

Antioxidant, Anti-obesity, and Lipid-lowering Properties of Philippine “Duhat” (*Syzygium cumini* L. Skeel) Freeze-dried Fruit Flesh

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Philippine indigenous berries are known as rich sources of antioxidants that may promote health and prevent the occurrence of diseases. Thus, the study investigated the nutritional, antioxidant, anti-obesity, and lipid-lowering properties of freeze-dried “duhat” (FDD) (*Syzygium cumini* L. Skeel) fruit using proximate analysis, *in vitro* antioxidant assays, and *in vivo* efficacy using obese diet-induced ICR mice, respectively. Mice were randomly allocated to five groups fed with various diets *ad libitum* for nine weeks as follows: Group 1 with normal diet (ND) and served as the control, Group 2 with high-fat diet (HFD) that served as the negative control, Group 3 with HFD + 10% w/w FDD powder, Group 4 with HFD + 20% w/w FDD powder, and Group 5 with HFD + 30% w/w FDD powder. Proximate composition of FDD fruit flesh includes $20.16 \pm 0.75\%$ moisture content, $2.64 \pm 0.06\%$ total crude fiber, $4.50 \pm 0.21\%$ crude fat, $7.10 \pm 0.20\%$ crude protein, and $62.22 \pm 1.11\%$ carbohydrate. It is a rich source of antioxidants with a total flavonoid content (TFC) of 0.02 mg QE/g FDD, total phenolic content (TPC) of 12.52 ± 0.02 mg GAE/ g FDD, and anti-oxidant activity of 96.07 ± 0.86 . Results of the *in vivo* study showed significant reduction in blood TG by 50% at 30% FDD supplementation (w/w) and by 30% at 20% FDD supplementation (w/w) ($p < 0.05$). Also, a significant increase of up to 45% in HDL-C in the 30% FDD-supplemented group was noted compared to the baseline mean (Week 0) at Weeks 3 and 6 of supplementation ($p < 0.05$). Meanwhile, no significant findings were noted in the blood total cholesterol (TC) levels. The improvement in lipid profile could be attributed to the nutritional and bioactive compounds found in Philippine duhat. Taken collectively, this study yielded interesting findings that can be further investigated at the cellular and molecular levels.

Keywords: anti-obesity, antioxidant, ICR mouse, lipid-lowering properties, Philippine duhat

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INTRODUCTION

The prevalence of overweight and obesity is increasing worldwide. It nearly tripled between 1975 and 2016 (WHO 2018) and presently affects 1.9 billion people globally (Saltiel and Olefsky 2017). Although its prevalence was reported to have slowed down in developed countries in the past few years, the prevalence in developing countries continues to increase and might have even tripled in some developing countries over the past few years (Hossain *et al.* 2007; Ng *et al.* 2014). The prevalence has steadily increased from 7.2–9.6% from 2015–2018 among Filipinos, more particularly adults 20 yr old and above (FNRI-DOST 2018). The rising concerns on obesity are correlated to the subsequent increase in the rates of various obesity-related metabolic disorders that are considered as major risk factors of cardiovascular diseases (CVDs), which are the leading causes of mortality globally (Roth *et al.* 2017). Elevated lipid profiles in obesity are a major determinant of risk to CVD. Obese people tend to develop dyslipidemia. Dyslipidemia is a blood lipid disorder characterized by increased blood levels of triglycerides (TG), TC, and low-density lipoprotein cholesterol (LDL-C), plus decreased level of high-density lipoprotein cholesterol (HDL-C) (Klop *et al.* 2013). In 2008, the World Health Organization (WHO) estimates the prevalence of dyslipidemia in Southeast Asia at 30.3% and 36.7% in the Western Pacific, while higher prevalence was recorded in Europe (53.7%) and in the Americas (47.7%) (Lin *et al.* 2018). In the Philippines, it was reported that around 47.2% of Filipino adults have borderline to high TC, 47.5% have borderline to high LDL-C level, around 71.0% had low HDL-C, and 38.7% have borderline to very high TG level (FNRI-DOST 2015a, b).

Obesity or excessive fat deposition, especially in the abdominal area, is a consequence of energy imbalance. This energy imbalance results from the interaction of several factors such as increased intake of energy-dense foods, decreased intake of food rich in micronutrients and bioactive compounds, decreased physical activity, as well as other contributing factors such as nutritional and hormonal status in early life, genetic, cultural, environmental, and economic factors (Greenberg and Obin 2006). Hence, obesity is a complex health issue to address. Nevertheless, the predisposing role of an unhealthy lifestyle in obesity is already clear and well-established. An unhealthy diet characterized by overeating particularly of foods rich in fats and sugars, combined with low levels of physical activity, results in excess weight and eventually obesity (Singh and Marar 2011). Hence, dietary intervention is a lifestyle modification and is widely known to effectively target obesity and its comorbidities. Specifically, intakes of fruits and vegetables are proven

dietary strategies to reduce the risk of obesity-related diseases –including CVDs, cancer, and diabetes mellitus (Netu 2007; Basu *et al.* 2010).

Among all fruits, berries are highly recognized for their distinct health benefits. Substantial evidence from studies mostly done abroad proved that *in vivo* supplementation of various varieties of berries such as lingonberry and bilberry have reduced body weight, insulin resistance, low-grade inflammation, and hepatic lipid (Heyman *et al.* 2014; Heyman Lindén *et al.* 2016). Philippine indigenous berries are found to be rich in both essential nutrients such as vitamins A, C, and E and non-nutrient compounds such as phenolic compounds with anti-oxidant properties (Gueco *et al.* 2017). An indigenous berry found in the Philippines is *Syzygium cumini* (L.) Skeels or locally known as duhat. Among its common names are black plum, purple plum, Java plum, Indian blackberry, “jambolan,” “jamun,” “jambolão,” “jambu,” “jambul,” “jambool,” “jamblang,” “naval,” Portuguese plum, Malabar plum, “jamaica,” and damson plum (Ayyanar and Subash-Babu 2012). It is found throughout the country and is considered to be one of the most popular summer fruits. Being a deep-colored fruit, it is considered to have high anthocyanin content, which exhibits good antioxidant characteristics. It also contains essential nutrients such as ascorbic acid, thiamine, riboflavin, nicotinic acid, iron, calcium, magnesium, sodium, potassium, and copper (Bijauliya *et al.* 2017). The health-promoting and disease-preventing activities of duhat have not been explored yet in the Philippine setting. Studies on its nutritional and health-promoting properties remain very few, while published studies on the efficacy of duhat in ameliorating the biomarkers of obesity and associated metabolic disorder are lacking. Hence, this study aimed to characterize the nutritional and antioxidant properties of Philippine duhat, as well as investigate its anti-obesity and lipid-lowering effects *in vivo*. The rationale is to find a cost-effective nutritional strategy in combating the risk factors of CVDs, which are placing social and economic burdens on both individuals and society in general.

MATERIALS AND METHODS

Characterization of FDD Fruit Flesh

Materials. Fully ripe duhat [*Syzygium cumini* (Linn.) Skeels] fruits that are dark purple in color were harvested in a local town in Batangas, Philippines. Duhat fruits were pulped at room temperature and fruit flesh, including peels, were freeze-dried at the Institute of Food Science and Technology, University of the Philippines Los Baños (UPLB). Freeze-drying of duhat fruit flesh and peels yielded 20% of the original weight. The FDD samples

were ground into a fine powder, packed in metalized bags, and stored at $-20\text{ }^{\circ}\text{C}$ until use.

Proximate composition. The moisture, crude protein, crude fat, crude fiber, ash, and nitrogen-free extract (NFE) contents of FDD fruit were determined according to the AOAC protocols (2000). Moisture content was determined by drying the sample in a hot-air oven at $105 \pm 5\text{ }^{\circ}\text{C}$ until constant. Crude protein was determined using the Kjeldahl method. Crude fat content was measured using the Soxhlet apparatus and hexane as solvent. The crude fiber was determined using acid and base hydrolysis. Ash was determined by direct incineration in a muffle furnace at $550\text{--}600\text{ }^{\circ}\text{C}$. All assays were carried out in triplicates.

DPPH free radical scavenging activity. DPPH assay is one of the most widely used methods for screening the antioxidant activity (AA) of plant extracts by investigating the free radical-scavenging activities of the present bioactive compounds (Rahman *et al.* 2015). The DPPH reagent (2,2-diphenyl-1-picrylhydrazyl) is relatively stable and can be reduced principally by more reactive reducing components such as phenolic substances (Morabbi-Najafabad and Jamei 2014). The DPPH radical scavenging activity was measured in triplicates according to the method of Shimada *et al.* (1992, as cited by Lizardo *et al.* 2015). One hundred grams (100 g) of each sample were weighed and placed in a test tube wrapped in aluminum foil. Five milliliters (5 mL) of 50% methanol solution was added to each test tube and mixed intermittently for 10 min using a vortex mixer. Solutions were transferred and filtered in another tube using filter paper. A 1-mL aliquot was obtained from each filtered solution, then 4 mL of distilled water was added. One milliliter (1 mL) of the previously prepared DPPH solution was transferred to each tube. Tubes were maintained for 30 min in a dark room. Spectrophotometric measurements were done at 517 nm using Labtronics microprocessor UV-Vis spectrophotometer (Labtronics Panchkula, Haryana, India). The DPPH scavenging activity, as well as the inhibition of the DPPH radical by the sample, was calculated based on the formula below:

$$\text{DPPH Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (1)$$

ABTS assay or TEAC. ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] assay, also known as Trolox equivalent antioxidant capacity (TEAC) assay, is one of the widely used method in studying both lipid-soluble and water-soluble antioxidants (Zhong and Shahidi 2015). The established procedure of Tomasina *et al.* (2012) was used in this analysis wherein a 0.25 M ABTS solution with 3.45 M potassium persulfate was prepared for ABTS radical cation. The mixture was stood

for 12–16 h in a dark environment at $4\text{ }^{\circ}\text{C}$ before. Dilutions were made until the absorbance reading reached 0.70 ± 0.05 at 732 nm. A 2.7-mL ABTS radical cation solution was added to a 0.3-mL diluted sample and incubated for 15 min in a dark environment at room temperature. The standards used were methanolic solutions of Trolox ($2\text{--}40\text{ }\mu\text{g/mL}$), and the absorbance was read at 732 nm. Analysis was done in triplicates and results were expressed as mg Trolox equivalents per g sample (mg TE/g). Trolox equivalents were calculated using the formula:

$$TE = cV/m \quad (2)$$

where TE refers to Trolox equivalents, c is the concentration ($\mu\text{g/mL}$) of Trolox established from the calibration curve, V is the volume (mL) of the extract solution, and m is the weight (g) of extract.

TPC. TPC was spectrometrically analyzed in triplicates using the Folin-Ciocalteu method (Esmaeili *et al.* 2015). To prepare the extract, 50 mg of dried sample in 5-mL absolute methanol were mixed for 30 min using a vortex mixer and centrifuged at 300 rpm for 10 min. Afterward, 200 μL of each methanol extract or gallic acid standard solution (0, 200, 400, 600, 800, 1000 $\mu\text{g/mL}$) was mixed with 2.8-mL distilled water, 1 mL of 2 M sodium carbonate solution, and 200 μL of Folin-Ciocalteu reagent, consecutively. The solution was placed in boiling water for 15 min and cooled to room temperature for color development. Absorbance was measured at 710 nm using Labtronics microprocessor UV-Vis spectrophotometer. Results are expressed as mg gallic acid equivalents (GAE) per gram of FDD.

TFC. TFC was measured with the aluminum chloride colorimetric assay in triplicates. Two-hundred-microliter (200- μL) aliquots and 200- μL standard quercetin solution (0, 100, 200, 400, 600, 800 $\mu\text{g/mL}$) were positioned into test tubes and 2 mL of distilled water and 0.3 mL of 5% sodium nitrite solution was added into each. After 5 min, 0.3 mL of 10% aluminum chloride was added. The solution was allowed to stand for 1 min for color development and then 1 mL of 1 M sodium hydroxide was added. The absorbance was measured at a 510 nm spectrophotometer using Labtronics microprocessor UV-Vis spectrophotometer. Data on TFC was expressed as mg of quercetin equivalents (QE)/ 100 g of FDD (Patel *et al.* 2012; Kamtekar *et al.* 2014).

FDD fruit flesh. All animal experiments performed were approved (2019-001) by the UPLB Animal Care and Use Committee.

Animals

A total of 35 6-wk-old male ICR mice weighing $22.0 \pm 2.0\text{ g}$ were obtained from the Laboratory Animal Facility of the Research Institute for Tropical Medicine at Alabang, Muntinlupa City, Philippines. Mice were

individually housed in commercial polycarbonate cages with stainless steel top and sterile corn cob bedding under 12 h: 12 h light/dark cycle lights on at 07:00 A. and off at 07:00 PM, 24 ± 2 °C, and 50–60% humidity at the laboratory animal experimental room, Department of Basic Veterinary Sciences, College of Veterinary Medicine, UPLB. The mice were acclimatized for 1 wk and were given commercial mouse maintenance diet (Dyets, Pennsylvania, USA) and distilled water *ad libitum*.

Diet Composition and Preparation

The HFD was formulated based on the composition of Dyets, Inc. (Pennsylvania, USA) with 45% calories from fat. Briefly, HFD was formulated by heating the agar solution in a microwave for 3 min. Pork lard was added, heated for another minute, then placed in an electric mixer and stirred until it reaches 40 °C. All the dry ingredients were then added to an agar-lard solution and mixed until homogenous. For HFD-duhat mixtures, exactly the same procedures were applied but the powdered FDD of different doses, *i.e.* 10, 20, and 30% w/w were added together with all the dry ingredients. These doses of FDD were based on the available published literature

that reported its lipid-lowering effects (Mykkänen *et al.* 2014; Heyman 2015). The prepared mice diet was then transferred in properly labeled clean containers and stored inside a freezer until use. Table 1 shows the composition and caloric content of the diets.

Induction of Obesity and Hypercholesterolemia

Obesity and hypercholesterolemia were induced by giving HFD *ad libitum* for 3 wk. Mice with more than 20% body weight gain were considered obese (Wang *et al.* 2010), while mice with at least a 20% increase in blood TC were considered hypercholesterolemic (Franciosi *et al.* 2009). Mice that met the criteria for these conditions were included in the intervention phase. For the intervention phase, mice were randomly allocated into four treatment groups (n = 7 each group), namely:

- normal control group: regular diet (Dyets, Inc. (Dyets # 110700, AIN-93G) + distilled water (DW);
- negative control group: HFD + DW;
- treatment group 1: HFD + 10% w/w FDD + DW;
- treatment group 2: HFD + 20% w/w FDD + DW; and
- treatment group 3: HFD + 30% w/w FDD + DW.

Table 1. Composition and caloric content of the different diets.

Ingredients	Kcal/g	ND (g)	HFD (g)	HFD + 10% FDD	HFD + 20% FDD	HFD + 30% FDD
Casein	3.58	100.0	116.54	112.57	108.60	104.64
L-cystine	4.0	1.5	1.75	1.75	1.75	1.75
Sucrose	4.0	50	100.69	100.69	100.69	100.69
Cornstarch	3.6	198.7	42.42	7.85	0.00	0.00
Dyetrose	3.8	66.0	58.27	58.27	32.96	0.21
Soybean oil	9.0	35.0	0	0	0	0
t-butylhydroquinone	0	0.007	0.003	0.00	0.00	0.00
Cellulose	0	25.0	29.13	29.13	29.13	29.13
Mineral mix #210025	0.88	17.5	0	0.00	0.00	0.00
Vitamin mix #310025	3.87	5.0	0	0	0	0
Choline bitartrate	0	1.25	1.17	1.17	1.17	1.17
Salt mix #210088	1.6	0	5.83	5.83	5.83	5.83
Dicalcium phosphate	0	0	7.57	7.57	7.57	7.57
Calcium carbonate	0	0	3.20	3.20	3.20	3.20
Potassium citrate H ₂ O	0	0	9.61	9.61	9.61	9.61
Vitamin mix #3000050	3.92	0	5.83	5.83	5.83	5.83
Pork lard	9.0	0	118.00	115.75	113.5	111.254
FDD	3.1778	0	0	50.00	100.00	150.00
Total		500.0	500.0	509.22	519.84	530.88
Kcal/g		3.76	4.59	4.51	4.42	4.32

ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat

Animals received their respective diets *ad libitum* for 9 wk of supplementation.

Food and Water Intake and Body Weight Measurement

The feed intake of mice was measured daily. Pre-weighed feeds were given to each mouse and daily refused was measured using a digital top loading balance (Shimadzu, Japan). The actual intake for the day was computed by subtracting the refused from the total feeds given. For the water intake, each mouse was given 10 mL of water per day. The actual water intake for the day was computed by subtracting the water refused from the total water given. Meanwhile, the weight of each mouse was measured weekly using a digital top loading balance (Shimadzu, Japan). The weight gain was calculated using the following formula:

$$\text{Weight gain (g)} = \frac{\text{Body weight (Day 90)} - \text{Body Weight (Day 0)}}{\text{Day 90} - \text{Day 0}} \quad (3)$$

Blood Lipid Measurement

One drop of eye anesthetic proparacaine hydrochloride was placed in the right eye of each mouse. Then, 250 μL of blood was collected from the retro-orbital sinus using heparinized hematocrit tubes (NRIS, Denmark). The collected blood was placed in a 1.5-mL microcentrifuge tube (Eppendorf tube, USA), labeled, and then analyzed for TC, HDL-C, and TG using Arkray SpotChem-EZ SP-4430 blood chemistry analyzer (ARKRAY Inc., Japan). Blood TC, HDL-C, and TAG were measured at baseline prior to the start of the intervention phase, then every 3 wk for 9 wk.

Euthanasia and Necropsy of Mice

Mice were anesthetized *via* intraperitoneal injection of pentobarbital sodium (Dolethal, Vetoquinol UK Ltd) at a dose of 80 mg/kg body weight. After intracardiac blood collection, fat pads such as the subcutaneous, epididymal, mesentery, abdominal, and heart fat were collected and weighed using an analytical balance (Shimadzu, Japan). Additionally, the liver, left and right kidneys, spleen, stomach, and heart were collected, weighed, and examined for gross pathologic changes. The relative organ weights were determined by dividing the weight of the organ (g) by body weight (g) and multiplied by 100. Meanwhile, the total adiposity index (AI%) was determined by calculating the sum of the subcutaneous, epididymal, mesentery, and abdominal fat weights (g) divided by body weight (g) and multiplied by 100.

Tissue Processing and Microscopic Examination

Liver and fat samples were processed using the paraffin technique, sectioned with a rotatory microtome, and

stained with hematoxylin and eosin. Stained sections were coded to prevent bias during examination under light microscopy (Zeiss Primostar). Furthermore, the mean diameter of abdominal white adipocytes from seven animals per group was calculated as an indicator of adipocyte hypertrophy in restricted view fields on a computer monitor using an automated image analysis system (Image J). The mean adipocyte diameter was expressed in μm and a minimum of 10 white adipocytes per fat pad was measured.

Human Dose Equivalent

The human dose equivalent was calculated using the method by Reagan-Shaw *et al.* (2008). It was determined by multiplying the animal dose (mg/kg) by 3 (animal Km factor for mouse) divided by 37 (human Km factor for an adult).

Statistical Analysis

For the characterization of the nutritional and functional properties of FDD fruit flesh, all assays were carried out in triplicate and their results were expressed as mean \pm standard deviation. Correlation coefficients (r) and coefficients of determination (r^2) were calculated using Microsoft Excel Professional Plus 2013. For the *in vivo* investigation, the anti-obesity and lipid-lowering properties were expressed as mean \pm standard error of the mean (SEM), while adipose diameter measurement results were expressed as mean \pm standard deviation (SD). Changes in lipid profiles across time periods were expressed as percentiles (%). The data were analyzed using, one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at $p < 0.05$ for differences between groups and within groups across time periods using SPSS (Statistical Package for the Social Sciences) version 20.0.

RESULTS

Proximate Composition and Antioxidant Properties of FDD Fruit Flesh

Freeze-drying is an effective method to preserve not just the desirable quality but also the antioxidant properties of fruits and other food materials (Gümüşay and Yalçın 2019). Results showed that FDD fruit flesh has a moisture content of $20.16 \pm 0.75\%$, the total crude fiber of $2.64 \pm 0.06\%$, crude fat of $4.50 \pm 0.21\%$, crude protein of $7.10 \pm 0.20\%$, and carbohydrate (NFE) value of $62.22 \pm 1.11\%$. It provides a caloric value of 317.78 kcal/ 100 g, making it a good energy source. For the antioxidant properties, the present findings indicate that FDD fruit flesh has a DPPH

AA of 96.07 ± 0.86 , ABTS value of 77.87 ± 5.43 mg TE/g FDD fruit flesh, TPC of 12.52 ± 0.02 mg GAE/g FDD fruit flesh, and TFC of 1.83 ± 0.02 mg QE/g FDD fruit flesh. Fruits with a DPPH free radical scavenging activity of 87% and above are considered to have an excellent scavenging effect against DPPH radical (Shofian *et al.* 2011). The AA and TPC of FDD fruit flesh were higher than other freeze-dried berries such as blueberry (49% AA; 4.26 mg GAE/g TPC) and cherry laurel (35% AA; 10.57 mg GAE/g TPC) (Islary *et al.* 2016; Shivembe and Ojinnaka 2017; Gümüşay and Yalçın 2019). Moreover, the ABTS value of FDD fruit flesh was higher than other red fruits such as pomegranate (76.10 ± 0.05 mg TE/g), strawberry (29.70 ± 0.02 mg TE/g), and raspberry (25.50 ± 0.02 mg TE/g) (Gramza-Michałowska *et al.* 2019).

***In Vivo* Investigawtion of the Anti-obesity and Lipid-lowering Properties of FDD Fruit Flesh**

Food and water intake. Mean feed and water intakes of different mice groups during the supplementation period are shown in Table 2. Results showed that all the mice from the different treatment groups consumed a normal amount of feeds. Control mice fed with ND consumed a

Table 2. Mean feed and water intake of mice during supplementation period.

Group	Mean feed intake (g)	Mean water intake (ml)
ND	4.24 ± 0.19^a	8.32 ± 0.43^a
HFD	5.17 ± 0.19^b	7.74 ± 0.49^a
HFD + 10% FDD	5.34 ± 0.20^b	7.31 ± 0.39^a
HFD + 20% FDD	5.32 ± 0.09^b	7.71 ± 0.48^a
HFD + 30% FDD	5.54 ± 0.17^b	7.48 ± 0.42^a

ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat

Values represent the mean \pm S.E. ($n = 7$).

Means in the same column followed by different letter(s) are significantly different at $p < 0.05$.

significantly lower amount of feeds compared with the HFD and HFD+ different concentrations of FDD mice groups. The highest feed consumption was observed in mice fed with HFD+30% FDD but not significantly different among other groups ($p < 0.05$). In terms of water intake, no significant difference was seen among mice groups, and water consumed was within the normal intake for mice.

Bodyweight. Table 3 presents the mean body weights of all mice groups which were statistically comparable at the start and end of the study. Weight gain was noted in all treatment groups with the HFD+20%FDD group having the least average weight gain and nearest with the control ND group weight gain, although not significantly different with other mice groups.

Macroscopic and microscopic observations of adipose tissues. Mean weights of adipose depots including the abdominal, subcutaneous, epididymal, mesenteric, and heart fat of each experimental group are shown in Figure 1. The HFD + 30% FDD group had the highest weights of adipose depots as well as %AI, a marker of central adiposity (Figure 2). These observations correspond to higher weight gain, which could possibly be due to the higher food consumption of this mice group. Interestingly, the HFD + 20% FDD group displayed the lowest weights of adipose tissues and %AI, although these values did not vary significantly ($p > 0.05$) with other mice groups.

Microscopic observations of abdominal adipose tissues between groups showed varying sizes of the adipocytes (Figure 3). Measurement of the diameter of the adipocytes from the HFD group showed a significant difference in adipocyte diameter compared to the other groups (Table 4). However, there was no significant difference between ND, HFD + 10% FDD, and HFD + 30% FDD, while HFD + 20% FDD showed a significant difference compared to ND (Table 4).

Macroscopic and microscopic observations of organs.

Table 3. Mean body weight and weight gain of mice during supplementation.

Group	Mean body weight (g)		Mean weight gain (g)
	Week 0	Week 9	
ND	31.24 ± 1.53^a	34.37 ± 2.47^a	3.13 ± 1.32^a
HFD	33.10 ± 0.76^a	37.35 ± 1.12^a	4.25 ± 0.74^a
HFD + 10% FDD	32.21 ± 0.91^a	37.92 ± 1.55^a	5.71 ± 0.88^a
HFD + 20% FDD	32.41 ± 0.70^a	36.28 ± 1.01^a	3.87 ± 0.82^a
HFD + 30% FDD	31.84 ± 1.63^a	37.54 ± 3.14^a	5.69 ± 1.70^a

ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat

Values represent the mean \pm S.E. ($n = 7$).

Means in the same column followed by different letter(s) are significantly different at $p < 0.05$.

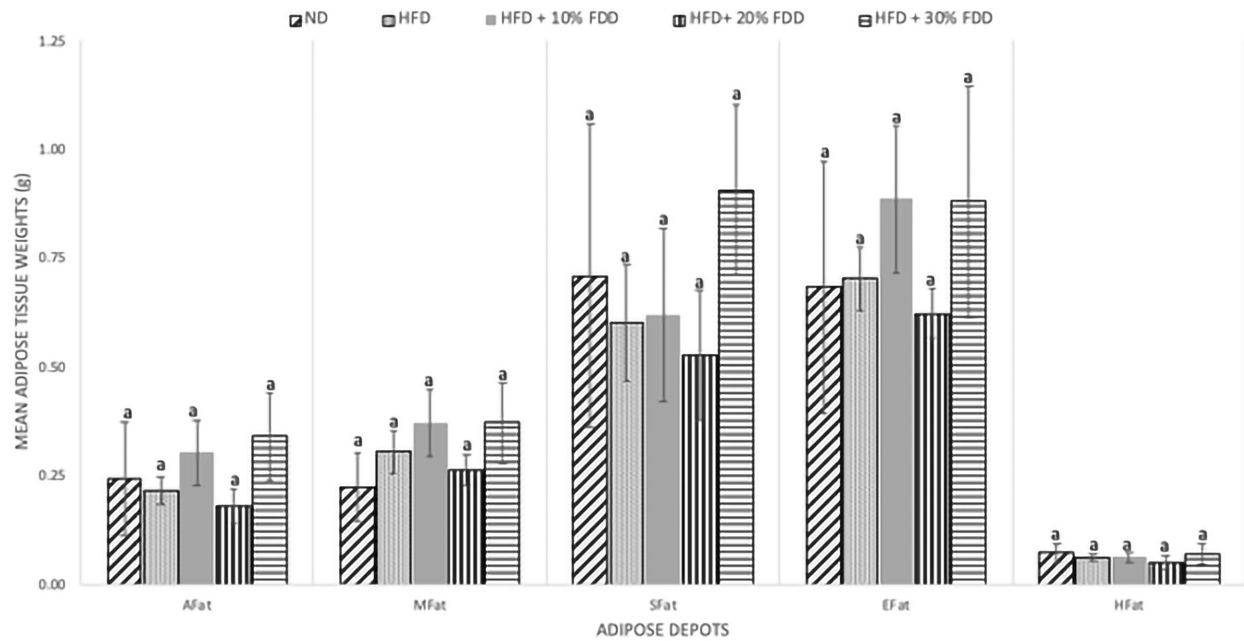


Figure 1. Mean adipose tissue weights (\pm SEM) of different treatment mice after supplementation. Bars with different letter(s) are statistically different at $p < 0.05$ using ANOVA. ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. AFat – abdominal fat; Mfat – mesentery fat; SFat – subcutaneous fat; EFat – epididymal fat; HFat – heart fat.

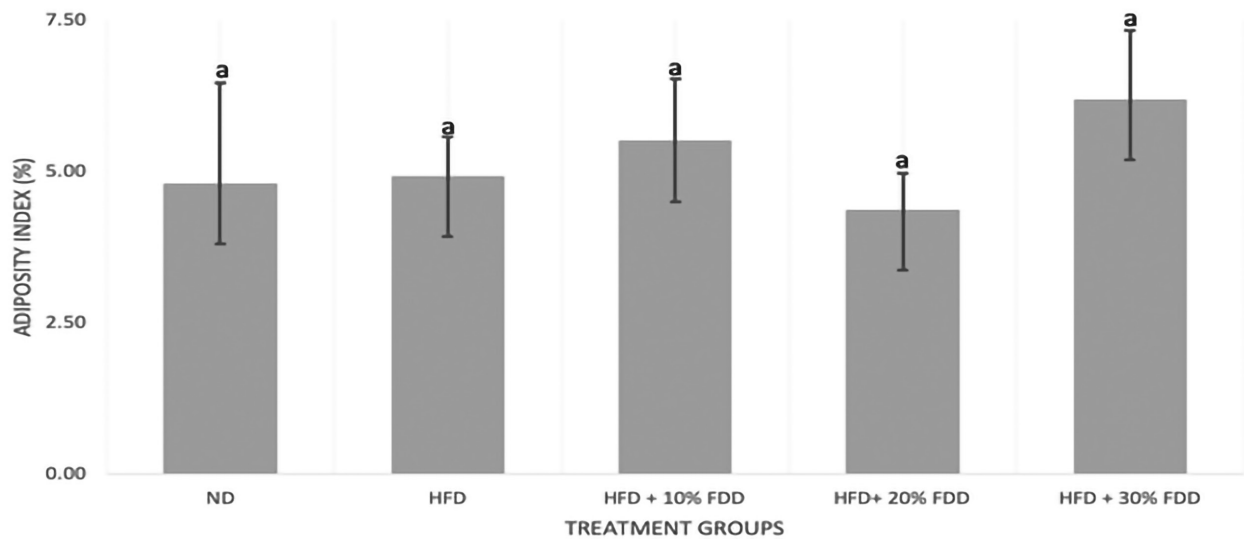


Figure 2. Mean AI% in mice after supplementation. Bars with the same letters are not statistically significant at $p < 0.05$ using ANOVA.

It was observed that the HFD group mice developed pale, soft, and enlarged livers, which were absent in other mice groups (Figure 4). Consistent with this result, the histopathologic analyses of the liver showed that the HFD group developed steatosis, characterized by

small intracytoplasmic fat vacuoles at the periacyinar and centrilobular areas with limited necrosis in the hepatocytes. Compared with the HFD group, normal appearance of liver architecture without any steatotic, fibrotic, and inflammatory changes were observed in the

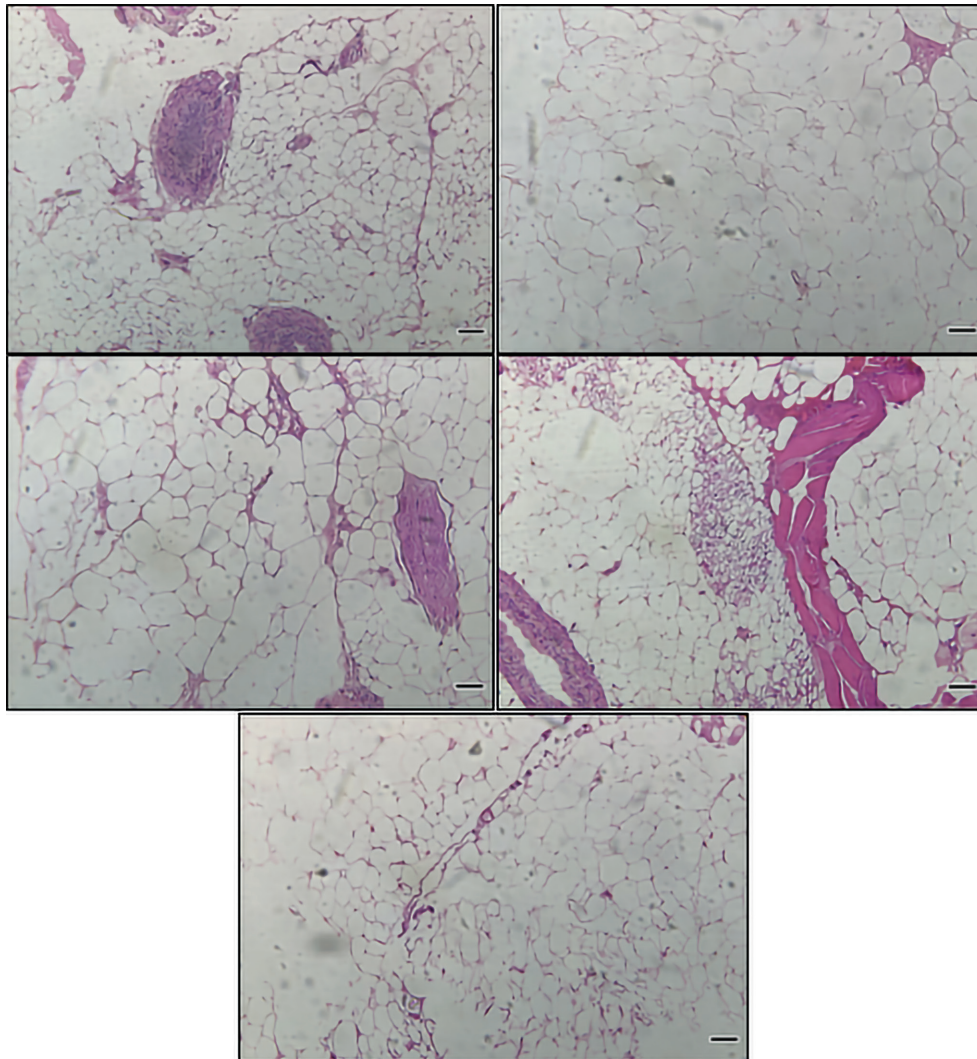


Figure 3. Effect of FDD on adipocyte size and architecture. Representative photograph of abdominal fat sections from each group of rats: ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. Representative histological sections of the abdominal fat from groups showed varying degrees of sizes of the adipocyte diameter. Hematoxylin and eosin; magnification = 10x, scale bar = 20 μ m.

Table 4. Mean adipocyte diameter (μ m) from mice abdominal adipose tissues with different diets.

Group	Adipocyte diameter (μ m)
ND	13.13 \pm 3.79 ^a
HFD	28.51 \pm 9.93 ^b
HFD + 10% FDD	14.93 \pm 6.30 ^{a,c}
HFD + 20% FDD	16.16 \pm 4.68 ^c
HFD + 30% FDD	13.75 \pm 3.08 ^{a,c}

ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat

Values represent mean \pm SD ($n = 60$)

Means with the same superscript are not significant at $p < 0.05$ using Tukey's HSD test.

ND group. Treatments with 10 and 20% FDD showed liver histology comparable with that of the ND group, while the HFD + 30% FDD group showed similar histological features in the HFD group but to a lesser extent (Figure 5). Meanwhile, no significant effect was observed in terms of relative weights of the left and right kidneys, spleen, pancreas, and stomach, which may indicate that these organs were not adversely affected by the HFD and HFD-FDD complexes (Figure 6).

Blood lipids. Prior to 9-wk supplementation, the HFD and HFD-FDD mice groups had significantly higher blood TC levels compared to the ND group (Figure 7), showing that HFD (45% fat) had induced hypercholesterolemia in male ICR mice during the 3-wk induction period.

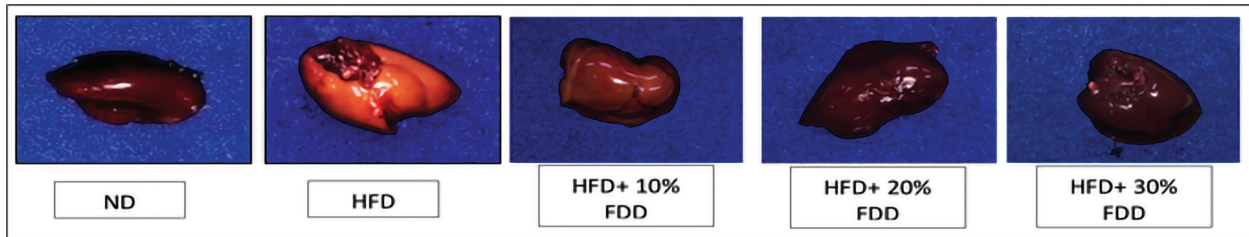


Figure 4. Gross appearance of the liver of representative mouse per treatment group. There was enlargement, softness, and paleness of the liver from the high-fat diet (HFD) group. Gross abnormalities of the liver were not observed in mice fed with normal diet (ND) and those that received HFD + different concentration of freeze-dried duhat (HFD + FDD).

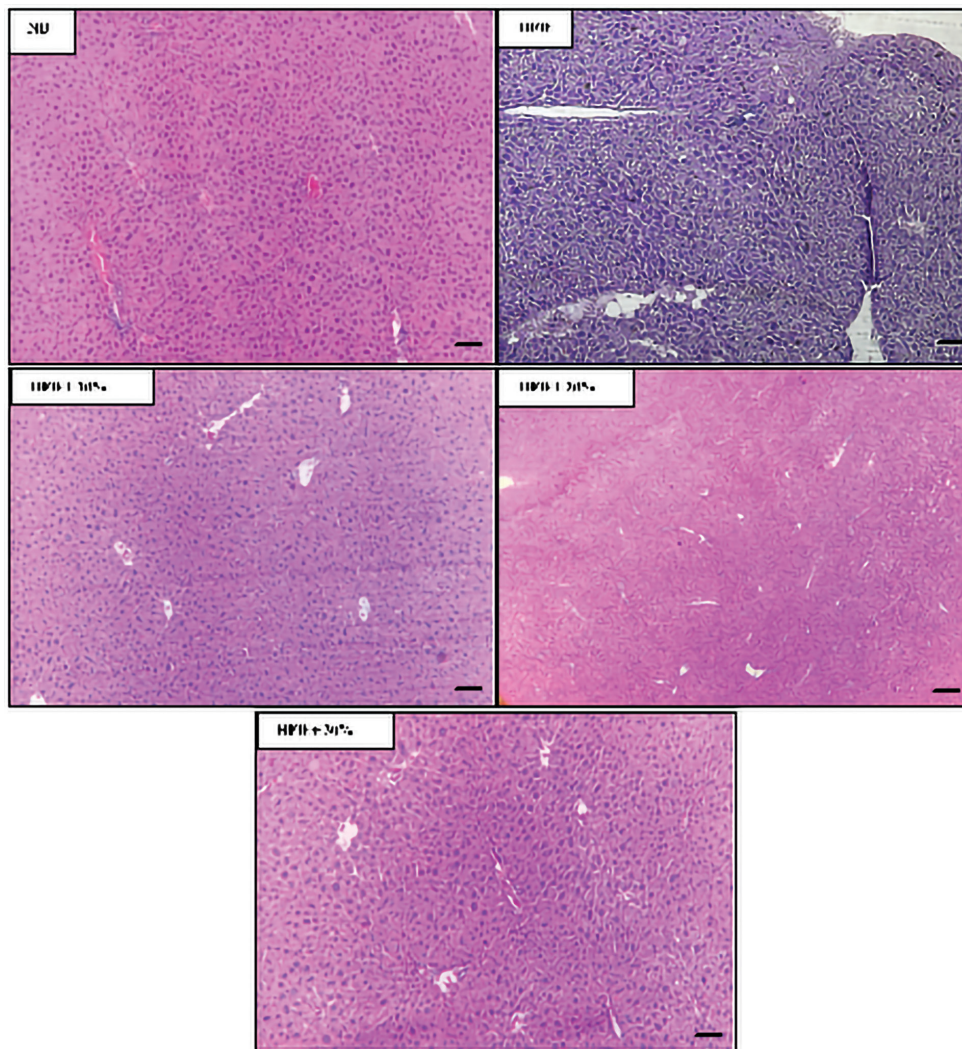


Figure 5. Effects of FDD on histopathological features of the liver. Representative photograph of liver sections from each group of rats: ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. The liver in ND group showed normal appearance of the liver architecture. However, mild infiltration of fat deposit in the liver parenchyma and degenerative changes such as mild necrosis in the hepatocytes were found in HFD group. HFD + 10% FDD and HFD + 20% FDD did not show fat deposits and showed normal appearance similar to ND group. HFD + 30% FDD showed occasional fat deposits and were decreased sometimes to zero. Hematoxylin and eosin; magnification 4x, scale bar = 200 μ m.

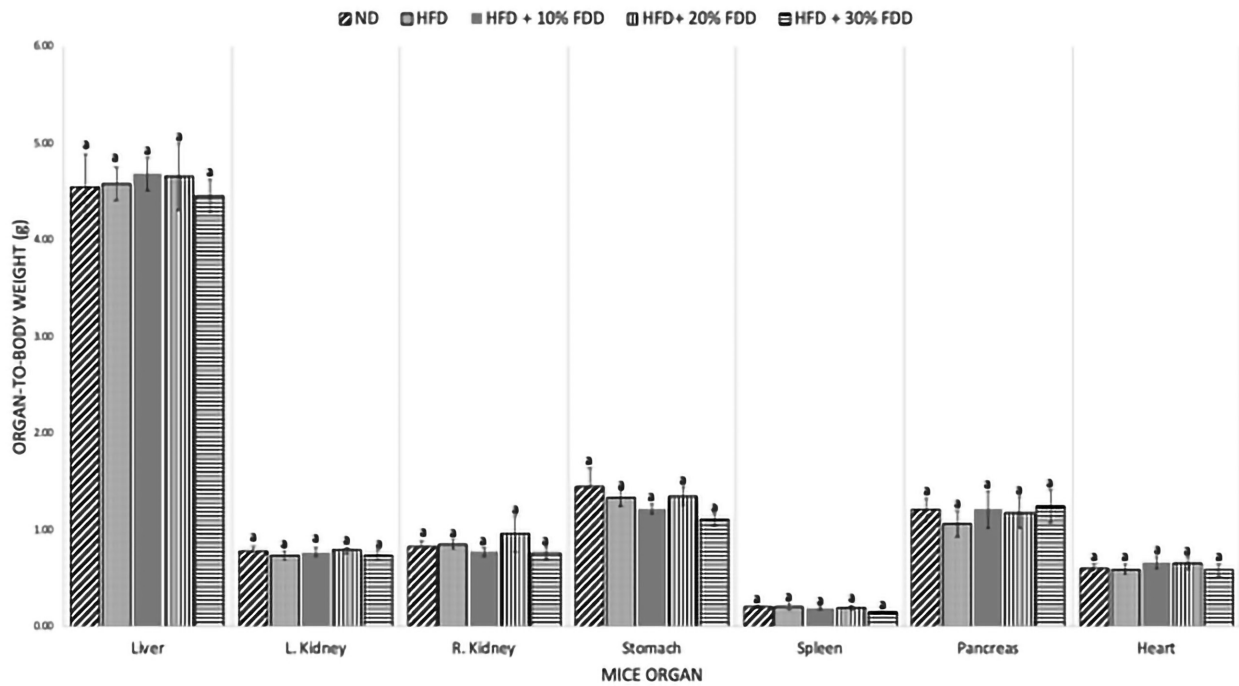
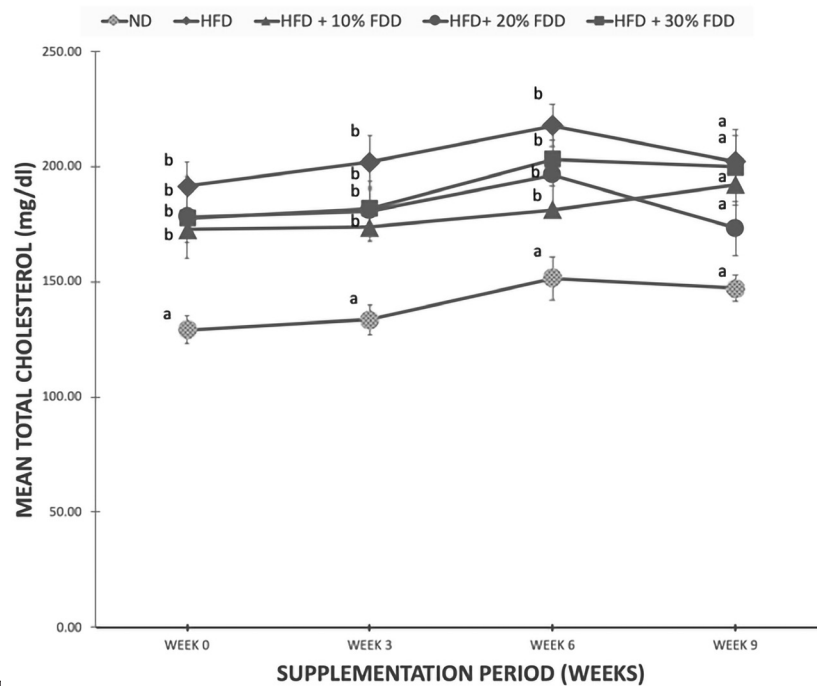


Figure 6. Mean (\pm SEM) organ-to-body weight ratios of different treatment mice after supplementation. Bars with the same letters are not statistically significant at $p < 0.05$ using ANOVA. ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat.



supplementation. ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. Different letters in the same week period are significantly different among the various treatment groups at $p < 0.05$ using ANOVA and Tukey's HSD test.

During the supplementation period, the mean blood TC levels of FDD-supplemented groups were lower but not significantly different compared to the HFD group. There was also no significant difference in the mean blood TC level across time periods within the same treatment group.

The effects of FDD supplementation on blood TG levels of mice are shown in Figure 8. From Weeks 0–9 of the supplementation period, the mean blood TG levels were statistically comparable among treatment groups. However, the trend shows that FDD-supplementation has decreased TG level from Weeks 0–9, with the 20 and 30% FDD groups having the highest reduction at Week 9, *i.e.* by 50.14 ± 8.96 and $30.86 \pm 8.01\%$, respectively. The percent change from the baseline mean TG levels of the said groups were significantly lower ($p < 0.05$) compared to ND ($-8.00 \pm 14.89\%$), HFD ($13.00 \pm 4.55\%$), and HFD + 10% FDD ($-23.43 \pm 10.96\%$) groups.

Meanwhile, results on the mean blood HDL-C levels were statistically comparable among mice groups from Weeks 0–9 (Figure 9). The trend shows that the levels were increasing throughout the study with the HFD + 30% FDD mice group having the highest percent increase of 34.86% compared to the ND (17.00%), HFD (6.00%), HFD + 10%

FDD (6.57%), and HFD + 20% FDD (3.43%) groups. Additionally, data shows that treatment with 30% FDD resulted in a significant increase from the baseline mean (Week 0) HDL-C levels of HFD fed mice immediately at Weeks 3 and 6 of supplementation.

The oral dose of 20% FDD w/w in mice that showed beneficial effects to blood TG represents a human oral dose of 2796 mg/kg/d FDD, which is approximately 302 g or around 1.5 cups of fresh duhat fruit daily for a typical adult weighing 60 kg. On the other hand, oral dose of 30% FDD w/w in mice that showed improvement in HDL-C levels represents 4291 mg/kg/d FDD for human consumption or approximately 463 g or around 2.5 cups of fresh duhat fruit daily for a standard 60-kg human.

DISCUSSION

The benefits of a diet rich in fruit and vegetables to human health and for the prevention of various degenerative diseases are shown in many epidemiological studies (Kim and Park 2011; Wu *et al.* 2013a, b). Berries that are rich in bioactive compounds may provide health benefits

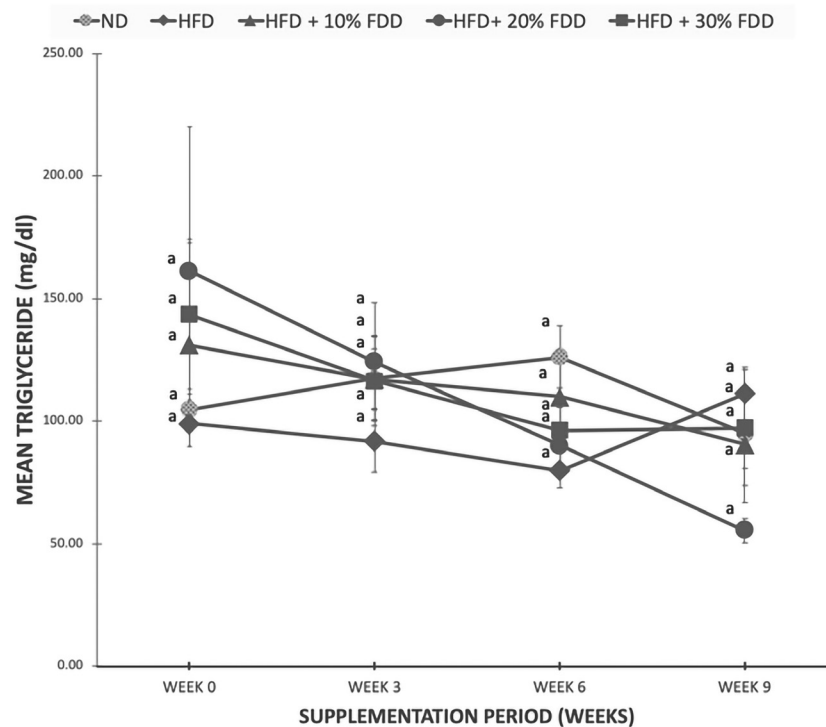


Figure 8. Mean (\pm SEM) blood TG levels of different treatment mice during supplementation. ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. Same letters in the same week period are not significantly different at $p < 0.05$ using ANOVA.

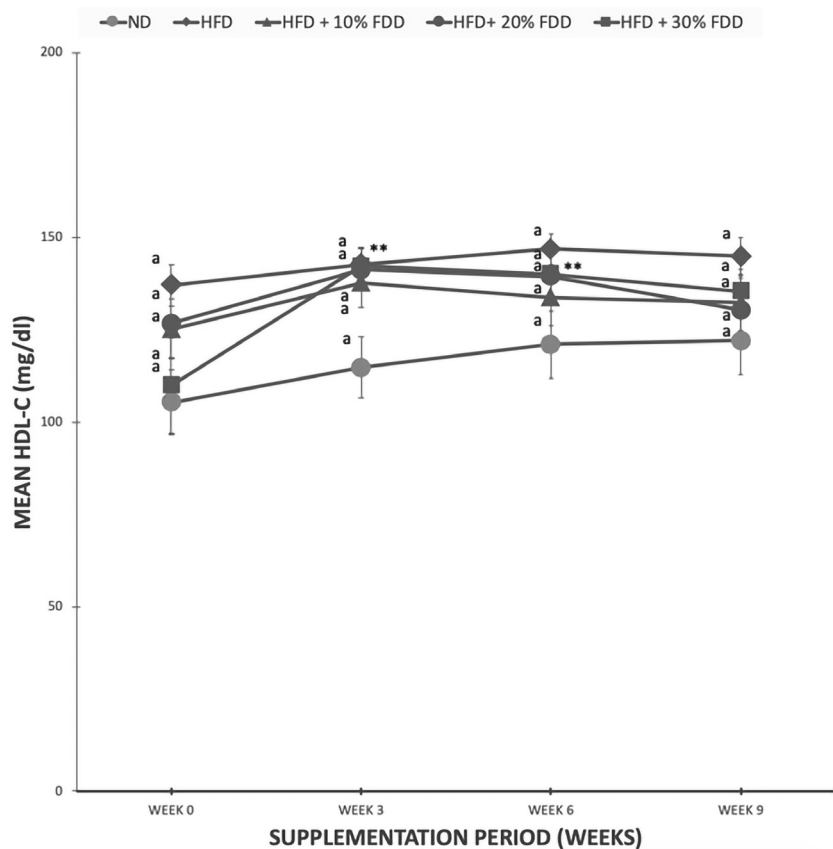


Figure 9. Mean blood HDL-C levels of different treatment mice during supplementation. ND – normal diet; HFD – high-fat diet; HFD + 10% FDD – HFD w/ 10% freeze-dried duhat; HFD + 20% FDD – HFD w/ 20% freeze-dried duhat; HFD + 30% FDD – HFD w/ 30% freeze-dried duhat. Same letters in the same week period are not significantly different at $p < 0.05$ using ANOVA. **Significantly different from week 0 at the same treatment, $p < 0.05$.

beyond basic nutrition (Heyman 2015). The findings of this study revealed that Philippine duhat is a good source of calories and high in AA. The presence of the phenolic compounds in FDD fruit flesh can be accounted for its AA, as previously reported for other fruits. Correlation analysis showed the significant contribution of the phenolic compounds to the AA of FDD fruit flesh. Specifically, TPC had a correlation value of 0.9987 contributing to about 79.8% ($r^2 = 0.798$, $p < 0.05$), while TFC had a correlation value 0.7279 contributing to about 88.5% ($r^2 = 0.885$, $p < 0.05$) of the DPPH radical scavenging activity in the FDD fruit. The remaining proportion of its AA can be attributed to other non-phenolic compounds such as antioxidant vitamins and minerals, as indicated in related studies on berries (Heyman 2015; Basu 2019).

In vivo investigation showed that FDD supplementation at different doses did not suppress weight gain in obese mice fed with HFD. A similar finding was reported by Prior *et al.* (2010), where the ingestion of black raspberries

did not significantly suppress body weight gain in mice fed an HFD (60% fat). This result could be attributed to higher feed intake of mice groups supplemented with FDD, which could possibly due to the natural sweetness of full-ripe duhat that enhanced appetite.

Body fat accumulation in obesity is said to play a role in disease risk. Generally, an HFD feeding is associated with the development of central obesity and fatty liver (Ulla *et al.* 2017; Eccleston *et al.* 2011). In the present study, histological analysis of the liver tissues showed that FDD supplementation prevented liver steatosis and controlled abdominal adipocyte hypertrophy. This result could indicate that FDD could regulate HFD-induced lipid accumulation in adipose tissues and the liver. Meanwhile, body fat distribution across major fat fads showed that 20% FDD supplementation prevented more body fat accumulation compared to the HFD group despite an increase in feed intake. The HFD + 20% FDD group had 18% lower abdominal fat, 13% lower subcutaneous fat,

11.67% lower epididymal fat, 11.4% lower mesenteric fat, and 16.67% lower heart fat than the HFD group. Consistent with this result, the HFD + 20% FDD group also had the lowest AI% among the groups, which was 11.36% lower as compared with the HFD group. This is in agreement with the results reported by Heyman *et al.* (2014), where the relative weight of epididymal fat pads was lower in groups supplemented with 20% w/w lingonberry and açai compared to the high-fat control. Meanwhile, the study on *S. cumini* seed and leaves also showed its limiting effects on retroperitoneal, mesenteric, and epididymal adipose fat pad accumulation in rat models (Benevides *et al.* 2019; Ulla *et al.* 2017).

Chronic dyslipidemia is believed to be a major clinical symptom associated with HFD feeding in animals and has been characterized as a major risk factor for CVDs (Ulla *et al.* 2017). In this study, HFD feeding in mice increased blood TC level. Among the treatment groups, 20% FDD supplementation prevented the rise of blood TC levels at Week 9. This result was in agreement with the *in vivo* evaluation of some berries, where mice groups fed with 20% w/w freeze-dried lingonberry and blackcurrant obtained a blood TC significantly lower compared to the control (Heyman *et al.* 2014). Rekha *et al.* (2008) investigated the possible antioxidant potential of aqueous extract of *Syzygium cumini* (pulp) in streptozotocin (STZ)-induced diabetic wistar female rats. Results reveal that animals fed with an aqueous extract of *Syzygium cumini* (100 and 200 mg/kg body weight) pulp had significantly lower TC compared to STZ-induced diabetic rats.

Blood TG levels for the FDD-supplemented groups were consistently lower throughout the supplementation compared to the HFD group. Interestingly, despite having the highest mean feed intake, the HFD + 20% FDD and HFD + 30% FDD mice groups were seen to have a significant reduction in the blood TG levels compared to ND, HFD, and HFD + 10% FDD at Week 9. Blood TG reduction brought about by the consumption of *S. cumini* fruit was in agreement with the findings of Rekha *et al.* (2008), where diabetic rats fed with aqueous extract of *S. cumini* pulp at 100 and 200 mg/kg body weight resulted in 28 and 46% lower TG levels respectively, as compared to the diabetic control group. Similar to the result in the blood TC levels, HFD + 20% FDD group also obtained the highest reduction in TG levels at Week 9. These findings suggest that 20% FDD supplementation was more prominent in suppressing blood TC and TG levels brought about by consumption of HFD.

In the present study, an increase in blood HDL-C levels from Weeks 0–9 was consistently displayed in all mice groups. It was noticeable, however, that the percent increase in HDL-C level of HFD + 30% FDD group resulted in a 5.6-fold increase compared to the HFD group

at the end of supplementation. Similar findings were found in high-fat-fed mice supplemented with freeze-dried açai berry, where the HDL-C level was 1.2-fold higher than the control at the end of supplementation (Heyman *et al.* 2014).

S. cumini fruits are known to contain bioactive compounds such as phenols, flavonoids, and tannins (Chagas *et al.* 2015). Antioxidant- and polyphenol-rich extract supplementation have a beneficial effect in HFD-fed obese animals in an experimental condition. Several reports suggested that the phenolic antioxidants present in *S. cumini* fruit powder could be responsible for less fat tissue accumulation (Taing *et al.* 2012; Chen *et al.* 2014). The lipid-lowering properties of *S. cumini* fruit may be due to mechanisms also related to the presence of phenolic compounds in it. Basu *et al.* (2010) reported that anthocyanins in berries have been shown to affect lipid metabolism in cellular and animal models of dyslipidemia. Moreover, naturally occurring polyphenols have been reported to inhibit pancreatic lipase, thereby influence fat digestion and affect energy intake (McDougall and Stewart 2005). There was evidence that polyphenol from fruit sources can also perform this action (Moreno *et al.* 2003). Polyphenols were also reported to lower the levels of cholesterol in the blood by decreasing the cholesterol micellar solubility (Kobayashi and Ikeda 2017) and by binding with bile acids (Kahlon and Smith 2007). The presence of flavonoids in *S. cumini* could be attributed to its cholesterol-lowering property. It has been described that these compounds increase the expression of cAMP-dependent phosphokinase, which inhibits HMG-CoA reductase – the rate-limiting enzyme of cholesterol biosynthesis (Havsteen 2002; Ravi *et al.* 2005; Sharma *et al.* 2008).

CONCLUSION

In conclusion, the study showed that FDD supplementation did not produce an anti-obesity effect in terms of body weight changes but could regulate HFD-induced lipid accumulation in adipose tissues and the liver, as shown in the histopathologic findings. FDD supplementation exhibited hypotriglyceridemic effects in high-fat-fed mice, more evidently at 20% w/w FDD, while the progressive effect on blood HDL-C level was demonstrated at 30% w/w FDD. As reported in previous studies on berries, the anti-obesity and lipid-lowering properties of FDD supplementation could possibly be due to nutritional and bioactive compounds found in FDD. Hence, Philippine duhat may have potential use in obese and dyslipidemic patients.

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