Physicochemical and Sensory Attributes of Trihoney Blend (*Trigona sp., Apis mellifera, and Apis dorsata*) for Enhanced Antioxidant Optimization using Response Surface Methodology (RSM)

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ABSTRACT

INTRODUCTION: The therapeutic applications of honey products have been extensively studied, but the various combination of honeys as a high-antioxidant product has not been explored. This study aimed to develop an optimized three honey formulation (Trihoney) with maximal antioxidant potency and physicochemical characteristics, as well as favourable among panellists. **MATERIALS AND METHODS**: The three types of honey studied are; i) *Trigona* sp. honey (TH), ii) *Apis mellifera* honey (MH), and iii) *Apis dorsata* honey (DH). Response surface methodology (RSM) was employed to design optimal Trihoney formulation for i) total phenolic content (TPC), ii) ferric-reducing ability of plasma (FRAP), and iii) 2,2'-diphenyl-1 picrylhydrazyl (DPPH) radical scavenging activity. Proximate and nutrient analyses, such as moisture, protein, fibre, carbohydrate, sugar, and gross energy were also conducted. Additionally, sensory evaluation was carried out to support the findings. **RESULTS:** Based on RSM, three optimal Trihoney formulation were developed; i) Trihoney 1 (MH15:DH10:TH45), ii) Trihoney 2 (MH15:DH10:TH25), and iii) Trihoney 3 (MH15:DH10:TH15). Trihoney 1 was the most promising formulation, exhibiting the highest TPC (0.50 mg/GAE/g), remarkable values of FRAP (230.85 AAE/g) and DPPH (86.32%). Physicochemical and sugar analysis indicated that all values complied with permitted quality standards. MH received the highest overall acceptability scores based on sensory evaluation. **CONCLUSION**: These findings warrant further extensive investigation of Trihoney formulation in animal studies to support its efficacy as a valuable food supplement.

INTRODUCTION

In food preservation, antioxidants play an important role in extending the shelf life by minimizing the detrimental effects of reactive oxygen species (ROS). When added to fatty foods, antioxidants prevent lipid oxidation, which can lead to browning and nutrient reduction.[1,2] Natural antioxidants including those found in honey, namely polyphenols and flavonoids, play a crucial role in food products for their health benefits and functional properties such as anti-inflammatory, anticancer, and cardioprotective properties.[3-5]

Trigona sp. refers to the stingless bee species within the Meliponini tribe, predominantly distributed in tropical regions like South America, Africa, and Asia, where they are known as 'Kelulut' in Malaysia.[6] Stingless bee honey, characterized by a sweet and sour taste, subtle fruity hint, distinct aroma, and a more liquid texture. On the other hand, *Mellifera* sp. and *Dorsata* sp. are subspecies of honeybees classified under the genus *Apis*, belonging to the tribe Apini. *Apis mellifera* is primarily found in Europe and Asia, while *Apis dorsata* is predominantly distributed in Southeast Asia.[7] Stingless bee honey is known for its multi-floral origin and is stored in clusters, whereas honey from *Apis* spp. is stored in hexagonal-shaped honeycombs.[8]

This study utilized the response surface methodology

(RSM) optimization technique to determine the most effective formulation of three distinct Malaysian honeys, aimed to maximize their antioxidant properties. Following this, the optimized formulation underwent validation by comparing predicted values from the model against actual experimental results. Subsequent steps involved conducting both physicochemical and sensory analyses to ensure that the optimized formulation demonstrated improved nutritional profiles and received favourable assessments in taste and aroma. This holistic approach aimed to comprehensively enhance the antioxidant properties of optimized Trihoney blends, physicochemical characteristics, and sensory attributes, thereby facilitating the development of a functional food product. Findings from this study will provide valuable guide to the food industry, aiding in the formulation of honey blends that are both nutritionally enhanced with desirable sensory characteristics.

MATERIALS AND METHODS

Chemicals

The analytical grade of chemicals and reagents such as sulfuric acid, methanol, hydrochloric acid, sodium carbonate, sodium phosphate, sodium hydroxide Folin-Ciocalteu reagent, FRAP reagent, DPPH solution, gallic acid, quercetin, ferric (III) chloride, ammonium molybdate and routine standards were obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, USA).

Honey samples collection

Honey samples from *Apis mellifera*, *Apis dorsata,* and *Trigona* sp. bee species were obtained from the east coast of the Peninsular region of Malaysia. The unprocessed honey from *Trigona* sp. (TH) was self-collected from the bee farm in Kota Bharu, Kelantan, Malaysia, while The Federal Agricultural Marketing Authority (FAMA), Pahang, Malaysia supplied the honey for *A. dorsata* (DH) and *A. mellifera* (MH). All samples were kept in sterile, airtight glass bottles at 15°C to avoid moisture absorption.

Experimental design and model verification

The optimized formulations for Trihoney were generated using response surface methodology (RSM) employing a three-factor inscribed central composite design (CCD) (Design-Expert® Version 6.0 software).[9] Three independent variables, TH (X_1: 15–45 ratio), MH (X_2: 15–45 ratio), and DH (X_3: 10-30 ratio) were investigated. The total phenolic content (Y_1), the ferric-reducing ability of plasma (Y_2), and the 2,2'-diphenyl-1 picrylhydrazyl (Y_3) were assigned as the response variables. Using CCD, a total of 20 randomized experiments, comprising six replicates were constructed and three levels of variables were coded (-1, 0, +1) as presented in Table I. The CCD applied the least-squares regression method to fit the data into a quadratic model.

Table I. The independent variables and their coded values used for the optimization

Independent variable	Unit	Symbol	Coded level				
			-1	0	1	Axial (-α)	Axial (+α)
TH	g	X_1	15	30	45	4.77	55.23
MH	g	X_2	15	30	45	4.77	55.23
DH	g	X_3	10	20	30	3.18	36.82

TH: *Trigona* sp. honey; MH: *Apis mellifera* honey; DH: *Apis dorsata* honey.

For the prediction of response variables, a second-order polynomial model was used as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1^2 + b_2{}^2X_2^2 + b_3{}^2X_3^2 + b_1b_2X_1X_2 + b_1b_3X_1X_3 + b_2b_3X_2X_3$$

Y = the predicted response
b_0 = a constant at the intercept
b_1, b_2, b_3 = the linear effect terms
$b_1{}^2$, $b_2{}^2$, $b_3{}^2$ = the quadratic effect terms
b_1b_2, b_1b_3, b_2b_3 = the interaction coefficient effects

Several numerical optimizations were conducted to establish the ideal ratios of independent variables that correspond to the desired responses (Y_1, Y_2, and Y_3). Finally, the projected values from the RSM model were compared to the experimental results.

Honey sample extraction

The honey samples were weighed and mixed with methanol to obtain a final concentration of 0.25 g/mL.[10] Then, the samples were set to pH 2 by adjusting with 0.1% hydrochloric acid (HCl). The honey was homogenized for 30 minutes in an incubator shaker at 300 rpm and 55°C. The samples were then filtrated through Whatman No. 1. and the extracts were stored at 4°C. Before analysis, the samples were pre-warmed for 5 minutes.

Total phenolic content (TPC)

Initially, 500 μL honey extracts were mixed with 2.5 ml of Folin-Ciocalteu reagent. The mixture was then incubated for 10 minutes at room temperature.[11] Next, 2 ml of 75 g/L sodium carbonate (Na_2CO_3) was added, and the mixture was vortexed briefly before incubating for 2 hours at room temperature in the dark condition. The absorbances (triplicate) were measured at 760 nm using UV/VIS spectrophotometer (Schott UVLine 9400, USA). The result was expressed as mg of gallic acid equivalents (GAEs) per g of honey.

Ferric-reducing ability of plasma (FRAP) analysis

Briefly, 200 μl of honey extracts were mixed with 1.5 ml of FRAP reagent. FRAP reagent was freshly made by combining 200 mL of 300 mM acetate buffer (pH 3.6) with 20 mL of 10 mM TPTZ solution, 20 mL of 10 mM ferric chloride ($FeCl_3.6H_2O$), and 25 mL of distilled water.[11] The reaction mixture was incubated for 5 minutes at 37°C in the dark. The absorbances (triplicate) were measured at 593 nm using UV/VIS spectrophotometer (Schott UVLine 9400, USA), with the FRAP reagent serving as a blank and ascorbic acid as the standard. The results were computed in μM of ascorbic acid equivalent per gram of honey (μM AAE/g).

2,2'-diphenyl-1 picrylhydrazyl (DPPH) scavenging assay

Briefly, 500 μL of the honey extracts were mixed with 100 μM methanol containing 1000 μL DPPH solution.[12] The mixtures were vortexed and allowed to remain in the dark for 15-50 minutes. Next, the measurements were taken in triplicate at 517 nm using a UV/VIS spectrophotometer (Schott UVLine 9400, USA). Ascorbic acid and quercetin were used as the reference and a standard calibration curve was constructed. Radical-scavenging activity (RSA) was computed using the following formula:

$$\% \text{ RSA} = ([A_x - A_y]/A_x) \times 100$$

A_x = the absorbance of the DPPH solution.
A_y = the absorbance of the extract's solution

Physicochemical analysis

The physicochemical characteristics of individual honeys (TH, MH, and DH) and Trihoney formulations (Trihoney 1, Trihoney 2, and Trihoney 3) were determined in triplicate, following the Association of Official Analytical Chemists and Harmonised Methods of the International Honey Commission standard procedures.[13,14] The pH value was measured using a pH meter (Mettler Toledo), while the water activity was determined using a water activity meter. For moisture content, the honey samples were homogenized and placed in a water bath at 50°C until all the sugar crystals were dissolved. After homogenization, the Abbe refractometer was used to measure the refractive index (triplicate). The moisture content was read by referencing a standard table. The table was derived from a formula developed from the previous report:[13]

$$W = 1.73190 - \log(R.I.\text{-}1)/ 0.002243$$

W is the water content in g per 100 g of honey and R.I. is the refractive index

Ash content was determined utilizing the dry ashing method by the incineration of 2 g of the sample in a muffle oven at 550°C for 12 hours. Then, the final weight was obtained and the value was expressed in g/100g.[13] Protein content was analysed using the Kjeldahl method, with digestion carried out using the KjelDigestor (K-446) and distillation by the Kjeldahl distillation unit.[15] In a Soxhlet extractor, 5 g of the sample was extracted with petroleum ether to estimate the total fat content in triplicates. A series of sulfuric acid and sodium hydroxide treatments were used to analyse crude fibre.[16] The residue was scraped into a pre-weighed porcelain crucible, and the weight was denoted as W_1. The crucible and residue were then incinerated for 2 hours at 600°C. The total weight was determined after being cooled for 30 minutes in the desiccator. The total fibre was calculated using the following formula:

$$\text{Total fibre (\%)} = (W1 - W2) / WS \times 100$$

W_1 = weight of crucible and residue after drying (g)
W_2 = weight of crucible and ash after incineration (g)
W_S = weight of the honey sample (g)

For the carbohydrate contents and gross energy values, the samples were calculated based on the following formula:[17]

$$\% \text{ Carbohydrate} = 100\% - (\% \text{ moisture} + \% \text{ crude protein} + \% \text{ crude fat} + \% \text{ ash})$$

$$\text{Energy}(kJ/100g) = 4.186[(\% \text{ carbohydrate} \times 4) + (\% \text{ crude protein} \times 4) + (\% \text{ crude fat} \times 9)$$

For sugar analysis, a 2690 Separations Module and a 2410 Differential Refractive Index Detector from the Waters Alliance HPLC System were employed, utilizing high-performance liquid chromatography (HPLC) with refractive index (RI) detection.[17] The value was expressed in g/100g.

Trihoney sensory evaluation

The sensory evaluation was conducted in the sensory room of the Nutrition Laboratory at the International Islamic University Malaysia in Kuantan, Malaysia.[9] Six samples of the honey were coded and presented randomly to fifty panellists. Seven sensory attributes namely colour, sweetness, sourness, odour, flavour, after-taste, and overall acceptability were evaluated. The panellists were given a brief description of these characteristics. A 5-point category hedonic scale, ranging from 1 (extremely dislike), to 3 (neither like nor dislike,) to 5 (extremely like), was employed for the evaluation process.

Statistical analysis

The data was analysed using Design-Expert® Version 6.0 software and presented as a mean ± standard deviation (SD). The means were compared using One Way ANOVA and Duncan's new multiple-range post hoc test ($p < 0.05$).[9] The regression coefficients were calculated using a response surface analysis, the statistical significance of model components was evaluated, and mathematical models were fitted to the experimental data. Regression (R^2) and ANOVA analyses ($p < 0.05$) were used to evaluate the model's suitability.

RESULTS

Determining the most optimal formulation

The central composite design (CDC) suggested three formulations that yielded highly optimized results for all three analyses (TPC, FRAP, and DPPH) in the honey combination. These optimal formulations were designated as i) Trihoney 1 (MH15:DH10:TH45), ii) Trihoney 2 (MH15:DH10:TH25), and iii) Trihoney 3 (MH15:DH10:TH15).

Fitting the response surface model

Table II displays the range and central point values of the three independent variables, along with the experimental and predicted values for response functions. A successful model is suggested by the similarities and closeness of the predicted values of TPC (Y_1), FRAP (Y_2), and DPPH (Y_3) with their experimental values. The coefficients of determination (R^2) for TPC, FRAP, and DPPH were 0.7923, 0.4777, and 0.8157, respectively, with $p > 0.05$. The R^2 value for TPC revealed that about 79.23% of the variability in TPC was accounted for by the model, indicating a robust fit. This suggests that the independent variables included in the model capture a significant portion of the observed variation in TPC. In the case of FRAP, the R^2 value signified a moderate level of explanation at 47.77%. Conversely, the R^2 value of 0.8157 for DPPH indicated that approximately 81.57% of the variability in DPPH activity can be attributed to the model.

Table II. The two-factor central composite design used for RSM experiment and predicted values for independent variables

SO	Factor 1 (X1) TH	Factor 2 (X2) MH	Factor 3 (X3) DH	TPC (mg/GAE/g) Y1		FRAP (AAE/g) Y2		DPPH (%) Y3	
				Exp.	Pred.	Exp.	Pred.	Exp.	Pred.
1	-1	-1	-1	0.43	0.42	245.85	207.12	81.38	78.42
2	1	-1	-1	0.50	0.51	230.85	252.84	86.32	87.72
3	-1	1	-1	0.31	0.30	183.63	190.74	68.78	69.07
4	1	1	-1	0.39	0.39	221.78	236.46	76.00	74.9
5	-1	-1	1	0.31	0.33	166.78	180.65	66.29	66.09
6	1	-1	1	0.41	0.43	234.56	226.37	81.65	80.06
7	-1	1	1	0.30	0.30	166.79	164.27	66.61	63.91
8	1	1	1	0.37	0.39	199.93	209.99	72.75	74.41
9	-4.77	0	0	0.26	0.26	123.26	170.11	59.07	61.75
10	55.23	0	0	0.44	0.41	235.11	247.00	79.26	78.41
11	0	-4.77	0	0.51	0.49	231.22	222.33	83.12	84.48
12	0	55.23	0	0.37	0.37	227.70	194.78	71.39	71.86
13	0	0	-3.18	0.41	0.42	246.22	230.81	78.79	79.58
14	0	0	36.82	0.37	0.34	206.59	186.30	67.74	68.79
15[a]	0	0	0	0.41	0.36	235.30	208.56	75.69	71.20
16[a]	0	0	0	0.34	0.36	198.07	208.56	68.28	71.20
17[a]	0	0	0	0.32	0.36	206.04	208.56	70.19	71.20
18[a]	0	0	0	0.34	0.36	196.78	208.56	68.04	71.20
19[a]	0	0	0	0.38	0.36	222.52	208.56	74.55	71.20
20[a]	0	0	0	0.33	0.36	192.15	208.56	70.79	71.20

SO: Standard order, [a]Centre point, TPC: Total phenolic content, FRAP: Ferric reducing ability of plasma, DPPH: 2,2-diphenyl-1-picrylhydrazyl, Y1: Response 1, Y2: Response 2, Y3: Response 3. TH: *Trigona* sp. honey, MH: *Apis mellifera* honey, DH: *Apis dorsata* honey, X1: 15–45 ratio, X2: 15–45 ratio, X3: 10-30 ratio.

Antioxidant capacities

Table II exhibits the TPC, FRAP and DPPH values of Trihoney combinations in this study. The honey samples showed TPC levels ranging from 0.26 to 0.50 mg/GAE/g. Notably, Trihoney 1 demonstrated the highest TPC in the 2nd run (0.50 mg/GAE/g), while the lowest TPC was observed in the 9th run (0.26 mg/GAE/g) with the TH-4.77:MH30:DH20 ratio. The FRAP values ranged from 166.79 to 246.22 AAE/g in this study, with the TH30:MH30:DH-3.18 ratio demonstrating the highest FRAP values at the 13th run. For DPPH, radical scavenging activity (RSA) ranged from 66.29 to 86.32% with Trihoney 1 also demonstrating the highest RSA in the 2nd run. Trihoney 1 is further compared with the reported antioxidant activities from the previous studies (Table III).

Table III. Comparison of antioxidant activities of Trihoney with previous studies

Sample	TPC	FRAP	DPPH Inhibition
Trihoney 1	0.50 mg GAE/g	230.85 AAE/g	86.32%
Apis sp.[18]	0.35 ± 0.81 mg GAE/g	576.91 ± 0.64 μM Fe (II)	59.89%
Trigona sp.[6]	0.60 ± 2.20 mg GAE/g	713.8 ± 20.10 μM Fe(II)	NA
Trigona carbonaria[19]	0.55 ± 6.11 mg GAE/g	NA	48.03%
Algerian honey[12]	0.46 ± 1.92 mg GAE/g	337.77 ± 1.01 μM Fe(II)	44.57%

NA=Not available

Physicochemical analysis

Table IV presents the physicochemical properties of various Trihoney formulation and individual honey samples. The recorded pH for all samples ranged from pH 2.45 to 2.99, but Trihoney samples contained lower pH than MH and DH. The moisture content was in the range of 23.00±0.00 - 30.24±0.20 g/100g, with Trihoney 1, Trihoney 2, and TH exhibiting the highest moisture content, which exceeded the maximum of 21g/100g recommended by The International Honey Commission. The moisture content was also significantly different between the honey samples. Water activity ranged from 0.69±0.00 to 0.71±0.00.

For ash content, no significant differences were found among the six honey samples. TH exhibited the highest value, followed by Trihoney 1 and Trihoney 2. All honey

Table IV. The physicochemical analysis of honey samples

Parameter	TH	MH	DH	Tri-honey1	Tri-honey2	Tri-honey 3	Mean
pH	2.47± 0.01	2.99± 0.01	2.87± 0.00	2.45± 0.03	2.47± 0.03	2.48± 0.01	2.62± 0.22
Moisture, g/100g	30.24± 0.20[d]	23.30± 0.00[a]	23.00± 0.00[a]	26.68± 0.04[c]	27.61± 0.07[b]	25.40± 0.00[e]	26.03± 2.58
Water activity, a_w	0.71± 0.00[d]	0.73± 0.00[a]	0.69± 0.00[c]	0.71± 0.00[b]	0.71± 0.00[e]	0.71± 0.00[d]	0.71± 0.01
Ash, g/100g	0.24± 0.01[a]	0.02± 0.00[e]	0.02± 0.00[e]	0.13± 0.00[b]	0.11± 0.00[c]	0.08± 0.00[d]	0.10± 0.07
Protein, g/100g	0.49± 0.08[a]	0.29± 0.00[bc]	0.33± 0.01[b]	0.27± 0.00[bc]	0.32± 0.00[b]	0.24± 0.02[c]	0.32± 0.08
Carbohydrate, g/100g	68.31± 0.16	75.64± 0.00	75.94± 0.00	72.18± 0.02	71.22± 0.04	73.55± 0.01	72.81± 2.70
Calorie, g/100g	275.21± 0.45	303.76± 0.01	305.11± 0.01	289.86± 0.11	286.22± 0.15	295.21± 0.00	292.56± 10.61
Fat, g/100g	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Crude fiber, g/100g	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

In each row, values with different letters (superscripts) indicate significant differences ($p < 0.05$). Data presented are mean ± SD. TH: Trigona sp. honey, MH: *Apis mellifera* honey, DH: *Apis dorsata* honey

samples analysed in this study complied with the limit value recommended by The International Honey Commission (≤0.6g/100g). For protein content, no significant differences were found among the six honey samples. The values ranged from 0.24 to 0.49 g/100g, with Trihoney formulations displaying protein contents between 0.24 and 0.32 g/100g.

Total carbohydrate content was also analysed and the result ranged from 68.31±0.16 to 75.94±0.00 g/100g, meeting the standard requirement of at least 60.0 g/100g. Notably, within the Trihoney formulation, Trihoney 3 demonstrated the highest carbohydrate content, whereas, among the individual honey samples, DH exhibited the highest. The honey samples also exhibited sufficient energy content with a mean value of calorie content of 292.56±10.61 g/100g. There was negligible values for both crude fibre and fat content.

As shown in Figure 1, significant differences were found in fructose and glucose contents (p<0.05). Maltose, however, was not detected in all samples. The sucrose content varied from 14.00±0.00 to 20.50±0.02 with Trihoney 3 depicting the highest value but in DH and MH, sucrose was not present. Trihoney 3 also produced the highest sum of fructose and glucose compared to other samples, while fructose content was detected to be higher in MH and DH samples.
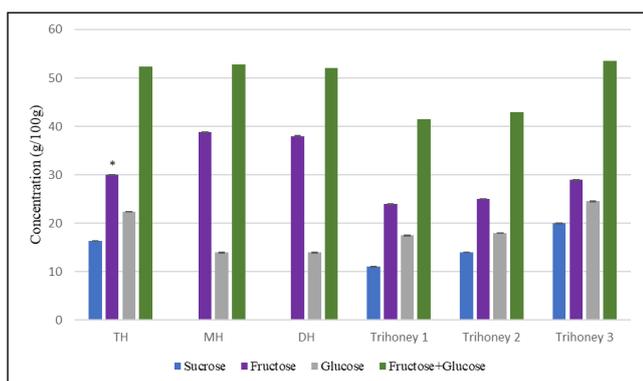
Figure 1: Reducing sugar contents of honey samples. Data are presented in mean±SD. TH=*Trigona* sp. honey; MH=*A. mellifera* honey=MH; DH=*A. dorsata* honey. *One-Way ANOVA showed significant differences in fructose and glucose contents between groups ($p < 0.05$).

Sensory evaluation

Other than antioxidant and physicochemical properties, the quality of honey also depends on its sensory attributes. The results of the sensory evaluation conducted in this study revealed no significant differences among the six honey samples concerning colour, sweetness, sourness, odour, flavour, aftertaste, and overall acceptability (Figure 2). All Trihoney formulations received overall acceptability scores below 5 with Trihoney 3 producing the highest overall acceptability score. Overall, compared to all other samples, MH not only exhibited the highest overall acceptability score but also secured the top rating for each attribute.
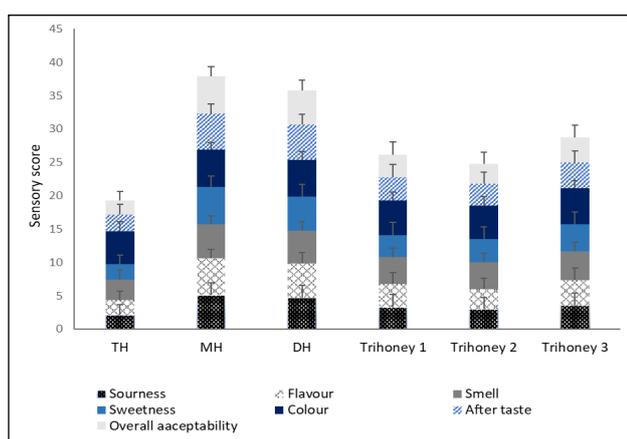

Figure 2. Sensory evaluation of honey samples. Data are presented in mean ± SD

DISCUSSION

As shown in Table III, the TPC level in Trihoney 1 surpassed the findings from prior studies on Algerian honey (0.46 ± 1.92 mg GAE/g) and *Apis* sp. honey (0.35 ± 0.81 mg/g).[12,18] Trihoney 1 also exhibited the highest

antioxidant activity in FRAP and DPPH assays among the samples, suggesting positive correlations between TPC and antioxidant capacity.[20] RSA values exhibited by the Trihoney 1 in this study exceeded those reported for Algerian honey (44.55%), Indian honey (57.5%), and *Apis* sp. honey (59.89%).[12,18] The elevated DPPH and FRAP values observed in Trihoney 1 can be attributed to the higher concentrations of *Trigona* sp. honey, known for its increased content of polyphenol compounds compared to other types of honey.[21] This aligns with the finding by Ismail et al. which revealed superior TPC, TFC, DPPH, and FRAP values of *Trigona* sp. compared to *Apis* sp.[6]

Generally, the pH of the samples reported in this study was lower compared to other Malaysian honey types, such as Tualang, Rubber, Acacia, and Kelulut, which generally exhibit pH values ranging from 3.1 to 4.3.[22] It was observed that Trihoney 1 was more acidic than the rest of the samples, primarily attributed to the inclusion of *Trigona* sp. honey in the formulations. *Trigona* sp. honey is distinguished from other honey types by having a lower pH (between 2.9 and 3.3), contributing to its distinct sour taste.[6]

Normally, honey exhibits an average water activity ranging between 0.5 to 0.65 a_w.[23] However, the water activity measured in this study was slightly higher. These findings align with data reported by Oddo et al. which indicated comparable water activity levels observed in Kelulut honey (0.76 a_w), as well as *Trigona* sp. honey from Australia (0.74 ± 0.01 a_w).[19] The elevated water activity and moisture content in the Trihoney blend were due to the high ratio of *Trigona* sp. honey in the formulations. Further optimization is essential to achieve lower water and moisture contents, crucial for enhancing shelf life and preventing fermentation during storage.

Typically, honey may contain a small amount of protein, ranging from 2 to 5 g/kg.[14] According to Moniruzzaman et al., Malaysian honey has been reported to have protein levels ranging from 2.04 to 4.83 g/kg.[18] A comparison with the findings of Ismail et al. revealed that the protein levels in *Apis* and *Trigona* honey were lower, falling within the range of 0.027 to 0.11 g/100g.[6] Anuar et al. reported a significantly high carbohydrate content (80%) in the

proximate analysis of *Trigona* sp. honey.[9] The carbohydrate content in honey is strongly correlated with energy and this assertion aligns with the observed gross energy values of Trihoney 3 and DH, where these samples, characterized by high carbohydrate content, also exhibited the highest gross energy levels. In the previous study, Buba et al. reported the average energy values for different honey types ranged from 303 kcal/100g to 337.37 ± 5.84 kcal/100g.[16]

The results for fibre and fat align with previous research findings by Kek et al. and Chua and Adnan which also reported the absence of dietary fibre and fat in honey samples.[21,24] The higher fructose levels observed in all honey samples in this study suggest the high quality of the samples. A higher fructose content contributes to sweetness, delays crystallization, and enhances the overall quality of honey.[25]

CONCLUSION

The optimized Trihoney formulation demonstrated superior antioxidant potential compared to individual honey samples, specifically for Trihoney 1. It is evident by its higher TPC, FRAP, and DPPH results and notable polyphenol levels such as flavonoids and phenolic acids. The findings offered compelling evidence that Trihoney formulations meet high-quality standards, supported by acceptable sensory scores and physicochemical parameters aligning with International Honey Commission specifications. Notably, the research highlighted that Trihoney formulation provided ample energy despite their low sugar content, making them a potentially beneficial food supplement for diabetic patients. Future studies on Trihoney formulation could explore their effects through animal studies by investigating its therapeutic potential in treating specific conditions or diseases in animal models. This could include examining its efficacy in wound healing, its anti-inflammatory effects, or its potential as an antihyperlipidemic.

CONFLICT OF INTEREST
The authors report no conflict of interest.

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