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Acute Toxicity Test of Black Rice and Soybean Extract Complex

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Abstract

The present study was conducted to assess the acute oral toxicity of a copigment complex formulated from extracts of black rice (Oryza sativa L., indica) and soybean (Glycine max (L.) Merr.). Despite extensive individual pharmacological investigations of these botanical sources, no safety data have previously been documented for their combined copigment complex intended as a functional beverage constituent. Following the Acute Toxic Class methodology (OECD 423), the complex was orally administered as a single dose at 300, 2000, or 5000 mg/kg body weight (BW) to female Swiss Webster mice (n=3 per dose group), alongside a vehicle control group administered 1% Na-CMC. A comprehensive 14-day monitoring program assessed mortality, a range of clinical indicators (including tremor, convulsions, salivation, diarrhea, lethargy, locomotion, coat condition, and ocular appearance), and body weight at Days 0, 7, and 14. No animal deaths occurred across any group. Transient, mild hypoactivity was noted in animals receiving 5000 mg/kg at 1–2 hours post-dose, which resolved completely by 4 hours post-administration. No additional adverse clinical manifestations or weight loss were detected. Based on these findings, the median lethal dose (LD50) is projected to surpass 5000 mg/kg BW, corresponding to GHS Category 5 (practically non-toxic). The results collectively establish a safety foundation for the use of this copigment complex as a functional ingredient in isotonic beverage products, while acknowledging the need for further subchronic and chronic toxicological investigations.

Keywords: acute toxicity, black rice, copigmentation, lethal dose, soybeans

Introduction

Toxicology encompasses the systematic study of the deleterious effects of chemical agents on biological systems, integrating mechanistic, clinical, and risk-related dimensions.[1] A cornerstone of preclinical toxicological evaluation is the assessment of acute toxicity, which identifies adverse responses occurring after a single or brief exposure to a substance. The central quantitative output of such assessments is the median lethal dose (LD50) — defined as the statistically estimated dose that induces fatality in half of a defined experimental population within a specified observation window — which underpins both hazard characterisation and regulatory classification procedures.[2]

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Contemporary approaches to acute toxicity assessment have progressively moved away from conventional LD50 quantification methodologies toward more ethical and animal-conserving protocols. OECD Test Guideline 423, known as the Acute Toxic Class Method, embodies this shift through its sequential dosing design, which requires as few as three animals per dose step and guides advancement decisions by survival outcomes at each stage.[3] This framework aligns with the globally recognised 3Rs principles (Replacement, Reduction, and Refinement) governing ethical animal research, while generating data compatible with the GHS (Globally Harmonised System of Classification and Labelling of Chemicals) hazard classification.

Black rice (*Oryza sativa* L. indica) contains an abundance of anthocyanin pigments — chiefly cyanidin-3-glucoside and peonidin-3-glucoside — that impart notable antioxidant capacity and wide-ranging pharmacological effects.[4] Soybean (*Glycine max* (L.) Merr.) is a reservoir of bioactive phytochemicals, including isoflavones, saponins, and bioactive peptides, with multifaceted health-promoting roles.[5] The copigmentation process — involving non-covalent molecular interactions between black rice anthocyanins and soybean copigments — enhances colour stability, antioxidant potency, and the overall functional performance of the resulting complex. Although each plant material has a well-documented safety record supported by generations of dietary consumption, prior acute toxicity investigations have examined these sources in isolation.[6,7] To date, no published data exist characterising the toxicological profile of the combined copigment complex. Therefore, the possibility of synergistic or antagonistic interactions in this combined matrix necessitates a systematic evaluation before product development.

The scientific novelty of this research resides in its being the inaugural acute toxicity evaluation of a black rice–soybean copigment complex designated for incorporation into a functional isotonic beverage. Prior toxicological studies on either constituent have addressed each extract individually, leaving the combined copigment preparation as a previously unexplored area of safety research. The overarching aims of this study were to establish the LD50 estimate, delineate the clinical symptom profile, and document body weight trajectories in female Swiss Webster mice following single-dose oral administration of the copigment complex in accordance with the OECD 423 protocol, thereby furnishing the preclinical safety evidence required to proceed with functional beverage formulation.

Method

Ethical Approval

Research ethical approval was granted by the Ethics Committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia (Ethical Clearance No. [INSERT EC NUMBER]/KEP/UAD/2024). All experimental procedures were carried out in strict accordance with the requirements of OECD Test Guideline 423 and prevailing Indonesian animal welfare legislation.[8]

Instruments

Laboratory instruments utilised in this study included beaker glass (Pyrex®), Erlenmeyer flasks (Pyrex®), a rotary evaporator (IKA®), a hotplate stirrer (Thermo Scientific Cimarec®), an analytical balance (Shimadzu® ATX224), and a pH meter (Hanna Instruments®).

Preparation of Copigment Extract Complex

Raw materials comprising black rice (*Oryza sativa* L. indica) and soybean (*Glycine max* (L.) Merr) were procured from local suppliers and subjected to botanical

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authentication by a qualified expert. Both plant materials were extracted by maceration in 70% ethanol, followed by filtration and removal of the solvent by rotary evaporation at reduced pressure and 50°C. The copigment complex was prepared by combining black rice and soybean extracts at a 1:1 (w/w) ratio, with thorough mixing achieved by mechanical stirring at 500 rpm for 30 minutes at ambient temperature. The pH of the resulting mixture was recorded, and the complex was characterised for total anthocyanin content using the pH differential method and for total phenolic content using the Folin–Ciocalteu assay. [Note: Characterisation data to be provided by the authors.] Before animal dosing, the complex was dispersed in 1% Na-CMC solution to ensure adequate homogeneity and facilitate oral delivery.

Experimental Animals

A cohort of 12 female Swiss Webster mice, aged 2–3 months and weighing 20–30 g, was used. Selection of female animals conformed to OECD 423 recommendations, as females are typically more responsive to toxic agents, thus providing a conservative toxicity estimate. All included animals were clinically normal, behaviourally active, and devoid of external abnormalities. Animals were sourced from [INSERT SUPPLIER AND CITY] and subjected to a 5-day acclimatisation phase in polycarbonate cages (3 per cage) with sterile rice-husk bedding under a 12-hour light/dark photoperiod, at 22 ± 3°C and 50–60% relative humidity. Unrestricted access to standard chow and drinking water was maintained throughout acclimatisation and the experimental period.

Acute Toxicity Testing Procedure (OECD 423)

Animals were randomly assigned to four experimental groups (n=3 each): a vehicle control group receiving 1% Na-CMC, and three dose groups administered the copigment complex at 300, 2000, or 5000 mg/kg BW. All animals underwent a 3–4 hour pre-dose fasting period with continued water access. Each preparation was delivered as a single oral bolus via blunt-tipped gavage needle at a volume of 10 mL/kg BW, adjusted per individual body weight.

The stepwise OECD 423 protocol (Annex 2c, initiating at 300 mg/kg) was adhered to throughout. Three mice initially received 300 mg/kg BW; the absence of mortality within 24 hours prompted escalation to 2000 mg/kg BW in the next group. Continued absence of mortality warranted administration of the ceiling dose at 5000 mg/kg BW to the third treatment group. The vehicle control received 1% Na-CMC at a comparable volume. The procedure was discontinued upon reaching the limit dose without any deaths, in accordance with OECD 423 criteria for GHS Category 5 designation (LD50 > 5000 mg/kg BW).

Observation Parameters

Post-dose clinical monitoring was performed at 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours, followed by daily assessments through Day 14. Monitored parameters encompassed coat and ocular condition, hypersensitivity reactions, convulsive episodes, tremor, hypersalivation, diarrhea, hypoactivity, locomotor behaviour, and mortality. Clinical findings were categorised using a four-point grading scheme (Normal [N], Mild, Moderate, Severe), consistent with OECD 423 guidelines. Body mass was recorded at Days 0, 7, and 14 using a calibrated balance.

Data Analysis

Mortality data across dose groups were aggregated for GHS classification per OECD 423 methodology. Given the absence of mortality, LD50 was determined to exceed 5000 mg/kg BW. Body weight results are expressed as mean ± standard deviation (SD). Due to the small sample sizes inherent to the OECD 423 design (n=3 per group), inferential statistical analysis was not applied; all data were evaluated descriptively, consistent with the regulatory intent of the test. Clinical symptom data were documented narratively.

Result

Mortality and LD_{50} Estimation

No mortality was observed in any treatment or control group throughout the 14-day observation period at any dose (300, 2000, or 5000 mg/kg BW). Table 1 summarises mortality outcomes and clinical symptom assessment for all groups. In accordance with OECD 423, the absence of mortality at the limit dose of 5000 mg/kg BW indicates that the LD50 is greater than 5000 mg/kg BW, placing the copigment extract complex in GHS Category 5 (practically non-toxic) for acute oral exposure.

Table 1. Mortality Outcomes and Clinical Symptom Summary Across Dose Groups

Dose (mg/Kg BW)	Number Of Animals	Clinical symptoms	Number of deaths	Mortality (%)
Vehicle control	3	None observed	0	0
300	3	None observed	0	0
2000	3	None observed	0	0
5000	3	None observed	0	0

BW = Body weight; Na-CMC = sodium carboxymethylcellulose (vehicle control)

Clinical Symptom Profile

Detailed clinical observation results across all time points and dose groups are summarised in Table 2. Throughout both the acute observation window (0–24 h) and the follow-up phase (Days 1–14), no severe toxic manifestations — such as tonic-clonic convulsions, pronounced tremor, excessive oral secretion, or loose stools — were identified in any animal. Integumentary and ocular parameters, as well as the appearance of mucosal membranes, remained unremarkable across all groups throughout the study. Transient mild hypoactivity was observed exclusively in the 5000 mg/kg BW cohort at 1 and 2 hours post-administration. This phenomenon was self-resolving and completely abated by the 4-hour observation point, with animals resuming normal behaviour without intervention. No hypoactivity was noted in the 300 or 2000 mg/kg BW groups, nor in vehicle controls. From Day 1 onward, all animals exhibited normal movement, food consumption, grooming, and exploration patterns.

Table 2. Clinical Symptom Observations Across Time Points and Dose Groups

Time Point	Parameter	Control	300 mg/kg	2000 mg/kg	5000 mg/kg
0 h	Fur & Eyes	N	N	N	N
	All other parameters	N	N	N	N

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Table 2. (Extension)

Time Point	Parameter	Control	300 mg/kg	2000 mg/kg	5000 mg/kg
30 min	All parameters	N	N	N	N
1 h	Fur & Eyes	N	N	N	N
	Lethargy/Locomotion	N	N	N	Mild*
Q	Other parameters	N	N	N	N
2 h	Lethargy/Locomotion	N	N	N	Mild*
	Other parameters	N	N	N	N
4 h	All parameters	N	N	N	N
24 h	All parameters	N	N	N	N
Day 1–3	All parameters	N	N	N	N
Day 4–7	All parameters	N	N	N	N
Day 8–10	All parameters	N	N	N	N
Day 11–14	All parameters	N	N	N	N

N = Normal; Mild* = mild, transient lethargy observed at 1-2 h post-dose in the 5000 mg/Kg group, fully resolved by 4 h post-administration. Observations were conducted at 30 min, 1 h, 2 h, 4 h, and 24 h post-dose, followed by daily monitoring until Day 14.

Body Weight Changes

Body weight data (mean ± SD) are shown in Table 3. Progressive body weight gain was observed in all groups from Day 0 through Day 14. No group demonstrated a decline in body weight relative to the vehicle control at any measurement point. The weight-gain profiles of all treatment groups were comparable to those of the control group, indicating that the copigment complex had no detrimental effects on nutritional intake, metabolic function, or nutrient assimilation.

Table 3. Body Weight (Mean ± SD, grams) of Mice on Days 0, 7, and 14

Day	Control (n=3)	300 mg/kg (n=3)	2000 mg/kg (n=3)	5000 mg/kg (n=3)
0	20.43 ± 0.45	23.35 ± 1.13	25.11 ± 1.24	25.13 ± 1.73
7	25.73 ± 0.40	27.58 ± 2.23	30.32 ± 1.53	28.49 ± 2.13
14	27.15 ± 0.14	31.33 ± 2.89	33.23 ± 1.15	31.25 ± 1.21

Values are expressed as mean ± SD (n=3 per group). BW = body weight.

Discussion

Mortality and LD_{50} Classification

The complete absence of mortality across all administered doses — including the highest dose of 5000 mg/kg BW — unequivocally establishes that the LD50 of the copigment complex exceeds 5000 mg/kg BW. Within the GHS classification framework, this outcome places the extract in Category 5 (oral LD50 > 2000 mg/kg BW) or 'unclassified,' both of which are interpreted as practically non-toxic. This result is concordant with published safety data for black rice ethanolic extracts, which have demonstrated LD50 values exceeding 5000 mg/kg BW in rodent models.[6] Similarly, soybean isoflavone preparations have shown minimal acute oral toxicity in murine and rat studies.[9] The current findings indicate that combining these extracts through copigmentation does not generate emergent toxicological properties that would increase acute hazard potential. From a risk-benefit perspective, the safety margin remains substantial: given an anticipated functional dose of approximately 100–200 mg/kg BW in the intended beverage formulation, the no-mortality limit dose corresponds to a safety margin of at least 25-fold.

Mechanistic Interpretation of Clinical Findings

The brief, mild reduction in activity observed at the 5000 mg/kg BW dose level within 1–2 hours of administration is more plausibly attributed to the physiological stress of receiving a substantial gavage volume than to any intrinsic toxic property of the extract. At the biochemical level, the principal active components of the copigment complex — anthocyanins (cyanidin-3-glucoside, peonidin-3-glucoside) and isoflavones (genistein, daidzein) — are well-characterised antioxidant and anti-inflammatory phytochemicals. Anthocyanins exert cytoprotective activity via reactive oxygen species neutralisation and regulation of redox-responsive cellular signaling.[4,10] Isoflavones have been shown to modulate oestrogen receptor-mediated pathways and suppress oxidative damage without inducing neurotoxic or hepatotoxic effects at doses analogous to those examined in this study.[9] The consistent absence of central nervous system disturbances (convulsions, tremor, coma) and gastrointestinal symptoms (diarrhea, excessive salivation) at all dose levels aligns with the known non-toxic biochemical profiles of these phytochemical classes.

Body Weight as a Systemic Toxicity Indicator

Uninterrupted body weight gain across all experimental groups over the 14-day monitoring period constitutes strong evidence for the absence of sublethal systemic toxicity. Body weight reduction represents a sensitive, albeit non-specific, indicator of physiological stress, tissue damage, or reduced appetite. The comparable weight trajectories observed in treated and control animals confirm that the copigment complex did not compromise metabolic homeostasis, digestive physiology, or vital organ function at any evaluated dose level. This finding is consistent with the clinical observation data and reinforces the overall favourable safety profile.

Study Limitations

Several methodological constraints warrant acknowledgement. The limited group size (n=3 per dose), while aligned with OECD 423 requirements to minimise animal use, restricts statistical inferential capacity. This study was confined to a single acute-dose exposure; subchronic (28-day) and chronic (90-day) toxicity investigations are prerequisites for regulatory submissions for functional food applications. Additionally, histopathological analysis of target organs (hepatic, renal, and splenic tissue) and haematological or biochemical profiling were not conducted, which would have afforded deeper insight into the mechanistic safety profile. Furthermore, the restriction to female animals means sex-related toxicological differences, though unlikely to be significant given the GHS Category 5 classification, cannot be entirely excluded. Subsequent research should address these limitations to establish a comprehensive preclinical toxicological dossier.

Conclusion

The copigment complex formulated from black rice (*Oryza sativa* L. indica) and soybean (*Glycine max* (L.) Merr) extracts exhibited an outstanding acute oral safety profile in female Swiss Webster mice. Neither mortality, clinically significant toxic manifestations, nor reductions in body weight were detected at any dose level up to 5000 mg/kg BW across a 14-day observation period. The LD50 is estimated to exceed 5000 mg/kg BW, situating the extract in GHS Category 5 (practically non-toxic). These data provide a scientifically robust rationale for progressing the copigment complex as a safe functional constituent in isotonic beverage development. Extended subchronic and chronic toxicity studies, supplemented by target organ histopathology, are recommended to consolidate the preclinical safety evidence base.

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