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The subacute toxicity effects of aged Tualang (*Koompassia excelsa*) honey with high 5-hydroxymethylfurfural content in rats

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Abstract

Tualang honey (TH) stored for more than 12 months is characterised as aged TH (ATH). Surprisingly, majority of the general public is unaware of the recommended consumption period for honey, since most honeys marketed locally have no determined expiry dates. The present work determined 5-hydroxymethylfurfural (HMF) content in four-year stored ATH, and evaluated the effects of ATH upon repeated dose during 28-day oral toxicity study. The HMF concentration in ATH was determined using HPLC. Animal toxicity study was conducted by treating Sprague Dawley rats with distilled water (control), 200, 1,000, 2,000 mg/kg/day ATH, or 2,000 mg/kg/day fresh TH for 28 days. The HMF concentrations were significantly high, exceeding the international maximum limit set for tropical honey (> 80 mg/kg). ATH did not affect the general conditions and behaviour of the rats. Nevertheless, female rats exhibited significant increase in creatinine and oestradiol levels when treated with 1,000 mg/kg/day ATH. The left kidney's weight of those treated with 200 mg/kg/day ATH also significantly decreased. Further, histological findings revealed that there were reductions in the Bowman's space and poorly defined glomerular boundary in female rats treated with 1,000 mg/kg ATH. In contrast, all parameters in male rats were statistically unaffected by ATH. The present work suggested that ATH with remarkably higher HMF concentrations modified the kidneys of female rats but not those of the male rats, which indicated sex-based variation. The no-observed-adverse-effect level was 2,000 mg/kg/day for male rats, and less than 200 mg/kg/day for female rats.

Introduction

Tualang honey (TH) is a precious national treasure that is deemed superior to other Malaysian honeys with infinite beneficial properties (Ahmed and Othman, 2013; Mohd Kamal *et al.,* 2021). The comprehensive scientific data available on TH has led to a resurgence of interest in its use as a dietary supplement and medicinal product to promote general health and well-being. Due to its popularity, it is not surprising that people still consume TH even after it exceeded its usual period of usage; most honeys marketed in Malaysia have no specified expiry dates. Moreover, majority of the general public is not even aware of the recommended period for honey consumption due to the orthodox belief that it can be consumed for a long period up to a lifetime as it contains high nutritional values.

According to Khalil *et al.* (2010), aged TH (ATH) is characterised as honey stored for more than 12 months, and should be consumed within one year. Relative to the fresh honey, ATH is physically darker due to the increase in certain polyphenols or pigments. Scientifically, ATH contains high 5-hydroxymethylfurfural (HMF), typically above the maximum limit set by the Codex Alimentarius Commission (2001) which is 40 mg/kg (with a higher limit of 80 mg/kg for honey originating from tropical regions). HMF is an organic neo-formed toxic contaminant which is a cyclic aldehyde produced by dehydration of fructose or glucose, especially fructose. It is typically absent or present in only small amount in fresh honey. Its concentration is generally recognised as a parameter affecting honey quality which can diminish due to overheating or poor storage conditions (Shapla *et al.,* 2018), adulteration with sugar additives (Keppy and Allen, 2009), and aging (Khalil *et al.,* 2010).

Our reason for investigating ATH of 4-year was because most local honeys in Malaysia are sold within the expiry date of four years. It is not strange that there are abundant of honeys including TH being

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marketed throughout the country with no determined expiry dates due to the lack of evidence on their safety profiles. Even if the expiry date is stated, it is usually within four years of the manufacturing date, which is also practiced in some other countries with the belief that honey has a long shelf life. Therefore, the safety profile of ATH is questionable, and of importance to be investigated to preserve its therapeutic values since the consumption of low-quality honey may cause detrimental health consequences.

The present work determined the HMF concentration in ATH, and evaluated its possible toxic effect upon repeated dose in 28-day oral toxicity study in animal model. The information obtained in the present work could be useful for further investigation on the safety of ATH. Apart from expanding new knowledge on TH, the obtained information can also indirectly enlighten consumers on the importance of consuming safe products while protecting their health and well-being.

Materials and methodology

Tualang honey

Malaysian TH (AgroMas®) assessed in the present work included ATH (stored for four years - harvested between July and September 2011) and fresh TH (FTH) (stored within six months - harvested in 2015) for comparison. Both types of honey were obtained from the Federal Agricultural Marketing Authority (FAMA), Kedah under the Ministry of Agriculture and Agro-Based Industry, Malaysia through Government-to-Government Research Collaboration (School of Medical Sciences, USM Health Campus & FAMA Kedah). The correct types and storage periods of the selected honey were authenticated prior to the commencement of the experiment. These honey samples were kept in different airtight glass bottles at room temperature (25-30°C), and protected from direct light in the Biomedical Laboratory, School of Health Sciences, Universiti Sains Malaysia (USM) Health Campus, Kelantan, Malaysia. Macroscopically, FTH had light amber golden colour, while ATH was dark brown. Both samples did not need any extraction but required only a simple dilution with distilled water prior to the analyses.

Analysis of HMF concentration

The high-performance liquid chromatography (HPLC) analysis was carried out for the determination of HMF concentrations in both ATH and FTH based on the methods prescribed by the International Honey Commission and Khalil *et al.* (2010). The TH samples were sent to the Acumen Scientific Sdn. Bhd, Penang, Malaysia for the analysis. The process began by dissolving each honey sample (10 g) in 25 mL of distilled water, and transferring the mixture into a 50 mL volumetric flask. Further dilution was done by adding extra water in the honey solution until it reached 50 mL, and then, the solution was filtered through a 0.45 µm membrane filter. Next, 20 µL of the sample was injected into the HPLC system, whereby, the standard for HMF solution was of 95% purity and the column used was Agilent Zorbax Eclipse XDB-C18. The mobile phase was in isocratic mode consisting of 90% water and 10% methanol, at a flow rate of 1.0 mL/min. The detection wavelength was 200-450 nm with specific monitoring at 285 nm, and all solvents used were of HPLC-grade. Finally, the HMF concentrations in honey were determined using a standard curve, and results were expressed in mg/kg.

Maintenance of test animals

The approval for the *in vivo* study was obtained from USM Institutional Animal Care and Use Committee [IACUC: USM/Animal Ethics Approval/2012/(78)(382]. A total of 50 healthy Sprague Dawley (SD) rats (9-10 weeks old) comprising equal numbers of male and female, weighing between 200-220 and 180-200 g, respectively, were used following 1 w of acclimatisation period. The selection of this range of age and weight was due to their suitable physiological and reproductive characteristics of fertile, young adults, as they reach young adulthood approximately two months of age. Our rats of 10 weeks old are equivalent to 21.21 human years, based on the puberty formula of one human year equals 3.3 rat days. As compared to humans, rats have an accelerated sexual maturity and adulthood, with the different phases of a rat's life providing an accurate correlation with human age.

The rats were individually caged in polypropylene cages under standard laboratory conditions, including temperature of 22 ± 3°C, humidity within 50-60%, and 12 h of light and dark cycle (light 0700-1900). For feeding, commercial rat pellets (Gold Coin Feedmills, Malaysia) were replenished daily except during food fasting, with distilled water available *ad libitum*. These rats were supplied and maintained at the Animal Research and Service Centre (ARASC), USM Health Campus, Kubang Kerian, Kelantan, Malaysia.

Dose selection and preparation

The selection of ATH doses was based on previous preliminary toxicological study performed by our research group to find the suitable dose range encompassing low, medium, and high doses for *in vivo* experiment. The preliminary study manipulated the pharmacological dose of 200 mg/kg, which is equivalent to one tablespoon of TH, commonly consumed by an adult. This was then set at three different dosages of 200, 1,000, and 2,000 mg/kg/day of ATH, whereas distilled water (DW) served as vehicle/diluent, as well as control. Additionally, only one dose of FTH was utilised in the present work which was obtained from Zaid *et al.* (2010) to provide a general comparison with ATH since the main concern of the present work was the effect of ATH in SD rats. The doses required were freshly prepared based on the average body weight of rats by diluting TH samples in 0.5 mL of DW every day.

Experimental protocol

Prior to the administration of doses, all rats were examined for any physical and behavioural abnormalities, whereas food consumption and body weights were measured daily. Female rats were observed for their oestrous cyclicity by performing daily vaginal smears. The procedure of obtaining vaginal smear was based on Wan Ezumi *et al.* (2017). Those showing abnormal patterns of cycle or health deficit were excluded from the experiments.

Following the OECD Guideline No. 407 (OECD, 2008b), 10 SD rats (5 males and 5 females) were assigned to each dose level; (DW - control), 200, 1,000, 2,000 mg/kg of ATH, and 2,000 mg/kg of FTH. Treatment began on a day after they reached the age of 9 w. They were dosed orally once daily using a syringe for 28 d. This duration was selected to provide information on possible health hazards of a substance likely to arise from a relatively limited period of time (OECD, 2008a). For female rats, the dose administration began after they entered dioestrus (luteal) stage of the oestrous cycle. The usual duration of treatment for subacute toxicity studies is 14-28 d (2-4 w) with the general aim of finding a dose range to be used in subsequent subchronic and chronic toxicity studies.

All rats were fasted overnight for 16 h prior to the scheduled sacrifice. At autopsy, as soon as the rats achieved a state of anaesthesia following intraperitoneal injection with 100 mg/kg sodium pentobarbital (Alfasan® Dorminal 20%, Holland), blood sample was collected from the inferior vena cava for further analysis. Male rats were sacrificed on the first day after the 28 d dose administration.

In contrast, laparotomy of female rats could be delayed up to several days until the rats reached the dioestrus stage (TH administrations were sustained) to standardise the timing of decease. This is important to normalise the diurnal hormonal variation in the females particularly when effects on the reproductive organs are concerned (OECD, 2008a; Wan Ezumi *et al.,* 2017). Throughout various stages of the oestrous cycle, the female reproductive tract undergoes numerous morphological, cytological, and histological changes. Therefore, it is essential to homogenise the timing for the tissues/organs and blood samplings in order to obtain accurate analysis and meaningful comparisons between animals (OECD, 2008a). Dioestrus stage that lasts for 48-72 h corresponds to a late secretory/luteal stage of the human reproductive cycle which encompasses a short period when the activities of reproductive organs gradually subsides following ovulation (Ajayi and Akhigbe, 2020).

Haematology

During blood collection, approximately 1.5 mL of rat blood was kept in heparinised K3EDTA tubes for full blood count measurement. Another 1.8 mL of blood was kept in coagulation sodium citrate 3.2% tube for blood coagulation test. The investigation included analysis on haemoglobin (Hb), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), packed cell volume (PCV), red cell distribution width (RDW), polymorph (POLYM), total white blood cell count, white blood cell differential count (lymphocytes, monocytes, eosinophils, basophils), platelet count, prothrombin time (PT), and activated partial thromboplastin time (APTT) (Siti Nurfarhana, 2019).

Serum biochemistry and hormones

Approximately, 3 mL of rat blood collected from each rat was extracted by centrifugation at 1,800 *g* for 15 min to obtain the serum which was then analysed for serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), gamma-glutamyl transferase (γ-GT), total bilirubin, albumin, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglyceride, total cholesterol, blood urea nitrogen (BUN), uric acid, creatinine, sodium, potassium, chloride, calcium, phosphorus, and blood glucose level. Additionally, the hormones analysed were free of thyroxine (T4), testosterone

(male rats), and oestradiol (female rats) (Siti Nurfarhana, 2019).

Gross assessment and histopathology

Following blood collection, full gross necropsy was immediately performed on the sacrificed rats. Careful observations and detailed gross assessments were carried out on the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities along with their contents. Subsequently, organs and tissues were removed from the body, cleared of any adhering tissues, blotted dry from normal saline, and weighed. Target organs were then fixed in 10% neutral buffered formalin (Thermo Scientific, USA) or Bouin's solution (Sigma Aldrich, USA) (for testes) prior to histological procedures.

The tissues of vital organs *i.e.* liver, kidney, epididymides, testes, ovaries, uterus, adrenal glands, vesicular, and prostate glands were routinely processed, embedded in paraffin, and sectioned at 3 - 5 µm. The sections were stained with haematoxylin-eosin (Labstain®, Malaysia) for microscopic examination (Wan Ezumi *et al.,* 2017; Siti Nurfarhana, 2019).

Statistical analysis

All numerical data were analysed using Statistical Package for Social Sciences (SPSS Inc. Chicago, Illinois, USA, version 21). The mean of HMF concentrations in ATH was compared to that of FTH and its international recommended values (IRV) using independent *t*-test and one sample *t*-test respectively. For data of animal study, GLM repeated measures were applied in analysing body weight and food consumption between control and treatment groups. Other parameters such as haematology, blood chemistry, hormonal levels, and organ weights were analysed using one-way ANOVA and *post-hoc* Tukey test. Data were expressed as mean ± standard deviation. The level of probability ($p < 0.05$) was considered as significant.

Results

HMF concentration

The HMF concentration of ATH (1,426 mg/kg) was significantly higher than that of FTH (27 mg/kg) and IRV (< 80 mg/kg) as shown in Figure 1.

General observations and behavioural changes

There were no signs of toxicity, morbidity, or mortality observed in the control and treatment groups throughout the study, in which all rats survived until the day of the scheduled necropsy. The vaginal smears of female rats demonstrated normal oestrous stages of a cycle completed within 4 to 5 d. This implied that ATH did not affect the progression of regular oestrous cyclicity in rats. Hence, the consumption of ATH for 28 days led to no deleterious effect on the general health and behavioural changes of SD rats regardless of the doses.

Body weights and food consumptions

There were no significant differences indicated in the body weights and food consumptions between control and treatment groups. All rats gained their body weights normally throughout the study period.

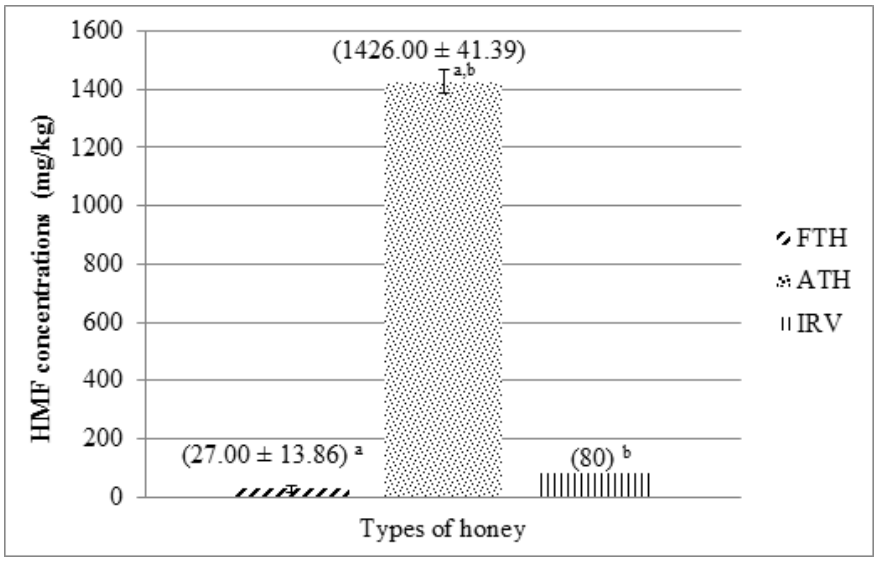

Figure 1. The HMF concentrations of ATH and FTH. The mean of ATH was compared to FTH, and significant difference ($p < 0.05$) is represented by (a). The mean of ATH was compared to IRV, and significant difference ($p < 0.05$) is represented by (b).

Clinical haematology

All haematological parameters showed that there were no statistically significant differences between control and treatment groups for both sexes.

Clinical biochemistry

The data of the clinical biochemistry of all rats are summarised in Table 1. The biochemical parameters seen in male rats did not exhibit any significant differences between control and treatment groups. In contrast, the creatinine values for female rats in Group 3 (1,000 mg/kg ATH) (0.74 mg/dL), indicated a significant increase as compared to the control group (0.60 mg/dL). However, the creatinine values significantly decreased in female rats of Group 4 (2,000 mg/kg ATH) (0.62 mg/dL), as compared to those in Group 3 (1,000 mg/kg ATH). Apart from the creatinine value, other parameters of female rats did not indicate any statistically significant differences.

Hormonal measurements

Referring to the data presented in Table 2, T4 and testosterone levels in male rats did not exhibit statistically significant differences between control and treatment groups. However, a statistical significance in the oestradiol level was indicated in female rats of Group 3 (1,000 mg/kg ATH) as compared to control (149.78 and 100.93 pmol/L, respectively).

Gross morphological assessments

The inspections through dissection of the abdominal cavities revealed that the internal organs of all rats were uniformly healthy with no gross lesions observed. There were no significant haemorrhagic or morphological changes on the macroscopic observation of all organs following the administration of ATH and FTH. These inspections were later elucidated by histopathological findings.

The absolute and relative weights of brain, heart, thyroid, spleen, lungs, liver, adrenals, kidneys, testes, epididymides, vesicular, and prostate glands of male rats (Table 3) showed no significant differences between control and treatment groups. Table 3 also shows the relative organ weights of female rats including brain, heart, thyroid, spleen, lungs, liver, adrenal glands, kidneys, uterus, and ovaries. There was a significant difference in the weights of the left kidney between Group 2 (200 mg/kg ATH) and Group 5 (2,000 mg/kg FTH) of the female rats.

Histology of organs

In male rats, no apparent abnormalities were noted on the histology of the liver, testes, epididymides, vesicular, prostate, and adrenal glands; these were therefore not discussed further in the present work. Additionally, the overall histology of the male rats' kidneys also revealed that there were no morphological changes, and they exhibited normal cellular arrangement and intact architecture. Nevertheless, in female rats, those of Group 3 (1,000 mg/kg ATH) showed some histological changes by which there were reductions in the Bowman's space and poorly defined glomerular boundary as compared to Group 1 (control) and other treatment groups (Figure 2). Other than that, no toxicity signs such as degeneration of proximal convoluted tubules, proteinaceous cast, or glomerular swelling were detected in all kidneys of female rats.

Discussions

The consumption of honey with high level of HMF has been debated since an earlier study successfully detected HMF in human urine after oral application of honey once a day (Hardt-Stremayr *et al.,* 2013). The raised concern on this issue is due to the conversion of HMF to sulfoxymethylfurfural (SMF), which is a stronger carcinogen than HMF itself. The present work was conducted to investigate the potential subacute toxicity of ATH with higher HMF concentrations in SD rats.

Results indicated that daily doses of ATH at 200, 1,000, or 2,000 mg/kg/day did not cause substantial deleterious effects in rats even with apparent higher HMF concentrations than in FTH. All rats showed an increase in food consumption, and had gained their body weight throughout the study duration. Although the rats treated with ATH showed an increment of body weight as compared to control, this was not statistically significant. This could be due to the fact that TH contained adequate calories which contributed to the normal body weight gain in all groups administered with it (Aziz *et al.,* 2014). Furthermore, the administration of ATH in rats for 28 days did not exhibit any substantial alteration in their general health status and behaviour.

Haematological data is of great importance for determining effects induced by treatment (Petterino and Argentino-Storino, 2006). According to an *in vivo* study, the reactive and genotoxic metabolite SMF was detected in the blood of mice after the administration of HMF (Monien *et al.,* 2009). This finding has stimulated concern that SMF

Table 1. Terminal clinical biochemistry of male and female SD rats after 28-day oral toxicity study with ATH.

Parameter	Tualang honey (mg/kg)					*p*-value
	DW/Control	200 ATH	1,000 ATH	2,000 ATH	2,000 FTH	
Male						
Liver function						
SGOT (U/L)	105.40 ± 33.44	111.00 ± 31.7	96.80 ± 26.15	84.40 ± 14.15	100.40 ± 22.93	n/s
SGPT (U/L)	44.40 ± 10.36	39.40 ± 4.50	40.80 ± 8.53	40.20 ± 5.50	38.20 ± 2.49	n/s
ALP (U/L)	214.20 ± 56.43	241.60 ± 65.14	212.60 ± 43.37	246.60 ± 54.81	195.60 ± 26.91	n/s
TBIL (mg/dL)	0.14 ± 0.05	0.14 ± 0.05	0.14 ± 0.05	0.12 ± 0.04	0.12 ± 0.04	n/s
Protein status						
TPRO (g/L)	69.00 ± 3.39	66.20 ± 3.11	65.60 ± 5.98	67.20 ± 3.03	66.60 ± 1.52	n/s
Albumin (g/L)	29.60 ± 1.34	30.00 ± 2.12	28.40 ± 2.07	29.40 ± 1.82	28.80 ± 3.35	n/s
Lipid profile						
LDL (mg/dL)	14.67 ± 4.25	9.27 ± 1.93	10.04 ± 8.10	9.27 ± 5.79	12.36 ± 8.88	n/s
HDL (mg/dL)	43.24 ± 6.95	41.70 ± 14.29	44.02 ± 8.88	47.88 ± 8.88	42.47 ± 16.22	n/s
TGL (mg/dL)	51.33 ± 24.78	61.95 ± 46.02	46.02 ± 34.51	58.41 ± 30.09	51.33 ± 19.47	n/s
TCHO (mg/dL)	67.95 ± 13.51	63.32 ± 21.62	63.32 ± 15.44	69.50 ± 16.60	64.09 ± 28.57	n/s
Renal profile						

BUN (mg/dL)	16.20 ± 1.64	17.40 ± 3.13	16.60 ± 2.07	18.40 ± 2.51	18.20 ± 2.39	n/s
Uric acid (mg/dL)	6.01 ± 2.17	6.25 ± 3.18	4.30 ± 0.81	6.58 ± 2.57	5.53 ± 1.15	n/s
Creatinine (mg/dL)	0.70 ± 0.07	0.70 ± 0.10	0.67 ± 0.05	0.70 ± 0.07	0.66 ± 0.05	n/s
Electrolyte and mineral						
Na (mmol/L)	139.60 ± 1.14	141.00 ± 2.83	141.60 ± 1.67	138.60 ± 2.07	139.20 ± 1.30	n/s
K (mmol/L)	6.76 ± 2.03	7.56 ± 1.96	7.70 ± 1.41	8.14 ± 0.60	6.96 ± 1.56	n/s
Cl (mmol/L)	99.80 ± 1.64	100.40 ± 3.85	100.40 ± 1.82	98.40 ± 3.51	100.20 ± 0.84	n/s
Ca (mmol/L)	2.74 ± 0.22	2.88 ± 0.44	2.72 ± 0.04	2.78 ± 0.13	2.78 ± 0.13	n/s
P (mmol/L)	4.72 ± 0.83	4.54 ± 1.03	4.50 ± 1.14	4.12 ± 0.31	4.38 ± 0.41	n/s
Blood glucose level						
Glucose (mmol/L)	9.70 ± 3.67	11.30 ± 5.43	8.00 ± 2.26	9.90 ± 2.80	9.34 ± 3.54	n/s
Female						
Liver function						
SGOT (U/L)	116.80 ± 47.52	137.60 ± 39.95	122.20 ± 33.25	115.20 ± 40.12	136.20 ± 33.42	n/s
SGPT (U/L)	39.20 ± 12.52	39.60 ± 6.88	40.60 ± 6.80	37.20 ± 5.07	36.80 ± 4.92	n/s
ALP (U/L)	95.20 ± 12.76	88.50 ± 24.84	100.80 ± 27.45	98.00 ± 20.12	115.40 ± 23.88	n/s
TBIL (mg/dL)	0.12 ± 0.04	0.10 ± 0.00	0.13 ± 0.04	0.10 ± 0.00	0.12 ± 0.04	n/s
Protein status						

TPRO (g/L)	74.80 ± 6.46	75.00 ± 10.22	72.60 ± 7.30	71.40 ± 2.88	72.40 ± 4.83	n/s
Albumin (g/L)	32.40 ± 2.97	32.20 ± 3.19	31.00 ± 2.35	28.80 ± 1.79	30.20 ± 0.84	n/s
Lipid profile						
LDL (mg/dL)	16.22 ± 8.88	20.85 ± 12.36	13.13 ± 5.02	12.36 ± 3.09	16.99 ± 1.93	n/s
HDL (mg/dL)	48.65 ± 3.47	42.47 ± 14.67	37.84 ± 9.27	41.70 ± 8.49	42.47 ± 7.72	n/s
TGL (mg/dL)	33.63 ± 3.54	31.86 ± 9.73	31.86 ± 4.42	31.86 ± 4.42	35.40 ± 10.62	n/s
TCHO (mg/dL)	71.04 ± 8.88	70.27 ± 21.62	57.92 ± 12.36	58.69 ± 11.58	65.64 ± 7.34	n/s
Renal profile						
BUN (mg/dL)	18.80 ± 3.90	20.80 ± 1.92	22.60 ± 5.64	20.00 ± 2.35	20.00 ± 3.32	n/s
Uric acid (mg/dL)	4.42 ± 0.76	3.58 ± 1.04	3.58 ± 1.39	3.72 ± 0.86	4.62 ± 1.07	n/s
Creatinine (mg/dL)	0.60 ± 0.00	0.68 ± 0.08	0.74 ± 0.09^{ab}	0.62 ± 0.04	0.66 ± 0.05	$p < 0.05$
Electrolyte and mineral						
Na (mmol/L)	136.60 ± 2.51	141.75 ± 2.50	140.00 ± 2.35	138.00 ± 2.74	137.60 ± 4.16	n/s
K (mmol/L)	7.50 ± 1.43	6.60 ± 2.10	6.80 ± 2.10	6.50 ± 1.49	7.28 ± 1.60	n/s
Cl (mmol/L)	102.80 ± 2.39	104.75 ± 1.71	104.20 ± 1.79	102.20 ± 1.64	103.60 ± 2.19	n/s
Ca (mmol/L)	2.68 ± 0.15	2.43 ± 0.10	2.50 ± 0.14	2.52 ± 0.19	2.34 ± 0.59	n/s
P (mmol/L)	3.92 ± 0.42	4.50 ± 1.20	3.92 ± 0.13	3.58 ± 0.85	4.30 ± 0.57	n/s
Blood glucose level						
Glucose (mmol/L)	6.44 ± 0.89	4.36 ± 1.13	7.04 ± 2.59	7.24 ± 2.11	5.72 ± 2.47	n/s

Values are mean ± standard deviation of five replicates ($n = 5$). n/s: No statistically significant differences between control and any of treatment groups. aSignificant difference ($p < 0.05$) in comparison with the control group. bSignificant difference ($p < 0.05$) in comparison with 2,000 mg/kg ATH group. Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), total bilirubin (TBIL), total protein (TPRO), low density lipoprotein (LDL), high density lipoprotein (HDL), triglycerides (TGL), total cholesterol (TCHO), blood urea nitrogen (BUN), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), and phosphorus (P).

Table 2. Terminal serum hormones of male and female SD rats after 28-day oral toxicity study with ATH.

Parameter	Tualang honey (mg/kg)					*p*-value
	DW/Control	200 ATH	1,000 ATH	2,000 ATH	2,000 FTH	
Male						
Free T_4 (nmol/L)	38.00 ± 4.05	35.50 ± 6.27	39.88 ± 4.47	36.32 ± 5.28	37.02 ± 10.39	n/s
Testosterone (nmol/L)	3.70 ± 2.81	12.08 ± 14.22	17.43 ± 7.82	4.86 ± 3.75	5.15 ± 5.05	n/s
Female						
Free T_4 (nmol/L)	34.34 ± 3.39	34.88 ± 6.48	34.42 ± 3.69	35.98 ± 2.79	30.06 ± 3.77	n/s
Oestradiol (pmol/L)	100.93 ± 7.04	118.92 ± 26.81	149.78 ± 27.88[a]	125.56 ± 20.19	145.93 ± 19.73	$p < 0.05$

Values are mean ± standard deviation of five replicates ($n = 5$). Serum thyroxine (T4). n/s: No statistically significant differences between control and any of treatment groups. aSignificant difference ($p < 0.05$) in comparison with the control group.

Table 3. Relative organ weights of female rats after 28-day oral toxicity study with ATH.

Organ	Tualang honey (mg/kg)					*p*-value
	DW/Control	200 ATH	1,000 ATH	2,000 ATH	2,000 FTH	
Brain	0.80 ± 0.05	0.84 ± 0.07	0.82 ± 0.06	0.79 ± 0.05	0.84 ± 0.06	n/s
Heart	0.31 ± 0.04	0.29 ± 0.03	0.29 ± 0.02	0.29 ± 0.02	0.30 ± 0.03	n/s
Thyroid	0.005 ± 0.003	0.006 ± 0.002	0.005 ± 0.002	0.004 ± 0.002	0.004 ± 0.001	n/s
Spleen	0.21 ± 0.03	0.23 ± 0.04	0.19 ± 0.02	0.21 ± 0.02	0.22 ± 0.03	n/s
Liver	3.62 ± 0.35	3.36 ± 0.30	3.15 ± 0.30	3.29 ± 0.31	3.63 ± 0.35	n/s
Lung (R)	0.42 ± 0.07	0.39 ± 0.06	0.36 ± 0.06	0.39 ± 0.05	0.42 ± 0.08	n/s
Lung (L)	0.24 ± 0.05	0.21 ± 0.04	0.20 ± 0.03	0.21 ± 0.04	0.23 ± 0.05	n/s
Adrenal gland (R)	0.013 ± 0.005	0.013 ± 0.004	0.013 ± 0.004	0.012 ± 0.004	0.014 ± 0.005	n/s
Adrenal gland (L)	0.016 ± 0.005	0.016 ± 0.004	0.015 ± 0.006	0.016 ± 0.005	0.016 ± 0.006	n/s
Kidney (R)	0.358 ± 0.06	0.333 ± 0.05	0.325 ± 0.04	0.346 ± 0.05	0.359 ± 0.05	n/s
Kidney (L)	0.358 ± 0.05	0.320 ± 0.04[a]	0.321 ± 0.06	0.346 ± 0.06	0.363 ± 0.07	$p < 0.05$
Uterus	0.16 ± 0.02	0.15 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	n/s
Ovary (R)	0.019 ± 0.010	0.018 ± 0.010	0.016 ± 0.009	0.017 ± 0.008	0.017 ± 0.009	n/s
Ovary (L)	0.018 ± 0.010	0.018 ± 0.009	0.014 ± 0.008	0.017 ± 0.010	0.019 ± 0.011	n/s

Values are mean ± standard deviation of five replicates ($n = 5$). n/s: No statistically significant differences between control and any of treatment groups. aSignificant difference ($p < 0.05$) in comparison with 2,000 mg/kg FTH group. Relative organ weight = (organ weight/body weight) × 100. Right (R); Left (L).

might react with the DNA in blood cells. However, our study has proven that the haematology and blood coagulation results following 28-day treatment established a normal or within the range of standard reference for all treatment groups. Hence, our study demonstrated that the repeated exposure of ATH in rats was unlikely to cause any adverse effect on the haematological parameters.

Clinical chemistry is another crucial data for determining treatment-related effects within living organisms. In the present work, the liver function test was performed to detect any possible hepatotoxicity that might arise due to the administration of ATH with a high level of HMF. It is known

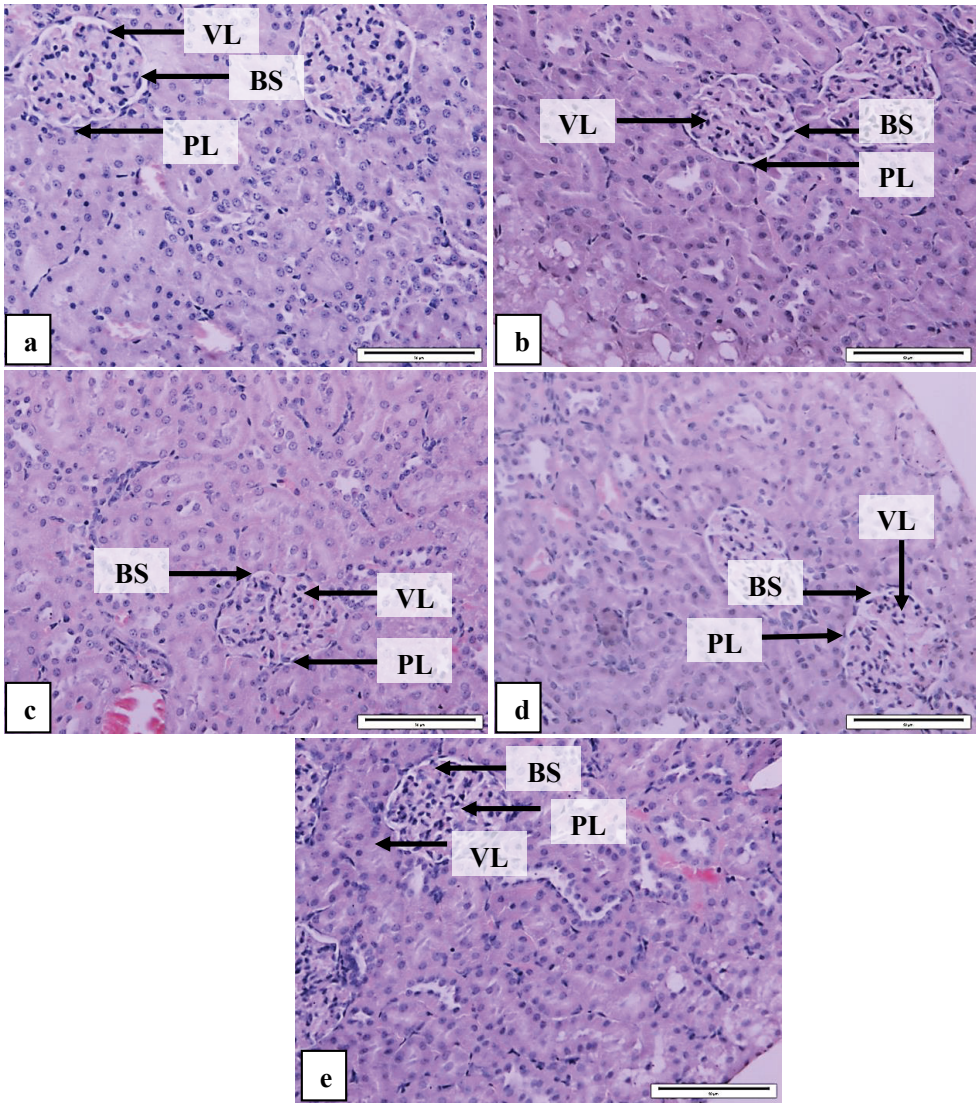

Figure 2. Histological sections of female rats' kidney stained with H&E. Photomicrographs of the representative longitudinal sections of kidney under 20× magnification of a light microscope. These were arranged in accordance to treatment groups (a) control, (b) 200 mg/kg ATH, (c) 1,000 mg/kg ATH, (d) 2,000 mg/kg ATH, and (e) 2,000 mg/kg FTH. The glomerular capsule consisted of the visceral layer (VL, modified epithelial cells) that surrounded the glomerular capillaries and parietal layer (PL, simple squamous epithelium layer). The capsular space between those layers was identified as Bowman's space (BS). The observation resulted in normal morphology and intact cellular architecture of the kidney between female rats. Nevertheless, BS reduction and poorly defined glomerular boundary were observed in the histological section of kidney from Group 3 (1,000 mg/kg ATH).

that the administration of HMF in rodents can be biotransformed to SMF by the SULTs enzyme, which is mainly expressed in the liver. Nevertheless, none of other parameters involved in the liver function test showed significant differences between control and treatment groups of both sexes. Hence, the administration of ATH within 28 days was unlikely to induce hepatotoxicity.

Honey is a well-known concentrated sugar solution. However, the administration of either ATH or FTH in the present work showed that both honey samples did not cause any significant elevation of blood glucose and cholesterol levels in the treated rats. This supports the previous finding by Erejuwa *et al.* (2011) who stated that the blood glucose concentrations in rats treated with TH were not significantly different from those in control rats. Moreover, for the total protein data, there were also no significant differences obtained between groups. This data is essential because the variation of its level may occur if the animals suffered from immunocompetence or other physio-pathological condition such as chronic progressive nephropathy (Petterino and Argentino-Storino, 2006).

Another parameter that is crucial to be highlighted in a toxicity study is the creatinine level. This is one of the important manifestations involved in renal function as the elevation of creatinine in peripheral blood signifies the inability of the kidney to efficiently remove creatinine from the body (Szekacs *et al.,* 2000; Shlipak *et al.,* 2013). In the present work, there was a statistically significant increase in the creatinine values of female rats administered with 1,000 mg/kg ATH as compared to the control group without affecting BUN and uric acid. However, the creatinine value in this group was within the normal range (0.4-1.4 mg/dL) (Sharp and La Regina, 1998). In contrast, there were no statistically significant differences between creatinine values of male rats assessed in the present work.

Evaluation of rat hormone treated with ATH was carried out to find any possible pharmacological interaction that might be related to underlying hormonal changes. All female rats treated with ATH had higher levels of oestradiol as compared to control; female rats treated with 1,000 mg/kg ATH had a significant increase in the oestradiol as compared to control. Nevertheless, the oestradiol value in this particular group fell into the normal range for rats (21.66-231.64 pmol/L) (Strom *et al.,* 2008) and did not cause a significant trend in the absolute and relative weights of the ovaries and uterus. The histological assessment on both organs also did not result in any abnormality such as hyperplastic or hypertrophic changes. A previous study reported that pure HMF compound could increase the oestradiol level, but the mechanism involved remains unclear (Elmaoğulları *et al.,* 2020). We postulate that the supplementation of ATH can reduce oxidative stress and ameliorate the effects of higher HMF content. It is based on the established information that among Malaysian honeys, TH has the highest source of phenolic acids and flavonoid compounds which have strong free-radical-scaveng-ing activities (Khalil *et al.,* 2011). Flavonoids particularly kaempferol and quercetin have been shown to have weak estrogenic activity (Zaid *et al.,* 2010). Apart from this, together with the increase in the creatinine value of the same dose group, this finding suggested that it might be a renoprotective effect of oestradiol by mediating the endothelium vasodilation to excrete the excessive creatinine (Szekacs *et al.,* 2000; Goldberg and Krause, 2016). Meanwhile for male rats, the administration of ATH did not cause any substantial fluctuation in the testosterone level.

Analysis of the selected organ weights and the histological assessment are sensitive indicators for any adverse effects of chemical exposure (Mohamad Zaid *et al.,* 2015). The present work revealed that there was a significant decrease in the left kidneys' weight of female rats treated with 200 mg/kg ATH as compared to those receiving 2,000 mg/kg FTH. A similar pattern of the kidneys' weight loss was also observed in the right kidney, but the results were not statistically significant. This finding will be further elucidated by looking at the microscopical examination of this particular organ.

The histology of the kidney showed that there were no apparent abnormalities noted on the overall cortex region. However, when observed deeper, it was revealed that there was Bowman's space reduction and poorly defined glomerular boundary of female rats receiving 1,000 mg/kg ATH. This finding, however, was not due to the serious mechanical pressure (*e.g.* glomerular hyperfiltration) as this could increase the Bowman's space volume, proximal tubular cells, and lumen volume (Chagnac *et al.,* 2019) which were not seen in the present work. Moreover, BUN and uric acid did not elevate significantly out of normal values which indicated that the kidney functioned normally (Cui *et al.,* 2018). In general, there was a connection between the changes in the kidney histology which attributed to the increase in the kidney weight and creatinine elevation. As kidney is the vital organ for the excretion of harmful products, it is also vulnerable to oxidative stress resulting from the imbalance between reactive oxygen species and defence mechanisms (Beshel *et al.,* 2014). Nevertheless, the liver of all treated rats did not demonstrate any bizarre changes which indicated a good sign of the absence of toxicity.

Apart from that, all other organs including the reproductive organs in the female rats showed uniformity of their respective histological patterns. Likewise, evaluation of the male rats' reproductive organs (testes, epididymides, vesicular, and prostate glands) also did not result in any visible histological defects.

Conclusion

Taking all the data together, the present work revealed that ATH contained remarkably higher HMF concentrations, exceeding the maximum standard limit (80 mg/kg for tropical honey). Oral treatment of ATH with high HMF at all dose levels demonstrated vital pharmacological responses *i.e.* modified the kidneys of female rats. Nevertheless, all male rats treated with ATH did not

show any definite toxicological effects, thus indicating sex-based variation. The no-observed-adverse-effect level was 2,000 mg/kg/day for male rats, and less than 200 mg/kg/day for female rats. Therefore, there is a need for further studies on ATH to elucidate the underlying mechanisms leading to these findings.

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