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Determination of 5-hydroxymethylfurfural in aged Malaysian Tualang honey and its effects on the female reproductive system of nulliparous Sprague Dawley rats

Harishini Rajaratinam[1], Nur Anissa Vidka Anjani[2] & Wan Ezumi Mohd Fuad[2,*]

[1]*Faculty of Medicine, Universiti Sultan Zainal Abidin, Medical Campus, Kuala Terengganu 20400, Terengganu, Malaysia.*
[2]*School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.*

**Correspondence:* wanezumi@usm.my

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ABSTRACT

This present study quantified 5-hydroxymethylfurfural (HMF) in ATH samples of different storage durations using UV-visible spectroscopy and evaluated the effects of these samples on the reproductive health of female Sprague Dawley (SD) rats. Rats with regular oestrous cycles were divided into 11 treatment groups (n=10), receiving either distilled water (control), fresh TH (FTH), ATH (24-, 36-, and 48-months old), purified HMF (low, medium, and high doses), or combinations of FTH and HMF. The rats were subsequently administered with test substances via oral gavage on the first day of dioestrus for four consecutive weeks. On the first dioestrus day upon completion of the four-week testing period, the rats (fasted overnight) were anaesthetised followed by blood collection and laparotomy. Blood samples were subjected to biochemical and hormonal analyses, and visceral reproductive organs were collected. The length of the oestrous cycles in control and treatment groups, in both pre (H-statistic=6.50, p=0.77) and during (H-statistic=11.48, p=0.32) treatment period showed no significant differences when evaluated using the Kruskal Wallis test. In addition, the one-way ANOVA test showed no significant differences in the body weights throughout the experimental period (F-statistic=0.195–0.978, p=0.468–0.996). As for the absolute and relative organ weights which were analysed using one-way ANOVA or Kruskal-Wallis analysis, only the absolute (H-statistic=18.87, p=0.042) and relative (H-statistic=20.62, p=0.024) weights of the right fallopian tube showed significant differences. Through the Kruskal-Wallis analysis, significant differences were observed in albumin (H-statistic=22.66, p=0.012), albumin/globulin A/G ratio (H-statistic=20.02, p=0.029), gamma-glutamyl transferase (GGT) (H-statistic=42.41, p<0.0001), and oestrogen (H-statistic=20.49, p=0.025) levels. These findings indicate that elevated HMF exposure may influence targeted biochemical parameters, regulation of oestrogen, and reproductive organ weight, even in the absence of marked changes in oestrous cyclicity and body weight. The inclusion of FTH may not attenuate high HMF effects, suggesting potential reproductive risks from prolonged honey storage.

Keywords: *5-Hydroxymethylfurfural (HMF); aged honey; female reproductive system and Tualang honey*

INTRODUCTION

The sweet natural product known as honey is created by honeybees, that collect nectar from blossoms and transform it into nutritious food (Shapla et al., 2018). In general, honey comprises a diverse array of significant bioactive compounds and numerous enzymes. Additionally, honey also contains various amino and organic acids, acid phosphorylase, phytochemicals, vitamins, minerals and carotenoid-like substances (Mohd Kamal et al., 2021). Honey is also generally rich in antioxidants which include phenolic acid, flavonoids, alkaloids, glucose oxidase, ascorbic acid, and others (Cianciosi et al., 2018; Ranneh et al., 2021).

In Malaysia, the utilisation of honey is vast and deemed beneficial for overall health and as complementary and alternative medicine (CAM) (Abd Wahab et al., 2017). The consumption of honey as a biologically based CAM is very well supported by the locals and it is reported that 56% of Malaysian respondents generally have favourable perceptions

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about honey as CAM (Abd Wahab et al., 2017). Tualang honey (TH), a precious national treasure is one of the common types of Malaysian honey (Azman et al., 2021), alongside others such as Gelam (Sujanto et al., 2021) and Kelulut (Haron et al., 2022). TH is a wild multifloral honey collected from the hives of wild honeybees, *Apis dorsata*, on Tualang trees found in the Malaysian rainforest. The colour of TH is dark brown, and it has a pH between 3.6 and 4.0 and a specific gravity of 1.34 (Azman et al., 2021). TH is a highly valued Malaysian natural product that is often regarded as superior to other local honey varieties due to its numerous beneficial properties (Ahmed & Othman, 2013; Mohd Kamal et al., 2021). According to Moniruzzaman et al. (2013), compared to Acacia, pineapple and Borneo honeys, TH displayed the highest concentration of total phenolics (352.73 ± 0.81 mg gallic acid/kg). The phenolic acids and flavonoids identified in TH, particularly caffeic acid, salicylic acid, luteolin, hesperetin, kaempferol, apigenin, and naringenin, contributed to enhanced radical scavenging activity and ferric reducing power (FRP) (Chew et al., 2018).

Given the fact of the widespread use of TH, a prominent concern surrounds the consumption of TH without an explicit shelf life. It is not surprising that people continue to consume TH even after the recommended time for use has passed (Sabireen et al., 2022). Most of the local honeys, including TH, are being sold in the market without having pre-determined expiry dates. Although an expiry date is provided, honey is generally marketed within four years of its manufacturing date. The locals are unaware that TH stored within 6 months is a fresh TH (FTH) and those stored for more than 12 months are considered aged TH (ATH) (Khalil et al., 2010). Relative to FTH, ATH is physically darker due to an increase in specific polyphenols or pigments (Sabireen et al., 2022). Khalil et al. (2010) further recommends that honey regardless of the type should be consumed within one year. The native Malaysian populace and old folks have always consumed aged honey as a nutritional supplement to enhance reproductive health and general well-being due to its antioxidant properties (Haron et al., 2014).

Despite the nutritional and therapeutic properties of TH, the general public is unaware that 5-hydroxymethylfurfural (HMF) together with its derivatives is present in TH. Particularly ATH, it comprises significant levels of HMF (Sabireen et al., 2020), frequently more than 80 mg/kg, an HMF limit for honey from tropical climates as specified by the Codex Alimentarius Standard. HMF, a cyclic aldehyde that is predominantly produced by the dehydration of fructose or glucose, is an organic neo-formed hazardous contaminant. It is typically absent or minimally present in fresh honey. The HMF content acts as a valuable parameter that indicates the quality of honey (Rajindran et al., 2022), which may be compromised by overheating or poor storage conditions (Shapla et al., 2018), adulteration with sugar additions (Fakhlaei et al., 2020), and ageing (Khalil et al., 2010).

Sabireen et al. (2020) reported that the HMF content in ATH stored for more than four years was 1426 mg/kg and it is deemed unsuitable for consumption. FTH in contrast, exhibited considerably low HMF content ranging from 4.19 mg/kg (Khalil et al., 2010) to 27.00 mg/kg (Sabireen et al., 2020). In agreement, Kishore et al. (2014) highlighted that TH harvested in 2005 exhibited an augmented HMF content of 449.89 ± 3.23 mg/kg compared to those harvested in 2010 (6.92 ± 0.02 mg/kg). The adverse effects of high HMF content may manifest in the forms of developmental toxicity (Jiang et al., 2022), oxidative stress (Hou et al., 2022) and indirect mutagenicity (Sommer et al., 2003; Severin et al., 2010).

Despite the fact that higher HMF contents have been demonstrated, scientific evidence regarding the potential effects of ATH remains scarce. Over the past decade, FTH has been the subject of extensive research, exploring for its various pharmacological benefits. Of these, research has established the beneficial effects of TH on both male (Hasan et al., 2022) and female reproductive systems (Zaid et al., 2010; Zaid et al., 2012; Ruslee et al., 2020) to support the historical claims that honey has long been traditionally utilised to enhance both men's and women's fertility. To date, studies and reviews have only focused on the superficial effects of honey on the reproductive systems, particularly in females. However, not much focus was given to the direct effects of high HMF content in aged honey on the female reproductive system.

The female reproductive system is governed by complex interactions involving the central nervous system (hypothalamus), pituitary gland and reproductive organs (Mikhael et al., 2019). This system is susceptible and require a conducive physiological environment for its normal operation. Administration of any detrimental agents, in this context is HMF or its metabolite may influence the reproductive-endocrine system, which may lead to profound harmful effects on the female reproductive functions. Elmaoğulları et al. (2020) demonstrated that peripubertal exposure to high-dose HMF was associated with precocious puberty and decreased anti-Müllerian hormone levels in female Wistar rats.

Considering the favourable consumption of TH in Malaysia and the relatively recent focus on the biological effects of HMF, this study aimed to determine the HMF contents in ATH and further investigate its effects on the female reproductive system of animal model. It was hypothesised that ATH, due to its elevated HMF content, disrupts hormonal balance and adversely affects the female reproductive system. In addition, FTH was proposed to mitigate the adverse effects of high-dose HMF on female reproductive health due to its antioxidant properties. By understanding this, the study will sequentially acknowledge the advantages and drawbacks in the pharmacological responses of HMF in ATH.

METHODOLOGY

Collections of ATH and FTH

In the present study, both Malaysian ATH and FTH of the Agromas® brand were provided by the Federal Agricultural Marketing Authority (FAMA), Kuala Nerang Kedah, under the Ministry of Agriculture and Food Industry, Malaysia. These TH samples were collected from the virgin, unpolluted Pedu Lake Forest Reserve and Muda Dam, Padang Terap, Kedah. The honey collected was subsequently sent to the Honey Processing Centre, FAMA Kuala Nerang, Kedah, for further analysis and processing in compliance with the Food Regulations 1985 (amendment 2014) of Food Act 1983 (Ministry of Health, Malaysia, 2014).

In general, TH in FAMA was processed through several stages, which include quality inspection, dehydration, packaging, and labelling. Upon collection, TH was examined for its water (moisture) and sugar contents as well as aroma and taste to ensure the authenticity of the product. The accepted TH should have an average percentage of sugar and water content of approximately 75% and 20%, respectively, abiding by the requirements that have been set by the MOH. TH was then weighed and filtered to remove foreign particles. Following that, TH was subjected to an evaporation process that took an average of 8 to 16 hours to remove about 3-5% of excess water. Evaporation ensured that the water content was maintained at a certain percentage, which could prevent TH from getting fermented and sour. Finally, TH underwent a second round of filtration before being bottled, weighed, and packaged under the Agromas® brand for distribution throughout Malaysia by FAMA.

The ATH obtained were categorised based on three different storage durations, 24-, 36- and 48-months old. FTH represents a fresh TH sample harvested and stored within 6 months and served mainly as a comparison to the contents and effects of ATH. Both ATH and FTH samples were stored in airtight bottles and kept at room temperature (25-30°C), away from direct sunlight.

Analysis of HMF concentrations

The HMF concentrations in a single batch of 24-, 36-, and 48-month-old ATH and FTH samples were determined using ultraviolet-visible spectroscopy analysis. The spectrophotometric technique described in AOAC official method 980.23 served as the foundation for determining the HMF content in honey with purified HMF (Thermo Scientific, USA) as the reference standard. Five grammes of honey were dissolved in 25 ml of water, quantitatively transferred into a 50 ml volumetric flask, supplemented with 0.5 ml of Carrez solution I and 0.5 ml of Carrez solution II, and diluted to a final volume of 50 ml with water. The solution was filtered through paper, discarding the initial 10 ml of the filtrate. Aliquots of 5 ml were transferred into two test tubes. In the first tube, 5 ml of distilled water was added (sample solution) whereas 5 ml of sodium bisulphite solution 0.2% was added into the second tube (reference solution). An ultraviolet–visible spectrophotometer (Lambda 365, PerkinElmer, USA) was utilised to determine the absorbances of the solutions at 284 and 336 nm. The linearity of the standard curve was used to determine the concentration of HMF in honey. The HMF content was calculated using the following equation:

$$\text{HMF (mg)/100g of honey} = \frac{(A284 - A336) \times 14.97}{5\ g\ of\ test\ sample}$$

A_{284}: the absorbance at 284 nm
A_{336}: the absorbance at 336 nm

Experimental animals

Healthy, nulliparous female Sprague-Dawley (SD) rats aged between 8 and 9 weeks old (bodyweight 220-230 g) were procured from the Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM), Health Campus. The conduct of this study was approved by the USM IACUC, approval number: USM/IACUC/2022/(137)(1222). Following institutional ethical approval, the experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of USM. At the commencement of the study, the weight variation of animals was ensured to be minimal and not exceeding ±20% of the total mean weight. All rats were housed together in a group of 5 rats per cage to maintain social interaction and improve their welfare. The cage is made of polypropylene material with a size of 480 x 375 x 210 mm (L x W x H). The females were individually dyed using an atoxic food colouring to avoid misidentification within the cage and were maintained at 20±2°C with a 12 h light / dark cycle (lights on from 0700 to 1900 hrs). The rats were given free access to commercially prepared pellets and tap water *ad libitum*. Cleanliness of the cage was maintained with regular bedding changes 2-3 times a week to allow good hygiene and minimise the risk of infection.

Experimental design and treatment groups

Following the acclimatisation period, the present study was initiated by looking at the oestrous cyclicity of female rats from 0800 to 0900 hours for one week prior to the start of treatment (referred to as the pre-treatment period) to provide baseline data for the regularity of cycling. The procedure of obtaining vaginal smear in this study was adopted with modifications from Marcondes et al. (2002) and Voipio & Nevalainen (1998). The oestrous cycle is divided into a sequence of phases, which consists of pro-oestrous (12-14 hours), oestrous (25-27 hours), metoestrus (or dioestrus I) (6-8 hours) and dioestrus (or dioestrus II) (55-57 hours), which are distinguished by the cytological proportions of cornified and nucleated epithelial cells, along with polymorphonuclear leukocytes (Fuad et al., 2017).

Female rats demonstrating 4 to 6 days oestrous cycle were judged to have a normal cycle. It was determined by counting the number of days from the first oestrus in a cycle to the next oestrus phase in a subsequent regular cycle. Females who experienced irregular cycles or health deficits during the pre-treatment period were not eligible to be included in the study (Fuad et al., 2017).

The fixed standard animal dose of 200 mg/kg honey was chosen for treatment as this dose is equivalent to one tablespoon, which is the daily amount of TH commonly consumed by an adult (Ruslee et al., 2020). The rats with normal, regular oestrous cycles during the pre-treatment period were randomly assigned to 11 treatment groups (n=10) as shown in Table 1.

Table 1

Experimental treatment groups and dosing regimen for fresh Tualang honey (FTH), aged Tualang honey (ATH), purified HMF, and combination treatments

Treatment groups	Treatment contents
A	control (vehicle/distilled water)
B	200 mg/kg FTH (within 6 months old)
C	200 mg/kg ATH 24-month (contains HMF low level)*
D	200 mg/kg ATH 36-month (HMF medium level)*
E	200 mg/kg ATH 48-month (HMF high level)*
F	16.1 mg/kg purified HMF low dose**
G	72.4 mg/kg purified HMF medium dose**
H	154.9 mg/kg purified HMF high dose**
I	200 mg/kg FTH + 16.1 mg/kg purified HMF low dose#
J	200 mg/kg FTH + 72.4 mg/kg purified HMF medium dose#
K	200 mg/kg FTH + 154.9 mg/kg purified HMF high dose#

*Note: *The actual levels of naturally occurring HMF in ATH depended on the quantification by UV-Vis spectrophotometer (groups A-E). **The actual doses of purified HMF (Sigma-Aldrich, USA) corresponded to the HMF levels measured in ATH (groups F-H). #To control the potential effect of other ATH components that may appear together with HMF during the ageing process of ATH (groups I-K).*

The animals were subsequently administered with test substances via gavaging on the first day of dioestrus at approximately the same time in the morning from 0900 to 1000 hours for four consecutive weeks. Vaginal samples were continuously inspected daily during this period. The number of animals that completed at least four regular oestrous cycles and the average oestrous cycle length were also documented. All female rats were also closely observed daily for mortality, moribundity, general physical health, behavioural changes, body weight and any signs of toxicity throughout the treatment period.

Laparotomy and gross assessment

On the first dioestrous day upon completion of the four-week dosing period, the overnight fasted female rats were anaesthetised with 60 mg/kg of sodium pentobarbital (Nembutal®) via intraperitoneal injection. Upon confirming that deep anaesthesia had been achieved by pinching the rat's toes to observe its response to stimuli, the rat was immediately laparotomised. The blood samples were collected via the cardiac puncture for biochemical and hormonal analyses. The rats were then euthanised under an overdose of sodium pentobarbital (100 mg/kg), by IP injection. The timing of euthanasia of all females was standardised between 1030 to 1130 hours in order to normalise the diurnal

hormonal variation in the females particularly when effects on the reproductive system are concerned (Fuad et al., 2017). They were subsequently subjected to macroscopical assessment of external and visceral organs. Only the visceral reproductive organs (hypothalamus, pituitary gland, cervix, uterus, fallopian tubes and ovaries) were resected and weighed. The absolute and relative organ weights based on equation below, were measured.

$$\text{Relative organ weight (\%)} = \frac{absolute\ organ\ weight\ (g)}{body\ weight\ of\ rat\ on\ sacrifice\ day\ (g)} \times 100\%$$

Serum biochemistry and hormonal analysis

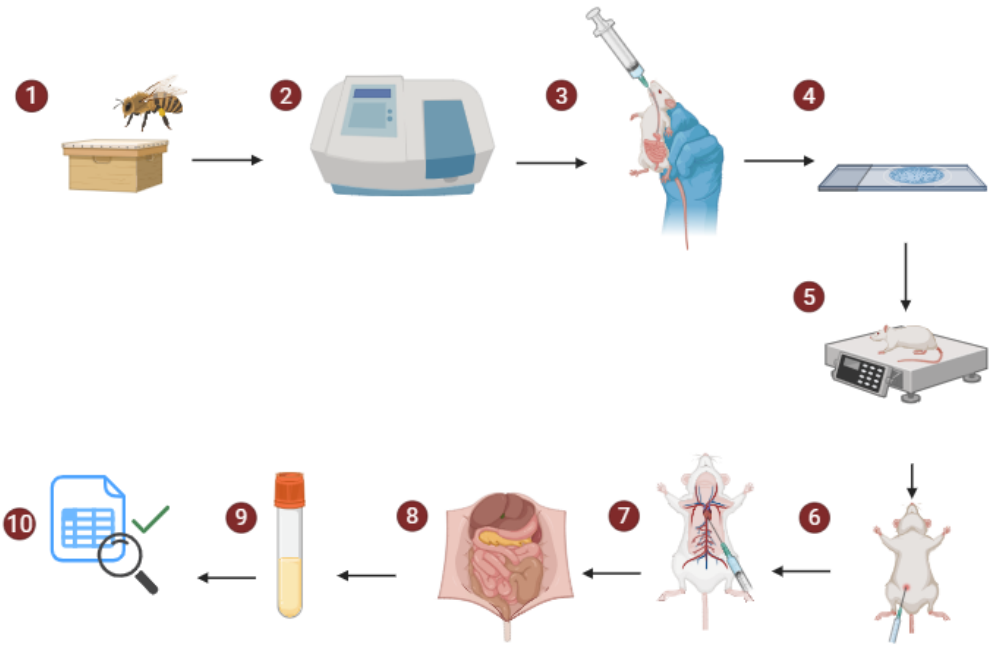
Approximately 3 mL of rat blood per specimen was collected and then later centrifuged at 1,800 g for 15 minutes to isolate the serum. The serum was subsequently analysed for various biochemical markers. The biochemical analysis has taken several parameters into consideration which include the levels of total protein (TP), albumin (AL), globulin (GL), AL/GL ratio (A/G R), alkaline phosphatase (AP), total bilirubin (TB), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine transaminase (ALT). The serum samples were also assessed to determine the levels of female reproductive hormones such as oestradiol and progesterone in the experimental female rats. These procedures were conducted in accordance with standard protocols at the Innoquest Pathology, a certified third-party laboratory service provider in Kota Bharu, Kelantan, Malaysia.

Statistical analysis

The selection of parametric or non-parametric analyses was determined based on the results of the normality test, together with the skewness and kurtosis distribution of the data. Statistical significance was set at $p<0.05$. Data are presented as either mean ± standard deviation (SD) or median (interquartile range, IQR). Error bars in the figures represent IQR (for non-parametric analyses). Statistical analyses and graph preparation were performed using SPSS version 29 and GraphPad Prism version 9 software. The overall experimental methodology has been summarised in the flow chart presented in Figure 1.

Figure 1

Experimental workflow of the study



Note: (1) Malaysian aged Tualang honey (ATH) and fresh Tualang honey (FTH) of the Apis dorsata origin were provided by FAMA, Kuala Nerang, Kedah, (2) HMF concentrations in 24-, 36-, and 48-month ATH and FTH samples were determined using UV-visible spectroscopy analysis, (3) followed by the administration of test substances via oral gavage, (4) vaginal smear collection to monitor oestrous cycle status, (5) body weight measurements were recorded, (6) rats were anaesthetised via intraperitoneal (I.P.) injection, (7) cardiac puncture was performed for blood collection, (8) laparotomy and gross morphological assessment of reproductive organs, (9) serum biochemical and hormonal analyses were conducted and (10) statistical analysis of collected data. Figure was created using the BioRender.com app.

RESULTS

Quantification of HMF in ATH and FTH samples

The level of HMF content increased as the storage duration progressed. The FTH, 24-, 36-, and 48-months old ATH samples contained 4.9, 16.1, 72.4, and 154.9 mg/kg of HMF, respectively.

Evaluation of body weights

The one-way ANOVA test was used to analyse any significant changes caused by different treatment modes in the body weights of female SD rats (groups A-K), at five time-points, days 1, 7, 14, 21 and 28 of the experimental period (Table 2). The rough observation of the body weights from day 1 to 28 for every group showed stable increments without the presence of body weight reduction. There was no significant difference (p>0.05) in the body weights of female SD rats of different treatment groups on days 1, 7, 14, 21, and 28 (Table 2).

General health and oestrous cyclicity

Throughout the treatment period, neither mortality nor morbidity were observed in the control and treatment groups of rats. No clinical signs of toxicity attributable to the test substances were noted upon initiation of treatment. It was found that the administration of test substances did not substantially alter the oestrous cyclicity of female rats in this study (Table 3). Within the respective pre-treatment and treatment periods that lasted for 28 days, the majority of animals (86.4%) completed 4 to 5 full cycles. An exception occurred for 15 females (13.6%) that displayed irregular cycles (either extended dioestrus, oestrous or shortened cycle) only once during the treatment period. The average length of their oestrous cycles was between 4 to 6 days and revealed no significant differences (p>0.05) between the control and treatment groups (Table 3).

Table 2

Body weights of SD rats during 28-days oral toxicity study

Body weights (g) of female rats					
Groups	**Day 1**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
A	225.9 ± 9.79	234.6 ± 17.56	239.3 ± 13.93	245.7 ± 18.28	256.6 ± 28.12
B	226.1 ± 9.49	236.7 ± 15.82	241.4 ± 15.29	246.2 ± 16.60	253.4 ± 18.25
C	224.9 ± 5.65	228.1 ± 10.14	232.1 ± 11.65	237.8 ± 13.06	246.2 ± 15.92
D	225.6 ± 5.21	229.4 ± 10.20	235.6 ± 12.45	236.8 ± 11.96	246.7 ± 15.99
E	225.7 ± 6.58	229.9 ± 10.06	237.3 ± 12.14	241.7 ± 13.23	250.2 ± 13.3
F	226.7 ± 3.65	230.8 ± 7.55	238.5 ± 12.4	241.8 ± 11.67	245.0 ± 11.26
G	225.1 ± 1.97	227.6 ± 6.40	233.7 ± 11.62	236.5 ± 9.19	240.5 ± 12.16
H	224.7 ± 2.83	228.2 ± 7.83	233.1 ± 7.98	236.2 ± 12.36	245.5 ± 12.90
I	227.0 ± 3.74	229.5 ± 8.96	232.8 ± 7.93	241.1 ± 9.71	243.6 ± 10.66
J	226.7 ± 2.58	225.5 ± 12.07	230.3 ± 13.70	237.3 ± 10.77	243.8 ± 7.80
K	226.5 ± 3.10	227.6 ± 7.24	236.3 ± 9.25	242.2 ± 8.65	247.1 ± 6.74
F-statistic	0.195	0.898	0.837	0.836	0.978
p-value	0.996	0.538	0.595	0.595	0.468

Note: The one-way ANOVA test was used for the analysis of the body weights on days 1, 7, 14, 21, and 28. The data were expressed as mean ± SD. Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose).

Evaluation of absolute and relative organ weights

The evaluation of the absolute and relative weights of uterus, cervix, vagina, ovaries, left fallopian tube, hypothalamus and pituitary gland weights showed no significant differences between the control and treatment groups (Table 4 & 5). However, the absolute and relative weight of the right fallopian tube showed a significant difference (p<0.05) between control and treatment groups with the lowest absolute weight observed in Group G (Table 4) and the lowest relative weight observed in Group E (Table 5).

Biochemical analysis

The biochemical results are tabulated in Table 6. The biochemical profile of the control group A was used as a baseline data for comparison. There were significant differences in the biochemical parameters, AL, A/G R and GGT of the female rats of different groups. Groups F, H, I, J and K displayed significantly lower levels of AL compared to the control group A. Furthermore, it was reported that groups F-K displayed significantly lower levels of A/G R and augmented levels of GGT compared to control group A. Interestingly, groups F-K were subjected to the administration of purified HMF, either solely or in combination with FTH, whereas none of the groups between B and E received purified HMF.

Aside from the significant results, group E which received ATH of 48-months revealed increased levels of TP, AL and AST when compared to other treatment groups, including the control group A. Groups H and K displayed the lowest A/G R ratios compared to other treatment groups. Additionally, group H which was subjected to the administration of high-dose HMF revealed the highest levels of AP compared to the other groups. The highest ALT value was detected in group K which received both FTH and high-dose HMF.

Hormonal analysis

Oestradiol levels

Based on the Kruskal-Wallis analysis, a significant difference (H-statistic=20.49, p=0.025) in the oestradiol levels in rats of different treatment groups was observed. It was noticed that the administration of FTH and purified HMF at a medium dose (Group J) for 28 days contributed to the highest oestradiol level (median (IQR): 169.20 (144.05) pmol/L) in rats. On the contrary, the administrations of ATH of 36-months old (medium level HMF) (Group D) and the combination of FTH and purified HMF at high dose (Group K) contributed to lower oestradiol levels in rats, 36.50 (75.25) pmol/L and 37.70 (51.82) pmol/L, respectively (Figure 2A). Groups D, G, and K displayed significantly lower levels of oestradiol compared to groups F, H, I, and J (p<0.05). Additionally, group G showed a significant decrease in oestradiol level compared to control group A (p<0.05).

Progesterone levels

Unlike the evaluation of oestradiol levels, the quantification and analysis of progesterone levels in female SD rats of different treatment modes revealed a non-significant difference (H-statistic=15.19, p=0.125), via the Kruskal-Wallis test (Figure 2B). The highest progesterone level (median (IQR): 57.85 (100.70) nmol/L) was detected in the serum of rats (Group B) that were subjected to the administration of only FTH for 28 days. In contrast, the lowest progesterone level (median (IQR): 10.59 (14.27) nmol/L) was recorded in the rats that received the combination of FTH and HMF at a high dose (Group K) for 28 days.

Key findings

HMF levels increased progressively with the storage duration of honey. Group G (medium-dose HMF) showed the shortest oestrous cycle. There were significant differences in the absolute and relative weights of the right fallopian tube, with the lowest absolute weight in Group G and the lowest relative weight in Group E. Groups F–K exhibited significant alterations in liver serum biochemistry. Notably, oestradiol levels were highest in Group J (FTH + medium-dose HMF) but markedly reduced in Group D (36-month ATH) and Group K (FTH + high-dose HMF). Additionally, Group G exhibited significantly lower oestradiol levels compared to Groups A, F, H, I, and J.

DISCUSSION

In the present study, the HMF contents in ATH were determined and its effects on the female reproductive system of nulliparous rats were further investigated. The HMF content in ATH increased with the duration of storage, at constant temperature. Compared to FTH, ATH stored for 24-, 36- and 48-months displayed a gradual increase in HMF content. According to the Codex Alimentarius Standard and the EU Honey Directive (international standards), it is recommended that honey from tropical regions have HMF content of less than 80 mg/kg (Bogdanov et al., 2002). Zaitsev

et al. (1975) determined the tolerable daily intake of HMF as 132 mg/day using a 40-fold safety margin. Malaysian honey samples stored between 3 to 12 months showed acceptable HMF levels (below the recommended limit) (Khalil et al., 2010; Moniruzzaman et al., 2013, Ya`akob et al., 2019; Sabireen et al., 2020; Ismail et al., 2021a).

Based on the recommended limit, the ATH stored for 48-months, utilised in the present study may not be safe for consumption, considering its high HMF content of 154.9 mg/kg. As per Zarei et al. (2019), there was a marginal decrease in the moisture content of honey after a year of storage, which led to an increase in the concentration of the constituents that cause the acidity of honey. This could account for the variations in pH and free acidity of TH that occur during storage (Zarei et al., 2019). Additionally, the natural acidity of honey (low pH) (Khalil et al. 2010) and its high fructose and glucose content (Cavia et al., 2002) may enhance HMF formation.

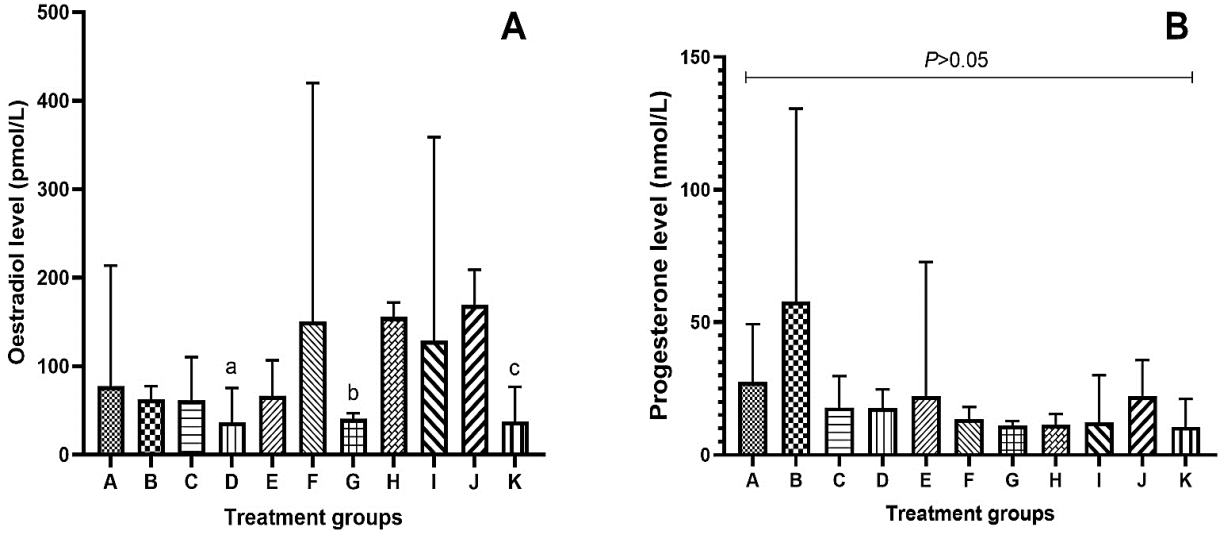
In the present animal experiment, Group G which received a medium dose of purified HMF displayed the shortest oestrous cycle (mean: 2 days, based on descriptive statistics). In a study by the National Toxicology Program (NTP) (2010), core groups and special study groups (for clinical pathology and neuropathologic evaluation) of 10 male and 10 female rats were administered 0, 94, 188, 375, 750, or 1,500 mg HMF/kg body weight in deionised water by gavage for 3 months. Some of the female rats displayed elongated oestrous cycles (National Toxicology Program (NTP), 2010). However, in the present study, the female SD rats showed a non-significant difference in terms of oestrous cyclicity. Female reproduction relies on optimal ovarian function since oestrous cyclicity, and oocyte maturation and release are predominantly governed by hormones produced by the ovary (Melin et al., 2016). A shorter oestrous cycle might be a sign of reproductive toxicity contributed by HMF. Endocrine-disrupting chemicals may alter ovarian function by interfering with hormone signalling via two mechanisms: (1) modifying the accessibility of ovarian hormones and (2) the binding and activity of the hormones at the receptor level (Craig et al., 2011; Ding et al., 2022).

A shorter or irregular oestrous cycle indicates that the ovaries are not functioning optimally. Impaired ovarian function may impact follicular development and ovulation. If the cycle is shortened, it may not allow enough time for proper follicle maturation or for the endometrium to become fully prepared for implantation, leading to decreased chances of successful conception. A shortened oestrous period is often a sign of reproductive toxicity, reflecting disruptions in hormonal balance, ovarian function, and overall reproductive health.

Apart from the oestrous cyclicity, the female SD rats subjected to various treatments showed no significant changes in body weight throughout the experimental period. Such observation is in line with previous study on the subacute toxicity effects of ATH with high HMF content in rats (Sabireen et al., 2022). The National Toxicology Program (NTP) (2010) study reported a loss in body weight of rats receiving HMF for three weeks or three months in doses exceeding 750 mg/kg/day. Considering the fact that the highest HMF value imposed in the present study was 154.9 mg/kg, relatively lower than the dose utilised in the NTP study, it may explain the absence of body weight loss amongst the experimental rats of the present study.

Figure 2

The [A] oestradiol ($p<0.05$) and [B] progesterone ($p>0.05$) levels in experimental rats (n=4) post 28 days of oral toxicity test using ATH, FTH and purified HMF



Note: Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose). Post hoc assessment for analysis [A]: [a]Group D showed significant differences in comparison with groups F, H, I, and J ($p<0.05$). [b]Group G showed significant differences in comparison with groups A, F, H, I, and J ($p<0.05$). [c]Group K showed significant differences in comparison with groups F, H, I, and J ($p<0.05$). No post hoc assessment was carried out for analysis [B].

Table 3

Summary of observations on general health and oestrous cyclicity of female rats after daily administration of test substances for four weeks

Parameter	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	***p*-value**
No. of females survived	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	-
Morbidity rate	0	0	0	0	0	0	0	0	0	0	0	-
Oestrous cyclicity												
Pre-treatment period												
No. of females with normal cycle	10	10	10	10	10	10	10	10	10	10	10	-
Length of cycles (days)	5.0 (0)	5.0 (0)	5.0 (0)	5.0 (0)	5.0 (1.0)	5.0 (2.0)	5.0 (1.5)	5.0 (0)	5.0 (1.25)	5.0 (1.25)	5.0 (0)	*p*=0.77 H=6.50
During treatment period												
No. of females with normal cycle	7	9	10	10	10	8	10	6	8	10	7	-
Length of cycles (days)	5.0 (6.0)	5.0 (1.25)	5.0 (0.25)	4.5 (5.0)	5.0 (2.0)	5.0 (2.25)	2.0 (5.25)	5.0 (6.0)	5.0 (2.25)	5.0 (0.25)	5.0 (5.0)	*p*=0.32 H=11.48

Note: The data were analysed using the Kruskal Wallis test and were expressed as median(IQR). Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose).

Table 4

The absolute weights of visceral organs

Absolute visceral organ weights									
Groups	**Uterus (g)[a]**	**Cervix (g)[b]**	**Vagina (g)[a]**	**Left ovary (mg)[a]**	**Right ovary (mg)[a]**	**Left fallopian tube (mg)[b]**	**Right fallopian tube (mg)[b]**	**Hypothalamus (g)[a]**	**Pituitary gland (g)[b]**
A	0.41±0.19	0.08(0.03)	0.17±0.21	13.9±5.4	19.26±11.37	9.15(3.75)	11.35(3.48)	0.13±0.19	0.01(0.01)
B	0.49±0.16	0.09(0.03)	0.12±0.03	20.0±9.3	19.08±11.04	7.15(6.23)	10.00(6.25)	0.07±0.01	0.01(0.00)
C	0.40±0.12	0.07(0.04)	0.11±0.03	25.1±24.1	17.15±7.44	7.30(7.33)	9.55(5.71)	0.12±0.19	0.01(0.00)
D	0.48±0.32	0.06(0.06)	0.11±0.04	13.7±8.5	15.70±13.23	10.65(4.11)	9.35(4.05)	0.06±0.01	0.01(0.01)
E	0.46±0.16	0.09(0.07)	0.09±0.04	19.5±12.4	20.33±10.15	10.60(5.11)	*7.65(4.45)	0.10±0.12	0.01(0.00)
F	0.38±0.12	0.07(0.04)	0.09±0.04	21.9±6.9	21.74±10.15	7.35(2.00)	*8.65(2.70)	0.07±0.02	0.01(0.00)
G	0.42±0.22	0.07(0.04)	0.10±0.02	23.8±14.0	19.63±9.64	8.75(5.86)	*7.45(2.15)	0.13±0.19	0.01(0.00)
H	0.44±0.14	0.08(0.04)	0.11±0.02	20.6±6.2	20.22±7.62	8.25(2.82)	9.15(2.83)	0.07±0.02	0.01(0.01)
I	0.40±0.14	0.07(0.10)	0.10±0.03	20.2±8.6	20.53±5.52	7.85(4.98)	9.10(4.41)	0.07±0.01	0.01(0.00)
J	0.39±0.15	0.06(0.02)	0.10±0.02	19.1±4.3	19.28±6.58	9.50(6.33)	11.25(12.20)	0.13±0.17	0.01(0.00)
K	0.36±0.06	0.07(0.02)	0.10±0.02	14.9±8.5	13.28±9.87	11.70(6.85)	*7.80(8.75)	0.07±0.01	0.01(0.01)
F- or H-statistic	0.611	5.151	1.211	1.148	0.654	13.330	18.87	0.724	10.44
p-value	0.801	0.881	0.294	0.335	0.764	0.206	0.042	0.700	0.403

*Note: [a]The data analysis carried out was one-way ANOVA and data were expressed as mean±SD. [b]The data analysis carried out was Kruskal-Wallis test and data were expressed as median(IQR). Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose). *Significant differences in comparison with the control group A ($p<0.05$ and $p<0.01$).*

Table 5

The relative weights (expressed in %) of visceral organs

Relative visceral organ weights (%)									
Groups	**Uterus[a]**	**Cervix[b]**	**Vagina[a]**	**Left ovary[a]**	**Right ovary[a]**	**Left fallopian tube[a]**	**Right fallopian tube[b]**	**Hypothalamus[b]**	**Pituitary gland[b]**
A	0.17±0.07	0.03(0.02)	0.07±0.07	5.95±2.54	7.92±4.15	3.92±1.15	4.78(1.47)	0.03(0.03)	0.00(0.01)
B	0.21±0.06	0.04(0.01)	0.05±0.01	8.41±3.63	7.98±4.08	3.29±1.56	4.42(1.89)	0.03(0.01)	0.01(0.01)
C	0.17±0.06	0.03(0.02)	0.05±0.01	10.55±9.86	7.27±2.90	7.17±11.96	4.08(2.86)	0.03(0.02)	0.00(0.003)
D	0.21±0.13	0.03(0.02)	0.05±0.02	5.93±3.35	6.74±5.31	4.72±0.85	4.08(1.51)	0.03(0.01)	0.01(0.01)
E	0.20±0.06	0.04(0.02)	0.04±0.02	8.31±4.98	8.70±4.09	4.35±1.32	*3.18(2.01)	0.03(0.01)	0.00(0.01)
F	0.16±0.05	0.03(0.01)	0.04±0.02	9.19±2.86	9.09±4.13	3.13±0.80	*3.59(0.93)	0.03(0.01)	0.01(0.01)
G	0.18±0.10	0.03(0.02)	0.04±0.01	10.50±6.29	8.58±4.21	3.88±1.39	*3.28(0.96)	0.03(0.01)	0.00(0.01)
H	0.19±0.06	0.04(0.02)	0.05±0.01	9.00±2.84	8.80±3.40	3.87±0.71	3.99(0.80)	0.03(0.01)	0.01(0.01)
I	0.18±0.07	0.03(0.05)	0.04±0.01	8.78±3.60	8.97±2.38	3.76±1.42	4.04(1.78)	0.03(0.00)	0.01(0.01)
J	0.17±0.06	0.03(0.01)	0.05±0.01	8.32±1.70	8.36±2.65	5.02±2.94	4.92(5.65)	0.04(0.01)	0.00(0.01)
K	0.16±0.03	0.03(0.01)	0.05±0.01	6.34±3.60	5.65±4.20	7.28±9.08	*3.32(3.75)	0.03(0.00)	0.00(0.01)
F or H statistic	0.705	5.131	1.217	1.221	0.763	0.902	20.620	13.180	8.539
p-value	0.718	0.882	0.290	0.287	0.664	0.534	0.024	0.214	0.576

*Note: [a]The data analysis carried out was one-way ANOVA and data were expressed as mean±SD. [b]The data analysis carried out was KruskalWallis test and data were expressed as median(IQR). Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose). *Significant differences in comparison with the control group A (p<0.05 and p<0.01).*

Table 6

The serum biochemical analysis of experimental SD rats

Biochemical parameters									
Groups	TP (g/L)	AL (g/L)	GL (g/L)	A/G R	AP (IU/L)	TB (mg/dL)	GGT (U/L)	AST (U/L)	ALT (U/L)
A	65.00(6.5)	43.00(21.50)	22.50(6.25)	1.90(0.50)	70.50(59.72)	1.90(0.00)	2.90(0.00)	152.00(70.20)	48.50(9.25)
B	67.00(10.25)	43.50(7.75)	25.50(6.00)	1.80(0.45)	74.50(22.75)	1.90(0.00)	2.90(0.00)	214.50(111.80)	50.00(25.50)
C	65.00(5.5)	42.00 (2.25)	22.50(4.75)	1.90(0.75)	66.50(49.25)	1.90(0.00)	2.90(0.00)	148.50(87.00)	44.00(20.00)
D	68.50(10.5)	44.00(4.75)	24.50(9.25)	1.90(0.75)	81.00(21.00)	1.90(0.00)	2.90(0.00)	201.50(63.80)	61.50(24.75)
E	69.00(9.5)	44.50(4.75)	24.50(6.25)	1.70(0.50)	77.50(67.30)	1.90(0.00)	2.95(0.85)	218.00(114.70)	56.50(34.75)
F	66.00(1.5)	[a]40.00(2.25)	26.00(3.75)	[b]1.50(0.30)	75.50(109.30)	1.90(0.00)	[c]6.90(0.00)	159.00(104.00)	56.50(34.75)
G	66.00(52.50)	40.00(3.75)	26.50(21.25)	[b]1.45(1.23)	71.50(35.50)	1.90(0.00)	[c]6.90(0.00)	191.00(51.50)	64.00(9.50)
H	67.50(2.5)	[a]39.50(2.50)	28.50(4.50)	[b]1.40(0.28)	101.50(36.00)	1.90(0.00)	[c]6.90(0.00)	166.00(36.80)	65.50(21.75)
I	65.50(1.75)	[a]39.00(1.50)	26.50(1.75)	[b]1.45(0.10)	69.00(16.25)	1.90(0.00)	[c]6.90(0.00)	175.00(47.30)	68.50(28.00)
J	66.50(9.0)	[a]39.50(3.25)	26.50(7.25)	[b]1.45(0.38)	89.50(45.05)	1.90(0.00)	[c]6.90(0.00)	163.50(28.00)	72.00(14.00)
K	63.00(7.5)	[a]37.00(6.00)	26.00(1.50)	[b]1.40(0.15)	79.50(29.00)	1.90(0.00)	[c]6.90(0.00)	176.50(36.00)	78.00(22.75)
H-statistic	6.751	22.660	9.614	20.02	8.791	-	42.21	13.06	18.18
p-value	0.749	0.012	0.475	0.029	0.552	>0.05	<0.0001	0.220	0.052

Note: The comparisons were carried out via Kruskal-Wallis analysis since the data were not normally distributed (n=4). The data were expressed as median(IQR). Biochemical parameters: total protein (TP), albumin (AL), globulin (GL), AL/GL ratio (A/G R), alkaline phosphatase (AP), total bilirubin (TB), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine transaminase (ALT). Treatment groups: A (control), B (FTH), C (ATH 24-month), D (ATH 36-month), E (ATH 48-month), F (purified HMF low dose), G (purified HMF medium dose), H (purified HMF high dose), I (FTH + purified HMF low dose), J (FTH + purified HMF medium dose) and K (FTH + purified HMF high dose). [a]Significant differences in comparison with the control group A (p<0.05 and p<0.01). [b]Significant differences in comparison with the control group A (p<0.05). [c]Significant differences in comparison with the control group A (p<0.01).

Apart from body weight, the absolute and relative weights of most resected visceral organs showed no significant differences among the treatment groups, except for the right fallopian tube, which demonstrated the lowest absolute and relative weights in Groups G and E, respectively. Since Groups G and E represented medium-to-high HMF content, the reduced right fallopian tube weights observed in these groups may indicate a possible HMF-related effect on reproductive tissues or organ. This contrasts with Ismail et al. (2021b), who reported that Gelam honey (HMF content=6.9mg/100g) attenuated uterine and vaginal epithelial atrophy and improved endometrial stromal and endothelial thickness. Although both Groups G and E contained higher HMF levels than the Gelam honey used by Ismail et al. (2021), several factors may account for the differing findings, including the type of honey and the form of HMF administered, as Group G involved purified HMF dose. In a study by Elmaoğulları et al. (2020), two dose levels of HMF (750 and 1500 mg/kg/day) were administered to immature female Wistar rats for three consecutive weeks. The study reported that higher dose-HMF-exposed rats displayed an enhanced number of secondary atrophic follicles and exhibited an early vaginal opening (Elmaoğulları et al. 2020).

HMF is known to form reactive intermediates. Following HMF consumption, it is transformed into 5-sulfoxymethylfurfural (SMF) in the liver and then released into the bloodstream to induce toxicity in other tissues. The high uptake of SMF into the liver may evoke hepatotoxicity (Bauer-Marinovic et al., 2012). In relation to biochemical analysis, the hepatic affection is characterised by the imbalance of the serum levels of TP, expressed by the deterioration of the A/G R. The decrease of the AL level is accompanied by the increase of the GL level, mainly of γ-GL (less frequently of α-GL or β-GL). The improvement of the A/G R involves the increase of serum AL, accompanied by a proportional decrease in the GL values (Andritoiu et al., 2014). The current study has observed that the administration of 48-months ATH (high level of HMF) (Group E) and the combination of FTH and purified HMF (high dose) (Group K) resulted in the highest and lowest AL levels, respectively, as compared to the control group A. The administration of heated honey (140 mg HMF/kg) and stored honey (181 mg HMF/kg) showed increased TP in male Wistar albino rats (Özkök & Silici, 2016). Increasing serum TP levels might result from increasing AL or GL functions (Özkök & Silici, 2016). According to El Bohi et al. (2020), mice treated with 250 mg/kg of HMF were reported with DNA damage, although it was noted that the toxicity may vary depending on the dose, animal species, and route of administration. In contrast to Özkök & Silici (2016), the study reported that overall protein, AL, and GL contents were reduced, which is thought to reflect the degenerative effect of HMF on the liver (El Bohi et al., 2020).

Groups H and K (both containing high doses of HMF, either solely or in combination with FTH) displayed the lowest A/G R ratios compared to other treatment groups. The ratio will decrease as AL decreases or GL increases, a change that may not be reflected in TP levels. As mentioned earlier, group K displayed the lowest AL level compared to the other groups. Decreased A/G ratios are often associated with inflammation (Chen et al., 2021). According to Xu et al. (2023), HMF may provoke persistent, chronic and systemic inflammation, hence hastening the advancement of frailty in mice via cellular senescence. Hence, this explains the possible association between low AL levels, reduced A/G ratio, inflammation, and high HMF levels.

GGT is extensively recognised as a marker for liver impairment, biliary disorders and excessive alcohol intake (Koenig & Seneff, 2015). Elevated serum GGT has been postulated as an independent marker of systemic inflammatory activation and enhanced oxidative stress, irrespective of its association with metabolic syndrome (Yamada et al., 2006; Cheraghi et al., 2019). In this current study, the groups that received purified HMF (regardless of doses) either solely or in combination with FTH showed increased serum GGT levels (groups F-K), compared to other groups. Interestingly, Qiu et al. (2022) reported that HMF imposes detrimental consequences on gastric mucosal epithelial cells via oxidative stress induction. Parallel with the concept of oxidative stress, Hasim et al. (2020) reported that the modulation of nociceptive responses in the prenatally stressed rat offspring by TH was linked with improvement in oxidative stress parameters and histology of the thalamus. It is evident that the dosage of HMF in TH plays an essential role in the regulation of oxidative stress, with regards to the wellbeing of the reproductive systems.

Besides reproductive organ structure, hormones such as oestrogen and progesterone also play important roles in follicular development. Oestrogen is known to affect granulosa cell growth and differentiation in association with gonadotropins. In the present study, the combination of FTH and purified HMF at a medium dose (group J) resulted in the highest oestrogen level compared to administrations of ATH of 36-months old (medium level HMF) (group D) and the combination of FTH and purified HMF at a high dose (group K). In BPA-exposed rats administered with TH (subjected to gamma irradiation), morphological deviations, such as the development of large antral cystic-like follicles (anovulation follicles), the inadequacy of the corpora lutea and preantral follicles and the number of atretic follicles, were marginally diminished (Zaid et al., 2014). Irradiation is known to reduce HMF levels in honey (Hussein et al., 2014). Zaid et al. (2015) further reported that in BPA-subjected rats, administration of TH (exposed to gamma irradiation) significantly alleviated ovarian and uterine morphological abnormalities and normalised oestrogen receptor (ER)-α, ERβ and C3 expression levels and distribution. According to Ahmed et al. (2018), the use of TH with 0.53 mg/100 g (5.3 mg/kg) of HMF level may ameliorate oestradiol at the serum level to inhibit its negative effects and modulate tumour necrosis factor-alpha (TNF-α) concentration. The inclusion of FTH with a lower HMF level may mask the medium dose of purified HMF and be beneficial for the upregulation of oestrogen, however, that might not be the case in the high dose purified HMF. Tsiapara et al. (2009) reported that thyme honey with high HMF level (203 mg/kg) displayed anti-oestrogenic and a weak oestrogenic effect in MCF-7 cells.

Earlier, it was reported that group G exhibited the shortest oestrous cycle compared to other treatment groups. Further evaluation of the oestrogen analysis showed that group G (subjected to a medium dose of purified HMF) exhibited significantly lower oestrogen level compared to groups A (control), F (purified HMF low dose), H (purified HMF high dose), I (FTH and purified HMF low dose) and J (FTH and purified HMF medium dose). The shorter oestrous cycle might be explained based on the lower oestrogen level in group G. This further supports that the inclusion of FTH may improve the effects of medium dose of purified HMF. While direct evidence is lacking, the observed hormonal changes in oestradiol level and oestrous cyclicity, may be linked to the disruption of the hypothalamic-pituitary-gonadal (HPG) axis, potentially triggered by the oxidative stress and liver dysfunction (Ullah et al., 2023; Papadimitriou et al., 2024) induced by high or continuous HMF exposure (Fan et al., 2021; Qiu et al., 2022).

The supplementation of ATH may help reduce oxidative stress and ameliorate the effects of elevated HMF content. Such absence of deleterious effect may be due to the fact that TH (among other Malaysian honey) has the most significant source of phenolic acids and flavonoid components which exhibit potent free-radical-scavenging and antioxidant activities (Kishore et al., 2011; Moniruzzaman et al., 2013). Phenolic compounds in honey are extensively known to influence reproduction. Phenolic compounds may induce alterations in the expression of genes and the activity of enzymes (aromatase, topoisomerases I and II, and ERKs) engaged in the regulation of reproductive processes (Hashem et al., 2021). However, HMF and its metabolites possess enzyme-inhibitory effects (Mizushina et al., 2006). Given that TH is rich in phenolic compounds, it might be able to overcome the enzyme-inhibitory effects of HMF.

The administration of FTH alone (group B) produced the highest progesterone level. Progesterone is an essential component in the female reproductive system for ovulation, implantation and sustenance of pregnancy. During ovulation, the Graafian follicle ruptures, releasing the ovum. Subsequently, the follicle undergoes remodelling to develop into a corpus luteum, which produces progesterone. The ovulatory process relies on progesterone (Dozortsev et al., 2020). According to Mosavat et al. (2014), TH imposed positive effects by decreasing the elevated cortisol and enhancing the reduced progesterone levels induced by different intensities of jumping exercises in female rats. In the study, however, the lowest progesterone level was detected in the group that received both FTH and a high dose of purified HMF. It could be postulated, that FTH may not be able to overcome the deleterious effects of a high dose of purified HMF. Although FTH contains beneficial bioactive compounds, it did not exert a protective effect against high-dose HMF in the study. This may be due to the overwhelming impact of the high HMF concentration, which could have surpassed the antioxidant and protective capacity of FTH. While initially it was hypothesised that a mitigating effect from FTH could be present, the results suggest that its protective potential is insufficient at higher toxicant levels. This finding highlights the dose-dependent nature of HMF toxicity and warrants further investigation.

Limitations of the current study include the absence of follicle-stimulating hormone (FSH) and luteinising hormone (LH) analyses, which restrict the ability to directly evaluate disruptions at the HPG axis. FSH and LH levels were excluded from reporting as their concentrations were below the detectable range of the assay. The low or undetectable FSH and LH levels observed in this study may be explained by the timing of blood collection during dioestrus, corresponding to the luteal phase in rats, during which gonadotropin levels are physiologically low. As these basal concentrations may fall below the detection range of some ELISA kits, kit sensitivity may have contributed to the inability to quantify FSH and LH in certain samples. As oestradiol and progesterone levels alone may not fully reflect endocrine disturbances, the lack of upstream gonadotropin data limits the mechanistic interpretation of hormonal dysregulation. Future studies incorporating comprehensive HPG axis profiling are warranted to clarify the pathways through which HMF exposure may contribute to female reproductive toxicity. The high standard deviations observed in Figures A and B are likely due to data variability arising from the limited availability of certain samples within each group. However, this does not imply that the data are inaccurate or unreliable. While this may have contributed to the observed variability, all experiments were conducted under standardised conditions to minimise technical inconsistencies. The team completely recognises the value of increasing sample size for improving reproducibility and will consider this in future studies as resources permit. Moreover, the absence of molecular (mechanistic) and histopathological analyses, which could have provided further validation of the observed biochemical and hormonal findings. Another limitation of the present study was the use of UV-visible spectroscopy as the sole method for HMF quantification. Although widely used for HMF determination, future studies incorporating high-performance liquid chromatography (HPLC) may improve the accuracy and reliability of HMF measurements. In the ongoing study, HPLC analysis has been included to further validate and strengthen the HMF measurements.

CONCLUSION

The administration of ATH and purified HMF (either solely or in combination with FTH) did not significantly affect the oestrous cycles and body weights of the female nulliparous Sprague Dawley rats. However, it impacted the absolute and relative weights of the right fallopian tube and the liver serum biochemistry of the rats. Group G (HMF-medium dose) showed pronounced impact with the shortest oestrous cycle, significant alteration in the reproductive organ and liver serum biochemistry and reduced oestradiol levels. The inclusion of FTH may not be able to mask the effects of purified HMF at high dose such as in the case of Group K with significant changes in liver serum biochemistry and oestradiol level. As for the ATH, Groups D (36-month) and E (48-month) with medium and high HMF levels contributed

to the alterations in oestradiol levels and relative organ weight, respectively, thus signifying the potential risk to the reproductive health following the consumption of ATH, particularly in 36- and 48 months old.

AUTHOR CONTRIBUTIONS

Harishini Rajaratinam contributed to formal analysis, visualization, methodology, investigation, data curation, writing-original draft, review and editing. Nur Anissa Vidka Anjani contributed to data curation, methodology, writing-review and editing. Wan Ezumi Mohd Fuad contributed to conceptualization, supervision, visualization, funding acquisition, writing-review and editing. All authors have given approval to the final version of the manuscript.

ETHICS APPROVAL

The conduct of this study was approved by the Institutional Animal Care and Use Committee (IACUC), USM, approval number: USM/IACUC/2022/(137)(1222).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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