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OPTIMIZATION OF EXTRACTION TEMPERATURE AND TIME ON PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF MALAYSIAN PROPOLIS *Trigona Spp*. AQUEOUS EXTRACT USING RESPONSE SURFACE METHODOLOGY

(Pengoptimuman Suhu dan Masa Pengekstrakan pada Sebatian Fenolik dan Aktivikiti Antioksidan daripada Ekstrak Akues Propolis Kelulut (*Trigona Spp.*) Malaysia menggunakan Kaedah Gerak Balas Permukaan)

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Abstract

Propolis is a natural product with rich bioactive constituents for medicinal, pharmaceutical, food, and cosmetic uses. It is considered a diet supplement to enhance health and prevent disease. The optimum extraction conditions used to obtain the highest yield of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant capacities for Trigona propolis aqueous extract was analyzed using response surface methodology and the central composite design. The effects of extraction temperature (X_1: 30 - 60 °C) and extraction time (X_2: 24 - 72 hours) on TPC (Y_1), TFC (Y_2), and antioxidant activities (DPPH (Y_3), $ABTS^{\bullet+}$ radical scavenging assay (Y_4), and ferric reducing antioxidant power (Y5) were investigated. The experimental data were satisfactorily fitted into a second-order polynomial model with regard to TPC ($R^2 = 0.9461$, $p = 0.0003$), TFC ($R^2 = 0.9110$, $p = 0.0015$), DPPH ($R^2 = 0.9482$, $p < 0.0001$), ABTS ($R^2 = 0.9663$, $p < 0.0001$), and FRAP ($R^2 = 0.9058$, $p = 0.0018$). The optimum extraction temperature and time were 43.75 °C and 52.85 hours. The predicted response values for TPC, TFC, DPPH, ABTS, and FRAP were 104.30 mg GAE/100g, 6.95 mg QE/g, 3.24 mMTE/g, 2.59 mMTE/g, and 4.34 mMTE/g, respectively. The experimental values were close to the predicted values 100.41 ± 2.74 mg GAE/100g, 6.74 ± 0.08 mg QE/g, 3.17 ± 0.08 mMTE/g, 2.76 ± 0.14 mMTE/g, and 4.60 ± 0.14 mMTE/g. As a result, the models generated are suitable, and RSM was successful in

optimizing the extraction conditions. Consequently, in this study, it was observed that the optimum extraction temperature and time provided the highest antioxidant yield of aqueous propolis extract which can be used as functional food ingredients.

Keywords: phenolic, antioxidant, propolis

Abstrak

Propolis merupakan hasil semulajadi yang kaya bahan bioaktif bagi perubatan, farmaseutikal, makanan dan kosmetik. Ia dianggap diet tambahan bagi kesihatan dan mencegah penyakit. Keadaan pengekstrakan optimum yang telah digunakan untuk hasil jumlah kandungan fenolik (TPC), jumlah kandungan flavonoid (TFC), dan kapasiti antioksidan tertinggi dianalis menggunakan pengekstrakan akues propolis kelulut melalui kaedah gerak balas permukaan, reka bentuk komposit berpusat. Kesan suhu pengekstrakan (X_1: 30 - 60 °C) dan masa pengekstrakan (X_2: 24 - 72 jam) pada aktiviti TPC (Y_1), TFC (Y_2) dan aktiviti antioksidan DPPH (Y_3), $ABTS^{\bullet+}$ (Y_4), dan FRAP (Y_5) telah diselidik. Data eksperimen diperolehi adalah sepadan bagi model polinomial peringkat kedua terhadap TPC ($R^2 = 0.9461$, $p = 0.003$), TFC ($R^2 = 0.9110$, $p = 0.0015$), DPPH ($R^2 = 0.9482$, $p < 0.0001$), ABTS ($R^2 = 0.9663$, $p < 0.0001$), dan FRAP ($R^2 = 0.9058$, $p = 0.0018$). Suhu dan masa pengekstrakan yang optimum ialah 43.75 °C dan 52.85 jam. Nilai tindak balas yang diramalkan untuk TPC, TFC, DPPH, ABTS, dan FRAP adalah 104.30 mg GAE/100g, 6.95 mg QE/g, 3.24 mMTE/g, 2.59 mMTE/g, dan 4.34 mMTE/g. Nilai eksperimen hampir dengan nilai yang diramalkan iaitu 100.41 ± 2.74 mg GAE/100g, 6.74 ± 0,08 mg QE/g, 3.17 ± 0.08 mMTE/g, 2.76 ± 0,14 mMTE/g, dan 60 ± 0,14 mMTE/g. Dalam kajian ini, diperhatikan bahawa suhu dan masa pengekstrakan yang optimum memberikan hasil antioksidan tertinggi ekstrak propolis dan dapat digunakan sebagai bahan makanan

Kata kunci: fenolik, antioksida, propolis

Introduction

Propolis is a resinous material collected by bees from plant exudates and the nectar of buds, tree gum, combined with wax and bee enzymes [1]. It is a natural product with rich bioactive constituents for medicinal, pharmaceutical, food, and cosmetic uses [2-4]. Propolis is considered a diet supplement to enhance health and prevent diseases [5, 6]. It has also been used as natural preservatives and is considered healthier and safer than synthetic preservatives [7, 8]. Propolis prolongs the shelf life, improves the quality of various food product compositions, and prevents undesirable changes in the physical and chemical characteristics of food [4, 9, 10]. Propolis extract is used as an antioxidant to enhance the antioxidant properties of honey, fruit, and juices during storage [4]. Thus, using ethanolic propolis extract or aqueous propolis extract is more economical. Alcoholic extraction, however, has certain disadvantages, such as heavy residual taste and alcohol sensitivity in some clients [7].

There is, however, limited data on the use of propolis aqueous solutions [4]. The issue of poor solubility of propolis in water at room temperature and lower amounts of the phenolic compounds are a disadvantage of aqueous extraction techniques compared to ethanol extraction [7, 11]. Nonetheless, higher solubility and diffusion coefficient of phenolic compounds can be achieved by increasing the extraction temperature [7, 12, 13]. The amount of total phenolic content increased with the increment in the extraction temperature. Higher extraction temperature leads to increased material transfer and therefore, increased penetration of solvent into propolis. Furthermore, when the temperature increased, the viscosity of propolis decreased and the entire process accelerated [14, 15].

A prolonged extraction time enhances polyphenolic compound extraction [15, 16]. However, extremely high temperatures may cause the degradation and/or volatilization of certain composites, resulting in decreased process efficiency [17, 18]. Mostly, a longer extraction period with high temperatures might lead to more polyphenol losses [14, 19]. Thus, extraction temperature and time are important factors that should be optimized to protect the active compounds, save process cost, and obtain extract rich in phenolic compounds and antioxidant activities [16, 19]. However, studies on the optimum temperature and time for the aqueous extraction of Malaysian Trigona bee

propolis are still limited. Thus, this study aims to use the response surface methodology (RSM) method for optimizing the extraction temperature and time to maximize the yield of total phenolic, total flavonoid content, and antioxidant capacities from Trigona propolis aqueous extract.

Materials and Methods

Chemicals and reagents

In the current study, analytical grade chemicals were utilized. Folin- Ciocalteu (FC) reagent, sodium carbonate, Iron(III) Chloride Hexahydride, aluminum chloride ($AlCl_3$), ethanol, methanol, were bought from Merck (Darmstadt, Germany). While, 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), HCl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were bought from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium persulfate and tetramethylchromane-2-carboxylic acid (Trolox) were purchased from Acros (New Jersey, USA). Gallic acid was from Merck (Hohenbrunn, Germany). Quercetin was bought from Nacalai-Tesque (Kyoto, Japan). Sodium Acetate Trihydrate was purchased from fisher scientific Loughborough, UK. Glacial Acetic Acid, was from MP Biomedicals, LLC, Parcd'innovation, IIIkirch, France.

Sample collection

In September 2017, 200 kg of Trigona propolis was collected from "TriBrothers Kelulut Farm", Kampung Kubang Kiat, Ketereh, Kota Bharu, Kelantan, Malaysia, and protected from the light and frozen at -20 °C until extraction.

Sample preparation and extraction

Propolis extraction was done using the method reported by Thusheva et al. [20], with some modifications. First, propolis was washed and cut using a knife. The cut samples were ground using mortar, pestle, and laboratory blender (Waring). Then 5 g of the ground propolis were extracted in 50 mL of distilled water (1:10 w/v) at different temperatures (23.79 - 66.21 °C), and various soaking times (14.06 - 81.94 hours) using RSM, central composite design (CCD), Design-Expert Version 6.0.10 (Minneapolis, MN) software for the optimization studies. The extracts were separated from the sediment through centrifugation at 1500 g for 5 minutes. The sediment was washed twice with 10 mL of the extract solution, then centrifuged at 1500 g for 5 minutes and added to the initial supernatant. The extracted propolis was concentrated by a rotary evaporator (Buchi, Flawil, Switzerland), under reduced pressure (72) at 60 °C and 50 rpm. Then the extracted soft propolis samples were preserved at 4 °C until analysis [21].

Estimation of total phenolic content

The FC reagent was utilized to evaluate the total phenolic content (TPC) in the various aqueous extracts of propolis (AEP) and standard [22]. About 20 µL of AEP (10 mg/mL) were mixed with 100 µL of FC reagent (1:10 v/v, reagent: water) in each well of a 96-well plate. This was followed by adding 75 µL of sodium carbonate (7.5%) to the mixture, then incubated for 40 minutes at room temperature in the dark and the absorbance recorded at 740 nm against a blank (distilled water) using a spectrophotometer [microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria)]. Gallic acid was used as a standard to plot the calibration curve at concentrations ranging from 20 to 160 µg/mL, R^2 = 0.9951. The mean and standard deviation of the triplicate (n = 3) were used, and the results were expressed as mg of gallic acid equivalents per 100 g of sample weight (mg GAE/100g).

DPPH free radical scavenging assay

The DPPH assay was conducted as stated by [23] with modifications using a 96-well microplate. Aliquots of 100 µL of the AEP (10 mg/mL), control, and standard were added to the wells, followed by 100 µL of 100 µM methanol solution of DPPH (3.94 mg in 100 mL methanol). The plate was incubated in the dark for 30 minutes and the absorbance was measured at 517 nm in a spectrophotometer [microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria)]. Aqueous (distilled water) was used as a blank (negative control) and Trolox was applied as a positive control. DPPH scavenging effect was calculated by DPPH discoloration percentage, using the equation 1:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100 \quad (1)$$

A_{blank} is the blank's absorbance (extraction solvent + DPPH solution) and the A_{sample} is the sample's absorbance (extracts + DPPH solution).

The decolorization is expressed as a percentage of absorbance inhibition, then plotted as a function of the antioxidant concentration in the sample. A standard curve of Trolox (5 - 60 µmol TE /mL, $R^2 = 0.9919$) was observed. The mean and standard deviation of the triplicate were used, and the outcomes were expressed as mmol Trolox equivalents per g of sample weight (mmol TE/g).

$ABTS^{\bullet+}$ radical scavenging assay

In the $ABTS^{\bullet+}$ radical scavenging assay (an electron transfer-based assay), the 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonate) radical cation ($ABTS^{\bullet+}$) was conducted to evaluate the antioxidant capacity in various AEP according to [25], with modifications. $ABTS^{\bullet+}$ radical stock solution was made by reacting 7 mM ABTS solution and 2.45 mM potassium persulfate solution in equal volumes and reacted for 16 hours in the dark at room temperature. Then 2 mL of $ABTS^{\bullet+}$ radical stock solution was diluted by 50 mL ethanol to get an absorbance of 0.706 ± 0.01 units at 734 nm using a spectrophotometer [microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria)]. Aliquots of 20 µL of standard Trolox, blank and AEP (10 mg/mL), and 180 µL of $ABTS^{\bullet+}$ radical solution were added to microplate wells and kept at room temperature in the dark. The absorbance was recorded 5 min after starting the oxidation at 734 nm. Distilled water was used as a blank. $ABTS^{\bullet+}$ scavenging influence was calculated by $ABTS^{\bullet+}$ discoloration percentage using equation 2 as follows:

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

where the blank was the mixture of distilled water and ABTS+ solution and the sample is the mixture of sample extract and ABTS+ solution. Trolox was employed as standard at concentrations ranging from 2.5 to 40µM/mL ($R^2 = 0.9931$). The mean and standard deviation (SD) of the triplicate were used and the results were expressed as mmol Trolox equivalents per g of sample weight (mmol TE/g).

Ferric reducing antioxidant power (FRAP)

The FRAP assay was modified from [26]. The fresh working solution of FRAP reagent (10:1:1) was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ, and 2.5 mL of 20 mM $FeCl_3.6H_2O$, and incubated in the dark at 37 °C. Then aliquots of 20 µL of AEP were added to 180 µL of FRAP reagent and the plate was incubated at 37 °C for 10 minutes. The absorbance was recorded using a spectrophotometer [microplate reader (Nano Quant Infinite M 200, Tecan, Grodig, Austria)] at 593 nm against a blank (distilled water). A calibration curve was created using Trolox (200 - 600 µM Trolox/mL, $R^2 = 0.9921$). The average and standard deviation of the three readings (n = 3) were used and expressed as mM Trolox equivalent (mM TE/g sample weight).

Experimental design

The central composite design was utilized to determine the optimum levels of temperature and extraction time for maximizing the antioxidant capacity of AEP on five responses, namely TPC, TFC, and antioxidant activities (DPPH, ABTS, and FRAP). Two factors, namely temperature (X_1: 23.79–66.21 °C) and extraction time (X_2: 14.06 - 81.94 hours) were coded into five levels (-1.414, -1, 0, 1, 1.414), from the lowest to the highest, respectively [21]. Thirteen different experiments were examined in terms of their responses (antioxidant properties). The coded and non-coded factors applied in the RSM design are presented in Table 1.

Table 1. Coded and actual values levels of factors used in the RSM design

	Factors Coded Levels	−1.414	−1	0	1	1.414
X_1	Temperature (°C)	23.79	30.00	45.00	60.00	66.21
X_2	Extraction time (hour)	14.06	24	48	72	81.94

Statistical analysis

The statistical analysis was implemented using the Design-Expert Version 6.0.10 software. The results for TPC, TFC, DPPH, ABTS, and FRAP were indicated as mean values of three replicates. The response surface analysis was carried out to validate the regression coefficients and the model statistical significance and for fitting the mathematical models of the experimental data to optimize the dependent variables. A second-order polynomial model was used to fit the data. As presented in the following equation 3:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_1^2X_1^2 + b_2^2X_2^2 + b_1 b_2 X_1X_2 \quad (3)$$

where the expected response is Y, while b_0 is a constant, the linear influence regression coefficients are b_1 and b_2, the quadratic impact is b_1^2 and b_2^2 and interaction influences are b_1 and b_2, respectively. The model quality was predicted by the ANOVA analysis (p <0.05) and the regression analysis (R^2). From the ANOVA analysis, only the significant coefficients were included. While the non-significant coefficients were omitted from the initial model. The relationship between the factors (X_1 and X_2) and the responses (Y_1, Y_2, Y_3, Y_4, and Y_5) is illustrated by the three-dimensional model graph. The desired aim was set in numerical optimization to produce the optimum condition and point predicted values of the responses.

Model verification

The experimental data for TPC, TFC, and antioxidant activities were determined according to optimum conditions predicted by the software. The experimental values were compared to the predicted values from the optimized model to confirm the validity of the model.

Results and Discussion

Fitting the model

The experimental values of TPC (Y_1), TFC (Y_2), and DPPH• scavenging ability (Y_3), ABTS•+ inhibition activity (Y_4), and FRAP (Y_5) were used in multiple regression analysis by applying response surface analysis to fit the second-order polynomial equations. The experimental values were close to the predicted values, demonstrating an adequate model (Table 2). The regression coefficients of determination (R^2), adjusted R^2 values, probability values (p), and lack-of-fit values for all dependent variables are shown in Table 3. The quality of fit to the second-order polynomial models was established based on the coefficients of determination (R^2), which were 0.9461, 0.9110, 0.9482, 0.9663, and 0.9058 for TPC, TFC, and antioxidant activities (DPPH, ABTS, FRAP), respectively; thus, indicating that approximately 91 to 97% of the variations were determined by the model. The fitness of the model was verified by the lack-of-fit test for all the responses but was insignificant (p >0.05).

Effect of extraction parameters on TPC, TFC, and Antioxidant activity

The effect of the two factors (X_1 and X_2) on the dependent variables (Y_1, Y_2, Y_3, Y_4, and Y_5) was established by the significant (p<0.05) coefficient of the second-order polynomial regression equation. For TPC (Y_1) and ABTS scavenging capacity (Y_4), the effect of extraction temperature and extraction time was significant (p <0.05) in the first-order linear effect (X_2), second-order quadratic effect (X_1^2, X_2^2), and interaction effect (X_1X_2), with a good regression coefficient (R^2 =

0.9461 and R^2 = 0.9663, respectively). The predicted models obtained for Y_1 and Y_4 are given in Table 3. Based on the polynomial equations for (Y_1) and (Y_4), both temperature and time affected TPC and ABTS scavenging capacity. The temperature was, however, the most important factor contributing to the increase in the extraction efficiency in terms of TPC and ABTS scavenging capacity. This is because increasing extraction temperature enhances the solubility of solute and increases the extraction coefficient, the TPC, and ABTS scavenging capacity. According to the literature [19], the extraction temperature plays a more critical role in comparison to the extraction time.

For TFC (Y_2) and FRAP (Y_5), the effect of temperature and time was significant (p <0.05) in the second-order quadratic effect (X_1^2, X_2^2) and no interaction effect, with a high regression coefficient of R^2 = 0.9110 and R^2 = 0.9058, respectively. The predicted models obtained for Y_2 and Y_5 are given in Table 3. Both the temperature and time affected TFC and FRAP, with the temperature affecting more than the time. The result is in agreement with the work of Yim et al. [19]. The extraction temperature plays a more critical role than the extraction time. The increasing temperature enhances the solubility of solute and higher TFC and FRAP.

However, for DPPH scavenging capacity (Y_3), the effect of temperature and time was significant (p <0.05) in the first-order linear effect (X_1) and second-order quadratic effect (X_1^2, X_2^2), with the regression coefficient (R^2 = 0.9482). The predicted model found for Y_3 is given in Table 3. High temperatures enhance phenolic compound recovery [15, 16, 32]. However, a quadratic influence was discovered with a longer extraction time and a higher temperature. Higher polyphenol losses may arise from longer extraction periods at high temperatures [13, 14, 19].

Figures 1a and 1d show the 3D response surface, with a quadratic effect of temperature, and linear increase, and a quadratic effect of extraction time, as well as a significant interaction impact between time and temperature on Y_1 and Y_4. Overall, a combination of moderate points of temperature (40.98 °C and 43.05 °C) and moderate time (58.09 and 55.13 hours) gave maximum yield (104.88 mg GAE/100g and 2.59 mMTE/g) of TPC contents and ABTS•+ inhibition activity, respectively. The TPC and ABTS•+ inhibition activity increased with an increase in temperature from 30 °C up to a certain point (40.98 and 43.05 °C) and time from 24 hours to 58.09 and 55.13 hours, respectively. It then decreased with further increase in temperature and prolonged time, producing a TPC and ABTS• scavenging capacity that ranged from 66.23 to 104.88 mg GAE /100g and 1.41 to 2.59 mMTE/g, respectively.

Similarly, Figures 1b and 1e show a 3D response, with a quadratic effect of both temperature and time on TFC and FRAP value. Generally, a combination of a moderate point of temperature (44.21 and 45.83 °C) and moderate time (53.14 and 51.70 hours) gave maximum values of TFC and FRAP (6.95 mg QE/g and 4.35 mMTE/g, respectively).

Figure 1c shows the response surface of the effect of temperature and time on DPPH• scavenging capacity. Overall, a combination of a moderate point of extraction temperature (42.65 °C) and moderate extraction time (49.72 hours) yielded a maximum DPPH• scavenging capacity value (3.24 mMTE/g). The antioxidative compounds may be subjected to decomposition and degradation upon longer extraction time and higher temperature [19].

The results are in agreement with previously reported studies. It illustrates that the increase in extraction temperature promotes higher solubility and diffusion coefficient of phenolic compounds and allows more phenolic extraction rate [7, 12, 27]. Higher extraction temperatures result in more material transfer and, as a result, more solvent penetration into propolis. Furthermore, when the temperature increased, the viscosity of propolis decreased, speeding up the entire process [14, 15]. However, extremely high temperatures might cause degradation and/or volatilization of some chemicals, resulting in a decreased process efficiency [17, 18]. Interestingly, there was a significant (p <0.05) negative interaction between extraction temperature and time for total phenolic contents and ABTS, which can be attributed to the decomposition of anti-oxidative compounds because of longer extraction time at higher

temperatures [14, 19]. The findings of this study are in line with the literature [14, 18]. The results are also consistent with previous studies on the beneficial effects of temperature on polyphenol extraction from various matrices [19, 28-31].

Concerning the effects of extraction time on TPC, TFC, DPPH, ABTS, and FRAP, the yield increased linearly with the increase in extraction time. A prolonged extraction time enhances polyphenolic compound extraction, giving enough time for solute exposure to the release medium. Moreover, the high temperature was known to enhance the capability of improving phenolic compound recovery [15, 16, 32]. A quadratic effect was observed with a more prolonged extraction time and a high temperature. A longer extraction period with high temperatures might lead to more polyphenol losses [13, 14, 19]. The result of the time effect on phenolic extracts is also in line with the literature [21]. The variations in the extraction conditions such as time and temperature should, however, be properly monitored and controlled to protect the active compounds from damage and to practically save the process cost. At optimum temperature, a longer extraction time should be avoided to prevent the loss, decomposition, and degradation of antioxidant compounds.

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

Extraction Parameters			**Responses**									
Standard Order	**Temperature (˚C)**	**Time (hour)**	**TPC (mgGE/100g) Y_1**		**TFC (mgQE/g) Y_2**		**DPPH (mMTE/g) Y_3**		**ABTS (mMTE/g) Y_4**		**FRAP (mMTE/g) Y_5**	
			Exp.	**Pred.**	**Exp.**	**Pred.**	**Exp.**	**Pred.**	**Exp.**	**Pred.**	**Exp.**	**Pred.**
1	-1	-1	62.15	66.23	4.01	3.88	2.22	2.35	1.34	1.41	3.06	3.26
2	1	-1	77.37	77.71	4.39	4.07	2.37	2.33	1.70	1.77	3.17	3.44
3	-1	1	92.57	97.65	4.79	5.16	2.58	2.67	2.16	2.21	3.43	3.45
4	1	1	69.52	70.86	4.35	4.54	2.24	2.18	1.59	1.65	3.48	3.57
5	-1.414	0	84.65	79.29	4.34	4.17	2.69	2.55	1.77	1.71	3.02	2.92
6	1.414	0	68.52	68.46	3.76	3.87	2.11	2.19	1.64	1.57	3.33	3.13
7	0	−1.414	75.67	73.66	3.85	4.18	2.39	2.34	1.71	1.64	3.99	3.71
8	0	1.414	94.47	91.04	5.81	5.43	2.47	2.46	2.18	2.12	3.96	3.94
9a	0	0	100.86	103.10	6.87	6.90	3.25	3.24	2.52	2.56	4.53	4.35
10a	0	0	98.16	103.10	7.55	6.90	3.42	3.24	2.76	2.56	4.23	4.35
11a	0	0	106.95	103.10	6.50	6.90	3.13	3.24	2.51	2.56	4.16	4.35
12a	0	0	101.61	103.10	6.12	6.90	3.08	3.24	2.46	2.56	4.49	4.35
13a	0	0	107.90	103.10	7.45	6.90	3.32	3.24	2.54	2.56	4.32	4.35

a Centre point.

Y_1 (TPC) = Total phenolic content, Y_2 (TFC) = Total flavonoid content

Y_3 (DPPH) = 2, 2-diphenyl-1-picrylhydrazyl radical scavenging ability,

Y_4 (ABTS) = 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical cation inhibition,

Y_5 (FRAP) = Ferric Reducing Antioxidant power, GAE = Gallic acid equivalent, QE = Quercetin equivalent,TE = Trolox equivalent, Exp. = Experimental value, Pred. =Predicted va--

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

Regression Coefficient	Polynomial Equation	R^2	R^2 (Adjusted)	Regression (*p* value)	Lack Of Fit
TPC (Y_1)	$+ 103.10 + 6.14X_2 - 14.61X_1^2 - 10.37X_2^2 - 9.5724X_1X_2$	0.9461	0.9075	0.0003	0.3029
TFC (Y_2)	$+ 6.90 - 1.44\ X_1^2 - 1.05X_2^2$	0.9110	0.8474	0.0015	0.6822
DPPH (Y_3)	$+ 3.24 - 0.13\ X_1 - 0.44\ X_1^2 - 0.42\ X_2^2$	0.9482	0.9112	< 0.0001	0.4619
ABTS (Y_4)	$+ 2.56\ + 0.17\ X_2\ - 0.46X_1^2 - 0.34X_2^2 - 0.23X_1X_2$	0.9663	0.9422	< 0.0001	0.5495
FRAP (Y_5)	$+ 4.35 - 0.66\ X_1^2 - 0.26\ X_2^2$	0.9058	0.8385	0.0018	0.1498

TPC (Y_1) = Tot al phenolic content, TFC (Y_2) = Total flavonoid content,
DPPH (Y_3) = 2, 2-diphenyl-1- picrylhydrazyl (DPPH) radical scavenging ability,
ABTS (Y_4) =2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonicacid) (ABTS) radical cation inhibition,
FRAP (Y_5) = Ferric Reducing antioxidant power

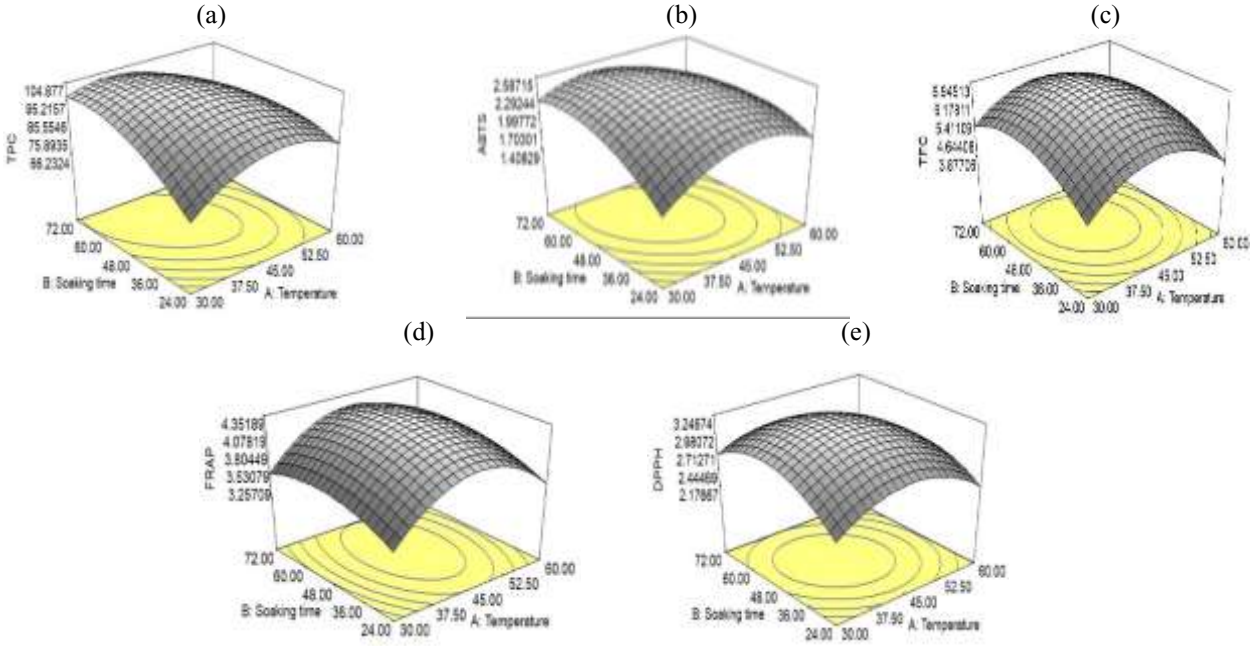

Figure 1. Response surface plot of temperature and extraction time on; (a) total phenolic content (mg GAE/100 g), (b) total flavonoid content (mg QE/g), (c) the DPPH (mM TE/g), (d) ABTS (mM TE/g), and (e) FRAP (mM TE/g) of Trigona propolis samples

Optimization of response and verification of model

In numerical optimization, the optimum covering criteria of temperature and time were 43.75 °C and 52.86 hours, respectively for propolis extraction (Figure 2). The predicted TPC, TFC, DPPH, ABTS, and FRAP were 104.30 mg GAE/100g, 6.95 mg QE/g, 3.24 mMTE/g, 2.58 mMTE/g, and 5.29 mMTE/g, respectively while the experimental values obtained were 100.41 ± 2.74 mg GAE/100g, 6.74 ± 0.08 mg QE/g, 3.17 ± 0.08 mMTE/g, 2.76 ± 0.14 mMTE/g, and 5.54 ± 0.14 mMTE/g, respectively. The experimental and predicted values were compared to verify the response surface model. The experimental values were close to the predicted values. The differences for TPC, TFC, DPPH, ABTS, and FRAP were 3.73%, 3.01%, 2.17%, 6.90%, 6.02%, respectively, as shown in Table *4*.

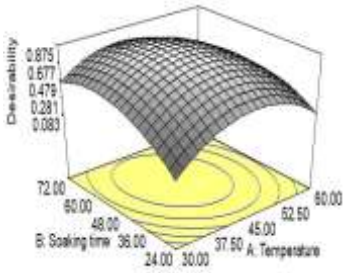

Figure 2. Response surface plot of the desirability as a function of extraction time and temperature

Table 4. Experimental data of the verification of predicted extraction parameters

Factors (Optimum)	**Temperature (ºC)**	**Time (hour)**	**Predicted Value**	**Experimental Value**	**% Difference**
Response	43.75	52.85			
TPC(mgGAE/100g)			104.30	100.41± 2.74	3.73
TFC (mgQE/g)			6.94	6.74±0.08	3.01
DPPH(mMTE/g)			3.24	3.17±0.08	2.17
ABTS(mMTE/g)			2.59	2.76±0.14	6.90
FRAP(mMTE/g)			4.34	4.60±0.14	6.02

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,
FRAP = Ferric reducing antioxidant power,
Results were expressed as mean ± standard deviation (n=3).

Conclusion

The optimum extraction time and temperature that yielded the highest TPC, TFC, and antioxidant activities from Trigona propolis aqueous extract were determined using central composite design, response surface methodology. Adequate model equations were obtained to predict the influences of the extraction temperature and time for aqueous propolis extraction. The high antioxidant capacity of the propolis aqueous extract was successfully verified through TPC, TFC, and DPPH

radical-scavenging assays, ABTS•+ inhibition activity, and FRAP. The extraction conditions for the optimized high antioxidant aqueous extract propolis were determined as extraction temperature and time at 43.75 °C and 52.86 hours, respectively. The optimum extraction conditions would ensure efficient energy use and process cost. The phenolic-rich extract with a high antioxidant capacity also provides many benefits for various potential applications. Though both temperature and time affect TPC, TFC, and antioxidant activities of Trigona propolis aqueous extract, the extraction temperature, however, plays a more critical role than the extraction time because the increasing temperature enhances the solubility of the solute. Future studies are suggested to evaluate the role of green solvents like natural deep eutectic solvents.

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