IRJIEST 7 (2021) 01-09
Available online at irjiest.batstate-u.edu.ph
International Research Journal on Innovations in Engineering, Science and Technology

Subacute oral toxicity evaluation of Philippine bignay [*Antidesma bunius* (L.) Spreng cv. 'common'] fruits in ICR mice

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ABSTRACT

The increasing prevalence of non-communicable diseases (NCDs) such as diabetes mellitus and dyslipidemia has prompted health research to investigate the use of indigenous crops as functional food ingredients. Bignay fruits are indigenous berries in the Philippines which have distinct health benefits. However, toxicity studies on these berries remain scarce, which are needed to ensure its safety for human consumption and development into food supplements. The primary objective of this study is to determine the possible toxic effects *in vivo* induced by oral subacute administration of bignay extracts. Using ICR mice of both sexes, aqueous bignay extracts were administered via oral gavage once daily for 28 days. The animals were observed daily for any abnormal behavior, and body weights and feed and water intake were also recorded. Gross and histopathology analysis were done to check for any abnormalities in key organs such as the brain, heart, lungs, GI tract, and kidneys. No significant changes were observed in body weight, feed and water intake, blood chemistry and hematology values in all mice groups and were comparable to published values and their respective controls. All mice groups also had appreciable body weight gain (10.89% to 21.52%) with zero morbidity and mortality. Gross and microscopic examination of the brain, heart, lungs, GI tract, liver, spleen, and kidneys showed normal architecture and histology, suggesting that the fruits did not induce any morphological abnormalities. The bignay fruits are non-toxic to both male and female ICR mice and have an LD_{50} value of greater than 2000 mg/kg body weight (BW).

Keywords: bignay, Philippine berries, subacute, toxicity, *Antidesma*

1. Introduction

The increasing prevalence of obesity and non-communicable diseases (NCDs) such as diabetes mellitus (DM) and dyslipidemia has led health research to explore the use of bioactive compounds naturally occurring in foods. Among fruits, berries have been a subject of interest because they contain bioactive compounds such as anthocyanins, flavonoids, phenolics, phenolic acids, tannins, essential oils, carotenoids, vitamins, and minerals [1-3] which can help alleviate these diseases. *In vivo* studies on mice indicated that dietary supplementation of red raspberries [4] and blueberries [5] have anti-obese effects while bilberry reduced blood glucose levels in diabetic mice [6]. Based on these studies, berries have the potential to be developed into food supplements. But there is a growing concern over the use of plant-derived drugs because of the presence of secondary metabolites and contaminants that can be harmful when consumed [7]. Thus, there is a need to assess the safety of the plant material before its further development for pharmaceutical use. One of the ways to address this is to perform oral toxicity tests *in vivo*.

Performing *in vivo* toxicity tests is one of the first steps in drug development. This is crucial in evaluating the characteristics and effects of a drug in a living organism [8]. These tests can be classified by test duration, wherein acute tests are done to determine the adverse effects that may occur after administration of a single dose or multiple doses of the test sample given within 14 days; subacute toxicity focuses on assessing the adverse effects after a single dose or multiple doses of a test sample per day for 28 days; while subchronic toxicity considers the adverse effects of the test sample given for 90 days, also in either a single dose or multiple doses per day [9]. Toxicity tests have been used to assess the safety of other berries such as goldenberries [10], black plum [11], and pigeon berry [12].

Bignay [*Antidesma bunius* (L.) Spreng cv. 'common'] is an indigenous berry in the Philippines which is also distributed in some parts of Southeast Asia to Northern Australia [13]. Its fruits are colored green when unripe and turn red to black as it ripens [14]. It gives a sweet-tart taste [15] and thus can be developed into fruit jams and wine. Bignay fruits are rich sources of several macro- and micronutrients such as vitamins A

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and C, minerals like calcium, phosphorus, and iron [13,16], as well as several phytochemicals [17]. Some studies have shown functional properties of its fruits and leaves, such as its antioxidant [18], antimicrobial [19], anti-inflammatory [20], and anti-angiogenic activities [21]. Studies on the acute toxicity of bignay fruits and seeds have also emerged [20,22, 23]. Despite studies on the acute toxicity of bignay fruits where it was found to be non-toxic at 14 days of exposure [23,24], its safety is still not clearly established because of the lack of long-term toxicity studies. Therefore, this study aimed to assess the possible toxic effects of bignay fruits in ICR mice upon subacute administration for 28 days.

2. Materials and methods

2.1. Plant collection and sample preparation

Fully ripe bignay [*Antidesma bunius* (L.) Spreng cv. 'common'] fruits were harvested from Los Baños, Laguna, Philippines and authenticated by the Botanical Herbarium, Museum of Natural History, UPLB. The fruit flesh and peels were freeze-dried, and the resulting samples were ground using mortar and pestle before passing through an 80-mesh standard sieve. Samples were stored in metallized bags at -20 °C until use.

2.2. Experimental animals

Healthy, 6-week-old, ICR mice were obtained from the Laboratory Animal Facility, Research Institute for Tropical Medicine (RITM), Department of Health, Alabang, Muntinlupa City, Philippines.

All mice, 10 males and 10 females, were acclimatized for one week prior to experimentation. The individual body weights, and feed and water intake were recorded daily. The mice were individually housed in standard polycarbonate cages with a stainless-steel top and maintained at a room temperature of 22±3 °C, 30 to 60% humidity, and a 12-hour light-dark cycle. The cages and beddings were changed once every week. A maintenance mouse pellet diet (Altromin, Germany) distilled water were provided *ad libitum*. All *in vivo* procedures were approved by the UPLB Animal Care and Use Committee (CHE-2019-002).

2.3. Subacute oral toxicity testing

The subacute toxicity test was done as described by the OECD Guideline 407 for Testing Chemicals: Repeated Dose 28-day Oral Toxicity Study in Rodents [25].

The test animals were randomly assigned to 2 groups: (1) control and (2) bignay (cv. 'common') (2000 mg/kg BW); with each group containing 5 males and 5 females. After acclimation, blood samples were collected from all test animals via the retro-orbital sinus and were subjected to blood chemical and hematological analyses. Mice in the treatment group received freshly prepared freeze-dried fruits reconstituted with distilled water at a dose of 2000 mg/kg BW every day for 28 days via oral gavage. Meanwhile, all mice in the control group received distilled water as the vehicle.

The vehicle and bignay extracts were given once a day using a 1-inch 22G stainless steel gavage needle (ThermoScientific, USA) and 1 mL sterile disposable syringe (Terumo, Japan). Food and water were withheld 1 hour before and 1 hour after treatment administration.

All animals were observed for the first 72 hours for any sign of regurgitation or behavioral signs of toxicity including convulsion, tremors, salivation, diarrhea, lethargy, or any changes in the skin, hair, eyes, mucous membranes, respiratory, circulatory, central, and autonomic nervous systems. The body weights, and feed and water intake of all animals were recorded daily. All mice were observed for 28 days for any sign of toxicity and morbidity. On day 29, all mice were anesthetized with the injection of tiletamine-zolazepam (Zoletil, Virbac Phils. Inc.) at a dose of 20 mg/kg BW IP. Blood samples were collected via cardiac puncture and were put into heparinized and EDTA-containing tubes for blood chemistry and hematological analyses.

2.3.1. Measurement of feed and water intake

Feed and water intake were measured by giving pre-weighed commercial feed pellets and distilled water. Briefly, 7-8 g of feeds and 10 ml of water were given to each mouse one hour after the administration of the vehicle and treatments. Leftover feeds and water were weighed daily using a digital top loading balance (Shimadzu, Japan) and graduated cylinder, respectively, for 28 days. To get the amount consumed, the weight of the leftover feeds and water were subtracted from the pre-weighed pellets and pre-measured water.

2.3.2. Measurement of body weight

All animals were weighed daily using a digital top loading balance (Shimadzu, Japan) before administration of the treatments. Weights were recorded to the nearest 0.001 gram and percent weight gains were computed using Eq. (1):

$$\%\, Weight\ gain = \frac{Final\ weight - Initial\ weight}{Initial\ weight} \times 100 \quad (1)$$

2.3.3. Morbidity and mortality

The morbidity and mortality rates for all groups were computed using Eq. (2) and Eq. (3):

$$Percent\ (\%)\ morbidity\ per\ group = \frac{No.\ of\ mice\ that\ showed\ toxicity\ signs\ per\ group}{Total\ no.\ of\ mice\ per\ group} \times 100 \quad (2)$$

$$Percent\ (\%)\ mortality\ per\ group = \frac{No.\ of\ mice\ that\ died\ per\ group}{Total\ no.\ of\ mice\ per\ group} \times 100 \quad (3)$$

2.3.4. Hematology and blood chemistry analysis

Before blood collection, a drop of ophthalmic anesthetic tetracaine (Alcaine®, Novartis, Philippines) was placed in the right eye of each mouse. After 2 minutes, 300 µl of blood were collected from the retro-orbital sinus of each mouse using a heparinized capillary tube (INRI, Netherlands). Blood samples were collected shortly before administration of the vehicle and berry extracts at day 1 and prior to euthanasia at

day 28. Blood collection on day 28 was performed via cardiac puncture. For the evaluation of hematology parameters such total red blood cell (RBC), total white blood cell (WBC), monocytes (MON), granulocytes (GRA), and lymphocyte (LYM) counts using an automated hematology analyzer (Orphee, Switzerland), 10 µl of blood were used. Meanwhile, 250 µl of blood was allotted for liver and kidney toxicity assessment by measuring the alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (CREA) levels using an automated blood chemistry analyzer (Arkway Inc., Japan).

2.3.5. Weighing, processing, macroscopic and microscopic examination of specific organs and tissues

After the 28-day study period, all mice were euthanized through intraperitoneal injection of 80 mg/kg pentobarbital sodium (Dolethal®, UK). A midventral incision at the thoraco-abdominal area was done using surgical scissors to exteriorize the esophagus, stomach, small and large intestines, liver, kidneys, spleen, heart, lungs, heart, and brain. All organs were macroscopically examined for any abnormalities and were flushed with 0.9% sodium chloride before weighing using a digital top loading balance (Shimadzu, Japan).

The relative organ weights were determined using Eq. (4):

$$Relative\ organ\ weight\ = \frac{Organ\ weight}{Mice\ body\ weight\ on\ sacrifice\ day} \times 100 \qquad (4)$$

After weighing, the organs were trimmed and then submerged in 10% buffered formalin solution for at least 72 hours for fixation. Tissue samples were processed using the paraffin technique, sectioned with rotatory microtome, one out of every four sections of each organ were collected, stained with hematoxylin and eosin (H&E) stain, and evaluated in a blind fashion under light microscopy (Zeiss Primostar, Germany). Photographs of the tissue sections were taken using a digital camera and a binocular research microscope. Analysis was carried out by a veterinary pathologist (U.P. National Institutes of Health, Manila).

2.4. Conversion of animal dose to human equivalent dose (HED)

The data gathered on the animal toxic dose were converted into HED using Eq. (5) [26]:

$$HED\ = Animal\ dose\ in\ \frac{mg}{kg} \times \left(\frac{Animal\ Km}{Human\ Km}\right) \qquad (5)$$

where Km is the correction estimated by dividing the average body weight (kg) of species to its body surface area (m^2) [27].

2.5. Data processing and analysis

Results were expressed as means ± standard error of the mean (SEM). Data were subjected to one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) to compare the mean values between the control and treatment groups at $p<0.05$. Paired sample t-test was used to analyze the difference of means within the group at day 1 vs. day 28, while two-sample-t-test was done to analyze differences between sexes within the same treatment group. All statistical analyses were performed using Minitab 19.0 Statistical Software for Windows (Minitab 19.0, Minitab LLC).

3. Results and discussion

3.1. Effect on feed and water intake

Subacute administration of bignay extracts did not adversely affect feed intake of male and female ICR mice (Figure 1). The mean feed intakes of all treatment mice were comparable to their controls. There were also no significant differences between male and female mice within each group. It was noted that male mice had generally higher intakes than female mice. All mice groups had increasing feed intake throughout the study period (weeks 1-4) compared to the acclimation week (week 0) except for male mice fed with bignay (BM) with decreased intakes at weeks 1 and 2, although these fluctuations were not significant and still within published values. The feed intakes of all groups were within the average daily feed consumption of 3-5 g for an adult mouse [28]. This indicates that consumption of bignay fruit extracts did not affect the feed intake of male and female ICR mice.

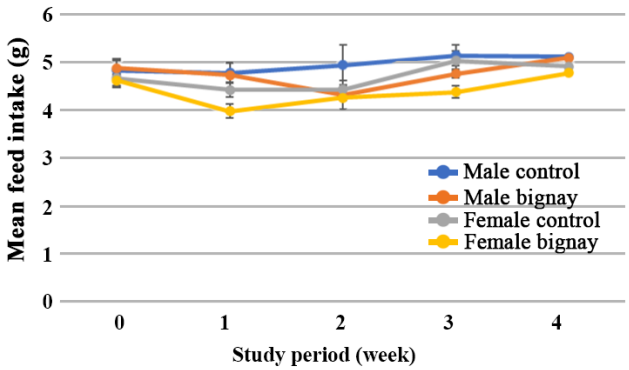

Figure 1. Mean feed intake (g) of male and female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/kg BW**.** Error bars indicate standard error of the mean (SEM).

Likewise, the water intakes of male and female mice of the treatment groups were comparable to their respective controls (Figure 2). It was also observed that water intake for all groups, except for females in the control group (CF), significantly decreased during the first week of the toxicity test. For the following weeks, a general increase in water consumption was noted for all groups. There was also a significant decrease in the water intakes of female mice of bignay (BF) at week 3, and BM at week 4. Under *ad libitum* conditions, 70% of the total water intake of mice are taken with meals. Hence, there is a positive correlation between feed and water intake [29]. This drop in water intake during week 1 may be due to the additional volume of liquid introduced by the start of vehicle and treatment

administration. Nonetheless, the mean weekly water intakes of all mice fall within the normal range of 3-5 ml per day [28]. Hence, the bignay extracts did not adversely affect the water intakes of male and female ICR mice.

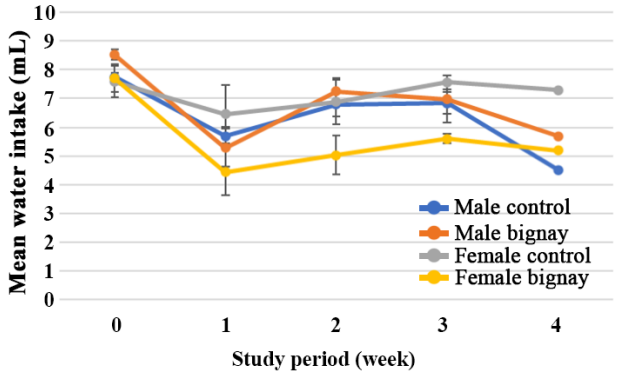

Figure 2. Mean water intake (ml) of male and female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/kg BW. Error bars indicate standard error of the mean (SEM).

3.2. Effect on body weight

As seen in Figure 3, the body weights of all test animals were not affected by subacute administration of bignay fruit extracts. The body weights of all treated mice were comparable to their controls. In general, the male mice were heavier than their female counterparts. This may be due to sex-specific differences in body composition [30] and increased feed intakes for male mice [31]. An increasing trend was observed for all mice weights for the duration of study, with no significant changes between the weekly body weights.

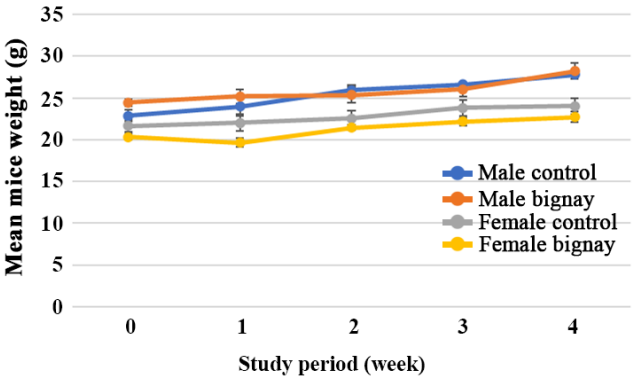

Figure 3. Mean weekly mice weights (g) of male and female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/kg BW. Error bars indicate standard error of the mean (SEM).

The weekly percent body weight gains with respect to week 0 are seen in Table 1. All mice groups had considerable weight gains throughout the experiment period. It was noted that the body weights of BF decreased in the first week of treatment administration, albeit insignificant. It was also observed that male mice in all groups gained weight faster than their female counterparts, which corroborates with a similar study in mice [32]. A noticeable decrease was observed in the body weight gain of BM compared to the control male group (CM). This might be caused by the stress brought about by oral gavage as previous research showed that a more viscous solution can cause more stress to the animal [33], as in the case with bignay. In some cases, decreases in body weight were reported [34, 35]. Despite this, body weights were significantly higher for all experimental groups through weeks 3 and 4 in comparison to week 0, suggesting that the mice have recovered from the stress possibly induced by oral gavaging.

Table 1. Percent (%) weight gain of mice treated with distilled water and bignay aqueous extracts.

Groups		Weight gain (%)			
		Wk 0 vs Wk 1	Wk 0 vs Wk 2	Wk 0 vs Wk 3	Wk 0 vs Wk 4
Control	Male	4.851	13.415	16.285*	21.518*
	Female	1.798	4.109	9.922*	10.887*
Bignay	Male	3.132	3.745	6.429*	15.413*
	Female	-3.423	5.397	8.919*	11.653*

Monitoring body weights during toxicity studies is crucial since these reflect the general health status of animals [36]. The insignificant changes in the body weights of all the animals suggest that the extracts did not interfere with normal body metabolism [37]. More importantly, body weights of the treated mice coincide with their respective controls, indicating that the bignay fruits did not suppress normal eating habits.

3.3. Effect on blood chemistry

Toxic compounds are known to affect the liver and kidney and impair their physiological functions [38]. Thus, the levels of ALT, BUN, and CREA are commonly used as renal and hepatic function tests. In the present study, no significant changes were observed for control and treatment groups in all parameters at day 1 vs. day 28 (Figure 4). All values for blood chemistry parameters are within published values [39], as summarized in Table 2 suggesting that the subacute administration of bignay fruit extracts did not cause damage to the liver and kidneys.

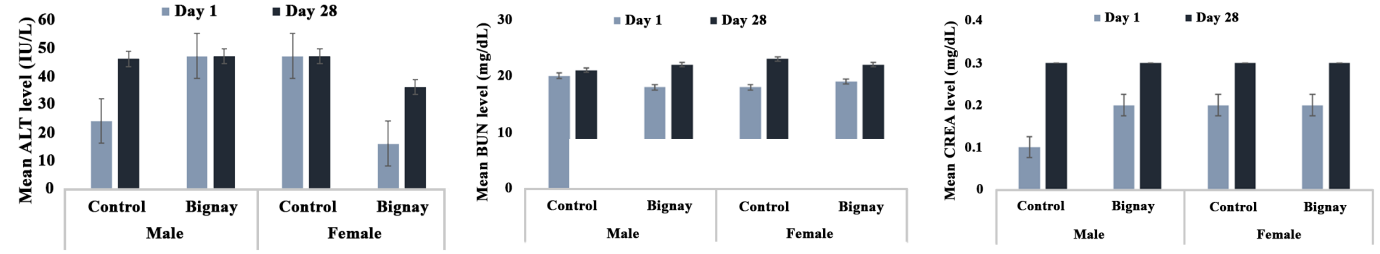

Figure 4. Mean a) alanine transaminase (ALT), b) blood urea nitrogen (BUN), and c) creatinine (CREA) levels of male and female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/kg BW. Error bars indicate standard error of the mean (SEM).

3.4. Effect on hematology

Hematological parameters are commonly used as an index of pathological and physiological status. In acute and chronic toxicological studies, changes in these parameters can be indicative of toxicity [40]. Results showed that there were no significant differences in hematological values between sexes within each group and same sex among control and treatment groups (Figure 5). All changes between baseline and endline values were also insignificant. Furthermore, levels were still within published values [39]. Hematology values obtained from all groups at day 28 were comparable to their respective baseline and control values, suggesting that bignay fruit extracts did not affect hematological parameters upon treatment for 28 days.

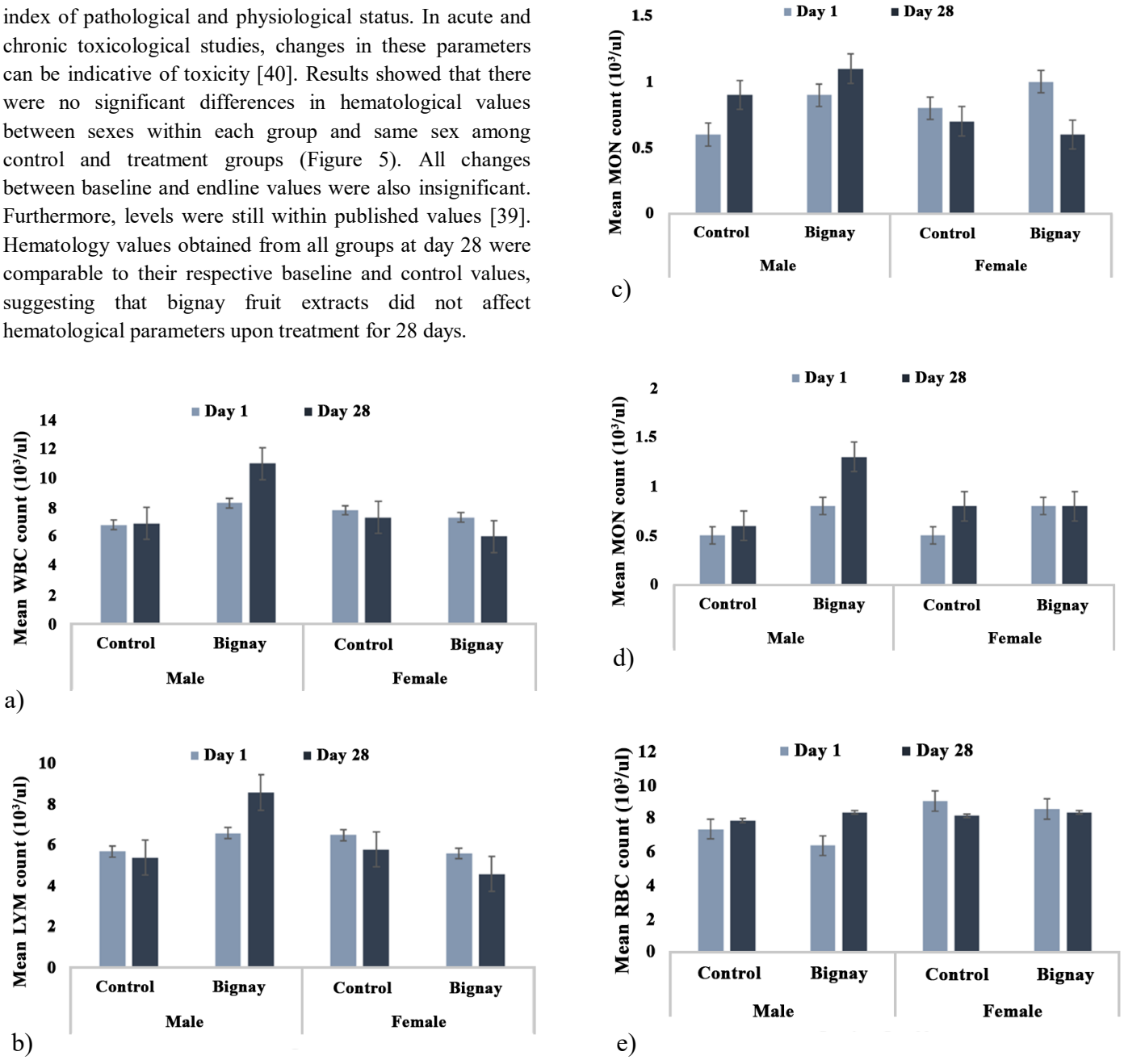

Figure 5. Mean a) total white blood cell (WBC), b) lymphocyte (LYM), c) monocyte (MON), d) granulocyte (GRA), and e) red blood cell (RBC) count of male and female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/kg BW. Error bars indicate standard error of the mean (SEM).

Table 2. Reference values for blood chemistry and hematological parameters.

Parameter	Range of accepted values	
	Male	Female
Blood chemistry		
ALT	26-59	15-41
BUN	18-29	14-22
CREA	0.2-0.4	0.2-0.4
Hematology		
WBC	8.97-18.33	6.55-24.25
LYM	6.91-15.58	5.57-20.55
MON	0-0.71	0-0.69
GRA	0-9.76	0-4.27
RBC	8.16-9.83	7.31-10.03

3.5. Effect on morbidity and mortality rates

The daily administration of 2000 mg/kg BW bignay fruit extracts for 28 days showed no mortality in all mice of the control and treatment groups. There were also no physical, behavioral, and physiological abnormalities observed in the mice of the control and treatment groups, suggesting that the bignay extracts did not cause any subacute toxic effect in ICR mice. Similar results were recorded by a previous acute toxicity study on the 'common' variety of bignay [23] and acute and subchronic studies on fruits and seeds of *Antidesma* species [22,40,29,41].

3.6. Effect on relative organ weights, and macroscopic and microscopic organ appearance

The relative organ weight, with corresponding histopathological examination, is a useful indicator of treatment-related changes in repeated toxicity studies [42]. It provides evidence for pathological changes as it can be used as a metric for atrophy and hypertrophy [43]. In the present study, the relative organ weights of bignay-treated groups were comparable to their corresponding controls (Figures 6-7). Moreover, there were no differences between opposite sex within each group.

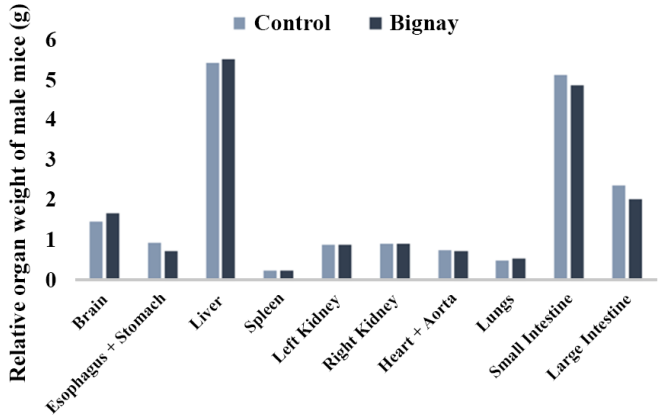

Figure 6. Relative organ weights of male ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/ kg BW.

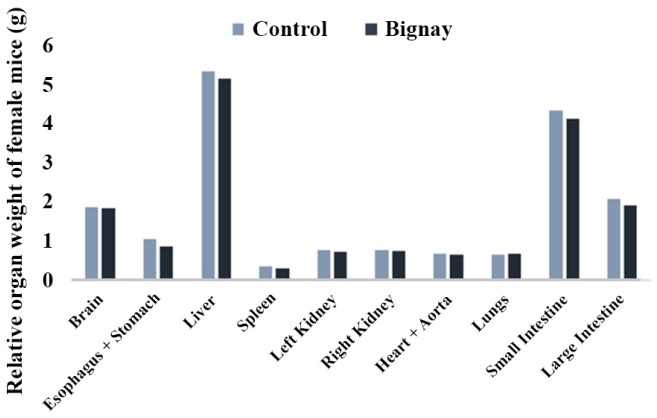

Figure 7. Relative organ weights of female ICR mice treated with distilled water and bignay aqueous extracts at 2000 mg/ kg BW.

Generally, female mice had heavier brain, esophagus, stomach, spleen, and lungs, while male mice had heavier liver and kidneys. These results coincide with similar toxicity studies wherein male mice had heavier kidney weights [44] and female mice had heavier brain, spleen, and lungs [45]. It was also observed that the heart and intestines of CM and BM were heavier than their female counterparts. These infer that bignay administration did not induce any effect on normal organ growth.

The organs of representatives from each group are shown in Figure 8. Findings showed no alterations in the morphology of the selected organs. All organs appeared normal in size, shape, and color. Gross examination revealed no visible lesions in all mice groups. No abnormalities were also observed in the selected organs upon microscopic examination as seen in Figure 9. The liver of the treated mice had normal cellular structures of hepatocytes, sinusoids, and central vein, and were similar to their controls. In the lungs, bronchiole, alveoli, alveolar duct, and blood vessel structures were normal. The heart showed normal cardiac muscle cells and fibers, while the kidney had normal glomerular architecture and binucleation. Similarly, the brain, spleen, stomach, and small and large intestines of all mice groups showed normal structure and histology. The results suggest that the bignay fruit extracts did not produce any histopathological changes in the mice organs.

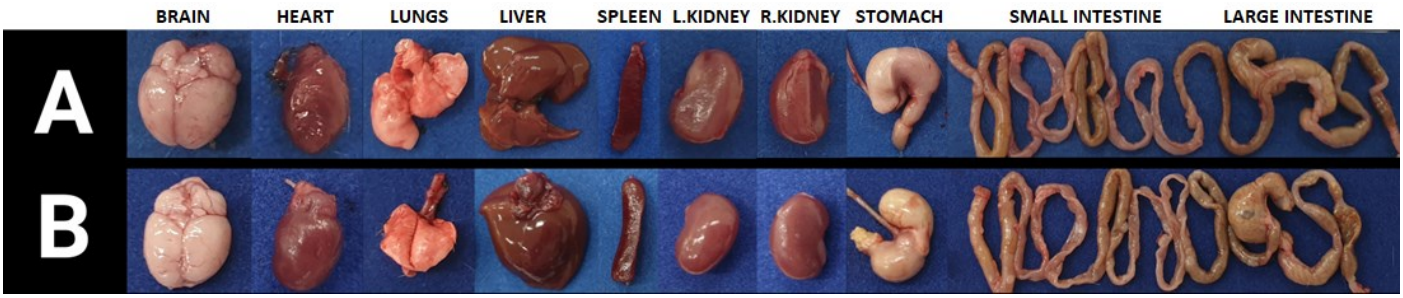

Figure 8. Digital image of representative organs of ICR mice treated with A) distilled water and B) bignay aqueous extracts at 2000 mg/kg BW.

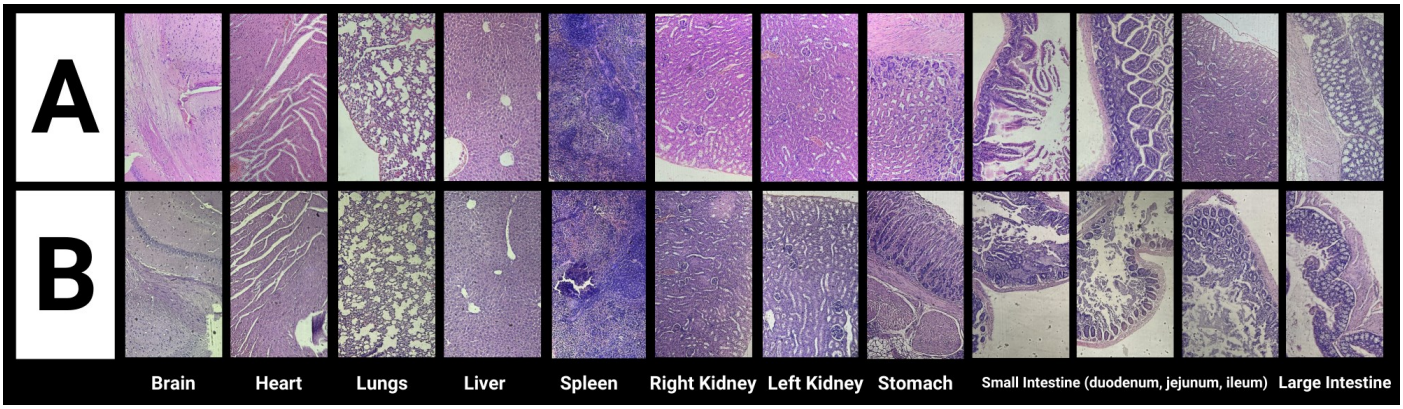

Figure 9. Micrographs of representative organs (10X) of ICR mice treated with A) distilled water and B) bignay aqueous extracts at 2000 mg/kg BW.

3.7. Conversion to human dose equivalent (HED)

The LD_{50}, also known as the median lethal dose, is the dose required to cause mortality to half of the tested population after a specific period [46]. It is usually expressed as the weight of the sample administered in milligrams per kilogram body weight of the animal. It is an important indicator for the prediction of the human lethal dose and for predicting the dose that would cause toxicity symptoms in humans [47]. The human equivalent dose (HED) is a dose in humans expected to provide the same degree of effect as that of the animals tested at a given dose [27]. In the context of toxicity studies, this provides an estimate as to what dose in mice will give the same effect in humans. Based on the guidelines provided by the U.S. Department of Health and Human Services – Center for Drug Evaluation and Research [26], the 2000 mg/kg animal dose used for bignay in human equivalent dose is 162.16 mg/kg BW, and is the amount considered safe for consumption by a typical 60 kg adult.

4. Conclusions

The subacute toxicity test of bignay [*Antidesma bunius* (L.) Spreng var. 'common'] fruit showed normal feed and water intake, as well as increased body weights in all experimental mice for the duration of the study. Generally, male mice were heavier and gained weight faster than their female counterparts. No significant differences were observed in the blood chemistry and hematology parameters between the control and treatment groups. Treatment administration also did not cause morbidity or mortality. Moreover, there were no significant differences in relative organ weights across all groups and sex. No gross and microscopic lesions were observed in all organs upon visual and histopathologic examination. Therefore, the aqueous extracts of Philippine bignay fruits are non-toxic at 2000 mg/kg dose or equivalent to 162.16 mg/kg human dose. The LD_{50} of bignay is greater than 2000 mg/kg BW.

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Acknowledgments

The authors are grateful for the financial support provided by the Department of Science and Technology (DOST) – Philippine Council for Health Research and Development (PCHRD) and DOST – Science Education Institute (SEI).

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