

## Optimisation of phenolic compounds and antioxidant capacity of *Trigona* honey and propolis using response surface methodology from fermented food products

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### Abstract

Honey and propolis are honeybee products that are becoming increasingly common as a result of their ability to improve human health. The optimal combination of honey and propolis for total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacity were analysed for *Trigona* honey and propolis aqueous extracts using response surface methodology and a central composite design. The effect of honey (X1: 15 - 16.5 g) and propolis (X2: 13.5 - 15 g) on the total phenolic content (TPC, Y1), total flavonoid content (TFC, Y2), antioxidant capacity (DPPH, Y3; ABTS, Y4), and FRAP (Y5) were tested. The experimental outcomes were adequately fitted into a second-order polynomial model regarding TPC ( $R^2 = 0.9539$ ,  $p = 0.0002$ ), TFC ( $R^2 = 0.9209$ ,  $p = 0.0010$ ), antioxidant activity (DPPH,  $R^2 = 0.9529$ ,  $p = 0.0002$ ; ABTS,  $R^2 = 0.9817$ ,  $p < 0.0001$ ), and FRAP ( $R^2 = 0.9363$ ,  $p = 0.0005$ ). The optimal percentage compositions of honey and propolis were 15.26 g (50.43%) and 15 g (49.57%), respectively. The predicted results for TPC, TFC, DPPH (IC<sub>50</sub>), ABTS, and FRAP were 162.46 mg GAE/100 g, 2.29 mgQE/g, 14.52 mg/mL, 564.27 μMTE/g, and 3.56 mMTE/g, respectively. The experimental outcomes were close to the predicted results:  $152.06 \pm 0.55$  mg GAE/100 g,  $2.21 \pm 0.05$  mg QE/g,  $13.85 \pm 0.34$  mg/mL,  $555.22 \pm 36.84$  μMTE/g, and  $3.71 \pm 0.02$  mMTE/g, respectively. It was observed that the optimal combination of honey and propolis provided the highest antioxidant yield and can be used as functional foods, cosmetics, and medical and pharmacological ingredients.

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### Keywords

phenolic content,  
flavonoid content,  
antioxidant,  
*Trigona propolis*,  
*Trigona honey*,  
response surface  
methodology

## Introduction

Natural products have been evaluated for their antioxidant activities. Propolis and honey contain high phenolic compounds that serve as natural antioxidants and support human health (Mouhoubi-Tafinine *et al.*, 2016). Honey is a natural, sweet, and viscous fluid that has been identified to contain about 200 components. Honey and propolis compositions, particularly their secondary metabolites, largely depend on their floral and botanical origins, in addition to the environmental factors, seasons, weather, and processing procedures (Anjum *et al.*, 2019).

Honey is commonly linked with high antioxidant activity due to its polyphenols, such as phenolic acids (cinnamic, coumaric, chlorogenic,

caffeic, ferulic, vanillic, ellagic, and benzoic acids) and flavonoids (kaempferol, pinocembrin, myricetin, apigenin, pinobanksin, hesperidin, luteolin, chrysin, galangin, and quercetin) (Nayik and Nanda, 2016; Biluca *et al.*, 2017). Stingless bee honey (*kelulut* honey) is assumed to have higher polyphenol content than other types of honey (Biluca *et al.*, 2016). For decades, honey has been utilised for its nutritious value as well as functional properties. For instance, it has therapeutic properties that can be used in the treatment of many diseases. It can be used alone or combined with additional ingredients, orally or topically, to manage or prevent certain diseases (Jaganathan *et al.*, 2014).

Propolis is a gummy material produced by bees from the nectar of buds and plant exudates, wax, and bee enzymes. It consists of 50% resins, 30%

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vegetable balms and beeswax, 10% essential oils, 5% pollen, and 5% other components (Oryan *et al.*, 2018). There are over 300 identified compounds in propolis (Anjum *et al.*, 2019). Malaysian propolis (stingless bee *Trigona* propolis) is a promising source of natural antioxidants due to its high polyphenols, ascorbic acid, flavonoids, tannins, and low sugar content. Many studies on the biological characteristics of Malaysian propolis have shown that it can be applied in wound healing (Jacob *et al.*, 2015), antioxidants, and cardio protection (Ahmed *et al.*, 2017).

For centuries, propolis and honey have been evaluated as therapeutic agents due to their functional activities (Cianciosi *et al.*, 2018; Olas, 2020; Santos *et al.*, 2020). Honey is a natural product that has been used for medicinal purposes since ancient times due to the remarkable antioxidant, anti-inflammatory, antimicrobial, and anticancer properties of its flavonoids and phenolic acids, which play an important role in human health (Cianciosi *et al.*, 2018). However, the phenolic contents in propolis are higher and show significantly higher antioxidant activities than that of honey (Mouhoubi-Tafinine *et al.*, 2016). Currently, propolis is used in many applications in biopharmaceuticals as a natural preservative, in cosmetic products, as a candy ingredient, and as a source of bioactive compounds in beverages and foods (Duman and Özpolat, 2015; Osés *et al.*, 2016; Pobjega *et al.*, 2019).

Nowadays, there is increasing interest in developing new food products with useful characteristics. Nevertheless, the scientific information about the probable advantages of including propolis in food products is still limited (Osés *et al.*, 2016). Mixtures of honey and propolis have demonstrated some improvements in the overall phenolic and flavonoid contents, antioxidant activities, and anti-inflammatory activities. A synergistic antimicrobial effect of honey and propolis combination has been reported (Juszczak *et al.*, 2016; Osés *et al.*, 2016).

Response surface methodology (RSM) is a useful technique for designing experiments and optimising various environmental processes (Karimifard and Alavi Moghaddam, 2018). Generally, the conventional technique for food product formulation is time-consuming and laborious (Quispe-Fuentes *et al.*, 2017). Though RSM has been used to optimise phenolic extraction from various natural products (Mat Alewi *et al.*, 2020; Pratami *et al.*, 2020), studies on the optimal mixture of Malaysian *Trigona* honey and propolis that produces

high polyphenolic and flavonoid contents, as well as antioxidant activities, remain limited. Therefore, the present work aimed to apply the RSM technique for optimising the Malaysian *Trigona* honey and propolis combination to maximise the yield of total phenolic content, total flavonoid content, and antioxidant capacities such as DPPH, ABTS, and FRAP.

## Materials and methods

### *Honey and propolis samples*

*Trigona* propolis and honey samples were collected from Kota Bharu, Kelantan, Malaysia. The honey was kept in a dry container at room temperature, while the propolis was frozen at -20°C before extraction.

### *Preparation and extraction of propolis*

The extraction of propolis was performed following Trusheva *et al.* (2007) with slight modification. Briefly, the propolis was washed, cut, and ground. Then, 5 g of ground propolis was extracted in 50 mL of distilled water (1:10 w/v) at 43.75°C and 52.85 h. The aqueous extracts were separated from the sediment via centrifugation at 1,500 g for 5 min. Fresh distilled water (10 mL) was used to wash the sediment twice, followed by centrifugation and addition to the initial supernatant. A rotary evaporator (IKA, RV10 control) was used to concentrate the extract at pressure (72), 60°C, and 50 rpm. Then, the soft extract of the propolis was kept at 4°C (Margretha *et al.*, 2012).

### *Chemicals and solvents*

Analytical grade chemicals were used. Sodium carbonate, Folin-Ciocalteu, iron (III) chloride hexahydrate, aluminium chloride (AlCl<sub>3</sub>), ethanol, and methanol were purchased from Merck (Darmstadt, Germany). Meanwhile, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), and HCl were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium acetate trihydrate was purchased from Fisher Scientific (Loughborough, UK). Glacial acetic acid was purchased from MP Biomedicals, LLC (Parc d'innovation, Illkirch, France). Tetramethyl-chromane-2-carboxylic acid (Trolox) and potassium persulfate were purchased from Acros (New Jersey, USA). Gallic acid was purchased from Merck (Darmstadt, Germany). Quercetin was purchased from Nacalai-Tesque (Kyoto, Japan).

### Experimental design

The central composite design of the RSM was utilised to determine the optimal levels of *Trigona* propolis and honey mixture according to five dependent variables, namely total phenolic content (TPC), total flavonoid content (TFC), DPPH, ABTS, and FRAP. The two independent variables, *Trigona* honey (X1: 14.69 - 16.81 g) and *Trigona* propolis (X2: 13.19 - 15.31 g) were coded into five levels (-1.414, -1, 0, 1, and 1.414), from the lowest to the highest. The dependent variables used to detect the impact of both independent variables (honey and propolis mixtures) were TPC, TFC, DPPH, FRAP, and ABTS. Thirteen experiments were investigated in terms of their responses. The coded and non-coded independent variables used in the RSM design are shown in Table 1.

### Estimation of total phenolic content

The TPC in various mixtures of *Trigona* honey and propolis were determined based on previous studies (Meda *et al.*, 2005; Alvarez-Suarez *et al.*, 2010; Socha *et al.*, 2017) with slight modification. Briefly, each sample (1 g) was diluted with 10 mL of distilled water and filtered by Whatman No. 1 paper. Then, 20  $\mu$ L of the solution was added to every well of the plate, and mixed with 100  $\mu$ L of 0.2 N Folin-Ciocalteu reagent for 5 min. Then, 80  $\mu$ L of 7.5% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added, and then the mixture incubated for 2 h at room temperature. The absorbance was measured at 760 nm against a distilled water blank using a microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria). A calibration curve was plotted using the gallic acid standard (80 to 200  $\mu$ g/mL,  $R^2 = 0.9904$ ). The mean and standard deviation ( $\pm$  SD) of the three replicates ( $n = 3$ ) were obtained, and expressed as mg gallic acid equivalents per 100 g (mg GAE/100 g of honey and propolis mixture sample).

### Estimation of total flavonoid content

A colorimetric aluminium chloride method was carried out to evaluate the TFC in various mixtures of *Trigona* honey and propolis, as reported by Meda *et al.* (2005) and Ahmed *et al.* (2015), with

slight modification. Briefly, 100  $\mu$ L of 2% aluminium trichloride ( $\text{AlCl}_3$ ) was mixed with 100  $\mu$ L of *Trigona* honey and propolis mixtures (0.02 mg/mL). After 10 min, the absorbance was measured at 415 nm (Nano Quant Infinite M 200, Tecan, Grodig, Austria) against a blank sample containing 100  $\mu$ L of honey and propolis solution, and 100  $\mu$ L distilled water without  $\text{AlCl}_3$ . The blank readings were deducted from the samples. A standard calibration curve with quercetin (20 - 80  $\mu$ g/mL,  $R^2 = 0.9938$ ) was used. The mean and standard deviation ( $\pm$  SD) of the three replicates ( $n = 3$ ) were obtained, and expressed as mg of quercetin equivalents (mg QE/g) of honey and propolis mixture.

### DPPH free radical scavenging assay

A DPPH free radical scavenging activity assay was conducted according to Socha *et al.* (2017), with modifications using 96-well microplates. Distilled water (10 mL) was used to dissolve 1 g of *Trigona* propolis and honey mixture samples, which were centrifuged at 4,350 g, and filtered through Whatman No. 1 paper. Then, aliquot (50  $\mu$ L) of the mixture (100 mg/mL), blank, standard, and 150  $\mu$ L of 100  $\mu$ M methanol solution of DPPH (3.94 mg in 100 mL absolute methanol) were added to the microplate wells, and incubated in the dark for 60 min. The absorbance was recorded at 517 nm using a microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria). A distilled water blank was used, and Trolox was used as a positive control. The DPPH discoloration percentage was used to calculate the DPPH scavenging, using Eq. 1 (Sakanaka *et al.*, 2005):

$$\% \text{Inhibition} = \left[ \frac{(C-CB)-(S-SB)}{C-CB} \right] \times 100 \quad (\text{Eq. 1})$$

where, S = sample absorbance (sample extracts + DPPH solution), SB = sample blank (sample extracts + methanol), C = control (solvent extract + DPPH solution), and CB = control blank (solvent extract + methanol).

The sample concentrations required to scavenge 50% of DPPH were also determined. The mean and standard deviation ( $\pm$  SD) of the three

Table 1. Coded and uncoded levels of independent variables used in the RSM design.

Independent variables coded levels		-1.414	-1	0	1	1.414
X1	<i>Trigona</i> honey	14.69	15	15.75	16.5	16.81
X2	<i>Trigona</i> propolis	13.19	13.5	14.25	15	15.31

replicates ( $n = 3$ ) were obtained, and expressed as  $IC_{50}$  mg/mL.

#### *ABTS free radical scavenging assay*

The antioxidant capacity of various mixtures of *Trigona* propolis and honey samples was determined using an ABTS assay, according to Hatano *et al.* (2012), with slight modifications. Briefly, distilled water (10 mL) was used to dissolve 1 g of propolis and honey mixtures, which were centrifuged at 4,350 g, and filtered through Whatman No. 1 paper. Then, an ABTS radical stock solution was prepared by reacting 2.4 mM potassium persulfate solution with 7 mM ABTS solution in equal amounts; this was left in the dark at room temperature for 16 h. The solution was then diluted by mixing 1 mL of ABTS working solution with 25 mL of ethanol to get an absorbance of  $0.706 \pm 0.01$  at 734 nm using a microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria). Aliquots (20  $\mu$ L) of various mixtures of propolis and honey samples or blank, and Trolox were mixed with 180  $\mu$ L of ABTS radical solution in a microplate, and incubated in the dark at room temperature. The absorbance was recorded after 5 min at 734 nm. The ABTS was determined using EQ. 2:

$$\text{ABTS radical scavenging activity (\%)} = \frac{(\text{A blank} - \text{A sample})}{\text{A blank}} \times 100 \quad (\text{Eq. 2})$$

where, blank = mixture of distilled water and ABTS+ solution, and sample = mixture of the sample extract and ABTS+ solution.

Trolox standard was used (20 to 100  $\mu$ M/mL,  $R^2 = 0.9902$ ). The mean and standard deviation ( $\pm$  SD) of the three replicates ( $n = 3$ ) were obtained, and expressed as  $\mu$ mol Trolox equivalent per gram of sample weight ( $\mu$ M TE/g).

#### *Ferric reducing antioxidant power (FRAP) assay*

A FRAP assay was also performed to assess the antioxidant activities in the *Trigona* honey and propolis mixtures using a method adapted from Alvarez-Suarez *et al.* (2010), with slight modification. Briefly, the FRAP solution (10:1:1) was prepared from a mixture of 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 20 mM  $FeCl_3 \cdot 6H_2O$ , 2.5 mL of 10 mM TPTZ, and incubated at 37°C in the dark. Then, aliquots (20  $\mu$ L) of honey and propolis mixtures were added to 180  $\mu$ L of FRAP reagent, and the plate was incubated at 37°C for 10 min. A microplate reader was used to read the absorbance at 593 nm against a distilled water blank.

A Trolox calibration curve (100 - 500  $\mu$ M Trolox/mL,  $R^2 = 0.9915$ ) was used. The mean and standard deviation ( $\pm$  SD) of the three replicates ( $n = 3$ ) were obtained, and expressed as mM Trolox equivalent (mM TE/g sample weight).

#### *Statistical analysis*

The Design-Expert Version 6.0.10 software was used for the statistical analysis. The results for TPC, TFC, DPPH, ABTS, and FRAP were reported as mean  $\pm$  standard deviation. The validation of the regression coefficients and the model statistical significance was done by employing response surface analysis. Furthermore, it was utilised to match statistical models of the experimental data for optimising the response variables. A second-order polynomial model was carried out to fit the data, as shown in Eq. 3:

$$Y = b_0 + b_1 X_1 + b_2 X_2 + b_{12} X_1 X_2 + b_{22} X_2^2 + b_{11} X_1^2 \quad (\text{Eq. 3})$$

where, the expected response = Y,  $b_0$  = constant, linear influence regression coefficients =  $b_1$ ,  $b_2$ ; quadratic impact =  $b_1^2$  and  $b_2^2$ , and interaction influences =  $b_1 b_2$ , respectively. The model quality was predicted by the ANOVA analysis ( $p < 0.05$ ) and regression analysis ( $R^2$ ). From the ANOVA analysis, only the significant coefficients were included. Meanwhile, the non-significant coefficients were omitted from the initial model. The relationship between the factors ( $X_1$  and  $X_2$ ) and the dependent variables ( $Y_1$ ,  $Y_2$ ,  $Y_3$ ,  $Y_4$ , and  $Y_5$ ) was illustrated by a three-dimensional model graph. The desired aim was set in numerical optimisation to generate the optimal conditions and point predicted outcomes of the responses.

#### *Model verification*

The TPC, TFC, DPPH, ABTS, and FRAP experimental values were detected as per the optimal combination predicted through the software. To validate the model's validity, the experimental results found from the independent samples in the optimal combination were matched with the expected results from the optimised model.

## **Results and discussion**

#### *Fitting the model*

The experimental data of TPC ( $Y_1$ ), TFC ( $Y_2$ ), and antioxidant capacities [DPPH• scavenging activity ( $Y_3$ ), ABTS•+ inhibition ability ( $Y_4$ ), and

Table 2. Experimental design and responses of the dependent variables to mixture.

Standard order	Mixture parameter			Response									
	Honey (g)	Propolis (g)	TPC (mgGAE/100 g) Y1	TFC (mgQE/g) Y2		DPPH (IC <sub>50</sub> mg/mL) Y3		ABTS (µMTE/g) Y4		FRAP (mMTE/g) Y5			
			Expt.	Pred.	Expt.	Pred.	Expt.	Pred.	Expt.	Pred.	Expt.	Pred.	
1	-1	-1	144.01	144.03	1.87	1.73	18.47	19.78	476.92	468.45	3.21	3.11	
2	1	-1	142.68	143.78	1.80	1.80	21.01	21.42	400.34	412.24	3.25	3.19	
3	-1	1	161.97	159.56	2.30	2.29	14.15	15.26	552.92	543.51	3.59	3.53	
4	1	1	150.52	149.19	1.82	1.87	23.38	23.59	411.66	422.62	2.98	2.96	
5	-1.414	0	145.54	146.96	1.99	2.03	20.76	19.35	453.49	466.65	3.15	3.24	
6	1.414	0	139.56	139.45	1.82	1.78	26.53	26.40	341.43	338.71	2.86	2.89	
7	0	-1.414	148.74	147.67	1.69	1.72	18.88	17.98	491.08	489.16	3.17	3.26	
8	0	1.414	160.11	162.48	2.19	2.16	16.94	16.32	550.15	549.57	3.37	3.40	
9 <sup>a</sup>	0	0	161.83	159.70	2.21	2.16	14.68	15.25	561.74	550.35	3.55	3.50	
10 <sup>a</sup>	0	0	156.09	159.70	2.02	2.16	16.19	15.25	545.71	550.35	3.52	3.50	
11 <sup>a</sup>	0	0	160.89	159.70	2.23	2.16	14.98	15.25	551.63	550.35	3.43	3.50	
12 <sup>a</sup>	0	0	159	159.70	2.12	2.16	14.68	15.25	541.53	550.35	3.49	3.50	
13 <sup>a</sup>	0	0	160.70	159.70	2.22	2.16	15.72	15.25	551.12	550.35	3.50	3.50	

<sup>a</sup> Centre point. Y1 (TPC): total phenolic content; Y2 (TFC): total flavonoid content; Y3 (DPPH): 2, 2-diphenyl-1-picrylhydrazyl radical scavenging ability; Y4 (ABTS): 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation inhibition; Y5 (FRAP): ferric reducing antioxidant power; GAE: gallic acid equivalent; QE: quercetin equivalent; TE: Trolox equivalent; Exp.: experimental value; and Pred.: predicted value.

Table 3. Polynomial equations and statistical parameters calculated following implementation of two-factor central composite experimental design.

Regression coefficient	Polynomial equation	R <sup>2</sup>	R <sup>2</sup> (Adjusted)	Regression (p-value)	Lack of fit
TPC (Y1)	+159.70 - 2.66 X <sub>1</sub> + 5.24X <sub>2</sub> - 8.25 X <sub>1</sub> <sup>2</sup> - 2.31X <sub>2</sub> <sup>2</sup>	0.9539	0.9209	0.0002	0.4321
TFC (Y2)	+2.16 - 0.088 X <sub>1</sub> + 0.16 X <sub>2</sub> - 0.13 X <sub>1</sub> <sup>2</sup> - 0.11X <sub>2</sub> <sup>2</sup> - 0.12 X <sub>1</sub> X <sub>2</sub>	0.9209	0.8643	0.0010	0.7845
DPPH (Y3)	+15.25 + 2.49 X <sub>1</sub> + 3.81X <sub>2</sub> <sup>2</sup> + 1.67 X <sub>1</sub> X <sub>2</sub>	0.9529	0.9193	0.0002	0.0859
ABTS (Y4)	+550.35 - 44.27 X <sub>1</sub> + 21.36 X <sub>2</sub> - 73.15 X <sub>1</sub> <sup>2</sup> - 15.49X <sub>2</sub> <sup>2</sup> - 16.17 X <sub>1</sub> X <sub>2</sub>	0.9817	0.9687	< 0.0001	0.0801
FRAP (Y5)	+3.50 - 0.12 X <sub>1</sub> - 0.22X <sub>1</sub> <sup>2</sup> - 0.085 X <sub>2</sub> <sup>2</sup> - 0.16 X <sub>1</sub> X <sub>2</sub>	0.9363	0.8907	0.0005	0.0566

TPC (Y1): total phenolic content; TFC (Y2): total flavonoid content; DPPH (Y3): 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability; ABTS (Y4): 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation inhibition; and FRAP (Y5): ferric reducing antioxidant power.

FRAP (Y5)] were used in the multiple regression analysis by applying response surface analysis for fitting the second-order polynomial equations. There was consistency between the experimental findings and the predicted outcomes, thus indicating an adequate model (Table 2). The probability values ( $p$ ), regression coefficients ( $R^2$ ), adjusted  $R^2$  values, and lack-of-fit values for all the response variables are shown in Table 3. The fit quality for the second-order polynomial models was defined based on the determination coefficients ( $R^2$ ), which were 0.9539, 0.9209, 0.9529, 0.9817, and 0.9363, for TPC (Y1), TFC (Y2), and antioxidant capacities (DPPH, ABTS, and FRAP), respectively. It was indicated that about 92 to 98% of the differences were determined through the model. The model fitness for the responses was confirmed by the lack-of-fit test, which was insignificant ( $p > 0.05$ ).

#### *Effect of combination parameters on total phenolic contents, total flavonoid contents, and antioxidant capacity*

The influence of a combination of factors (X1 and X2) on TPC (Y1), TFC (Y2), and antioxidant capacities [DPPH• scavenging activity (Y3), ABTS•+ inhibition ability (Y4), and FRAP (Y5)] responses was stated by the significant coefficient ( $p < 0.05$ ) of the second-order polynomial regression equation.

For TPC (Y1), the impact of honey and propolis was significant ( $p < 0.05$ ) in first-order linear influence (X1, X2), second-order quadratic impact (X1<sup>2</sup>, X2<sup>2</sup>), and with no interaction influence (X1X2) on TPC (Y1), with a regression coefficient ( $R^2$ ) of 0.9539. The predicted model found for Y1 is provided in Table 3. Though both honey and propolis affected the TPC, the propolis was the most significant factor contributing to the TPC yield of the combination, as shown in the polynomial equations for (Y1) in Table 3.

A significant ( $p < 0.05$ ) difference was obtained for TFC (Y2) and ABTS scavenging activity (Y4), while the influence of honey and propolis had a first-order linear effect (X1, X2), second-order quadratic effect (X1<sup>2</sup>, X2<sup>2</sup>), and interaction effect (X1X2), with regression coefficients ( $R^2$ ) of 0.9209 and 0.9817, respectively. The predicted models found for Y2 and Y4 are shown in Table 3. Both honey and propolis affected the TFC and ABTS scavenging activity.

However, for DPPH scavenging capacity (Y3), the impact of honey and propolis was significant ( $p < 0.05$ ) in the first-order linear effect (X1), second-order quadratic effect (X1<sup>2</sup>), and

interaction effect (X1X2), with a regression coefficient ( $R^2$ ) of 0.9529. The predicted model for Y3 is given in Table 3. Both honey and propolis affected the DPPH scavenging capacity.

For FRAP (Y5), the influence of honey and propolis was significant ( $p < 0.05$ ) in the first-order linear effect (X1), second-order quadratic effect (X1<sup>2</sup>, X2<sup>2</sup>), and interaction effect (X1X2), with a regression coefficient ( $R^2$ ) of 0.9529. The predicted model found for Y5 is given in Table 3. Both honey and propolis affected FRAP.

Figure 1A shows the 3D response surface with a linear increase and a quadratic influence of both honey and propolis on the TPC (Y1). A combination of a moderate amount of honey (15.63 g) and propolis (14.83 g) gave the maximum yield (163.44 mg GAE /100 g) of TPC. The TPC increased with the increase in honey, up to a certain point. However, a decrease in TPC was obtained with a further increase in the honey ratio. Meanwhile, with the propolis, the TPC increased with an increase in the propolis ratio, ranging between 143.78 and 163.44 mg GAE /100 g. The TPC also dropped with a further increase in the honey to propolis ratio.

Figures 1B and 1D show the 3D responses with a linear increase and a quadratic influence of both honey and propolis on the TFC and ABTS scavenging activity. Overall, a combination of honey (15.12 and 15.45 g, respectively) and propolis (15 and 14.92 g, respectively) gave a maximum yield of TFC and ABTS scavenging activity of 2.29 mgQE/g and 568.85  $\mu$ MTE/g, respectively. The TFC and ABTS scavenging activity increased with a slight increase in honey, up to a certain ratio. However, there was a drop in TFC and ABTS scavenging activity with a further increase in the honey ratio. Similar trends were observed with the propolis, in that the TFC and ABTS scavenging capacity increased with an increase in the propolis ratio, producing TFC and ABTS scavenging activity from 1.73 to 2.29 mg QE/g and 412.24 to 586.85  $\mu$ MTE/g, respectively. Subsequently, the values decreased with an additional increase in the honey to propolis ratio.

Figure 1C shows the 3D response with a linear increase and a quadratic impact of honey, and the interaction effect of both honey and propolis on DPPH• scavenging capacity. A combination of a certain amount of honey (15.38 g) and propolis (14.81 g) produced a maximum DPPH• scavenging capacity ( $IC_{50} = 14.43$  mg/mL). The DPPH• scavenging capacity increased with a slight increase in the amount of honey, up to a certain ratio. However, a decrease in DPPH• scavenging capacity

was observed with a further increase in the honey ratio. Similar trends were observed with the propolis, in that the DPPH• scavenging capacity increased with an increase in the propolis ratio, generating a DPPH• scavenging capacity ( $IC_{50}$  values ranging from 14.43 to 23.59 mg/mL). The scavenging capacity, however, decreased with a further increase in the honey to propolis ratio.

Figure 1E shows the 3D response with a linear increase and a quadratic influence of honey, as well as a quadratic effect of propolis and the interaction impact of both honey and propolis on

FRAP. Overall, a certain combination of honey (15.30 g) and propolis (14.90 g) gave a maximum FRAP value of 3.56 mMTE/g. There was an increase in FRAP values with a slight increase in honey, up to a certain ratio. However, a decrease in FRAP value was observed with a further increase in the honey ratio. Similarly, with the propolis, the FRAP value increased with the increase in the propolis ratio, producing FRAP values of 2.96 to 3.56 mMTE/g. Subsequently, FRAP decreased with a further increase in the honey to propolis ratio.

The findings in the present work are in

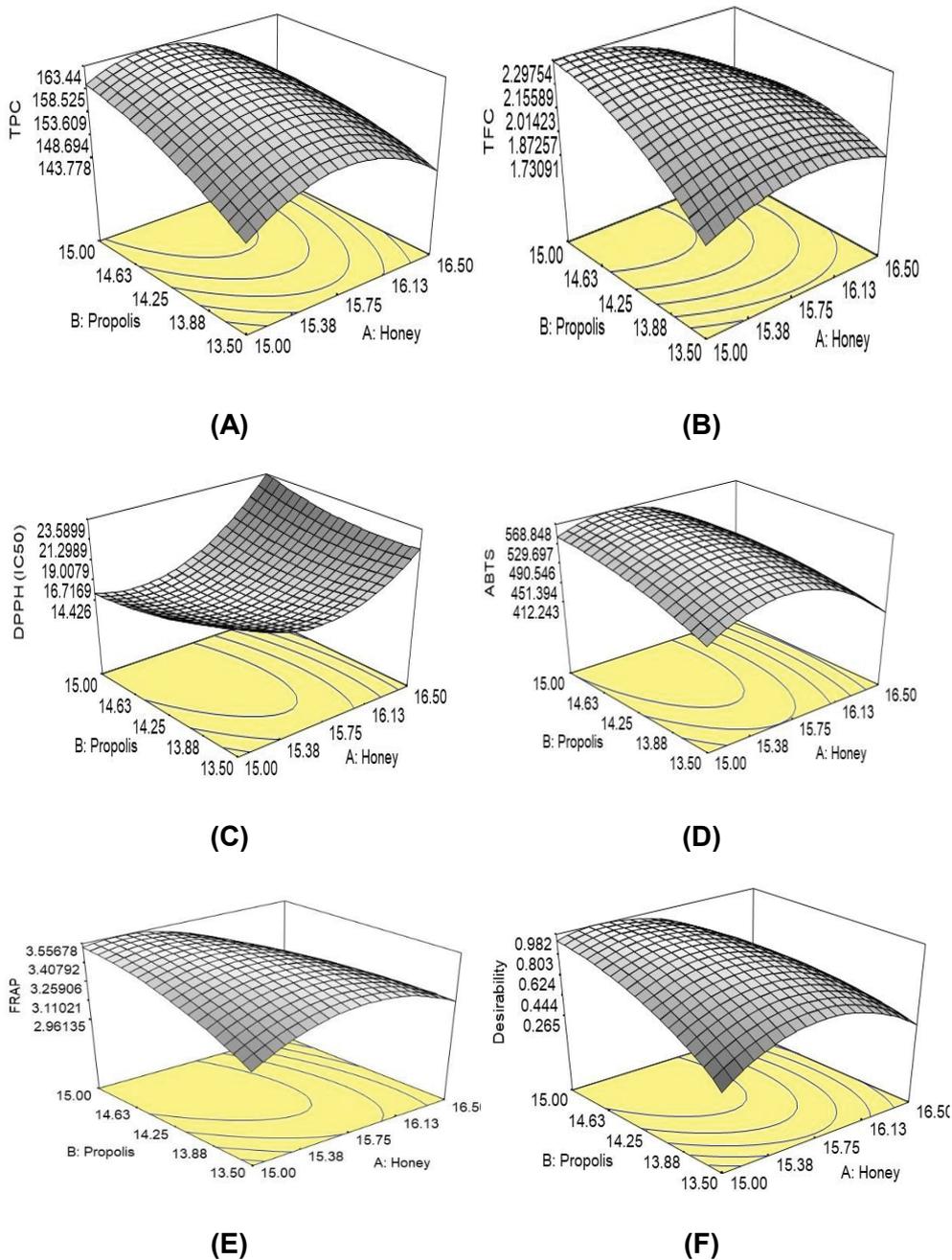


Figure 1. Response surface plot of *Trigona* honey and propolis mixture on (A) total phenolic content (mg GAE/100 g); (B) total flavonoid content (mg QE/g); (C) the DPPH ( $IC_{50}$  mg/mL); (D) ABTS ( $\mu$ M TE/g); (E) FRAP (mM TE/g); and (F) response surface plot of the desirability as a function of honey and propolis mixture.

Table 4. Predicted and experimental values of optimised honey and propolis composition.

Optimisation	Honey (g)	Propolis (g)	Predicted value	Experimental value	%Difference
Factors (optimised)	15.26 (50.43%)	15 (49.57)			
<b>Responses</b>					
TPC (mgGAE/100 g)			162.46	152.06 ± 0.55	6.04
TFC (mgQE/g)			2.29	2.21 ± 0.05	3.49
DPPH (IC <sub>50</sub> mg/ml)			14.52	13.85 ± 0.34	4.6
ABTS (µMTE/g)			564.27	555.22 ± 36.84	1.60
FRAP (mMTE/g)			3.56	3.71 ± 0.02	4.21

TPC: total phenolic content; TFC: total flavonoid content; DPPH: 2, 2-diphenyl-1-picrylhydrazyl radical scavenging ability; ABTS: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation inhibition; and FRAP: ferric reducing antioxidant power. Values are mean ± standard deviation of triplicate ( $n = 3$ ).

agreement with previously reported studies. An increase in the propolis ratio led to an increase in the TPC, TFC, and antioxidant capacity (Juszczak *et al.*, 2016; Osés *et al.*, 2016). This is because propolis contains higher phenolic content than honey, and thus, higher antioxidant activity (Banskota *et al.*, 2001; Meda *et al.*, 2005; Yildiz *et al.*, 2014; Sime *et al.*, 2015; Socha *et al.*, 2015).

Furthermore, *in vitro* and *in vivo* studies have also revealed that pure compounds give better results than those obtained from consuming raw materials (Rowland, 1999). The active compounds are more concentrated in the propolis extract than in the raw honey, which might increase the TPC and antioxidant capacity in the combination of propolis and honey. The results are consistent with previous study (Osés *et al.*, 2016). Soft extracts of propolis added to honey, even at a low concentration of 0.1%, can increase the antioxidant, anti-inflammatory, and antimicrobial activities of the base honey. If the propolis extracts are added at higher concentrations to honey, this improves the bioactive properties of honey. Honey enriched with extracts of propolis has shown an increase in TPC and antioxidant activity (Juszczak *et al.*, 2016; Osés *et al.*, 2016).

In terms of honey, it showed an increase in TPC, TFC, and antioxidant capacity, up to a certain ratio, which was then followed by a decrease. This might be due to the lower honey components than the propolis. Propolis contains higher phenolic content and antioxidant properties (Banskota *et al.*, 2001; Meda *et al.*, 2005; Yildiz *et al.*, 2014; Sime *et al.*, 2015; Socha *et al.*, 2015). The decrease in the TPC might be due to the increase in the raw honey ratio to propolis, which contains lower phenolic and flavonoid content than propolis extract. Therefore,

the ratio of honey to propolis is very important because a high ratio of honey with a low ratio of propolis produces a lower TPC, TFC, and antioxidant capacity. Furthermore, this inconsistency of *Trigona* honey concentrations, in terms of antioxidant activity may be due to the pro-oxidant activity, which could have occurred because of various factors (Mohd Nur Nasyriq *et al.*, 2019). Our findings are in line with previous study (Aissat *et al.*, 2015). Honey is a complex mixture of compounds that can act as a pro-oxidant or antioxidant when used in various concentrations. The *Trigona* honey used in the present work is tropical multiflora honey, which could act as peroxide-producing honey, and thus, might contribute to free radical formation (Erejuwa *et al.*, 2014). Furthermore, the *Trigona* honey used in the present work was collected from Kelantan. Thus, the transportation and handling factors could have exposed the honey to heat and air, which in turn might have activated hydrogen peroxide production (Aoshima and Ayabe, 2007). Similarly, the propolis extract was dissolved in water, which might have triggered the production of hydrogen peroxide produced by the action of glucose oxidase, thereby influencing the decrease in antioxidant capacity in the formulated combination (Henriques *et al.*, 2006).

#### *Optimisation of responses, and verification of model*

To obtain a combination of honey and propolis with high levels of TPC, TFC, and antioxidant activity, the optimal level of the overall desirability from the five responses was determined based on the influence of the honey and propolis ratio in the mixture. In numerical optimisation, the factors were applied in a range, whereas the responses were maximised, except for the DPPH, which was kept at

a minimum. The optimal criteria for the content of honey and propolis were 15.26 g (50.43%) and 15 g (49.57%), respectively, for the combination of honey and propolis (Figure 1F). The predicted values for TPC, TFC, DPPH (IC<sub>50</sub>), ABTS, and FRAP were 162.46 mg GAE/100 g, 2.29 mg QE/g, 14.52 DPPH (IC<sub>50</sub> mg/mL), 564.27 µMTE/g, and 3.56 mMTE/g, respectively. Meanwhile, the experimental values were 152.06 ± 0.55 mg GAE/100 g, 2.21 ± 0.05 mg QE/g, 13.85 ± 0.34 mg/mL, 555.22 ± 36.84 µMTE/g, and 3.71 ± 0.02 mMTE/g, respectively. The response surface model was verified by comparing the experimental and expected values. The experimental outcomes were close to the expected values. The variances for TPC, TFC, DPPH, ABTS, and FRAP were 6.04, 3.49, 4.6, 1.60, and 4.21%, respectively (Table 4).

## Conclusion

The optimal combination of honey and propolis obtained from the central composite design and response surface methodology produced a high TPC, TFC, and antioxidant capacity (DPPH• scavenging efficiency, ABTS•+ inhibition ability, and FRAP). A satisfactory model equation was found to predict the effects of the factors (honey and propolis) and the optimal combination of honey and propolis. The high antioxidant ability of the honey and propolis combination was effectively verified by the TPC, TFC, DPPH radical-scavenging efficiency, ABTS•+ inhibition ability, and FRAP. The honey and propolis composition for the optimised high antioxidant properties was 15.26 g (50.43%) and 15 g (49.57%), respectively. The optimal combination could offer efficient energy use and reduce process costs. The combination of phenolic-rich honey and propolis could benefit many potential applications.

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