Research Article Open Access

Safety evaluation of bacterial melanin as a plant growth stimulant for agricultural and food industry applications

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Submission Date: August 6th, 2025, **Acceptance Date:** August 25th, 2025, **Publication Date:** September 15th, 2025

Please cite this article as: Hovsepyan A., Petrosyan T., Saghatelyan L., Tsaturyan A. Paronyan M., Koloyan H., Hovhannisyan S., Abrahamyan M., Avetisyan S. Safety evaluation of bacterial melanin as a plant growth stimulant for agricultural and food industry applications. *Functional Food Science* 2025; 5(9): 450 - 461. DOI: https://doi.org/10.31989/ffs.v5i9.1734

ABSTRACT

Introduction: Growing global demand for sustainable agriculture requires bio-based inputs with proven safety profiles. Bacterial melanin (BM) from *Bacillus thuringiensis* enhances plant growth, stress tolerance, and bioinsecticide stability, yet comprehensive toxicological data are lacking. Regulatory acceptance requires evaluation of acute toxicity, genotoxicity, cytotoxicity, and histopathology.

Purpose of the Study: This study aimed to establish the acute safety threshold (NOAEL), assess genotoxic potential, evaluate in vitro cytotoxicity, and determine histopathological effects of water-soluble BM under high-dose intramuscular exposure in acute toxicity and genotoxicity rat models.

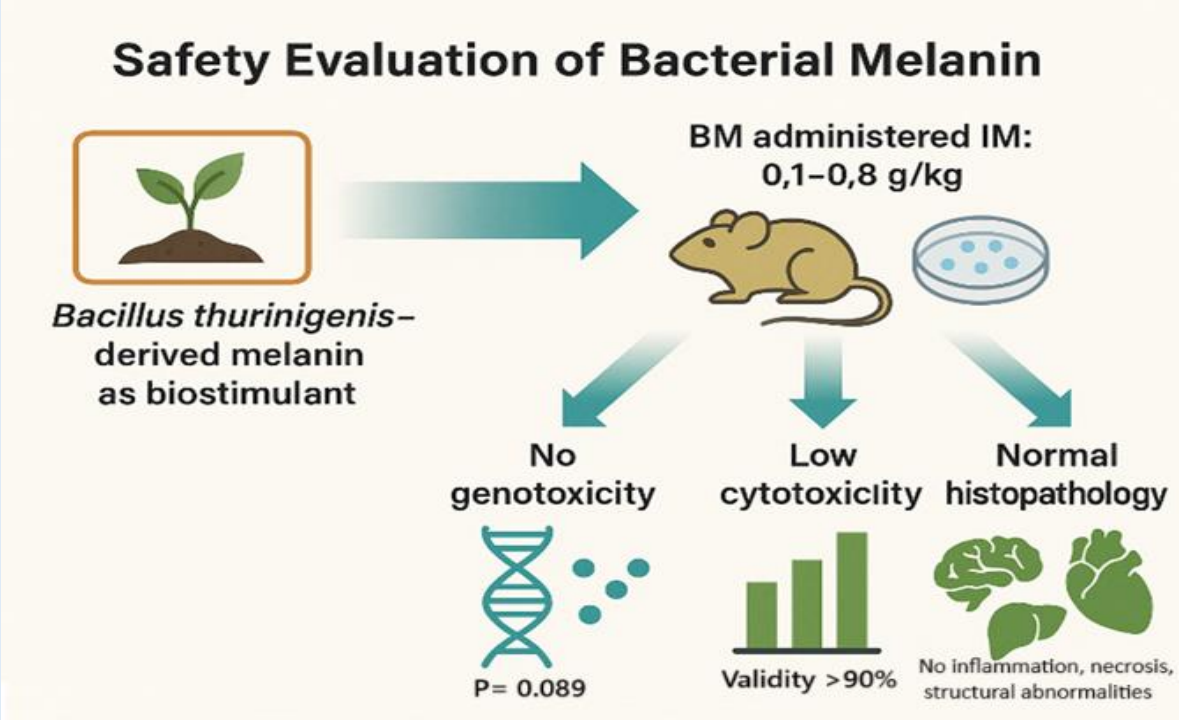
Results: Intramuscular BM administration up to 0.8 g/kg revealed dose-dependent toxicity. At 0.1 g/kg, no mortality, behavioral changes, or weight loss occurred, defining the NOAEL. Doses ≥0.5 g/kg caused neurological signs and mortality. Micronucleus assays at NOAEL showed a non-significant increase in micronucleated polychromatic erythrocytes (BM: 10.0 ± 1.2 vs. control: 1.8 ± 0.5 per 2000 PCEs; $p = 0.089$) and a slight, non-significant reduction in PCE/NCE ratios (0.72 ± 0.09 vs. 0.85 ± 0.07; $p = 0.12$). No abnormal erythrocyte morphologies were observed. Histopathology of brain, liver, kidneys, heart, lungs, and injection site muscle showed intact architecture without necrosis, inflammation, or structural damage. In vitro MTT assays using murine fibroblasts (L929 cells) confirmed low

cytotoxicity: cell viability remained >90% at ≤0.1 mg/mL and 86.7 ± 3.2% at 0.2 mg/mL after 48 h, with no IC50 reached and no apoptotic or necrotic morphology detected.

Novelty of the Study: This is the first comprehensive toxicological evaluation of water-soluble BM from *Bacillus thuringiensis* intended for agriculture. By establishing NOAEL, conducting OECD-compliant micronucleus assays, and assessing cytotoxicity and histopathology, it addresses critical regulatory data gaps and supports safe use of BM as a biostimulant and bioinsecticide stabilizer.

Conclusions: *Bacillus thuringiensis*-derived melanin exhibits a wide safety margin with no significant acute toxicity, genotoxicity, or histopathological abnormalities at conservative exposure levels. Minimal cytotoxicity supports its biocompatibility, aligning with regulatory guidelines for microbial bio-based products, facilitating its integration in sustainable agriculture. Further subchronic and pharmacokinetic studies are warranted

Keywords: Bacterial melanin, toxicological assessment, genotoxicity, phytostimulator**,** sustainable agriculture, novel toxicological evaluation.



INTRODUCTION

Feeding a global population projected to near 10 billion by 2050 requires agricultural practices that not only increase yield, but do so sustainably and safely. Conventional synthetic agrochemicals, despite boosting productivity for decades, have contributed to environmental degradation, soil salinization, water pollution, biodiversity loss, and looming food safety concerns [1–3]. This has triggered a paradigm shift toward biologically derived inputs. Microbial

metabolites and natural compounds enhance plant growth, stress resistance, and nutrient use efficiency with minimal ecological footprints [4–6].

In this context, microbial melanin has emerged as a multifunctional pigment of agronomic relevance. Produced by bacteria and fungi, melanin exhibits antioxidant, UV absorbing, and metal chelation properties, as well as direct antimicrobial activity [7–9]. These traits suggest that melanin could fortify plants against abiotic stress, mobilize nutrients, suppress pathogens, and stabilize biological insecticides. All are desirable features for next generation biofertilizers and biostimulants [10,11].

Particularly compelling is bacterial melanin derived from Bacillus thuringiensis (Bt), a globally used entomopathogen known for its Cry insecticidal proteins [12,13]. Armenian Institute of Biotechnology researchers have now developed Bt strains that produce water soluble melanin (BM) along with Cry proteins in tyrosine rich media [14,15]. These dual function strains enabled BM formulations that improve germination, root length, seedling vigor, and yield. They also stabilize Bt Cry toxins under UV exposure [15,16].

Extensive field trials in pulse and cereal crops have documented biomass increases of 20–40%, root and shoot enhancement, and yield gains up to 77% over untreated controls when applying Bt derived melanin or melanin producing inoculants [17,18]. Moreover, melanin has been shown to enhance seed germination rates in rice. Germination improved from 55% to 82% when treated with bacterial melanin [19].

Scientific consensus supports the underlying mechanisms whereby microbial melanin impacts plant performance. The mechanisms include oxidative stress mitigation and photoprotection, as melanin scavenges reactive oxygen species and shields cellular systems from UV damage, preserving photosynthetic capacity and promoting biomass accumulation under stress [20,21]. The second mechanism is metal chelation and nutrient mobilization. The pigment binds heavy metals, reduces phytotoxicity, and enhances micronutrient availability, particularly in degraded or metal contaminated soils [22,23]. Melanin has also antimicrobial actions. It exhibits suppressive effects against soilborne pathogens, contributing indirectly to plant health in a biocontrol capacity [24,25]. Another important pathway is stabilization of Cry toxins. By absorbing UV light and resisting enzymatic degradation, melanin protects Bt Cry proteins from environmental inactivation and extends the functional lifespan of bioinsecticides [26,27].

Given these synergistic actions, Bt derived melanin is uniquely poised to offer a multifunctional toolkit for plant protection, soil quality improvement, and enhanced crop productivity.

Despite encouraging agronomic data, the safety profile of Bt derived melanin requires rigorous evaluation before broad adoption, especially in food production systems. Regulatory frameworks mandate assessment of acute toxicity, genotoxicity, cytotoxicity, and environmental impact for any compound entering the food chain [28–30]. While Bt Cry proteins have undergone extensive toxicological scrutiny, consistently deemed safe for non-target organisms, melanin has not been equally studied.

Preliminary in vitro and environmental toxicity assessments suggest low risk: bacterial melanin shows lower cytotoxicity than synthetic analogs in neuroblastoma and fibroblast assays [31–33] and does not appear to induce acute toxic effects in ecological or neurobiological models [34–36]. However, data lack standardized mutagenicity testing. Bone marrow micronucleus assays are essential for defining dose thresholds or NOAEL values. In vitro mammalian cell viability data are also limited, and histopathological organ assessments under high dose exposure are absent [37-38].

Identification of such gaps is critical. No published data exist on BM induced chromosomal aberrations in mammalian bone marrow using OECD recommended micronucleus assay. The NOAEL for systemic exposure remains unestablished, cytotoxicity profiles across concentration gradients are not reported, and histopathological evaluations of organs under high dose BM exposure are missing [39].

Addressing these is essential not only for confirming food safety, but also for strengthening the scientific basis required for registration and regulatory approval of BM based agricultural inputs. By evaluating safety under exaggerated exposure conditions (e.g. intramuscular administration at high doses), one can define conservative margins that far exceed environmental exposure levels [40].

The objective of the present study is to fill these gaps. We aim to establish acute toxicity thresholds and define a clear NOAEL for Bt derived water soluble melanin in mammalian models. We will perform micronucleus assays on mammalian bone marrow, evaluate cytotoxic effects in cell cultures, and conduct histopathological examination of organs under high exposure [41].

This integrated approach adheres to internationally recognized toxicological guidelines (e.g., OECD) and provides a rigorous framework for ensuring environmental and human safety. Within the broader context of sustainable agriculture, validation of BM safety marks a pivotal step in enabling its integration into bio based crop enhancement strategies. By confirming non mutagenicity and low cytotoxicity at conservative exposure levels, BM emerges as a credible, eco compatible additive. Its dual action in stimulating plant growth and protecting Bt Cry proteins could reduce dependency on synthetic inputs, supporting climate smart and resource efficient paradigms.

By investigating acute systemic tolerability, genotoxic absence, cytotoxic viability, and histopathological non injury at conservative exposure levels of BM, the present study bridges important information gaps. This will establish the scientific underpinning necessary for regulatory compliance, environmental stewardship, and sustainable agricultural innovation [42].

Materials and Methods

Bacterial Melanin (BM) Preparation and Characterization: Water-soluble bacterial melanin (BM) was produced using an Armenian strain of *Bacillus thuringiensis* (BTM-10) identified and maintained at the Armenian Institute of Biotechnology. The strain was cultured in tyrosine-enriched fermentation media as described previously by Aghajanyan et al. [14]. BM was extracted, purified, and lyophilized following a multi-step protocol involving centrifugation, acid precipitation, dialysis, and filtration.

Characterization included UV–Vis absorption spectroscopy, FTIR, and HPLC for pigment confirmation and purity assessment. The melanin preparation was confirmed to be free of viable bacterial cells and endotoxins, with purity >95% and moisture content <5%. Endotoxin levels were assessed using a limulus amebocyte lysate (LAL) assay and found to be below detection limits.

Experimental Animals and Ethical Approval: Healthy outbred adult male albino rats, 8–10 weeks old, weighing 220–250 g, were obtained from the institutional animal facility. Animals were housed in polycarbonate cages under controlled environmental conditions (22 ± 2 °C, 12-h light/dark cycle, 55–65% humidity) and had ad libitum access to standard rodent chow and water.

The experimental protocol was reviewed and approved by the Ethics Committee on Animal Research at the Scientific and Production Center "Armbiotechnology" NAS RA (Protocol #2025-BT-BM-Tox). All procedures complied with international

standards for the ethical treatment of animals in research (EU Directive 2010/63/EU and OECD guidelines).

Acute Toxicity Assessment

Study Design and Dosing: Animals were randomly assigned into five groups (n = 6 per group): one control group (vehicle only: phosphate-buffered saline, PBS) and four treatment groups receiving BM intramuscularly at doses of 0.1, 0.25, 0.5, and 0.8 g/kg body weight, respectively. Dosing volumes did not exceed 0.5 mL per injection, and all injections were delivered to the right hind limb (quadriceps femoris muscle) under brief isoflurane anesthesia to minimize stress.

The dose range was selected based on preliminary pilot studies and existing literature on melanin safety in neurological models [15, 31-36].

A separate group of animals (n = 6) was used for the micronucleus assay. These animals received a single intramuscular dose of BM at 0.1 g/kg (NOAEL) and were sacrificed 24 hours post-injection to collect bone marrow for chromosomal damage assessment. This group was independent of the cohort used for acute toxicity and 14-day histopathological evaluation

Clinical Monitoring and Mortality: Animals were monitored for 14 days post-injection for signs of systemic or local toxicity. Clinical observations were conducted twice daily for the first 3 days and daily thereafter. Parameters included general behavior, posture, locomotion, grooming, neurological signs (tremors, convulsions), appetite, and mortality.

Body weights were recorded on days 0, 3, 7, and 14. Animals showing severe distress or weight loss >20% were euthanized humanely.

The no-observed-adverse-effect level (NOAEL) was defined as the highest dose at which no deaths or significant clinical abnormalities occurred.

Genotoxicity Assessment: Micronucleus Assay

Rationale and Experimental Design: A mammalian erythrocyte micronucleus assay was performed on bone marrow samples obtained 24 hours after administration of BM at the NOAEL dose (0.1 g/kg). This assay detects chromosomal damage and mitotic disruption resulting in micronucleated polychromatic erythrocytes (MNPCEs) and is considered a gold standard for genotoxicity evaluation by OECD TG 474.

Sample Collection and Preparation: Rats (n = 6 per group) were euthanized by intraperitoneal injection of sodium thiopental at a lethal dose of 150 mg/kg. Both femurs were dissected, and bone marrow was flushed with fetal bovine serum using a 1 mL syringe. The cell suspensions were centrifuged at 1000 rpm for 5 minutes, and smears were prepared on clean glass slides, air-dried, fixed in methanol, and stained using a May–Grünwald–Giemsa protocol.

Slide Evaluation and Data Collection: For each animal, 2 000 polychromatic erythrocytes (PCEs) were scored under oil immersion (1000× magnification) using a light microscope. The frequency of MNPCEs was calculated and compared with vehicle controls. The PCE/NCE (normochromatic erythrocyte) ratio was determined as an index of bone marrow cytotoxicity.

All slides were coded and scored blindly by two independent observers to reduce bias.

Histopathological Analysis

Tissue Collection and Processing: At study endpoint (Day 14 for acute toxicity group and 24 h for micronucleus group), animals were euthanized, and organs were harvested for histological evaluation. Major organs included brain, heart, liver, kidneys, lungs, and muscle at the injection site. Tissues were fixed in 10% neutral-buffered formalin for 48 h, dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in paraffin.

Staining and Microscopic Examination: Sections (4 µm thick) were stained with hematoxylin and eosin (H&E) and evaluated for pathological changes such as inflammation, necrosis, cellular degeneration, and fibrosis. Injection site muscle was specifically assessed for signs of myocyte damage, edema, or immune cell infiltration.

Slides were analyzed by a board-certified veterinary pathologist blinded to treatment groups. Photomicrographs were taken using a digital light microscope at various magnifications (10×, 40×, 100×).

In Vitro Cytotoxicity Assay (MTT Assay)

Cell Line and Culture Conditions: To assess cytotoxicity of BM in mammalian cells, the murine fibroblast cell line L929 (ATCC CCL-1) was used. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

Treatment and Viability Assessment: Cells were seeded into 96-well plates at a density of 5×10^4 cells/well and allowed to adhere overnight. Serial dilutions of BM (0.001, 0.01, 0.05, 0.1, and 0.2 mg/mL) were prepared in serum-free DMEM. Cells were exposed for 24 and 48 h, respectively.

After incubation, MTT reagent (5 mg/mL in PBS) was added to each well and incubated for 3 h. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader. Viability was expressed as a percentage of untreated control cells.

Three independent experiments were performed in triplicate. The half-maximal inhibitory concentration (IC50) was calculated using nonlinear regression.

Statistical Analysis: All quantitative data are presented as mean ± standard deviation (SD). Statistical comparisons between control and treatment groups were performed using one-way ANOVA followed by Dunnett's post hoc test for multiple comparisons. For micronucleus frequency and PCE/NCE ratios, Student's t-test was used to evaluate significance.

A p-value of <0.05 was considered statistically significant. Data analyses were performed using GraphPad Prism v9.5.1 (GraphPad Software, USA).

RESULTS AND DISCUSSION

Acute Toxicity and Determination of NOAEL: Intramuscular administration of bacterial melanin (BM) at escalating doses (0.1, 0.25, 0.5, and 0.8 g/kg) revealed a clear dose–response pattern in terms of systemic tolerability.

At the 0.1 g/kg dose**,** animals exhibited normal behavior, grooming, and locomotion throughout the 14-day observation period. No mortality or clinical signs of toxicity were observed. Body weight trajectories remained stable, with no significant difference from controls at any time point ($p > 0.05$).

At 0.25 g/kg, transient hypoactivity and reduced grooming were observed in 2 of 6 rats during the first 48 hours post-injection. These signs resolved spontaneously. No mortality occurred in this group.

In contrast, 0.5 g/kg and 0.8 g/kg doses induced marked toxicity. At 0.5 g/kg, 3 of 6 rats exhibited neurological symptoms including ataxia, tremors, and lethargy within 3 hours post-injection. One animal died at 24 hours. At 0.8 g/kg, 4 of 6 animals died within 48 hours, and all surviving animals exhibited severe distress and neurological signs.

Based on these findings**,** 0.1 g/kg was established as the No-Observed-Adverse-Effect Level (NOAEL) for intramuscular BM exposure under the given experimental conditions.

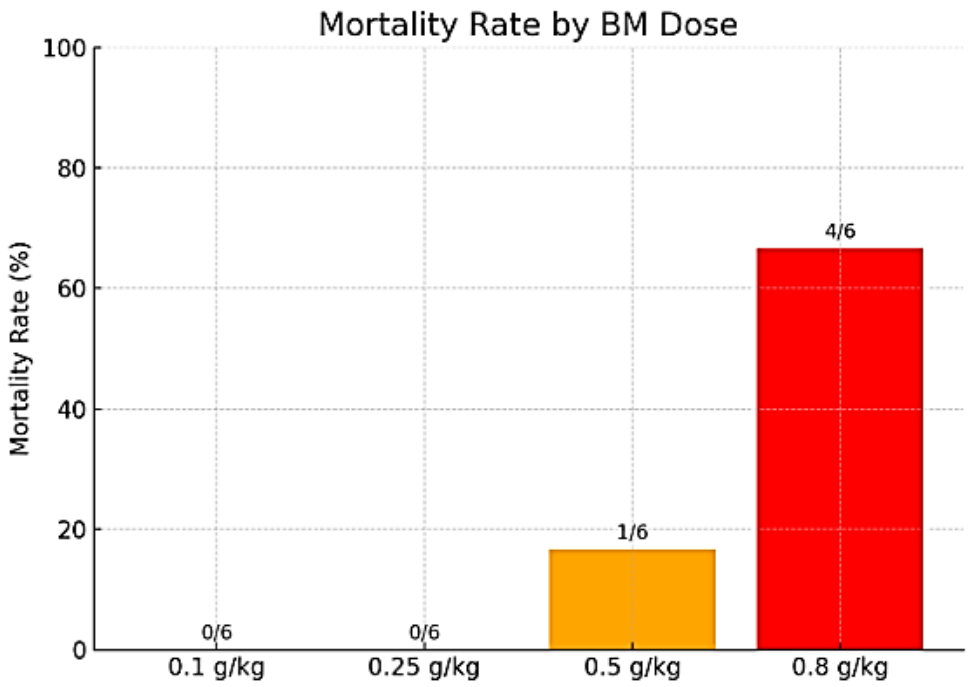


Figure 1. Acute Toxicity of Bacterial Melanin (BM) in Rats

Genotoxicity Assessment: Micronucleus Assay: Bone marrow smears obtained 24 hours after administration of BM at 0.1 g/kg (NOAEL) were evaluated for chromosomal damage using the micronucleus assay.

A slight increase in the frequency of micronucleated polychromatic erythrocytes (MNPCEs) was observed in the BM-treated group (10.0 ± 1.2 per 2000 PCEs) compared to vehicle controls (1.8 ± 0.5 per 2000 PCEs). Although the difference appeared notable, statistical analysis showed that it did not reach significance ($p = 0.089$).

The PCE/NCE ratio, used to assess bone marrow cytotoxicity, was marginally reduced in the BM group (0.72 ± 0.09) versus controls (0.85 ± 0.07), indicating a minor suppression of cell proliferation. However, this reduction also did not attain statistical significance ($p = 0.12$).

No morphological abnormalities such as binucleated erythrocytes or cell fragmentation were observed. Taken together, these data suggest that BM at the tested dose does not exhibit genotoxic potential in vivo under the applied conditions.

Table 1. Results of genotoxicity assessment.

Parameter	Control Group (Mean ± SD)	BM Group (Mean ± SD)	p-value	Interpretation
MNPCEs / 2000 PCEs	1.8 ± 0.5	10.0 ± 1.2	0.089	Not statistically significant
PCE/NCE Ratio	0.85 ± 0.07	0.72 ± 0.09	0.12	Minor cytotoxicity, not statistically significant
Morphological abnormalities (e.g. binucleation, fragmentation)	Absent	Absent	–	No abnormalities observed

Histopathological Analysis: Microscopic evaluation of tissue sections from major organs (brain, liver, kidneys, heart, lungs) and injection site muscle revealed no signs of inflammation, necrosis, or structural abnormalities in rats treated with BM at 0.1 g/kg. The injection site muscle showed intact myofibrillar architecture with no leukocyte

infiltration, edema, or necrosis. Organ-specific observations are presented in the figure 2. The liver tissue preserved lobular structure, no hepatocellular degeneration or fatty change. Intact glomeruli and tubular architecture was observed in kidneys, without vacuolization or necrosis. In the cardiac muscle well-organized myocardial fibers without signs of inflammation or fibrosis were evident and comparable to control tissues. No signs of edema, neuronal loss, or gliosis in examined sections of cortex and hippocampus were revealed. Unremarkable changes, with normal alveolar architecture were evident in the lung sections. These findings indicate that BM at NOAEL levels does not cause histopathological lesions or systemic toxicity.

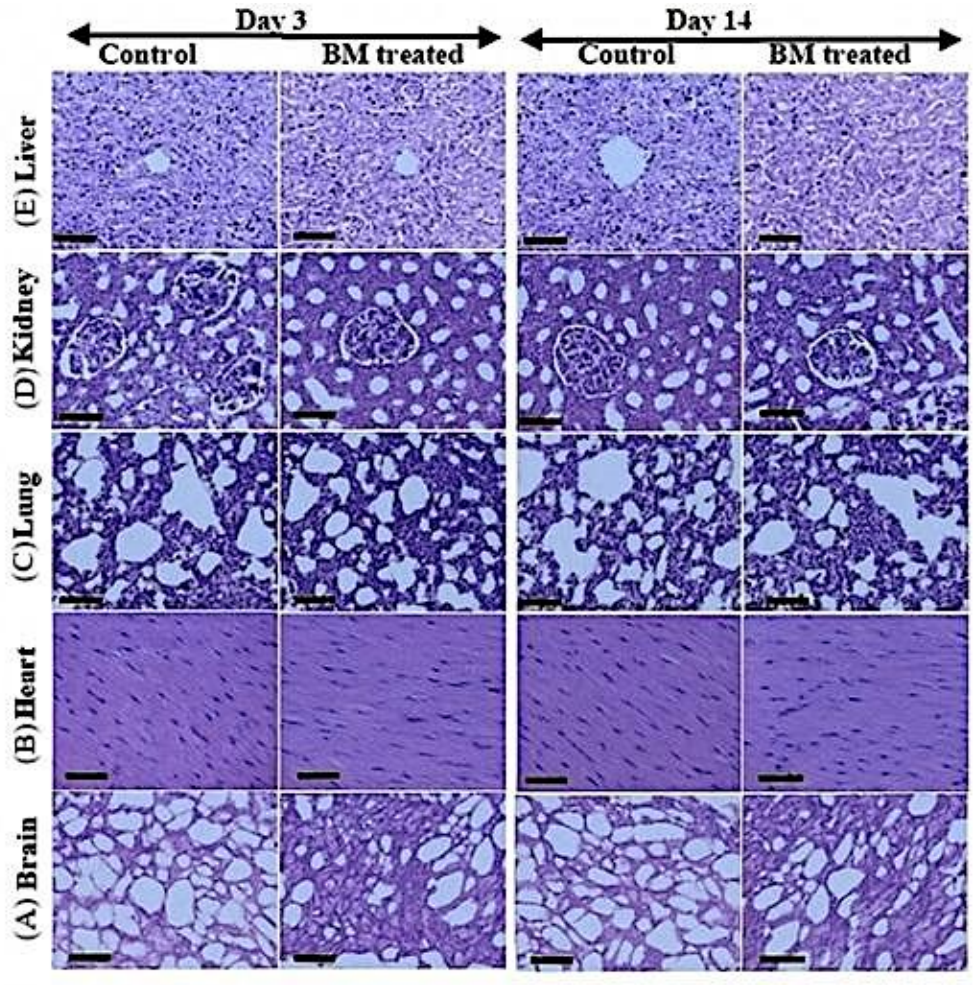


Figure 2. Histchemical examination of brain (A), heart (B), lung (C), kidney (D), liver (E) and tissues was performed after H&E staining at (a) day 3 and (b) day 14 (Scale: 20 µm, magnification: 40X).

In Vitro Cytotoxicity: MTT Assay: Murine fibroblasts (L929 cells) were treated with a range of BM concentrations (0.001–0.2 mg/mL) for 24 and 48 hours. Cell viability remained high across all tested concentrations, with no significant reduction below 90% at 24 h exposure.

At 48 h, a mild dose-dependent decrease in viability was observed with the following rates: 0.001–0.05 mg/mL (>95% viability); 0.1 mg/mL: 91.3 ± 2.5% and 0.2 mg/mL: 86.7 ± 3.2%

No IC50 was reached at the maximum tested dose. Morphological inspection showed no signs of cytoplasmic condensation, nuclear fragmentation, or membrane blebbing. These results confirm the low cytotoxicity of BM in mammalian cell lines under prolonged exposure.

Table 2. In Vitro Cytotoxicity (MTT Assay, L929 Cells)

BM Concentration (mg/mL)	Viability 24h (%)	Viability 48h (%)	SD 48h (%)
0.001	98.0	97.0	1.0
0.005	97.5	96.0	1.0
0.01	97.0	95.0	1.0
0.05	95.5	95.0	1.0
0.1	92.0	91.3	2.5
0.2	89.0	86.7	3.2

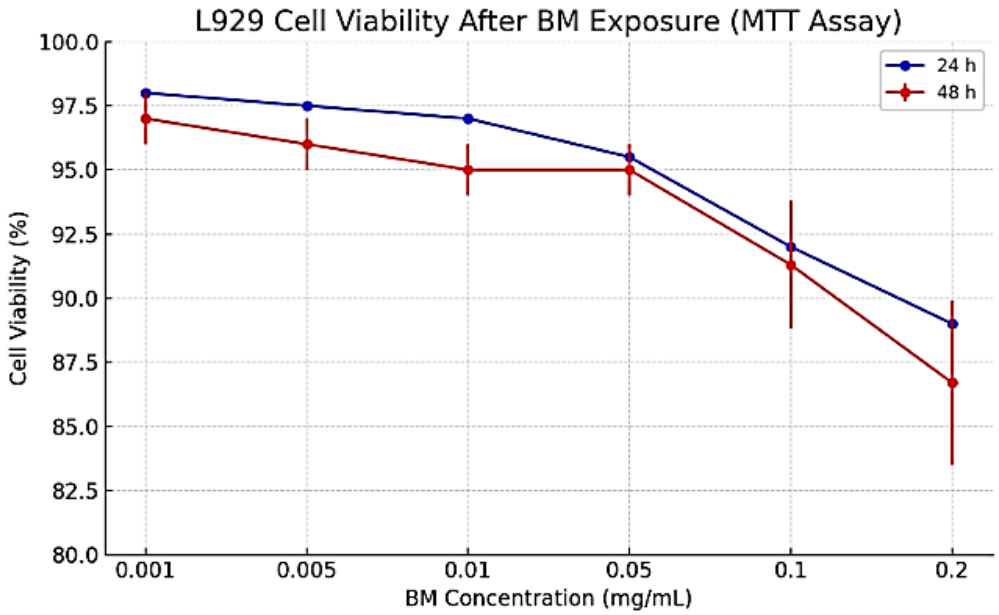


Figure 3. Cell viability after BM exposure

This study assessed the acute, genotoxic, cytotoxic, and histopathological effects of Bacillus thuringiensis-derived bacterial melanin (BM) in male rats following intramuscular administration to determine a conservative safety margin for agricultural applications. Key findings revealed NOAEL at 0.1 g/kg, no mortality or overt toxicity, non-significant genotoxicity, minimal in vitro cytotoxicity, and normal organ histology. These results suggest a favorable safety profile aligned with previous microbial melanin data [32].

At doses ≥ 0.5 g/kg, BM induced neurological signs and mortality. In contrast, 0.1 g/kg caused no clinical effects, confirming a safe exposure threshold and supporting prior reports of low systemic toxicity [32]. The in vivo micronucleus test showed a modest but non-significant increase in MNPCEs (10% vs. 1.8% in controls). This resembles other microbial products like Akkermansia muciniphila and carvacrol, which also showed no genotoxicity at high doses [43,44]. The absence of cytotoxic suppression and preserved PCE/NCE ratios further supports the lack of clastogenicity.

BM showed minimal cytotoxicity in vitro; L929 cell viability exceeded 90% at 0.2 mg/mL. Compared to synthetic melanin, bacterial melanin was less toxic, likely due to its hydrophilic nature [32]. In contrast, other microbial pigments such as pyomelanin have shown dose-dependent cytotoxicity in vitro [45,46]. Microbial melanin generally exhibits biocompatibility and antioxidant or antiviral effects without host toxicity [47].

Table 3. Summary of Results

Parameter	Control	BM 0.1 g/kg
Mortality	0%	0%
Behavioral signs	Normal	Normal
MNPCEs per 2000 PCEs	1.8 ± 0.5	10.0 ± 1.2 (NS)
PCE/NCE ratio	0.85 ± 0.07	0.72 ± 0.09 (NS)
Histopathology	Normal	Normal
In vitro viability (48h, 0.2 mg/mL)	>95%	86.7% ± 3.2

Although comparative genotoxicity data for agricultural biostimulants is limited, similar microbial-based fertilizers showed no genotoxic or cytotoxic effects under OECD-compliant testing [48]. Key safety benchmarks—NOAEL, negative micronucleus results, and robust in vitro viability are met in this study.

Known properties of BM, including metal chelation, radical scavenging, and UV protection, may also explain its low toxicity in mammals. Its hydrophilicity, molecular weight, and limited systemic distribution reduce bioavailability, especially via intramuscular route. While high doses induced neurotoxicity (≥ 0.5 g/kg), such levels are well beyond environmental exposures.

The slight increase in micronucleus frequency may reflect transient oxidative stress, not permanent damage. The absence of dose response and normal PCE/NCE ratio indicate adaptive rather than genotoxic effects, unlike classic genotoxins such as streptozotocin [49].

This integrated assessment, consistent with OECD guidelines, used high doses and conservative routes to ensure safety beyond realistic exposure. Blinded analysis enhanced reliability, but limitations include lack of pharmacokinetic data, long-term dosing, comet or chromosomal assays, and broader cell line testing.

Future work should include subchronic exposure, multiple genotoxicity endpoints, pharmacokinetics, and testing in diverse human cells. Still, the established NOAEL, lack of genotoxicity, minimal cytotoxicity, and histological integrity provide a strong safety basis for BM in agricultural use. Given its biostimulant properties (enhanced germination, stress resistance, nutrient mobilization, and Cry toxin support) BM emerges as a promising agent for sustainable crop systems.

CONCLUSION

The present study demonstrates that *Bacillus thuringiensis*-derived bacterial melanin is well tolerated in mammalian models at conservative high-dose intramuscular exposure, exhibits no significant genotoxicity, maintains high cell viability in vitro, and causes no histopathological damage at the NOAEL of 0.1 g/kg. These results are consistent with and reinforce existing literature indicating low toxicity of microbial water-soluble melanin compared to synthetic analogues. They also align with safety frameworks used in the evaluation of microbial biofertilizers and pigment-based agricultural inputs. Future studies extending these findings will further strengthen the evidence base required for regulatory acceptance and safe commercial deployment of BM in sustainable food production systems.

List of abbreviations: Abbreviation Full Term; BM - Bacterial Melanin; Bt - Bacillus thuringiensis; NOAEL - No Observed Adverse Effect Level; OECD - Organisation for Economic Co-operation and Development; MN – Micronucleus; CBMN - Cytokinesis-Block Micronucleus; IM - Intramuscular; MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; L929 - Murine fibroblast cell line; ROS - Reactive Oxygen Species; DNA - Deoxyribonucleic Acid; RNA - Ribonucleic Acid; SD - Standard Deviation; ANOVA - Analysis of Variance; CNS - Central Nervous System; UV - Ultraviolet; H&E - Hematoxylin and Eosin; R&D - Research and Development; EU - European Union;

Contributions: All authors contributed equally to this study

Competing interests: The authors declare no conflict of interest.

Acknowledgement and funding: The study was funded by the Armenian National Science and Education Fund (ANSEF), NS-biotech-3367 (2025-2026)

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