally controlled chamber at a temperature degree of 23±2°C, relative humidity 40-70% and a light /dark period of 12 hrs.

2.3. Acute oral toxicity study:

Acute oral toxicity study was carried out according to the USEPA guidelines [12].

2.4. Sub-acute toxicity study:

After a period of acclimatization, male rats were divided into five groups with four rats in each group. The first group, referred to as the control group (P1), received distilled water as their treatment. The second (P2), third (P3) were orally intubated with $2X10^7$, $8X10^7$ cfu's/kg as low and high doses of MR1 formulation, respectively. While the fourth (P4) and fifth (P5) groups were orally intubated with $2.6X10^7$, $1X10^8$ cfu's/ kg as low and high doses of MR2 formulation, respectively. The intubation period was 28 days as sub-acute toxicity study according to EPA No (EPA712–C–00–366). The doses were calculated based on body weights that were recorded every week. At the end of the experiment, all rats were fasted overnight then anesthetized. Blood samples were collected from the retro-orbital venous plexus using a fine sterilized glass capillary tube into tubes containing EDTA-2K as an anticoagulant, to be used for hematological studies. Additional blood samples were collected in dry clean centrifuge tubes. Theses

samples were centrifuged at 3600 rpm for 15 minutes to obtain the serum. The supernatant was collected and preserved in sterilized Eppendorf tubes at -40 ºC until further use in the biochemical studies. The kidney and liver were removed, immediately washed in saline solution (0.9% Nacl) and then used for the histopathological examination [13].

2.4.1. Determination of Hematological Parameters

The complete blood picture (CBC) test was conducted using an automated electronic Coulter MAXM system (manufactured by Beckman Coulter, Inc., Fullerton, CA) at the end of the treatment after 28 days [14-16].

2.4.2. Determination of liver functions:

Liver function biomarkers: Alanine aminotransferase (GOT / ALT), Aspartate aminotransferase (GPT / AST) were determined according to Reitman and Frankel [17], while alkaline phosphatase (ALP) activity was determined [18], total protein (TP), albumin levels (Alb) and gamma-glutamyl transferase (GGT) were determined according to Szasz [19] and Whitfield et al. [20].

2.4.3. Determination of kidney functions:

Levels of creatinine, urea, and uric acid were measured by colorimetric methods according to Henry [21], Barham and Trinder [22] and Fossati et al. [23].

2.4.4. Histopathological Studies:

Livers for both control and *Bt* formulations -treated rats were taken and fixed in formalin (10%) as a fixative for 24 hours. Liver tissues were trimmed, dehydrated in a sequence of graded ethanol (70%, 80%, 90%, and 100%) twice, and then treated with xylene before being embedded in the paraffin blocks. Using a rotary microtome (Leica, model RM2125 RTS), sections with a 7- µm thickness were cut, deparaffinized in xylene, and stained with hematoxylin and eosin (HE) for general histology [24].

2.5. Ethics approval and consent to participate

The protocols and regulations employed in this work were approved by Cairo University's Institutional Animal Care and Use Committee (CU-IACUC) (No. CU/I//F/34/23).

2.6. Statistical analysis

The LD_{50} values of Wettable powder formulation of both *Bt* formulations (MR1 and MR2) were determined. All collected data were subjected to analysis of variance (ANOVA) one-way test using Statistical Package for Social Science (SPSS), version 20 and the data were presented as means ± SE, then the Duncan multiple tests was used to assess the differences between means.

3. Results and Discussion:

3.1. Acute Oral LD_{50} Study:

The acute LD_{50} values of MR1 and MR2 formulations in rats were over $1X10^8$ cfu's/kg body weight (BW). Furthermore, none of the studied animals showed signs of infection, pathogenicity, mortality, or toxicological effects.

3.2. Signs of toxicity in rats exposed to Bt based formulations:

The present study was carried out to determine any toxic effects of the two new formulations of the novel isolates of *Bt* that were administered orally to male rats at two dose levels for repetitive treatments for 28 days. No obvious signs of toxicity were noted during the experimental duration in behavioral activity or external appearance in any of the treated rats. Furthermore, no mortality was recorded.

3.3. Biochemical parameters:

3.3.1. Hematological bioindicators in rats exposed to Bt formulations:

The current data Table 1 indicated the impact of tested biopesticides on hematological parameters. The results indicate a significant increase in RBCs, Hb level in rats exposed to the high dose (P2) of MR1compared to the control. The same trend was noticed in MCV of rats exposed to both doses of MR1 and the high dose of MR2. While rat exposed to both formulations exhibited a significant decrease in white blood cell (WBC) when compared to the control. On the other hand, the means of other hematological parameters including hematocrit (PCV) and MCH in this group did not show any significant alterations compared to the control group except MCHC which declined in rats exposed to the high dose of MR1.

3.3.2. Liver biomarkers in rats exposed to Bt formulations:

The data presented in Table 2 showed the impact of biopesticides formulation on serum liver functional biomarkers. The data indicated that there was no significant differences in the liver biomarkers in rats exposed to the tested doses of *Bt* formulations except the significant increase in AST activity and total protein level in rats exposed to MR1 formulation. Also, the significant decrease in ALT activity was

noticed in serum of rats treated with the high dose of MR1.

3.3.3. Renal biomarkers in rats exposed to Bt based formulations:

As indicated in Table 3, the data showed the effect of the two biopesticides on nephrotoxicity biomarkers, urea, creatinine and uric acid. The data indicated that the two formulations did not cause any nephrotoxicity, except some significant changes that were noticed in creatinine at high doses of MR1 and MR2.

3.3.4. Histopathological effects in rats exposed to Bt formulations:

The liver of rats treated with the formulations showed mild vacuolar degeneration of hepatocytes (Fig. 1P2) at the low dose of MR1. While rats treated with the high dose of MR1 showed nearly normal hepatocytes (Fig. 1P3). Vacuolar degeneration, pyknotic nuclei of hepatocytes and moderated vacuolar degeneration are shown in Fig. 1P4, 1P5. B. thuringiensis biopesticide administered orally caused renal histological problems. Such pathological complications were noticed as engorged dilated renal blood vessels for the treated group of low doses of MR1 (Fig. 2P2). Meanwhile the MR2 treated group exhibited a mild to moderate necrotic tubular epithelium with nearly normal renal tubules on high dose (Fig. 2P5).

Table 1 Hematological effects of wettable powder formulations (MR1 and MR2) on albino rat males.

Parameters	Treatments				
	P1	MR1		MR2	
		P2 (2×10^7)	P3 (8×10^7)	P4 (2.6×10^7)	P5 (1×10^8)
Hb (g/dl)	12.35 ± 0.25 [a]	12.55 ± 0.26 [a]	13.27 ± 0.20 [b]	11.92 ± 0.20 [a]	12.20 ± 0.19 [a]
WBC ($X10^3$ /µl)	12.125± 0.23 [a]	12.275± 0.44 [a]	10.925± 0.16 [b]	11.275± 0.43 [b]	11.050± 0.16 [b]
RBCs ($X10^6$ /µl)	6.24 ± 0.06 [a]	6.44± 0.14 [a]	6.77 ± 0.01 [b]	6.15± 0.10 [a]	6.23± 0.07 [a]
PCV (%)	40.25± 0.48 [a]	43.50± 1.70 [a]	43.50±1.32 [a]	40.75± 1.10 [a]	42.25± 0.62 [a]
MCV (Fl/cell)	64.86± 0.49 [a]	67.90± 0.83 [b]	66.84± 0.54 [b]	66.17± 0.63 [a]	66.99± 0.66 [b]
MCH (Pg/cell)	19.79± 0.42	19.48± 0.11	19.60± 0.21	19.38± 0.10	19.58± 0.14
MCHC (g/dl)	30.52± 0.87 [a]	28.67± 0.34 [b]	29.34 ± 0.20 [a]	29.28± 0.31 [a]	29.22 ± 0.13 [a]

n = 5, MR1 (*Bt*, OM666062) and MR2 (*Bt*, OM666063), P1: Control, P2, P3: Low and high dose; cfus / ml for MR1, P4, P5: Low and high dose; cfus / ml for MR2.
All values are mean ±SE, means having the same letters are not significantly different from each other, $p < 0.05$.

Table 2 Effect of wettable powder formulations on Liver function of albino rat males

Parameters	Treatments				
	P1	MR1		MR2	
		P2 (2×10^7)	P3 (8×10^7)	P4 (2.6×10^7)	P5 (1×10^8)
AST (u/l)	26.25 ± 1.97 [a]	44 ± 1.08 [b]	26.00 ± 2.17 [a]	28.50 ± 2.59 [a]	25.50 ± 2.17 [a]
ALT (u/l)	18.37 ± 0.23 [a]	14.12 ± 0.42 [b]	16.25 ± 0.47 [b]	18.00 ± 0.70 [a]	18.75 ± 1.65 [a]
ALP (u/l)	90.50 ± 2.02 [a]	88 ± 3.02 [a]	84.25 ± 2.39 [a]	84.75 ± 3.11 [a]	89.50 ± 1.04 [a]
T.P (gm/dl)	6.46 ± 0.16 [a]	7.53 ± 0.25 [b]	6.40 ± 0.05 [a]	6.70 ± 0.32 [a]	6.17 ± 0.29 [a]
Albumin (gm/dl)	3.43 ± 0.11 [a]	3.70 ± 0.05 [a]	3.58 ± 0.04 [a]	3.52 ± 0.075 [a]	3.26 ± 0.150 [a]
GGT (u/l)	25 ± 0.81 [a]	24 ± 0.40 [a]	27.75 ± 1.10 [a]	25.50 ± 0.64 [a]	25.25 ± 1.10 [a]

n = 5, MR1 (Bt, OM666062) and MR2 (Bt, OM666063), P1: Control, P2, P3: Low and high dose; cfus / ml for MR1, P4, P5: Low and high dose; cfus / ml for MR2.
All values are mean ±SE, means having the same letters are not significantly different from each other, $p < 0.05$.

Table 3 Effect of wettable powder formulations on Kidney function of albino rat males.

	Parameters	Treatments			
	P1	MR1		MR2	
		P2 ($2X10^7$)	P3 ($8X10^7$)	P4 ($2.6X10^7$)	P5 ($1X10^8$)
Urea (Mg/dl)	38.27 ± 1.44 [a]	34.75 ± 1.18 [b]	35.92 ± 0.39 [b]	34.75 ± 1.86 [b]	37.52 ± 2.38 [b]
Creatinine (Mg/dl)	0.62 ± 0.02 [a]	0.54 ± 0.03 [a]	0.52 ± 0.01 [b]	0.56 ± 0.03 [a]	0.73 ± 0.037 [c]
U. A (mg/dl)	3.80 ± 0.40 [a]	4.17 ± 0.85 [a]	4.00 ± 0.10 [a]	3.62 ± 0.12 [a]	3.70 ± 0.07 [a]

n = 5, MR1 (*Bt*, OM666062) and MR2 (*Bt*, OM666063), P1: Control, P2, P3: Low and high dose; cfus / ml for MR1, P4, P5: Low and high dose; cfus / ml for MR2.

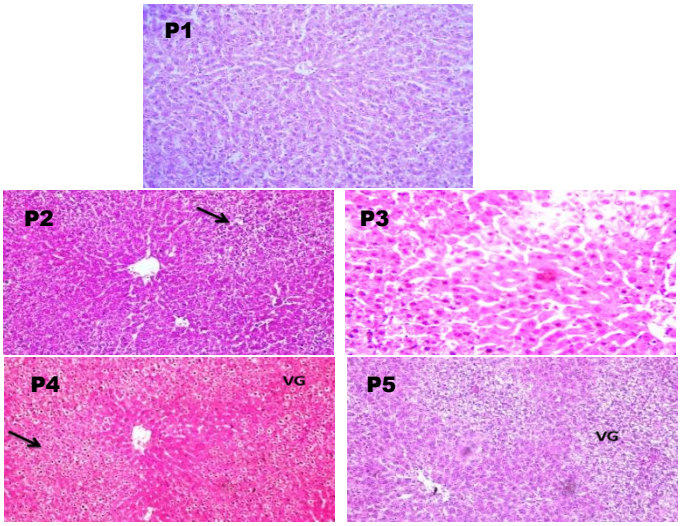

Fig. 1 Pathological effect of MR1 and MR2 on Liver of male albino rats (P1: Control, P2: mild vacuolar degeneration of hepatocytes for low dose of MR1, P3: nearly normal hepatocytes for high dose of MR1 P4: vacuolar degeneration and pyknotic nuclei of hepatocytes (black arrow) for low of MR2, P5: moderated degree vacuolar degeneration for high dose of MR2)

2- Discussion

Most pesticide pollutants endanger not only humans but also affect the ecosystem integrity and function [25]. A decrease in a plant's photosynthetic ability and seed production occurs when synthetic pesticides enter the environment through a variety of channels including indiscriminate disposal, vapor movements, droplet drift, erosion, and leaching [26]. In most cases, synthetic pesticides cause the soil to become brittle, lower soil respiration, and decrease the activity of certain macro-organisms such as earthworms [27, 28]. It also lessens animals' immunity against infections, maintains vigor, and increase the likelihood that an animal will successfully mate [29]. Since the 1980s, microbial pesticides, especially those based on entomopathogenic bacteria and fungi, emerged as a sustainable alternative for pest control [30, 31]. The mode of action of bio-pesticides includes regulation of gut disruption, pest metabolism and pest growth.

These actions contribute to their efficacy in pest management. Bio-pesticides function by releasing neuromuscular toxins and bioactive chemicals, denaturing protein, inducing metabolic disorders and paralysis. Compared to conventional pesticides, bio-pesticides have the potential to change the trajectory of insect resistance because of their various effects [32–34]. Complete blood count (CBC) offers detailed information about red blood cells (RBC's), white blood cells (WBC's) and platelets. The bone marrow produces these blood cells. Hematological characteristics have been used to diagnosis many diseases triggered by industrial compounds, heavy metals, dyes, drugs, pesticides and many others.

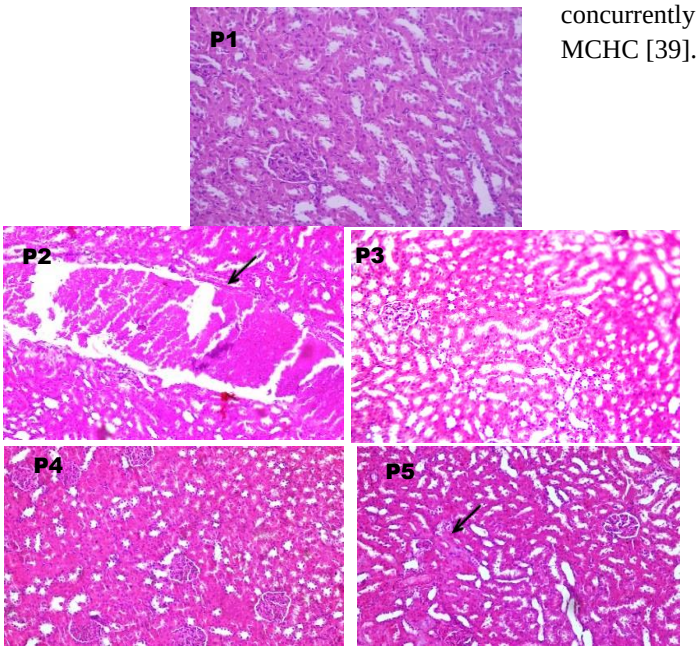

Fig. 2 Pathological effect of MR1 and MR2 on Kidney of male albino rats (P1: Control, P2: engorged dilated renal blood vessels (black arrow) for low dose of MR1, P3: normal kidney for high dose of MR1, P4: normal kidney for low dose of MR2 and P5: mild to moderate necrotic tubular epithelium with nearly normal other renal tubules for high dose of MR2**).**

RBC's (erythrocytes) are essential for the transportation of oxygen from the lungs to the tissues and hemoglobin concentration is directly correlated with RBC's count. Increase in PCV, RBC and Hb indicates that the rats were not anemic. PCV measures the percentage by volume of packed RBC in a whole blood sample after centrifugation, measures the amount of Hb in the blood and provides an estimate of oxygen carrying capacity of the RBCs. This notable increase in PCV, RBC and Hb may result from blood regeneration in the circulatory system, since there was a decrease in stress brought on by the organism's decreased activity. This helps in increasing the oxygen carrying capacity and tissue oxygenation. The obtained results from this research work agree with those found by many authors who stated that Bt increase RBCs counts and hemoglobin concentration in rabbits and rats [35, 36].

Furthermore, Eissa and Zidan [37] and El-Saadany et al. [38] observed that male rats treated with Bt had significantly lower white blood cell counts than usual. Immunosuppression symptoms might be represented by this reaction [37]. Our findings further imply that, in reaction to hemolysis, the bone marrow may be releasing immature red blood cells early. As a result, reticulocytes increased concurrently with increases in MCV and decreases in MCHC [39].

The kidney and liver are vital and crucial organs that are very vulnerable to any harm caused from external sources. Both organs are responsible for metabolism, excretion, detoxification, and storage of different bacteria and their metabolites. The release of cytosol enzymes into the blood takes place when the hepatic cell membrane is damaged. These enzymes include alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT). It should be noted that both AST and ALT are intracellular enzymes, and their presence in the bloodstream is suggestive of cellular damage. Because of this, it's critical to assess the toxicity that particular bacteria create in such target organs based on specific characteristics. In fact, physiological values of AST and ALT could rise to 3.5 and 6-fold the average value, respectively, according to a review of historical data from Sprague-Dawley rats that took reference values of biochemical parameters into consideration [40]. Peng et al. [41] reported that the histopathological findings of an experiment to study effect of an oral administration of GM Bt with vegetative insecticidal protein gene in rats showed no significant differences between control and treated groups. According to Sun et al. [42], Bt can alter the defense system of liver cells. They can also produce

oxidative stress enzymes and stimulate lipid peroxidation because free radicals are formed and cause damage to the cell membranes of hepatocytes in rats, thus eventually leading to an inflammatory response. The histopathological results of this study showed no alterations in the kidney. These results could be associated with the hepatic activity in the detoxification process as it seemed that the hepatic activity did not allow the toxin to spread to the kidneys. The appearance of engorged dilated renal blood vessels when the tested animals were exposed to low dosages of the biological insecticide could be due to the effect of the oxidative stress enzymes produced by the Bt formulation (fig. 2P2). Also, no differences in albumin and total protein levels were detected among the groups of treated animals. Moreover, the lesions found in liver histopathological samples were mild, even in those animals treated with the higher dose of the formulations. It is well known that albumin produced by the hepatic parenchymal cells, is considered one of the most prevalent plasma protein [43] that regulates osmotic blood pressure and facilitates the transportation of fatty acids, hormones, and drugs to all tissues in different body organs. Increased binding ligands to the albumin are associated with altered ligand distribution, elimination, and metabolism [44]. Uric acid excreted by the kidney is the final product of protein metabolism. Creatinine and uric acid are crucial parameters for detecting nephrotoxicity caused by exogenous compounds. These parameters are considered indices of renal damage in living organisms [45]. High levels of urea and creatinine in serum of treated male albino rats might be attributed to the reduction in glomerular filtration in the kidney. It might also indicate a renal tubule malfunction [46].

Biosafety studies have confirmed that exposure to *Bt* cry might cause slight toxic impacts to vertebrates at doses above 10^8 and 10^{11} cfu's/kg for both mice e and humans, respectively [47]. The absence of harmful effects from Cry enterotoxins in mammals can be attributed to stomach acidification and the lack of specific binding sites for Cry toxins in the intestine. Notably, these proteins undergo rapid degradation in gastric fluid, with approximately 90% degradation occurring within 2 minutes. Although the degradation process is slower in the intestine, its significance for the organism is minimal because, upon ingestion, these proteins are nearly entirely hydrolyzed by pepsin in the stomach. Additionally, studies have reported that Cry toxins do not engage with any particular region of the digestive tract [48].

Mild vacuolar degeneration of hepatocytes and engorged dilated renal blood vessels in treated groups with biopesticides formulations agree with Soumya et al. [49]. However, the material tested in this study was a commercial formulation, which might include other potentially toxic ingredients such dispersants and surfactants. Pesticide formulations might also contain toxic adjuvants that are more toxic than their active principles and that could be the reason for secondary toxic effects [50].

2. Conclusions

In conclusion, the outcomes of this study showed that novel *Bt*-based wettable powder formulations are relatively safe. However, the oral administration of these formulations caused some biochemical, hematological and pathological abnormalities in mammals. Further studies are needed to thoroughly investigate the blood and liver parameters that were affected by the oral administration of both formulations in order to assess the toxicological risks for humans.

3. Conflicts of interest

The authors declare that they have no competing interests

4. Formatting of funding sources

Not applicable

5. Acknowledgments

Not applicable

6. References

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