Pharmacogn. Res., 2023; 15(3):504-513.
https://www.phcogres.com

Original Article

Subacute Oral Toxicity Evaluation of Freeze-Dried Fruits of Lipote (*Syzygium polycephaloides* (C.B. Rob.) Merr.) in ICR Mice

Ann C. Cayetano[1], Liezl M. Atienza[1,*], Maria Amelita C. Estacio[2], Katherine Ann T. Castillo-Israel[3], Mark Joseph Desamero[2], Roxanne P. Gapasin[2], Jonna C. Maniwang[2], Rohani C. Navarro[4], Dianne Jane A. Sunico[1,5], James Ryan D. Aranzado[1], Loraine C. Bainto-Ancheta[3], Joan I. Delomen[1], Jonina Marie J. Tengco[1]

[1]Institute of Human Nutrition and Food, College of Human Ecology, University of the Philippines Los Baños (UPLB), Laguna, PHILIPPINES.
[2]Department of Basic Veterinary Sciences, College of Veterinary Medicine, UPLB, Laguna, PHILIPPINES.
[3]Institute of Food Science and Technology, College of Agriculture and Food Science, UPLB, Laguna, PHILIPPINES.
[4]Institute of Molecular Biology and Biotechnology, National Institute of Health, University of the Philippines Manila, Manila, PHILIPPINES.
[5]Department of Science and Technology-Science Education Institute DOST Compound, Bicutan, Taguig City, PHILIPPINES.

ABSTRACT

Background: Lipote (*Syzygium polycephaloides* (C.B. Rob.) Merr.) is an indigenous fruit in the Philippines with potential functions against obesity and non-communicable diseases, but limited literature was available on its safety. **Objectives:** This study investigated the subacute toxicity effects of freeze-dried fruits of lipote (*Syzygium polycephaloides* (C.B. Rob.) Merr.) on ICR mice. **Materials and Methods:** Ten male and ten female 6-week-old ICR mice were divided into two groups: (1) control (vehicle), and (2) lipote group, given with 2000 mg/kg body weight (BW) dose of freeze-dried lipote fruit powder reconstituted in distilled water. **Results:** After 28 days of oral gavage, the lipote group showed no significant changes on feed and water intake, and hematology and blood chemistry parameters were comparable with those of control group and published normal values. Body weights of all experimental animals also increased significantly ($p<0.05$), and no mortality, morbidity, or gross and microscopic morphological abnormalities of internal organs were noted. **Conclusion:** These results showed that 28-day oral consumption of freeze-dried lipote fruits is safe and has LD_{50} >2000 mg/kg.

Keywords: Indigenous berries, Lipote, Subacute, *Syzygium*.

Correspondence:
Prof. Liezl M. Atienza, RND, PhD
Institute of Human Nutrition and Food, College of Human Ecology, University of the Philippines Los Baños (UPLB), Laguna 4031, PHILIPPINES.
Email: lmatienza@up.edu.ph

Received: 06-02-2023;
Revised: 19-03-2023;
Accepted: 23-04-2023.

INTRODUCTION

Lipote is a member of the Myrtaceae family and is known in various scientific names including *Syzygium polycephaloides, Eugenia polycephaloides*, and *Syzygium curranii*.[1] It is believed to be native in South and Southeast Asian countries, including the Philippines, and is considered an endangered species in Sulawesi according to the Ecosystems Research and Development Bureau of the Department of Environment and Natural Resources.[2] In the Philippines, lipote trees are found in Bicol, Laguna, Samar, and Quezon and can be propagated by seeds or through grafting. Its trees usually reach about 15 m in height and 30 cm in diameter and have angular twigs, and alternate, oblong leaves and usually flowers during December and fruits can be harvested around July. They usually grow at low and medium altitudes and in forested areas and bears within 4 to 5 years.[1]

The Food and Fertilizer Technology Center of Taiwan described the lipote tree with glossy green leaves and white flowers which turn into round, purple fruit clusters.[3] The naturally sweet fruits are usually eaten fresh or are cooked into preserves such as jams and jellies or turned into beverages such as wines. The fruit is also used as an ingredient in making cakes while the tree itself is also used as an ornamental plant due to its brightly colored fruits. It is also used as a shade tree due to the leaves' resistance to moth larvae and as a reforestation species due to its economic and ecological contributions. Lipote trees also do not require much agricultural inputs as they are seldom attacked by pests and diseases, therefore limiting the amounts of chemical insecticides and pesticides being used.[3]

In 2011, Coronel studied the nutritional content of lipote fruit and found that it contains rich amounts of carbohydrates and water, as well as vitamins A, B1, B2, B3, and C, and essential minerals such as calcium, phosphorus, and iron.[2] Numerous

DOI: 10.5530/pres.15.3.053

studies have also showed the anti-microbial, anti-cholesterol, anti-obesity, anti-diabetic, anti-inflammatory, and anti-tumor properties of lipote;[4-9] however, literature investigating the safety of Philippine grown lipote fruits for consumption is not well-established. Therefore, this study evaluated the subacute oral toxicity potential of freeze-dried fruits of lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.).

With lipote's high nutritional value and minimal agricultural inputs, findings of this study can help promote the use and utilization, as well as development of practical and sustainable processes for local berries, particularly lipote, and contribute to the achievement of the second 2030 Sustainable Development Goals (SDG), "end hunger, achieve food security and improved nutrition and promote sustainable agriculture".[10] It can also aid in the fight against obesity and non-communicable diseases by developing functional food products for both preventive and therapeutic purposes, supporting the attainment of the third SDG, which is to "ensure the healthy lives and promote well-being for all at all ages".[10]

MATERIALS AND METHODS

Plant Collection and Extract Preparation

Fully riped lipote fruits were harvested from the province of Laguna and were confirmed for authenticity by the Botanical Herbarium, Museum of Natural History, University of the Philippines - Los Baños (UPLB). Fruits were then pulped at room temperature and freeze-dried at 20°C and 40 mTorr pressure using VirTis Co. (Gardiner, NY) at the Institute of Food Science and Technology, UPLB. The freeze-dried lipote fruits were then powdered, passed through an 80-mesh US standard sieve, and stored in metallized bags at -20°C until use.

The toxicological potential of the sample was evaluated via *in vivo* subacute oral toxicity study. All procedures were approved by the UPLB Animal Care and Use Committee with assigned protocol number: CHE-2019-002.

Experimental Animals

Ten (10) each of male and female 6-week-old ICR mice were obtained from Laboratory Animal Facility, Research of the Institute for Tropical Medicine (RITM), Department of Health, Alabang, Muntinlupa City, Philippines. Because of its rapid growth rate and high productivity, this albino, outbred mouse strain named after the Institute of Cancer Research (ICR) USA is utilized as a general-purpose model for a wide range of research, including toxicity.[11] To investigate sex differences in toxicity sensitivity, male and female mice were used.[12]

Acclimation

Acclimation was conducted a week prior to actual experiment to ensure the use of healthy animals and allow them to adjust to the new environment. Individual mice body weights were recorded, and detailed physical examinations were performed. Individual standard polycarbonate cages with stainless steel tops were utilized to house the animals, which were kept in a controlled environment with a 12-hr light-dark cycle, a temperature of 22-24°C, and a relative humidity of 30-60%. Every week, the cages and beddings were changed. All experimental mice were also given a maintenance mouse pellet diet (Altromin, Germany) and free access to distilled water.

Subacute Oral Toxicity Testing

The Organization for Economic Cooperation and Development (OECD) guideline 407 for testing chemicals was followed in conducting the subacute 28-day oral toxicity investigation. The experiment was carried out in the Laboratory Animal Room of the UPLB College of Veterinary Medicine's Department of Basic Veterinary Sciences.

The experimental animals were randomly assigned into two (2) groups: (1) control; (2) lipote (2000 mg/kg BW), with each group containing 5 males and 5 females. Blood samples were collected from all experimental animals via the retro-orbital sinus after acclimatization and an overnight fasting, and blood chemical and hematological tests were performed.

The animals in the treatment group were given freshly prepared freeze-dried berries reconstituted with distilled water every day for 28 days via oral gavage at a dose of 2000 mg/kg BW. The control group, on the other hand, received only the vehicle or distilled water by oral gavage. These were administered using a 1-inch 22G stainless steel gavage needle (Thermoscientific, USA) and 1 ml sterile disposable syringe (Terumo, Japan). Food and water were withheld an hour before and after administration.

For the first 72 hr, all animals were monitored for signs of regurgitation or poisoning, such as salivation, diarrhea, convulsions, tremors, lethargy, or alterations in the skin, hair, eyes, mucous membranes, circulatory, central, respiratory, and autonomic nervous systems, among others. Feed and water intakes, body weights, signs of toxicity and morbidity, and mortality were documented daily for 28 days. All mice were anesthetized on the 29th day with a 20 mg/kg BW IP injection of tiletamine-zolazepam (Zoletil, Virbac Phils. Inc.). Blood samples were collected through cardiac puncture and placed in heparinized and EDTA-containing tubes for hematological and blood chemistry analyses.

Measurement of Feed and Water Intake

The intake of pre-measured commercial mouse pellets and distilled water was measured daily for each mouse in each treatment group. For 28 days, residual pellets and water were measured daily with a digital top loading balance (Shimadzu, Japan) and a graduated cylinder, respectively.

Measurement of Body Weight

A digital top loading balance was used to weigh all animals daily, with weights recorded to the closest 0.001 gram (Shimadzu, Japan). The percent weight gains were calculated using the formula:[13]

$$\%\ Weight\ gain = \frac{final\ weight - initial\ weight}{initial\ weight}\ x\ 100 \quad (1)$$

Morbidity and Mortality

Morbidity and mortality rates for all treatment groups were recorded and computed.

Morbidity rate per group:

$$\%\ Morbidity = \frac{Total\ number\ of\ mice\ that\ showed\ toxicity\ signs\ per\ group}{Total\ number\ of\ mice\ per\ group}\ x\ 100 \quad (2)$$

Mortality rate per group:

$$\%\ Mortality = \frac{Total\ number\ of\ mice\ that\ died\ per\ group}{Total\ number\ of\ mice\ per\ group}\ x\ 100 \quad (3)$$

Hematology and Blood Chemistry Analysis

Prior to blood collection, a drop of anesthetic tetracaine (Alcaine®, Novartis, Philippines) was applied in the right eye. After two minutes, a heparinized capillary tube (INRI, Netherlands) was used to draw blood from the retro-orbital vein. At Day 1 and Day 28, 300 microliters (300 µl) of blood were collected from each mouse in each treatment group prior to gavage administration.

Hematology parameters including total red (RBC) and white blood cell (WBC), and differential lymphocyte (LYM), monocyte (MON), and granulocyte (GRA) counts were measured using ten microliters (10 µl) of blood and an automated hematology analyzer (Orphée, Switzerland), while serum alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine (CREA) levels were analyzed using 250 µl of blood and an automated blood chemistry analyzer (Arkray Inc, Japan). All analyses were also performed at the College of Veterinary Medicine, UPLB.

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

All mice in each treatment group were euthanized via intraperitoneal injection of 60 mg/kg sodium pentobarbital (Dolethal®, UK) after the 28-day experimentation period. The brain, lungs, heart, esophagus, stomach, small and large intestines, kidneys, liver, and spleen were all exteriorized by a midventral incision at the thoraco-abdominal area. All organs were examined for gross abnormalities and flushed with 0.9% sodium chloride solution. The organs were then weighed using a digital top loading balance (Shimadzu, Japan) and relative organ weights were then computed as follows:

$$\%\ Relative\ organ\ weight = \frac{Organ\ weight}{Mice\ body\ weight}\ x\ 100 \quad (4)$$

After weighing, the organs were trimmed and fixed for at least 72 hrs in 10% buffered formalin, then processed using the paraffin technique, and sectioned at 4 µm thickness using a rotary microtome. For histopathologic examination, one out of every four sections of each organ were collected and stained with Hematoxylin and Eosin (H&E) stain and viewed under light microscopy (Zeiss Primostar). Histopathologic changes such as presence or absence of cellular inflammatory, healing processes, neoplasia, degenerative and proliferative responses, and other changes were noted and photographed using a digital camera. Semi-quantitative scoring was applied when histopathological lesions were present, then histopathological analysis and interpretation was conducted by a veterinary pathologist.

Translation of Animal Dose to Human Equivalent Dose (HED)

The human equivalent dose (HED) was computed using the formula and table from the U.S. Department of Health and Human Services-Center for Drug Evaluation and Research.[14]

$$HED\ \left(\frac{mg}{kg}\right) = animal\ dose\ in\ \frac{mg}{kg}\ x\ \left(\frac{Animal\ Km}{Human\ Km}\right) \quad (5)$$

Data Processing and Analysis

All analyses were performed in triplicates. Results were expressed as means ± standard error of the mean (SEM) and were analyzed using IBM Statistical Package for the Social Sciences (IBM SPSS v. 20). Independent *t*-tests were applied for differences between different sex within a group and between same sex from different groups at $p<0.05$. Meanwhile, paired sample t-test was performed to determine differences before and after treatment within a group.

RESULTS

Effect of Subacute Toxicity Testing on Feed and Water Intakes

Based on the results (Figure 1), the subacute administration of reconstituted freeze-dried lipote fruits at a dose of 2000 mg/kg BW did not have an impact on the feed consumption of male and female ICR mice. All mice fed with lipote had comparable feed intakes with their respective control counterparts. Moreover, despite the significantly lower feed intakes of lipote-fed male mice during the first two weeks of the experiment, an increasing trend was still observed throughout the study period. All experimental animals also had normal feed intakes of at least 4-5 g a day[15] suggesting that reconstituted freeze-dried lipote fruit powder did not induce any negative effect on feed intake.

In terms of water intake, Figure 2 shows that all mice exhibited normal water intakes of at least 3-5 ml per day[15] indicating that the reconstituted freeze-dried lipote fruit powder had no adverse effects on water consumption. It is important to note, however, that in reference to acclimation period (week 0), except for control

females, significantly lower water intakes were observed among all groups starting at week 1. Also at week 1, it can be observed that male and female mice in the control group had significantly higher water intake than lipote-fed mice of corresponding sex. These observations may be due to the additional water and fiber present in the gavage solution. Nevertheless, all experimental animals showed an increasing water intake throughout the experimentation period and that lipote-fed mice exhibited comparable water intakes with those of control.

Effect of Subacute Toxicity Testing on Body Weight

Figure 3 shows that all mice groups had increasing body weights throughout the experimentation period, with lipote-fed male and female mice having comparable body weights with their corresponding control groups. Compared to acclimation period (week 0), significantly higher body weights were recorded starting at week 2 for all mice groups except among control females, which started at week 3. As seen in Table 1, significant changes in the body weight of all mice groups were also observed starting at week 2, with notable similarities in weight gain patterns of lipote and control mice.

Comparing opposite sexes, male mice were significantly heavier than females at weeks 2-4, which may be attributed to the observed higher feed intakes among males, as well as sex-related differences in body composition.[16] Relatively higher water intakes among female mice may also cause early satiety thus lower feed intake, leading to lower weight gain.

On the other hand, mice of the same sex did not differ significantly between groups. These results indicate that the reconstituted freeze-dried lipote powder did not induce any adverse effect on body weight and concur with the results of related oral toxicity studies on lipote[17] and other *Syzygium* species.[18]

Effect of Subacute Toxicity Testing on Blood Chemistry and Hematology

In terms of blood chemistry, results showed no significant differences in the serum ALT, BUN, and CREA levels of all mice, regardless of sex and treatment group (Figure 4). No significant changes between baseline and endline values of these blood chemical parameters were also found. While higher endline values were observed, these were within the published normal ranges,[19] indicating that lipote did not induce negative effects on the liver and kidneys.

Similarly, comparing baseline and endline values, no significant changes in the mean GRA, LYM, MON, RBC, and WBC counts were observed (Figure 5). No significant differences in all hematological parameters were also noted between male and female mice within groups and between same sex from treatment and control groups. It is good to note, however, that male mice from control and lipote groups exhibited increased MON values while female counterparts exhibited otherwise. These increased endline MON values among male mice are considered elevated than normal ranges; nevertheless, these changes are insignificant when compared with their respective baseline values. Meanwhile, all changes in GRA, LYM, RBC, and WBC values were within the

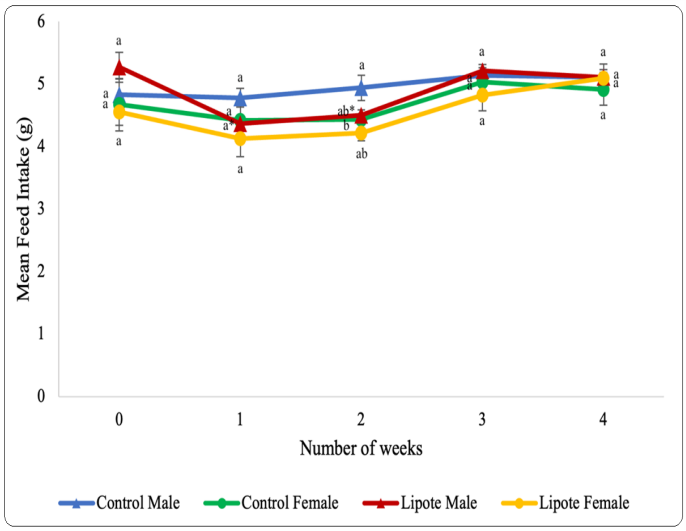

Figure 1: Mean weekly feed intake (g) of male and female ICR mice given with distilled water and 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Means in the same column with different superscript(s) are significantly different at $p<0.05$; Means with asterisk (*) denotes significant difference at $p<0.05$ compared to Week 0. Comparable feed intakes between control and lipote groups.

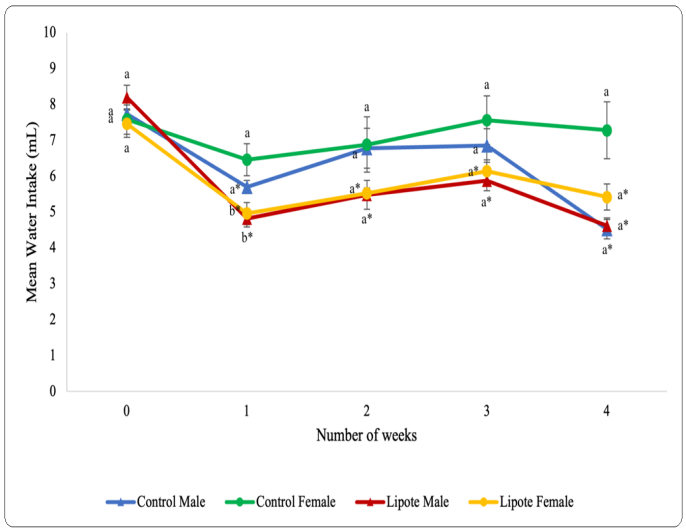

Figure 2: Mean weekly water intake (ml) of male and female ICR mice given with distilled water and 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Means in the same column with different superscript(s) are significantly different at $p<0.05$; Means with asterisk (*) denotes significant difference at $p<0.05$ compared to Week 0. All mice groups exhibited normal water intakes of at least 3-5 ml per day.

Table 1: Mean weekly body weight gains (g) and percent changes (%) of male and female ICR mice given with distilled water, and 2000 mg/kg BW dose of freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruit extract. All mice groups had increasing body weight gain throughout the experimentation period.

Treatment groups		Wk 0 Vs. Wk 1		Wk 0 Vs. Wk 2		Wk 0 Vs. Wk 3		Wk 0 Vs. Wk 4	
		Mean BWG[a] (g)	% change	Mean BWG (g)	% change	Mean BWG (g)	% change	Mean BWG (g)	% change
	Male	1.110	4.851	3.069	13.415[b]	3.726	16.285[b]	4.923	21.518[b]
Control									
	Female	0.390	1.798	0.890	4.109	2.149	9.922[b]	2.358	10.887[b]
	Male	0.151	0.632	1.474	6.185[b]	2.826	11.860[b]	3.683	15.455[b]
Lipote									
	Female	0.497	2.394	1.591	7.663[b]	2.277	10.969[b]	3.536	17.032[b]

[a] BWG: body weight gain; [b]with significant difference at $p<0.05$ compared to Week 0.

published normal ranges,[19] suggesting no adverse effects in the hematological parameters in mice.

Effect of Subacute Toxicity Testing on Morbidity and Mortality Rates

Throughout the experimentation period, no morbidity nor mortality due to possible toxicity was recorded. All mice did not produce toxicity-related physical, behavior, and somatomotor changes, indicating that subacute administration of reconstituted freeze-dried lipote fruits was not harmful to mice. This is consistent with the findings of a study conducted by Estacio and colleagues (2020) on acute toxicity of lipote.[17]

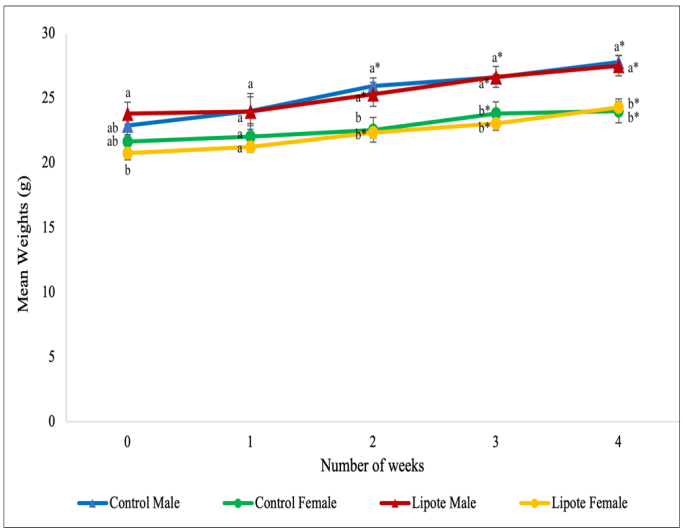

Figure 3: Mean weekly body weights (g) of male and female ICR mice given with distilled waterand 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Means in the same column with different superscript(s) are significantly different at $p<0.05$; Means with asterisk (*) denotes significant difference at $p<0.05$ compared to Week 0. All mice groups had increasing body weight gain throughout the experimentation period, with significant changes starting at Week 2.

Effect of Subacute Toxicity Testing on Relative Organ Weights, and Macroscopic and Microscopic Organ Appearance

Figure 6 shows that regardless of sex and treatment group, no significant differences in relative weights of the brain and small and large intestines were observed. On the other hand, comparing male and female mice within groups, lipote-fed female mice had significantly heavier stomach (1.05 ± 0.22g) than males (0.72 ± 0.06 g) while female mice from control group had significantly heavier lungs (0.65 ± 0.12 g) than control males (0.49 ± 0.07). Female mice from control and lipote groups also had significantly heavier spleen (0.36 ± 0.08 g and 0.34 ± 0.05 g, respectively) than male counterparts (0.23 ± 0.21 g and 0.25 ± 0.02 g, respectively) while lipote-fed male mice had significantly heavier liver (5.72 ± 0.52g), left (0.91 ± 0.033g) and right kidneys (0.98 ± 0.04g), 300 and heart (0.71 ± 0.08g) than female counterparts (4.97± 0.25g, 0.73 ± 0.08g, 0.72 ± 0.06g, 0.58 ± 0.07g, respectively). These observations concur with other studies showing heavier liver and heart among males than female.[20,21] Meanwhile, comparing same sex from treatment and control groups, males from control group had heavier stomach (0.92 ± 0.14g) than lipote-fed male mice (0.72 ± 0.06g), but the latter had significantly heavier lungs (0.59 ± 0.03g) than the former (0.49 ± 0.07g).

Despite the differences in relative organ weight, no significant differences in the cellular structures were observed; all organs of male and female mice from control and lipote groups had normal color, shape, and size, and without gross (Figure 7) or microscopic lesions (Figure 8). Specifically, the brain, intestines, spleen, and stomach appeared in normal structures and histology while normal structures of the alveoli, alveolar duct, bronchioles, and blood vessels in the lungs were observed. Moreover, all mice had normal cardiac muscle, glomerular architectures, and hepatocyte, sinusoids, and central vein structures in the liver. These suggest that subacute administration of reconstituted freeze-dried lipote

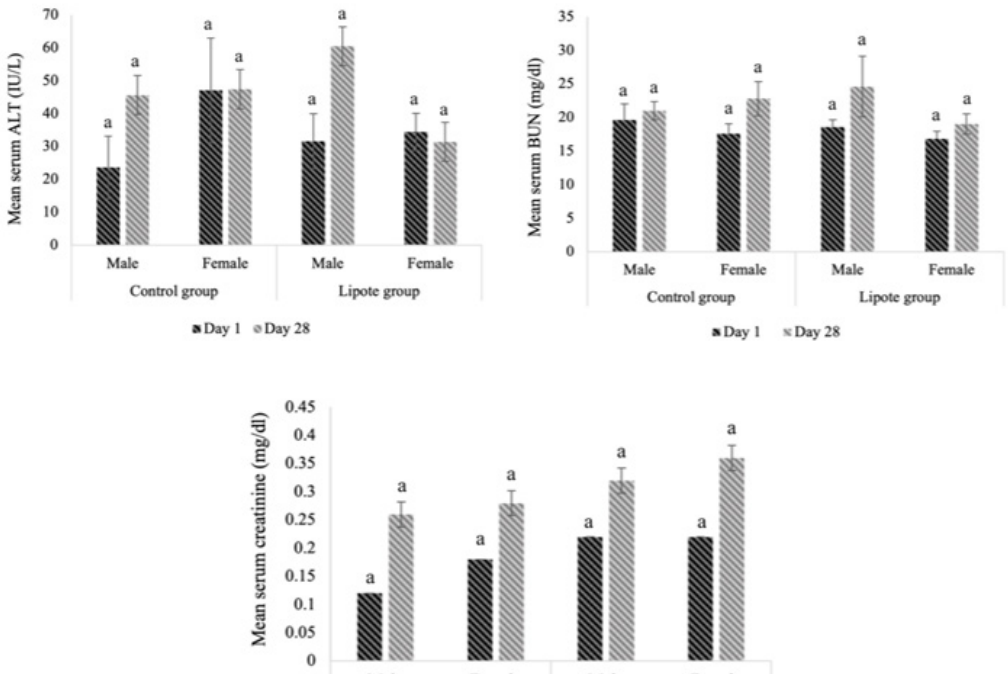

Figure 4: Mean Alanine Transaminase (ALT), Blood Urea Nitrogen (BUN), and serum creatinine levels of male and female ICR mice given with distilled water and 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Error bars denote mean ± SEM. Bars with different letter(s) denote significant difference at $p<0.05$(independent and paired t-test). No significant changes were observed between baseline and endline ALT, BUN, and CREA levels.

fruit did not produce any negative alterations on the organ morphology of mice.

Human Dose Translation

Translating the 2000 mg/kg animal dose to human equivalent dose using the established formula by the US Department of Health and Human Services-Center for Drug Evaluation and Research,[14] this study shows that 162.16 mg/kg body weight (BW) freeze-dried lipote fruit is safe for consumption for a typical 60 kg adult.

DISCUSSION AND CONCLUSION

In the present study, all male and female mice from control and lipote groups continued to gain weight throughout the experiment period, accompanied by an increase in feed intake. These results suggest that subacute administration of freeze-dried lipote fruit did not affect the animals' normal growth pattern as previous studies showed that experimental animals exposed to potentially toxic substances had reduced body weight gains.[22,23]

In most animal experiments, male mice are usually used than females due to the possible effects of hormones that are highly expressed in the latter.[24] However, recent studies showed that the difference between male or female mice is not significant.[25] Female rodents are also found to be more sensitive to toxic substances and their pathological changes can be easily revealed; therefore, making them suitable for toxicity studies.[26] In this study, male and female mice are subjected to oral toxicity testing and it was observed that females have relatively lower feed intakes and body weight gains than males. This may be due to a higher estrogen expression among females, which decreases energy intake and increases energy expenditure[27] as female mice were observed to be more active than males[28,29] due to estrogen-mediated dopamine release.[30,31] Weight gain may also be influenced by age, sex steroid hormones, hepatic lipid metabolism, and systemic metabolism.[32] Numerous reports showed that males show greater changes during diet or genetic manipulation and that adipose tissue distribution between male and female mice greatly differs due to their sex hormones and metabolism.[33]

The study also tested biochemical parameters, particularly of liver and kidneys since these organs are found to be very sensitive to toxic substances.[34] The parameters include the enzymes serum ALT, BUN, and CREA, which indicate a high degree of organ injury severity and are used for kidney and liver function tests.[35] In the present study, results showed no significant d ifferences in all biochemical parameters of treatment groups compared to the control. Although there is an increase or decrease in the level of biochemical parameters, all values are still within the normal range and the changes are not significant.

The results on biochemical parameters were congruent with the results on relative organ weights and gross and microscopic organ appearances. Relative organ weight was used in the study since

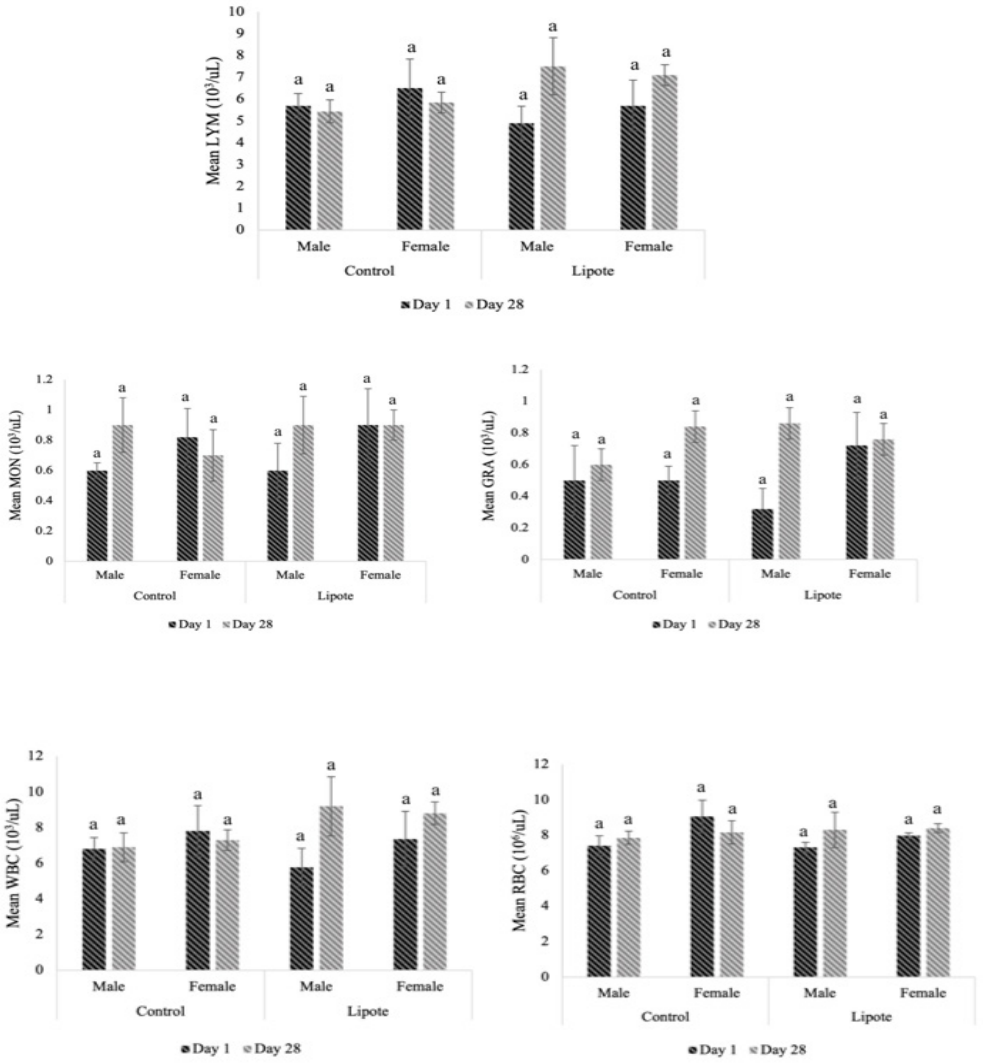

Figure 5: Mean white blood cell (WBC), lymphocyte (LYM), monocyte (MON), granulocyte (GRA), and red blood cell (RBC) counts of male and female ICR mice given with distilled water and 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Error bars denote mean ± SEM. Bars with different letter(s) denote significant difference at $p<0.05$ (independent and paired t-test). No significant changes were observed between baseline and endline hematology values.

it was reported to be more indicative of toxicity than absolute organ weight.[36] The present study observed no significant differences in relative organ weights of animals from treatment groups when compared with the control. Although a significant difference between some male and female relative organ weights were observed, gross and microscopic organ observations did not reveal any toxic effect.

Assessment of hematological parameters is also important in toxicity studies since potential toxins, or its metabolites can interact and induce significant alterations in the structure and function of target tissues. In this study, the hematological parameters evaluated include leukocytes (white blood cells, lymphocytes, monocytes, and granulocytes), which are essential components of the body's immune response, and erythrocytes (red blood cells), which are important in the transportation of nutrients and oxygen throughout the body. For all hematological parameters, no significant differences between opposite sex within each group and between same sex from each group were observed. No significant change in all parameters was also showed. These results showed that subacute toxicity testing of freeze-dried fruits of lipote did not produce any sign of toxicity at 2000 mg/kg BW dose, which is equivalent to 162.16 mg/kg human dose (26 grams fresh intact berries). The LD_{50} of lipote for subacute toxicity is >2,000 mg/kg BW.

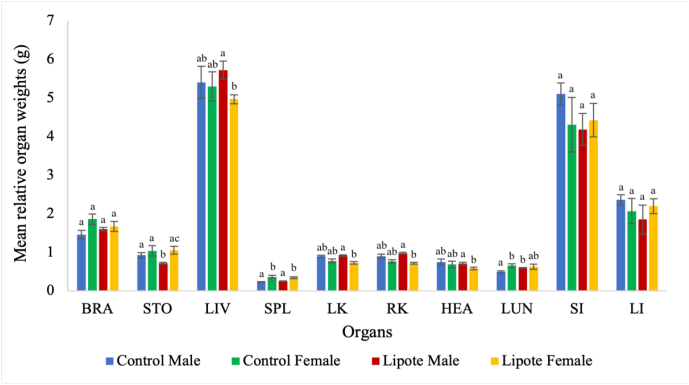

Figure 6: Mean relative organ weights (g) of male and female ICR mice given with distilled water and 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. Error bars denote mean ± SEM. Bars with different letter(s) denote significant difference at $p<0.05$ (independent and paired *t*-test). No significant differences in relative weights of the brain and small and large intestines; heavier spleen among females than males; heavier liver, left and right kidneys, and heart among lipote-fed males than lipote-fed females; and heavier lungs among control females than males.

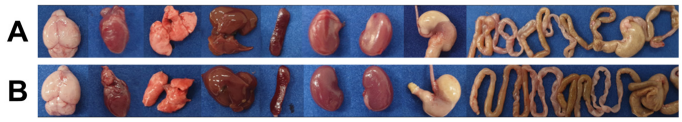

Figure 7: Organs of representative ICR mice given with (A) distilled water and (B) 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. (Left to right: brain, heart, lungs, liver, spleen, right kidney, left kidney, stomach, small and large intestines). All mice from control and lipote groups had normal gross organ morphology.

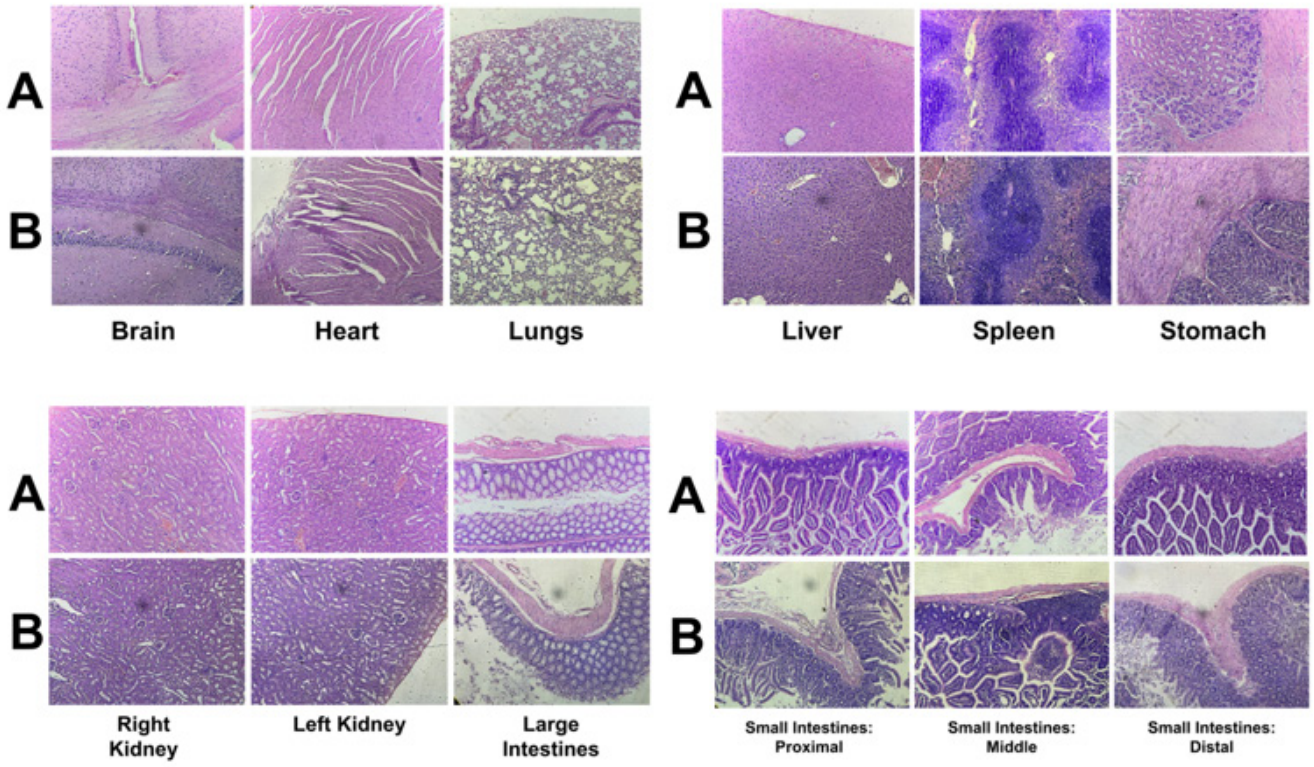

Figure 8: Representative photograph (10x) of organs from each group of ICR mice given with (A) distilled water and (B) 2000 mg/kg BW dose of reconstituted freeze-dried lipote (*Syzygium polycephaloides* (C. B. Rob.) Merr.) fruits. (Left to right: brain, heart, lungs, liver, spleen, stomach, right kidney, left kidney, large intestines, and parts of small intestines). All mice from control and lipote groups had normal organ histopathology.

ACKNOWLEDGEMENT

This study was supported by the Institute of Human Nutrition and Food in collaboration with the Department of Basic Veterinary Sciences and Institute of Food Science and Technology, University of the Philippines, Los Baños. This study was funded by the Philippine Council for Health Research and Development-Department of Science and Technology (PCHRD-DOST) and the Enhanced Creative Work and Research Grant-Office of the Vice Chancellor for Academic Affairs (ECWRG-OVCAA), University of the Philippines.

CONFLICT OF INTEREST

The authors of this study declare no conflict of interest.

FUNDING

This study was funded by the Philippine Council for Health Research and Development-Department of Science and Technology (PCHRD-DOST) and the Enhanced Creative Work and Research Grant-Office of the Vice Chancellor for Academic Affairs (ECWRG-OVCAA), University of the Philippines.

ABBREVIATIONS

BW: Body weight; **SDG:** Sustainable Development Goals; **UPLB:** University of the Philippines - Los Baños; **RITM:** Research of the Institute for Tropical Medicine; **ICR:** Institute of Cancer Research; **OECD:** Organization for Economic Cooperation and Development; **EDTA:** Ethylenediaminetetraacetic acid; **SD:** Standard deviation; **IBM SPSS:** IBM Statistical Package for the Social Sciences; **RBC:** Red blood cell; **WBC:** White blood cell; **LYM:** Lymphocyte; **MON:** Monocyte; **GRA:** Granulocyte; **ALT:** Alanine transaminase; **BUN:** Blood urea nitrogen; **CREA:** Creatinine.

SUMMARY

In the current study, oral safety of freeze-dried lipote fruits was evaluated through a subacute oral toxicity test using ICR mice. Results revealed that mice given with 2000 mg/kg body weight of reconstituted freeze-dried lipote fruits had comparable body weight gain and feed and water intakes with the control group across the 28-day period. Normal levels of biochemical and hematological parameters were also observed after 28 days, and these levels were comparable with those of control group. No toxicity-related morbidity or mortality was also recorded. Relative organ weights, and gross and microscopic morphological observation also revealed normal organ architecture without lesions related to toxicity. These data therefore suggest that LD_{50} of lipote for subacute toxicity is >2,000 mg/kg BW.

REFERENCES

1. Florido HB, Cortiguerra FF. Lesser known edible tree species. Research Information Series on 399 Ecosystems. 2003;15(3):1-8.
2. Ecosystems Research and Development Bureau (ERDB-DENR). Lipote (*Syzigium polycephaloides* [C.B. Rob.] Merr.). Research Information Series on Ecosystems. Vol. 29(2); 2017.
3. Food and Fertilizer Technology Center (FFTC). Fruits and vegetables from the tropical forest [updated]; 2013. cite202Jan16. Available from: http://www.fftc.agnet.org/library.php?func=view&style=type&id=20110913150737.
4. Jemi R, Syafii W, Febrianto F. Hanafi M. Sifat anti Jamur Kayu kupa (*Syzygium polycephalum* (Mig))(antifungal properties of kupa wood (*Syzygium polycephalum* Mig.)). J Ilmu Teknol Kayu Tropis. 2010;8(2):93-108.
5. Baldo LA, Puma EJ. Preliminary assessment of the hypoglycemic effects of anthocyanin-rich *Syzygium curanii* L. Lipote fruit extract on male albino rats (Doctoral dissertation, Undergraduate thesis).
6. Basilan PC, Rosanes KJ. Hypocholesteric and antiobesity effect of anthocyanin-rich *Syzygium curanii* L. lipote fruit extract on male rat fed with a high fat and high cholesterol diet ([doctoral dissertation]. Dasmariñas: De La Salle University).
7. Blanca MA, Cayabyab SM. Antiangiogenic effect in chorioallantoic membrane (Cam) of 10-day old duck embryo of anthocyanin-rich extract from Syzygium Curanii L.(Lipote) fruit ([doctoral dissertation]. Dasmariñas: De La Salle University).
8. Ragasa CY, Torres OB, Shen C-C, Lachica MKEG, Sulit AB, Chua DBDL, *et al*. Triterpenes from the leaves of *Syzygium polycephalum*, *S. cumini*, and *S. samarangense*. Chem Nat Compd. 2014;50(5):942-4. doi: 10.1007/s10600-014-1126-2.
9. Juanda D, Aligita W, Elfahmi HR, Musaad S. Antioxidant and alpha glucosidase inhibition activity of kupa (*Syzygium polychepalum* Miq.) cortex. Int J Pharm Phytopharmacol Res (eIJPPR). 2018;8(3):33-8.
10. United Nations. The 17 goals [cited Jan 16 2021]. Available from: https://sdgs.un.org/goals.
11. Johnson M. Laboratory mice and rats. Mater Methods. 2012;2:(10.13070) doi: 10.13070/mm.en.2.113.
12. Pohjanvirta R, Miettinen H, Sankari S, Hegde N, Lindén J. Unexpected gender difference in sensitivity to the acute toxicity of dioxin in mice. Toxicol Appl Pharmacol. 2012;262(2):167-76. doi: 10.1016/j.taap.2012.04.032, PMID 22564538.
13. Ugwah-Oguejiofor CJ, Okoli CO, Ugwah MO, Umaru ML, Ogbulie CS, Mshelia HE, *et al*. Acute and sub-acute toxicity of aqueous extract of aerial parts of Caralluma dalzielii NE Brown in mice and rats. Heliyon. 2019;5(1):e01179. doi: 10.1016/j.heliyon.2019.e01179, PMID 30775575.
14. US Department of Health and Human Services. Center for Drug Evaluation and Research. Estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. In: Guidance for industry. MD; 2005. p. 1-27.
15. The Johns Hopkins University Animal Care and Use Committee. [homepage on the Internet]. The mouse [cited Mar 7 2021]. Available from: http://web.jhu.edu/animalcare/procedures/mouse.html#general.
16. Reed DR, Bachmanov AA, Tordoff MG. Forty mouse strain survey of body composition. Physiol Behav. 2007;91(5):593-600. doi: 10.1016/j.physbeh.2007.03.026, PMID 17493645.
17. Estacio MA, Atienza L, Gapasin R, Maniwang JR, Aranzado JR, Mercado CJ, *et al*. Acute oral toxicity test of selected Philippine indigenous berries as potential food supplements. Curr Dev Nutr. 2020;4(Supplement_2):684-. doi: 10.1093/cdn/nzaa050_007.
18. Sumiwi SA, Zuhrotun A, Hendriani R, Rizal M, Levita J, Megantara S. Subchronic toxicity of ethanol extract of *Syzygium polyanthum* (Wight) Walp. Leaves on Wistar Rat. Indones Biomed J. 2019;11(1):30-5. doi: 10.18585/inabj.v11i1.458.
19. Serfilippi LM, Stackhouse Pallman DR, Russell B, Spainhour CB. Serum clinical chemistry and hematology reference values in outbred stocks of albino mice from three commonly used vendors and two inbred strains of albino mice. J Am Assoc Lab Anim Sci. 2003;42(3):46-52.
20. Bouwknecht JA, van der Gugten J, Hijzen TH, Maes RA, Hen R, Olivier B. Male and female HT1B receptor knockout mice have higher body weights than wildtypes. Physiol Behav. 2001;74(4-5):507-16. doi: 10.1016/s0031-9384(01)00589-3, PMID 11790410.
21. Prakash C, Deopa D, Thakkar HK. Study of internal organ weight and its correlation to body weight in Kumaon Region of Uttarakhand. J Indian Acad Forensic Med. 2013;35(1):29-32.
22. Teo S, Stirling D, Thomas S, Hoberman A, Kiorpes A, Khetani V. A 90-day oral gavage toxicity study of D-methylphenidate and d, l-methylphenidate in Sprague–Dawley rats. Toxicology. 2002;179(3):183-96. doi: 10.1016/s0300-483x(02)00338-4, PMID 12270592.
23. Arfat Y, Mahmood N, Tahir MU, Rashid M, Anjum S, Zhao F, *et al*. Effect of Imidacloprid on hepatotoxicity and nephrotoxicity in male albino mice. Toxicol Rep. 2014;1:554-61. doi: 10.1016/j.toxrep.2014.08.004, PMID 28962268.
24. Pennell LM, Galligan CL, Fish EN. Sex affects immunity. J Autoimmun. 2012;38(2-3):J282-91. doi: 10.1016/j.jaut.2011.11.013, PMID 22225601.
25. Crain JM, Nikodemova M, Watters JJ. Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult central nervous system in male and female mice. J Neurosci Res. 2013;91(9):1143-51. doi: 10.1002/jnr.23242, PMID 23686747.Demma J.

Demma J, Gebre-Mariam T, Asres K, Ergetie W, Engidawork E. Toxicological study on glinus lotoides: A traditionally used taenicidal herb in Ethiopia. J Ethnopharmacol. 2007;111(3):451-7. doi: 10.1016/j.jep.2006.12.017, PMID 17210235.

26. Yuet Ping K, Darah I, Chen Y, Sreeramanan S, Sasidharan S. Acute and subchronic toxicity study of *Euphorbia hirta* L. methanol extract in rats. BioMed Res Int. 2013;2013:182064. doi: 10.1155/2013/182064, PMID 24386634.
27. Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. Proc Natl Acad Sci U S A. 2000;97(23):12729-34. doi: 10.1073/pnas.97.23.12729, PMID 11070086.
28. Lightfoot JT. Sex hormones' regulation of rodent physical activity: a review. Int J Biol Sci. 2008;4(3):126-32. doi: 10.7150/ijbs.4.126, PMID 18449357.
29. Taylor GT, Lerch S, Chourbaji S. Marble burying as compulsive behaviors in male and female mice. Acta Neurobiol Exp (Wars). 2017;77(3):254-60. doi: 10.21307/ane-2017-059, PMID 29182616.
30. Beeler JA, Faust RP, Turkson S, Ye H, Zhuang X. Low dopamine D2 receptor increases vulnerability to obesity via reduced physical activity, not increased appetitive motivation. Biol Psychiatry. 2016;79(11):887-97. doi: 10.1016/j.biopsych.2015.07.009, PMID 26281715.
31. Ruegsegger GN, Booth FW. Running from disease: molecular mechanisms associating dopamine and leptin signaling in the brain with physical inactivity, obesity, and type 2 diabetes. Front Endocrinol. 2017;8:109. doi: 10.3389/fendo.2017.00109, PMID 28588553.
32. Nishikawa S, Yasoshima A, Doi K, Nakayama H, Uetsuka K. Involvement of sex, strain and age factors in high fat diet-induced obesity in C57BL/6J and BALB/cA mice. Exp Anim. 2007;56(4):263-72. doi: 10.1538/expanim.56.263, PMID 17660680.
33. Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, *et al*. Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. Br J Nutr. 2013;109(10):1755-64. doi: 10.1017/S0007114512003923, PMID 23110765.
34. Andjelkovic M, Buha Djordjevic A, Antonijevic E, Antonijevic B, Stanic M, Kotur-Stevuljevic J, *et al*. Toxic effect of acute cadmium and lead exposure in rat blood, liver, and kidney. Int J Environ Res Public Health. 2019;16(2):274. doi: 10.3390/ijerph16020274, PMID 30669347.
35. El-Shenawy SM, Hassan NS. Comparative evaluation of the protective effect of selenium and garlic against liver and kidney damage induced by mercury chloride in the rats. Pharmacol Rep. 2008;60(2):199-208. PMID 18443381.
36. Demma J, Gebre-Mariam T, Asres K, Ergetie W, Engidawork E. Toxicological study on Glinus lotoides: a traditionally used taenicidal herb in Ethiopia. J Ethnopharmacol. 2007;111(3):451-7. doi: 10.1016/j.jep.2006.12.017, PMID 17210235.

Cite this article: Cayetano AC, Atienza LM, Estacio MAC, Castillo-Israel KAT, Desamero MJ, Gapasin RP, *et al*. Subacute Oral Toxicity Evaluation of Freeze-Dried Fruits of Lipote (*Syzygium polycephaloides* (C.B. Rob.) Merr.) in ICR Mice. Pharmacog Res. 2023;15(3):504-13.