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EFFECTS OF HIGH DOSE OF *Heterotrigona itama* KELULUT (STINGLESS BEE) HONEY FOR SHORT-TERM ORAL CONSUMPTION

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Abstract: Recently, Kelulut honey has gained popularity and has become a preferred and most sought-after honey for its medicinal properties. However, only some systemic studies are available with regards to the curative and medicinal properties of the honey, this leaves consumer without a safety net or quality guarantee. Therefore, this study was designed and undertaken in order to evaluate the effect of kelulut honey, consumption harvested from the *Heterotrigona itama* species of stingless bee, an acute oral toxicity study, which adopted the Organisation for Economic Co-operation and Development (OECD) test guideline 423, which tested rats with two high doses of honey at 2,000 mg/kg and 5,000 mg/kg for 14 days. The rats were observed for signs of toxicity and other abnormalities after the first dose was consumed. The rats were observed and analysed both physically, biochemically, and histopathology. There were no observable toxic effects on the rats dosed with kelulut honey, as evidenced by the fact that even at the highest dose (5,000 mg/kg) the rats did not show any signs of toxicity or mortality in the rats and did not significantly differ physically or biochemically when compared to rats in the control group on a diet of distilled water. Kelulut honey's lethal dose surpassed 5,000 mg/kg and was categorised as being in Category 5 according to the Globally Harmonised System and was safe to consume it in high doses. However, further subacute toxicity studies are needed to evaluate the effects of long-term daily consumption of kelulut honey.

Keywords: Acute oral toxicity, kelulut honey, *Heterotrigona itama*, Malaysian honey, stingless bee.

Introduction

Kelulut is a stingless bee species from the Meliponinae subfamily of Apidae family. These bees are thought to be the oldest bee species in existence as a fossil of a female stingless bee encased in amber had been discovered. Carbon dating marked the fossil as being at least 80 million years old (Crane, 1999).

However, honey harvested from these stingless bees has not been investigated as thoroughly as honey from stinging bees, especially those of the *Apis* sp. Moreover, *Apis* sp. bees produce honey in larger quantities, which is easier to harvest and has high economic potential, making them the primary choice for commercial use and research.

In contrast, stingless bees are mostly found in tropical regions near the equator and produce smaller quantities of honey, resulting in less scientific focus especially at the international level (Grüter & Grüter, 2020). In spite of that, stingless bees or kelulut honey is in great demand these days due to its being more beneficial than honey from the *Apis* sp. bee species (Melia *et al*., 2024). Additionally, kelulut honey possesses a higher nutritional and therapeutic value (Gadge *et al*., 2024; Krishnappa & Sekarappa, 2024; Zulkifli *et al*., 2024).

In general, kelulut honey contains approximately 70%-75% carbohydrates, 20%-25% water, a lower pH of 3.00-3.27, and a higher free acidity of 107.50-246.25 meq/kg of honey compared to *Apis* honey (Shamsudin *et al*., 2019). Both honeys have high fructose and glucose concentrations with lower amounts of organic acids, phenolic acids, amino acids, vitamins, minerals, enzymes, and other phytochemicals, making honey the main source of carbohydrates for the bees (Shamsudin *et al*., 2019; Sujanto *et al*., 2021).

The medicinal effects of honey are greatly enhanced by the presence of numerous beneficial compounds. One of these is trehalulose, a glucose disaccharide that has the ability to prevent obesity by browning white adipose tissue and preventing the formation of white adiposity (Zulkifli *et al*., 2023).

Another research paper by Zulkifli *et al*. (2024) found that honey is a viable alternative to prescription medications for improving sleep patterns because of its distinct makeup and calming properties. The presence of various beneficial compounds gives honey a wide range of therapeutic effects that can benefit consumers. One of these benefits is a disaccharide glucose known as trehalulose that can prevent obesity by causing browning of white adipose tissue and inhibition of white adiposity growth (Zulkifli *et al*., 2023). Honey also has significant antioxidant properties as well. Furthermore, honey retains its antioxidant capabilities even when stored for an extended period of time, provided it is stored properly (Monggudal *et al*., 2018). The antioxidant qualities of honey can help with the wide array of disease prevention strategies, including ageing, cancer, heart disease, inflammation, neurological degeneration, wound healing, and infections (Pimentel *et al*., 2021).

As a result, honey is a safe-to-consume food product. However, the effects of high concentrations of kelulut honey consumption are still unknown.

At this point of time, Kelulut honey is considered new and untested. Therefore, no international standard is available to measure the quality or safety of honey. Esa *et al*. (2022) also noted that kelulut honey often falls short of existing quality standards, underscoring the need for a distinct set of criteria tailored specifically for it (Biluca *et al*., 2016). Moreover, all stingless bee honey from Malaysia, Brazil, Thailand, and Venezuela showed significant variations from the CODEX Standard for Honey (CODEX Stan 12-1981, 2001) values (Omar *et al*., 2019). Meanwhile, Braghini *et al*. (2021) stated that numerous reports indicate that kelulut honey does not meet these quality standards, highlighting the need for a distinct standard of its own. With the increase in consumers' interest in the use and benefits of kelulut honey, in addition to significant promotional activities by government agencies, a systemic toxicity study needs to be conducted on the honey urgently to ensure consumer health and safety.

Moreover, the data are useful as it adds value to the local honey and will be used for the future commercialisation of the honey at a higher level (Ismail *et al*., 2018). Thus, this study was conducted to observe the acute toxicity effects on the consumption of stingless bee honey collected from the local farm using animal models.

Materials and Methods

Sample Collection

Local kelulut honey from the stingless bee species *Heterotrigona itama* was obtained from Kelulut Park in the Malaysian Agricultural Research and Development Institute (MARDI) Centre, Serdang, Selangor, Malaysia. The honey was kept at 4°C in glass bottles, away from direct sunlight. Abscisic acid (ABA), diethyl ether, sodium chloride, sodium hydroxide, Harris haematoxylin, and eosin (H&E) were purchased from Sigma-Aldrich. Xylene, formalin, and ethanol were obtained from MERCK. Paraffin plasticised pellets were purchased from R&M Chemical.

Preparation of Test Samples

Based on the rats' body weight, an acute oral toxicity test of kelulut honey at 2,000 mg/kg and 5,000 mg/kg was conducted orally using an oral gavage (No *et al*., 2002; Samat *et al*., 2014).

The dose selection followed the Organisation for Economic Co-operation and Development (OECD) 423 guidelines for oral toxicity, starting with 2,000 mg/kg (a potentially lethal dose). An additional dose of 5,000 mg/kg was allowed to assess safety for consumers, animal health, or the environment. Distilled water was used as a negative control to mimic the current rats' conditions (handled identically) and 0.014 mg/kg of abscisic acid was used as a positive control. Abscisic acid was chosen to serve as a biomarker for quality control of the honey (Silva *et al*., 2013).

Experimental Animal Husbandry

The experimental protocol was approved by the Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Research Ethics Committee (UAPREC), Reference No. UAPREC/04/041. The test model was obtained from the Animal Laboratory, Faculty of Bioresources and Food Industry, UniSZA, Besut, Terengganu, Malaysia. At the commencement of the study, each rat was eight weeks old and weighed between 190 g and 230 g. Both genders male and female rats were used in this study since the different genders may have different tolerances and exhibit signs of toxicity differently (Haghighi *et al*., 2012). The rats were housed in a one rat per cage basis and kept in standard environmental conditions at an ambient temperature of between 21 ± 3°C and 40%-65% relative humidity, with a 12-hour light/dark cycle, fed with certified rodent food from Australia, and drinking water was available *ad libitum*. The animals were acclimatised for a week before and each test model was given doses according to their body weight.

Acute Oral Toxicity Test

With modifications, this study performed an acute oral toxicity test based on the OECD guidelines for testing chemicals, TG 423 (No *et al*., 2002). It measures adverse effects after a single dose of oral substance administration. From this experiment, the median lethal dose was determined using a fixed dose procedure and the value was expressed in terms of the weight of the test substance per unit weight of the test animal (mg/kg) (No *et al*., 2002).

Initially, rats were made to fast overnight, except for water, and weighed before the experiment. About 24 healthy male and female Sprague-Dawley (SD) rats were used to test for acute toxicity single dose of the sample being consumed. The rats were divided into four groups with six sample sizes for male (n = 6) and female (n = 6) rats. The four groups were (1) distilled water; negative control (C1), (2) abscisic acid 0.014 mg/kg (ABA concentration as per ratio of 5,000 mg/kg) calculated from the identification and quantification of the marker compound abscisic acid in kelulut honey from Ultra Performance Liquid Chromatography (UPLC) screening; positive control (C2), (3) the concentration of 2,000 mg/kg (the highest fixed levels chosen), which are most likely to result in mortality according to OECD TG 423; treatment 1 (T1) and (4) the concentration of 5,000 mg/kg (in case 2,000 mg/kg of kelulut honey may not produce mortality); treatment 2 (T2) (No *et al*., 2002; Samat *et al*., 2014).

Physical Observation

Mortality and the clinical signs of toxicity, including changes in the skin, fur, eyes, mucous membranes, and behavioural patterns were systematically recorded individually by observation for 0.5, 1, 2, 3, and 4 hours after administration and then once per day for the next 14 days (No *et al*., 2002). In addition, observations of convulsions, tremors, diarrhoea, salivation, lethargy, sleep, and coma were also monitored (Al-Afifi, 2018).

Body Weight and Meal Pattern Analysis

Each rat's Body Weight (BW) was recorded once per week and the differences in BW were noted. Each body weight was calculated using the following equation (Al-Afifi, 2018):

$$\text{BW} = \frac{[\text{Body weight at the end of each week - Initial body weight}]}{\text{Initial body weight}} \times 100\% \quad (1)$$

The commercialised pellets were weighted daily and given without honey in the meal pattern analysis. The pellet food consumed was measured by subtracting from the initial quantity of food and summing up the total weekly (Samat *et al*., 2014).

Anaesthesia of Rats and Blood Collection

The rats were generally anaesthetised to collect blood and sacrificed using diethyl ether following the method outlined by Tuffery (1995). The rats were fasted overnight for blood collection on day 14 and the anaesthesia procedure was conducted the following day. For the anaesthesia procedure, 20 mL of 20% diethyl ether was soaked in a pad or gauze and then placed in the euthanasia chamber. The chamber was closed for five minutes to let the anaesthetic agent vaporise. The animal was placed in the chamber individually for 10 minutes until it was rendered unconscious, with no tickle on its whiskers and no pain reaction when pressed on its paws. As soon as the animal's unconscious state was verified, blood was collected from the specimens immediately. The anaesthetised rats were given cardiac punches to collect blood samples of between 4 mL and 5 mL using a 5 mL syringe and 18 G needles for complete blood count (haematological test for red blood cells, platelet, lymphocyte, monocyte, and eosinophil) and serum biochemical analysis (urea, total cholesterol, triglyceride, and enzyme for liver function test). Then, the rats were sacrificed via cervical dislocation (Al-Afifi, 2018).

Organ Collection

The vital organs of each rat were collected, including the heart, kidney, lung, spleen, and liver to measure their Relative Organ Weight (ROW). A gross observation was conducted for any signs of abnormality and lesions due to any effects of the honey administration (Samat *et al*., 2014). The organs were then carefully dissected, cleaned of any fat, and weighed. The ROW of each organ was calculated using the following equation (Balijepallim *et al*., 2015):

$$ROW = \frac{\text{Absolute organ weight (g)}}{\text{Body weight of rat on sacrifice day (g)}} \times 100\% \qquad (2)$$

Then, kidney and liver specimens (the major internal body filter for toxic substances) were fixed in 10% buffered formalin for histopathological processes (Tuffery *et al*., 1995). The samples were fixed for 24 hours before the tissue processor machine proceeded with the dehydration process. The hardened specimens were embedded into moulds with paraffin wax sectioned into 5 µm using a microtome and stained with H&E staining (Junquira *et al*., 2005).

Statistical Analysis

Data was analysed using Statistical Package for Social Sciences (SPSS) version 20.0. The differences between group means were determined by Student's t-test and one-way ANOVA, followed by Tukey post hoc test. $P < 0.05$ is considered significant.

Results and Discussion

Physical Analysis

In Malaysia, kelulut honey is commonly consumed by locals as it is believed that its medicinal properties are better than that also mostly available honey produced by honeybees (common bees from the *Apis* sp. (Kek *et al*., 2014). However, scientific data on the medical effectiveness of kelulut honey still needs to be elasticated in comparison to the honey harvested from the honeybees (Omar *et al*., 2019). Therefore, using an animal model, this study evaluated the impact of oral consumption in high doses for a short period on bodily systems. The results of this test can be used as supportive research for medicinal treatments in the future. This study chose abscisic acid (ABA) as a positive control because the plant hormone was an indicator of antioxidant compounds in kelulut honey and may act as a biomarker for the quality control of the honey (Silva *et al*., 2013; Stefanis *et al*., 2023).

ABA acts as a central regulator for many plants and is a phytohormone that signals the removal of Reactive Oxygen Species (ROS) when the plant is stressed by environmental changes (Sah *et al*., 2016). Many studies reported that ABA was consistently present in kelulut honey from different continents (Silva *et al*., 2013; Massaro *et al*., 2014; Al-Hatamleh *et al*., 2020).

Kelulut honey dosages in this study was set at 2,000 mg/kg and 5,000 mg/kg body weight. The dose selection was based on the OECD 423 for oral toxicity study, according to which a dose expected to cause death should be considered a starting dose (2,000 mg/kg). Apart from that, the OECD guideline also stated that another dose of 5,000 mg/kg was permitted for testing on the safety of the consumer, animal health, or the environment. The high 5,000 mg/kg dose may persist in curing various illnesses. Thus, the 5,000 mg/kg dose was chosen as another dose conducted for this study (No, 2002). In addition, the 2,000 mg/kg kelulut honey dose was equal to 324.32 mg/kg for human consumption by adults (Reagan-Shaw *et al*., 2008). Meanwhile, the 5,000 mg/kg dose of kelulut honey was similar to the maximum dose of 810.8 mg/kg kelulut honey that may be taken by an adult (Reagan-Shaw *et al*., 2008).

From the first day after the honey consumption until day 15 when the test subjects were euthanised, no abnormal signs were observed physically in any of the specimens. As such, there were no toxicity signs such as vomiting, diarrhoea, lethargy, and panting. There was also no eye and fur colour changes or excessive fur shedding. The outcome coincides with the study by Samat *et al*. (2014) and Saiful Yazan *et al*. (2016), which showed no physical abnormalities throughout the 15 days of the study. The study by Samat *et al*. (2014) also found no abnormality, no signs of toxicity, and no differences in the behaviour of the rats treated with the highest dose (2,000 mg/kg) of honey of *Apis* sp., respectively.

Another study by Saiful Yazan *et al*. (2016), with a 1,183 mg/kg body weight dose of kelulut honey, which mimics double the intake of usual human consumption of kelulut honey was found to have no toxicity in the rats. According to Mohd Azam *et al*. (2022), honey at a daily dose of 2,000 mg/kg had no harmful effects on rats and was categorised as safe for consumption in high doses at Category 5 of the Globally Harmonised System of Classification and Labelling of Chemicals. Honey consumption has also been linked to improved immunological function, normal weight increase, and food intake control (Mohd Azam *et al*., 2022).

Interestingly, this study found that T1 male rats, who consumed 2,000 mg/kg of kelulut honey were immediately very active around five minutes after consumption while T2 male rats, who consumed 5,000 mg/kg of kelulut honey were active around 15 minutes after the consumption. The T1 and T2 female rats were slightly active after about half an hour after consumption. At the same time, the positive and negative control of both genders were inactive having curled almost immediately after consumption. The lack of increased activity levels of the rats was assumed to be due to the presence of simple sugars (monosaccharides fructose and glucose) in kelulut honey, which can provide instant energy (Eteraf-Oskouei & Najafi, 2013; Baglio & Baglio, 2018).

Curling up is also a normal behaviour for rats, especially when they are resting or trying, to conserve body heat, feel secure, and relaxed (Suckow *et al*., 2019).

Body Weight and Food Intake

The motivation percentage of the tested rats was calculated in accordance with the body weight of each specimen. The data in Figure 1 showed an increment in body weight throughout the 14-day study period. Overall, the consumption of honey had no damaging or toxic effects. However, the effects of toxins can only be inferred if a rat loses at least 20% of its original body weight (Olfert & Godson, 2000). From the data, the percentage of body weight increment for C1 (negative control) was the highest, followed by C2 (positive control), T1 (2,000 mg/kg kelulut honey), and T2 (5,000 mg/kg kelulut honey) for male rats [Figure 1 (A)].

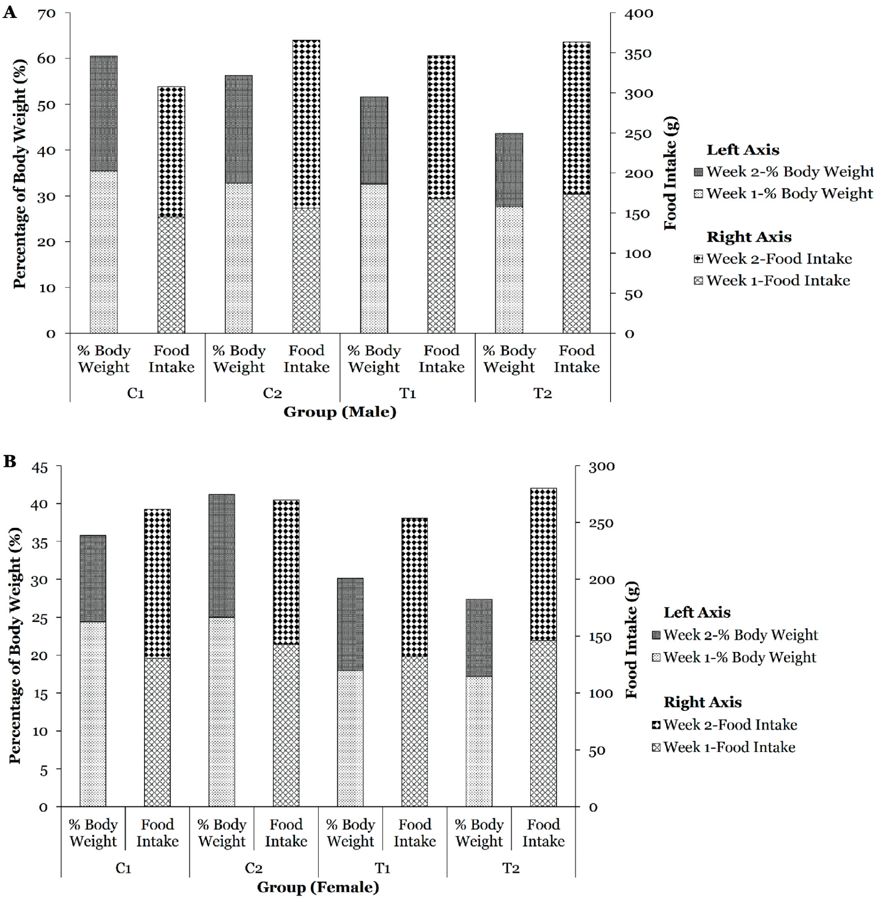


Figure 1: Percentage body weight (%) and total food intake (g) for acute toxicity of honey consumption, (A) male rats and (B) female rats; C1: distilled water; C2: 0.014 mg/kg abscisic acid; T1: 2,000 mg/kg kelulut honey; T2: 5,000 mg/kg kelulut honey for 14 days on both gender

Interestingly, the data obtained showed that the food intake of C2 and T2 was higher compared to C1. This suggests that the consumption of kelulut honey is a body weight control even with an increased or high food intake (Rafie *et al*., 2018). These results were the same for females as well with the lowest percentage of body weight increment, but the highest food intake was observed in T2 [Figure 1 (B)]. However, no significant difference was seen in the one-way ANOVA analysis for both body weight percentages and food intake in both genders.

A study by Rafie *et al*. (2018) supported this outcome as it showed a decrease in body weight and a percentage of body weight gain for rats induced with a high-fat diet, which were treated with the same kelulut honey samples from *H. itama*.

Furthermore, the test subjects' food intake also acts as an indicator of toxicity. A toxic substance may affect the metabolism and is often detected with differences in clinical pathology and histopathology (Moriyama *et al*., 2008). A study by Soti *et al*. (2019) unexpectedly found that a dietary phytochemical of ABA can act as an appetite control and energy balancer in mammals. They found that microinjection of phytohormone ABA reduced body weight while not disturbing food appetite. However, in this study, kelulut honey also contained other compounds that might explain the negative and positive effects on food intake after consumption.

A study by Teff *et al*. (2004) discussed the effect of fructose on suppressing ghrelin less than glucose, which may be one of the reasons for the increase in food appetite. Besides, kelulut honey also contains another compound such as sucrose, which also suppresses ghrelin less than glucose and may also cause an increase in food intake.

Honey has a lower Glycaemic Index (GI) than many processed sweets, despite the fact that it contains simple sugars. A lower GI causes blood glucose and insulin levels to rise more gradually, which may lessen fat accumulation while still satiating the desires for sweets (Zamanian & Azizi-Soleiman, 2020; Abu Bakar, 2024). In short, by consuming honey, we can increase appetite while lowering rich chances of ingesting too many calories or fats over time.

Relative Organ Weight

A tissue profile is a crucial evaluation used to determine the toxicity of a substance since it often makes direct contact with the substances in question. Abnormalities in ROW, whether significantly higher or lower than that of the negative control group may indicate the toxic effects of the substances being consumed. In this study, the kidney and liver of all treatments showed insignificant differences in ROW. However, the data (Figure 2) discovered a reduction in liver ROW in both genders on a kelulut honey diet, hinting at the possibility of an anti-obesity effect (Rafie *et al*., 2018; Arora & Marwaha, 2022). However, female T2 liver ROW was slightly higher compared with the control group, C1, but the gains were not significant. This outcome raises a few questions since kelulut honey is rich in antioxidants and is more likely to improve liver function (Silva *et al*., 2013; Stefanis *et al*., 2023).

Coincidently, Samat *et al*. (2014) also reported a higher ROW with honey intake compared to the control and the differences were suggested to be due to the variation in the size of the internal organ of each rat. However, additional histopathology observations need to be conducted to confirm if kelulut honey is safe for consumption.

Haematological Analysis

An analysis of the components in the blood of the specimens gave this researcher a better understanding of the effects of kelulut honey on the body as blood is the primary means of transportation throughout the body (Al-Afifi *et al*., 2018). From the results (Figure 3), all

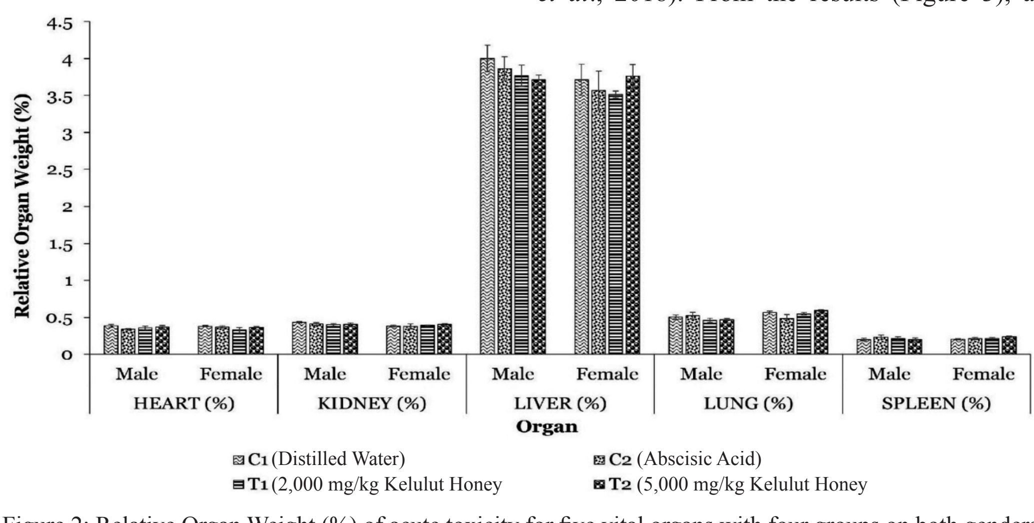


Figure 2: Relative Organ Weight (%) of acute toxicity for five vital organs with four groups on both genders (14 days). Values are expressed as mean ± SEM (n = 5)

blood parameters were in the physiological normal range except for the Total Red Blood Cell (TRBC) of groups C2 and T1 female rats. The TRBC data showed that C2 had the lowest count compared to other groups, indicating that consumption of ABA may reduce the TRBC count [Figure 3 (A)].

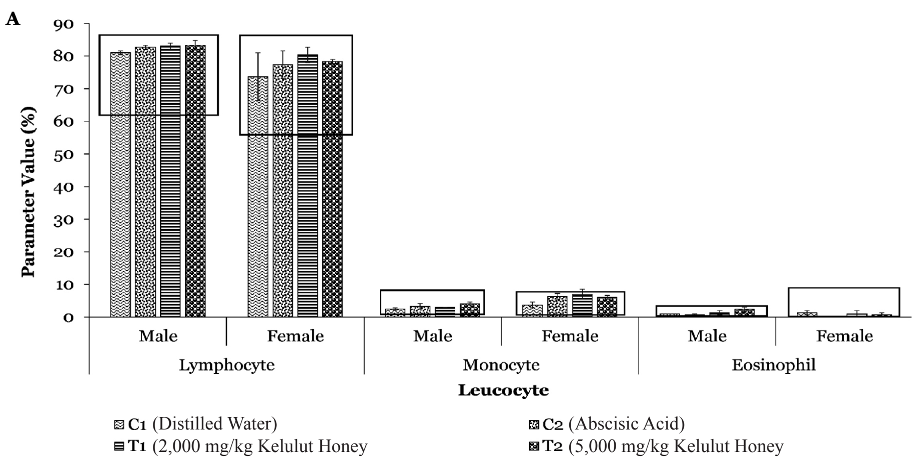
However, the TRBC count increased in the groups on a diet of kelulut honey. In fact, the TRBC count rose in tandem with the increase in kelulut honey intake. This outcome can be explained by the presence of iron in kelulut honey (Ajibola *et al*., 2012), which is an important component in erythropoiesis (Abbaspour *et al*., 2014).

Generally, honey is also known to improve anaemic conditions by increasing haemoglobin concentrations, escalating red blood cell count, improving haematocrit value, and boosting the immune system (Chepulis, 2007; Abbaspour *et al*., 2014; Ajibola, 2015). Additionally, the study by Saiful Yazan *et al*. (2016) reported higher TRBC, haemoglobin, and white blood cell counts after the consumption of kelulut honey at 1,183 mg/kg compared to the negative control (distilled water). This outcome coincides with the findings in this research paper, which used a higher dose of kelulut honey at 2,000 mg/kg, T1 and 5,000 mg/kg, T2. Al-Zail *et al*. (2023) also found that the consumption of honey improved haematological changes in the albino rats after being exposed to cigarette smoke. Other than the TRBC and platelet count, the consumption of kelulut honey resulted in a higher differential white blood cell count compared to C1 (negative control, distilled water) [Figure 3 (B)]. Since honey is a massive source of antioxidants, it helps to remove ROS, boosts the immune system, and prevents cell damage (Tuksitha *et al*., 2018).

Various diseases linked to ROS activity, including heart disease, diabetes, cancers, and ageing can be treated with honey. Antioxidants such as those available in kelulut honey can provide an electron to unpaired valence electrons within free radicals to prevent the name from harming the cells (Silva *et al*., 2013; Kek *et al*., 2014). This would help with the regulation and function of leucocytes and improve overall body health (Al-Kafaween *et al*., 2022).

Serum Biochemical Analysis

A serum biochemical analysis was conducted on the blood, collected on day 15 of the experiment. The serum was collected to perform liver and kidney function tests as well as a lipid profile analysis on the blood, following kelulut honey consumption. Lipids are one of the most important macromolecules in the body measured in the analysis. Lipids act as energy storage units, cellular communication devices, insulators, and protectors of internal organs. It is also a component of the phospholipid bilayer (Henne *et al*., 2020).



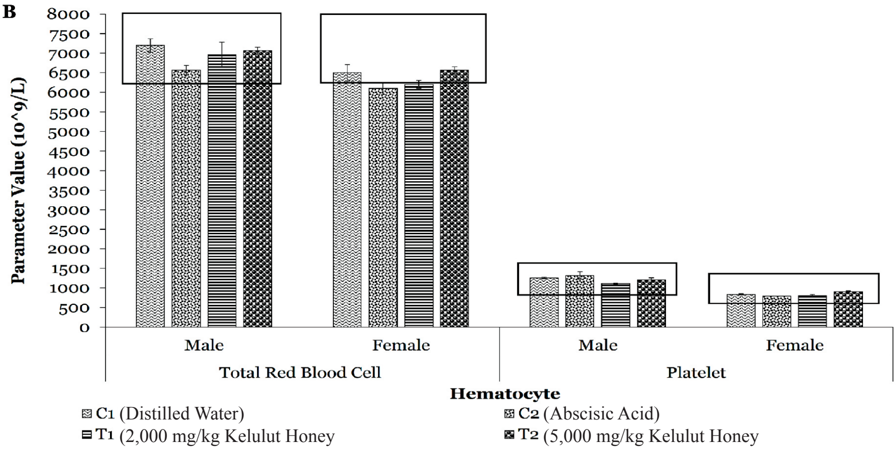


Figure 3: Effect of consumption of kelulut honey and the controls (A) haematology parameter of lymphocyte, monocyte, and eosinophil, (B) haematology parameter of Total Red Blood Cells (TRBC) and platelet for acute toxicity of each treatment for 14 days on both genders. Values are expressed as mean ± SEM (n = 5). The boxes indicate the normal range, TRBC: 6.39 - 8.01 x 10^9/L (both gender); platelet: 735 - 1418 x 10^9/L (male), 674 - 1373 x 10^9/L (female); lymphocyte: 61% - 86% (male), 55% - 86% (female); monocyte: 0.20% - 8.0% (male), 0.20% - 7.5% (female); eosinophil: 0.30% - 3.25% (male), 0.30% - 10.0% (female)

Lipids are derived from foods that contain excess glucose conversion and stored for energy usage (Henne *et al*., 2020). In this study, the lipid becomes one of the toxicity indicators since honey is one of the high sugar-containing foods and has been speculated to cause obesity and the failure of vital internal organs such as the liver and kidneys.

However, our data showed the effectiveness of kelulut honey consumption (T1 and T2) in lowering the value of total blood cholesterol and triglyceride of the rats [Figure 4 (A)]. This outcome coincides with a study by Rafie *et al*. (2018), which found a reduction in adiposity index for high-fat diet induced-specimens after consuming kelulut honey (adiposity index: 4.6 ± 0.5%) compared with those that did not consume kelulut honey (adiposity index: 10.1 ± 0.5%). The possible mechanism elaborating the anti-obesity effect was due to the presence of coumaric and caffein acid in kelulut honey which has been reported to possess anti-obesity properties and can regulate the G1 cell cycle arrest in 3T3-preadipocytes (Henne *et al*., 2020). Zulkifli *et al*. (2022) also suggested that the consumption of honey can reduce glucose, triglycerides, cholesterol, and LDL via adipolysis, enhance insulin sensitivity, and improve glucose homeostasis in adipocyte cells within the bloodstream. Kelulut honey also reduces the amount of urea in the rats, which can cause bodily intoxication at high concentrations [Figure 4 (A)].

Apart from that, the liver, which acts as a filter in the body, significantly influences the results of the toxicity study. Therefore, the serum was also collected for liver function tests, which measure the value of enzymes such as alkaline phosphatase (ALP), alanine transaminase (ALT), and aspartate transaminase (AST), that indicate the performance of the liver (Samat *et al*., 2014). These enzymes serve as a functional indicator for cellular tissue damage and help to identify structural tissue damage apart from histology.

As a result, the lower value of these enzymes indicates the good condition of the liver while the high value displays the damage and enzyme leakage from the cellular membranes (Al-Afifi *et al*., 2018). Based on the obtained data, the values of those parameters for rats with kelulut honey consumption, T1 and T2 showed lower liver enzymes than the negative control, C1 [Figure 4 (B)]. Therefore, this data suggests that kelulut honey at a dose of 2,000 mg/kg (T1) and 5,000 mg/kg (T2) may aid in improving liver function. A previous study by Rafie *et al*. (2018) reported that treatment of rats induced with a high-fat diet and 1,000 mg/kg dose of kelulut honey showed a reduction in the enzyme precursor compared with the untreated control. Interestingly, our study with a higher intake of honey dosage showed lower enzyme accumulation in blood serum compared to the negative control, C1, indicating the safety of Kelulut honey.

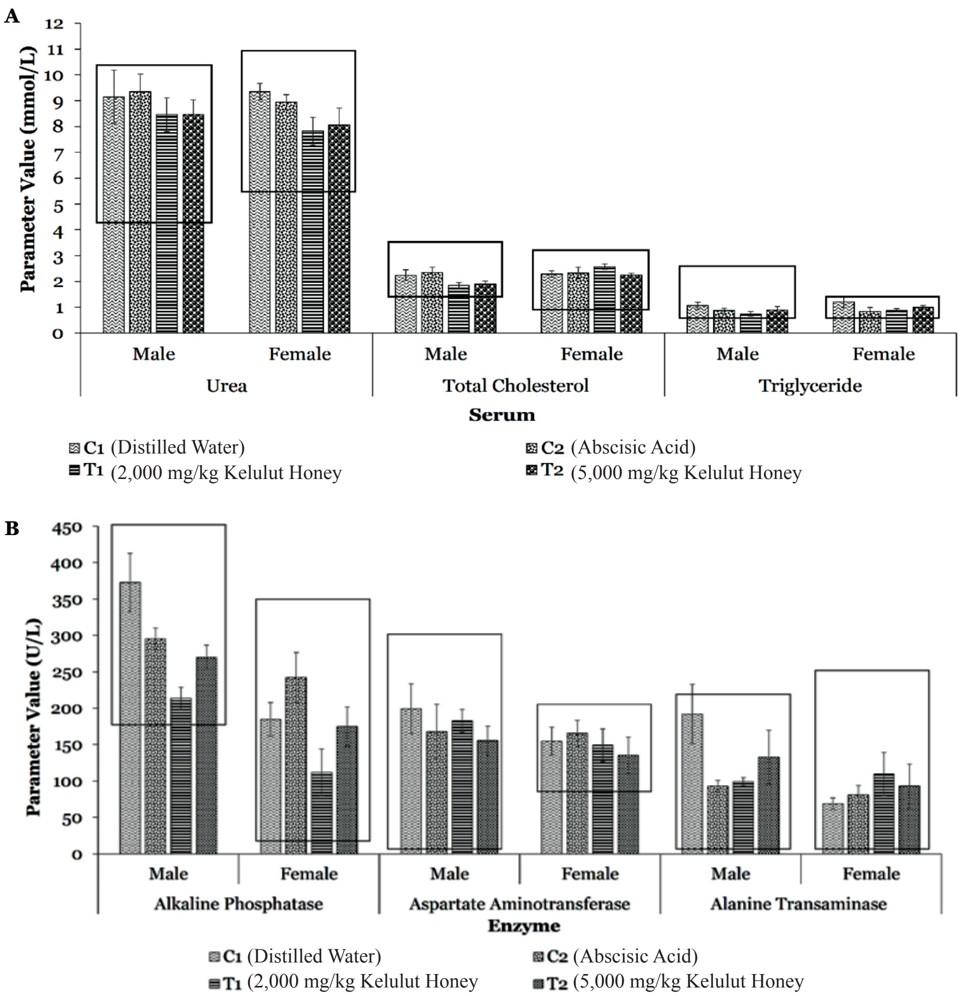


Figure 4: Analysis of rat serum from blood samples (A) some biochemical parameters of urea, total cholesterol, and triglyceride, (B) parameter of liver function test for acute toxicity of each treatment for 14 days on both genders. Values are expressed as mean ± SEM (n = 5). The boxes indicate the normal range, urea: 4.32 - 10.30 mmol/L (male), 5.56 - 11.01 mmol/L (female), total cholesterol: 1.14 - 3.60 mmol/L (male), 0.83 - 3.26 mmol/L (female), triglyceride: 0.60 - 2.86 mmol/L (male), 0.52 - 1.50 mmol/L (female), alkaline phosphatase: 180 - 450 U/L (male), 26 - 350 U/L (female), aspartate aminotransferase: 2 - 300 U/L (male), 80 - 203 (female), alanine transaminase: 1 - 223 U/L (male), 2 - 250 (female)

Organ Histopathological Analysis

The basic unit of a liver is a hepatocyte, generally called the liver cell. Hepatocytes comprise most of the liver density and have a specific function to detoxify metabolites, synthesise proteins, and build biochemicals necessary for growth and digestion (Nahmias *et al*., 2006; Ramli *et al*., 2019). Hepatocyte damage is usually associated with intoxication due to heavy drinking, which causes the inability of hepatocytes to expel toxins from the body (Tallarico *et al*., 2020). The condition also causes liver steatosis or steatohepatitis which increases liver weight and causes the visible accumulation of fats in the liver cells (Tallarico *et al*., 2020). This condition is also prone in diabetes patients due to high fat in the body.

Fortunately, there were no visible fat droplets in any of the rats' livers from our study. However, based on the liver histology (Figure 5), signs of mononuclear cell infiltration in the central vein for all rats' liver were found except for the negative control, C1 (male and female) and T2 male. According to Krishna (2017), mononuclear cell infiltration is one of the precursors of liver damage.

However, mononuclear cell infiltration was observed less in liver organs in Figures 5 (d) and (f). Thus, kelulut honey can improve liver function at different doses for different genders. However, the data was obtained after the 14th day of consumption, which may have caused the effect of kelulut honey to become less effective. According to Rafie *et al*. (2018), the daily consumption of 1,000 mg/kg of kelulut honey in induced-obese rats was demonstrated to reduce adipocyte hypertrophy compared to non-treated obese rats. Therefore, further study on the daily consumption of kelulut honey may need to be conducted to evaluate more detailed effects of kelulut honey consumption on liver cells.

For the histological analysis of the kidneys, the segments analysed were the renal corpuscles, each comprising the glomerulus and Bowman's capsule with the proximal convoluted tubules (Figure 5). The Bowman's capsules were all in fine round shapes, indicating no cell damage had occurred. Overall, there were no visible toxicity signs on the kidney structure such as necrosis of proximal and distal tubular or sloughing of tubular epithelial cells in both genders of rats in all groups. There were also no visible signs of kidney toxicity or degeneration, for instance, interstitial inflammatory cells, glomerular atrophy, and capillary congestion (Abdel-Salam *et al*., 2014). A study from Abdel-Moneim and Ghafeer (2007) also revealed the protective effect of natural honey against renal tubule toxicity from CdCl2 by lessening the lipid peroxidation and improving the tissue activity of enzyme glutathione (GSH) and glutathione peroxidase (GPx).

Conclusions

In conclusion, the medium lethal dose (LD_{50}) for kelulut honey exceeds 5,000 mg/kg of rats' body weight. Therefore, Kelulut honey is classified as being in Category 5 or unclassified according to the Globally Harmonised System and Chemical Labelling, which is the safest level in acute oral toxicity. Generally speaking, there was no significant difference between rats consumed kelulut honey and rats in the control group, which indicates that kelulut honey does not cause any undesirable effects on the physical or chemical nature of an organism while improving health.

Based on the data, this research paper proposes that kelulut honey may have an anti-obesity effect, improve immune function, and act as an energy source. Remarkably, the effect of kelulut honey consumption may vary depending on gender and dosage. The dosage of kelulut honey may differ depending on the specific targeted function. The *in vivo* study also suggests the practical application of kelulut honey as a supplement to improve body health.

Therefore, further study must be done to evaluate the long-term effect of daily consumption of kelulut honey and its ability to

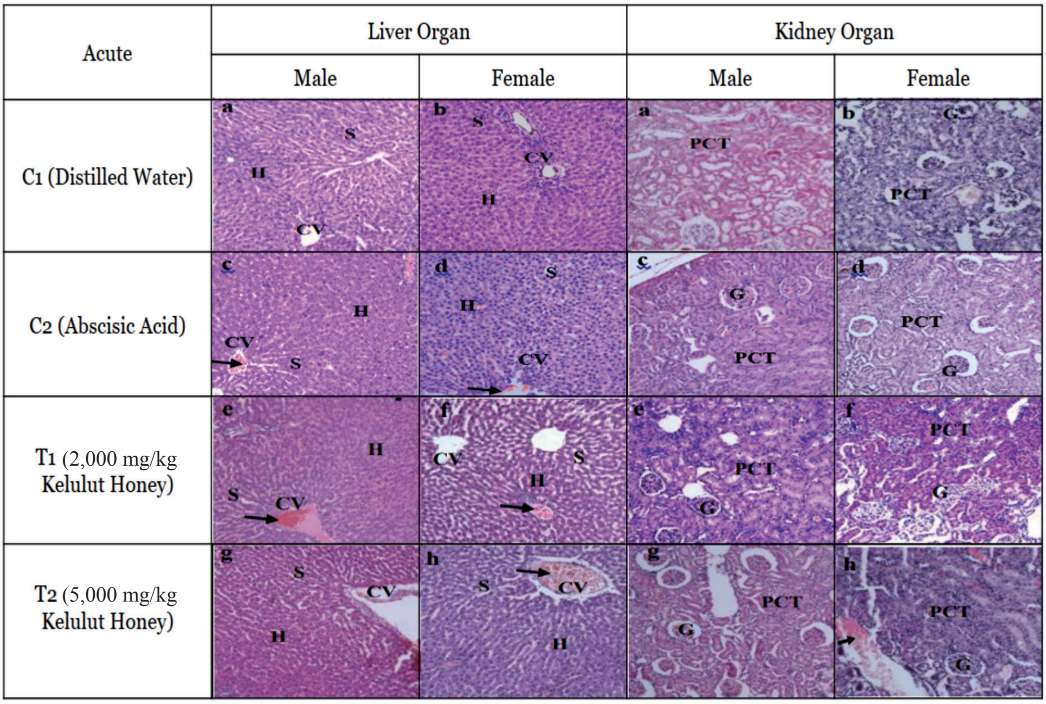


Figure 5: Acute toxicity of haematoxylin and eosin (H&E) staining of liver and kidney organs, with each left panel representative for male rats while right panel female rats – **a**, **b** kidney and liver sections from rats treated with distilled water (negative control) showing normal histological structure with hepatocytes (H), sinusoids (S) and Central Vein (CV) for liver while for kidney glomerulus (G), and Proximal Convoluted Tubules (PCT); **c**, **d** treated with abscisic acid with c and d in liver organs showed mononuclear cell infiltration (long arrow); e, f treatment of 2,000 mg/kg Kelulut Honey showed mononuclear cell infiltration and normal histology of kidney structure; g, h treated with 5,000 mg/kg Kelulut Honey with h (female liver, T2) also visible of mononuclear cell infiltration while kidney organs showed normal structure of kidney tissue g while h (female kidney, T2) showed haemorrhage in some parts of the interstitial tissue (long arrow) [H&E, 100x]

improve health. Furthermore, the mechanism of action of honey in treating various diseases is still not well understood and requires extensive exploration.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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