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Effect of Daily Supplementation of Malaysian Jungle Tualang Honey and Australian/New Zealand Manuka honey on Hematological and Some Biochemical Variables in Female Rats

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29

30 **Abstract**

31 Honey as a traditional medicine has been used to cure several ailments since ancient

32 times. This study was conducted to investigate the potential effects of acute administration of

33 Malaysian jungle Tualang honey (TH) and Austrian/New Zealand Manuka honey (MH) in albino

34 rats model. Thirty nulliparous female rats were divided into three groups: Group 0 (negative

35 control) and Groups 1 and 2 received 1.0 g/kg body weight/day of TH and MH respectively.

36 After 120 days of treatment, necropsy was executed followed by samples collection. The body

37 weight, fasting blood glucose, haematological parameters and serum level expression of proteins

38 such as Apaf-1, IFN-γ, TNF-α and E2 were determined. Results show that an increased body

39 weight was observed as the administration progressed over days ($p>0.05$). TH and MH showed a

40 potentiating effect on the level of haematological parameters such as red blood cells (RBC),

41 haemoglobin (Hb) and packed cell volume (PCV) ($p>0.05$), as well as lymphocytes, TWBC,

42 RDW, eosinophils, monocytes and platelets ($p<0.05$). TH and MH presented a slightly

43 hypoglycemic effect ($p>0.05$). The treatments also showed an elevated serum level of Apaf-1,

44 IFN-γ, TNF-α and a reduced level for E2. Thus, TH and MH may act as immune-stimulant agent

45 at haematological and serological level.

46 **Keywords:** Biochemical variables, Haematological parameters, Tualang honey, Manuka honey

47 **Introduction**

48 Traditional medicines have been of pivotal importance in the treatment of various ailments over
49 centuries (Banerjee et al., 2003). Honey as a traditional medicine is referred in the utmost ancient
50 written archives. It is produced by bees *Apis mellifera* (*A. mellifera*) (Banerjee et al., 2003,
51 Bogdanov et al., 2008). Its traditional or folklore use to treat bacterial infections and wounds is
52 dated back in ancient cultures of Malaysia, India, China, Japan, Egypt, Romans, Spain and many
53 others, around 2, 500 BC (Mandal and Mandal, 2011, Ahmed et al., 2003). Recently, it has been
54 proven to be of medicinal significance both at invigorative and defensive level (Aliyu et al.,
55 2012). It is recognized as a complementary and alternative treatment in modern medicine(Ahmed
56 and Othman, 2013a). It has shown promising pronounced anti-cancer, anti-angiogenic, ant-
57 imetastatic, antibacterial, anti-inflammatory, immune-stimulant and antiulcer effects (Bogdanov
58 et al., 2008, Ahmed and Othman, 2013a). It is also considered as a natural phytoestrogen (Al-
59 Rahbi et al., 2014). It is composed of more than 181 substances and primarily fabricates sugars, the
60 fructose (38%) and glucose (31%). It also comprises flavonoids, phenolic acids, enzymes, amino acids,
61 proteins and a miscellaneous group of compounds (Ahmed and Othman, 2013a).

62 Tualang honey (TH) is a multi-floral jungle honey. It is produced by bees "*Apis*
63 *dorsata*" which build their hives on Tualang trees (*Kompassia excelsa*) in Malaysian tropical
64 rainforests (Ahmed and Othman, 2013b, Bashkaran et al., 2011). The Tualang trees (common
65 name *Mengaris*) are tall trees which could reach up to 250 feet in height. In Malaysia, the trees
66 are plentiful in the north eastern region, in the state of Kedah. Tualang honey combs can reach
67 up to 6 feet across with as many as 30,000 bees and more than 100 nests producing about 1000
68 tons of honey. In Malaysia, it is used traditionally as a health supplement, anti-aging and libido-
69 promoting agent. Research has shown that TH exhibits antimicrobial, wound-healing, anti-

70 oxidant, anti-inflammatory, anti-diabetic and anticancer effects (Ahmed and Othman, 2013b).
71 Manuka honey (MH) unlike Tualang honey is a mono-floral honey, produced by honey bees
72 from nectars of Manuka bush (*Leptospermum scoparium*) throughout New Zealand and Australia
73 (Yao et al., 2003). Published literature on Manuka honey indicates its numerous therapeutic
74 properties against several ailments (Old, 2013). However, many potential health effects of these
75 two honeys need to be fully understood.

76 This study was undertaken to investigate the effects of acute administration of TH and
77 MH on body weight, fasting blood glucose level, hematological and some biochemical
78 parameters in female SD rats with a view to ascertain and validate whether it is safe to take
79 honey on daily basis and what are its modulatory effects?

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81 **Material and Methods**

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83 ***Animals and source of honey***

84 The experimental protocol used in this study was approved by the animal ethics
85 committee of Universiti Sains Malaysia, Malaysia [USM/Animal Ethics Approval/2011/ (68)
86 (306). Sprague-Dawley (SD) female rats aged between 28-33 days old were obtained from
87 Animal Research and Service Centre (ARASC), Universiti Sains Malaysia (USM). Tualang honey
88 was supplied by Federal Agricultural Marketing Authority (FAMA), Ministry of Agriculture and
89 Agro-based Industry, Malaysia. The honey samples were filtrated, evaporated at 40 ^{0}C (to
90 achieve 20% water content) and were subjected to gamma irradiation at 25 kGy for sterilization
91 by STERILE GAMA™, Selangor, Malaysia. Manuka honey was purchased from the market (Packed

92 under licence No. 1003 for Vitaco Health (NZ) Ltd, New Zealand and imported and distributed by
93 Cambert (M) Sdn.Bhd, Malaysia).

94

95 ***Quality Assessment of the TH and MH Samples***

96 The quality of TH and MH samples was assessed through hydroxylmethylfurfural (HMF)
97 level and diastase number (DN) using spectrophotometric method (Thermo Scientific™
98 Evolution 60S UV-Visible) as described by(Aliyu et al., 2012) using following formulas:
99 mg of HMF/100g of honey = (A284nm - A336nm) × 14.97 × 5/g of test sample
100 DN (units/g of honey) = 28.2 × change in A660nm + 2.64

101

102 ***Treatment plan and study design***

103 A total of 30 nulliparous female SD rats were divided into 3 groups with 10 animals in each
104 group. These rats were housed in a standard cage with commercial pine chip bedding in a well-
105 ventilated animal room with a 12 h day/night cycle, maintained on standard and balanced rat feed
106 diet and had free access to water ad libitum. Honey treatment by oral feeding was started to rats
107 at age 40 days old. The treatment was planned to be continued till day 120th. The grouping of the
108 rats was as follows;
109 a) Group 0: Negative control (normal rats).
110 b) Group 1: treated group; rats receiving TH 1.0 g/kg body weight/day treatment.
111 c) Group 2: treated group; rats receiving MH 1.0 g/kg body weight/day treatment.

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113 The total body weight of rats was measured using a digital analytical balance (Sartorius AG,
114 Germany) weekly from start of treatment till day terminated. The percentage body weight

115 changes were calculated at the end of study (week 16) using following formula: Percentage body
116 weight change or gain (BW change %)= [(FBW – IBW) × 100] /IBW
117 Legends: BW=weight, IBW=initial body weight, FBW=final body weight.

118 After 120th day of honey treatment, all the rats used in the present study were sacrificed
119 after intra-peritoneal (i.p) injection of pentobarbital 100mg/kg body weight. The blood samples
120 were collected into EDTA and plain tubes by cardiac puncture using 10ml syringe and 23G
121 needle. Blood samples in plain tubes were left to clot for 2 hours prior to centrifugation for 15
122 minutes at 4000 rpm (Eppendorf centrifuge, Germany). The serum was collected and stored at -
123 $80C^0$ until assayed. FBC was carried out using an automated cell count analyzer (Sysmex KX-
124 21, Japan) via non-cyanide hemoglobin analysis for parameters such as Hb, PCV, RBCs, MCV,
125 MCHC, MCH, platelet and WBCs counts. The equipment of sampling probe aspirated 20 µl with
126 well mixed blood samples and the result of analysis was obtained accordingly. A total of 8-9
127 samples were run for FBC for each group.

128 Seven to eight serum samples per treatment and control group were analyzed to
129 determine the level of Apaf-1, IFN-γ, TNF-α and E2 in 50µl serum using Apaf-1, IFN-γ, TNF-α
130 and E2 ELISA kits (Catalog no. BG-RAT10190, Inc., Novate in Bio Sciences; CSB-E04579r;
131 CSB-E11987r and CSB-E05110r Inc., COSMO BIO, USA respectively). Standards comprised
132 serum of known concentrations of Apaf-1, IFN-γ, TNF-α and E2 and a serum blank. The ELISA
133 procedure was performed according to the manufacturer's instructions. The results were obtained
134 by calculating the mean absorbance at 450nm (Spectrophotometer, Thermo Fisher Scientific Inc,
135 Waltham, MA, USA) for each of the duplicate standards, controls and samples as stated by the
136 manufacturer. A standard curve was created by plotting with the absorbance value as the
137 dependent variable (Y-axis) and concentration as the independent variable (X-axis), results in an

equation formatted as follows: $y = ax^2 + bx + c$, with best-fit straight line, where solving for x determined the protein concentration of the sample.

Statistical analyses

Data were analysed using IBM SPSS, Statistics version 23. Mixed model two way repeated measures ANOVA was conducted to evaluate the effect of treatments on the rats body weight gain. The time main effect and the experimental groups x time interaction effect were tested using the multivariate criterion of Wilk's lamda (Λ). Comparison of the median values between groups was done by Kruskal-Wallis H test followed by Benferroni's correction. P value <0.05 was considered statistically significant.

Results

Diastase number and hydroxylmethylfurfural level

The diastase number or activity was 3.8 and 2.9 units/g of TH and MH, respectively. The level of hydroxylmethylfurfural was 0.53 and 0.65 mg/100 g of TH and MH, respectively (Table 1). This indicates that TH and MH were of good quality as the values are far below the imposed limit of > 8 units/g of honey for DN and > 15 mg/100 g of honey for HMF.

Table 1. Diastase number and hydroxylmethylfurfural level for TH and MH.

Honey	Diastase number (units/g)	hydroxylmethylfurfural level (mg/100 g)
TH	3.7	0.55
MH	2.9	0.65

207 **Body weights and haematological parameters**

208 In general, body weights of the rats in all groups (non-treated negative control & TH, and
209 MH treated groups) were found to be increased throughout the experimental period over time
210 (Figure 1). Data for median body weights of rats in each group at week 1 and 16 is presented in
211 Table 2. At week 16, all the rats in TH and MH treated groups showed a higher BW change %
212 with no weight loss compared to the negative control (Table 2). The tested dose of MH presented
213 a higher body weight change % than similar doses of TH (Table 2). For haematological
214 parameters, treatment with TH and MH showed a slightly potentiating effect on Hb, RBC, PCV,
215 lymphocytes RDW, eosinophils, monocytes and platelets compared to the non-treated negative
216 control. While, it was observed that the level of MCV, MCH, MCHC and polymorphs was
217 almost comparable for TH and MH compared to the non-treated negative control. The detailed
218 results with statistical analyses are presented in Table 3.

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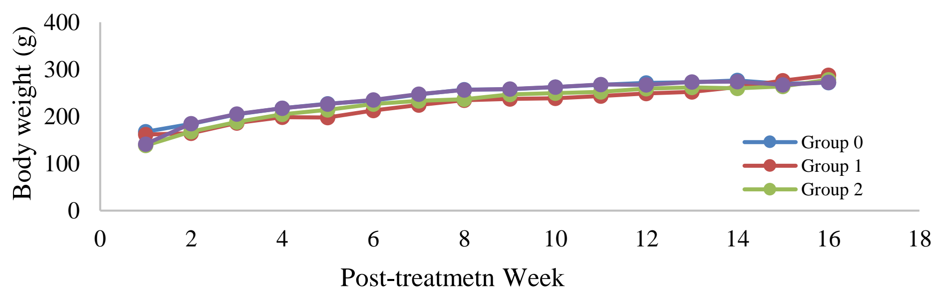

240 Figure 1: Body weight progression among all groups of rats during 16 weeks of experimental period. Data is
241 presented as mean ± SEM and a mixed model two-way repeated measures ANOVA was conducted to analyze the
242 results. A positive body weight progression was observed over time (p>0.05). Legends: TH= Tualang honey, MH=
243 Manuka honey, Group 0= negative control (normal rats), Group 1= 1.0 g/kg TH and Group 2= 1.0 g/kg MH.

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262 Table 2: Body weight measurements of rats among all groups at week 1 and week 16.

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	Groups			
Body weight	0 -ve control	1 (1.0 g/kg TH)	2 (1.0 g/kg MH)	P value[a]
BW at week 1	167.5 (32.25)	162.5 (94.5)	138 (60.25)	0.300
BW at week 16	272 (32.25)	284.5 (31)	278 (55.75)	0.392
BW change (%)	62.38 (37.16)	81.01 (90.05)	101.44 (18.42)	0.182

269 [a]Kruskal-Wallis test. Data are expressed as median interquartile range (IqR). Values are statistically
270 significant when $p \leq 0.05$. Legends: BW= Body weight, TH= Tualang honey, MH= Manuka honey, -ive
271 control=normal rats.

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290 Table 3: The haematological parameters of TH and MH treated groups compared to the negative control.

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	Groups			
	1 -ive control	2 (1.0 g/kg TH)	3 (1.0 g/kg MH)	P value[a]
RBC (10^{12}/L)	7.25 (0.42)	7.92 (3.32)	8.25 (2.75)	0.003
Hb (g/dl)	14.97 (0.77)	16.1 (5.95)	17.35 (4.45)	0.003
PCV (%)	46 (3.25)	46.05 (17.75)	47.1 (12.25)	0.009
MCV (fl)	68.5 (3.25)	68 (11.75)	68.4 (10.25)	0.013
MCH (pg)	20.6 (1)	22 (3)	22 (3.5)	0.169
MCHC (g/L)	32 (1)	32.5 (3.5)	31.9 (2.25)	0.062
RDW (%)	11.4 (1.57)	12.85 (2.17)	12.65 (2.1)	0.01
TWBC (10^9/L)	4.75 (1.75)	6.15 (8.75)	7.35 (6.85)	0.02
Polymorphs (%)	32 (8.75)	31.71 (11.25)	32.01 (9.5)	0.01
Lymphocytes (%)	68 (8)	68.9 (9.75)	69.5 (4.5)	0.014
Monocytes (%)	1 (1.5)	1.5 (1)	1.5 (4.25)	0.231
Eosinophils (%)	0 (1)	1 (1)	1.5 (0.25)	0.102
Basophils (%)	0	0	0	1
Platelets (10^9/L)	839 (225.75)	861.5 (229.25)	852.5 (324.75)	0.01

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311 [a]Kruskal-Wallis test. Data are expressed as median interquartile range (IqR). Values are statistically significant
312 when p ≤0.05. Legends: FBC=full blood count, RBC= Red blood cells, Hb= Haemoglobin, PCV= Packed cell
313 volume, MCV= Mean corpuscular volume, MCH= Mean corpuscular haemoglobin, MCHC= Mean corpuscular
314 haemoglobin concentration, RDW= Red cell distribution width, TH= Tualang honey, MH= Manuka honey, -ive
315 control= normal rats.

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Fasting blood glucose level and Serum level concentration of Apaf-1, IFN-γ, TNF-a and E2

The rats treated with TH and MH (Groups 1 and 2) showed a higher median concentration of Apaf-1, IFN-γ and TNF-α, but a lower E2 concentration compared to the those of non-treated negative control. MH presented a slightly higher concentrations when compared to a similar dose of TH. While, TH showed a slightly more decreasing effect on E2 compared to MH. A significant statistical difference was observed between all groups ($p<0.05$). Treatment with TH and MH presented a slightly reducing effect on fasting blood level compared to the non-treated negative control (Figure 2.5). The statistical difference between treated and non-treated negative control groups, and the treated groups among themselves was observed statistically non-significant ($p>0.05$).

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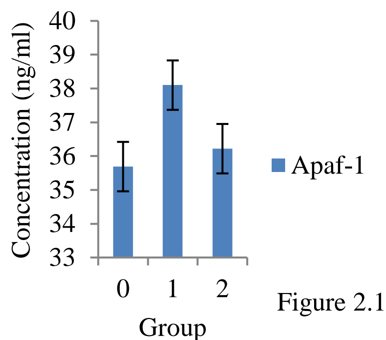

Figure 2.1

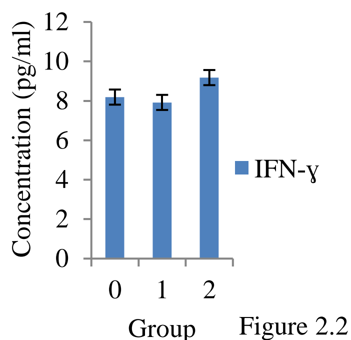

Figure 2.2

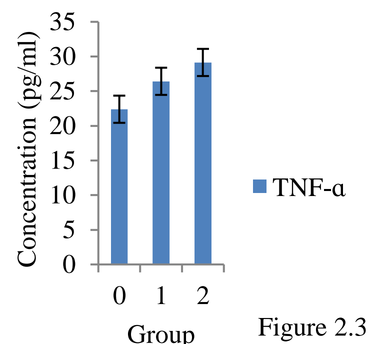

Figure 2.3

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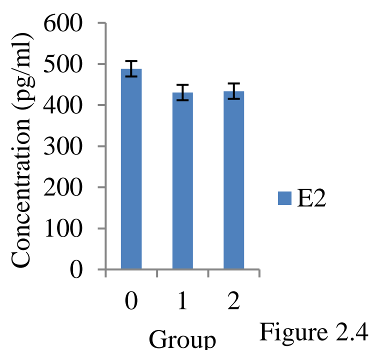

Figure 2.4

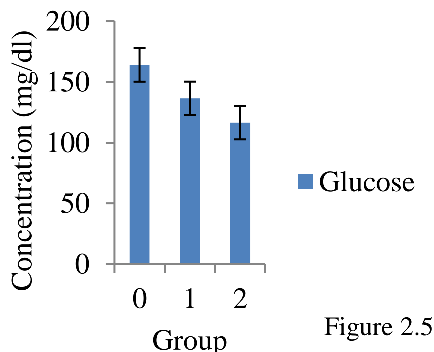

Figure 2.5

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357 Figure 2 (2.1, 2.2, 2.3, 2.4 and 2.5): The serum level concentration of Apaf-1 (ng/ml), IFN-γ (pg/ml), TNF-α
358 (pg/ml) and E2 (pg/ml);figure 2.5 is showing fasting blood glucose level (mg/dl) in the rats of TH and MH groups
359 compared to the rats of negative control. Group 0= negative control (normal rats), Group 1= 1.0 g/kg TH and Group
360 2= 1.0 g/kg MH. Data are expressed as median interquartile range (IqR) using Kruskal-Wallis test. Values are
361 statistically significant, $p<0.05$. Legends: Apaf-1=Apoptotic protease activating factor 1, IFN-γ=interferon gamma;
362 TNF-α =tumour necrosis factor alpha; E2=estradiol; TH= Tualang honey, MH= Manuka honey.
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372 **Discussion**

373 Honey has been used to cure several ailments since ancient times. Its medicinal and
374 nutritional values are getting scientific re-appraisal (Ahmed and Othman, 2013a). It is often
375 named based either on geography, floral source or the trees on which hives are found and varies
376 in composition and physicochemical properties based on origin (Ahmed and Othman, 2013a,
377 Ahmed and Othman, 2013b). It has been shown to have several medicinal effects such as anti-
378 inflammatory, anti-microbial, anti-mutagenic, antioxidant, antidiabetic and anti-tumoural effects
379 (Ahmed and Othman, 2013a, Ahmed and Othman, 2013b). Our study highlights intriguing
380 findings regarding the utilization of Tualang honey and Manuka honey as potential natural
381 medicinal agent on body weight hematological and biochemical variables at various
382 concentrations tested in normal albino female rats.

383 Quality assessment of honey has pivotal importance to interpret its activity and efficacy
384 as a therapeutic agent. Thus, the honeys used in this study were of very fine quality to rebut the
385 parameters analyzed. Our study signposts that all the tested strengths of TH and MH showed a
386 positive effect on percentage body weight gain compared to the non-treated negative control
387 (Figure 1). Based on the results, it can be presumed that the higher percentage of body weight
388 gain in the treatment groups could be attributed to TH and MH treatments. This weight gain can
389 be of great importance to make the use of honey in diseases such as in cancer where weight loss
390 leads to worst prognosis, recurrence and death (Caan et al., 2008). It has been reported that
391 honey exhibits androgenic property to modulate the serum level androgens (Nervey et al., 2012).
392 The observed increase in body weight in our study could be due to the androgenic properties of
393 the honey and its nutritional value. One of the other mechanisms explains that sugars in honey
394 trigger a small spike in insulin levels, and insulin stimulates the release of tryptophan in the

395 brain. Tryptophan is converted to serotonin, which is then converted into melatonin at night.
396 Melatonin in turn inhibits the release of insulin, thus further stabilizing blood sugar levels. This
397 implication causes to down regulate the aerobic glycolytic pathway that is believed to play a vital
398 role in lipogenesis, which may ultimately lead to an increase in body weight (Ron, 2007).

399 Full blood count is a prerequisite investigation in different diseases and poor blood
400 parameters affect the outcome and prognosis of diseases (Akinbami et al., 2013). Research has
401 shown that the functioning of the immune system at haematological level has a direct influence
402 on diseases (Akinbami et al., 2013). We observed that treatment with similar strengths of TH and
403 MH showed intriguingly a slightly potentiating effect on the haematological parameters such as
404 Hb, RBC, PCV, lymphocytes, RDW, eosinophils, monocytes and platelets compared to the non-
405 treated negative control (Table 2). Research has reported an abnormal level of RBC, Hb, PCV,
406 MCV, RDW, TWBC, platelets and lymphocytes in diseases like cancer with acute anaemia
407 (Akinbami et al., 2013). Our findings suggest that TH and MH may alter or modify these
408 parameters to ameliorate different ailments. Exclusive honey feeding in the absence of any
409 disease significantly modifies the haematological parameters (Aliyu et al., 2012).

410 Apoptotic protease-activating factor-1 (Apaf-1) is a key regulator of the mitochondrial
411 apoptotic pathway (Zou et al., 1997). Loss of Apaf-1 expression can aid cells to evade immune
412 attack-induced death and programmed cell death or apoptosis in diseases, especially in cancer
413 (Satyamoorthy et al., 2001). Our data reported that TH and MH cause to increase the
414 concentration of Apaf-1 at serum level (Figure 2.1). We can assume that TH and MH may act as
415 therapeutic agents to modulate the expression of Apaf-1 and thus can be used against different
416 diseases to enhance Apaf-1 level.

417 IFN-γ a cytokine is secreted by antigen activated lymphocytes or NK cells (natural killer
418 cells). It is critical for innate and adaptive immunity against various types of diseases and its
419 higher concentrations predict a favourable outcome in diseases (Zhu et al., 2014). Our results
420 show that TH and MH potentiate IFN-γ level (Figure 2.2), are consistent with a research
421 reporting that honey can modulate level of IFN-γ (Salih et al., 2009). Thus, honey acts by
422 enhancing immunological activity of IFN-γ to make a profound effect. It can be a potential
423 preventive immune-stimulating agent against diseases. IFN-γ is produced by lymphocytes
424 (Gutterman, 1994), and our study also shows that TH and MH cause to increase the level of
425 lymphocytes. This validates that TH and MH hinder this signaling pathway by increasing IFN-γ
426 as well as lymphocytes.

427 TNF-α has been shown to play both beneficial and deleterious role in the promotion or
428 inhibition of diseases (Schluter and Deckert, 2000), but the primary role of TNF-α is to regulate
429 immune cells. The increased concentration of TNF-α in TH and MH treated groups of our study
430 may be assumed to be due to effect of these treatments. Thus, TH and MH treatments may tend
431 to increase TNF-α concentration at serum level to ameliorate diseases. Pasture, jelly bush, and
432 Manuka honeys (at concentrations of 1%w/v) stimulate monocytes to release TNF-α (Tonks et
433 al., 2003). TNF-α is produced by monocytes, lymphocytes and eosinophils(Idriss and Naismith,
434 2000), and our study also shows that TH and MH cause to increase the monocytes, lymphocytes
435 and eosinophils level in blood. This validates that TH and MH hinder this signaling pathway by
436 increasing TNF-α as well as monocytes, lymphocytes and eosinophils, ultimately resulting in
437 enhancing immunity.

438 Estradiol (17β-estradiol or E_2), a female sex hormone, acts as a key regulator of growth,
439 differentiation and immune processes (Jansson and Holmdahl, 1998). Its prolonged exposure to

440 target tissues or cells to results in cancer (Jansson and Holmdahl, 1998). The findings of our
441 study demonstrate a reducing effect on E2 concentrations after treatment. Research has shown
442 that honey modulates estrogen through its antagonistic action (Tsiapara et al., 2009). This effect
443 is attributed to its phenolic content (Tsiapara et al., 2009). It is also possible that honey, which is
444 a natural phytoestrogen (Al-Rahbi et al., 2014), plays its role in modulating the endogenous
445 estrogen by stimulating immune system and other signaling pathways. TNF-α regulates the
446 balance of activating and deactivating pathways of estrogen metabolism. TH and MH seem to
447 modulate both E2 and TNF-α concentration, as observed in our study. Our study further suggests
448 that TH and MH may module E2 at serum level to inhibit its negative effects.

449 Considering honey as a sugar or sweetener, we would expect that the blood glucose level
450 would rise after honey treatment, but our study shows otherwise. The rats of TH and MH treated
451 groups showed a slightly lower fasting blood glucose level or hypoglycemic effect compared to
452 those of non-treated control group (Figure 2.5). Elevated serum and fasting blood glucose in
453 patients are associated with recurrence and worse outcomes (Minicozzi et al., 2013). Thus, honey
454 does not raise blood glucose level which may be a favourable factor to use honey against
455 diseases, with no hyperglycemic effects. Research has shown that honey exhibits hypoglycemic
456 or anti-diabetic effects (Erejuwa et al., 2012). The proposed mechanism for hypoglycemic effect
457 of honey may be through the role of honey in modulating the insulin signaling pathway
458 (Batumalaie et al., 2013). The effect of Malaysian Gelam honey extracts on activated insulin
459 signaling pathway in pancreatic cells was recently investigated under hyperglycemic condition,
460 in which honey showed a hypoglycemic effect (Batumalaie et al., 2013). Thus, our findings
461 suggest that TH and MH may modulate this insulin signaling pathway to pose hypoglycemic
462 effect. The factors which can influence the effectiveness of honey to act as immune-stimulating

463 can be its acidic PH, enzymes, minerals, osmotic properties and vitamins (Biswal et al., 2003). It
464 can also be hypothesized that the phenolic acids and flavonoids in honey can also contribute to
465 its protective effects against pathological conditions.

466 **Conclusion**

467 Oral administration of Tualang and Manuka honeys ameliorate body weight, fasting
468 blood glucose level, hematological and biochemical variables such as Apaf-1, IFN-γ, TNF-α and
469 E2. Our study also suggests that daily consumption of honey can be safe as a health supplement
470 and most reliable study on the usefulness of honey is to conduct research in clinical trials.

471

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