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Immunomodulatory Properties of Tualang Honey in BALB/c Mice.

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ABSTRACT

Local natural products have been gaining great focus from research and therapeutic aspects. The outcome of studies on one of nation's renowned Tualang honey (TH) (honey bee collected from *Koompassia excels* tree) has profoundly anchored its broad aptitude in anti-inflammatory, anti-oxidant and anti-microbial properties. This study was aimed to investigate the immunomodulatory properties of TH in BALB/c mice. TH was orally administered daily for 14 days to male BALB/c mice (5/group) in dose ranging from 0.5 g/kg, 1.5 g/kg and 3.0 g/kg per group. Pre-treatment and post-treatment body weights were measured. Upon sacrificing, the spleen was weighed and then homogenized. The splenocytes were stained with various surface markers antibodies namely $CD3^+/CD4^+$ (T helper), $CD3^+/CD8^+$ (T cytotoxic), $CD14^+$ (macrophage) and CD19+ (B lymphocyte) and immune cell populations were obtained by using flow cytometer. On the other hand, proliferation assay of splenocytes were done by using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS). Body weight of mice showed increment after the treatment but body weight of mice treated with 0.5 g/kg decreased upon completion of TH treatment. BALB/c mice treated with TH showed increased populations of $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD14^+$ and $CD19^+$ compared to the control group. Proliferation analysis of splenocytes and spleen weight obtained from TH-treated mice also showed increment. The results revealed the immunostimulant potential of TH in mice by enhancing lymphocyte populations especially T helper ($CD3^+/CD4^+$), cytotoxic ($CD3^+/CD8^+$) and B ($CD19^+$) cells.
Keywords: immunomodulation, natural product, food, Tualang honey

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INTRODUCTION

Immunomodulatory through dietary approach had been shown to be effective. Nowadays, people begin to gain their interest and confidence on natural products and foods for their immunomodulatory effect as food and natural products contain many substances that can control the physiological functions of the body and modulating immune response [1]. In addition, these food and natural products can regulate and boost our immune system to fight against pathogens.

Many foods that being taken daily by human have been found to be immunomodulating. Fruits such as strawberry was found to contain high phenolics, including flavonoids which able to stimulate splenocyte proliferation [2]. Soybean's extracts significantly stimulated the proliferation of human peripheral blood mononuclear cells (PBMC) and the secretion of IFN-γ [3]. Besides, water extraction of green soybean also found to be immunomodulating by inhibiting IgE production and regulated B cell apoptosis [4]. A study on garlic's active substance allicin showed inhibition of IL-8, IFN-γ-inducible protein of 10 kD (IP-10), monokine induced by INF-γ (MIG) and IL-1β from intestinal epithelial cells which involved in inflammatory bowel diseases [5].

Honey is commonly used to promote general health and boost immune system since ancient times. The typical composition of honey is consists of moisture, 17.7%; total sugars, 76.4%; ash, 0.18%; and total acid (as formic acid), 0.08%. Honey also contained approximately 200 substances, including a mixture of sugars (fructose, glucose, maltose, and sucrose) and small amounts of other constituents, such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes, and other phytochemicals [6]. Besides serving as food products, honey also found to exhibit some healing properties including anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, anti-ulcerous, anti-lipid and anti-cancer properties [7-12]. One of the most precious types of honey that can be found in Malaysia is Tualang honey (TH). TH is collected from wild honey bees' hives on Tualang trees found in the Malaysian rain forest. TH has been reported to exhibit various medicinal properties [13]. A study on the anti-oxidative compounds of TH has found that TH had the highest total phenolic and protein content compared to other types of honey; Gelam, Indian forest and Pineapple honey [14]. Previous studies also reported the antibacterial properties of TH against various microorganisms e.g. *Pseudomonas* spp. and MRSA [13, 15, 16]. Besides, TH was also reported to induce apoptotic cell death in oral squamous cell carcinoma and osteosarcoma cell lines [17] and also human breast and cervical cancer cell lines [18]. However to date, no studies has been reported on immunomodulatory properties of TH. Our present study was aimed to evaluate the immunomodulatory properties of TH in BALB/c mice.

METHODOLOGY

Tualang honey

The TH (AgroMas) used in the study was supplied by the Federal Agricultural Marketing Authority (FAMA), Ministry of Agriculture and Agro-Based Industry, Malaysia. For the preparation of treatment, TH was diluted by dissolving 10 mg, 30 mg and 60 mg of TH stock into 0.2 mL of distilled water respectively for final dosages of 0.5g/ kg/ day, 1.5 g/ kg/ day and 3.0 g/ kg/ day. The dosages were modified based on previous study carried out on rats [19].

Experimental animals

Male BALB/c mice, age 6- to 7-week old were obtained from the Animal Research and Service Centre (ARASC) of Universiti Sains Malaysia (USM) Health Campus, Kelantan. All animals care and experimental procedures were approved by the Animal Ethics Committee, USM Health Campus (Ethic approval: 2010/ (59)(208)). Food pellets and water were provided *ad libitum* to the animals throughout the study.

Treatment of mice and sacrifice

The mice were allocated into 5 mice per groups (0.5g/ kg/ day, 1.5 g/ kg/ day and 3.0 g/ kg/ day groups). The mice were allowed to acclimatize for 5 days before the oral administration of TH was started. Pre-experimental weight was recorded and the mice were administered daily for 14 days continuously with vehicles (distilled water), 0.5, 1.5 or 3.0 g/ kg/ day of TH intragastrically via oral gavage. Post-treatment weight

was recorded after the course of oral feeding completed. Relative spleen weights are calculated based on the formula below:

Relative Spleen Weight = $\dfrac{\text{Spleen weight}}{\text{Final body weight}}$ X 100

At the end of the experiment, the mice were sacrificed by cervical dislocation. Following cervical dislocation, the spleen was removed and harvested by mechanical dispersion in sterile RPMI. The splenocytes were suspended in RPMI, counted and stained with surface staining markers for cell subsets analysis or resuspended at 1×10^6 cells/ml in complete media [RPMI 1640 supplemented with 10% FBS, and 1 unit/ml penicillin/streptomycin] and cultured for 72 hours for proliferation and phagocytosis assays.

Cell subset analysis by using flow cytometry

Splenocytes suspensions from treated and untreated groups were used for preparation of cell subset analysis using flow cytometry. Antibodies (FITC Rat Anti-Mouse CD4, PE Anti-Mouse CD8a, PerCp Hamster Anti-Mouse CD3e, FITC Anti-Mouse CD14 and FITC Rat Anti-Mouse CD19) were added to labelled flow tubes under dark condition. Then, 100 µL of cell suspension was aliquot into respective flow tubes containing appropriate antibody. The flow tubes were incubated for 20 minutes at 4°C in dark condition. Cells were then washed two times with PBS. Flow tubes containing cell suspension were then centrifuged at 300 g, 10°C for 5 minutes. Supernatant was discarded and cells were resuspended with 500 µL of PBS. Cells were then analyzed by FACS Canto II analyzer.

Phagocytosis assay

Splenic phagocytes were measured using Phagocytosis Assay Kit (IgG FITC) (Cayman, USA). Splenocytes suspensions prepared earlier were used in this assay. The cells were cultured in 24-well plates at a density of 5×10^5 cells/ml overnight. The next day, Latex Beads-Rabbit IgG FITC solution were added into each well and incubated for 48 hours. After 48 hours the cells were harvested and washed 3 times. The samples were immediately analyzed in the FL1 channel of a flow cytometry.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20 statistical package (SPSS inc., USA) for windows employing Wilcoxon signed-rank, Mann-Whitney U and Kruskal Wallis tests. Differences were considered statistically significant when p-value <0.05. Values represented the mean±SD.

<div align="center">

RESULTS

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Comparison between pre- and post- treatment body weight

Body weight of the treated and control mice were measured before and after completion of the treatment. The bar chart (Figure 1) represents the pre-treatment and post-treatment body weight of mice of control groups and TH treatment groups at three different dosages; 0.5 g/kg/day, 1.5 g/kg/day or 3.0 g/kg/day respectively. The data plotted is the mean body weight of 5 mice of each group. The post-weight of the control group was significantly higher than the pre-weight. There was slight reduction of the post weight from the group treated with 0.5 g/kg/day TH compared to the pre-weight. For mice administered with 1.5 g/kg/day TH, the weight was significantly increased throughout the study. The mice administered with 3.0 g/kg/day TH showed post-weight higher than the pre-weight but the increment was not statistically significant.
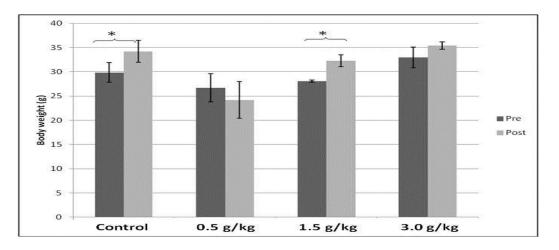
Figure 1: The pre-treatment and post-treatment body weight of mice of control group and treatment groups of different dosage. * Significantly different compared to pre-treatment body weight of the same group. (*p*<0.05)

Analysis of relative spleen weight of control and treatment mice

Spleen weight of each mouse was measured prior to sacrifice. Relative spleen weight is a better way to compare the spleen weight of mice from different groups due to different body weight and size of the mice. Relative spleen weight and standard deviation (SD) was calculated. From the results obtained (Table 1 and Figure 2), the relative spleen weight was increasing at a dose-dependent manner from low to higher dosages. The relative spleen weight of mice administered with 0.5 g/kg/day TH was about the same with the relative spleen weight of the control group. In addition, relative spleen weight from mice administered with 3 g/kg/day TH was significantly increased compared to the control group. Statistical analysis was performed by using Mann-Whitney test as the data was not normally distributed in groups. * indicates significantly different compared to control group (p<0.05).

Table 1: The average spleen weight and mean relative spleen weight of mice from control group and TH treatment groups. * Significantly different compared to control group (*p*<0.05).

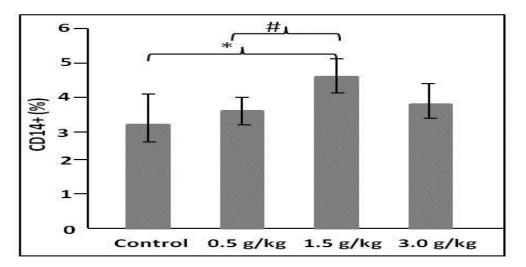| Groups (n=5)	Average Spleen Weight (g)	Relative Spleen Weight (g)
Control	0.089±0.009	0.265±0.039
0.5 g/kg/day	0.068±0.004	0.263±0.034
1.5 g/kg/day	0.097±0.009	0.298±0.013
3.0 g/kg/day	0.108±0.005	0.312±0.011*

Figure 2: Effect of TH on the percentage of splenic CD14^{+} cells on control group and TH treatment groups. * Significantly different compared to control group. (*p*<0.05). # indicates significant difference between treatment groups.

Flow cytometry analysis of splenocytes population

Table 2 showed the CD3+/CD4+, CD3+/CD8+ cell population as well as CD4+/CD8+ ratio of control groups and TH treatment groups; 0.5 g/kg, 1.5 g/kg and 3.0 g/kg respectively. The values represent the mean±SD of T cell subpopulations cell counts of 5 mice per groups which were analyzed by using Kruskal-Wallis test to compare mean value between treatment groups and Mann-Whitney test to compare values between control group and TH treatment groups. From the results, the CD4+ cells populations showed a dose-dependent increment from control to mice administered with higher dosages though none of the value was statistically significant when compared to the control. For CD8+ cell population, the percentage showed a dose-dependent increment from low to higher dosage groups. Interestingly, the CD4+/CD8+ ratio increased at dose-dependent manner.

Table 2: CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ cells population and CD4$^+$/CD8$^+$ ratio of control groups and TH treatment groups. * Significantly different compared to control group (p<0.05).

Groups (n=5)	CD3$^+$CD4$^+$ populations (%)	CD3$^+$CD8$^+$ populations (%)	CD4$^+$/CD8$^+$ ratio
Control	12.86±1.51	7.00±1.16	1.87±0.28
0.5 g/kg/day	12.9±2.66	6.95±1.32	1.87±0.34
1.5 g/kg/day	13.45±1.20	7.15±0.97	1.90±0.31
3.0 g/kg/day	14.88±1.96	7.22±0.54	2.07±0.31

Percentage of splenocyte populations positively expressing the CD14+ surface marker is shown in Figure 2. The data plotted was the mean CD14+ of 5 mice from each group. The mice administered with TH showed higher percentage of splenic CD14+ cells compared to the control group. However, only mice administered with 1.5 g/kg/day TH showed a significant increase compared to the control group and the group received 0.5 g/kg daily.

Analysis of splenic phagocyte

Figure 3 illustrated the percentage of splenic phagocytes in control group and three different TH treatment groups; 0.5 g/kg, 1.5 g/kg and 3.0 g/kg daily. The percentage of splenic phagocytes is increase dose-dependently from low to higher dosages. Interestingly, the percentage of splenic phagocytes showed a significant increase when comparison made to the control group.
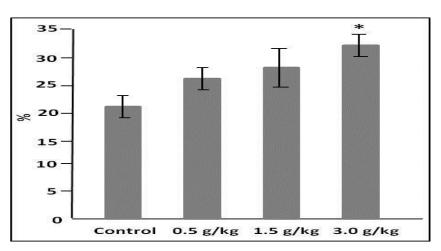

Figure 3: Percentage of splenic phagocytes from mice administered with TH. * Significantly different compared to control group.

DISCUSSION

Pre-treatment and post-treatment weights of the mice were measured and recorded. It was found that the body weight of mice from the control group and mice administered with 1.5 g/kg showed significant (p <0.05) increment upon completion of treatment. Post-treatment body weight of mice from mice

administered with 3.0 g/kg also showed increment but was not statistically significant. However, the post-treatment body weight of mice from mice administered with 0.5 g/kg reduced after completion of TH treatment. Previous studies reported that oral TH treatment for four weeks increased body weight of rats as honey was considered a good source of energy as it provided 313 calories per 100g [19, 20]. This study showed that honey diet was able to induce body weight gain when consumed in proper amount. In contrast, a previous study on TH found that ovariectomized rats fed with 0.2 g/kg of TH daily for six weeks showed reduction in body weights compared to ovariectomized rats without TH treatment which showed normal body weight increment [19]. Furthermore, a previous study by Nemoseck *et al.* [21] showed that honey might reduce weight gain and adiposity due to lower food intake, promote lower serum triglycerides but higher non-high density lipoprotein cholesterol concentrations. Based on this previous research, the reduction in body weight in mice administered with 0.5 g/kg might be caused by lower food intake; however the food intake was not measured in the current study.

The spleen of each mouse was dissected and weighed after 14 days of oral administration with TH. From the results obtained in this study, the relative spleen weight showed an increasing pattern. This was corresponded to the result of splenic cell subset analysis. The splenic weight of mice administered with 1.5 g/kg showed a higher relative spleen weight at 0.298±0.013 g, however, the relative spleen weight increment was not statistically significant compared to the control group. In mice administered with 3.0 g/kg, the relative spleen weight showed a significant difference ($p<0.05$) compared to the control group with a value of 0.298±0.013 g. In a previous study, honey treatment on rats showed no difference on the relative spleen weight [22]. However, this research showed that there was increment of relative spleen weight and the increment was statistically significant at high dose of TH treatment. The contrast results might be due to the different type of honey used in these two studies. Compositions of honey can be different from one type to another. This might explain the difference in its effect on spleen weight.

CD4^{+} cells or also known as T helper (Th) cells are type of T cells that functioning in mediating activity of other immune cells, either stimulating or depressing the activity and proliferation of them. From the results obtained in this study, CD4^{+} cell population (12.9 ±2.66%) of mice treated with 0.5 g/kg of TH daily was about the same with the control group (12.86±1.51%). Splenocytes CD4^{+} population increased dose-dependently with the percentage of 13.45±1.2% and 14.88±1.96% in mice administered with 1.5 g/kg and 3.0 g/kg daily doses of TH respectively. This result indicated that consumption of TH at higher dose might increase CD4^{+} populations. It is possible for natural products such as honey to be able to modulate CD4^{+} population since some of natural products have shown this ability. For instants, marine oligopeptide preparation from Chum Salmon has levelled up CD4^{+} cells count in mice in four weeks of treatment [23]. In addition, a flavanoid called genistin, a constituent of soybean was able to significantly increased CD4^{+} T cells [24].

CD8^{+} also known as cytotoxic T lymphocytes (CTL), are cells that possess cytotoxic ability which able to destroy intracellular pathogen upon stimulated by APCs. The populations of CD8^{+} in this study has the same pattern with CD4^{+} population where the CD8^{+} cells population in 0.5 g/kg group increased slightly compared to CD8^{+} cells population of the control group, with percentage of 6.95±1.32% and 7.00±1.16% respectively. In mice administered with 1.5 g/kg and 3.0 g/kg TH daily, CD8^{+} cells populations showed an ascending pattern from 7.15±0.97% to 7.22±0.54%. However, the increment of CD8^{+} population was not statistically significant in this study. Several studies showed that the ratio of the two T lymphocytes subsets (Th cells and CTL cells) were the most meaningful parameters to determine the balance state of immunomodulatory and homeostatic response of the instrinsic immune system [25]. From the results obtained, the CD4^{+}/CD8^{+} ratio of 0.5 g/kg/day group was exactly the same with that of the control mice, both had ratio of 1.87. On the other hand, the ratio showed slightly increment from mice administered with 1.5 g/kg to 3.0 g/kg TH daily, with ratio of 1.90±0.31 and 2.07±0.31 respectively. Similarly, previous studies also included CD4^{+}/CD8^{+} as a parameter to evaluate the immunomodulating effect where results showed there was no different in CD4^{+}/CD8^{+} in treatment group of soybean peptides [26, 27].

The CD14^{+} population in this study showed an increment pattern after 2 weeks daily administration with TH. The CD14^{+} population in the control group was 3.26±0.79% while in TH treated mice, the percentage of CD14^{+} were 3.63±0.36%, 4.48±0.51% and 3.70±0.44% by the mice administered with 0.5 g/kg, 1.5 g/kg and 3.0 g/kg TH daily respectively. Interestingly, administration of 1.5 g/kg TH daily has induced higher CD14^{+} population compared to the other treated groups. The results suggested that TH has the ability to trigger production of splenic macrophages in mice. Previous studies showed that honey was able to stimulate

macrophages to secrete TNF-α, IL-1β and IL-6 [28, 29]. In addition, a previous study showed that propolis treatment was able to stimulate IFN-γ production in BALB/c mice [30].

As splenocytes cells were used in this phagocytosis assay, the targeted phagocytes are such as red pulp macrophage and white pulp macrophage. These macrophages play important role in clearance of senescent blood cells apoptotic B cells [31]. The percentage of splenic phagocytes is increase dose-dependently from low to high dosage with the mice administered with 3.0 g/kg TH daily has significant different compared to the control group. A previous study showed that honey has the ability to activate phagocytes even at the concentration as low as 0.1% [32].

The present study has demonstrated that TH has the potential to non-specifically enhanced the immune response. Moreover, the positive immunomodulation by TH is most likely attributed to the stimulation of helper T cells; as indicated by increasing ratio of CD4$^+$/CD8$^+$ cells. In addition, daily administration of TH has also improved CD14$^+$ population in mice. Our results also demonstrated that consumption of low dose of TH was able to reduce body weight of the treated mice. It was suggested that for future research, the anti-lipid effect of honey at certain dosages should be studied. In conclusion, TH may potentially improve general immune function when consumed in optimal portion and regularly as a natural supplement.

ACKNOWLEDGEMENT

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