ORIGINAL ARTICLE

Tualang Honey Supplementation Alleviates Obesity and Dyslipidaemia in High Cholesterol Diet Induced Non-Alcoholic Steatohepatitis Animal Model

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ABSTRACT

INTRODUCTION: Dyslipidaemia and obesity are two main features of non-alcoholic steatohepatitis (NASH). This study aimed to investigate the effects of Tualang honey (TH) supplementation on bodyweight, liver weight, and lipid profiles in high cholesterol diet (HCD) induced NASH animal model. **MATERIALS AND METHODS:** Sixteen Sprague-Dawley rats were given 12% HCD for 16 weeks to induce NASH. These animals were divided into 4 groups; Group 1 (continued HCD), Group 2 (changed to normal diet), Group 3 (normal diet and TH 1.2g/kg) and Group 4 (normal diet and TH 3.0g/kg) for the following 4 weeks. Bodyweight was measured daily. At the end of the study, blood was collected via retro-orbital bleeding and the rats were sacrificed to harvest their liver. **RESULTS:** The group 4 rats had significantly lower mean final bodyweight than rats in group 1, 2 and 3 (478.0±24.4 vs. 641.5±25.1, 593.8 ±29.3, 552.0±72.9 g, $p<0.05$). Animals in group 4 were also found to have a significantly lower mean liver weight compared to groups 1 and 2 (12.9±0.9 vs 20.1±2.2, 15.7±1.2 g, $p<0.05$). In comparison to controls, the mean concentration of total cholesterol was significantly lower in all the other groups and the lowest mean concentration of triglycerides was recorded in group 4 with significant difference when compared to the controls (0.9±0.4 vs 3.6±0.4 mmol/L, $p<0.05$). **CONCLUSION:** The change from HCD to a normal diet coupled with TH supplementation has been shown to reduce bodyweight, liver weight, total cholesterol and triglyceride levels in the 12% HCD NASH induced animal models.

Keywords
Non-alcoholic steatohepatitis, obesity, tualang honey, dyslipidaemia, animal model

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has been recognised as one of the leading causes of chronic liver disease worldwide.[1] NAFLD is an umbrella term describing excessive accumulation of fat in hepatocytes that progresses in the absence of secondary causes such as excessive alcohol intake, medications or certain inherited genetic disorders.[1] It is considered to be the hepatic manifestation of metabolic syndrome as individuals with components of metabolic syndrome, such as obesity, insulin resistance, and hyperlipidaemia, have an increased risk of developing NAFLD.[2] It is a multisystem disease, with several subtypes ranging from simple steatosis or non-alcoholic fatty liver (NAFL) to non-alcoholic steatohepatitis (NASH), which is distinguished by inflammation and hepatocellular injury, in addition to the steatosis, with or without fibrosis.[2] Although NAFL commonly has a benign clinical course, NASH can gradually progress to cirrhosis, end-stage liver disease and has an increased risk of developing hepatocellular cancer (HCC).[3]

Prevalence of NAFLD worldwide is estimated at around 25% of the global population, bearing in mind that there are significant variations between countries and racial/

ethnic populations.[4] Globally, the highest rates occur in the Middle East and South America (>30%) and lowest rates in Africa (13%).[4] In Malaysia, although studies have been limited, overall prevalence rate of NAFLD has been estimated to be at around 22-37%, with older Indians and Malays making up most of the cases.[6] Meanwhile, prevalence of NASH at the population level has been a bit more difficult to be estimated as diagnosis requires a liver biopsy, which is an invasive procedure and only performed if absolutely necessary.[5] Through indirect extrapolation of data from liver biopsy case series involving patients with NAFLD and living donors for liver transplants, estimates suggest that about 20% of all patients with NAFLD will have NASH histological findings.[6]

Despite the high prevalence, in parallel with the nationwide increase of obesity and diabetes, there are no approved drugs for treating NAFLD/NASH.[7] Nutritional counselling with the aim of reducing body weight, combined with regular physical activity remains the first line of treatment.[8] Several studies have reported that a weight reduction of more than 7% body weight could not only reduce hepatic steatosis, but eventually resolve steatohepatitis.[9] Pairing this with any type of exercise, intensity or amount has shown to reduce hepatic fat, thus improving liver inflammation and liver cell injury.[9] It is crucial to note that while no drugs have been approved to treat NASH, several drugs have been tested and has shown some beneficial effect. Among the drugs that have been used include Pioglitazone, Vitamin E and Liraglutide.[10] However, the beneficial effects from these drugs are not sustained and its efficacy has yet to be proven.

In recent years, demand for safe and effective natural products to treat chronic illnesses has been on the rise. This need has led to a reconsideration of the therapeutic use of many plants and plant-based products, including honey. Honey has been used for nutritional aims and for curing ailments since ancient times by most ancient civilisations such as the Greeks, Romans, Egyptians, and Babylonians.[11] It is also mentioned in several religious text, including the holy Quran, where there is an entire chapter called an-Nahl (the Bee). Honey is largely composed of fructose, glucose and other sugars, including more than 180 other substances such as amino acids, vitamins, minerals and enzymes.[11-12] One particular honey which is the primary interest of this present study is Tualang honey (TH). TH is a Malaysian wild multifloral honey, collected by giant Asian rock bees (Apis dorsata) which build their hives on the tall branches of Tualang trees.[10] TH tall tree can reach up to heights of around 75 meters (250 feet), and could house to more than 100 nests, yielding an average of about 450 kilograms of honey.[12]

Various studies have thus far shown that TH has anti-inflammatory, antioxidant, antimicrobial, antidiabetic properties, and wound healing essentials.[11] Furthermore, when compared to several other local honey samples, such as Gelam and Indian forest, TH was found to have the highest content of antioxidants such as phenolics and flavonoids, as well as having the best free radical scavenging properties.[12] It has a total of six phenolic acids (syringic, benzoic, gallic, p-coumaric, trans cinnamic and caffeic acids) and five flavonoids (kaempferol, catechin, luteolin, apigenin and naringenin).[14-15] This study was performed to investigate the effects of TH supplementation on NASH induced rats, particularly looking into the changes in bodyweight, liver weights, lipid profile and histology.

MATERIALS AND METHODS

Animals

Sixteen Sprague Dawley rats (weighing 300-330g) purchased from A-Sapphire Enterprise (Seri Kembangan, Selangor) were used in this study. All rats were housed individually in polypropylene cages at 60 ± 5% relative humidity and 24 ± 2°C, with a 12-hour light/dark cycle, with free access to water and food. The experimental protocols were approved by the Animal Care and Use Committee, International Islamic University Malaysia (IIUM/504/14/2/IACUC).

High Cholesterol Diet

The 12% high-cholesterol diet (HCD) was prepared by

mixing 1kg of powdered commercial rat pellets (Gold Coin, Malaysia) with 120 grams of analytical pure cholesterol powder (Nacalai-Tesque Inc., Kyoto, Japan). Three grams of cholic acid (Nacalai-Tesque, Japan) was then added to the powdered mixture to enhance cholesterol absorption. This 12% HCD model was used based on a previous study by Ghani et al[16] that proved to show NASH findings in animal liver samples after 6 weeks. To prevent oxidative modification of the cholesterol, the cholesterol/cholic acid mixture was prepared twice weekly.

Tualang Honey

Tualang Honey (TH) was provided by the Federal Agricultural Marketing Authority (FAMA), Ministry of Agriculture and Agro-Based Industry, Malaysia. The water concentration of the TH supplied was standardised by FAMA at 18%. Two doses of TH were prepared; low dose (1.2g/kg/daily) and high dose (3.0g/kg/daily). The honey dose was calculated by conversion of human equivalent dose to rat dose using Km factor, applying the following formula:

Human Equivalent Dose (HED) = Animal Km factor/Human Km factor x Animal dose.[17]

Experimental procedure

The experiment was divided into 2 phases. Phase 1 (induction phase) lasted for 16 weeks, while phase 2 (supplementation phase) was conducted for 4 weeks (28 days). After 14 days of acclimatisation, the animals were split into 4 groups (4 animals per group).

Group 1 animals were given HCD and drinking water throughout the experimental period (20 weeks). No additional supplements were given. This group served as the control group. Group 2, 3 and 4 animals received HCD during the induction phase but was then changed to a normal diet of commercial rat pallets for phase 2. While group 2 rats received no extra supplements, group 3 and 4 rats were supplemented with TH (1.2g and 3.0g/kg respectively) daily via oral gavage for phase 2.

Body weight and total food intake was documented daily from day 1. At the end of the study period, the final body weight was recorded, and blood was collected via retro-orbital bleeding. The serum of the collected blood samples was separated and stored at-70°C, before being sent for biochemical analysis. All rats were sacrificed, and the livers were harvested, cleaned, weighed and preserved in formalin. Both absolute liver weights and relative liver weights were used during the analysis. Absolute liver weight refers to the actual weight of the whole liver after harvesting, while relative liver weight is calculated against the final bodyweight using the following formula:

Relative liver weight = [Liver weight (g)/Final bodyweight (g)] x 100

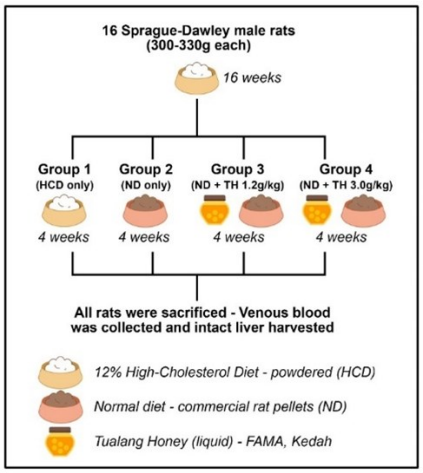

Figure 1: Experimental workflow for phase 1 (induction) and phase 2 (supplementation)

Biochemical analysis

The biochemical parameters analysed was the lipid profile which includes total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c). The analysis was performed using the Cobas Integra 400 Plus Analyser (Roche, Switzerland) based in Sultan Ahmad Shah Medical Centre (SASMEC).

Histological study

The liver specimens were cut into smaller size slices of not more than 4 mm thickness, and representative samples were placed in cassettes to be loaded into an

automated tissue processor (Leica Biosystems, USA). After processing, the samples were removed from their cassettes and put into wax moulds to ease trimming and sectioning. Trimming and serial sectioning of the samples into 4 μm thick ribbons were performed using a semi-automated rotator microtome (Leica RM2245, Leica Biosystems, USA). The ribbons of liver samples were then mounted onto frosted glass microscope slides after being dried in the warm oven overnight. Afterwards, one set of slides were stained with haematoxylin and eosin (H&E) and another set were stained with Masson's Trichrome, using the Leica ST5010 Autostainer XL (Leica Biosystems, USA). After mounting with glass cover slips, the slides were assessed by two pathologists who were blinded to the samples.

NASH grading and staging

The assessment of the grade and stage of NASH was performed based on Brunt Schema[12] for grading and staging. The Brunt Schema for grading (Table 1) covers 4 aspects; steatosis, ballooning, lobular inflammation and portal inflammation, with each aspect being either mild (grade 1), moderate (grade 2) or severe (grade 3). While staging is based on the scoring system that was refined by the NASH Clinical Research Network (CRN), that looks at the location and extent of fibrosis (Table 2).

Table 1 : Brunt schema for grading NASH

	Grade 1 (Mild)	Grade 2 (Moderate)	Grade 3 (Severe)
Steatosis	Primarily macrovesicular involving less than 33-66% of the lobules	A mix of macrovesicular and microvesic-ular	Typically, more than 66% (pan acinar) and commonly mix steatosis
Ballooning	Sometimes detected in zone 3 hepatocytes	Apparent and present in zone 3 hepatocytes	Marked ballooning and predominantly in zone 3
Lobular inflammation	Mild acute inflammation and occasional chron-ic inflammation	Polymorph may be noted with ballooned hepatocytes and pericellular fibrosis	Scattered acute and chronic inflammation. Polymorph may appear focused in zone 3 and perisinusoidal fibrosis
Portal inflammation	None or mild	Mild to moderate	Mild to moderate

Statistical analysis

Statistical analysis was performed using SPSS version 26. A value of $p<0.05$ was considered to be significant. The numerical data were normally distributed and were presented as mean and standard deviation (SD). Comparison of means were accomplished using one way analysis of variance (ANOVA). The difference between each of the groups were identified by LSD post-hoc test.

Table 2: Fibrosis staging of NASH

Stage	Description
0	None
1a	Mild zone 3 perisinusoidal fibrosis
1b	Moderate zone 3 perisinusoidal fibrosis
1c	Portal fibrosis only
2	Zone 3 periportal and perisinusoidal fibrosis
3	Bridging fibrosis
4	Probable or definite cirrhosis

RESULTS

Bodyweight and liver weights

Table 3 shows the baseline characteristic of animals in all groups in terms of body and liver weight. At the beginning of the study, the mean initial bodyweight of rats in all groups were homogenous as there was no significant difference between the groups ($p > 0.05$). At the end of the study, the final bodyweight of rats in the control group (group 1) were recorded to have the greatest increase (641.5 ± 25.1g) with 50.4% of weight gain, and the lowest in group 4 with only a 34.7% increase.

Table 3: The effects of TH on bodyweight, absolute and relative liver weights

Grouping Treatment	Group 1 HCD only	Group 2 ND only	Group 3 ND + 1.2g TH	Group 4 ND + 3.0g TH
Initial Bodyweight (g)	317.8 ± 9.4	298.5 ± 17.5	305.00 ± 10.1	312.0 ± 14.1
Final Bodyweight (g)	641.5 ± 25.1	593.8 ± 29.3	552.0 ± 72.9[a]	478.0 ± 24.4[a,b]
Bodyweight gain (%)	50.4 ± 2.9	49.7 ± 0.9	44.1 ± 7.2[a]	34.7 ± 0.5[a,b]
Absolute liver weight (g)	20.1 ± 2.2	15.7± 1.2[a]	15.3 ± 1.9[a]	12.9 ± 0.9[a,b]
Relative liver weight (g)	3.1 ± 0.4	2.6 ± 0.1[a]	2.8 ± 0.1[a]	2.7 ± 0.2[a]

Data expressed as mean ± standard deviation (SD). a indicates a significant difference ($p<0.05$) when compared to control (group 1), b indicates significant difference ($p<0.05$) when compared to the normal diet only (group 2) using One-Way ANOVA.

When compared to the controls, the mean final bodyweight of the animals that were changed to a normal diet and treated with low dose (1.2g/kg) and high dose (3.0g/kg) TH showed a significantly reduced final bodyweight (641.5 ± 25.1 vs. 552.0 ± 72.9, 478.0 ± 24.4

g, p<0.05). The rats in group 4 also had a significantly lower mean final bodyweight as compared to the rats with the diet change alone (478.0 ± 24.4 vs. 593.8 ± 29.3 g, p <0.05). As for the liver weights, significantly lower relative liver weights were seen as compared to the control group (3.49 ± 0.47 vs. 2.63 ± 0.08 vs. 2.78 ± 0.07 vs. 2.68 ± 0.19 g, p <0.05). However, no significant difference was found when comparing between the changed diet groups (group 2, 3 and 4).

Blood biochemistry

Table 4 shows the blood biochemistry results which include lipid profile parameters. The mean concentration of total cholesterol was noted to be significantly lower in all the other groups when compared to the control (2.8 ± 0.8 vs 1.8 ± 0.2, 1.5 ± 0.2, 1.7 ± 0.2 mmol/L, p <0.05). Similarly, when comparing to the control group, the mean concentration of triglycerides was also significantly lower in the groups with diet change (3.6 ± 0.4 vs 1.1 ± 0.4, 1.22 ± 0.5, 0.9 ± 0.4 mmol/L, p <0.05) (Figure 2). It could also be noted that the differences were dose dependent as the higher dosage of TH resulted in lower triglycerides levels. Nevertheless, no significant difference could be seen when comparing between animals in groups 2, 3 and 4. No significant differences were found when comparing between the groups with regards to HDL-c and LDL-c levels.

Table 4: Blood biochemical results

Laboratory parameters	Group 1	Group 2	Group 3	Group 4
	HCD only	ND only	ND + 1.2g TH	ND + 3.0g TH
Total cholesterol (mmol/L)	2.8 ± 0.8	1.8 ± 0.2*	1.5 ± 0.2*	1.7 ± 0.2*
Triglyceride (mmol/L)	3.6 ± 0.4	1.1 ± 0.4*	1.2 ± 0.5*	0.9 ± 0.4*
HDL-c (mmol/L)	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
LDL-c (mmol/L)	0.1 ± 0.6	0.4 ± 0.3	0.2 ± 0.2	0.4 ± 0.1

*Data expressed as mean ± standard deviation (SD). * indicates a significant difference (p<0.05) when comparing total cholesterol and triglyceride levels to controls.*

Histology

Figure 2 demonstrates representative pictomicrograph of liver sections between all four groups. The H&E sections shows tightly packed, pink staining plates of hepatocytes. The liver histology of the animals in the control group which were given high fat diet showed evidence of NASH establishment, with generalised microvesicular and macrovesicular steatosis indicated by the presence of multiple fat droplets. Ballooning of zone 3 hepatocytes were also observed, together with mild lobular and portal inflammation.

Following the change to normal diet, the liver histology in the animals of Group 2 showed no evidence of steatosis with the absence of fat droplets. Similar findings were observed in the liver histology of Group 3 and 4 which were given low dose and high dose of TH respectively. Yet, lobular and portal inflammation persisted in these liver sections, ranging from mild to moderate in severity. In terms of staging, there were no significant difference between the groups as overall there were no fibrosis detected.

DISCUSSION

The current study evaluated the effects of two doses of TH supplementation on the bodyweight, liver weights, histological changes and lipid profiles of NASH induced rat model. Following 12% HCD for 20 weeks, animals in the control group demonstrated the highest bodyweight, relative liver weight, total cholesterol and triglycerides levels in comparison to other groups. Ghani et al[16] and Mohamed et al[18] have reported hypercholesterolemia and histological changes that were consistent with NASH using the same induction method, but at a shorter induction period of 6 weeks. In this study, the induction period was extended to 16 weeks to enhance the establishment of NASH in the animal model. To optimise the outcome, the dose of TH was also increased into a higher dose (3.0g/kg), instead of 2.4g/kg as documented in those previous studies.[19] Additionally, the effects of TH supplementation when coupled with a diet change was also investigated, based on the current NASH management of nutritional counselling to reduce body weight and improve lipid profiles.

The main findings of this study showed that animals which were subjected to both a diet change and TH supplementation had the lowest bodyweight, relative liver weight, total cholesterol and triglycerides levels when

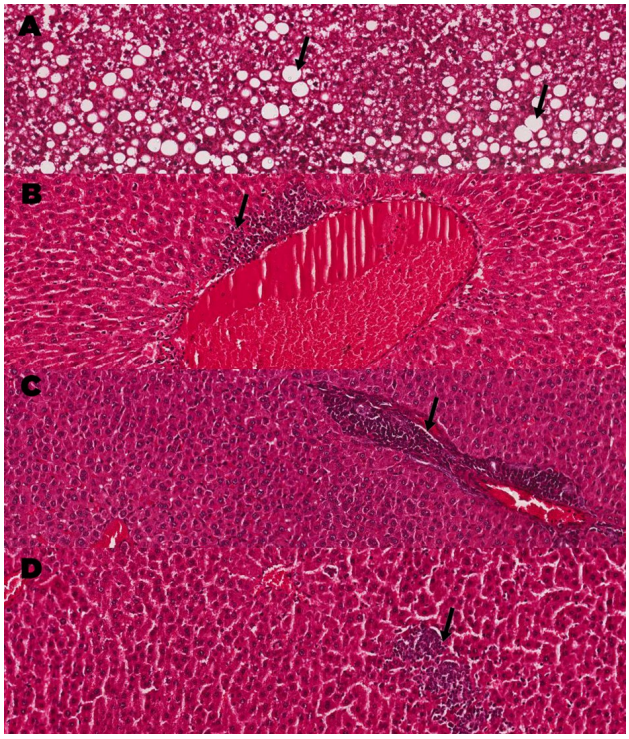

Figure 2: Microscope images (10× magnification, scale bar 300 µm) of liver section between the 4 groups; A (Group 1 – arrow shows macrovesicular and microvesicular steatosis), B (Group 2 – arrow shows focal areas of lobular inflammation), C (Group 3 – arrow shows areas of portal inflammation), D (Group 4 – arrow shows areas of portal inflammation). Steatosis not apparent in groups 2, 3 and 4.

compared to the controls that were continued with HCD and no TH supplementation. The animals which underwent diet change and was given TH at a higher dose had significantly lower bodyweight as compared to the rats in the diet change only group. This finding highlights the potential role of TH supplementation to further augment any weight reduction in the NASH animal model in addition to diet change alone. The significant reduction in the bodyweight of the rats with the change of diet and supplementation with TH, is supported by several other studies that focused on hypoglycaemic properties of honey.[20-23] Honey in general and TH in particular is essentially composed of fructose, glucose and other sugars. Previous study has proven that intake of fructose in moderate amount can prolong gastric emptying, which in turn delays intestinal absorption and lowers food intake to result in reducing weight gain.[23] Samat et al[24] has also reported similar findings in obesity induced rats but using Gelam and Acacia honey instead. The recent finding may suggest the potential of TH to be recommended as a supplement for weight reduction. Meanwhile, the significant low relative liver weight in rats treated with TH as shown in this study may indicate the reduction of fatty liver which is the main feature of NASH. Consumption of honey may stimulate conversion of excess food into energy instead of being converted into fat storage. Consistently, honey is known as a natural source of flavonoids that has been shown to have positive effects on fatty liver disease.[24]

In this study, the rats that underwent diet change coupled with TH supplementation were also recorded to have the lowest total cholesterol and triglyceride levels when compared to the controls. These findings are consistent with Nurmasitoh et al[25] that documented reduction in total cholesterol, triglyceride and LDL-c levels in diet-induced hypercholesterolaemic rats following treatment Kapok Tree honey for seven days. The effect of honey on lipid metabolism was further reinforced by Bezerra et al[26] whereby rats fed with honey from stingless bees (Malícia honey) showed lower food consumption and decreased total cholesterol. Several human clinical studies have also been conducted to look into the effects of honey on lipid profile. Among them, Waili et al[27] reported a decrease in total cholesterol and triglyceride in healthy subjects, and a decrease in triglyceride levels in patients with hypertriglyceridaemia. While Whitfield et al[28] showed that Kanuka honey formulated with cinnamon, magnesium and chromium significantly reduced the total cholesterol. All these studies propose a functional role credited to the non -sugar components of honey, especially the phenolic antioxidants, that have been reported to be helpful in improving lipid profile.[29-31] Even so, larger clinical studies need to be carried out to further explore the mechanism of this effect as the underlying reason have not yet provided strong evidence.

Undeniably, the diet change alone is seen to have significant changes to all the parameters measured in this study, as seen when comparing between groups 1 and 2. This finding suggest that diet modification remains essential in improving obesity and dyslipidaemia. However, TH supplementation in this model may further enhance the effects, with a higher dose required to produce a more significant effect.

CONCLUSION

The change of diet from HCD to a normal diet consisting of commercial rat pallets, coupled with high dose TH supplementation has shown to improve bodyweight, liver weight, total cholesterol and triglyceride levels in the 12% HCD NASH induced animal models.

CONFLICT OF INTEREST

The authors affirm that the research was performed in the absence of any commercial or financial associations that could be construed as a potential conflict of interest.

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