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Acute Toxicity Test of Healthy Drink of Anthocyanin Pigmented Polyphenols from Black Rice and Sprouts Soybean

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Abstract: Black rice and sprouted soybean (BRiSS) can be formulated into a healthy drink BRiSS. The safety of this novel combination must be rigorously tested. Therefore, this study was designed to conduct a comprehensive acute toxicity test of the healthy drink flavylium anthocyanins from black rice (*Oryza sativa* L. indica) pigmented polyphenols from soybean sprouts (*Glycine max* L.). The black rice was extracted with a 96% ethanol solution in 3% citric acid. The pigmentation process was achieved by adding hydroxyl groups from soybean polyphenols. The active material was mixed with 20% maltodextrin, shaken, poured into a pan, and dried with a cabinet dryer (60° C). The mixture was stirred with 0.3% sodium, 0.1% citric acid, 50% active ingredients, 5% sucrose as a sweetener, 0.05% sodium benzoate, and water, 100 ml. The five groups of male Wistar rats were acclimatized. Group 1 was a control, and groups 2, 3, 4, and 5 were sequentially treated with a healthy drink containing extract doses of 5, 50, 300, and 2000 mg/kg BW. The acute toxicity test results at 30 minutes, 3 to 24 hours, day 3, and day 14 were written on the acute toxicity test clinical symptom observation sheet. All test animals, including both the control and test groups with various dosage variations, did not exhibit changes in skin, fur, eyes, behavior, respiratory systems, and did not experience weight loss, seizures, tremors, lethargy, or diarrhea. The limitation was that the macropathology examination of internal organs (kidneys, liver, heart, brain, stomach, intestines, pancreas) was not performed. Not all animals have mortality in a 5-3000 (mg/kg BW) extract dose.

Keywords: acute toxicity, black rice, herbal drink, sprouted soybean

1. INTRODUCTION

Health drinks are currently very popular [1]. Therefore, it is usually said that pigmented drinks help maintain freshness. Healthy beverages are primarily intended for individuals who are physically active or engage in mentally demanding work that involves significant stress s [2]. Healthy drinks have a different effect; they are not categorized as drugs, but other beverages because they are rich in antioxidants [3].

The formation of free radicals can continuously protect the human body; however, the body requires an external antioxidant system. Antioxidants function to prevent chain reactions from free radicals. Free radicals produced continuously during normal metabolism can cause damage to the function of body cells, leading to degenerative diseases. The body needs antioxidants to maintain health [4].

Black rice is a local variety that has the best color. Its color is genetically regulated and can change due to differences in genes that control the color of the aleurone and endosperm, as well as starch composition in the endosperm. Black rice aleurone and endosperm produce high-intensity anthocyanins, resulting in rice that is dark purple to almost black in color [5].

Anthocyanins act as antioxidants that protect the body from various degenerative diseases because the content of black rice can be used as a base ingredient for making healthy drinks [6]. Previous research has stated that black rice has a high anthocyanin content, ranging from 19.4 to 140.8 μg/100 g. The anthocyanin content of red rice is only 0.3-1.4 μg/100 g [7].

Copigmentation is a method that can increase anthocyanin stability. Anthocyanin is referred to as "intermolecular copigmentation" when anthocyanin pigments and copigments, especially phenolic acids and flavonoids, are directly mixed in a solution through non-covalent bonds, so that the copigmentation reaction can increase the stability of anthocyanin color and increase its half-life [8].

Soybeans contain functional components that are beneficial for health, such as peptides, anthocyanin isoflavones, phytosterols, phenols, saponin bioactive proteins, and others, which provide nutrients including vitamins, fatty acids, phytosterols, phenolic acids, and proteins [9]. Increasing soybean polyphenols through the germination process can alter gene expression and enhance the synthesis of specific metabolites through a dynamic biochemical process during germination, thereby increasing the concentration of phenolic compounds, flavonoids, and other secondary metabolites [10].

The 3-day germination period of soybeans produced polyphenols at a concentration of 15.5127 μmol/g and an antioxidant activity of 2.9875 μmol/g [11,12]. Based on previous research related to the development of functional foods, this study developed a healty drink product. The formulation of this drink has a reasonably complete content to maintain body fluids and is rich in antioxidants. The development in the manufacturing process with a copigmentation process to produce antioxidants that are pretty high and stable, so that this can be a novelty in the development of safer and quality functional foods for the needs of the community [13,14] Therefore, it was necessary to prove its safety with a toxicity test. This study examines the potential toxicity of healthy drinks of anthocyanin-pigmented polyphenols from black rice (*Oryza sativa* L. indica) and sprouted soybean (*Glycine max* L.)(BRiSS).

2. MATERIALS AND METHODS

2.1. Chemicals

Ethanol 96% (Emsure®), citric acid 3% (Merck), aqua, sucrosa (Merck), NaCl (Merck), natrium benzoate (Merck).

2.2. Method of preparing the BRiSS

Black rice, sieved to a 40-mesh size of about 500 g, was macerated in a 96% ethanol and 3% citric acid solution for 24 hours in a dark room and evaporated at 40 °C [15]. Therefore, the 500 g germinated soybean sprouts powder was macerated with 96% ethanol for 24 hours [16]. The co-pigmentation process was carried out intermolecularly by adding materials containing hydroxyl groups from the most active soybean polyphenols to anthocyanin pigments, with a 1:1 ratio. The mixture was then heated at 90 °C for 30 minutes. [17]. A total of 300 ml of black rice extract from polyphenol co-pigmentation was mixed with fillers and foaming agents. The variation of maltodextrin filler used was 20%. After the ingredients were mixed, they were shaken using a mixer for 10 minutes to ensure an even mixture. The mixture was then poured into a baking pan and dried in a cabinet dryer for 3 hours at a temperature of 60°C to produce the extract powder, which serves as the active ingredient in the drink [18]. In making BRiSS healthy drinks), the main ingredients consist of active anthocyanin polyphenols derived from co-pigmentation; additional ingredients include minerals and real honey, serving as a natural sweetener [19]. The formula for the BRiSS healthy drink was shown in Table 1.

Table 1. The BRiSS Healthy Drink Formulation

Material	F1 (g)	F2 (g)	F3 (g)	F4 (g)	Function
Sucrose	11.250	11.250	11.250	11.250	Sweetener + carbohydrate supplier (energy) for the body
NaCl	0.625	0.625	0.625	0.625	Rapid fluid replacement
Citric acid	0.250	0.250	0.250	0.250	Regulates ph
Copigmentation complex	0.005	0.050	0.300	2.000	Active substance antioxidant
Natrium benzoate	0.125	0.125	0.125	0.125	Preservative
Distilled water	250	250	250	250	Solvent

BRiSS: black rice and soybean sprouts. F1: Formula 1; F2: Formula 2; F3: Formula 3; F4: Formula 4

The BRiSS healthy drink mixture was stirred well using a stirring rod. It contained 0.25% Sodium, 0.1% citric acid, active ingredients of a flavylium anthocyanin and polyphenol complex (50%), 4.5% sucrose sweetener, 0.05% sodium benzoate, and an aqueous solvent. The volume of the preparation was increased by 250 mL, and filtered under vacuum. After filtering the solution, 4.5% sweetener is added, adjusted to the desired sweetness level. The drink was filled in hot conditions (hot filling) using a filler machine into Polypropylene (PP) glass packaging. The BRiSS healthy drinks were put into the bottle and closed (sealed) [20]. The BRiSS healthy drinks were pasteurized for 15 minutes at 85 °C and then cooled to room temperature. The primary process in making commercial BRiSS healthy drinks involves dissolving or mixing ingredients according to a predetermined composition, and preserving the product through the application of a thermal process at the optimal temperature and time, with packaging that protects the product from the risk of recontamination [21].

2.3. Method of preparing the animals

The animals used were 2-3-month-old male Wistar white mice (*Mus musculus*) weighing 20-30 grams, totalling 25 (divided into 5 groups). The mice were acclimatized for 7 days by being given sufficient food and water, to help adapt the test animals. Preparation of the samples at doses of 5, 50, 300, and 2000 mg/kg body weight (BW, which were given orally. The mice were divided into 5 test groups, each with 5 mice. Group 1 served as a control, receiving oral administration of distilled water, while groups 2-5 received treatment BRiSS healthy drink at doses of 5, 50, 300, and 2000 mg/kg BW. Treatment was conducted by counting the deaths of test animals within 24 hours after a single administration of the test preparation in each group. Observation of toxicity symptoms was carried out 3 hours after administration of sample by observing symptoms of convulsions (seizures), tremors (shaking), lethargy, diarrhea, and death [22].

2.4. Data analysis

The data from the acute toxicity test were statistically analyzed using IBM SPSS Statistics 27.0. The parametric test was used if the data were normally distributed. The one-way ANOVA continued a post-hoc test if significant at 95% confidence [23].

3. RESULTS AND DISCUSSION

3.1. Organoleptic tests and pH of the black rice and sprouted soybean (BRiSS) healthy drink

The formulation of the black rice and sprouted soybean (BRiSS) healthy drink, resulting from the copigmentation process of flavylium anthocyanin with soybean sprout polyphenols, is presented in Table 2.

Table 2. Organoleptic Test and pH o BRiSS Healthy Drink

Formula	Color	Flavour			pH
		Black Rice	Sweet	Sour	
F1		+	+++	+++	3.07 ± 0.006
F2		++	+++	+++	3.41 ± 0.006
F3		+++	+++	++	3.8 ± 0.03
F4		++++	+++	++	3.98 ± 0

BRiSS: the black rice and sprouted soybean. F1: Formula 1; F2: Formula 2; F3: Formula 3; F4: Formula 4
+ : a little; ++ : a quiet; +++ : a lot of; ++++: very much

The pH of the BRiSS healthy drink was measured based on co-pigmentation. The results range from 3.07 to 3.98 at doses of 5, 50, 300, and 2000 mg in 250 ml BRiSS healthy drink. The pH results for the preparation drink were within normal limits according to the requirements determined by SNI (No. 01-4452, 1998), which states that the maximum pH value for an isotonic drink is pH 4,0.

3.2. The acute oral toxicity test

The acute oral toxicity test has received approval from the Research Ethics Committee (KEP) of Ahmad Dahlan University on January 16th, 2025, number 012412374. A test to detect toxic effects that appear within a short time after administration of the test preparation given orally in a single dose or repeated doses. Acute toxicity testing in this study was conducted on male Wistar rats, and the test preparation involved administering several doses to the test animal group. Acute toxicity testing in this study was carried out using the Fixed Dose Method. This method, according to the preclinical test protocol guidelines for acute toxicity. A group of test animals of the same sex at fixed doses: 5, 50, 300, and 2000 mg/kg body weight, with one group serving as a control. The following are the results of acute toxicity testing on test animals 30 minutes after exposure.

Observations of the animals, including both the control and test groups, were conducted before the provision of the sample and compared after the test at 30 minutes, 3 to 24 hours, day 3, and day 14, as recorded on the acute toxicity test clinical symptom observation sheet. Observations of the skin and fur were conducted to assess for any abnormalities, such as redness, red spots, blue bruises, dry, scaly skin, or fur falling out, standing up, or becoming sticky. While observing the eyes and breath, if abnormal conditions occur, such as shortness of breath or breathing stopping. The animal's behaviour, particularly its gait, was observed to determine whether it walks normally or with a slant. It is also monitored whether the test animal appears frightened, weak, or even hyperactive. Did the animals experience tremors and seizures? Did the animals experience lethargy, limp, diarrhea, loss of weight, and death? The results of the 14-day acute toxicity observations are presented in Table 3.

Table 3. Clinical signs of acute toxicity of BRiSS healthy drinkobservation for 14 days

Clinical Symptoms	Evidence	Day Observation													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Skin	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fur	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Eye	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Respiratory system	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Behaviour	Normal	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tremor	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Seizures	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lethargy	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diarrhea	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
limp	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Weight loss	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Death	No	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Observation of acute toxicity symptoms in test animals at 30 minutes, 24 hours, 3 days, and 14 days after administration did not reveal any abnormal symptoms, and these findings were consistent across animals in the control and test groups. Furthermore, animal deaths were shown in Table 4.

Table 4. The mortality and toxicity symptoms of animal tests in the acute toxicity of BRiSS healthy drink

Group	Treatment	Mortality				Total of deaths
		30′	24 hours	3 days	14 days	
P1 (control)	Aquadest	C	C	C	C	0
P2	5 mg/kg BW BRiSS Healthy Drinks	C	C	C	C	0
P3	50 mg/kg BW BRiSS Healthy Drinks	C	C	C	C	0
P4	300 mg/kg BW BRiSS Healthy Drinks	C	C	C	C	0
P5	2000 mg/kg BW BRiSS Healthy Drinks	C	C	C	C	0

Symptom Category: A: Dead; B: Showing Toxicity Symptoms; C: No Toxicity Symptoms

In assessing chemical toxicity, it is undoubtedly the best test species for humans because accurate extrapolation of animal data directly to humans may not be guaranteed due to interspecies variations in anatomy, physiology, and biochemistry. However, for ethical reasons, chemicals must be tested using animal models before being tested in humans [24].

Black rice (*Oryza sativa* L. Indica) and sprouted soybeans (*Glycine max* L.) were used in BRiSS healthy drink, and their safety has been proven through acute toxicity tests. Black rice is a common ingredient in daily food. Soybean sprouts are also powdered [23,25]. The process, including extraction and formulation, is safe in animal studies. The results were obtained from toxicity tests on soybeans, which are rich in isoflavones. The results obtained in the acute toxicity test indicate that germinated soybean sprout powder has a high margin of safety, as evidenced by the No-Observed-Adverse-Effect Level (NOAEL) for rats in this study [26],

4. CONCLUSION

The black rice and sprouted soybean (BRiSS) healthy drink, made from black rice and soybean sprouts, was a common ingredient in daily food. The process, including extraction and formulation, was safe in animal studies, with no adverse effects observed on skin, fur, eyes, breathing, behavior, tremors, seizures, weakness, lethargy, limpness, diarrhea, or weight loss. Maximizing the extract did not cause mortality in animal tests. The LD_{50} was up to 2000 mg/kg BW.

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