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Subacute oral toxicity study of freeze-dried fruits of duhat (*Syzygium cumini* (L.) Skeels) in ICR mice

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ABSTRACT

Duhat is one of the indigenous fruits in the Philippines that had been traditionally considered in prevention and management of non-communicable diseases including diabetes and hypertension. These underutilized fruits are rich in antioxidants and bioactive substances; however, studies investigating the possible toxic effects of duhat fruits grown in the Philippines using *in vivo* procedures are insufficient. This study investigated the subacute toxic effects of freeze-dried duhat (*Syzygium cumini* (L.) Skeels) on ICR mice. A total of 10 male and 10 female 6-week-old ICR mice were divided into groups: control (vehicle) and duhat group given with freeze-dried duhat fruit powder reconstituted in distilled water at 2000 mg/kg BW dose through oral gavage for 28 days. Results revealed no significant changes on feed and water intake, blood chemistry and hematology parameters when compared with the control and published values. All mice groups also exhibited significant increases in body weights with zero morbidity and mortality. Gross and microscopic examination of the brain, heart, lungs, GI tract, liver, spleen, and kidneys also showed normal architecture suggesting no morphological abnormalities. The study concluded that repeated oral administrations of freeze-dried duhat fruits for 28 days are safe for consumption and has LD50 >2000 mg/kg or 162.16 mg/kg BW in human equivalent dose.

Keywords: duhat, indigenous berries, subacute, *Syzygium*

1. Introduction

The continuous increase in the prevalence of obesity and non-communicable diseases (NCDs) such as diabetes mellitus (DM), cancer, and cardiovascular disease (CVD) lead numerous research to focus on bioactive compounds found naturally in food commodities or medicinal plants for preventive and therapeutic uses against these disease conditions. Berries are among the natural plant foods that are currently being studied as rich sources of bioactive compounds such as phenolic compounds and antioxidants [1-4]. Historically, most berries were used in folk remedy for various illnesses [5,6] while recent scientific reports indicate solid evidence on their nutritional and health benefits such as treatment for cancer [7-9], DM [10,11], and inflammation [12,13]. A genus of berries known as *Syzygium* is of particular interest due to their antioxidative properties, phenolic and flavonoid contents [14] which can help ameliorate the biomarkers of obesity, associated metabolic disorders, and inflammation, among others [15]. Fruits under this genus are believed to be indigenous to Southeast Asia including the Philippines. One of the underutilized but important indigenous fruits under this genus is "duhat" (*Syzygium cumini* (L.) Skeels) [16], also known as Java plum or black plum. Duhat trees grow in tropical and subtropical climates with low and medium altitudes and are usually planted around February to March and flowers around May to June [17]. The trees can grow up to 30 meters tall and 11 meters in diameter, and produce oval, dark purple to nearly black ripe fruits with green or brown seeds [18].

Duhat fruits are rich sources of macro and micronutrients including vitamins A, B1, B2, B3, and C, and minerals such as iron, calcium, and phosphorus [19]. Duhat fruits were also observed to demonstrate scavenging and antioxidative activities using deoxy-d-ribose assay and linoleic acid pre-emulsion system, respectively, and contain certain phytoconstituents including phenols and flavonols [14]. The effectiveness of duhat and other *Syzygium* fruits and other plant parts such as leaves and stem barks on the prevention and management of NCDs were already documented [20,21]. Duhat has also been traditionally considered as an antidiabetic remedy, which has been confirmed by several scientific reports linking several components (alkaloid, jambosine, and glycoside jambolin or antimellin) present in duhat seeds that hinder the conversion of starch into sugar [6,22-25]. Moreover, an earlier acute toxicity study on freeze-dried duhat fruits revealed safety levels up to 5,000 mg/kg body weight [26]. Other available *in vivo* acute toxicological studies on duhat had focused on the seeds and methanolic fruit extracts, which showed a median lethal dose (LD50) of more than 2000 mg/kg and 3000 mg/kg, respectively, without adverse effects to organs [27,28]. Similarly, no adverse effects were observed through subacute toxicity study at 750 mg/kg and 1500 mg/kg doses of methanolic fruit extract [27], but alteration in blood lipids and liver damage were observed

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using 1000 mg/kg ethanolic seed extract [28]. However, studies investigating the possible toxic effects of Philippine duhat fruits using *in vivo* procedures are insufficient. Therefore, this study aimed to assess the possible subacute toxic effects of freeze-dried duhat (*Syzygium cumini* (L.) Skeels) fruits on Institute of Cancer Research (ICR) mice to establish its safety level for consumption, potentially increasing the current utilization of duhat for food applications and nutraceuticals. The results of this study can also augment existing acute studies on toxicity of duhat and can provide bases for efficacy studies.

2. Materials and methods

The safety for consumption of duhat fruits were assessed through a subacute oral toxicity study. All *in vivo* procedures were approved by the UPLB Animal Care and Use Committee with assigned protocol number CHE-2019-002.

2.1. Plant collection and extract preparation

Duhat fruits were harvested at a fully ripe stage from the province of Batangas, Philippines. Sample was verified through evaluation of its morphological characteristics including fruit weight, shape, and thickness and authenticity was confirmed by the Botanical Herbarium, Museum of Natural History, UPLB. Fully matured fruits were selected through visual observation (i.e., fruits that are bluish black or deep purple in color) [29]. Fruits were pulped at room temperature, seeds were extracted, and the remaining fruit flesh and peels were freeze-dried altogether at the Institute of Food Science and Technology, UPLB using VirTis Co. freeze dryer (SP Gardiner, NY) at 20 ˚C and 40 mTorr pressure. Freeze dried duhat samples were finely ground, and then passed through an 80-mesh US standard sieve, packed in metallized bags and stored at -20 ˚C until use.

2.2. Experimental animals

Obtained from the Laboratory Animal Facility, Research of the Institute for Tropical Medicine (RITM), Department of Health, Alabang, Muntinlupa City, Philippines were 10 male and 10 female 6-week-old ICR mice. This outbred mouse strain named after the Institute of Cancer Research (ICR) USA is known for their docile nature, rapid growth rate, and high productivity. Therefore, it is commonly used as a general-purpose model for a wide range of studies including toxicology [30]. Male and female mice were used to consider the possible sex variations in toxicity sensitivity [31,32].

2.3. Acclimation

The animals were acclimatized for a week prior to experimentation and recording of individual body weights and detailed physical examinations were performed during the acclimation period to ensure the use of healthy animals. The animals were housed in individual standard polycarbonate cages with stainless steel tops, and with housing conditions of the controlled system of 12-hour light-dark cycle, temperature of 22-24 ˚C, and relative humidity of 30-60%. The cages and the beddings were changed once every week. The experimental animals were also provided with a maintenance mouse pellet diet (Altromin, Germany) and distilled water ad libitum.

2.4. Subacute oral toxicity testing

The subacute 28-day oral toxicity study was performed based on the OECD guideline 407 for testing chemicals [33]. This experiment was conducted at the Laboratory Animal Room at the Department of Basic Veterinary Sciences, College of Veterinary Medicine, UPLB. The experimental animals were randomly assigned into 2 groups: (1) control (distilled water as vehicle); (2) duhat group (2000 mg/kg BW), with each group containing 5 males and 5 females. Blood samples were extracted and analyzed at baseline and endline. After acclimation and an overnight fasting, baseline blood samples were collected from all experimental animals via the retro-orbital sinus and were subjected to blood biochemical and hematological analyses. Animals in the treatment groups received freshly prepared freeze-dried berries reconstituted with distilled water at a dose of 2000 mg/kg BW every day for 28 days through oral gavage. Meanwhile, all animals in the control group only received the vehicle or distilled water through oral gavage.

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

All animals were observed 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, among others. Daily feed and water intake were recorded, and body weights were also determined daily. All experimental animals were observed for 28 days for any sign of toxicity and morbidity. Mortality among the treatment groups was also recorded.

After 28 days, all mice were anesthetized with the injection of tiletamine zolazepam (Zoletil, Virbac Phils. Inc.) at a dose of 20 mg/kg BW IP. Endline blood samples were then collected via cardiac puncture and were put into heparinized and EDTA-containing tubes for hematological and blood chemistry analyses.

2.4.1. Measurement of feed and water intake

Feed and water intake of each mouse per treatment group were measured daily by providing premeasured commercial mouse pellets and distilled water. Daily leftover pellets and water were weighed using a digital top loading balance (Shimadzu, Japan) and a graduated cylinder, respectively, for 28 days.

2.4.2. Measurement of body weight, morbidity and mortality

All animals were weighed daily using a digital top loading balance (Shimadzu, Japan). Weights were recorded to the nearest 0.001 gram and the percent weight gains were computed [34]. Morbidity and mortality rates for all treatment groups were also recorded and computed.

2.4.3. Hematology and blood chemistry analysis

A drop of ophthalmic anesthetic tetracaine (Alcaine®, Novartis, Philippines) was placed in the right eye prior to blood collection. After 2 minutes, blood was collected through the retro orbital vein using a heparinized capillary tube (INRI, Netherlands). There were 300 µl of blood collected from each mouse per treatment group prior to administration of vehicle or duhat freeze-dried extracts at day 1 and then after day 28. To measure the hematology parameters including tRBC, tWBC, differential WBC, MON, GRA and LYM counts using an automated hematology analyzer (Orphée, Switzerland), 10 µl of blood were used while 250 µl of blood were used to measure the ALT,

creatinine, and BUN levels using an automated blood chemistry analyzer (Arkray Inc, Japan). All analyses were also performed at the College of Veterinary Medicine, UPLB.

2.4.4. Weighing, processing, macroscopic, and microscopic evaluation of specific organs and tissues

After the 28-day experimentation period, all mice per treatment group were euthanized via intraperitoneal injection of 60 mg/kg sodium pentobarbital (Do lethal®, UK). A midventral incision was made at the thoraco-abdominal area to exteriorize the esophagus, stomach, small and large intestines, liver, kidneys, spleen, lungs, heart, and brain. All organs were examined for any gross abnormalities and flushed with 0.9% sodium chloride solution before weighing using a digital top loading balance (Shimadzu, Japan). The relative organ weights of each organ were then computed.

The organs were trimmed, fixed in 10% buffered formalin for at least 72 hours, processed using the paraffin technique, and sectioned at 4 µm in thickness using a rotary microtome. One out of every four sections of each organ were collected and stained with Hematoxylin and Eosin (H&E) for histopathologic evaluation, observed under light microscopy (Zeiss Primostar), and assessed using blinded evaluation. 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 present, only then the semi-quantitative scoring was opted. Histopathological analysis and interpretation were carried out by a veterinary pathologist.

2.4.5. Translation of animal dose to human equivalent dose (HED)

The collected data on animal toxic dose were translated into HED as shown in Eq. (1) [35].

$$HED = animal\ dose\ in\ \frac{mg}{kg} \times \left(\frac{Animal\ Km}{Human\ Km}\right) \quad (1)$$

where Km is the correction factor.

2.5. Data processing and analysis

All analyses were performed in triplicates and were expressed as means of replicates ± standard error of the mean (SEM). Statistical analyses were performed using IBM Statistical Package for the Social Sciences (IBM SPSS v. 20). Data were subjected to one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests to determine any significant difference between the samples at p -value of <0.05. Independent and paired t-tests were also performed to determine the significant difference between male and female mice within each group, and before and after treatment of each group, respectively.

3. Results and discussion

3.1. Effect of subacute toxicity testing on feed and water intake

Subacute administration of 2000 mg/kg BW dose of freeze-dried duhat fruit extracts did not affect the feed intake of male and female ICR mice (Figure 1). The mean feed intakes of all treatment mice were comparable to their corresponding control mice. No significant difference in the feed intakes between male and female mice within and across groups were also noted except within the control group at week 2. It is also interesting to note that male mice had generally higher feed intake than their female counterparts and that male mice fed with duhat (DM) had higher feed intakes than control male mice (CM). All animal groups also exhibited increasing feed intakes throughout the oral toxicity testing period (weeks 1-4), with DM showing significant increase at week 3. All mice had feed intakes within the usual amount of 4-5 grams a day or 12 grams/100 grams body weight/day [36]. This could indicate that duhat fruit extracts did not induce any negative effect in terms of feed intake among male and female mice.

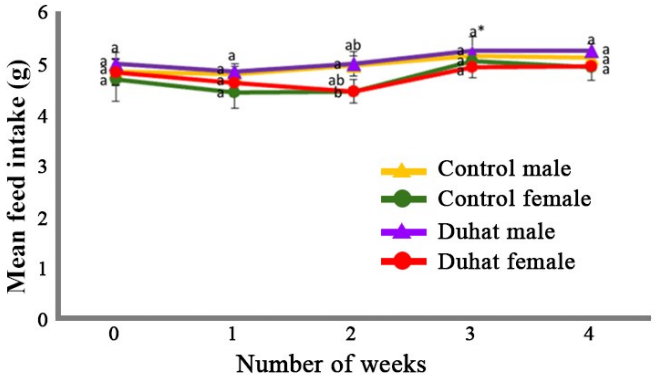

Figure 1. Mean weekly feed intake (g) of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Point values with different superscript (s) in a week are significantly different at p<0.05. Asterisk (*) denotes significant difference at p<0.05 in reference to week 0 (baseline).

Similarly, no effect on water intake of all experimental animals was observed. The water intakes of treated male and female mice were also comparable with those of control animals except for DM which had significantly higher water intake than CM at week 4, possibly due to the fiber content of duhat extract that can induce thirst due to its high capacity in binding water [37]. Furthermore, no significant difference in water intakes was also noted between male and female mice within each group. Figure 2 shows that all mice groups, except females in the control group (CF), had significantly lower water intake during the experimental period than acclimation, which could be due to the increased feed intake and the additional weight of fiber and water administered through gavaging. The relatively higher water intakes among CF, on the other hand, could be due to better adaptation as female mice are found to have better water intakes than male rodents subjected to similar stressful procedures such as gavaging [38]. Nonetheless, all mice exhibited a generally increasing water intake throughout the experimental period and within the normal range of 3-5 ml per day or 1.5 ml/10 g body weight per day [36], which suggests that the duhat fruit extracts did not have negative impact on water intake.

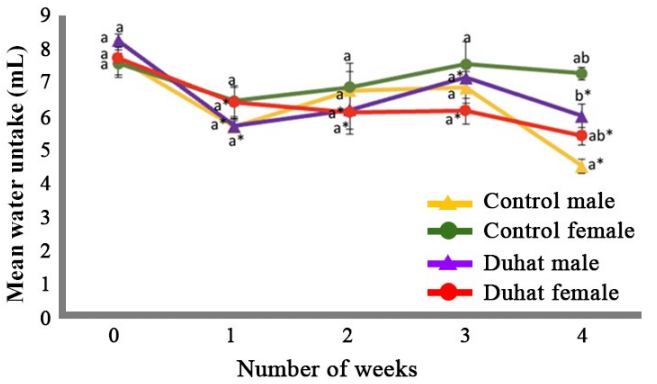

Figure 2. Mean weekly water intake (ml) of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Point values with different superscript(s) in a week are significantly different at $p<0.05$. Asterisk (*) denotes significant difference at $p<0.05$ in reference to week 0 (baseline).

3.2. Effect of subacute toxicity testing on body weight

Results show that the subacute oral administration of duhat fruit extracts did not induce any negative effect on the body weights of all experimental mice. As seen in Figure 3, the mean body weights of male and female mice fed with duhat are comparable with their corresponding control groups. Generally, male mice had significantly heavier body weights than their female counterparts, which may be due to their body composition differences brought about by sex [39] and higher feed intake as also observed by other studies [40]. All animals exhibited an increasing trend in body weight throughout the experiment period, with significant changes in comparison to week 0, but not when weekly weights were compared to the week before.

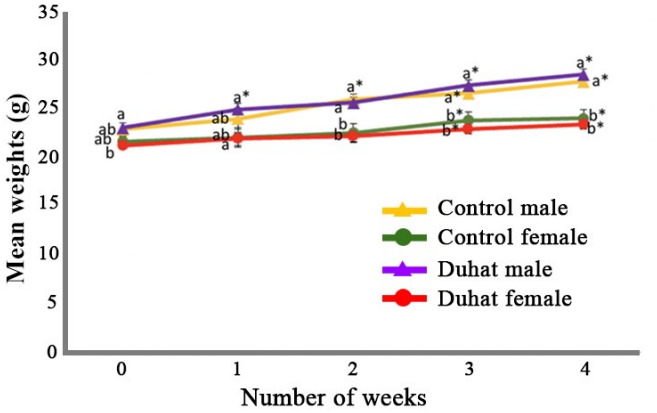

Figure 3. Mean weekly body weights (g) of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Point values with different superscript(s) in a week are significantly different at $p<0.05$. Asterisk (*) denotes significant difference at $p<0.05$ in reference to week 0 (baseline).

Table 1 shows the mean weekly body weight gains and corresponding percent changes against week 0. Notably, there were modest weight gains from all mice groups across time and most of the significant increase in body weights among male and female mice were observed starting at weeks 2 and 3, respectively. Generally, male mice exhibited an earlier increase in body weights than female mice as also observed in a previous study [41]. It is important to note that the weight pattern of male and female mice from the treatment groups follow that of the control groups, suggesting that the administration of duhat extracts has no significant adverse effect on weight. This agrees with the results of related oral toxicity studies on duhat [42-44] and other *Syzygium* species [26,45,46], which showed increased body weight of treated mice, indicating that the berry extracts were, therefore, safe for consumption.

Table 1. Mean weekly body weight gains in percent changes (%) of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kgBW dose.

Treatment Groups	Sex	Weekly change in weight (%)			
		1	2	3	4
Control	M	4.851	13.415*	16.285*	21.518*
	F	1.798	4.109	9.922*	10.887*
Duhat	M	8.055*	11.110*	18.836*	23.680*
	F	3.605	4.533	8.198*	10.391*

**Significantly different at p<0.05 in reference to week 0 (baseline)*

3.3. Effect of subacute toxicity testing on blood chemistry

The means and percent changes in alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (CREA) levels of mice are presented in Figure 4. Regardless of sex and group, no significant differences in the ALT, BUN, and CREA values were observed throughout the experimentation period (day 1 vs day 28). No significant difference was also noted between male and female mice within groups and within same sex of different groups.

Although relatively higher blood chemical values were observed, the changes were insignificant, and all values were still within the normal range [47], indicating that the subacute oral administration of duhat fruit extracts is not liver or kidney toxic in treated animals.

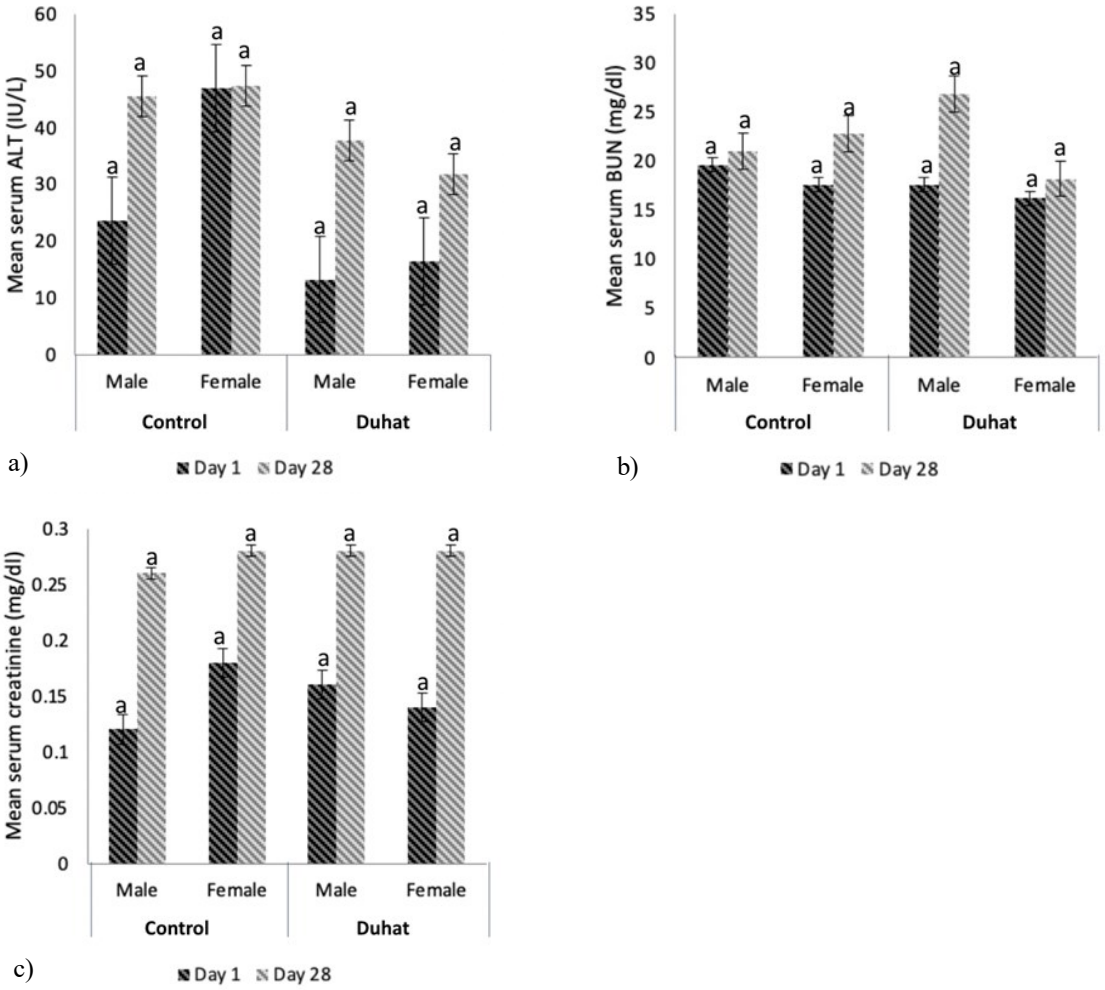

Figure 4. Subacute toxicity testing on blood chemistry, a) serum ALT, b) serum BUN, and c) serum creatinine levels of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Bars with different superscript(s) are significantly different at $p<0.05$. Bars with asterisk (*) denotes significant difference at $p<0.05$ in reference to week 0 (baseline).

3.4. Effect of subacute toxicity testing on hematology

Results showed no significant differences in all hematological parameters between opposite sex within each group and between same sex of treatment and control groups (Figures 5). All changes in hematology parameters from day 1 to day 28 were also statistically insignificant except for the tRBC counts of female mice fed with duhat (DF), which exhibited a significant increase. Generally, all mice groups exhibited an increased tWBC, GRA, and tRBC levels except for CF which exhibited a decrease for WBC and RBC parameters. Meanwhile, mice fed with duhat exhibited an increase in LYM counts while control groups exhibited otherwise. For MON count, all male groups exhibited an increase while all female groups showed a decrease. These MON values were considered elevated when compared with the published normal ranges; however, these changes are non -significant when compared with their respective baseline and control values. Meanwhile, all changes in WBC, LYM, GRA, and RBC values were still within the normal published values [45]. These results suggest that administration of duhat extracts had no adverse effects in the hematological parameters in mice.

3.5. Effect of subacute toxicity testing on morbidity and mortality rates

Assessment of the morbidity and mortality rates through the subacute toxicity study provides the possible harmful effects of the measured variables. In this study, no morbidities nor mortalities were observed among all treatment groups throughout the experimentation period. No changes in physical appearance, somatomotor, and behavior were also observed in all treatment groups. This agrees with the results of similar acute and 28-day subacute toxicity studies on duhat and other *Syzygium* species [26,42,48,49]. With this, the estimated median lethal dose (LD50) of duhat fruit extract is greater than 2000 mg/kg BW as zero mortality was observed.

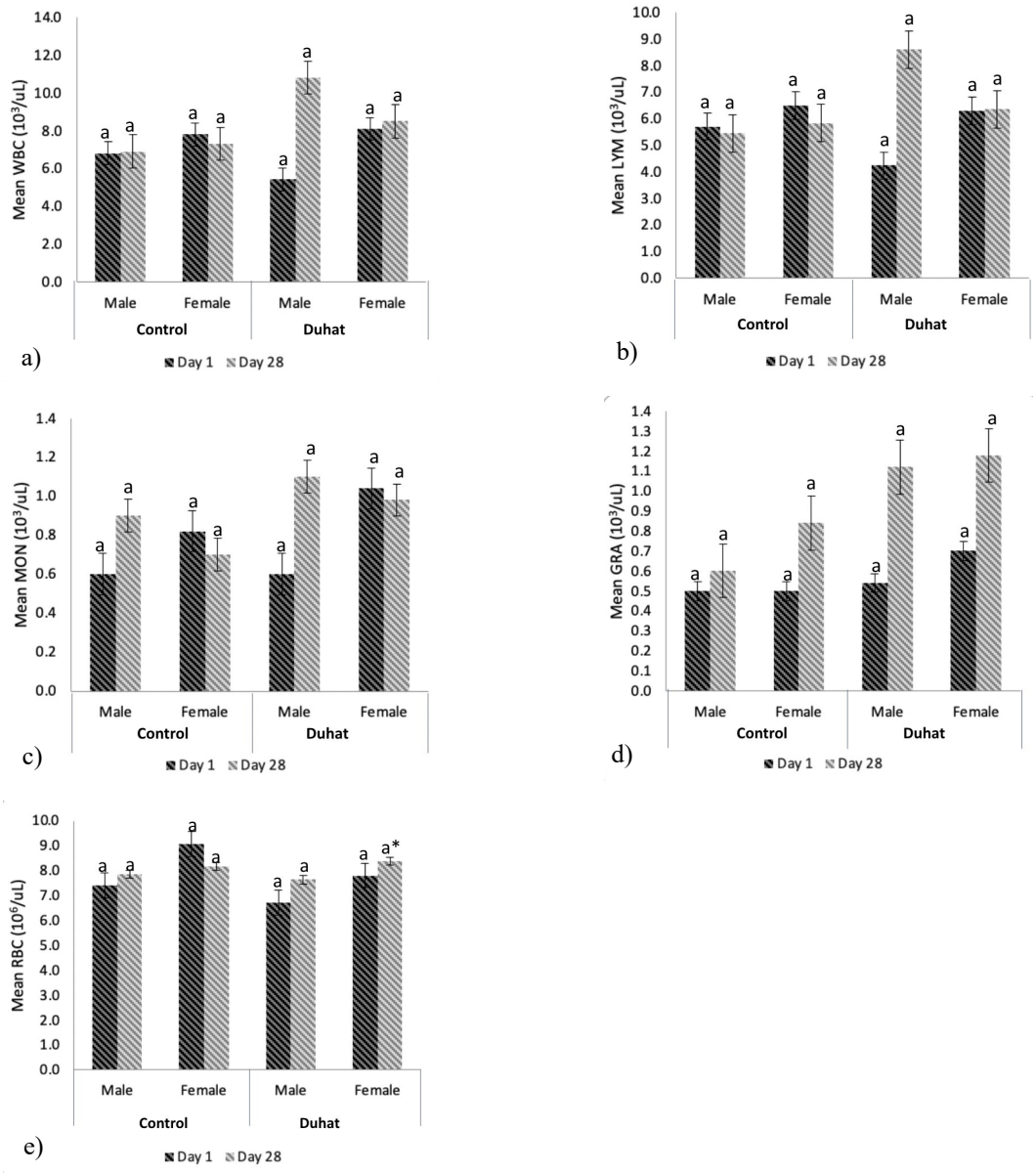

Figure 5. Subacute toxicity testing on hematology, a) WBC, b) LYM, c) MON, d) GRA, and e) RBC counts of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Bars with different superscript(s) are significantly different at $p<0.05$. Bars with asterisk (*) denotes significant difference at $p<0.05$ in reference to week 0 (baseline).

3.6. Effect of subacute toxicity testing on relative organ weights, and macroscopic and microscopic organ appearance

All relative organ weights of male and female mice fed with duhat were comparable with their corresponding control groups (Figure 6). Moreover, no significant differences on the relative weights of brain, heart, and small and large intestines were also observed between opposite sex within groups. Notably, DF had significantly heavier stomach (0.954 ± 0.080) and spleen (0.318 ± 0.026) than DM (0.681 ± 0.024 and 0.230 ± 0.010, respectively), while CF had significantly heavier spleen (0.362 ± 0.036) and lungs (0.652 ± 0.053) than their male counterparts (0.229 ± 0.009 and 0.489 ± 0.032, respectively). Moreover, significantly heavier liver and right and left kidneys were observed among DM (5.919 ± 0.162, 0.980 ± 0.050, and 0.949 ± 0.016, respectively) than DF. Control males also had significantly heavier stomach (0.921 ± 0.064) and large intestines (2.358 ± 0.135) than DM (0.681 ± 0.024 and 1.71 ± 0.143, respectively).

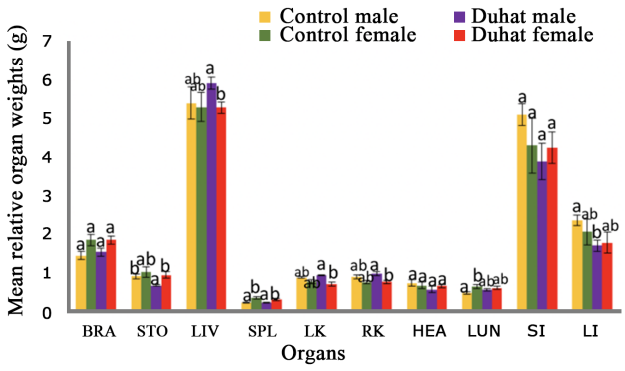

Figure 6. Mean relative organ weights (g) of male and female ICR mice given with distilled water and duhat fruit extract at 2000 mg/kg BW dose. Error bars represent standard errors. Bars with different superscript (s) are significantly different at $p<0.05$. Bars with asterisk (*) denotes significant difference at $p<0.05$ in reference to week 0 (baseline).

Generally, female mice had a relatively heavier brain, esophagus and stomach, spleen, and lungs while male mice had heavier liver and kidneys. Female mice from duhat groups also exhibited heavier small and large intestines than DM, while the opposite was observed among those of control groups. With CM exhibiting heavier small and large intestines than CF. Heavier heart weight, however, was observed among CM and DF compared to their counterparts. This concurs with previous studies in which heavier brain weight was observed among females [50] while heavier liver [51] and heart [52] were recorded among males.

Figure 7 shows the organs of representative ICR mice from different treatment groups. All organs appear normal in size, shape, and color.

No gross or microscopic lesions on individual organs were observed in all mice from all groups (Figure 8). All treatment groups did not show any significant difference from each other.

The lungs had normal cellular structures of bronchiole, alveoli, alveolar duct, and blood vessels while the liver had similar cellular structures of hepatocytes, sinusoids, and central vein with the control group. Normal cardiac muscle cells structures and glomerular architectures were observed in the heart and kidneys, respectively. Normal brain, small and large intestines, spleen, and stomach structures and histology were also observed. These suggest that duhat administration for 28 days at 2000 mg/kg dose did not induce any adverse alterations on the morphology of mice organs.

3.7. Human dose translation

Based on the guidelines of the US Department of Health and Human Services- Center for Drug Evaluation and Research [35], the 2000 mg/kg animal dose in human equivalent dose is 162.16 mg/kg body weight.

4. Conclusions

The subacute toxicity study showed that all groups had increased body weights and feed intakes, with male groups showing higher gains in both variables than their female counterparts. All groups also exhibited normal water intakes, with zero morbidity and mortality. No significant changes in blood chemistry and hematology parameters across all groups and sexes were also observed, except for a significant increase in tRBC count of DF group. Furthermore, no significant differences on the relative organ weights among same sex was observed except for the heavier stomach and large intestines among CM than DM. Comparing opposite sex, heavier stomach, spleen, and lungs were observed among female mice than their male counterparts while significantly heavier liver and right and left kidneys were observed among males than females. No gross and microscopic abnormalities of all organs were also examined. Therefore, these results suggest that aqueous extracts of duhat (*Syzygium cumini*) fruits did not induce toxic effects, estimated LD50 value greater than 2000 mg/kg in mice and HED of 162.16 mg/kg (26 grams fresh intact berries).

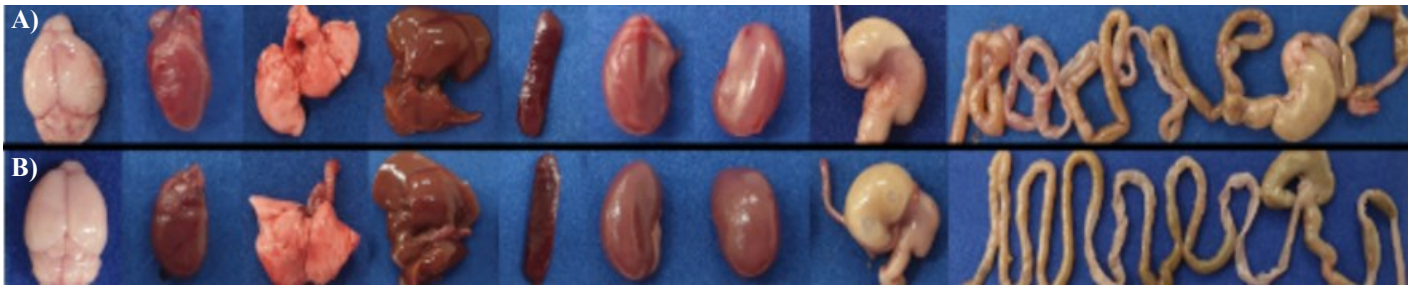

Figure 7. Organs of representative ICR mice given with (A) distilled water and (B) duhat fruit extract at 2000 mg/kg BW dose (Left to right: brain, heart, lungs, liver, spleen, right kidney, left kidney, stomach, small and large intestines).

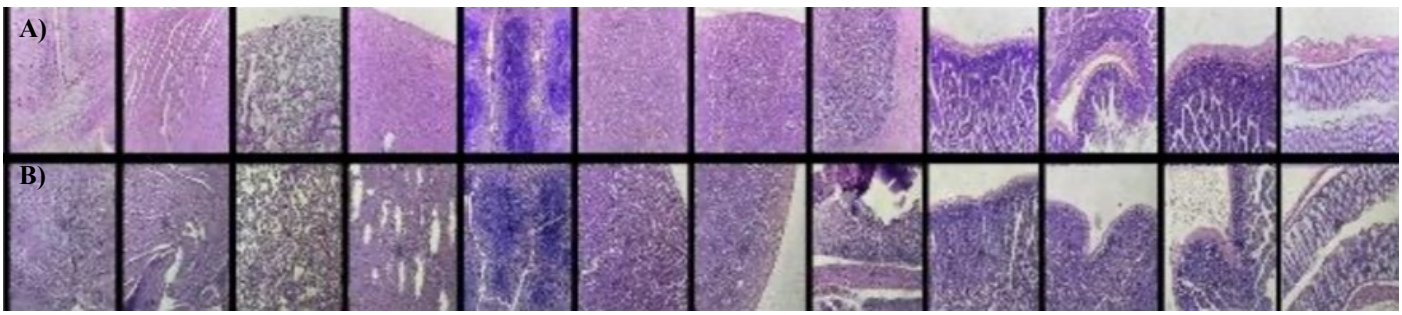

Figure 8. Representative photograph (10x, stained with Hematoxylin and Eosin) of organs from each group of ICR mice given with A) distilled water and B) duhat fruit extract at 2000 mg/kg BW dose (Left to right: brain, heart, lungs, liver, spleen, right kidney, left kidney, stomach, small and large intestines).

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