In vivo study of subacute oral toxicity of kelulut honey


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Abstract
Kelulut honey or also known as stingless bee honey is favoured for its tremendous nutritional benefits. However, the lack of systematic safety studies leads to it having no quality control or safety guarantee for the consumers. Consequently, the present work was designed to assess the effect of daily kelulut honey consumption. Subacute oral toxicity study was conducted following the Organization for Economic Co-operation and Development (OECD) test guideline 407. Sprague Dawley rats were administered with kelulut honey at the concentrations of 500, 1,000, and 2,000 mg/kg for four weeks, and observed for any changes or toxicity signs following daily consumption. The rats were physically and biochemically analysed, and the serum of highest honey concentration (2,000 mg/kg) consumption underwent metabolite analysis. Histopathology observations on the kidney and liver were also performed. The highest concentration of kelulut honey did not show any mortality or toxicity. Overall, there were no significant differences in all parameters, physically and biochemically, as compared to the control (distilled water), thus indicating the absence of toxicity of kelulut honey daily consumption. It was found that kelulut honey consumption demonstrated generally good health effects, such as in controlling food intake, weight gain, and increasing immune function. The honey’s lethal medium dose surpassed 2,000 mg/kg, thus classified in category 5 according to the Globally Harmonized System of Classification and Labelling of Chemicals, which means that it is safe to consume at a high dose.

Keywords
subacute oral toxicity, kelulut honey, stingless bee honey, honey biomarker, in vivo study

Introduction
Stingless bee honey, also known as kelulut honey in Malaysia, is well-known for its high medicinal properties (Abu Bakar et al., 2017). Generally, honey such as Apis sp. honey, contains sugars, amino acids, organic acids, vitamins, and minerals (Abu Bakar et al., 2017). However, the sugars in kelulut honey are lower than that in common honey from Apis sp., whereas moisture and free acidity are higher (de Almeida-Muradian, 2013; Monggudal et al., 2018). Kelulut honey is less sticky, and has a tangy smell and distinct sour taste. Kelulut honey is produced by different stingless bee species such as Heterotrigona itama, Geniotrigona thoracica, and Tetragonula laeviceps. As the name implies, they have a reduced sting which makes them accessible among the beekeepers and in the honey production business which is called meliponiculture (Jaapar et al., 2016).

Kelulut honey is usually stored in a propolis pot-shaped storage container known for its high
antioxidant and anti-inflammatory properties (Abdullah et al., 2019). The propolis composition of fibres, proteins, and minerals indirectly diffuse and increase the nutritional value of kelulut honey (Abdullah et al., 2019). Unique preferences of stingless bees which forage on polyfloral sources in the lowest shrub contribute to this honey’s high medicinal value (Jaapar et al., 2016). Tiny body of stingless bees also allow them to sip nectar from the inner part of a flower, thus allowing for more nutrient content (Ab Hamid et al., 2016). This condition increases phenolic acids and flavonoids in it, thus leading to high antioxidant content (Ab Hamid et al., 2016). Furthermore, the watery feature enables the fermentation of osmophilic yeast carried by the bees, thus leading to the sour taste and antibacterial properties of kelulut honey (Abdullah et al., 2019). The fermentation of glucose in kelulut honey produces glycolic acid and hydrogen peroxide which kills the bacteria (Yaacob et al., 2018). Kelulut honey is also a good wound dresser due to these properties. The moisture in honey also helps prevent dehydration, and improves angiogenesis and collagen synthesis which further enhance wound healing (Zulkhairi Amin et al., 2018), while giving aesthetic effect and reducing wound pain (Yaacob et al., 2018). In honey, antibacterial properties fight bacterial infection, and antioxidant controls oxidative stress, thus accelerating wound healing (Zulkhairi Amin et al., 2018).

Apart from all the benefits of kelulut honey, it is not recorded in the Codex Alimentarius Standard due to its lack of information, especially in the systematic safety study. Differences in physicochemical properties and chemical compositions as compared to Apis sp. honey cause it to have different quality standards, thus stressing the demand for its individual standards. Thus far, there is no proper study regarding the toxicity of kelulut honey; thus, there is no safety guarantee on its consumption. Therefore, a daily systematic safety study is crucial, and was conducted in the present work to provide information regarding consumer health. The present work also provides baseline data for further medicinal and clinical studies in the future.

Materials and methods

Chemicals and reagents
Abscisic acid (ABA), diethyl ether, sodium chloride, sodium hydroxide, Harris haematoxylin and eosin (H&E), deuterium oxide (D₂O), and 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP) were purchased from Sigma-Aldrich (UK). Xylene, formalin, and ethanol were purchased from Merck (Germany). Paraffin plasticised pellets were purchased from R&M (UK).

Sample collection
Malaysian kelulut honey from H. itama was obtained from the Kelulut Park of Malaysian Agricultural Research and Development Institute (MARDI) Centre, MAEPS, Serdang, Selangor, Malaysia. The honey was then refrigerated at 4°C away from direct sunlight in amber glass bottles.

Preparation of kelulut honey
The kelulut honey was administered orally using oral wire gavage which entered directly into the rats’ stomach. Three different treatments at the doses of 500 (T1), 1,000 (T2), and 2,000 mg/kg (T3) were given based on the rats’ body weight (BW). Distilled water served as the negative control (C1), whereas 0.0056 mg/kg of ABA (C2) served as the positive control. Identification and quantification of the marker compound abscisic acid in kelulut honey was performed using ultra performance liquid chromatography (UPLC) according to Fu et al. (2012) with modifications.

Experimental animal husbandry
The study protocol was approved by the Universiti Sultan Zainal Abidin (UniSZA) Animal and Plant Research Ethics Committee (ref. no.: UAPREC/04/041; dated 04/01/2019). The rats were obtained from the Animal Laboratory, Faculty of Bioresources and Food Industry, UniSZA. Both male and female Sprague Dawley (SD) rats were used as the test model. Both genders were used due to differences in toxicity tolerance towards substances. Thirty male rats and 30 female rats were divided into five groups with six rats (n = 6) in each (following the resource equation calculation) for each gender. The rats used were eight weeks old (age of young adult), and weighed from 190 - 230 g (Samat et al., 2014; 2018). The rats were individually caged in standard environmental conditions under ambient temperature of 21 ± 3°C and 40 - 65% relative humidity, with a 12-h light/dark cycle, and fed with certified rodent food (Rodent Diet Specialty Feeds, Glen Forrest,
Australia) and water ad libitum. The animals were acclimatised a week before the beginning of the study, and individually labelled.

Subacute administration test and physical observation

The subacute oral test was performed based on the guidelines of the Organization for Economic Co-operation and Development (OECD) for the testing of chemicals, TG 407 (OECD, 2008) with modifications. This method measures the adverse effects after daily oral administration of a substance on several parameters such as general behaviour, reflexes, motor activities, respiratory patterns, and macroscopic changes on the skin and fur. From this experiment, the median lethal dose (LD<sub>50</sub>) was obtained, and the value was expressed with respect to the weight of the test substance per unit weight of the test animal (mg/kg).

Initially, rats were deprived of food every 3 h before dose consumption, and water was excluded to avoid any food scrap disturbance. For the subacute toxicity study, 30 healthy male and female SD rats were used. The rats were divided into five groups of six animals (following the resource equation calculation) for each gender. The dose selected was based on a previous study which revealed the safety of the highest dose of 2,000 mg/kg in an acute study (Samat et al., 2014). However, the effect of kelulut honey using this highest dose for daily consumption was still unknown. The next dose was reduced by half. Mortality and clinical toxicity signs were systematically recorded. Observations for severe effects including convulsions, tremors, diarrhoea, salivation, lethargy, sleep, and coma were also done at 0.5, 1, 2, and 4 h daily for four weeks following kelulut honey administration. Food was given after the 4 h observation period (OECD, 2008). The movement of the rats were also recorded upon dose consumption.

Body weight (BW) and meal pattern analysis

The BW of each rat was recorded weekly, and the changes in BW were recorded. The BW was calculated as the percentage of BW increment using Eq. 1 (Al-Afifi et al., 2018):

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

(Eq. 1)

In meal pattern analysis, the amount of food consumed was measured weekly by subtracting from the initial food supply (Samat et al., 2014).

Anaesthesia of rats

The rats were anesthetised using diethyl ether on day 29 following the method by Tuffery (1995). For the rat’s anaesthesia, 20 mL of 20% diethyl ether was soaked in a pad, and placed in the euthanasia chamber. The euthanasia agent was allowed to vapourise for 5 min. The rats were then individually placed in the chamber for 10 min until unconscious, as evidenced by no tickle on their whiskers, and no pain reaction when pressed on their leg palms. Then, blood was immediately drawn from the rats.

Blood collection

The rats were deprived of food overnight beforehand. The cardiac puncture was done on day 29 after the rats were confirmed to be anesthetised, blood samples of approximately 4 - 5 mL each were collected using a 5-mL syringe and 18 G needles for complete blood count (haematological test) and serum biochemical analyses.

Serum analyses by proton nuclear magnetic resonance

Rats’ serum from subacute toxicity treatment 3, T3 (2,000 mg/kg kelulut honey) for both genders was analysed using a nuclear magnetic resonance (NMR) spectrometer (Bruker Avance 400 MHz, Germany) at the Institute of Marine Biotechnology, Universiti Malaysia Terengganu following the method by Lin et al. (2016) with modifications. Rats’ serum (200 µL) was transferred into a clean vial and added with 400 µL of D<sub>2</sub>O containing 0.1% TSP, and the mixture was vortexed until completely dissolved. Then, the mixture was transferred into an NMR glass tube until filling 3 - 4 cm of the tube, capped, and labelled. Next, the tube was inserted into the NMR spinner. A pre-saturation and the Carr-Purcell-Meiboom-Gill pulse sequence were applied to suppress water signal at 4.70 - 4.90 ppm, and broad protein resonances of the serum samples. The processed NMR spectrum was analysed using MestReNova 8.1 software (Mestrelab Research S.L, Spain) and Chenomx NMR Suite Profiler (Chenomx Inc., Canada).
Relative organ weight and histopathological observation

The five vital organs, namely heart, lung, kidney, liver, and spleen were collected from each gender to measure their percentage of relative organ weight (ROW). A gross observation was conducted on the internal organs to check for any signs of abnormality and the presence of lesions due to any effects following kelulut honey administration. The organs were carefully dissected, removed of any fats, and weighed. The ROW of each organ was obtained using Eq. 2 (Samat et al., 2014):

\[
\text{ROW} = \left( \frac{\text{absolute organ weight (g)}}{\text{BW of rat on sacrifice day (g)}} \right) \times 100\% \quad \text{(Eq. 2)}
\]

The liver and kidney specimens were then subjected to histopathology since they play a crucial role in filtering the body wastes/metabolites. The organs were fixed 24 h in 10% neutral buffered formalin for histopathological processes. Then, the organ samples were cut and followed by dehydration using a tissue processor. The hardened specimens were embedded into moulds with paraffin wax, sectioned into 5 µm using a microtome, and stained with H&E staining. The histological procedure was adapted from Junqueira and Carneiro (2002).

Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using Statistical Package for Social Sciences (SPSS) version 22.0 and student's t-test One-way ANOVA, followed by Tukey’s post-hoc test. Values with a confidence level of \( p < 0.05 \) were considered significant.

Results

Physical analysis

Observation on the daily consumption of kelulut honey on rats showed no mortality throughout four weeks of the study period. Rats also displayed no toxicity signs such as abnormal urine or stool, changes in skin or eye colour, excessive shedding of fur, or abnormality in their behaviours for example vomiting, convulsion, and tremor. Subacute study of kelulut honey up to 2,000 mg/kg showed no mortality; thus, we could not determine the LD\text{50}, which is greater than the used highest dose. Therefore, kelulut honey could be classified in category 5 or unclassified, which is the safest category in subacute oral toxicity according to the Globally Harmonized System of Classification and Labelling of Chemicals.

Body weight and total food intake

After four weeks of the study period, male rats’ (Figure 1) showed a significant difference between C2 and C1 for food intake in Week 3, C2 and T1 also had a significant difference as compared to C1 in Week 4. T1 yielded the lowest total food intake (517.07 ± 13.24 g) whereas C1 yielded the highest (556.48 ± 9.69 g). Interestingly, C2 (547.72 ± 25.12 g) had relatively similar food intake with T3 (546.09 ± 5.66 g). For male percentage BW increment, C2 yielded the lowest of only 38.97 ± 14.67% for the four weeks of ABA consumption, whereas T3 the highest (89.33 ± 15.57%). We may assume that ABA consumption caused high food intake in male rats but lower BW increment. Female rats showed the lowest percentage of BW for C1 (9.74 ± 1.11%), whereas the highest was for T1 (22.00 ± 4.53%) (Figure 1). For total food intake, C2 yielded the lowest (360.17 ± 17.47 g), whereas C1 the highest (440.72 ± 24.02 g).

Haematological analysis

In male rats, analysis of erythrocyte (normal range = 6390 - 8010 \times 10^6/L for both genders) (He et al., 2017; Figure 2) showed a significant decrease in T2 (6880 ± 220 \times 10^6/L) as compared to C1 (7520 ± 70 \times 10^6/L), whereas in female, T1 (6800 ± 110 \times 10^6/L) and T3 (6780 ± 210 \times 10^6/L) significantly decreased as compared to C1 (7320 ± 310 \times 10^6/L). Meanwhile, thrombocyte count (normal range, male = 638 - 1177 \times 10^6/L; female = 674 - 1373 \times 10^6/L) (Han et al., 2010) showed a significant increase in male, T3 (1093.60 ± 83.24 \times 10^6/L) as compared to C1 (848.40 ± 70.35 \times 10^6/L), whereas thrombocyte count of female T1 (906.60 ± 108.21 \times 10^6/L) and T2 (881.60 ± 44.74 \times 10^6/L) also had a significant decrease as compared to C1 (1289.40 ± 70.19 \times 10^6/L). Our data also recorded the increment of leukocytes with kelulut honey consumption (Figure 2). For instance, lymphocytes (a type of leukocytes) of group T2 (83.00 ± 1.05%) recorded the highest count in male, and T3 (80.20 ± 0.20%) in female rats.

Serum biochemical analyses

Serum urea (normal range, male = 4.32 - 8.97
Figure 1. Percentage of body weight and total food intake for male rats (A) and female rats (B) (C1 = distilled water; C2 = abscisic acid; T1 = 500 mg/kg; T2 = 1,000 mg/kg; and T3 = 2,000 mg/kg) for the subacute study following daily administration of kelulut honey for four weeks. Values are mean of each week. *p < 0.05 for week 3, *p < 0.05 and **p < 0.01 for week 4 in male rats indicate significant difference as compared to negative control (C1).
Figure 2. Haematology parameters of erythrocyte and thrombocyte (A) and some differential white blood count (B) (lymphocyte, monocyte, and eosinophil) of male and female rats for the subacute study following daily administration of kelulut honey for four weeks. Boxes indicate the normal range for each parameter in both genders. Values are mean ± SEM (n = 5) *significant difference (p < 0.05) as compared to negative control (C1).
mmol/L; female = 5.56 - 12.67 mmol/L) was analysed for kidney function test, total cholesterol (normal range, male = 0.80 - 4.53 mmol/L; female = 1.13 - 4.86 mmol/L), and triglyceride (normal range, male = 0.15 - 2.65 mmol/L; female = 0.48 - 3.37 mmol/L) to analyse blood parameters and liver enzymes to monitor the kelulut honey effect toward the liver function (Han et al., 2010; He et al., 2017; Delwatta et al., 2018). Figure 3 shows the higher value of urea with kelulut honey consumption than the control. C1 (male = 6.05 ± 0.18 mmol/L; female = 7.21 ± 0.20 mmol/L) and T2 (7.34 ± 0.27 mmol/L) yielded the highest value in male, and T3 (7.45 ± 0.49 mmol/L) in female rats, but not significant. The total cholesterol in both male and female rats showed that T1 (1.34 ± 0.04 and 1.71 ± 0.17 mmol/L, respectively) as having the lowest value, but not significant in male rats (Figure 3).

Figure 3. Biochemical parameters of urea, total cholesterol, and triglyceride (A), and some parameters of liver function tests (B) of male and female rats for the subacute study following daily administration of kelulut honey for four weeks. Boxes indicate the normal range for each parameter in both genders, respectively. Values are mean ± SEM (n = 5) *significant difference (p < 0.05) as compared to negative control (C1).
Serum analyses of enzymes alkaline phosphatase (ALP, normal range, male = 62.0 - 230.0 U/L; female = 30.1 - 116.0 U/L), alanine transaminase (ALT, normal range, male = 30.8 - 73.4 U/L; female = 2.1 - 426.9 U/L), and aspartate transaminase (AST, normal range, male = 64.1 - 168.1 U/L; female = 20.8 - 470.2 U/L) (De Biasio et al., 2006) somehow revealed the condition of the liver following kelulut honey consumption. Figure 3 shows that T1 male and female rats (153.40 ± 12.87 and 212.2 ± 8.21 U/L, respectively) having the highest value in AST but not significant in male rats as compared to C1 (male = 121.60 ± 7.23 U/L; female = 114.80 ± 4.71 U/L). ALT in male rats also showed that T1 as having the significantly highest value (68.80 ± 3.87 U/L) as compared to C1 (55.00 ± 4.00 U/L), whereas in female rats, T3 (59.80 ± 4.10 U/L) had the highest value but not significant.

Detection of biomarkers in rats’ serum after four weeks of kelulut honey consumption

Analysis of proton nuclear magnetic resonance (1H NMR) for rats’ serum of the highest concentration of kelulut daily consumption (2,000 mg/kg) allowed for the detection of any defect or abnormal metabolites in the rats’ body (Figure 4). Analysis of the rats’ serum grouped the compounds such as amino acids (betaine and glycine), protein (creatinine), organic acids (acetate, lactate, succinate, and glycolate), and organic compounds (acetone, glucose, trimethylamine-N-oxide (TMAO), and imidazole). There were no visible unfamiliar compounds following kelulut honey daily administration.

Relative organ weight (ROW)

Figure 5 shows a significant increase in the ROW of male rats’ lungs for C2 (0.87%) and T3 (0.89%) as compared to C1 (0.70%). A significant decrease for female liver ROW of T2 (2.08%) and T3 (3.07%) as compared to C1 (3.81%) was also observed. Meanwhile, C2 female kidney ROW (0.43%) showed a significant increase as compared to C1 (0.40%).

Organ histopathological analyses

Histology results of the liver and kidney following kelulut honey daily administration showed normal tissue structure with no visible abnormality as compared to C1 (Figure 6). However, in female rats, T2 showed the presence of mononuclear cell infiltration but was absent in T1 and T3. This condition is questionable since both doses lower and higher than T2 displayed normal and healthy liver tissue. In addition, there were no visible toxicity signs on the kidney structure for kidney organs such as necrosis of proximal and distal tubular or sloughing of tubular epithelial cells. The kidney structure was also free from any injury such as the formation of interstitial inflammatory cells, glomerular atrophy, or capillaries congestion (De Biasio et al., 2006).

Discussion

Kelulut honey has demonstrated various medicinal properties such as antioxidant, anti-obesity, antibacterial, wound-healing, and anticancer (Saiful Yazan et al., 2016; Mohd Rafie et al., 2018; Biluca et al., 2020). These benefits come from its polyfloral sources, thus contributing to its antioxidant properties (Jaapar et al., 2016). Plants naturally produce antioxidants as protection under abiotic and biotic stresses. A plant hormone that explicitly controls antioxidant production is ABA. Sah et al. (2016) found ABA to be a phytohormone that acts as a central regulator that signals the emergence of reactive oxygen species, and activates the genetic makeup for antioxidant production to discard it in plants (Sah et al., 2016). Therefore, ABA is a prominent natural occurrence in plants. ABA has also been found to be present in all kelulut honey from different continents, thus serving as its biomarker (de Almeida-Muradian, 2013; Abu Bakar et al., 2017).

According to the OECD 407 for oral subacute toxicity study, a highly expected dose to cause death based on the previous study should be considered as a starting dose. Samat et al. (2014) reported no toxicity signs on rats for the highest dose of 2,000 mg/kg for gelam and acacia honey. Therefore, the highest dose of 2,000 mg/kg of kelulut honey was selected as the starting dose, and reduced by half to evaluate its daily consumption effectiveness.

Based on the observation, no abnormal signs were observed on the movement and physical of each rat from the first day until the sacrifice day (day 29). The outcome agrees with the study from Samat et al. (2014), which showed no physical and behaviour abnormalities throughout the study period for the common Apis sp. honey. In contrast to BW and total...
Figure 4. $^{1}H$ NMR spectra of male and female rat’s serum fed with 2,000 mg/kg kelulut honey for four weeks. Identified signals: 1 = lactate; 2 = acetate; 3 = acetone; 4 = succinate; 5 = trimethylamine-N-oxide; 6 = creatinine; 7 = glycine; 8 = glycolate; 9 = betaine; 10 = glucose; and 11 = imidazole.

Figure 5. Relative organ weights of male and female rats following daily administration of kelulut honey for four weeks. Values are mean ± SEM ($n = 5$) *significant difference ($p < 0.05$) as compared to negative control (C1).
<table>
<thead>
<tr>
<th>Groups</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 (Distilled water)</td>
<td><img src="example1.png" alt="Image" /></td>
<td><img src="example2.png" alt="Image" /></td>
</tr>
<tr>
<td>C2 (Abscisic acid)</td>
<td><img src="example3.png" alt="Image" /></td>
<td><img src="example4.png" alt="Image" /></td>
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<tr>
<td>T1 (500 mg/Kg Kelulut Honey)</td>
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<td><img src="example6.png" alt="Image" /></td>
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<tr>
<td>T2 (1000 mg/Kg Kelulut Honey)</td>
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<td><img src="example8.png" alt="Image" /></td>
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<tr>
<td>T3 (2000 mg/Kg Kelulut Honey)</td>
<td><img src="example9.png" alt="Image" /></td>
<td><img src="example10.png" alt="Image" /></td>
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(A)
Figure 6. Subacute toxicity of haematoxylin and eosin (H&E) staining of liver (A) and kidney (B) after four weeks of kelulut honey administration. Left panel is representative of male rats, while right panel is representative of female rats: a and b = kidney and liver sections from rats fed with distilled water (negative control, C1) which show normal histological structure with hepatocytes (H), sinusoids (S), and central vein (CV) for liver, while glomerulus (G) and proximal convoluted tubules (PCT) for kidney; c and d = consumption of abscisic acid (C2); e and f = consumption of 500 mg/kg kelulut honey (T1); g and h = consumption of 1,000 mg/kg kelulut honey (T2); i and j = consumption of 2,000 mg/kg kelulut honey; both liver and kidney show the normal structure of the organs (H&E, 100× magnification).
food intake, different concentrations of honey showed distinctive effects on the rats. This might have been caused by the individual variation in the rats’ metabolism (Hsu et al., 2015). BW is a crucial indicator in analysing toxicity with losses of ≥ 20% of the original BW is regarded as severe damage, and recorded as a humane endpoint in some international guidelines (OECD, 2008). Food intake is associated with BW. However, the percentage of BW increment for different individuals might not be directly proportional to food intake because other factors such as energy expenditure or body metabolism also affect BW (Nordin et al., 2018). This condition might be due to the increase in sugar intake by the rats while the energy expenditure is less due to the limited space of the rats. Meanwhile, in female rats, the percentage of BW increment varied in groups, probably due to hormonal effects. Deibert et al. (2010) also pointed out that the complicated effect of BW on honey consumption may be due to different metabolisms and body hormones in male and female rats.

ABA as the biomarker for kelulut honey may play an important role in the medicinal properties since it is constantly found in the honey. Soti et al. (2019) found that male rats treated with ABA have significantly decreased BW and increased appetite, which also correlates with our result on male rats. The finding was due to ABA’s significant impact on insulin secretion, which decreases the concentration of fasting blood glucose while improving glucose tolerance in mice (Guri et al., 2007). The condition results in low glycaemia that induced gluconeogenesis and increased food intake (Soti et al. 2019). However, BW change is still mainly affected by the caloric intake and energy expenditure of the organisms (Hsu et al., 2015). Meanwhile, female rats did not exhibit the same effect as male rats. This could have been due to the variation in the modulation of hypothalamic regulatory on the basal metabolic rate that could cause changes in energy expenditure without food restriction controlling the different psychological and physiological factors, including the change in mood, emotional state, and activity (Saiful Yazan et al., 2016; Nordin et al., 2018). Since ABA is a hormone that may participate in body metabolism and food appetite, disturbance and change in hormone regulation may also be affected by the effect of ABA since both genders had contrast body conditions, and the substance may interact with the body differently (Deibert et al., 2010).

Blood parameter data play a significant role in determining the possible adverse effect since blood serves as the primary medium for substance transfer in the body (Abotsi et al., 2011). Robust changes in blood components are inimical to normal bodily function (Abotsi et al., 2011). Low erythrocyte counts are usually associated with anaemia or erythropoietin deficiency resulting from chronic kidney diseases and deprivation of the body’s oxygen. In contrast, high erythrocyte counts indicate the increase in oxygen needs of body cells due to some conditions that need oxygen. However, the erythrocyte count in the present work was still in the normal range (He et al., 2017), thus indicating that daily kelulut honey administration did not alter the erythrocyte counts. Meanwhile, thrombocyte counts were different in male and female rats due to daily kelulut honey administration. Saiful Yazan et al. (2016) also reported a decrease in thrombocyte count but in male rats after being treated with kelulut honey. The decrease in the body’s thrombocyte needs might be due to the antioxidant and wound healing effect of kelulut honey that improves body cell function (Mohd Rafie et al., 2018; Nordin et al., 2018; Biluca et al., 2020). The effect was different for different doses and genders, thus suggesting different probabilities of the optimum dose of kelulut honey. Differential leukocytes also play a vital role in protecting the body. Any adverse effect of a substance will affect the prognostic value of leukocytes. Apart from being rich in antioxidants, kelulut honey is also found to improve the immune system by increasing the number of leukocytes. Saiful Yazan et al. (2016) again supported our finding with the high leukocyte count for kelulut honey treatment (10.12 ± 0.95 × 10⁹/L) as compared to rats fed with distilled water (7.45 ± 0.88 × 10⁹/L). Al-Waili and Haq (2004) also found that natural honey stimulates the antibody production for the primary and secondary immune responses against thymus-dependent and thymus-independent antigens, thus suggesting that honey has a significant effect on the body immune responses. However, high leukocyte count can also induce anxiety since it may indicate body infections or diseases (such as leukaemia, immune system disorder, or stress), which is in contrast with our data since all the blood parameters were in the normal range (Han et al., 2010; Delwatta et al., 2018).

Analysis of blood serum can also be one of the specific and essential precursors for the adverse effect
of substances (Samat et al., 2014). Nitrogen waste product results from the break of protein by the liver, combined with other elements such as carbon, hydrogen, and oxygen, thus forming urea. This molecule is then transferred to the kidney to be filtered and flushed out from the body (Stepanova et al., 2013). Therefore, high blood urea may indicate the ineffectiveness of kidney function. Apart from that, an increase in urination elaborates the diuretic properties of kelulut honey which mimicks furosemide. Furosemide increases the urinary excretion of certain ions such as potassium and chloride in the thick ascending limb of the Henle loop, thus causing hypokalaemia, a contrast effect as compared to honey (El-Guendouz et al., 2017). An incease in urea value may denote an increase in urine volume, which removes toxins and reduces water retention problems. A previous study had also reported an increase in urea value upon kelulut honey consumption (Saiful Yazen et al., 2016). Other than that, lipid is one of the critical elements in the body that functions as cushions for internal organs and prominent energy storage. However, hyperlipidaemias is toxic to the body by promoting many severe diseases such as atherosclerosis, hypothyroidism, and liver and kidney failure (Stepanova et al., 2013). Lipid acts as a crucial toxicity indicator in honey quality assessment since honey is generally speculated as an obesity agent. Conversely, our data found that kelulut honey consumption reduced the total cholesterol and triglyceride values, which showed the anti-obesity effect. Many preceding studies also reported the anti-obesity effect of natural honey such as in our data. Our results coincide with Mohd Rafie et al. (2018) who found a significant decrease in low-density lipoprotein cholesterol and triglyceride in rats following kelulut honey consumption. The anti-obesity properties of kelulut honey were found due to the insulin-mimetic effect of hydrogen peroxide in the honey, which increased the rats’ metabolism (Mohd Rafie et al. 2018).

Liver is a vital organ that functions to filter blood, detoxify chemicals, and metabolise drugs in the body. Therefore, abnormality of liver performance also marks the adverse effect of a substance. Elevation of these enzymes might occur due to the enzyme leakage from the damaged cellular membrane. Currently, the increment in ALT and AST levels is commonly associated with drugs, chronic viral infection, chronic alcohol consumption, and non-alcoholic steatohepatitis (NASH) (Stepanova et al., 2013). NASH can occur with the abnormality in the bodies’ metabolic performance due to diseases such as obesity, type II diabetes, or hyperlipidaemia (Bailey et al., 2004; Stepanova et al., 2013; Craig et al., 2015; El-Guendouz et al., 2017). NASH can be severe and lead to further complications, for instance, cirrhosis and hepatocellular carcinoma. This happens because of high toxicity in the blood, increased liver burden to remove the toxins, thus leading to damage and reduced liver function. Mohd Rafie et al. (2018) also recorded a high value of ALP for high-fat diet male rats (obese-induced) treated with 500 mg/kg kelulut honey (140.5 ± 9.0 U/L) as compared to control (89.8 ± 4.8 U/L). Other than that, Samat et al. (2014) reported a high AST value after consumption of 2,000 mg/kg acacia honey (154.00 ± 5.48 U/L) as compared to control (112.00 ± 4.47 U/L) for female rats. Although our data found high value of the enzymes following kelulut honey administration, the value was still within the normal range (Figure 3) (Han et al., 2010). Therefore, the rats’ liver was subjected to $^1$H NMR and histopathology to confirm any abnormality of the metabolites and organs.

Apart from serum analyses, the rats’ serum also underwent $^1$H NMR to detect any abnormal serum metabolites following kelulut honey administration. The $^1$H NMR analysis is vastly used for structure determination since it can elaborate complex structure in a complicated system, has a non-invasive nature, has rapid data elimination, presents a broad range of metabolites in one analysis, and above all, requires only a small amount of sample (Lin et al., 2016). One of the metabolites found in our analysis was glycine. Glycine usually represents 11.5% of the total amino acids in the human body, which functions in protein synthesis and collagen formation (Razak et al., 2017). Collagen is essential to sustain the normal structure, strength of connective tissue, and elasticity of the skin, and also a precursor for several key metabolites of low molecular weight (Li and Wu, 2018). Supplementation of glycine is also able to enhance the quality of sleep and neurological functions, and prevent disease including cancers (Razak et al., 2017). Succinate serves as a critical metabolite intermediate in the tricarboxylic acid cycle, and connects directly with the electron transport chain of the mitochondria, thus allowing the direct production of adenosine triphosphate (ATP) via oxidative metabolism (Giorgi-Coll et al., 2017). A reduced level of succinate might decrease the production of ATP and increase the oxidative stress.
of body cells. Next, oxidation of serum glycolate by glycolate oxidase to glyoxylate, and then to oxalate, produces hydroxyl acids, a carbohydrate precursor, and serves as an energy source (Kubiń et al., 2014). Other than that, acetone (product of fat breakdown) and glucose (product of carbohydrate breakdown) are also energy providers (Abu Bakar et al., 2017), bearing a pivotal role to kick-start oxidative metabolism. Interestingly, TMAO peak was higher in female rats and lower in male rats. Elevation in TMAO level is usually associated with cardiovascular disease. Wang et al. (2011) reported that a diet containing choline, TMAO, or betaine can induce the activeness of multiple macrophage scavenger receptors that are vulnerable to atherosclerosis. Nevertheless, no harm was observed in the female rats. Last but not the least, the imidazole ring is ubiquitous and significant as a build-up for histamine and histidine in humans and many organisms. Histidine can be found in many proteins, and is essential as the iron binder for haemoglobin in red blood cells (Krishna Deepak and Sankararamakrishnan, 2016). Decarboxylation of histidine produced histamine, an allergic agent alleviating inflammatory responses in immunity (Krishna Deepak and Sankararamakrishnan, 2016). All these metabolites are essential in maintaining optimum bodily functions. All the metabolites found in the rat serum are needed by the body, and promoted their normal condition.

Other than that, analysis of organ weight is also critical in determining xenobiotics’ effect on the animals’ body performance. ROW can be the more sensitive detector for a substance effect since ROW significance may occur without any morphological changes (Bailey et al., 2004). Interestingly, data showed a significant increment of male rats’ lung ROW with ABA intake, and T3 indicated that ABA might have an effect in increasing lung ROW. However, Hontecillas et al. (2013) reported the effectiveness of ABA as an immune-modulator for respiratory infection without any severe side effects. Apart from that, the result also showed an increment in male liver ROW, an intriguing concern since hepatomegaly is usually associated with fat accumulation. Despite that, the increment in lung and liver ROW might also be due to the different individual organ sizes (Samat et al., 2014). The decrease in female ROW can suggest the liver’s water loss or reduction of original liver fats (Mohd Rafie et al., 2018). Coincidently, our results were supported by Mohd Rafie et al. (2018) who found a significant reduction in adiposity index and liver ROW after being treated with kelulut honey. The resulted condition suggested that kelulut honey highly possesses the anti-obesity effect, which was found to be affected by the ABA that ameliorates glucose tolerance in rats (Soti et al., 2019). Other than that, the significant increase of ROW does not necessarily cause defects in histopathology as compared to the absolute organ weight of the organs (Craig et al., 2015).

Observation of histology has a deep relation with xenobiotics’ effect on the body (Samat et al., 2014). Moreover, liver and kidney serve as the crucial organs in maintaining body homeostasis by eliminating unnecessary and waste compounds. Therefore, histological analyses of these organs may help explain the exact body condition upon consumption of substances (Craig et al., 2015). Hepatocytes are the basic unit of the liver with a specific function to synthesise protein, detoxify metabolites, and channel biochemicals for body growth and digestion (De Biasio et al., 2006). Meanwhile, kidney is another filtration organ, and will also be damaged by severe toxins. As expected, all groups display normal morphology of the kidney and liver cells based on the histology analysis. However, the abnormality of female rats’ liver for T1 and T3 tissues somehow might be associated with specific autoantibodies inducing mononuclear cell infiltration and immunoglobulin G (De Biasio et al., 2006). Saiful Yazan et al. (2016) also reported the visibility of cancerous cells in their negative control (treatment with distilled water, C1) that was assumed due to cell mutation. In addition, other studies also reported the effectiveness of kelulut honey consumption in eliminating cancer cells in the colon (De Biasio et al., 2006; Saiful Yazan et al., 2016; Mohd Rafie et al., 2018; Nordin et al., 2018; Biluca et al., 2020). According to Abdel-Moneim and Ghafeer (2007), the treatment of rats with honey on renal tubular toxicity from cadmium chloride reduces lipid peroxidation, and improves tissue activity for enzyme glutathione and glutathione peroxidase.

Conclusion

The medium lethal dose for daily consumption of kelulut honey was higher than 2,000 mg/kg rats’ BW. Therefore, it is classified in category 5 or unclassified according to the Globally Harmonized
System of Classification and Labelling of Chemicals, which is the safest in subacute oral toxicity. Generally, there was no abnormality in rats’ behaviour and individual performance. In the present work, we found that kelulut honey had a prognostic value in decreasing BW (anti-obesity) while increasing food intake (increase appetite). Haematological analyses showed an increment in lymphocytes necessary for antibody formation in the immune system to fight pathogens, and blood serum analyses showed normal value for each serum and enzyme analysed. Results also indicated general good health effects of kelulut honey consumption. ¹H NMR analysis revealed the metabolites in male and female rats treated with kelulut honey, and those metabolites in the rats’ serum are essential for bodily functions. Kelulut honey consumption also significantly decreased female liver ROW. At the same time, histopathology observation showed normal tissue structure for both the liver and kidney, the prominent organs in filtering toxins, thus further indicating the safety of kelulut honey. Nevertheless, the optimum doses for males and females might be different due to hormone regulation in the body. The effect on individuals may also vary based on the current condition of each individual. Overall, kelulut honey at a concentration up to 2,000 mg/kg (which is equal to 518.912 mg/m² for human adults and 384.000 mg/m² for children) is safe for daily consumption, and does not cause any adverse effect while improving healthy bodily function on individuals (Reagan-Shaw et al., 2007). However, the effect of honey might vary with individuals, and the dose needed for specific treatment might also be different. The long-term effects for 90 days also need to be investigated. Therefore, in the future, toxicity safety study can be pursued with a clinical study, especially in highlighting the medicinal effects of kelulut honey.

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References


