



Preliminary Study on Malaysian Honey as Anti-Stress Agent

Qamarul Hafiz Zainol Abidin¹, Wan Iryani Wan Ismail², Nahlah Elkudssiah Ismail³, Zolkapli Eshak^{1*}

¹ Department of Pharmacology and Chemistry, Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam Campus, 42300, Puncak Alam, Selangor, Malaysia

² School of Fundamental Science (PPSA), Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Terengganu, Malaysia

³ Faculty of Pharmacy, MAHSA University, Bandar Saujana Putra, 42610, Jenjarum Jaya, Selangor, Malaysia

*Corresponding author: Tel. +60332584744; Fax. +60332584602;

E-mail address: zolkapli_eshak@puncakalam.uitm.edu.my

Keywords: Chronic stress, Acacia Honey, Tualang Honey, Stress-disorder

Introduction

Stress has become a common problem of modern everyday life. Exposure to stressors triggers cascading of nervous, endocrine and neurological systems that attempt to maintaining homeostasis and towards the biological changes. Prolonged stress causes significant physiological disorders and can contribute to other morbid diseases such as hypertension, cardiovascular disease, obesity, diabetes and cancer. Stress is also a major contributing factor which can leads to psychosomatic disorder:- illness and disease that resulted from aggravated psychological disorder, namely emotional stress such as depression ¹.

Recent studies showed that honey might contain compounds that can alleviate stress-disorder effects. For example, Tualang honey have shown to exhibit antinociceptive effect and reduced pain response during stress ². Another study has reported that Tualang honey normalised cortisol levels when tested in jumping stressed rats ³. As Tualang honey is just one of many types of honey exist in Malaysia, it is still unsure whether the effects are honey specific or can be applied to all honey types. Moreover, since most of these studies do not employ a wide range of stressors that simulate real life stress, the practicality of using honey as stress-disorder treatment is still unconvincing. Thus, in this study, commonly consume Malaysian honey, Tualang and Acacia honey were screened for its anti-stress effects by using chronic stress animal experiment.

Methods

Honey Analysis

Acacia honey was collected from *Apis mellifera* sp. bee hives nested near the Acacia (*Acacia mangium*) plantation located in Kota Tinggi, Johor whereas Tualang honey was collected from *Apis dorsata* sp. bee hives nested on Tualang (*Koompassia excelsa*) tree in Pedu Lake Forest Reserve, Kedah. Honey was collected using sterile 50 mL syringe and kept in sealed tube stored in a dark chiller at 2 °C to 4°C. Moisture content, pH and free acidity of honey were tested according to Harmonized Methods of International Honey Commission ⁴.

Modified version of the folin-ciocalteu method and colorimetric assay method using 96-well plate was used for total phenolic compound (TPC) and total flavonoid compound assays. Briefly, 50 µL honey and gallic acid (0 – 125 µg / mL, Sigma-Aldrich, Germany) solutions were pipetted into the 96-wells plate (Corning, New York, USA). Subsequently, 80 µL 15% Folin was pipetted into all wells before adding 80 µL NaCO₃. The plate was incubated at 37°C. For TFC assay, catechin (Sigma-Aldrich, Germany) at varying concentration (0 – 1000 µg / mL) was prepared. Then, 25 µL honey solution and catechin were pipetted into 96-well plate before 30 µL 1.25 % w/v of NaNO₂ was added into all wells. Next, 30 µL 2.5 % w/v AlCl₃ and 50 µL 1M NaOH were pipetted into each well. Colour changes in the multi-wells plates were observed at 756 nm for TPC and 510nm for TFC using Infinite M200 microplate reader (Tecan, Zurich, Switzerland). Results were expressed as gallic acid equivalents (GAE) and catechin equivalent (CE).

Anti-oxidants activity of tested honey was measured using oxidative haemolysis inhibition assay (OxHLIA) method with modification⁵. Honey were prepared at various v/v concentrations (6.25%, 12.5%, 25%, and 50%) and pipetted into 96-wells plate (Corning, New York, USA). Then, 10 mL rats' erythrocytes was suspended in PBS at 3.0% (v/v) and 50 µL rats' erythrocytes were pipetted into each well and incubated at 37°C in shaking incubator with 1200 rpm for 10 minutes. Then, 50 µL 160 mM of 2,2-azobis(2-amidinopropane) dihydrochloride (APPH) diluted in the PBS was immediately added and further incubated at 37°C shaking

incubator (1200 rpm). The optical density of the plate was measured at every 10 minutes at 660 nm by using microplate reader Infinite M200 and percentage of survival erythrocytes population were calculated. Antioxidant activities of honey were determined as time to reach 10% haemolysis for each honey concentration ⁵.

Chronic Stress Experiment

A total 16 adult male Sprague-Dawley rats (weighing 200 ± 50 g) were obtained from Laboratory Animal Facility and Management (LAFAM), UiTM. The rats were maintained at 24 ± 1 °C, $45 \pm 15\%$ relative humidity, and 12h/12h light/dark cycle with food and water provided *ad libitum*. Honey was administered daily using oral gavage. The maximum volume of liquid given at one time does not exceed 1 mL/100g body weight. Honey was diluted with distilled water to get the required dosage of 1.2 g/Kg.

Chronic stress procedure was conducted as previously reported ⁶. Rats were divided into 4 groups; Normal control (NC) group, Stress-Model (SM) group, Stress-Acacia honey (SA) group, and Stress-Tualang honey (ST) group. The chronic stress experiment was conducted for 31 days with 28 days of stress experiment and three days of assessment on physical, behavioural and blood parameters. Stressors were randomised using Research Randomiser software version 4.0. One random stressor out of seven selected stressors was given each day to each stress rats group (i.e. Overnight illumination, forced swimming, fasting, novel environment, white noise, cage tilting & restricted movement). All stressor were conducted between 9:00 AM to 12:00 PM. Animal study was approved by Research Committee on the Ethical Use in Research, Universiti Teknologi MARA (UiTM Care No.: 108/2015).

Behaviour, Weight, and Blood Evaluation

Open field test (OFT) was conducted at day-29 during the study. The OFT procedure was conducted by allowing each individual rat to open field test box (200 cm x 150 cm) for 5 minutes and the locomotors activities were recorded and analysed using Any-MAZE software version 4.80 (Stoelting Inc, Illinois, USA). Body weight changes and food intake analysis were conducted at the end of the animal experiment.

Blood sampling was conducted on day-31 of the study. Rats were anesthetized with 100 mg/kg of ketamine and 10 mg/kg xylazine at 0.2 mL/100 gram of body weight. Blood was collected using retro-orbital bleeding and was analysed using Coulter LH 500 haematology analyzer (Beckman Coulter, California, USA). Blood sample for serum chemistry was collected using anticoagulant tubes. Blood samples were centrifuged at 10,000 rpm and serum were pipetted into 1.5 mL centrifuge tube and analysed at Veterinary Laboratory Service Unit, Faculty of Veterinary, Universiti Putra Malaysia. Serum cortisol was measured by using cortisol (rodent) enzyme-linked immunosorbent assay kit from Abnova, Taiwan (kit no.: KA2328).

Results and Discussion

Table 1: Physicochemical and antioxidant activity.

Types of Honey	Moisture Content (%)	pH	Free acidity (meq / kg)	Gallic acid equivalent (mg GAE / kg)	Catechin equivalent (mg CE / kg)	ΔT (min) of 10% Haemolysis
Acacia	19	3.87 ± 0.009	36.0 ± 0.578	111.2 ± 1.65	$14.16 \pm 1.15^*$	80.393
Tualang	21	4.51 ± 0.003	36.3 ± 0.882	123.2 ± 4.72	$29.74 \pm 4.12^*$	129.481

Moisture content of Tualang honey was higher compared to standard (< 20%) set by the International Honey Commission (IHC) but this value is not uncommon for honey originates from a tropical country like Malaysia (Table 1) ⁷. Nevertheless, moisture content value of Acacia honey and Tualang honey was comparable with other Malaysia's Acacia and Tualang honey studies (17% - 26%) ⁸. The pH and free acidity values of the honey were within the IHC standard and correlates with the others Malaysia's honey.

Both Acacia honey and Tualang honey showed high phenolic and flavonoid content with Acacia honey (Table 1). Moreover, both honey possessed high antioxidant activity in term of erythrocytes protective effects from APPH-derived peroxy radicals observed from OxHLIA test. Both honey prevent 50% of erythrocytes haemolysing even after 180 minutes of incubation with Tualang honey has high antioxidant activities compared to Acacia honey (Table 1). This finding correlate with other researchers' antioxidants results that used ferric reducing/antioxidant power (FRAP) assay and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) antioxidant assay where high anti-oxidant activities for Tualang honey were observed in comparison to Acacia honey ⁹

Table 2: Physical and behavioral parameters results.

Treatment group	Body weight change (g)	Total calorie intake per weight increase (Kcal/day/gram)	Total distance travel (m)	Total time mobile	Total climbing duration (s)
NC	86.78 ± 8.327	1.045 ± 0.098	45.30 ± 4.011	54.18 ± 8.050	48.88 ± 8.867
SM	75.46 ± 26.416	1.536 ± 0.150 [*]	42.58 ± 12.554	48.08 ± 18.423	41.50 ± 17.243
SA	109.12 ± 52.057	1.252 ± 0.056	69.59 ± 6.112	82.55 ± 24.647	76.23 ± 15.732
ST	54.33 ± 4.520 [*]	1.451 ± 0.061 [*]	50.60 ± 8.740	64.15 ± 6.444	76.10 ± 17.408

Note: Value are expressed as ± SEM (n = 4). *Significant difference (p < 0.05) versus NC group.

No statistically significant results were observed among all groups in OFT. SA rats group was observed to record the highest reading for all OFT parameters tested with total distance travel, total time mobile and total climbing duration values (Table 2). Possible explanation for increased activity SA group was because of honey neutrophilic content that has been reported to boost brain function in many other honey studies ¹⁰. SM group's total distance travelled (42.58 ± 12.554 m), total time mobile (48.08 ± 18.423) and total climbing duration (41.50 ± 17.243 s) values recorded the lowest reading among all experimented group. This concordant with others finding that showed lower horizontal activity in stressed induced rats in comparison to normal control rats ¹¹.

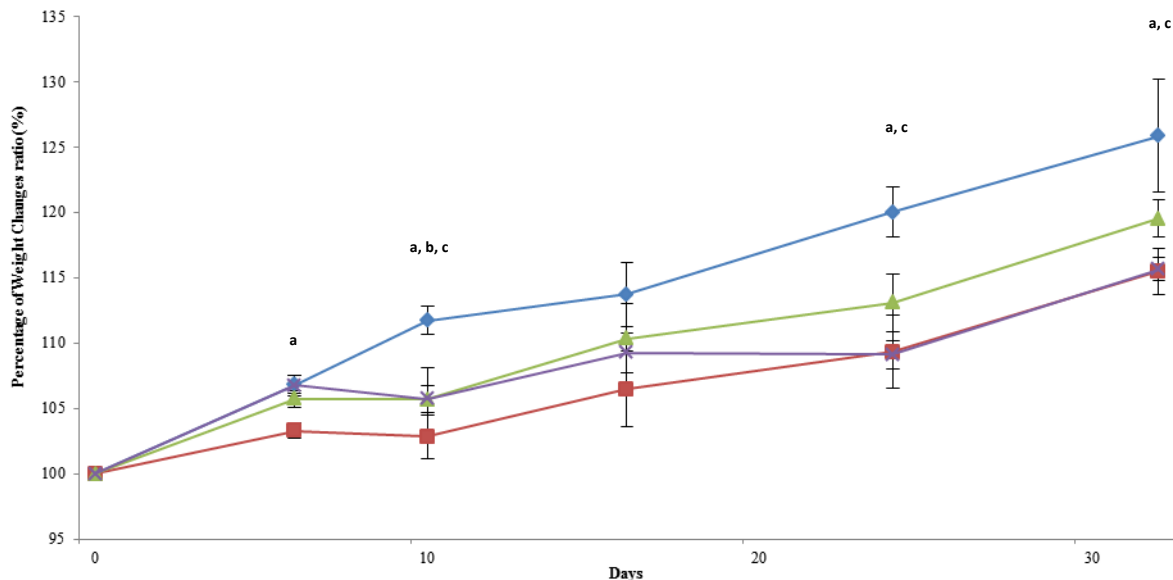


Figure 1: Percentage of weight changes (%) at specific time (n = 4). ^a SM group significant difference (p < 0.05) versus NC group. ^b SA group significant difference (p < 0.05) versus NC group. ^c ST group significant difference (p < 0.05) versus NC group. (♦: NC group, ■: SM group, ▲: SA group, x: ST group)

Body weight analysis (Figure 1) shown that all rats experienced weight increased but weight gained in rats group exposed to chronic stress was significantly less compared to NC group. Statistically significant (p < 0.05) increased was observed in SM group at all weeks except week-3 and ST group in week-2, week-4 and week-5 but not in SA group. Food intake analysis showed that total calorie intake per weight increases (Kcal/day/gram) for both SM group (1.536 ± 0.150 kcal/day/g) and ST group (1.451 ± 0.061 kcal/day/g) values were statistically significant difference (p < 0.05) compared to NC group which indicate inefficient food to energy mechanism in SM and ST group but not in SA group. Our finding in term of body weight was in line with other rodents-stressed study which showed reduces weight gain in stressed rats in comparison to non-stressed rats ¹². Finding by Karagiannides et al. (2014) identified that impaired adipocytes are what causing the low weight gained in stressed rats. Chronic stress effects, through inflammation-related mechanism cause glucose metabolism dysregulation and insulin resistance in stressed rats causing impaired food storage ¹². Thus, impaired adipocytes function might occur in SM and ST group rats but the effect was not severed in SA group rats which might indicate Acacia honey consumption may has protective effects against this effect.

Table 3: Blood serum parameters results.

Treatment group	RBC (10 ⁶ /μL)	Creatinine (umol/L)	Urea (umol/L)	Na (umol/L)	K ⁺ (umol/L)	Cl (umol/L)	Cortisol (ng/mL)
NC	9.62 ± 0.12	64.5 ± 5.09	7.50 ± 0.68	144.3 ± 1.28	5.70 ± 0.25	101.9 ± 0.81	0.45 ± 0.02
SM	7.21 ± 0.52*	59.0 ± 4.35	7.50 ± 0.48	143.0 ± 1.73	7.78 ± 2.26	102.7 ± 1.88	1.13 ± 0.32
SA	9.00 ± 0.70	61.8 ± 3.87	8.18 ± 0.73	144.0 ± 0.73	5.60 ± 0.50	103.4 ± 0.59	0.80 ± 0.11
ST	8.90 ± 0.17	59.5 ± 2.77	7.83 ± 0.63	145.7 ± 1.35	5.48 ± 0.23	104.7 ± 0.70	1.26 ± 0.11

Note: Result are reported as mean ± SEM (n = 4). *Significant difference (p < 0.05) versus NC.

As shown in the table 3, SM group showed significant variation (p < 0.05) in term of red blood cell (RBC) count (7.21 ± 0.52 10⁶/μL) in comparison to NC group (9.62 ± 0.12 10⁶/μL). Low RBC count triggered the rats' physiology homeostasis reaction towards low RBC by increasing K⁺ in blood circulation so as to have better control over it RBC count through blood pressure regulation¹³. Blood K⁺ is also a vital blood biomarker that can gives information on the extent of muscle and tissue cell damage apart from it functions as blood pressure regulation signals¹³. In this study, SM group K⁺ was the highest in comparison to other group. Thus, it can be said SM group suffered from fatigue condition but the effect was not observed in SA and ST group.

In this study, cortisol was measured as stress-related hormone. Cortisol level (Table 3) in SM group (1.13 ± 0.32 ng/mL) and ST group (1.26 ± 0.11 ng/mL) show significant increased (p < 0.05) in comparison to NC group (0.447 ± 0.023 ng/mL) but not in SA group. This concurred with other studies that saw a significant elevation in physiological cortisol level between stressed and unstressed rodents³. For example, plasma cortisol was significantly increased in Wistar rats undergo chronic stress compare to non-stressed rats¹⁴. Thus, it can be postulated that stress with Acacia honey supplement such as in SA group does not increase cortisol level.

Conclusion

From the experiment we observed that stress rats exhibited decreased weight gain and increase cortisol level while daily administration of Acacia honey at 1.2 g/kg seemed to be able to mitigate these effects in comparison to Tualang honey administration. Furthermore, high antioxidant content and activities in Tualang honey do not necessarily can be translated into better anti-stress effects. Thus elucidating the mechanism of Acacia honey's anti-stress properties are proposed for future investigation.

Acknowledgements

This study was supported by the Research Acculturation Grant Scheme (RAGS):- 600-RMI/RAGS 5/3 (191/2013) provided by Malaysia's Ministry of Higher Education.

References

- Aschbacher K, Kornfeld S, Picard M, et al. Chronic stress increases vulnerability to diet-related abdominal fat, oxidative stress, and metabolic risk. *Psychoneuroendocrinology*. 2014;46:14-22. doi:10.1016/j.psyneuen.2014.04.003.
- Badariah C, Aziz A, Aishah C, et al. Effects of Different Doses of Tualang Honey on Pain Behavior in Rats with Formalin-Induced Inflammation. *J Physiol Biomed Sci*. 2014;27(2):39-43.
- Mosavat M, Ooi FK, Mohamed M. Stress hormone and reproductive system in response to honey supplementation combined with different jumping exercise intensities in female rats. *Biomed Res Int*. 2014;2014:123640. doi:10.1155/2014/123640.
- Codex Alimentarius. Codex Standard For Honey. *Codex Stan 12-1981*. 2001:1-8.
- Takebayashi J, Iwahashi N, Ishimi Y, Tai A. Development of a simple 96-well plate method for evaluation of antioxidant activity based on the oxidative haemolysis inhibition assay (OxHLIA). *Food Chem*. 2012;134(1):606-610. doi:10.1016/j.foodchem.2012.02.086.
- Willner P, Moreau J-L, Nielsen CK, Papp M, Sluzewska A. Decreased hedonic responsiveness following chronic mild stress is not secondary to loss of body weight. *Physiol Behav*. 1996;60(1):129-134. doi:10.1016/0031-9384(95)02256-2.
- Bogdanov S, Ruoff K, Persano Oddo L. Physico-chemical Methods for The Characterisation of Unifloral Honeys: A Review. *Apidologie*. 2004;35:S4-S17. doi:10.1051/apido.
- Moniruzzaman M, Sulaiman SA, Azlan SAM, Gan SH. Two-year variations of phenolics, flavonoids and antioxidant contents in acacia honey. *Molecules*. 2013;18(12):14694-14710. doi:10.3390/molecules181214694.
- Moniruzzaman M, Khalil MI, Sulaiman SA, Gan SH. Physicochemical and antioxidant properties of Malaysian honeys produced by Apis cerana, Apis dorsata and Apis mellifera. *BMC Complement Altern Med*. 2013;13(1):43. doi:10.1186/1472-6882-13-43.
- Rahman MM, Gan SH, Khalil MI. Neurological Effects of Honey: Current and Future Prospects. *Evid Based Complement Alternat Med*. 2014;2014. doi:10.1155/2014/958721.
- Grønli J, Murison R, Fiske E, Bjorvatn B, Sørensen E. Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions. 2005;84:571-577. doi:10.1016/j.physbeh.2005.02.007.
- Karagiannides I, Golovatscka V, Bakirtzi K, et al. Chronic unpredictable stress regulates visceral adipocyte-mediated glucose metabolism and inflammatory circuits in male rats. *Physiol Rep*. 2014;2(5):e00284. doi:10.14814/phy2.284.
- Hall JE, Guyton AC. *Textbook of Medical Physiology*; 2006. doi:10.1136/pgmj.51.599.683-c.
- Jameel MK. Effect of Various Physical Stress Models on Serum Cortisol Level in Wistar Rats. *J Clin Diagnostic Res*. 2014;181-183. doi:10.7860/JCDR/2014/7210.4116.