ABSTRACT

Aim: This study aimed to examine the effects of tualang honey against the high cholesterol diet induced biochemical and histological changes in the liver and pancreas. Methods: Female Sprague-Dawley rats were used, where the control group (n = 5) was fed with commercial rat pellet; the high cholesterol diet (HCD) group (n= 5) was given 12% cholesterol diet; while the HCD with tualang honey (HCD+TH) group (n =5) was fed with 12% cholesterol diet with daily 1.4 g/kg/day of tualang honey. Blood biochemical analysis were analysed at 1 week and 6 weeks. The liver and pancreas tissues were harvested at the end of 6 weeks for histological examination. Results: The cholesterol diet induction resulted in dyslipidaemia and abnormal liver function. The HCD+TH group have shown an increase in total cholesterol, LDL-c, ALP levels and decreased TG, HDL-c and AST levels significantly at the end of 6 weeks compared to HCD group. The mean plasma glucose level was elevated at 1 week in the HCD group. Plasma insulin levels were higher in HCD+TH group at both 1 and 6 weeks. The HOMA-IR was also higher at 1 week in the HCD+TH group. The liver histology of both HCD and HCD+TH groups showed steatohepatitis with minimal hepatocyte degeneration while the pancreatic sections revealed no abnormalities. Conclusion: The cholesterol diet of 6 weeks duration in this study did induce some features of NASH with dyslipidaemia with abnormal liver profile. Low dose of Tualang honey supplementation exhibited no effect on dyslipidaemia and histopathological changes.
KEY WORDS: High Cholesterol Diet, Tualang Honey, Non Alcoholic Steatohepatitis (NASH).

INTRODUCTION
Nonalcoholic fatty liver disease (NAFLD) is characterised by the presence of hepatic steatosis in the absence of significant use of alcohol or other liver disease. It is considered as the hepatic manifestation of the metabolic syndrome.\[1] It is a spectrum of disorders that include nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH).\[2] The progressive form NASH is diagnosed based on histological features of steatosis, inflammation and hepatocellular ballooning on liver biopsies.\[3] Insulin resistance, excessive oxidative stress and inflammatory response are believed to play important roles in the development and progression of NASH.\[4] Nonalcoholic steatohepatitis is deemed to be a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.\[5] There is presently no established treatment for NASH beyond weight loss and associated metabolic co-morbidities management.\[6,7,8,9] It is essential therefore that the possible treatment options for NASH be explored.

Honey which has been used since ancient times to treat several diseases\[10] has been shown to have a good anti-inflammatory\[11] and also significant antioxidant activities.\[12] Additionally it has been proven to have good anti-bacterial\[12], cardioprotective\[13] and renoprotective\[14] properties as well as a considerable effect on the healing process of different types of wounds.\[15] Other studies have demonstrated its anti-neoplastic activity\[16] and revealed a potential role in the improvement of learning and memory.\[17] Tualang honey is a type of Malaysian polyfloral wild honey.\[17] It is rich in phenolic acids and flavonoid compounds, known for their strong free radical-scavenging activities.\[18]

The aim of this study was therefore to determine the effects of high cholesterol diet on the liver and pancreas biochemically and histologically. It also examined the protective effects of tualang honey against the high cholesterol diet induced biochemical and histological changes.

MATERIALS AND METHOD
Animals
Fifteen female Sprague-Dawley rats (age 6-8 weeks) weighing 140-170 grams were used in this study. The rats were purchased from A-Sapphire Enterprise, Seri Kembangan, Selangor. Two rats were housed in each cage under standard experimental conditions of 20-26°C at 50
- 70% humidity with 12 hours light/dark cycles. Throughout the experiment, the animals were given free access of water and food. The experimental protocols were approved by the Institutional Animal Care and Use Committee, International Islamic University Malaysia (IACUC-IIUM) No. of IACUC Approval: IIUM / IACUC Approval / 2016/ (12) (83).

**High cholesterol diet**

Twelve percent cholesterol diet was prepared by mixing 1kg of commercial rat pellet in powder form with 120 grams of analytical pure cholesterol powder (Nacalai-Tesque, Kyoto, Japan. Lot No. M4T5494. Code 08721-75). Three grams of cholic acid (Nacalai-Tesque, Kyoto, Japan. Lot No. M6H9123. Code 08805-56) were added to the preparation in order to produce stable hypercholesterolemia. In order to avoid oxidative modification of the cholesterol, the preparation of the high cholesterol diet was carried out on a weekly basis.

**Tualang honey**

Tualang honey (AgroMas, Malaysia) was supplied by Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia. The nutritional composition and specifications of tualang honey are as shown in table 1. The honey dose was calculated by conversion of human equivalent dose to rat dose using Km factor according to as the following:

Human equivalent dose (HED) = Animal dose × Animal Km factor/ Human Km factor.

**Table 1: Nutritional composition and specifications of tualang honey.**

<table>
<thead>
<tr>
<th>Parameter, Unit</th>
<th>Result</th>
<th>Standard (Food Reg 1985,Reg. 130)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing Sugar (g/100g):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>38.0</td>
<td>&gt;60.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>36.9</td>
<td></td>
</tr>
<tr>
<td>Sucrose (g/100g)</td>
<td>Not detected (&lt;0.01)</td>
<td>&lt;10.0</td>
</tr>
<tr>
<td>Ash (g/100g)</td>
<td>0.02</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Moisture (g/100g)</td>
<td>23.1</td>
<td>&lt;20.0</td>
</tr>
</tbody>
</table>

**Experimental design**

Following 10 days of acclimatization, the animals were randomly divided into three groups. Group I served as a control group (n=5) and was fed with commercial rat pellet. Group II served as the high cholesterol diet (HCD) group (n=5) and was fed with 12% cholesterol diet. Group III (n=5, HCD+TH) was fed with 12% cholesterol diet along with oral daily dose of 1.4 g/kg/day of tualang honey by gavage. The experimental diets were administered for 6 weeks.
Biochemical study

Blood specimens collected, after overnight fasting, at 1 week and 6 weeks were analysed for liver function test, lipid profile (Siemen Xpand Plus, USA), fasting plasma glucose (MEDISAFE MINI Blood Glucose Reader, Japan) and fasting serum insulin (radioimmunoassay method). The homeostasis model assessment of insulin resistance (HOMA-IR) index was calculated using the formula below:

\[ \text{HOMA-IR} = \frac{\text{fasting serum insulin (µU/ml)} \times \text{fasting plasma glucose (mmol/L)}}{22.5} \]  

[21]

Histological study

At 6 weeks, all rats were euthanized and the liver and pancreas of each of the animals were harvested and fixed in 10% neutral buffered formalin for histological examination. The liver and pancreas tissues were processed using automated tissue processor (Leica TP 1020). The tissues were embedded into paraffin blocks (Leica EG1160). They were sectioned at 4 µm thickness and stained with hematoxylin and eosin (H&E) and Masson trichrome.

Statistical analysis

Statistical analysis was performed using ANOVA (SPSS version 20.0) to compare the biochemical blood results of the study groups. A value of \( p < 0.05 \) was considered to be significant. The histological sections were analysed by two pathologists.

RESULTS

Lipid profile

Table 2 shows the lipid profile results. There were significant increments for both the serum total cholesterol (TC) \( (p = 0.013) \) and low-density lipoprotein (LDL-c) \( (p = 0.011) \) in the HCD group as compared to the control group at 6 weeks. The serum TC \( (p = 0.003) \) and LDL-c \( (p = 0.003) \) of HCD+TH group were also significantly higher as compared to the control group at 6 weeks. There was no significant difference in the TC and LDL-c levels between the HCD and the HCD+TH groups at both 1 week and 6 weeks intervals. As for the high density lipoprotein (HDL-c), both groups, the HCD \( (p = 0.001) \) and the HCD+TH \( (p < 0.001) \) exhibited significantly lower values than the control group at 6 weeks with no difference was observed between the HCD and the HCD+TH groups. The serum triglyceride (TG) was lower in the HCD+TH group at 1 week as compared to the HCD group \( (p = 0.035) \) and the control group \( (p = 0.208) \). At 6 weeks although the TG levels in the HCD group \( (p = 0.019) \) and HCD+TH group \( (p = 0.019) \) were significantly lower than the control, there was no significant difference between the HCD group and the HCD+TH group.
Table 2. Lipid profile.

<table>
<thead>
<tr>
<th>Lipid Profile parameter (mmol/L)</th>
<th>1 week</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HCD</td>
</tr>
<tr>
<td>TC</td>
<td>1.90±0.25</td>
<td>2.84±0.68</td>
</tr>
<tr>
<td>LDL-c</td>
<td>0.01±0.06</td>
<td>0.31±0.35</td>
</tr>
<tr>
<td>HDL-c</td>
<td>1.82±0.24</td>
<td>2.45±0.65</td>
</tr>
<tr>
<td>TG</td>
<td>0.36±0.08</td>
<td>0.40±0.10*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. Significant differences were analysed using ANOVA test, and indicated as *p<0.05 when comparing control with HCD and HCD+TH groups.

Liver function test

Table 3 shows liver profile results of the three experimental groups. Both the HCD (p= 0.006 at 1 week, p= 0.044 at 6 weeks) and HCD+TH (p= 0.008 at 1 week, p= 0.024 at 6 weeks) groups demonstrated higher serum alkaline phosphatase (ALP) levels than the control group at 1 week and 6 weeks. There was however no significant difference in the levels between the HCD and HCD+TH groups. Both groups also had significantly higher levels of serum aspartate aminotransferase (AST) than control at 6 weeks (HCD group p= 0.002, HCD+TH group p= 0.218). The HCD+TH group however showed a significantly lower mean level than HCD group at 6 weeks with p= 0.021.

Table 3. Liver function test.

<table>
<thead>
<tr>
<th>Liver profile parameters</th>
<th>1 week</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HCD</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>13.4±0.55</td>
<td>12.80±1.79</td>
</tr>
<tr>
<td>Total bilirubin (umol/L)</td>
<td>3.40±0.55</td>
<td>3.40±0.89</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>231.40±42.88</td>
<td>343.40±77.80*</td>
</tr>
<tr>
<td>Gamma glutamyl transferase (U/L)</td>
<td>3.40±2.51</td>
<td>3.00±1.41</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/L)</td>
<td>279.20±31.14</td>
<td>374.40±99.28</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>53.60±3.65</td>
<td>48.00±23.84</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. Significant differences were analysed using ANOVA test, and indicated as *p<0.05 when comparing control with HCD and HCD+TH groups.
Fasting plasma glucose, serum insulin, and HOMA-IR

The fasting plasma glucose, serum fasting insulin, and HOMA-IR results are as shown in Table 4. Overall there were no significant differences between the groups in the fasting blood glucose levels except for the mean blood glucose level in the HCD which was significantly higher than the control group at 1 week (p=0.037). As for the fasting insulin levels they were significantly higher in HCD+TH group in comparison to the control group both at 1 week (p=0.006) and 6 weeks (p=0.041). Meanwhile, the fasting insulin level did not show any significant difference between HCD and HCD+TH group at 1 week (p = 0.052) and 6 weeks (p = 0.077). The HOMA-IR revealed a significantly higher mean level in the HCD+TH group at 1 week (p= 0.008) in comparison to the control group.

Table 4. Fasting glucose, Insulin, and HOMA-IR

<table>
<thead>
<tr>
<th>Parameters</th>
<th>1 week</th>
<th>6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>HCD</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>6.02±0.69*</td>
<td>7.82±1.80*</td>
</tr>
<tr>
<td>Serum insulin (uU/ml)</td>
<td>0.34±0.17*</td>
<td>0.46±0.15</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.09±0.06*</td>
<td>0.16±0.06</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. Significant differences were analysed using ANOVA test, and indicated as *p<0.05 when comparing control with HCD and HCD+TH groups.

Liver histology

The control group showed normal liver histology. The sections of the livers from the HCD group and HCD+TH group showed areas of microvesicular steatosis with mild lobular and portal inflammation (Figure 1). Hepatocyte degeneration was minimal. Sections of the liver stained with Masson Trichrome stain from all the groups revealed no areas of fibrosis (Figure 1).
Figure 1: (A): Section of the liver from control group (Haematoxylin and Eosin stain, x20 objective). The section showed normal liver histology. (B): Section of the liver from high cholesterol diet group (Haematoxylin and Eosin stain, x20 objective). The section showed microvesicular steatosis (arrow) with lobular and portal inflammation (arrow head). (C): Section of the liver from high cholesterol diet with Tualang honey group (Haematoxylin and Eosin stain, x20 objective). The section showed microvesicular steatosis (arrow) with mild lobular inflammation (arrow head). (D): Section of the liver from control group (Masson Trichrome stain x10 objective). The section showed no area of fibrosis. (E): Section of the liver from high cholesterol diet group (Masson Trichrome stain x10 objective). The section showed no area of fibrosis. (F): Section of liver from high cholesterol diet with Tualang honey group (Masson Trichrome stain x10 objective). The section showed no area of increased of fibrous tissue formation.

**Pancreatic histology**

Sections of the pancreatic tissues from the control group, HCD group, and HCD+TH group all revealed normal pancreatic glands. Sections of the pancreatic tissue stained with Masson Trichrome stain from the three groups showed no areas of increased amount of fibrous tissue formation (Figure 2).
DISCUSSION
In the current study, the consumption of 12% cholesterol diet by the experimental animals resulted in dyslipidaemia and abnormal liver function. The group that received tualang honey supplementation also displayed dyslipidaemia and abnormal liver function profile with no difference observed when compared to the group that did not receive honey supplementation except for lower TG and AST levels at 1 week and 6 weeks respectively. Plasma glucose...
level was elevated at 1 week in the HCD group but not in the group receiving tualang honey. Plasma insulin levels were higher than the control in the supplemented group, both 1 and 6 weeks. The HOMA-IR was also was higher at 1 week in the group. The liver histology of both the HCD and HCD+TH groups showed steatohepatitis with minimal hepatocyte degeneration while the pancreatic sections revealed no abnormalities.

Khalili et al. (2009) in their study also revealed a significant increase in the TC level and decrease in HDL level at 4 weeks following hypercholesterolemic diet. Similar findings were documented by Matos et al. (2005). Alsaif et al. (2007) however reported a significant elevation in plasma HDL-c, cholesterol and TG levels after 13 weeks from 1% cholesterol diet administration. As for the TG levels in this study, they were however lower than the control group at 6 weeks. No similar results have been reported previously. Earlier studies reported either an increase in serum TG levels with high cholesterol diet administration, or lack of significant difference in the TG with high cholesterol diet supplementation. Tualang honey supplementation with the high cholesterol diet in this study did not have protective effects against the dyslipidaemia except for the reduction in the TG level at 1 week when compared to HCD group. Erejuwa et al. (2011) have also demonstrated that the administration of tualang honey (1.0g/kg body weight once daily for 4 weeks), significantly decreased the level of TG in diabetic rats compared to diabetic control rats. The tualang honey effect on the plasma TG in this study was however not sustainable which could perhaps be attributed to the high cholesterol diet model diet and the Tualang honey concentration used in this study.

In the current study 12% cholesterol diet administration resulted in elevation of the ALP and AST levels but not the ALT. Chen et al. (2016) reported similar results with the exception of ALT levels which were elevated in their study. Another study carried out by Kim et al. (2014) on rabbits showed similar results to our study for both AST and ALT where the AST levels were higher in the rabbits fed a high-cholesterol diet than those fed the control chow while the ALT levels did not differ significantly among the groups. The ALP elevation in the study is perhaps due to intrahepatic obstruction resulting from the steatohepatitis. The elevated AST levels could indicate infiltrative effect of cholesterol on the hepatocytes resulting in hepatocyte damage. This however could not explain why the ALT levels were not significantly elevated. Concurrent honey supplementation appeared not to have any protective effect against the most probable intrahepatic obstruction that led to the increased
ALP levels. It however resulted in a significant reduction of AST level which was in agreement with Erejuwa et al. (2012)[29]. In their study on diabetic rats, they used tualang honey at a dose of 1g/kg/day. They also showed reduction in both the levels of ALT and ALP with the honey supplementation.[29]

As for the liver histology, our study findings concurred with that by Kim et al. (2014)[27] and Chen et al. (2016)[28] where the liver histology in the animals fed with high cholesterol diet revealed steatosis, lobular and portal inflammation with hepatocyte degeneration. However in our study the steatosis was of the microvesicular type with mild inflammation and minimal hepatocyte degeneration. This could be attributed to the relatively short duration of HCD administration to the experimental animal model. Also contrary to our findings, their studies revealed the presence of fibrosis in the liver sections. Tualang honey in this study did not protect against the histological of the liver caused by the high fat diet although it is known to have anti-inflammatory as well as anti-oxidative effects. Abdul Ghani et al. (2016) however demonstrated the ability of tualang honey in improving the histological grading and NAFLD activity score in the NASH animal model. The dosage as well as the duration of experiment differed significantly from this study.[30] Although Erejuwa et al. studied the hepatoprotective effect of honey, they did not analysed the histological sections of the liver and had relied solely on the liver function profile.[29]

Impaired insulin sensitivity and compensatory hyperinsulinemia have been suggested to contribute to the development of NAFLD. Using the homeostasis model assessment method, Marchesini et al. (1999) showed that NAFLD is associated with insulin resistance and hyperinsulinemia even in non-obese subjects with normal glucose tolerance.[31] Israt & Liaquat reported that, NAFLD subjects had significantly higher fasting serum insulin and HOMA-IR than those without NAFLD indicating the association between insulin resistance and NAFLD.[32] In the present study the administration of high cholesterol diet resulted in elevation of blood glucose in HCD group at 1 week but not at 6 weeks while the insulin levels did not exhibit any significant elevation as compared to the control group. Abdel Hamid (2014) however demonstrated the ability of 4% cholesterol diet to significantly elevate blood glucose and insulin levels in rats after 8 weeks of administration.[25] Tualang honey supplementation in the current study surprisingly resulted in significantly higher insulin levels both at 1 and 6 weeks with the HOMA-IR index being significantly higher at 1 week when compared to the control group. These findings were however not observed with
the HCD group. We could not provide possible explanation for the above findings in view of the fact that insulin is known not to response to fructose which is present in significant amount in honey. This would therefore need more clarification with future studies.

CONCLUSION
The 12% cholesterol diet of 6 weeks duration in the animal model did induce some features of NASH with dyslipidaemia and abnormal liver profile but no effects were documented on the plasma glucose, serum insulin and HOMA-IR index. The pancreas also revealed no abnormality histologically. Tualang honey supplementation for the 6 weeks duration along with the 12% cholesterol diet resulted in improvement of the liver enzymes but exhibited no effect on the dyslipidaemia and did not improve the histopathological changes of the liver. Further studies are needed to explain the higher serum insulin levels and HOMA-IR with the honey supplementation. Also a longer duration of the high cholesterol diet administration would perhaps results in more obvious histological changes compatible to NASH. Additionally the dosage and duration of honey supplementation are subjected to further studies.

FUNDING
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