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Research article

Green honey of Banggi Island: A preliminary anti-diabetic study on zebrafish model

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ABSTRACT

Zebrafish is a developing vertebrate model with several advantages, including its small size, and high experimental efficiency. Malaysia exhibit one of the highest diabetes rates in the Western Pacific and incurring an annual cost of 600 million US dollars. The objective of the study is to determine the antidiabetic properties of green honey (GH) using a zebrafish model. Adult zebrafish, aged 3–4 months, were subjected to overfeeding and treated with streptozotocin (STZ) through intraperitoneal injection (IP) on days 7 and 9. The study assessed the oral sucrose tolerance test (OSTT) and the anti-diabetic effects of green honey. The evaluation was conducted at three time points: 30, 60, and 120 min after treatment and sucrose administration. The study utilised a model with a sample size of 5. The study was performed in six groups. These groups are (1) Normal control (non-diabetic, no intervention), (2) Normal control + GH (non-diabetic, supplemented with GH 3 µl), (3) DM control (diabetic, no intervention), (4) DM Gp1 (diabetic, 3 µL GH), (5) DM Gp2 (diabetic, 6 µ L GH), (6) DM Acarbose (diabetic, treated with acarbose). Fasting blood glucose levels for non-diabetic (non-DM) and diabetic (DM) groups were evaluated before and after the 10 days of diabetic induction. DM groups (excess of food and two injections of STZ) have caused a significant increment in the fasting blood glucose to 11.55 mmol/l ($p < 0.0001$). Both GH treatments effectively decreased postprandial blood glucose levels and the area under the curve in the oral glucose tolerance test (OSTT). Based on these results, it is concluded that green honey could play a role in hyperglycemia management and show potential as a natural alternative to conventional diabetes therapy. The underlying mechanisms need to be clarified, and their potential use in human diabetes therapy needs to be investigated.

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1. Introduction

Diabetes mellitus (DM) is a metabolic condition characterised by insufficient production of insulin or an insufficient insulin response, leading to elevated blood sugar levels. This medical condition has emerged as a substantial worldwide health issue, affecting not just adults but also children and adolescents. According to the estimation provided by the International Diabetes Federation, it is projected that more than 10% of the global adult population will be afflicted with diabetes by the year 2040 [1–3]. Diabetes mellitus (DM) is classified into two distinct types: type 1 (T1DM) and type 2 (T2DM). Type 1 diabetes mellitus (T1DM) is defined by the partial or total death of β-pancreatic cells, leading to the inability to make insulin. This condition is caused by an autoimmune disease. T2DM, the most common kind, is characterised by insufficient insulin secretion and/or resistance to insulin hormones [4]. The prevalence of diabetes may be influenced by genetic factors or might manifest at any stage of life. This disease does not discriminate based on age; however, scientific research indicates that it is more prevalent in developing nations than the global average, including both developed and third-world countries [3].

Models using zebrafish have been developed to investigate several human diseases, such as genetic abnormalities and diabetes [5, 6]. The current significance of zebrafish in the disciplines of developmental biology and disease modelling can be attributed to several key traits. This organism is categorized as a vertebrate and displays notable genetic, morphological, and physiological similarities to the human species. The organism in question possesses a high reproductive capacity, making it suitable for large-scale maintenance. Additionally, it has a relatively short lifespan, early adult transparency, and reduced housing costs, enabling convenient genetic and chemical genetic screening processes [7,8]. Alternatively, non-genetic techniques for inducing diabetes are more desirable due to their potential for wider accessibility, lower cost, or simpler execution [6].

The global utilisation of natural products as an adjunctive therapy for type 2 diabetes mellitus (T2DM) is on the rise. An alternative strategy for treating diabetes involves utilising herbal medicines, dietary constituents or supplements, and other natural goods such as honey [9]. Most of the biological properties of honey are connected to the presence of bioactive compounds in honey, like flavonoids (Phenolic compound), ascorbic acid, catalases, and peroxidase contribute to antioxidants in honey [10–12]. The phenolic profile or bioactive compound of honey is variable depending on the floral source of honey and also on the entomological origin of honey [13, 14].

Many reports have been made to assess the antidiabetic potential of various Malaysian honey. A study was conducted to assess the

Fig. 1. Raw green honey collected from Banggi Island, Sabah. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antidiabetic properties of stingless honey derived from various floral sources [15]. Another study was conducted to investigate the antidiabetic properties of tualang honey [16].

Green honey, recently discovered on Banggi Island, Sabah, has garnered attention owing to its striking green color, primarily attributed to its high chlorophyll content and distinctive chemical composition [17]. Additionally, its significant and beneficial microbial composition has been outlined [18]. However, despite these revelations, the potential antidiabetic effects of green honey remain unexplored. Hence, our present research objectives to examine the antidiabetic characteristics of green honey by utilising a STZ-induced zebrafish diabetics' model. This study aims to fill a critical knowledge gap in understanding the therapeutic potential of this remarkable natural resource.

2. Material and method

2.1. Green honey and preparation

The green honey was a crude honey and collected from Banggi Island of Sabah, Malaysia between January and February 2023. Honeybee, inhabiting the rainforest was responsible in producing the green-colored honey (Fig. 1). The physicochemical properties of the honey were evaluated and reported elsewhere [17]. To preserve the integrity of the honey, it was kept in cold and dry storage until required. Due to the high viscosity for low volume handling, the green honey was diluted to 3% (v/v) in sterile normal saline solution (0.9% (w/v) NaCl). The dosage was adjusted to be equivalent of 1 (15 mL) and 2 (30 mL) tablespoon green honey per normal adult of 68 kg [19].

2.2. Zebrafish and hyperglycemic induction

The use of Zebrafish in research was granted approval by the Institutional Animal Care and Use Committee (IACUC) of the International Islamic University Malaysia (IIUM) under the reference number IIUM/504/14/2/IACUC.

2.2.1. Zebrafish maintenance

A total of 36 mixed male and female zebrafish of the age of 5 months with the average weights 0.517 ± 0.033 g from a single breeding batch was purchased from Aquatics Sdn. Bhd., Shah Alam, Malaysia. Upon arrival, the fish were quarantined and acclimatized to the 10 h dark/14 h light cycle photoperiod laboratory condition for 10 days at the density of five fish for every 1 L of water. The average water temperature was maintained at 25 ± 2 °C, conductivity between 800 and 1200 μS/cm, pH 7 ± 1, ammonia <0.1 mg/L. The water quality was maintained by periodic water change by 40% of total volume every 2 days. Throughout the acclimatization, the fishes were fed twice a day (Otohime B2, Marubeni Nisshin Feed, Japan). No death was recorded during 10 days of quarantine and acclimatization, indicating the fish are fit for experimentation.

2.2.2. Induction of diabetics by STZ

Following a period of 10 days for quarantine and acclimatization, the fishes were categorized into 6 groups, with each group consisting of 6 individuals (n = 6). Two groups were classified as normal non-diabetic groups, whilst the other groups were deliberately caused to develop fat and hyperglycemia. The induction for diabetic condition was adopted from Abdullah et al. [6], for a total of ten days course. Briefly, the diabetic groups were fed with excess amount of food with high carbohydrate four times per day for 10 days. On day 7 and 9 of the induction course, the diabetic groups were given 350 mg/kg body weight (bw) of STZ *via* intraperitoneal injection using 29G syringe (Fig. 2).

2.2.3. Repeated blood sampling

Small size of adult zebrafish ranging 3–4 cm limits the volume of blood that can be safely collected from them. Most of the protocols

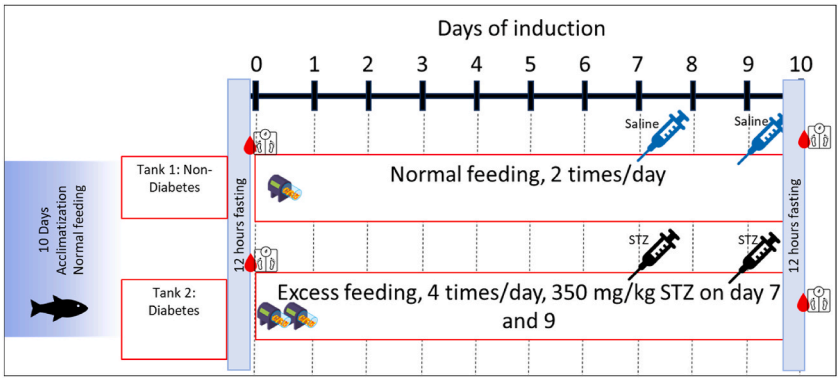

Fig. 2. The timeline of the diabetic induction using two doses of STZ on days 7 and 9 with excess feeding regime.

are single sampling and require the sacrifice after each time of blood collection. Abdullah et al. [6], has established a method that allows repeated blood sampling from an adult zebrafish without sacrificing them. The method permits the longitudinal monitoring from an individual fish. The volume of blood collected in this method is about 5 μL and sufficient for the use of standard glucometer.

The diabetic condition was confirmed by the level of fasting blood glucose on day 10 of the induction course, after the fish were fasting for at least 12 h. Blood glucose was measured using glucometer (AccuCheck Instant S meter) on the zebrafish blood collected from caudal vein as stated by Abdullah et al., [6]. By using that method, repeated blood sampling made possible from a same fish without sacrificing them.

2.2.4. Oral sucrose tolerance test

Immediately after the measurement of the fasting blood glucose and while the stomach of the zebrafish empty following the 12 h of fasting, the fish were given treatment orally (Fig. 3), a 3 or 6 μL of prepared green honey were administered to the following groups Normal control + GH (non-diabetic, supplemented with GH 3 μl), DM Gp1 (diabetic, 3 μL GH), and DM Gp2 (diabetic, 6 μ L GH). Oral administration of acarbose at 300 mg/kg bw (body weight) serves as positive control of α-glucosidase inhibitor and normal saline was given to the DM Control. The oral sucrose tolerance test (OSTT) was conducted by giving 1250 mg/kg bw to each fish orally 30 min of post treatment administration. Subsequently, blood glucose levels were assessed at 30, 60, and 150 min after the post treatment.

2.2.5. Zebrafish pain management and euthanasia

Intraperitoneal injection of STZ and caudal vein blood collection was done under anesthesia condition. The individual fish was exposed to MS-222 (150 mg/L) in cold water bath (12–14 °C) for 1 min or until the fish lost its motor responses. The injection procedure was done on ice-cold sponge to secure the position and anesthesia state of the fish. Following IP injection or blood sampling, slight compression was applied to the affected site to reduce the bleeding. The fish was then transferred into a recovery tank containing anti-bacterial agent at temperature of 20 ± 4 °C. Fully recovered fish then returned into experimental tank. Finally, all fish were euthanized in ice-cold water at temperatures between 0 and 4 °C for at least 10 min.

3. Statistical analysis

The graphics and data were produced via GraphPad Prism version 8.0 (GraphPad Software Inc.). The data were reported as the average value ± standard error of the mean (SEM). The significant differences were assessed using One-way Analysis of Variance (ANOVA), whereas a nonparametric *t*-test was employed to identify significant contrasts among the groups at certain time intervals. A *p-value* less than 0.05 was deemed to indicate statistical significance.

4. Results

4.1. STZ-induced diabetics conditions in zebrafish model

Upon completion of quarantine and acclimatization, all fishes were weight, and their first fasting blood glucose was measured. There was no significant difference in fish weight between the DM and non-DM groups prior to the induction of STZ and the intake of excessive diet (Fig. 4). Following the ten days of induction course, there is slight increase in the body weight of the DM group as compared to non-DM group ($p < 0.01$, paired *t*-test). The excess feeding, twice frequency than given to the DM group and administration of STZ has cause significant increase in the fish body weight ($p < 0.001$).

Fig. 5 represents the average of fasting blood glucose level in the non-DM and DM control groups that were measured before and after the ten days of induction course. Both groups were started at the similar average blood glucose level of 5.10 ± 0.5 mmol/L. Interestingly in the DM groups, the induction for diabetics by excess of food and two injections of STZ has causes significant increment in the fasting blood glucose to 11.55 ± 0.9 mmol/l ($p < 0.0001$). No significant changes in the fasting blood glucose for non-DM indicating the normal diet and injection with normal saline did not affect the fasting blood glucose. The result shows evidence of the successful induction of diabetics by 2 doses of 350 mg/kg bw of STZ and excess feeding over the course.

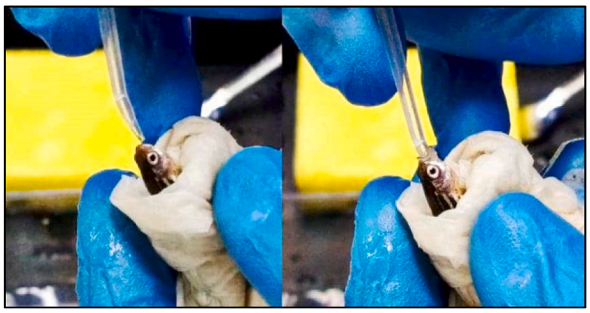

Fig. 3. Force feeding (Oral administration) of GH, acarbose and sucrose.

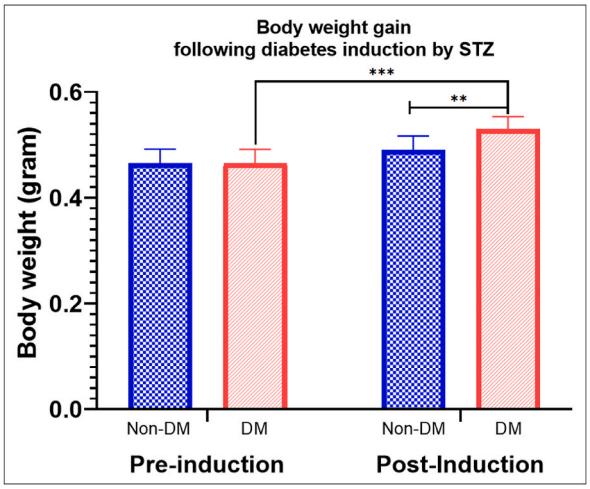

Fig. 4. The changes of body weight between the Non-DM and DM, groups at pre- and post-induction of diabetes using excess diet and STZ.

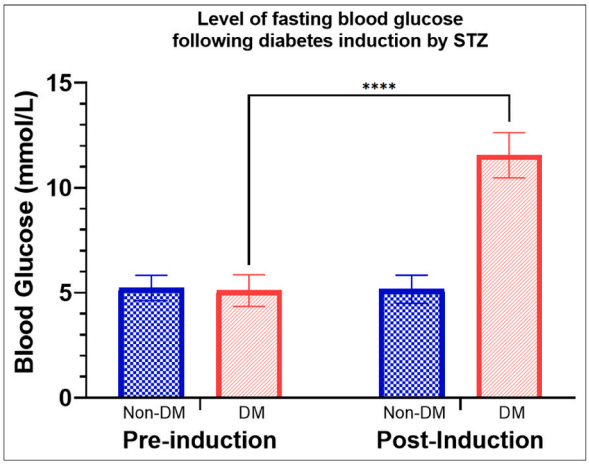

Fig. 5. The differences of the fasting blood glucose level between the non-DM and DM groups during pre- and post-induction of diabetes. (n = 24, paired *t*-test).

4.2. Oral sucrose tolerance test OSTT

Following a 12-h fasting period, the zebrafish were treated with an oral administration of green honey, to 3 groups namely normal control + GH, DM Gp1 and DM Gp2. Serving as solvent control, 1 group of fish (DM control) was given with same volume of normal saline. The positive control group received acarbose (DM acarbose), while the normal control group was administered a normal saline orally.

Fig. 6 shows the blood glucose level (BGL) of zebrafish after oral administration of sucrose over two and half hours. DM groups start at higher BGL 11.55 mmol/L, which is very significant ($p < 0.0001$) as compared to non-DM groups which starts at 5.10 mmol/L 30 min later, the fish were loaded with 1250 mg/kg of sucrose, and the second blood glucose level was measured. At times 30 min a slight increase in BGL was observed in all groups after treatment of GH, but for normal control group the BGL remain same.

Following a 30-min period of sucrose loading, the DM control group had the highest peak of blood glucose level at 13.58 mmol/L. There were extremely significant differences while comparing the blood glucose level of DM control groups and the treated groups DM Gp1 (diabetic, 3 μL GH), DM Gp2 (diabetic, 6 μL GH), and DM Acarbose. After a duration of 1 h, there was a significant difference in between the DM control groups and those treated with DM Gp1 ($p < 0.0001$), DM Gp2 ($p < 0.0001$), DM Acarbose ($p < 0.0001$). The significant reduction of BGL in all groups treated with GH were found after 2.5 h of OSTT course. The postprandial blood glucose level (BGL) analysis demonstrates that both GH treatments and acarbose had a substantial lowering effect on BGL compared to the control groups with diabetes mellitus (DM).

Area under the curve for the treatment and control group is shown (Fig. 7). This study highlights the efficiency of zebrafish in the regulation of postprandial blood glucose levels after sucrose delivery, following treatment with normal saline, GH and acarbose.

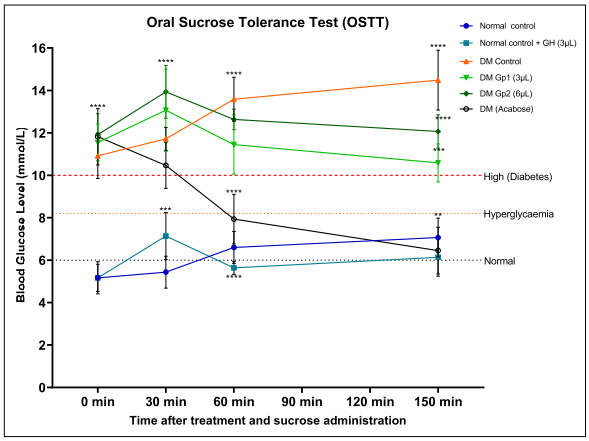

Fig. 6. The effect of GH treatment and acarbose on Oral Sucrose Tolerance Test (OSTT) treatment was orally administered to zebrafish for 30 min before sucrose loading. The postprandial blood glucose level was observed for 2.5 h, are presented as mean ± SEM, with a sample size of n = 15. A nonparametric *t*-test was employed to assess significant differences between various groups at distinct time intervals. Significance levels were denoted as follows: ****p < 0.0001, indicating variations in means when compared to the control group of healthy, non-diabetic zebrafish.

Fig. 7 shows the reading of the total amount of glucose in the bloodstream over the entire testing period. DM Gp1, DM Gp2 and DM Acarbose group shows a decrease in blood glucose levels (BGL) and prevented the abrupt increase in BGL for a duration of 2 h during the oral sucrose tolerance test (OSTT), in comparison to the DM control.

The data comparison presented in Fig. 7 for two normal control groups and normal control + GH, did not indicate any significant differences when compared to both these two normal control groups and the group treated with the conventional medicine, acarbose. The results obtained from measuring the overall effect of sucrose loading using the area under the curve (AUC) indicate that there was no significant difference ($p = 0.9643$) between these two groups normal control group and normal control + GH.

The observed lack of substantial distinctions in the comparison between the normal control and normal control + GH groups, in contrast to the DM control group, implies that both interventions have a beneficial influence on blood glucose regulation in zebrafish after sucrose administration. Notably, a statistically significant difference is evident when comparing the normal control + GH group to the DM control group, with a p-value of (0.0311), followed by the normal control group treated with DM control, which has a p-value of (0.0165).

Oral administration of GH didn't worsen the BGL in any groups of treatment. Interestingly, GH only caused a slight increase in the immediate BGL but steadily reduced after that. In comparison, the normal control and DM control groups that receive saline, instead of GH shows significant increase in the BGL across OSTT duration.

5. Discussion

The present study involved the development and validation of the STZ-induced zebrafish model to assess the anti-diabetic potential of green honey recently discovered on Sabah's Banggi island. Inducing the zebrafish confirms the finding of a previously established model [6,20]. However, the excessive feeding, twice as frequently as the non-DM group, and STZ administration resulted in a

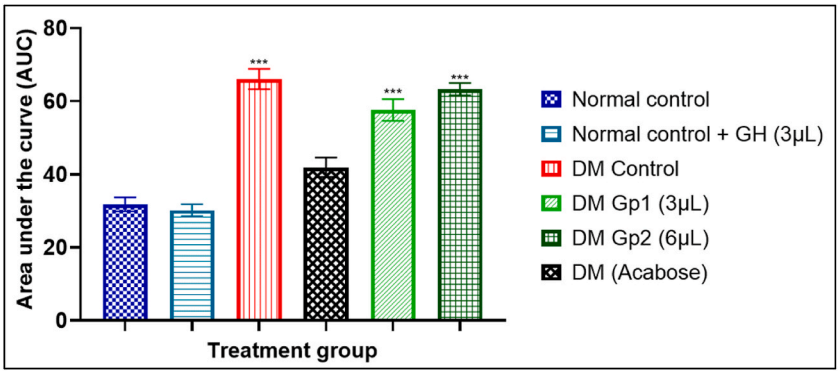

Fig. 7. Area under the curve (AUC) of the zebrafish blood glucose level after oral sucrose administration. Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used to carry out the significant differences with a *t*-test using Dunnett's Multiple Comparison. The significant difference was considered at ***p < 0.001 between the means of groups compared to the control normal healthy group.

considerable increase in fish body weight in the DM group ($p < 0.001$).

The antidiabetic potential of green honey is the primary finding of the current research. The customary dosage administered to zebrafish is about the same quantity as consumed by an adult person which is one to two tablespoons as outlined by Ali et al., [21]. The determination of specific volumes of green honey used in this study aligns with the weight of the zebrafish specimens (0.4–0.5 g).

This research demonstrates that zebrafish efficiency in controlling the blood glucose level after sucrose oral administration with specific treatment of green honey (3 μl and 6 μl). The OSTT data showed that DM Gp1, DM Gp2 as well as DM Acarbose groups has effectively reduced the blood glucose levels, compared to the normal control groups.

Acarbose or Alpha-glucosidase inhibitors (AGI) particularly has a major role in lowering blood glucose levels by prolonging carbohydrate breakdown into glucose [6]. A slight elevation in fasting blood glucose levels was found in DM zebrafish after administering volumes of 3 μl and 6 μl of green honey. This could be attributed to the presence of simple sugars in green honey, as previously described [17].

At time 30, after loading sucrose the increment stops and then starts to decrease for DM Gp1 and DM Gp2 compared to DM control and keep decreasing until 1.5 h. We hypothesize, the increment of blood glucose at early stage is because of the presence of simple sugar in the GH, but soon after that, the complex sugars were not converted into simple sugar for absorption in the GH showing the potential of inhibition of complex sugar metabolism. It is worth exploring the effect of green honey on alpha glucosidase activity.

The finding of Elmazar et al. [22], revolved that phytol has the potential to play a role in the management of insulin resistance and metabolic diseases that are commonly associated with diabetes and/or obesity. This potential is due to its ability to activate RXR through its metabolite and regulate other parameters that play a role in metabolic disorders. In addition, the application of molecular docking techniques to investigate the interaction of phytanic acid with two crystal structures of PPARγ binding protein, as well as the RXRα/PPARγ heterodimer, demonstrated a favorable correlation with experimental observations. This alignment further supported the well-established antidiabetic properties of phytol and its metabolite, phytanic acid.

The results of the docking investigation demonstrated that phytanic acid exhibits a strong affinity for interacting with PPARγ in a manner that closely resembles the pattern observed with TZD agonists. This interaction involves the formation of four hydrogen bonds with specific amino acids, namely Ser-289, His-323, His-449, and Tyr-473. Therefore, these four amino acids are deemed essential for the molecular identification, activation, and antidiabetic biological activity of PPARγ [22]. Some amino acid from these were already confirmed in our green honey [17,23] that might be a reason of green honey sample to possess antidiabetic properties. Another study performed by Semaan et al. [24], showing the antidiabetic effect of pheophytin *a* and *b*, via *in vitro* study. These evidence the beneficial use of raw green honey as a natural product in controlling the postprandial blood glucose in diabetic and non-diabetic individuals.

6. Conclusion

The findings of our research on the anti-diabetic properties of green honey in zebrafish offer important new information about the potential medical benefits of the green honey sample. Our studies showed a considerable decrease in blood glucose levels in zebrafish treated with green honey 3 μl, and 6 μl, demonstrating its effectiveness in controlling hyperglycemia, a defining feature of diabetes. The current research strongly recommended to study the effect of green honey on alpha glucosidase activity. The study also strongly recommended additional research using molecular docking and molecular simulation to reconfirm or identify a specific bioactive component in green honey which activate specific pathway like PPARγ.

Data availability

The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Animal ethics

The Institutional Animal Care and Use Committee (IACUC) of the International Islamic University Malaysia (IIUM) approved the *in vivo* study of adult zebrafish. The study was conducted in compliance with the Malaysian Animal Welfare Act 2015, which governs the treatment and utilisation of animals for scientific purposes.

CRediT authorship contribution statement

Saeed ullah: Methodology, Investigation, Formal analysis, Data curation. **Fahrul Huyop:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Nurul Huda:** Methodology, Data curation, Conceptualization. **Roswanira Ab Wahab:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Azzmer Azzar Abdul Hamid:** Validation, Supervision, Methodology, Data curation. **Mohd Azrul Naim Mohamad:** Methodology, Data curation, Conceptualization. **Hajar Fauzan Ahmad:** Methodology, Data curation, Conceptualization. **Amir Husni Mohd Shariff:** Methodology, Formal analysis. **Mohd Hamzah Mohd Nasir:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be

construed as a potential conflict of interest.

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