Original article

Biosafety evaluation of two Beauveria bassiana products on female albino rats using acute oral test

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Abstract

Application of bio-pesticides in agriculture has been developed as alternative agents to conventional pesticides due to residues accumulating which causing detrimental effects to human and environment. The aim of this investigation is to evaluate biosafety of a bio-insecticide Beauveria bassiana using two products in female rats by single oral dose through hepato- and renal toxicity, hematotoxicity and lipid profile. The two products from B. bassiana (AUMC 9896) were metabolic crude (MC), and wettable powder formulation (WP) of the local isolate. Results showed a significant increase in values of erythrocytes (RBCs), leucocytes (WBCs), platelet count (Plt) and the absolute differential WBC counts. Liver enzymes (AST, ALT, and ALP) and globulin (Glb) content were reduced in the exposed female rats with both types of B. bassiana in comparison to controls. While ratio of AST/ALT and A/G, total protein level (TP) and albumin (Alb) were raised in Beauveria bassiana-treated rats (Bb-treated rats). Urea and creatinine concentrations decreased or increased significantly in treated rats. Moreover, there was a decline in the serum of lipid profiles in WP-treated rats, but LDL levels increased in all treated animal. Additionally, no mortality or toxicity in all treated. All animals treated showed non-significant modifications in body weight gain and a slight change in relative liver weights when compared to controls. These results suggest that both treatments effect markedly on function and somatic index of the liver and slight effects on CBC and lipid profile aspects of treated female rats.

1. Introduction

Unfortunately, synthetic chemical pesticides are detrimental to humans and environment; consequently, the investigators are exploring alternative methods less harmful to protect agricultural products (Sandhu et al., 2012). One of them is biological control with entomopathogenic agents of pests (EMPA), which is safe to non-targeted pests, plants, humans, and animals (eco-friendly pesticides) (Wu et al., 2014).

Microbial Pest Control Agents (MPCAs) or biopesticides are pathogenic microorganisms such as bacteria, viruses, nematodes, fungi and protistes agents (Ahirwar et al., 2013). Entomopathogenic fungi (EPF) have an essential role in pest control at agriculture, veterinary medicine, and medicine around the world (Aboelhadid et al., 2018; Eads et al., 2021). Up to now, over 1000 species of EPF and around 100 mycoinsecticides have been recorded worldwide in the Hyphomycetes class (Shang et al., 2015). B. bassiana (Balsamo-Crivelli Vuillemin – Deuteromycota: Hyphomycetes) is a widely used fungus (based mycopesticides) in the biocontrol of agricultural pests (700 insect species) and disease vectors (Zamania et al., 2013; Biswas et al., 2015; Ebani and Mancianti, 2021). Due to the insecticidal effect of entomopathogenic agents, it has been evaluated as a bioinsecticide against several insect pests (Litwin et al., 2020). Different formula-
tions of entomopathogenic fungus *B. bassiana* were evaluated against *Spodoptera littoralis*. Ali, (2016) reported that the dust formulation was the most virulent formulation to *S. littoralis* larvae. As well as Jordan et al., (2021) identified the fungal strains of *Beauveria* spp. and *Metarhizium anisopliae* with the highly virulence to *Goniperta platensis*. Despite the widespread use of microbial control agents, little information is available about their effect on the environment and non-target organisms, mainly vertebrates (Jonsson and Maia, 1999). Owing to this fact, microbial control agents should be evaluated on animals for safety in all possible conditions (Vestergaard et al., 2003).

While several countries produce mycoinsecticides commercially, not all their bio-safety protocols have been published. Before conducting any practical application of a microorganism for biocontrol, investigations on risk assessment and biosafety are required (Brunner-Mendoza et al., 2017; Strasser and Kirchmair, 2006). The acute oral toxicity experiment on an organism model required (Brunner-Mendoza et al., 2017; Strasser and Kirchmair, 2006). The acute oral toxicity experiment on an organism model should be assessed, to provide information on health risks (Brunner-Mendoza et al., 2017).

Therefore, this study aims to estimate oral LD50 of *Beauveria bassiana* culture (AUMC 9896) local strain as metabolic crude (MC) and wettable powder formula (WP) also, assess its biosafety to female rats during acute oral dosing through hepato- and renal toxicity, hematotoxicity and lipid profile parameters.

2. Materials and methods

2.1. Bio-insecticide used

In the recent study, the entomopathogenic fungus, *Beauveria bassiana* (AUMC 9896) was used as metabolic crude (MC) and wettable powder (WP) formulation (Biosissana, 2.5% WP, 1 × 10^6 Conidia/ml) and obtained from Bio-insecticide Production Unit (BIPU), Institute of Plant Protection Research, Center of Agricultural Research, Giza, Egypt.

2.2. Fungal culture

*Beauveria bassiana* (AUMC 9896) was isolated from soil and identified in Mycological Center, Faculty of Science, Assiut University (Ali and Moharram, 2014). This isolation was cultured on Czapek-dox agar (CZA) with 1% yeast extract plates in Petri dishes (9 cm in diameter) and were cultivated for 15 days at 25 ± 1 °C and 70% relative humidity (RH). Conidia were collected by gently scraping the surface of the 14-15th day old culture with an inoculation needle.

2.3. Preparation of crude extract

Flasks with 100 ml Czapek-dox liquid medium were adjusted to a concentration of 1×10^7 conidia/ml. The flasks were maintained for 5 days in shaking incubator (150 rpm), at 27 ± 1 °C and dark to produce the Blastospores stage (primary inoculation). Ten ml of primary inoculation added to 250 ml of Sabouraud's dextrose with 1% yeast extract liquid medium, and inoculated for 21 days at 240 rpm/min and 27 ± 1 °C. Each flask was filtered twice through Whatman 1 filter paper to remove the mycelia and conidia (Valencia et al., 2011). The fungus viability was evaluated before the experiment.

2.4. Mass production of conidia and formulation

Boiled rice was autoclaved for 20 min at 121 °C in Erlenmeyer flasks (500 ml). The flasks were inoculated with 1 ml of suspension (10^7 conidia /ml), then incubated in the dark for 2–3 weeks at 25 ± 1 °C (Posada-Flórez, 2008). After spores, grown left to dry at room temperature into the cardboard bags for 15 days then the conidia were separated by sieves. The conidia were added with the powdered additives to produce the wettable powder (2.5% WP) formulation that contains to 1 × 10^6 conidia/gm (Ali, 2016).

2.5. Experimental design

Thirty adult Sprague Dawley (SD), *Rattus norvegicus albinus*, female (nulliparous and non-pregnant) rats were purchased from the Egyptian Company for Biological Products and Vaccines (Helwan Farm). The females were 6–8 weeks of age and weighting 180 ± 20 g when the studies began. The animals were allowed to adapt for one week prior to the experiment under laboratory conditions: 12 h light/12 h dark, 25 ± 5 °C, 40–70% (70 ± 5%) RH and supplied with a commercial diet and water ad libitum.

The tested animals were splitted into two main groups; the 1st main group for estimate the oral LD50 of two products (MC and WP) from *Beauveria bassiana* (AUMC 9896), and the 2nd main group for assays biosafety study during acute oral toxicity. All animals were fasted overnight before dosing by orally via stomach gavage tube and were observed closely for any symptom of toxic effects.

2.6. Estimation of oral LD50

The estimation of oral LD50 was conducted according to Organization for Economic Cooperation and Development (OECD, 2008). The onset and duration of toxicological symptoms observed in the treated animals were recorded. Observation on the rats that survived was conducted daily for 21 days. Then the acute oral LD50 values were calculated.

2.7. Acute oral toxicity study

After estimating the acute oral LD50 value, the biosafety study was done according to USEPA (1996). Five animals for each two *Bb* products (MC and WP) were gavage with single dose (=LD50 values) of *Bb* MC (0.5 ml/100 g BW) and 1 ml WP/100 g BW (containing 1.1 × 10^10 conidia/100 g BW). The treated animals were allowed under observation for 21 days. Observation on the rats that survived, and the body weights were conducted daily.

At the end of the testing periods, the animals were weighed, sacrificed, and dissected. Some vital organs (livers, kidneys, brains, spleen, heart, and lung) from both control and treated animals were dissected out carefully, immediately washed with physiological saline (0.9% NaCl), then dried and weighed separately (absolute organ weight). The relative organ weight (organ body weight ratio) or the tissue somatic index was calculated according to Stanley et al., (2005); Nelli et al., (2013). The % of changes in body weight were determined according to equation of Mansour et al., (2008).

2.8. Blood sample collection

At the end of observation period, the blood sample for hematological analysis (Complete blood picture, CBC) was taken from the retro - orbital plexus into a specially prepared (commercially available) EDTA-treated tube. After filling the tube to the 1-ml mark, it was carefully shaking several times. Although the minimal amount of EDTA in the tube keeps the blood from clotting, it can be safely ignored in the quantitative analysis (Dacie and Lewis, 1991).

Also, other part from blood samples from each animal was collected into non - heparinized clean dry centrifuge tubes according to (Schmer mer, 1967). To obtain the serum, the blood samples were left to clot for about 20 min at room temperature, then centrifuged at 3600 rpm for 15 min. The supernatants were immersed in dry
clean-capped tubes and stored at –40 °C until performing the biochemical analysis.

2.9. Hematological determination

Hematological method described by Theml et al., (2004) was used for hematological analysis by Auto Hematology Analyzer (Counterpoint 20+, SFRI SAS, France). The hematological profile includes measurement of total cell counts platelets (plt), red blood corpuscles (RBCs) and white blood corpuscles (WBCs), hemoglobin (Hgb), packed cell volume (PCV), red cell indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW) and absolute differential WBC counts, or differentiation of leucocytes counts (DLC)]. Erythrocyte indices values were calculated using standard formulae (Schalm et al., 1975).

2.10. Biochemical assays

In the clinico-biomarkers for liver and kidney damage and lipid profiles in serum were determined using the commercial diagnostic kits. Transaminases (AST and ALT), and alkaline phosphatase (ALP) activities were assayed according to (Reitman and Frankel, 1957; Roy, 1970) respectively. According to (Bradford, 1976; Doumas et al., 1971), total protein (TP) and albumin concentrations (Alb) were measured, respectively. As well as the AST/ALT ratio, globulin (Glb) concentrations and albumin / globulin ratio (A/G ratio) were calculated. The method of (Fawcett and Scott, 1960) was used for determining the urea level, whereas the creatinine level was determined using the kinetic method of (Siest et al., 1985). The lipid profiles such as total cholesterol (TC), triglyceride (TG) and high-density lipoprotein (HDL) cholesterol levels were measured by spectrophotometer (Allian et al., 1974; Buccolo and David, 1973; Friedwald et al., 1972) respectively. From the triglycerides, the concentration of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) can be calculated using Friedewald's equation:

\[
LDL = \frac{TC - HDL - \frac{VLDL}{5}}{}
\]

\[
VLDL = \frac{TC}{5}
\]

2.11. Ethical statement

The Institutional Animal Care and Use Committee of Zagazig University (No. ZU-IACUC/2/F/156/2020) approved the ethical procedures and policies used in this study.

2.12. Statistical analysis

The oral LD50 values of B. bassiana (AUMC 9896) at both products (MC and WP) was determined using the software AOT425S-tatPgm “Acute Oral Toxicity (Guideline 425) Statistical Program”. For the biosafety acute oral toxicity study, all data as percentage were transformed into angular transformation values (arcsin √percent) data to carry out the analysis. All data were analyzed using Statistical Package for Social Science (SPSS) for Windows (IBM), version 25, Chicago, USA) and presented as means ± S.E. ANOVA one-way test followed by the Duncan multiple tests were applied to assess the differences between means. All values of results data were considered significant (*) at p ≤ 0.05 significant from the control.

3. Results

3.1. Acute oral LD50 study

Our results showed that the estimated acute oral LD50 values of B. bassiana strain AUMC 9896 in female rats were more than 5000 mg / kg BW and greater than 1.1 × 10^10 conidia/kg with crude (MC) and formulation (WP) products respectively. Additionally, no mortality, toxicological symptoms, pathogenicity or infection were observed in the tested animals.

3.2. Acute oral toxicity study

3.2.1. Hematological studies

Hematological results are represented in Table 1 showed significant increases in RBCs in treated animals with both Bb products, and WBCs of females treated with Bb formulation while, MCH values significantly decreased in all Bb products treated animals. As well as the absolute differential WBC counts (DLC) such as lymphocytes, monocytes, eosinophils, and neutrophils were significantly increased with Bb formulation treated females. Otherwise, no significant changes in Hgb, PCV, MCV, and MCHC induced in all treated groups comparing to control females.

3.2.2. Clinico-biochemical studies

Based on clinico-biochemical of liver function vis transaminases (ALT, AST), ALP and Glb were reduced in the exposed female rats with both type of B. bassiana in comparison to control group as

| Table 1 |
|---|
| **Effects of B. bassiana as metabolic crude (MC) and wettable powder formulation (WP) on hematological parameters of female rats after acute exposure.** |
| Items | Control | Beauveria bassiana (MC) | Beauveria bassiana (WP) |
| RBCs (10^6/µl) | 6.292 ± 0.0225 | 6.558 ± 0.0815* | 6.78 ± 0.0912* |
| WBCs (10^6/µl) | 7.538 ± 0.2542 | 8.247 ± 0.287 | 8.764 ± 0.5129* |
| Hgb (g/dl) | 14.2 ± 0.2191 | 14.28 ± 0.2538 | 14.88 ± 0.1855 |
| PCV (%) | 39.1 ± 0.5013 | 39.28 ± 0.1231 | 40.17 ± 0.5391 |
| MCV (fl/cell) | 63.16 ± 1.139 | 61.12 ± 0.6499 | 61.38 ± 0.68731 |
| MCH (Pg/cell) | 22.58 ± 0.2577 | 21.72 ± 0.1261* | 21.92 ± 0.1319* |
| MCHC (g/dl) | 35.72 ± 0.0205 | 35.52 ± 0.1513 | 35.78 ± 0.1666 |
| RDW (%) | 21.9 ± 0.2225 | 22.61 ± 0.1849 | 22.13 ± 0.2262 |
| Platelet (10^3/mm³) | 665.6 ± 23.82 | 680.8 ± 28.96 | 924.2 ± 38.74* |
| Differentiation of WBCs |
| Lymphocytes (k/µl) | 6.322 ± 0.2335 | 6.81 ± 0.2499 | 7.534 ± 0.2057* |
| Monocytes (k/µl) | 0.2344 ± 0.0086 | 0.2844 ± 0.0224* | 0.3296 ± 0.0141* |
| Eosinophils (k/µl) | 0.1168 ± 0.0044 | 0.1464 ± 0.0041* | 0.1436 ± 0.0039* |
| Neutrophils (k/µl) | 0.7416 ± 0.0304 | 0.9068 ± 0.0383 | 1.172 ± 0.0808* |

n = 5 (M ± SE) * significant at P ≤ 0.05. The percentages were transformed into angular transformation values (arcsin √percent).

| Table 2 |
|---|
| **Effects of B. bassiana as metabolic crude (MC) and wettable powder formulation (WP) on liver function of female rats after acute exposure.** |
| Items | Control | Beauveria bassiana (MC) | Beauveria bassiana (WP) |
| AST (U/l) | 13.77 ± 0.6768* | 14.16 ± 0.2304* |
| ALT (U/l) | 55.42 ± 1.727 | 56.32 ± 0.5588* | 13.86 ± 0.4031* |
| ALP (U/l) | 3.758 ± 0.135 | 3.909 ± 0.1535* | 8.426 ± 0.054* |
| TP (g/dl) | 4.705 ± 0.0678 | 7.838 ± 0.1738* | 6.337 ± 0.0928* |
| Glb (g/dl) | 2.883 ± 0.1474 | 2.253 ± 0.0462* | 2.088 ± 0.0903* |
| A/G | 1.64977 ± 0.09 | 3.488 ± 0.1376* | 3.063 ± 0.1698* |

n = 5 (M ± SE) * significant at P ≤ 0.05.
shown in Table 2. While AST/ALT ratio, TP, Alb, and A/G ratio were increased in all treated female rats.

Data obtained from kidney functions (Fig. 1A) showed urea concentration which significantly decreased in treated rats MC product, conversely it elevated in rats treated with WP formulation in comparison to control. Moreover, creatinine levels (Fig. 1B) were increased in females treated with MC but decreased with WP formulations.

Data presented in Fig. 2 illustrates the lipid profile analysis in MC and WP treated females. Serum cholesterol, triglyceride, HDL and VLDL levels in Bb WP- treated rats were reduced compared to control group (Fig. 2 A, B, C, E). On contrary, LDL levels (Fig. 2 D) were markedly raised in all treated animal compared to untreated group.

3.2.3. Body and relative organ weight studies

Body weight changes and tissue somatic index (relative organ weights) have been observed (Tables 3 and 4) as physiological status of control and treated females. Results showed that these products as bioinsecticides did not cause any significant changes in the body weight gain (Table 3) and tissue somatic index (Table 4) of exposed animals with products of B. bassiana compared with control rats. Meanwhile, slight increase was observed in somatic index of liver in treated group with MC product.

4. Discussion

Results of LD_{50} study showed that no mortality, or toxicological symptoms, occurred in any group throughout the experiment con-
B. bassiana, as metabolic crude (MC) and wettable powder formulation (WP) on body weight gain of female rats after acute exposure.

| Treatments | Parameters | Beauveria bassiana Controls | Beauveria bassiana WPs |
|------------|------------|------------------------------|------------------------|
| Initial    | 187.0 ± 5.148 | 198.0 ± 6.042 | 208.0 ± 4.359 |
| Final      | 204.0 ± 4.583 | 217.0 ± 7.176 | 224.0 ± 4.848 |
| BWG        | 17.0 ± 1.225  | 19.0 ± 1.871  | 16.0 ± 1.0 |
| BWG/W      | 0.0305 ± 0.0027 | 0.0119 ± 0.0027 | 0.0256 ± 0.0014 |
| % BWG/W    | 10.02 ± 0.4527 | 10.26 ± 0.4524 | 9.199 ± 0.251 |

\[ n = 5 \text{ (M ± SE) } * \text{ significant at } P \leq 0.05. \]

The percentages were transformed into angular transformation values (arc sin, %). The same result of increased values of WBC and Plt was obtained by Kasmi et al. (2018) who demonstrated that treatment of difenconazole affected fibrinolysis systems and blood coagulation of rats. Generally, the exposure to any toxicants triggers modifications in leukocyte system exhibiting heterophilia and lymphopenia in leukocytosis form (Ahmad, 2012).

According to hematological markers which reflects the physiological sensitivity of the animal (Kreutz et al., 2011). According to hematological aspects, the abundance in the WBC’s count probably can be because of inducing the animal’s immune system (Yousef et al., 2003; Lamfon, 2013), and/or the transformation from the spleen to peripheral blood in leukocytic pool (Lamfon, 2013). Additionally, the leukocytes increase occurred due to the inflammation resulted from pesticide toxicity (Dinis-Oliveira et al., 2007).

The toxicology of various chemicals was evaluated by TP and A/G ratio test. In the present experiment an elevation of TP and A/G ratio of Bb–treated female rats were observed. The increase of A/G ratio with the decrease of serum globulin levels (Glb) reflected the high levels of TP in the tested female rats. These increases in TP levels and A/G ratio in Bb-treated rats are likely due to disturbance in the function of liver and kidneys (Mansour and Mossa, 2005).

The kidney has a multifunctional role in the elimination of waste and toxic substances from blood, so it is the primary organ that damaged after exposure (Harris and Neilson, 2012; Mossa et al., 2018). Our data exhibited a significant elevation of creatinine level in MC- treated rats. Similarly, Eissa and Zidan (2010) reported that creatinine levels were elevated in the rats treated with abamectin and Bacillus thuringiensis. In addition, Walmsley and White (1994) demonstrated that serum treated female rats have an increase of creatinine level and urea concentrations. These findings might be due to nephrotoxicity and malfunction of kidney tubules (Eissa and Zidan, 2010).

Cholesterol is an essential component of lipids, which is synthesized in the liver to serve as a precursor to steroid hormones in cell physiology (Kuzu et al., 2016). Cholesterol is one of the main causes of death and diseases resulted in free radicals. It is well-known that natural antioxidants such as plants and microbial metabolites, bacteria and fungi is considered a source of antioxidants which can scavenge the free radicals in the body (Zohri et al., 2017; El-Saadony et al., 2020, 2021, 2022; Abdel-Moneim et al., 2022; Saad et al., 2015, 2021). Significant decrease of cholesterol in this experiment may be due to hyperthyroidism (Rizos et al., 2011).

In toxicological studies, the body and organ weight are an indicator for the metabolic state, normal growth and development of the organism, and an indicator of side effects of different chemicals such as organ dysfunction, detoxification process, and organ toxicity (Mukinda and Eagles, 2010; Mossa et al., 2018). The relative liver weights in rats exposed to B. bassiana increased significantly compared to the untreated animals, possibly due to the potential toxicity of B. bassiana acute dose exposed. This increase is in accordance with research from EL-Gendy et al. (2015) by abamectin in mice. Moreover, Eweis et al. (2015) found that the weights of rats and organs (liver, brain, and kidney) were changed in rats treated with B. bassiana Vuillemin.

In recent studies, several investigators have been interpreted these adverse effects of B. bassiana strains on hematological, clinico-biochemical (liver and kidney functions and lipid profile) parameters in treated albino rats. For example, Strasser et al., (2000); Fuguet & Vey (2004) reported that Beauveria spp. strains have the potential to produce several secondary metabolites with various biological and chemical properties (bassianin, tenellin, etc.).
bassicrin, beardocin, bassianolide, beaveruloids, and oosporin) after the application. As well as Fuguet et al., (2004); EFSA (2018) demonstrated that the metabolites of Bb such as low molecular weight compounds, non-peptide pigments (bassianin and tenellin), cyclodepsipeptides (beaverulcin and bassianolide), high molecular weight proteins, and a hydrophilic, chitosanase-like protein have different potential effects on humans and the environment according to the metabolite type. The scientific opinion of EFSA regarding the health hazardous to human and animal was attributed to beaverulcin existence in food (EFSA, 2013; EFSA Contam Panel, 2014; EFSA Biohaz Panel, 2020).

Particularly, beaverulcin is toxic to mammals’ cell lines since it is a specific inhibitor of cholesterol acyltransferase, inducing apopptosis and cytosis (Vey et al., 2001; Pascale et al., 2002). Besides, lipid profiles (triglyceride, cholesterol, and high-density lipoprotein) were inhibited in females treated with Bb formulation. This decrease may be related to the ability of beaveruloids metabolite to repress the accumulation of lipids. This hypothesis was further supported by Namatame et al. (2004) who illustrated the beaveruloids from Beavervia sp. FO-6979 suppress the lipid droplet aggregation in primary peritoneal macrophages of rats. Also, Kozłowska et al., (2018) informed that B. bassiana is considered a biocatalyst for steroid compounds transformation such as dehydroepiandrosterone (DHEA, synthesized from cholesterol). As well as Jeffs and Khachatourians (1997) demonstrated that bassianin and tenellin metabolites inhibit the erythrocyte membrane ATPases.

Some metabolites of most beavervia strains were assessed, and no available information on the others and their potential effect on humans or the environment (EFSA Biohaz Panel, 2020). Regarding registration and risk evaluation, these metabolites toxicity and their actions are of concern (Zimmermann, 2007).

5. Conclusion

The present study could be concluded that each product type of B. bassiana –ALIMC 9896 (MC and WP) affected on function and somatic profiles (triglyceride, cholesterol, and high-density lipoprotein) have different potential effects on humans or the environment (EFSA Biohaz Panel, 2020). Regarding registration and risk evaluation, these metabolites toxicity and their actions are of concern (Zimmermann, 2007).

Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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