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Acute Oral Toxicity Test of Philippine "Bignay" [*Antidesma bunius* (Linn.) Spreng cv. 'Common'] in ICR Mice

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Acute oral toxicity test of "bignay" 'common' [*Antidesma bunius* (Linn.) Spreng cv. 'Common'] fruit extract (BCFE) was conducted in male and female ICR mice following the OECD Guidelines 425 (2008) to evaluate its safety profile. Irrespective of the sex, physiological [body weight (BW) and BW gain; feed and water intake], hematological [total and differential white blood cell (WBC) and red blood cell (RBC) counts], and biochemical [creatinine and blood urea nitrogen (BUN) levels] parameters were generally unaffected except for the observed decrease in BW and increase in BUN in female mice treated with 2000 and 175 mg/kg BCFE, respectively. In addition, only slight alterations in absolute WBC counts were accounted in BCFE-treated female mice given 55, 175, and 2000 mg/kg doses, as well as in treated male mice administrated with 550 mg/kg BCFE, as opposed to their corresponding controls. Corroborating the absence of mortality, no overt signs of treatment-related toxicity were noted upon gross, biometric, and histological assessment of major organs including the gastrointestinal tract, lungs, liver, kidneys, spleen, heart, and brain. Taken together, the cumulated findings of the present study suggest that oral supplementation of BCFE is relatively safe for consumption up to 5000 mg/kg in treated ICR mice.

Keywords: acute oral toxicity, *Antidesma bunius*, Bignay 'common', mice

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INTRODUCTION

Berries are widely consumed for their taste, nutrition, and health benefits. Known berries under the family Rosaceae (such as strawberry, blackberry, and raspberry), as well as the family Ericaceae (including blueberry and cranberry), are considered to be the best source of bioactive compounds (Skrovankova *et al.* 2015). These include tannins, polyphenols such as anthocyanin and flavonoids, and phenolic acids, all of which have been documented to possess therapeutic properties (Nile and Park 2014; Skrovankova *et al.* 2015). Anthocyanin exhibits anti-obesity, anti-diabetes, and anti-cancer properties, as well as preventive effects against cardiovascular diseases such as atherosclerosis (Tsuda 2016; Khoo *et al.* 2017). Hypolipidemic and hypocholesterolemic activities are also reported in different berries such as blueberry (Wu *et al.* 2013; Kim *et al.* 2016), blackberry and blackcurrant (Kim *et al.* 2016), mulberry (Wu *et al.* 2013), black chokeberry (Baum *et al.* 2016), and gooseberry (Yokozawa *et al.* 2007; Krishnaveni *et al.* 2010). The progress of studies regarding these different therapeutic effects of known berries indicates that indigenous berries in the Philippines may potentially exhibit these properties as well.

"Bignay" [*Antidesma bunius* (Linn.) Spreng] is one of the indigenous berries in the Philippines typically seen in northern Luzon and Mindanao (Castillo *et al.* 2005). The two dominant varieties of bignay in the country are the 'Kalabaw' and 'Common' cultivars with the former having a higher percent edible portion, heavier weight, and bigger size (Castillo-Israel *et al.* 2020). Bignay is a fruit-bearing tree that grows from 6–30 m tall (Islam *et al.* 2018). It has small, green, and odorous flowers (Grijaldo *et al.* 2019), which bloom in the month of March (Janick and Paull 2008), and leaves that are small, 8–20 cm in length, dark green, shiny, alternate, pointed at the tip, and rounded or pointed at the base (Grijaldo *et al.* 2019). It bears 30–40 ovoid fruits clustered together in a bunch (Belina-Aldemita *et al.* 2013), which appear green to pale yellow when unripe (Islam *et al.* 2018) then turns red or black as it matures (Belmi *et al.* 2014; Islam *et al.* 2018). Its fruits are good sources of calcium and iron (Alberto and Galvez 2004) and other minerals like potassium, magnesium, manganese, copper, and zinc (Khomdram *et al.* 2017). Aside from minerals, bignay fruits also contain vitamins such as thiamine, riboflavin, and niacin (Morton 1987). Bignay fruits are usually consumed as a healthy drink, juice concentrate, and wine (Jorjong *et al.* 2015). A 100-g serving of the edible portion of bignay contains an energy value of 134 kJ, 90–95 g water, 6.3 g carbohydrates, 0.8 g fat, 0.7 g protein, 37–120 mg calcium, 22–40 mg phosphorus, 8 mg vitamin C, 10 IU vitamin A, and 0.1–0.7 mg iron (Islam *et al.* 2018). Other phytochemical components of bignay include polyphenols, which has been previously reported to possess therapeutic properties. For instance, the bignay fruit flesh of both 'Kalabaw' and 'Common' cultivars at all maturity stages were shown to exhibit lipid lowering properties through cholesterol binding of bile acids (Crieta *et al.* 2021). In another study, bignay seed exhibited hypolipidemic activity by decreasing total cholesterol, triglyceride, and low-density lipoprotein cholesterol but increasing high-density lipoprotein cholesterol in streptozotocin (STZ)-induced diabetic rats (Chowtivannakul *et al.* 2016). In addition, hypoglycemic activity was also observed in different plant parts of bignay such as its fruits (Herrera *et al.* 2010; Quiming *et al.* 2017; Tanquilut *et al.* 2019), leaves (El-Tantawy *et al.* 2015; Grijaldo *et al.* 2019) and seeds (Chowtivannakul *et al.* 2016). The antioxidant activity of bignay is also of interest. Udomkasemsab *et al.* (2018) reported that bignay fruit acts as a free radical scavenger and Fe^{3+} chelator reducing malondialdehyde levels, which are associated with lipid peroxidation inside tissues. Recently, our group has demonstrated that the antioxidant activity of indigenous bignay 'common' fruit is influenced by factors such as maturity and heat processing treatments. In particular, we have shown that higher antioxidant activity is associated with ripe fruit and blanching treatment in converse to the unripe and steaming process (Castillo-Israel *et al.* 2020). The same trends were observed in the bignay 'Kalabaw' cultivar (Sartoga *et al.* 2021).

The above-mentioned information implies that this locally available berry can be further developed as a potential functional food supplement. However, to date, the safety of this particular berry is still not established due to the lack of oral toxicity studies. Hence, we would like to perform a comprehensive acute oral toxicity test of BCFE and determine its effect on various parameters including mortality and morbidity rate, BW changes, food and water intake, physical and behavioral alterations, hematology profile, biochemical levels of kidney-filtered substances, and gross and microscopic features of harvested organs.

MATERIALS AND METHODS

Preparation of BCFE

Fully ripe bignay 'Common' fruit samples were harvested in Los Baños, Laguna, Philippines from May–June 2019. The authenticity of this plant specimen was certified by the curator of the Botanical Herbarium, Museum of Natural History, University of the Philippines Los Baños (was). For the preparation of BCFEs, the fruits were deseeded using a pulper (Kiya Keisakusho, Japan). The pulp together with the peel was then freeze-dried at 20 °C and 40 mTorr pressure using VirTis Co. (Gardiner, NY).

Freeze-dried BCFE samples were finely ground and then passed through an 80-mesh US standard sieve. Contingent on mouse BW, each BCFE concentration (55, 175, 550, 2000, and 5000 mg/kg BW) was individually prepared by reconstituting the powder of the freeze-dried fruit samples in 1 mL of distilled water and vortexed for 1 min. The reconstituted BCFE samples were then coded at the Institute of Human Nutrition and Food (IHNF), College of Human Ecology (CHE), UPLB by an independent member of the group who was not directly involved in the conduct of the toxicity experiment and data analysis to preclude any possible research bias.

Animals

Ten (10) male and 10 female 6-wk-old ICR mice were obtained from Laboratory Animal Facility, Research of the Institute for Tropical Medicine, Department of Health, Alabang, Muntinlupa City, Philippines. All procedures in mice were approved by the UPLB Animal Care and Use Committee with assigned protocol number CHE-2019-002. Mice were individually caged in standard polycarbonate cages with stainless tops and maintained at 22 °C (± 2 °C), 30–60% humidity, and 12-h: 12-h light-dark period in the laboratory animal room of the Department of Basic Veterinary Sciences (DBVS), College of Veterinary Medicine (CVM), UPLB. Commercial maintenance mouse pellets (Altromin, Germany) and distilled water were provided *ad libitum*. Mice were acclimatized for 1 wk prior to experimentation.

Acute Oral Toxicity Test

The acute oral toxicity test up-and-down method was performed following the OECD Guideline 425 (2008). In brief, 10 male and 10 female ICR mice were randomly distributed into five groups (n = 1 male, 1 female per group) – namely, Group 1 was treated with 55 mg/kg BW BCFE, Group 2 was treated with 175 mg/kg BW BCFE, Group 3 was treated with 550 mg/kg BW BCFE, Group 4 was treated with 2000 mg/kg BW BCFE, and Group 5 was treated with 5000 mg/kg BW BCFE. A corresponding control animal given distilled water was assigned for each BCFE treatment group, hence resulting in five control groups. Food and water were withheld 1 h before and 1 h after the administration of the treatment. One (1) mL of the vehicle distilled water and berry extracts were given once using a 1-in 22G stainless steel gavage needle (Thermoscientific, USA) and 1 mL sterile disposable syringe (Terumo, Japan) on Day 1 of experimentation. Treatment per group was instituted successively from lower to higher doses with at least 48-h intervals to confirm the absence of mortality.

The BW of each mouse per treatment group was measured using a digital top loading balance (Shimadzu, Japan) weekly, starting on Day 1 prior to treatment administration and then on Days 7 and 14 of the experimentation period. The feed and water intake of each mouse per treatment group were measured daily by providing pre-measured commercial mouse pellets and distilled water. Daily leftover pellets were weighed using a digital top loading balance and water using a graduated cylinder for 14 d.

The mice were closely observed during the first 30 min post-administration treatment, then for 24 h and daily thereafter for 14 d. The presence of physical changes such as abnormalities in the skin, fur, eyes, and mucous membranes was noted. Moreover, it was imperative to look out for signs of tremor, convulsion, salivation, diarrhea, lethargy, sleep, and coma. In this study, physical and behavioral changes were not numerically scored but only categorically noted whether present or absent. All animals identified to be in a moribund condition and experienced severe pain and distress were humanely killed, and the time of death were noted as precisely as possible.

Morbidity and mortality rates for all treatment groups were recorded and the formulas used for the computation of these rates are shown below.

Morbidity rate per group:

$$\frac{Total\ number\ of\ mice\ that\ showed\ toxicity\ signs\ per\ group}{Total\ number\ of\ mice\ per\ group} \times 100$$

Mortality rate per group:

$$\frac{Total\ number\ of\ mice\ that\ died\ per\ group}{Total\ number\ of\ mice\ per\ group} \times 100$$

Hematology and Blood Chemistry Analysis

A drop of the ophthalmic anesthetic tetracaine (Alcaine®, Novartis, Philippines) was placed in the right eye prior to blood collection. After two mins, blood was collected through the retro-orbital vein using a heparinized capillary tube (INRI, Netherlands). Three hundred (300) µL of blood were collected from each mouse per treatment group prior to administration of vehicle or BCFE on Day 1 and then on Day 14. Ten (10) µL of blood were used to measure the total RBC count, WBC count, and differential WBC count using an automated hematology analyzer (Orphée, Switzerland), whereas 250 µL of blood were used to measure the creatinine and BUN levels using an automated blood chemistry analyzer (Arkray, Japan).

Collection, Weighing, and Histopathological Processing of Organs, and Macroscopic and Microscopic Evaluation of Specific Tissues and Organs

After the 14-d experimentation period, all mice per treatment group were euthanized *via* intraperitoneal injection of 60 mg/kg sodium pentobarbital (Dolethal®, UK; AVMA 2013). A midventral incision was made at the thoraco-abdominal area to exteriorize the esophagus, stomach, small and large intestines, liver, kidneys, spleen, heart, lungs, and brain. All organs were examined for any gross abnormalities and were weighed using a digital top-loading balance (Shimadzu, Japan). The organs were trimmed, fixed in 10% buffered formalin for at least 72 h, processed using the routine paraffin technique, and sectioned at 4 μm in thickness using a rotary microtome. One out of every four sections of each organ was collected and stained with hematoxylin and eosin (H&E) stain for histopathologic evaluation using an inverted microscope (Nikon, Japan). The presence or absence of cellular inflammatory, degenerative and proliferative responses, healing processes, neoplasia, and other possible histopathologic changes were noted and photographed using a digital camera. When histopathological lesions were detected, then semi-quantitative scoring using the following parameters would be employed: [1] 0–10% of the section showing histopathological lesions, [2] 11–25% of the section showing histopathological lesions, [3] 26–50% of the sections showing histopathological lesions, [4] 51–75% of the sections showing histopathological lesions, and [5] > 75% of the sections showing histopathological lesions. Histopathological analysis and interpretation were independently carried out by a veterinary pathologist, who was completely blind to the treatment assignment.

Statistical Analysis

The mean daily water and feed intake were presented as mean ± SD and analyzed using the independent sample t-test. All analyses were performed using SPSS v.23 (IBM Corp., Armonk, NY, USA), and significant differences were determined at $p < 0.05$.

RESULTS

Morbidity and Mortality

Administration of various concentrations of BCFE did not induce any changes in the physical appearance and behavioral pattern of mice, regardless of sex and treatment group (Tables 1A and B). Furthermore, no mortality was observed in all control and BCFE-treated mice of both sexes.

Body Weight (BW)

Treatment with 55–5000 mg/kg of BCFE did not affect the mean BW of male and female ICR mice in comparison to their corresponding controls except for the female mouse that received 2000 mg/kg dose, which showed a slight decrease in BW (Figures 1A and B).

Feed and Water Intake

Mean feed (Figure 2A) and water intake (Figure 2B) were relatively indistinguishable between male and female mice that were orally administered with distilled water or BCFE. More importantly, increasing the dose of the treatment did not negatively affect both consumption profiles; in fact, all animals registered values within or even higher than normal.

Hematology

The total WBC (Figure 3A) and total RBC (Figure 3B) count of both male and female mice treated with different concentrations of BCFE fell within the normal range of the control groups and published data for ICR mice. As for the differential WBC count, apart from the male mice depicting a slight increase in monocyte count following 550 mg/kg treatment, as well as a higher Day 1 granulocyte count that normalized to the ideal range after the 14th day 2000 mg/kg treatment; the female mice displayed a modest increase in monocyte count after 500 mg/kg dose at Day 14 and a greater granulocyte count after supplementation with 55–2000 mg/kg dose. All remaining parameters were within the acceptable range for the strain (Figures 3C–E).

Creatinine and BUN Levels

The blood creatinine levels of male and female mice given distilled water and various concentrations of BCFE were within the published normal range for ICR mice (Suckow *et al.* 2001; Serfilippi *et al.* 2003) (Figure 4A). Likewise, BUN levels for BCFE-treated male and female ICR mice were within the normal range of the control mice and published range values for ICR mice, as reported by Suckow *et al.* (2001), and Serfilippi *et al.* (2003) with the exemption of a moderate increase in female mouse treated with 175-mg/kg dose (Figure 4B).

Macroscopic and Microscopic Observation of Organs

The organ weights of the collected tissues such as the esophagus, stomach, small and large intestines, lungs, liver, kidneys, spleen, heart, and brain of both male (Table 2A) and female (Table 2B) mice were comparable to their corresponding control group regardless of the administered dose of BCFE. Grossly, the organs of the control and BCFE-treated male and female mice appeared to be normal wherein no obvious macroscopic lesions were deciphered.

Table 1A. Effect of varying doses of BCFE on the physical appearance and behavior of male ICR mice.

Parameters	30 min post-administration						24 h					
	Control	55 mg/ kg BC	175 mg/ kg BC	550 mg/ kg BC	2000 mg/ kg BC	5000 mg/ kg BC	Control	55 mg/ kg BC	175 mg/ kg BC	550 mg/ kg BC	2000 mg/ kg BC	5000 mg/ kg BC
Abnormal skin	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal fur	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal eyes	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal mucous mem.	A	A	A	A	A	A	A	A	A	A	A	A
Tremor	A	A	A	A	A	A	A	A	A	A	A	A
Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A	A	A	A	A	A
Lethargy	A	A	A	A	A	A	A	A	A	A	A	A
Sleep	A	A	A	A	A	A	A	A	A	A	A	A
Coma	A	A	A	A	A	A	A	A	A	A	A	A
Parameters	**7 d**						**14 d**					
	Control	55 mg/ kg BC	175 mg/ kg BC	550 mg/ kg BC	2000 mg/ kg BC	5000 mg/ kg BC	Control	55 mg/ kg BC	175 mg/ kg BC	550 mg/ kg BC	2000 mg/ kg BC	5000 mg/ kg BC
Abnormal skin	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal fur	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal eyes	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal mucous mem.	A	A	A	A	A	A	A	A	A	A	A	A
Tremor	A	A	A	A	A	A	A	A	A	A	A	A
Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A	A	A	A	A	A
Lethargy	A	A	A	A	A	A	A	A	A	A	A	A
Sleep	A	A	A	A	A	A	A	A	A	A	A	A
Coma	A	A	A	A	A	A	A	A	A	A	A	A

Legend: [BC] bignay 'common'; [P] present; [A] absent

Table 1B. Effect of varying doses of BCFE on the physical appearance and behavior of female ICR mice.

Parameters	30 min post-administration						24 h					
	Control	55 mg/ kg BC	175 mg/kg BC	550 mg/kg BC	2000 mg/kg BC	5000 mg/kg BC	Control	55 mg/ kg BC	175 mg/ kg BC	550 mg/ kg BC	2000 mg/ kg BC	5000 mg/ kg BC
Abnormal skin	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal fur	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal eyes	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal mucous mem.	A	A	A	A	A	A	A	A	A	A	A	A
Tremor	A	A	A	A	A	A	A	A	A	A	A	A

Table 1B Cont.

Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A	A	A	A	A	A
Lethargy	A	A	A	A	A	A	A	A	A	A	A	A
Sleep	A	A	A	A	A	A	A	A	A	A	A	A
Coma	A	A	A	A	A	A	A	A	A	A	A	A
Parameters	**7 d**						**14 d**					
	Control	55 mg/kg BC	175 mg/kg BC	550 mg/kg BC	2000 mg/kg BC	5000 mg/kg BC	Control	55 mg/kg BC	175 mg/kg BC	550 mg/kg BC	2000 mg/kg BC	5000 mg/kg BC
Abnormal skin	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal fur	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal eyes	A	A	A	A	A	A	A	A	A	A	A	A
Abnormal mucous mem.	A	A	A	A	A	A	A	A	A	A	A	A
Tremor	A	A	A	A	A	A	A	A	A	A	A	A
Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
Salivation	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A	A	A	A	A	A
Lethargy	A	A	A	A	A	A	A	A	A	A	A	A
Sleep	A	A	A	A	A	A	A	A	A	A	A	A
Coma	A	A	A	A	A	A	A	A	A	A	A	A

Legend: [BC] bignay 'common'; [P] present; [A] absent

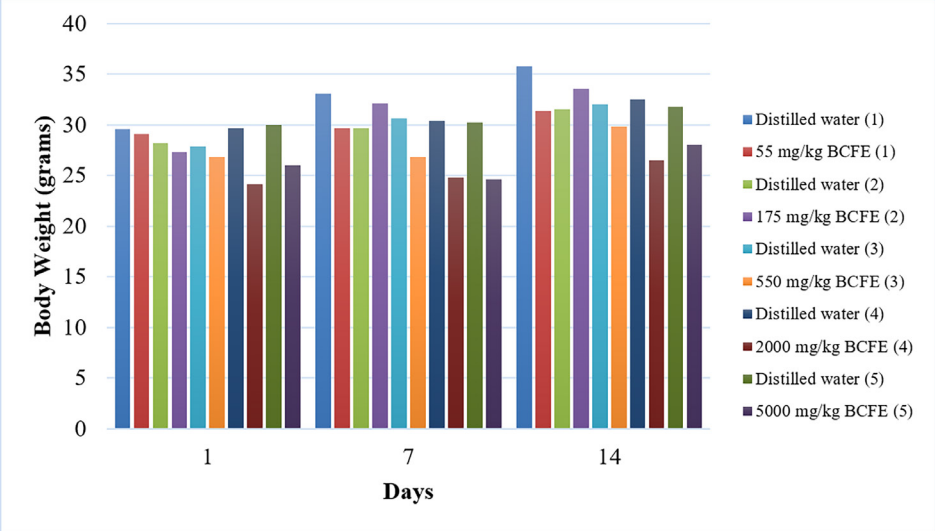

Figure 1A. Body weight (in g) of male ICR mice given distilled water and varying doses of BCFE. The number enclosed in parenthesis signifies the corresponding control and treatment group.

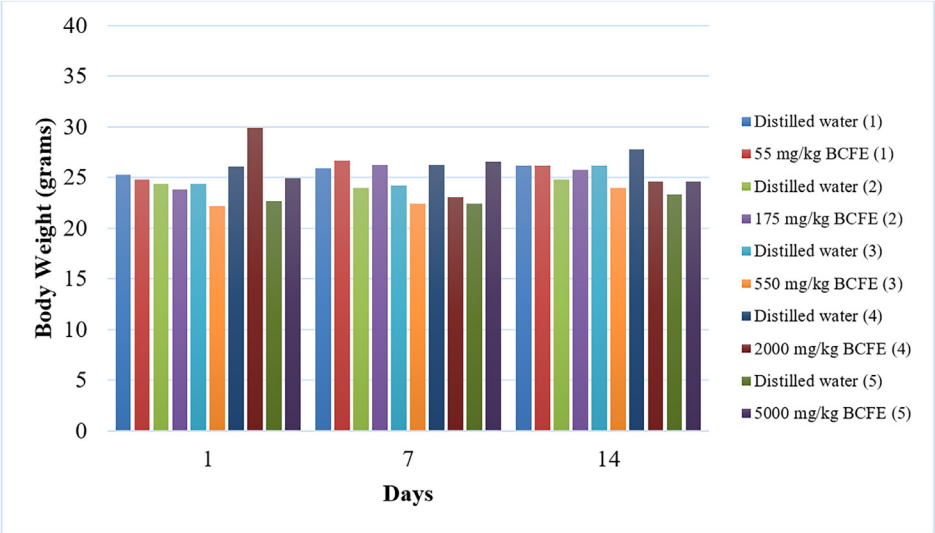

Figure 1B. Body weight (in g) of female ICR mice given distilled water and varying doses of BCFE. The number enclosed in parenthesis signifies the corresponding control and treatment group.

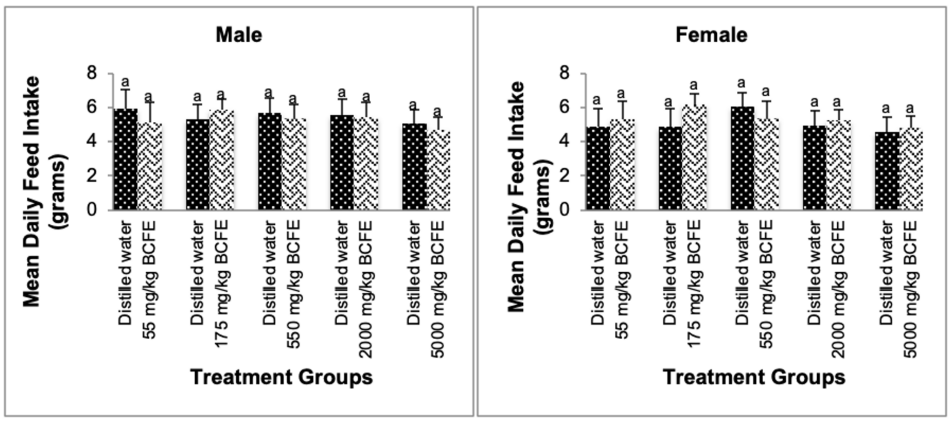

Figure 2A. Mean daily feed intake of male and female ICR mice given distilled water and varying doses of BCFE. Means with similar letters are not statistically significant ($p > 0.05$) using independent sample t-test.

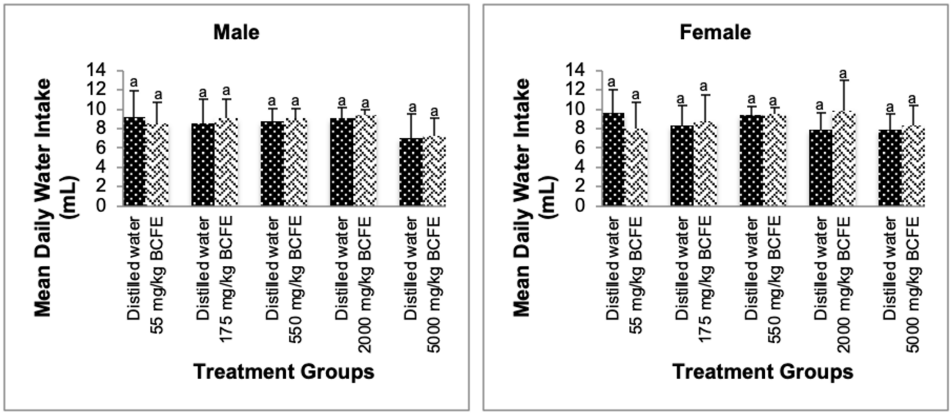

Figure 2B. Mean daily water intake of male and female ICR mice given distilled water and varying doses of BCFE. Means with similar letters are not statistically significant ($p > 0.05$) using independent sample t-test.

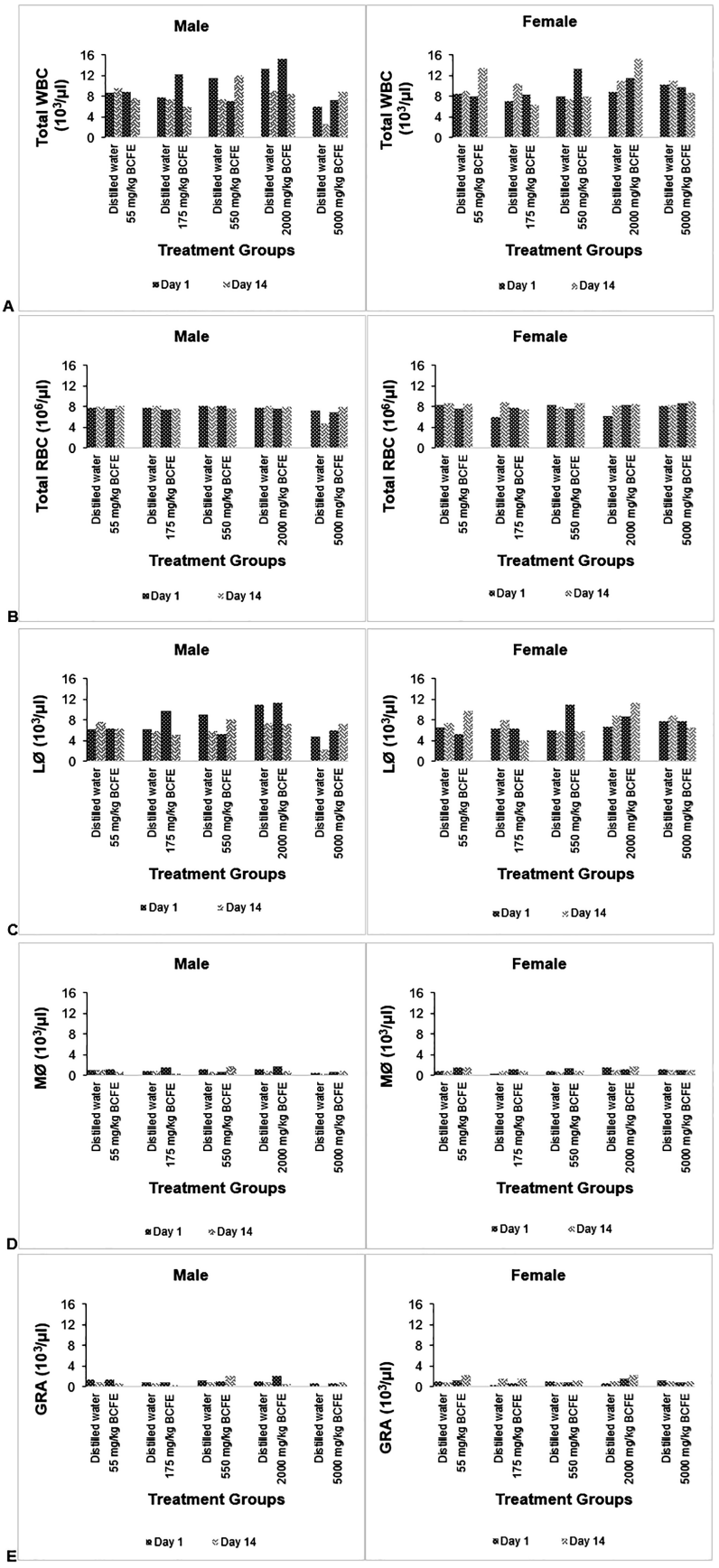

Figure 3. Effects of varying doses of BCFE in the hematological profile of male and female ICR mice. Legend: [A] total WBC count (10^3/µL), [B] total RBC count (10^6/µL), [C] lymphocyte count (10^3/µL), [D] monocyte count (10^3/µL), and [E] granulocyte count (10^3/µL).

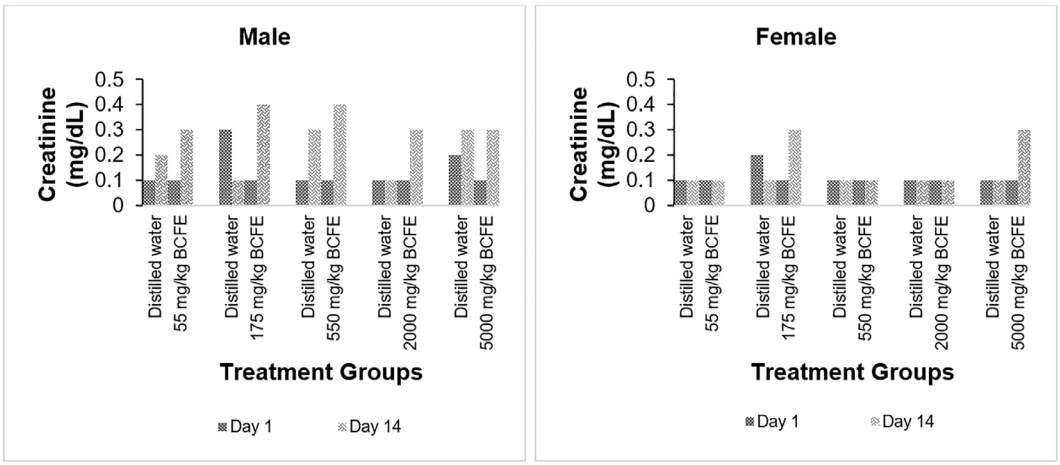

Figure 4A. Blood creatinine profile of male and female ICR mice given distilled water and varying doses of BCFE.

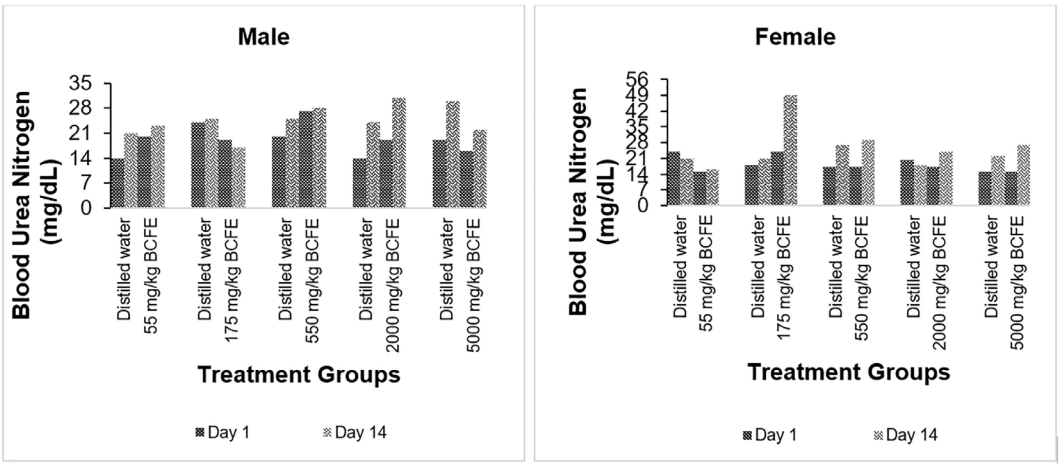

Figure 4B. Blood urea nitrogen level of male and female ICR mice given distilled water and varying doses of BCFE.

Table 2A. Weight of selected organs (g) of different treated male ICR mice.

Treatment	Brain	Heart	Liver	Spleen	Stomach	Large intestine	Small intestine	Right kidney	Left kidney	Lungs
Distilled water	0.46	0.30	1.85	0.08	0.18	0.32	0.51	0.28	0.26	0.37
55 mg/kg BC	0.47	0.20	1.90	0.06	0.17	0.26	0.59	0.28	0.26	0.15
Distilled water	0.46	0.18	1.67	0.06	0.30	0.33	0.60	0.22	0.22	0.14
175 mg/kg BC	0.48	0.20	1.90	0.09	0.23	0.24	0.43	0.28	0.28	0.17
Distilled water	0.48	0.24	1.87	0.08	0.19	0.30	0.85	0.26	0.27	0.17
550 mg/kg BC	0.50	0.21	1.53	0.11	0.17	0.24	0.50	0.19	0.19	0.16
Distilled water	0.47	0.23	1.70	0.09	0.23	0.31	0.82	0.29	0.29	0.18
2000 mg/kg BC	0.44	0.21	1.32	0.08	0.13	0.24	0.64	0.23	0.21	0.14
Distilled water	0.42	0.22	1.94	0.10	0.17	0.28	0.53	0.29	0.26	0.17
5000 mg/kg BC	0.46	0.18	1.34	0.08	0.16	0.31	0.69	0.26	0.22	0.13

Legend: [BC] bignay 'common'

Table 2B. Weight of selected organs (g) of different treated female ICR mice.

Treatment	Brain	Heart	Liver	Spleen	Stomach	Large intestine	Small intestine	Right kidney	Left kidney	Lungs
Distilled water	0.51	0.22	1.62	0.10	0.21	0.24	0.43	0.17	0.15	0.18
55 mg/kg BC	0.52	0.19	1.48	0.08	0.17	0.22	0.46	0.22	0.20	0.18
Distilled water	0.46	0.19	1.36	0.06	0.19	0.30	0.61	0.22	0.22	0.14
175 mg/kg BC	0.46	0.16	1.64	0.05	0.19	0.31	0.55	0.15	0.15	0.15
Distilled water	0.51	0.18	1.33	0.11	0.20	0.50	0.76	0.20	0.19	0.15
550 mg/kg BC	0.43	0.19	1.38	0.06	0.19	0.20	0.46	0.18	0.16	0.15
Distilled water	0.51	0.18	1.50	0.08	0.20	0.32	0.74	0.23	0.17	0.13
2000 mg/kg BC	0.44	0.18	1.32	0.07	0.18	0.31	0.66	0.17	0.16	0.17
Distilled water	0.48	0.22	1.26	0.08	0.13	0.18	0.59	0.19	0.17	0.15
5000 mg/kg BC	0.50	0.19	0.99	0.06	0.18	0.21	0.56	0.18	0.15	0.14

Legend: [BC] bignay 'common'

Microscopic examination of stained tissue sections from all the male and female mice treated with BCFE and distilled water did not show any abnormalities. In particular, the liver appeared normal with the central veins and hepatic sinusoids lined with endothelial cells with normal radiating hepatocytes; the kidneys had normal histologic features; the heart showed normal cardiac muscle fibers; whereas the lungs displayed normal alveolar structure with no treatment-related inflammatory response. No identified histological alternations were also perceived in the remaining organs studied such as the small and large intestines, spleen, and brain (Figure 5).

DISCUSSION

Due to the increasing knowledge of their therapeutic properties, berries such as bignay have piqued the interest of many researchers. Unfortunately, accounts on their safety and toxicity left unexplored, especially in the context of locally occurring cultivars. In the present study, we comprehensively described the acute toxicity profile of Philippine bignay 'Common' – specifically, its fruit extract on various physiological, physico-behavioral, hematologic, biochemical, and gross, biometric, and microscopic parameters in close adherence to the recommendations stipulated in the OECD Guideline 425.

The overt signs of toxicity such as physical, neurologic, cardiorespiratory, and behavioral changes were absent in male and female ICR mice treated with different concentrations of BCFE. These, in conjunction with zero mortality observed in all treatment groups, suggest that oral ingestion of bignay 'Common' fruit is relatively innocuous when consumed up to 5000 mg/kg. Similarly, Tanquilut *et al.* (2019) earlier reported that oral administration of fresh and ethanolic bignay fruit extract up to 1000 mg/kg in db/db diabetic mice elicited no prominent signs of toxicity, as well as the absence of mortality. However, in stark contrast to our present study, the group only adopted a significantly lower high-dose limit coupled with a considerably shorter assessment period of 48 h. Moreover, the apparent safety of bignay consumption has also been described in a few lines of acute toxicity studies, although these works preferentially employed different plant parts of bignay such as its leaves (Grijaldo *et al.* 2019; Tanquilut *et al.* 2019) and seeds (Chowtivannakul *et al.* 2016). Further reinforcing these above-mentioned data, related species of bignay such as *Antidesma acidum* (Sireeratawong *et al.* 2012) and *Antidesma ghaesembilla* (Habib *et al.* 2012) have been previously recounted to produce no perceptible indications of morbidity and mortality. Taken together, it may be inferred that Philippine bignay 'Common' possess a satisfactory degree of safety and this may hold true for other related berries belonging to the same genus.

In general, oral ingestion of various concentrations of BCFE did not affect the physiologic state of ICR mice. Specifically, male and female mice treated with BCFE had normal BWs throughout the duration of the experiment based on the 20–40-g normal weight range of adult mice (Suckow *et al.* 2001). In addition, gain in BW of BCFE-treated mice and distilled water-treated controls were comparably stable except for the female mouse given 2000 mg/kg BCFE, which registered a BW decrement of 17.59% – a value slightly exceeding the recommended tolerable weight loss of not more than 10% BW (Pingale *et al.* 2011; Musa *et al.* 2019). However, considering the findings that this particular mouse did not continually manifest significant deterioration of BW and clinical condition and was able to maintain BW within the acceptable limit; therefore, it counters the impression that supplementation of BCFE may

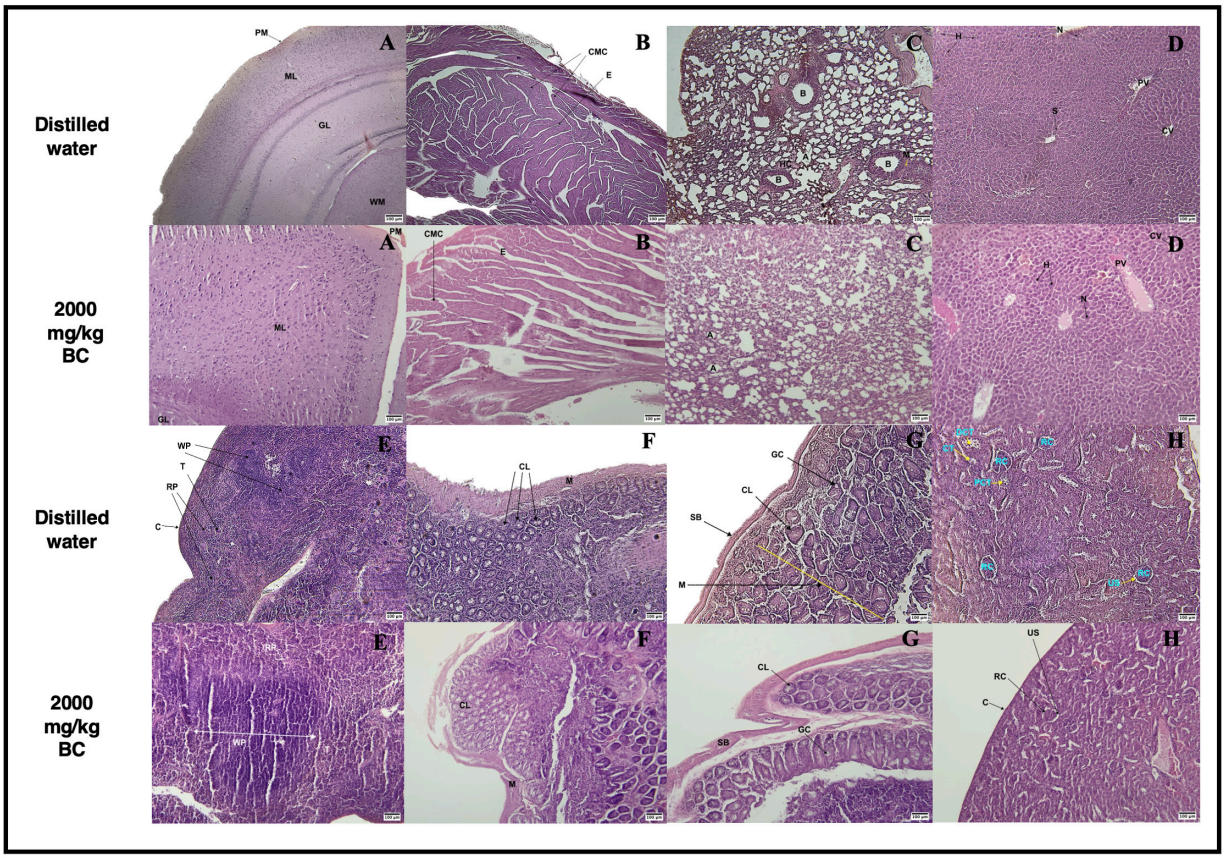

Figure 5. Histological findings of the brain (A), heart (B), lungs (C), liver (D), spleen (E), small intestine (F), large intestine (G), and kidney (H) of ICR mice given the control (distilled water) and BCFE (2000 mg/kg BW). Hematoxylin and eosin (H&E) stain. Scale bar: 100 µm. Legends: pia mater (PM), molecular layer (ML), granular layer (GL), white matter (WM), cardiac muscle cell (CMC), endomysium (E), alveolus (A), brochiole (B), hyaline cartilage (HC), muscularis (M), nucleus of hepatocyte (N), sinusoid (S), hepatocyte (H), portal vein (PV), central vein (CV), capsule (C), red pulp (RP). Trabecula (T), white pulp (WP), crypt of Lieberkühn (CL), Mucosa (M), mucosa (M), crypt of Lieberkühn (CL), goblet cell (GC), striated border (SB), renal corpuscle (RC), proximal convoluted tubule (PCT), collecting tubule (CT), disctal convoluted tubule (DCT), and urinary space (US).

yield adverse toxic repercussions. To bolster further this argument, female mice receiving the highest BCFE dose of 5000 mg/kg displayed only a negligible BW reduction of 1.09%. On the other hand, the consumption of feeds and drinking water by both BCFE-treated and untreated mice were within or even higher than the average normal range of 12–18 g/ 100 g BW/d and 15 mL/ 100 g BW/d, respectively (Suckow *et al.* 2001). The high palatability of mouse pellets fed to the test mice possibly led to high consumption of water. Of particular interest was the female mouse administrated with 2000 mg/kg BCFE that, as mentioned above, exemplified a fluctuation in BW gain. The result unveiling that this particular animal managed to consume feeds and water at a relatively higher level than its untreated counterpart lends substantial support to the notion that BCFE is virtually safe and non-toxic and coincided with the fact that female mice are generally more sensitive than male mice when subjected to acute toxicity test (Lipnick *et al.* 1995; OECD 2008).

Hematological analysis revealed that the total RBC and WBC counts of male and female ICR mice treated with BCFE were within the normal range of the controls and the established range of Serfilippi *et al*· (2003) and Suckow *et al.* (2001). In consonance, Chowtivannakul *et al.* (2016) reported that the total RBC counts of albino Wistar rats given bignay seed extract did not differ from their corresponding control group. However, the total WBC counts were significantly higher in those treated *versus* the control group. As for the differential WBC count, a slightly elevated monocyte count was observed in male and female mice given 550 and 2000 mg/kg doses, respectively. Moreover, increased granulocyte count was observed in females treated with 55, 175, and 2000 mg/kg doses. These notable changes in differential WBC profiles in females, specifically those supplemented with higher concentrations of BCFE reaffirm the more pronounced predisposition of female mice than of male mice to toxic agents (Lipnick *et al.* 1995; OECD 2008).

Lastly, the increased granulocyte count exhibited by the male mouse on Day 1 before the commencement of treatment with 2000 mg/kg BCFE may possibly be due to stress observed during blood collection since no treatment-related abnormalities were observed in this particular animal. Accordingly, O'Connell *et al.* (2015) emphasized that stress and excitement are known to cause increased granulocyte count, especially in male mice.

The blood levels of kidney-filtered substances such as creatinine and BUN of the male and female ICR mice treated with BCFE were within the normal range of their corresponding control group and published normal range by Suckow *et al.* (2001) and Serfilippi *et al.* (2003) except for the high BUN level observed in the female mouse treated with 175 mg/kg dose (Suckow *et al.* 2001; Serfilippi *et al.* 2003). This is in agreement with the study done by Quiming *et al*. (2017), which reported normal BUN and creatinine levels in alloxan-induced diabetic ICR mice treated with aqueous and ethanolic bignay leaf extract. Concordantly, STZ-induced diabetic rats treated with bignay seed extract showed decreased BUN and normal creatinine levels (Chowtivannakul *et al.* 2016). An explanation cannot be offered for the discrepancy in BUN and creatinine levels of female mice treated with a 175 mg/kg dose; nevertheless, the same blood sample was utilized and both assays were done simultaneously using an automatic blood chemistry analyzer. Between BUN and creatinine assays, the latter is recognized to be more sensitive in monitoring kidney toxicity (Washington and Hoosier 2012). Additionally, gross and microscopic examination of the right and left kidneys consistently demonstrated absence of pathologic lesions across all treatment regimens evaluated. Collectively, the blood chemistry, gross, and histopathologic results imply that BCFE does not cause kidney damage.

The gross and organ-weight data of harvested tissues like GIT, lungs, liver, kidneys, spleen, heart, and brain from BCFE-treated male and female mice disclosed comparable results with respect to their controls revealing the absence of any discernible signs of abnormalities, which appreciably correlated with subsequent histological findings. As deemed compatible with our study, one investigation discovered that the administration of bignay seed extracts induces inoffensive effects in critical organs including the liver, kidneys, heart, and lungs (Chowtivannakul *et al.* 2016), whereas no striking lesions were found in the brain heart, lungs, liver, kidneys, spleen, adrenal glands, and sex organs of Sprague Dawley rats treated with 5000 mg/kg root ethanolic extract of *Antidesma acidum* in another experimental study (Sireeratawong *et al.* 2012).

CONCLUSION

Based on the cumulated test results and the absence of demonstrable mortality in male and female ICR mice, the present study suggests that bignay 'Common' fruit is relatively non-toxic when ingested orally with increasing dose of up to 5000 mg/kg. Since there were certain alterations in weight gain and absolute differential WBC counts in females given high BCFE doses, it is recommended that subchronic and chronic oral toxicity tests should be conducted to ensure safety of continuous oral consumption of bignay 'Common' fruit.

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STATEMENTS

Author Contributions

L.M.A., K.A.T.C.I., and M.A.C.E. contributed to conceptualization, supervision, project administration, and funding acquisition. L.M.A., and M.A.C.E contributed to methodology and resources. L.M.A., M.A.A.D.C., R.P.G., J.R.C.M., D.J.A.S., J.R.D.A., J.I.D., L.C.B.A., R.B.C.N., and M.A.C.E. contributed to the investigation. M.A.A.D.C., R.P.G., and M.A.C.E. contributed to the original draft. L.M.A., M.A.A.D.C., R.P.G., J.R.C.M., M.J.M.D., and M.A.C.E. contributed to the validation and formal analysis. M.A.A.D.C., R.P.G., J.R.C.M., M.J.M.D., and M.A.C.E. contributed to the review and editing. M.A.A.D.C., R.P.G., J.R.C.M., M.J.M.D., R.B.C.N., and M.A.C.E. contributed to the visualization. R.P.G., and J.R.C.M. contributed to the curation of data. All authors gave final approval and agree to be accountable for all aspects of work in ensuring intergrity and accuracy.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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