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Safety Assessment of a Fungal-based Red Colorant Produced by *Monascus purpureus* MTCC 25436

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Pigment production by *Monascus* species for application to a wide array of food products has been explored in many countries. However, possible contamination with citrinin, a mycotoxin, has been a concern with different *Monascus*-based products leading to a regulated usage of the microbial food additive worldwide. In the Philippines, there is no known local large-scale producer of microbial pigments. A red colorant from *Monascus purpureus* MTCC 25436 was successfully produced and patented by the National Institute of Molecular Biology and Biotechnology–University of the Philippines Los Baños (NIMBB-UPLB). The produced colorant was subjected to various safety assessment tests to determine its safety for food applications. The AMES test determined that the product was not capable of inducing reverse mutations in *Salmonella typhimurium* mutant strains, and this non-mutagenicity of the colorant is further confirmed by the mammalian micronucleus test. Establishing the LD_{50} was done to determine the lethal dose of the colorant. No model animal died, even after feeding with the highest allowable experimental concentration. Based on the dermal sensitization test, the colorant was a very weak sensitizer. Lastly, detection of citrinin was done using high-performance liquid chromatography. According to the generated chromatograms, citrinin was at non-detectable levels. In conclusion, the colorant obtained from the local strain *M. purpureus* MTCC 25436 is safe for consumption. The results of the study can be used by the local regulating agencies as a baseline for possibly crafting regulations for the registration of microbial-derived food colorants, which the Philippines currently lacks.

Keywords: citrinin, microbial pigment, *Monascus*, natural colorant, safety assessment, toxicity tests

INTRODUCTION

The industrial production of pigments from *Monascus* species is one of the most successful applications of microbial products. There are more than 50 patents issued in Japan, the United States (US), France, and Germany concerning the identification of *Monascus* strains, production processes, and *Monascus* products. The application of the red mold *Monascus* traces its history – even to the early Chinese era when it was believed to have a therapeutic effect. This mold was used as a food preservative, food colorant, and additive to make rice wine (Childress *et al.* 2013). Red yeast rice (RYR), a product derived from *Monascus*, is now a common dietary supplement being sold with no regulations in the market due to its claimed ability to lower blood cholesterol (Childress *et al.* 2013). Fermentation of *Monascus* for RYR production leads to the production of secondary metabolites – more specifically, the monacolin family. Monacolin K is recognized by the US Food and Drug Administration as lovastatin and is an inhibitor of

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hydroxymethylglutaryl-coenzyme A reductase involved in the biosynthesis of cholesterol (Childress *et al.* 2013). In a study conducted by Li and colleagues (1998), quails and rabbits were used as model organisms. Hyperlipidemia was induced in the test animals and thereafter fed with a dietary supplement containing RYR. Results showed a significant lowering of the cholesterol levels, which proved that *Monascus* could really lower the presence of lipids *via* the action of monacolin. Apart from lowering cholesterol levels, *Monascus* colorant was also regarded as a possible therapeutic agent against cancer. In research done by Zheng and team (2010), six pigments of *Monascus* were extracted and evaluated for cytotoxicity effects on several human cancer cells. Positive results were obtained, and the pigment that showed the greatest anticancer effect was rubropunctamine, a red pigment. This pigment has the capacity to induce cell death on human gastric epithelial cells without causing much harm to normal cells. In another related study, Lee and co-authors (2013) were able to prove that extracts from RYR could also induce apoptosis in human breast cancer cells, which could suggest that RYR extracts could be developed into a potential anticancer drug. Other secondary metabolites can be found along with the production of *Monascus* pigments. Some of these metabolites were positively acting on the maintenance of blood glucose levels. Shi and team (2011) fed diabetes-induced rats with RYR, and a significant reduction in urine sugar and urine protein levels was observed.

Issues on the Safety of *Monascus* as Food Additive

According to a review done by Pattanagul and co-authors (2007), some species of *Monascus* produce a certain type of mycotoxin called citrinin. This toxin damages the kidney and liver and has also been claimed to induce cancer formation. This toxin is also produced by other mold species like *Penicillium* and *Aspergillus* and is a rare contaminant in food products. This mycotoxin is usually found together with another toxin, ochratoxin A (Flajs and Peraica 2009). The antimicrobial activity of *Monascus* colorant is also attributed to the production of citrinin (Blanc 1995; Pattanagul *et al.* 2007). This was proven by the disk-diffusion test performed by Sroykesorn and colleagues (2011), wherein the toxin showed inhibitory effects on the growth of *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Staphylococcus aureus*. The researchers were able to establish the antimicrobial potency of pure citrinin at various concentrations. The researchers also related these values with the zones of inhibition that they had obtained through disk diffusion assays of citrinin extracted from three ultrasonic-induced mutant generations of *Monascus purpureus* TISTR 3541.

Regulations

In China, there are existing national standards for *Monascus* products. The GBI5961-22005, which was enacted in 2005, assesses the sensory requirement and physicochemical indexes of *Monascus* products. However, it does not include the regulation of citrinin. In 2008, another category was added to the previous regulation. The GB-4946-2008 for Red Kojic rice powder was added, then followed by an assessment of the health requirement. The regulation included limiting aflatoxin, but – once again – not the amount of citrinin. In Japan, *Monascus* color is already included in the list of approved additives by the Japan External Trade Organization. The regulatory requirement for *Monascus color* has been established in the Japan specifications and standards for food additives with citrinin level at 0.2 ppm. This is to address the possible health concerns associated with the presence of the fungal toxin.

In the Philippines, the first *Monascus* large-scale production patent was awarded to researchers from the BIOTECH-UPLB. Out of a multimillion investment of the government for more than a decade, researchers developed a method of producing the microbial-based colorant. Through an optimized submerged fermentation, the pigment yield can reach 920.16–1288.50 U/g on an industrial scale. The good solubility of *Monascus* Red™ in water, oil, and alcohol attracted local food manufacturers, thus pushing the researchers to lobby for its inclusion on the list of approved additives regulated by the local Food and Drug Administration (FDA). The effort resulted in the categorization of *Monascus* Red as a "novel ingredient" in the Philippines. This research study contains the comprehensive safety assessment of the *Monascus* colorant produced in the Philippines.

MATERIALS AND METHODS

***Monascus* Red Colorant**

The *Monascus* Red colorant was produced at the BIOTECH-UPLB in College, Laguna using the optimized liquid fermentation production parameters. The harvested liquid colorant was concentrated using an ultrafiltration set-up (ROMICON™ 3" Hollow Fiber Ultrafiltration, Koch Membrane System, Wilmington, Massachusetts, USA) and converted into powdered form through spray drying. The color value of the produced *Monascus* colorant was determined using a CM-5 chromameter (Konica-Minolta, Japan).

Micronucleus Test

The micronucleus test is an *in vivo* method used to screen chemicals/samples for chromosome-breaking effects (Fenech 2000). The micronucleus test of the produced

Monascus red colorant was done at the Assay Services Laboratory of the Institute of Chemistry, College of Science, University of the Philippines Diliman (UPD). The mice used were 4–5-wk-old Swiss Webster ICR albino mice obtained from the FDA in Alabang, Muntinlupa City. The animals were acclimatized for 1 wk in standard cages in groups of 4–5 mice per cage. The cages were kept in a well-ventilated room with an average temperature of 22–25 °C. A 12 h: 12 h light-and-dark cycle was observed. The mice were fed with commercial pellets and purified drinking water regularly.

Mice weighing 25 ± 5 g were administered with two doses of samples, 24 h apart, at a dose of 0.2 mL/ 20 g body weight. Oral sample administration was done using a gavage needle. Eight treatments were prepared for this experiment. Treatments were as follows: negative control (distilled water), spontaneous control (no sample administered), positive control (tetracycline; dose at 55 mg/kg body weight), pure concentrated liquid *Monascus* red colorant (10,000 ppm administration), and four colorant powder samples (500 ppm administration). The powder samples had different components: [1] colorant powder with ethylenediaminetetraacetic acid (EDTA) and tricalcium phosphate (TCP), [2] colorant powder with TCP only, [3] colorant powder with EDTA only, and [4] colorant powder without additive. Six (6) h after the second administration of the test samples, the mice were sacrificed *via* cervical dislocation. Both the femora were removed by cutting through the pelvis and tibia. The tissues were removed, and the marrow canal was exposed. The marrow was flushed several times with approximately 0.2 mL of fetal calf serum. The samples were collected and centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was decanted until about 50 µL was left. The remaining solution was mixed and then smeared onto glass slides. Three glass slides per mouse were prepared. The smeared glass slides were air-dried overnight and were stained using the following procedure: [1] 3 min in undiluted May-Grunwald stain, [2] 2 min in 50% May-Grunwald stain solution diluted with methanol, and [3] 10 min in aqueous Giemsa Stain solution. The slides were allowed to dry overnight. The slides were viewed under the microscope at 1000X magnification and were scored for the number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 polychromatic erythrocytes (PCE). Data were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test using SPSS version 16.0 to determine the statistical significance of differences between groups ($\alpha < 0.05$).

Ames Test

The Ames test was developed to screen substances for their ability to act as mutagens in bacteria (Mortelmans and Zeiger 2000). The Ames test for the *Monascus* red colorant was conducted at the Assay Services Laboratory of the Institute of Chemistry, College of Science, UPD. *Salmonella typhimurium* tester strains TA 98, TA 100, TA 1535, and TA 1537 were used in the assay, and each strain was grown overnight in nutrient broth. Standard plate incorporation was employed, which exposed the tester strain to the test samples directly on a minimal glucose agar (MGA) plate in the absence of a metabolic activation system. The samples were added to 2-mL molten top agar supplemented with a trace amount of histidine-biotin solution (0.05 mM), which was mixed and poured into MGA plates. The top agar was allowed to harden and then inverted. The plates were incubated at 37 °C for 120 h to allow efficient bacterial growth. The number of revertant colonies per plate was counted. Samples submitted for the Ames test were as follows: pure liquid colorant sample, colorant powder with EDTA and TCP, colorant powder with EDTA only, and colorant powder without additive. The positive controls used were daunomycin (for TA 98), methyl methanesulfonate (for TA 100), sodium azide (for TA 1535), and ICR 191 (for TA 98). A spontaneous control was also used, as well as distilled water as negative control. The data were analyzed for statistical significance using ANOVA. Mean differences were compared using Dunnett Test at 5% level.

Microbial Analyses

Microbial analyses of the produced *Monascus* red colorant were conducted at the Philippine National Collection of Microorganisms at BIOTECH-UPLB. Analyses included bacterial count, yeast count, mold count, detection of *Salmonella* species, detection and count of total coliforms, detection and count of fecal coliforms, and detection and count of *Escherichia coli.* The US FDA Bacteriological Analytical Manual was used as the source of the reference protocols. Two batches of powdered colorant samples were analyzed.

Heavy Metal Analysis

Heavy metal analysis of the produced *Monascus* red colorant was done at the Central Analytical Services Laboratory at BIOTECH-UPLB. Lead (Pb) and arsenic (As) contents of the powdered colorant were quantified using atomic absorption spectrophotometry (Perkin Elmer AAnalyst 400 Spectrophotometer with MHS-15 attachment, USA) (AOAC 972.25 19th ed. for Pb and AOAC 986.15 19th ed. for As).

Acute Oral Toxicity Test

Acute oral toxicity – specifically, the lethal dose 50% (LD_{50}) test – was conducted at the Standards and Testing Division, Industrial Technology Development Institute, Department of Science and Technology (STD-ITDI, TSR

052016-PTS-0034). Powdered colorant samples were submitted for analysis. Male ICR mice (27-31 g body weight) were obtained. Three preliminary increasing dosages of the samples were serially given to the animals *via* the oral route. Ten (10) mice were assigned for each dose. Any abnormal signs and behaviors were closely observed and noted for the first 2 h of dosing. Monitoring was continued up to 14 d. Thereafter, animals were sacrificed and necropsied to determine any adverse effects on vital organs.

Allergenicity Test (Dermal Sensitization Test)

An allergenicity test was also conducted at STD-ITDI (PTS ITDI-052016-PTS-0033). Powdered red colorant samples from *Monascus* were submitted for testing. The method used was the dermal sensitization method – specifically, the modified Buehler test (Robinson *et al.* 1990). Three set-ups involving 20 guinea pigs per group were done. The samples were normal saline solution for the blank group, 0.05% dinitrochlorobenzene (DNCB) for the positive control, and 300 mg/mL colorant for the test group. Hair from the area between the shoulder blades was removed by shaving and clipping. The sample was applied topically under 2 cm^2 occlusive patch conditions for a 6-h exposure period on Days 0, 7, and 14 for the induction phase. No application of the test sample was made for the 2-wk resting stage. The challenge application was then done on the 28th day under occlusive patch conditions for a 24-h contact period. Scoring of the reaction was done on the 24th and 48th h after removal of the patch during the sensitizing doses and challenge phase. The scale for scoring for skin reactions was as follows:

1. Erythema formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (but redness) to slight eschar formation (injuries in depth)	4
2. Edema formation	
No edema	0
Slight edema	1
Moderate edema (raised more than 1 mm and extending beyond area of exposure	2
Severe edema	3
Overall maximum score	7

The mean responses were computed using the following formula:

$$Mean\ response = \frac{\sum\ [(Erythema) + (Edema)]}{Total\ number\ of\ animals} \quad (1)$$

Quantification of Citrinin

The quantification of citrinin content of the produced *Monascus* red colorant was done at the Tissue Culture Laboratory of BIOTECH-UPLB. Two batches of samples were tested for the presence of citrinin using Shimadzu® Prominence LC 20 HPLC System (Shimadzu Corporation, Japan). Samples were passed through Wako Nucleosil ODS-5 (4.6 x 200 mm) C18 column with 100% methanol (isocratic) as the mobile phase at a flow rate of 1 mL/min. Detection was done at 250 nm. Citrinin standard from Sigma-Aldrich® was used for the test.

RESULTS AND DISCUSSION

Micronucleus Test

The micronucleus assay is a mutagenic test system for the detection of chemicals that can induce the formation of small membrane-bound DNA fragments (*i.e.* micronuclei) in the cytoplasm of interphase cells (Fenech 2000). These micronuclei may arise from acentric fragments, which are centromere-lacking chromosome fragments or whole chromosomes, which are unable to migrate with the rest of the chromosomes during anaphase. The purpose of the micronucleus assay is to determine if the colorant can modify chromosome structure and segregation. Harvested and stained interphase cells were then analyzed microscopically for the presence of micronuclei. Micronuclei were scored in cells that complete nuclear division following exposure to the test substance. The appearance of MNPCE is shown in Figure 1. The results of the samples tested for *Monascus* red colorant are tabulated in Table 1. The possible mutagenic effect of the addition of EDTA and TCP was also investigated in the analysis. EDTA is a popular chelating agent in the food industry that prevents the discoloration of pigments (*e.g.* betanins) by preventing metal-catalyzed degradation (Sadowska-Bartosz and Bartosz 2021). The TCP, on the other hand, is an anticaking agent that prevents or delays the formation of lumps in powdered product systems, thereby prolonging the shelf-life and stability of packaged powders (Lipasek *et al.* 2011). Based on the statistical analysis, all colorant samples tested were non-mutagenic upon comparison with the negative control (distilled water). This was further confirmed when the samples were compared with the mutagenic positive control (tetracycline). No spontaneous mutation was also observed in untreated cells. In essence, the *Monascus* red colorant did not cause mutation in living cells, thus proving the safety of the produced colorant to be used for food and other applications.

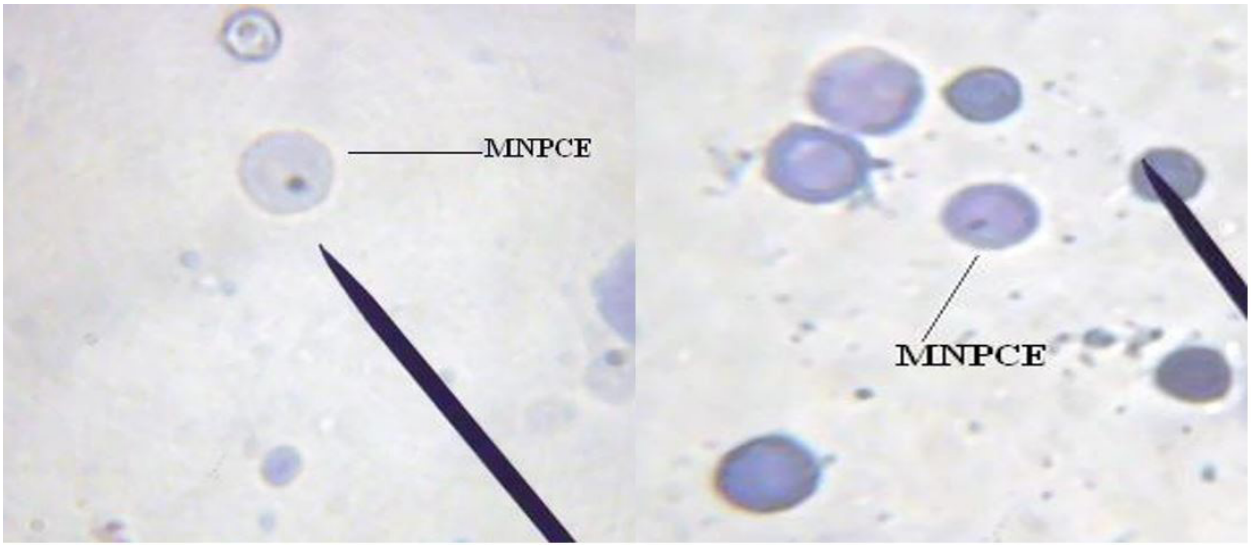

Figure 1. MNPCE from positive control tetracycline under 1000x magnification.

Table 1. Mutagenicity of the *Monascus purpureus* MTCC 25436 red colorant samples by micronucleus test.

Sample	Mean no. of MNPCE per 1000 PCE (MNPCE/PCE% ± SEM)[+]	Remarks
Negative control (distilled water)	1.78 ± 0.40	Non-mutagenic
Positive control (tetracycline)	7.52 ± 0.36*	Mutagenic
Spontaneous	1.47± 0.26	Non-mutagenic
Sample 1 (Concentrated liquid colorant:10,000 ppm)	1.59 ± 0.26	Non-mutagenic
Sample 2 (Colorant powder + EDTA +TCP: 500 ppm)	1.03 ± 0.13	Non-mutagenic
Sample 3 (Colorant powder +TCP and no EDTA: 500 ppm)	1.84 ± 0.28	Non-mutagenic
Sample 4 (Colorant + EDTA and no TCP: 500 ppm)	1.43 ± 0.18	Non-mutagenic
Sample 5 (Colorant, no EDTA and TCP: 500 ppm)	1.87 ± 0.021	Non-mutagenic

Based on Dunnett's *post hoc* test
[+]MNPCE – micronucleated polychromatic erythrocytes; PCE – polychromatic erythrocytes
*Mean difference is significant at 0.05 level upon comparison with the negative control

Ames Test

The Ames test is a rapid bacterial mutagenicity procedure that can determine the capacity of substances to cause genetic damage leading to reverse mutations (Mortelmans and Zeiger 2000). Mutagens can also cause genetic alterations leading to infertility and cancer. Thus, the Ames test is vital in safety assessment and is considered part of international guidelines for the registration of various products. The method involves the use of *Salmonella* strains that have different gene mutations within the histidine operon leading to histidine dependence. These mutations are significantly targeted by mutagens for inducing DNA damage. When *Salmonella* tester strains are inoculated on a minimal medium with trace amounts of histidine, no colonies will be formed. However, upon exposure to a mutagen, the tester strains will regain the capacity to produce histidine, allowing the reversion to histidine independence (Mortelmans and Zeiger 2000). Table 2 shows the result of the Ames test. The positive control plates were significantly different from their respective spontaneous control group at $p < 0.05$; therefore, all the positive controls were mutagenic at 100 mg/mL. On the other hand, the colorant samples, which were prepared at 25 µL/mL of top agar, were not significantly different from the spontaneous control groups at $p < 0.05$. Thus, the given samples were non-mutagenic when compared to the respective positive control on each tester strain.

Heavy Metal Analysis

As one of the common requirements for the regulation of food products, the red colorant produced by *M. purpureus* MTCC 25436 was tested for the presence of heavy metals

Table 2. Ames test for the determination of the mutagenicity of the *Monascus purpureus* MTCC 25436 red colorant.

Sample	Concentration	Revertant colonies per plate				Remarks
		TA 98	TA 100	TA 1535	TA 1537	
Negative control	–	15.4 ± 1.33	296.6 ± 5.97	247.4 ± 7.73	143.0 ± 4.36	Non-mutagenic
Positive control	100 mg/mL	49.8 ± 1.28*	529.8 ± 3.34*	555.6 ± 6.29*	352.8 ± 1.28*	Mutagenic
Spontaneous control	–	22.0 ± 0.894	288.6 ± 3.63	244.4 ± 9.85	135.2 ± 2.41	Non-mutagenic
Sample 1 (concentrated liquid colorant: 10,000 ppm)	25 µL/mL	16.8 ± 0.374	302.0 ± 11.2	253.2 ± 3.83	141.1 ± 3.80	Non-mutagenic
Sample 2 (colorant powder +EDTA +TCP: 500 ppm)	25 µL/mL	19.2 ± 1.07	274.0 ± 6.75	238.6 ± 2.25	138.0 ± 2.39	Non-mutagenic
Sample 3 (colorant powder +EDTA and no TCP: 500 ppm)	25 µL/mL	13.8 ± 0.583	271.0 ± 4.97	264.2 ± 6.77	140.2 ± 5.22	Non-mutagenic
Sample 4 (colorant, no EDTA and TCP: 500 ppm)	25 µL/mL	12.8 ± 0.663	274.2 ± 3.99	235.2 ± 4.10	140.2 ± 6.57	Non-mutagenic

Based on Dunnett's *post hoc* test at $\alpha < 0.05$
*Mean difference is significant at the 0.05 level upon comparison with the negative control

– specifically, lead and arsenic. These metals were tested since these are the usual contaminant of food components with similar processing methods as the produced colorant. Acute lead exposure adversely affects the brain, kidneys, and gastrointestinal tract, whereas constant exposure impacts the circulatory and central nervous systems, blood pressure, and vitamin D metabolism (Tchounwou *et al.* 2012). The ability of lead to cross the placental barrier and accumulation in a developing fetus, even at low-level exposure, critically affects the intellectual growth of young children (Madkour 2020). Arsenic, on the other hand, is a known carcinogen, and diet is the main source of exposure (Tchounwou *et al.* 2012). Arsenic affects almost all organs in a time- and dose-dependent manner. Results from the analysis of colorant for heavy metals are shown in Table 3. The standards for the lead and arsenic content set by the Code of Regulation by the USA FDA (FDA CFR 21) and European Union (EU) (EC 231/2012) are also provided in the table. The standards for lead and arsenic set by China, Japan, and South Korea are also presented. The values obtained from the analysis indicated that the *Monascus* red colorant passed the standards for the lead and arsenic content. Thus, the specific heavy metal contaminants were not introduced throughout the whole production process – from raw material preparation to product packaging. Atomic absorption spectrophotometry was used in detection since it is a highly sensitive, convenient, and cost-effective analytical technique that can detect heavy metals even at very low levels (Assubaie 2015).

Table 3. Heavy metal analysis of the red colorant produced by *Monascus purpureus* MTCC 25436 against the heavy metal content standards of different countries.

Sample	Lead (Pb) content (ppm)	Arsenic (As) content (ppm)
Monascus red colorant	**1.02 ± 0.00**	**0.816 ± 0.048**
Set standard		
US	< 10	< 3
EU FDA	< 3	< 3
China	2	1
Japan	< 10	< 4
Korea	< 3	< 3

Microbial Analyses

Comprehensive microbial analyses of the red colorant produced by *M. purpureus* MTCC 25436 were conducted on two production batches of the product. Results showed

that the produced colorant has satisfactory microbial characteristics and can be safely applied on food products. The common foodborne illness-causing microorganisms such as *Salmonella*, coliforms, and *Escherichia coli* were not detected or assumed to be at very low and safe levels. Table 4 shows the results of the microbial tests done for the produced *Monascus* red colorant.

Acute Oral Toxicity Test

The toxicity of the colorant was assessed using the mean lethal dose (LD_{50}), wherein the volume of a substance that will kill half of the test animals in a fixed time is measured (Walum 1998). The results of the toxicity assay are shown in Table 5. No death was recorded across all doses even at the highest dose of 45 g/ kg body weight. Behavioral observations and toxidrome after oral administration of the colorant sample to the test animal are shown in Table 6. To determine the LD_{50} value, the sample administered must be increased. However, further increase in the amount of colorant sample to be administered will already surpass the maximum limit a mouse normally consumes. Thus, the LD_{50} value was not determined. Nevertheless, it can be concluded that the *Monascus* red colorant is non-toxic and does not cause lethal effects on mice, even at high dosages. Necroscopy also showed no abnormal effects of the colorant on the vital organs of the laboratory animals.

Table 4. Results of the microbial analyses of the produced *Monascus purpureus* MTCC 25436 red colorant against the standard limit for *Monascus* colorant.

Test conducted	Method/culture medium used/ incubation period	Result
Bacterial count	Plate count agar incubated at 30 °C for 24–48 h	< 10 CFU/g
Yeast count	Dichloran rose Bengal chloramphenicol agar incubated at 25°C for 48 h	< 100 CFU/g
Mold count	Potato dextrose agar supplemented with chloramphenicol incubated at 25 °C for 5–7 d	< 100 CFU/g
Detection of *Salmonella* sp.	Chapter 5: *Salmonella*: in US FDA Bacteriological Analytical Manual (Andrews *et al.* 2021)	Not detected
Total coliforms	Chapter 4: enumeration of *Escherichia coli* and the coliform bacteria: in US FDA Bacteriological Analytical Manual (Feng *et al.* 2021)	< 3.0 MPN/g
Fecal coliforms	Chapter 4: enumeration of *Escherichia coli* and the coliform bacteria: In US FDA Bacteriological Analytical Manual (Feng *et al.* 2021)	< 3.0 MPN/g
Detection and count of *Escherichia coli*	Chapter 4: enumeration of *Escherichia coli* and the coliform bacteria: In US FDA Bacteriological Analytical Manual (Feng *et al.* 2021)	Not detected

Table 5. Summary of the mortality ratio of mice administered orally with *Monascus purpureus* MTCC 25436 red colorant at increasing dosages.

Group number	Dose (g/kg body weight)	Number of animals	Mortality ratio				
			Day 1	Day 2	Day 3	Day 7	Day 14
I	25	10	0/10	0/10	0/10	0/10	0/10
II	35	10	0/10	0/10	0/10	0/10	0/10
III	45	10	0/10	0/10	0/10	0/10	0/10

Table 6. Behavioral observation/toxidrome after oral administration of *Monascus purpureus* MTCC 25436 red colorant to male ICR mice in increasing dosage.

Dose (g/kg body weight)	Number of animals	Observation
25	10	10 min after dosing, the mice manifested grooming, increased motor activity, hyperemia, followed by decreased motor activity and respiratory rate, piloerection, and urination (after 5 h of observation)
35	10	
45	10	All mice were normal after 24 h. No other adverse/abnormal signs or death occurred within the 14-d period of observation

Allergenicity Test (Dermal Sensitization Test)

The modified Buehler test was used to determine the capability of the colorant to elicit an allergic response in test animals. The dermal sensitization test is an important procedure for evaluating the allergenic potential of chemicals prior to human exposure (Robinson *et al.* 1990). It efficiently detects strong, moderate, and most weak sensitizers. Topical exposure enables the assessment of dose responses, interaction among chemicals, and the sensitization potentials of contaminants. Results of the allergenicity test are shown in Table 7. The positive control used to demonstrate the susceptibility of the model animals to a known allergen was 0.05% DNCB. The reaction in the retest (*i.e.* challenge phase) was compared with the average of the readings taken after each of the original patch applications (*i.e.* induction phase). A higher value for the retest suggests that a substance could produce a sensitization effect. As a rule, two or more clear positive responses in a group of 20 animals meant that the substance can be a sensitizer; on the other hand, a negative response inferred that the sample is not a strong sensitizer. Both the positive control and *Monascus* colorant had a higher mean response in the challenge phase than in the induction test. However, the reaction values for the positive control were significantly higher than those for the colorant. The mean response readings for *Monascus* colorant were also less than 1, indicating a very slight sensitization effect (*i.e.* barely perceptible erythema) in accordance with the scale for scoring. Thus, the colorant is a very weak sensitizer and hardly elicits an allergic response.

Detection and Quantification of Citrinin

One major concern in the production of metabolites from fungi like *Monascus* is the production of mycotoxins. *Monascus* species are known to produce a type of mycotoxin called citrinin (Pattanagul *et al.* 2007). Citrinin is a nephrotoxin that severely affects the proximal tubules of the kidney, leading to the disruption of renal functions (Flajs and Peraica 2009). High-performance liquid chromatography (HPLC) is a powerful analytical technique that separates the components of a sample into individual constituents based on the interaction with the stationary phase (Gika *et al.* 2016). HPLC analysis of the *Monascus* red colorant samples indicated that citrinin was at non-detectable levels based on the absence of a peak in the chromatogram of both the liquid and powder samples (Figure 2).

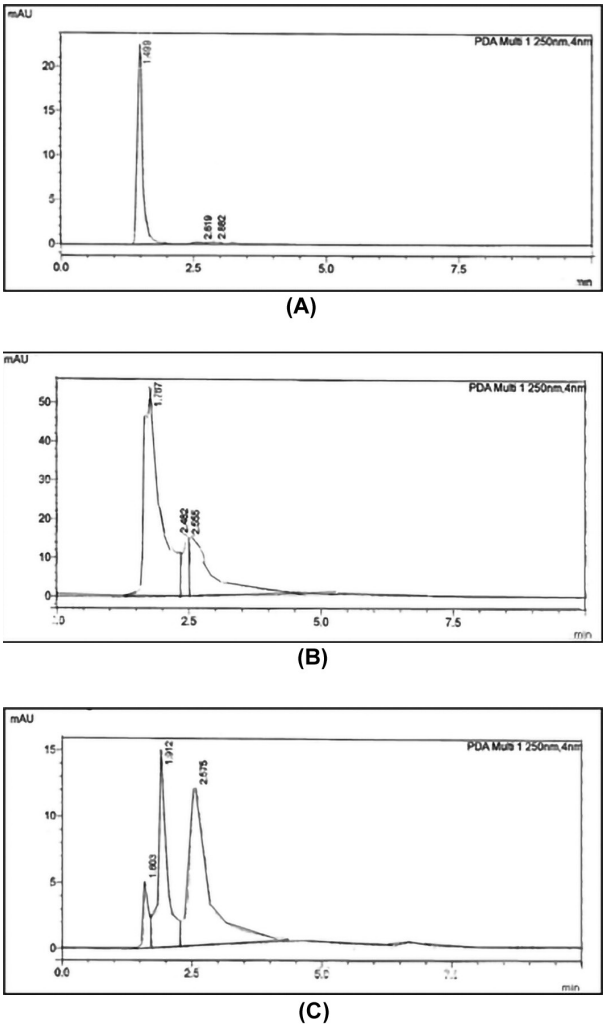

Figure 2. Chromatograms from the HPLC analysis of the *Monascus* red colorant for the detection of citrinin at 250 nm (A – citrinin standard; B – liquid colorant; C – colorant powder).

Table 7. Mean response readings from skin reaction scores of *Monascus purpureus* MTCC 25436 red colorant as compared to positive control for the dermal sensitization test.

Groups	Mean response (MR) readings (skin reaction score)				
	Induction phase				Challenge phase
	Day 0	Day 7	Day 14	Average	
Negative control	0	0	0	0	0
Positive control (0.05% DNCB)	4.80	6.80	7.65	6.42	7.40
Monascus red colorant	0	0	1.05	0.35	0.60

CONCLUSION

The produced *Monascus* red colorant was subjected to various safety assessment tests to determine its capacity to induce mutagenic, allergenic, or toxic side effects. Several *Salmonella typhimurium* strains were used in the Ames test, and the colorant did not produce reverse mutations. The non-mutagenicity of the colorant was also confirmed by the micronucleus assay, as seen in the low occurrence of MNPCE. The colorant also conforms to standards set for lead and arsenic contents and microbial quality. LD_{50} test was used to determine the lethal concentration of the colorant. No deaths were recorded for the colorant sample, even at the highest allowable concentration for testing. The colorant is also a very weak sensitizer agent and, therefore, can be explored for possible cosmetic applications. Lastly, citrinin – which is the mycotoxin usually associated with *Monascus*-based colorants – was at non-detectable levels. In conclusion, the colorant is safe for consumption. The results of the study can be used as basis for possible upscaling of a microbial-based colorant in the Philippines.

Natural products could exhibit differences in activities and characteristics. Thus, it is recommended that future productions of colorant will be tested randomly for toxicity as part of the quality control to establish the safety of the colorant.

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STATEMENT ON CONFLICT OF INTEREST

There is no conflict of interest.

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