3. Material and Methods

3.1. Preparation of Jaboticaba Seed and Peel Extract

Jaboticaba fruits were washed with tap water to remove any surface attachment and debris. The seeds and peels were separated from the fruits manually and dried. The seeds were dried using hot air dryer at < 40°C 7 for hours, while the peels were dried using freeze-dryer for 72 hours. The dried samples were extracted with 95% ethanol, 50% ethanol and water, individually. Ratio of sample (both seed and peel) to solvent was 1:9. The seed and peels were ground and maceration was done at 4°C for 24 hours. The mixture was then centrifuged at 7000 rpm at 4°C for 10 minutes. Vacuum filtration was done to separate the residue from the supernatant. In order to remove the solvent, the supernatant was evaporated using a rotary evaporator and then freeze-dried. The sample was kept at -20°C until used. Extraction yield of peel is ±20 gram from 500 gram of fresh peel (0.04%). While for seed, the extraction yield is ±10 gram from 100 gram of fresh seed (0.1%).

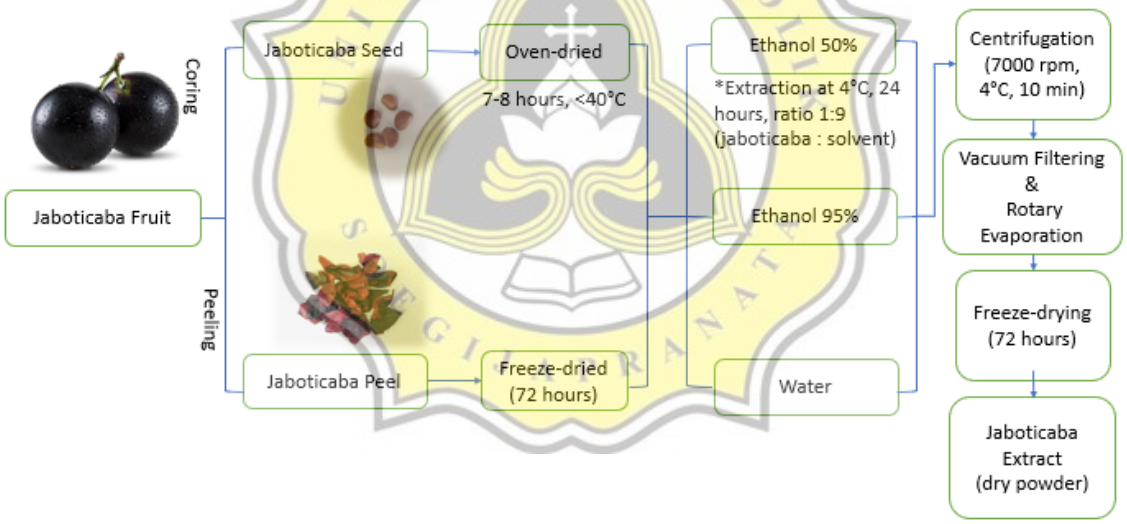

Figure 2. Flowchart of Jaboticaba Extraction

3.2. Zebrafish Breeding

The AB-Wild-type zebrafish were purchased from GENDANIO BIOTECH INC., Taipei, Taiwan. Zebrafish, males (15) and females (9), were kept in room B14, Guerin Hall, Department of Food and Nutrition, Providence University. Zebrafish was controlled in the following conditions: water temperature was maintained at 28 ± 1°C, the blast volume was 30 L/ min, and environment was kept the 14 hours of light and 10 hours of darkness. Twenty-four hours before the collection of fertilized eggs, the male and female fish were

placed in a breeding box with mesh and kept in the dark. The fertilized eggs were collected the next morning. Fertilized eggs were incubated in 28.5℃ water, which contained sea salt (60 mg/L) and methylene blue (2 mg/L).

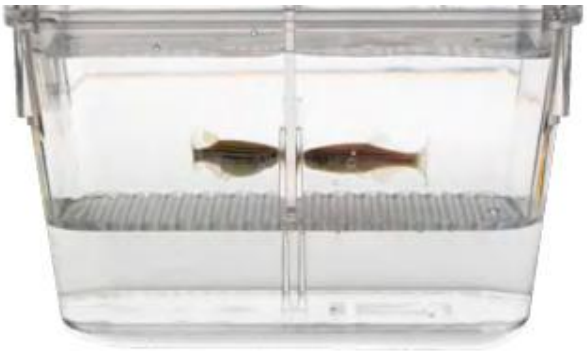

Figure 3. Breeding Box of Zebrafish

3.3. Zebrafish Grouping

Embryos are divided into nine groups, namely (1) control group, (2) LPS-induced group, (3) LPS + 500μM L-NMMA (NG-nitro-L-arginine methyl ester hydrochloride drug control group), and (4) LPS + 0.3 % Peel low dose group, (5) LPS + 0.6% peel medium dose group, (6) LPS + 1% peel high dose group, (7) LPS + 0.3% seed low dose group, (8) LPS + 0.6% seed medium dose group, (9) LPS + 1% seed high dose group, There were 48 embryos in each group. After the drug treatment, NO determination, neutral red staining and Sudan black staining analysis were carried out, and 8 juveniles were used for each analysis.

3.4. LPS Zebrafish Treatment

Since drugs dissolved in water can enter the body directly through the skin or gills of zebrafish, the administration of LPS (Sigma-Aldrich Chemical Co.) or sample of this study was to add to the zebrafish breeding water or culture medium. The embryos are soaked in 10 μg/mL LPS for 24 hours, with a total volume of 2 mL. Control embryos were immersed in the original incubation fluid. Juveniles were raised in an environment of 28.5 ℃ and observed for signs of disease and death (e.g. embryo turning white in color).

3.5. Nitric Oxide Determination (Griess Method)

Determination of nitric oxide in zebrafish embryos is done using Griess Reagent. First, the embryos were washed with PBS, homogenized with 500 µl PBS, and centrifuged 4000 rpm for 5 minutes. Using 1x Griess reagent, the supernatant was mixed in a ratio of 1:1. The absorbance of each sample was recorded with a spectrometer at 540 nm after incubation for 15 minutes (Xiong et al., 2019).

Standard curve was plotted with the concentration of nitrite sodium ranging from 0-100 µM in the X-axis and absorbance in the Y-axis. The concentration of each sample was calculated from the equation of the standard curve based on its absorbance.

3.6. Neutral Red Staining (NR)

Neutral red (Sigma-Aldrich Chemical Co.) is an important dye, which is mainly accumulated in lysosomes by cell endocytosis. The best staining of macrophages in living embryos is 2.5 µg/mL NR solution, containing 0.003% PTU (Phenylthiourea), and stained in a dark environment at 28.5 °C for 6-8 hours (Herbomel et al., 2001). After staining, the movement of macrophages was observed with a dissecting microscope (IX71, Olympus, Tokyo, Japan).

3.7. Sudan Black Staining (SB)

Sudan Black B is often used to stain the granules of granulocytes. After staining, the granules are easier to be differenced from any other cell structures. Neutrophils, the granules of granulocytes, can be stained by SB and then washed with a large amount of 70% ethanol, so neutrophils show highly specific staining (Sheehan & Storey, 1946)

Stock solution was formulated as 0.6 g of SB powder dissolved in 200 mL of pure ethanol, filtered and stored at 4 °C. Buffer solution was formulated as 16 g of phenol, dissolved in 30 mL of ethanol, and 0.3 g of $Na_2HPO_4 \cdot 12H_2O$ was added to dissolve in 100 mL of distilled water. Working staining solution was formulated as 30 mL stock solution and 20 mL buffer solution, mixed and filtered. Embryos were fixed with 4% methanol-free paraformaldehyde (PFA) (dissolved in PBS) and allowed to react for 2 hours at room temperature. After washing with PBS, samples were stained with SB for 40 minutes, and then washed with 70% ethanol. After washing, the sample rehydrated to

PBS containing 0.1% Tween 20, and then image J 1.46r software was used to quantify the results. (Le et al., 2008).

3.8. Statistic Analysis

Statistical analysis of data was done by using SPSS 24.0 software and all data were presented in means ± standard deviation. Significant differences of data among groups were determined using two-way ANOVA and Duncan's new multiple-range test as post hoc. A value of $P < 0.05$ was accepted as indication of significant difference.