

Response surface optimisation and antioxidant characterisation of high antioxidant soft jelly prepared from *Baccaurea angulata* fruit juice and *Trigona* sp. honey using central composite design

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Abstract

The optimum combination of *Baccaurea angulata* fruit juice (X_1 : 15 - 85 ratio) and *Trigona* sp. honey (TH) (X_2 : 15 - 85 ratio) in developing a high antioxidant soft jelly was investigated based on the antioxidant capacity (Y_1), phenolic (Y_2), and flavonoid (Y_3) content. Response surface methodology (RSM), via central composite design (CCD), was used to produce optimal combination effects of the two independent variables (*B. angulata* fruit juice and TH) for highest recovery of antioxidant capacity (AC), total phenolic content (TPC), and total flavonoid content (TFC). The polynomial models generated were satisfactory. The lack-of-fit test were higher than $p > 0.05$ for all three analyses, signifying the suitability of the models in accurately predicting the variations. Predicted values of the analysis agreed with those of the experimental values. An optimum combination of *B. angulata* fruit juice and TH was developed (ratio 40:40). The sample also exhibited significant FRAP and DPPH radical scavenging activities. Several polyphenols were identified for the samples through UHPLC-MS/MS. In conclusion, *B. angulata* and *Trigona* sp. honey have high potentials to be used in fortifying the soft jelly samples, making them prospective food supplements due to their nutritional and health benefits.

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Keywords

Antioxidants,
Baccaurea angulata,
Trigona sp. honey,
Response surface
methodology,
Radical scavenging
activity

Introduction

Many literatures have stated that due to cellular metabolic reactions, reactive oxygen species (ROS) are naturally formed in the human body, and can induce negative modifications in cell components when they are in high concentration in the body (Ahmed and Othman, 2013). The occurrence of adverse characteristic alterations against cell mechanisms and components could lead to oxidative stress damages and various pathological conditions such as hypercholesterol, cancer, and diabetes (Ahmed and Othman, 2013; Ahmed *et al.*, 2015). To date, many researchers have attempted to find various alternative treatments and therapeutic approaches to control these diseases through improving and implementing natural resources. Most medicinal plants are known to produce a variety of antioxidants that react against cellular oxidative damages by delaying the oxidation process as well as inhibiting the polymerisation chain reaction initiated by free radicals, which then results in the removal of

ROS and reactive nitrogen species (RNS) (Erejuwa *et al.*, 2012; Di Meo *et al.*, 2016). Examples of such natural sources include honey and *Baccaurea angulata*.

For centuries, honey has been used for its medicinal properties to treat a wide variety of ailments. It may be used alone or in combination with other substances, and administered orally, or topically, for the eradication of certain ailments (Jaganathan *et al.*, 2015). *Trigona* sp. bee, also known as 'lebah kelulut' in Malaysia, is a stingless bee species. There are nearly 150 species of *Trigona* bees such as *T. apicalis*, and *T. thorasica*. Depending on its quality, *Trigona* sp. honey (TH) can contribute to twice the normal nutritional content as compared to ordinary honey due to its many vitamins and minerals that heighten its antioxidant and anti-inflammatory potentials (Khalil *et al.*, 2011). Many of its curative properties are the basis for some traditional medicinal treatments and have since been proven through numerous research studies (Erejuwa *et al.*, 2012; Jaganathan *et al.*, 2015). Erejuwa *et al.* (2012) indicated that the redox and scavenging properties of

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honey are due to its various bioactive compounds. The presence of multiple types of polyphenols in honey such as caffeic acid, cinnamic acid, apigenin, quercetin, and kaempferol may also contribute to its high antioxidant activities (Nayik and Nanda, 2016; Biluca *et al.*, 2017).

Most plants and fruits, including *B. angulata*, contain various types of antioxidants that are of interest to the field of research as they are beneficial to the human body (Mikail *et al.*, 2016). Antioxidants assist the body in balancing the ratio of free radicals through methods such as decomposing peroxides and scavenging free radicals (Di Meo *et al.*, 2016). *B. angulata*, locally known as 'belimbing dayak', is from the Euphorbiaceae family, and one of the underutilised tropical fruits from the Island of Borneo, Malaysia. The trees of *B. angulata* (6 - 21 m high) grow in the tropical primary and secondary riverine, and non-riverine rain forests in the Island of Borneo, especially in Sarawak (Lim, 2012). The fruits are seasonal, and can be found at the end of the year (November to February). The star-shaped fruit is obovoid with a tapering apex, a pericarp of 1 - 2 mm thick and seeds that are 16 - 23 × 7 - 16 × 4 - 9.5 mm. As compared to the deep red colour of an unripe *B. angulata* fruit, it has a lighter red colour and a sweet and sour taste when it has ripened (Lim, 2012).

The *B. angulata* fruit has been reported to contain nutritional and chemical compositions such as ash (3.68 - 7.28%), protein (3.11 - 3.89%), total fat (0.28 - 5.15%), carbohydrate (64.58 - 74.12%), total dietary fibre (3.6 - 6.3%), moisture (11.37 - 19.09%), and water activity (0.41 - 0.44 a_w) (Ibrahim *et al.*, 2013; Jauhari *et al.*, 2013). The evaluation of its antioxidant properties through various analyses including TPC (3.48 - 8.62 mg/g), TFC (7.93 - 19.12 mg QE/g), TAC (0.33 - 1.20 mg c-3-g/100 g), FRAP (13.31 - 50.86 mM TE/g), and DPPH (46.23 - 78.54 mg AA/100 g) revealed high antioxidant activities (Jauhari *et al.*, 2013). The phenolic constituents of *B. angulata* include quercetin, kaempferol, rutin, caffeic acid, cinnamic acid, and 4-hydroxybenzoic acid (Ahmed *et al.*, 2015; 2017). Its antioxidant and physicochemical properties have been found to be effective in inducing the increase of antioxidant enzyme activities and inhibiting lipid peroxidation (Ahmed *et al.*, 2015; Mikail *et al.*, 2016).

Response surface methodology (RSM) is a tool for statistical and mathematical analysis that is suitable in dealing with statistical modelling, multi-variant experimental design strategy, and process optimisation (Bezerra *et al.*, 2008). This method is also useful in developing data-driven models, and is often utilised to explore the simultaneous effects of controllable factors on the responses, which later develop into the factor settings that optimise the response (Nagendra Prasad *et al.*, 2011; Nayik and Nanda, 2016). Among the possible

approaches in RSM, central composite design (CCD) was applied in the present work, as it was found to be more practical than other available methods (Bezerra *et al.*, 2008). In general, the conventional method of formulating food product is acknowledged as a laborious and time-consuming process (Quispe-Fuentes *et al.*, 2017). Hence, a design of experimental and statistical analysis generated from RSM was employed to determine the effects between several independent variables and the responses/dependent variables, with the aim of obtaining the optimal conditions for maximum recovery of antioxidant content present in the soft jelly samples.

In recent years, many have acknowledged food safety and the effectiveness of natural products when compared to synthetic products. The acceptability of natural products has greatly improved as consumers' preference for naturally processed, additive-free, and safe products rises. This change has been attributed to several concerns such as the toxicity levels and carcinogenicity nature of synthetic antioxidants that are extensively used as food additives (Ahmed and Othman, 2013). Subsequently, various natural sources have been receiving attention as both complementary and alternative sources in the medicinal fields. In view of this, the potential of *B. angulata* fruit juice and TH as a source of natural nutritional supplement and bioactive phytochemicals should be explored. Hence, the present work was conducted with the aim of (i) developing the optimal combination of *B. angulata* and TH through a soft jelly medium using RSM, and (ii) examining their potential antioxidant properties.

Materials and methods

Sample materials and chemicals

B. angulata fruits were obtained from the forest of Bau, Sarawak, Malaysia, and were authenticated by the Forest Research Institute Malaysia (FRIM). The fruits were collected from trees at full ripeness, and preserved at -20°C upon arrival. *Trigona* sp. honey samples were obtained from Ketereh, Kota Bahru, Kelantan, Malaysia, and kept at 25°C. The honey sample was authenticated by employing a preliminary phenolic compounds analysis as described by Soares *et al.* (2017), with slight modifications.

The chemicals used were of either analytical or chromatographic grade, and were from Sigma-Aldrich (Germany) or Merck (Germany): sodium hydroxide (NaOH), hydrochloric acid (HCl), methanol, sulphuric acid, sodium phosphate, ammonium molybdate, ascorbic acid, Folin-Ciocalteu, gallic acid, sodium carbonate, sodium nitrate, aluminium chloride, quercetin, methanol (HPLC grade, 100%),

2,2-diphenyl-1-picrylhydrazyl (DPPH), TPTZ (2,4,6-tripyridyl-s-triazine), acetate buffer, kaempferol, rutin, maleic acid, caffeic acid, cinnamic acid, coumaric acid, salicylic acid, sinapic acid, *p*-hydroxybenzoic acid, vanillic acid, iron(III) chloride hexahydrate (FeCl₃.6H₂O), formic acid, ammonium formate, and acetonitrile.

Soft jelly formulation using RSM

Preliminary analysis and experimental design

A two-factor inscribed central composite design (CCD) was used to identify the relationship between the responses and the variables, as well as to determine the conditions (the ratios of *B. angulata* and TH) that optimise the antioxidant capacity (AC), total phenolic content (TPC), and total flavonoid content (TFC) of the soft jelly samples. A preliminary analysis was conducted using the same method as the AC analysis for the RSM in order to determine the range for the independent variables/factors (ratios of *B. angulata* fruit juice and TH). The ratios were randomly set at 0:0, 25:25, 50:50, 75:75, and 100:100 (*B. angulata* fruit juice:TH). From the results, the ratios for the independent variables/factors were established. Subsequently, the present work investigated the effects of the independent variables/factors, which were the amount of *B. angulata* fruit juice (X_1 : 15 - 85 ratio) and TH (X_2 : 15 - 85 ratio) to be incorporated in the soft jelly, on the recovery of the response variables/factors (antioxidant (Y_1), phenolic (Y_2), and flavonoid content (Y_3)). Table 1 states the values of the independent

process variables formulated by the CCD and their converted values, which were based on a 1:5 ratio.

Preparation of the soft jelly

Soft jelly was chosen as the medium as it would allow for easier consumption and dispersion of the samples. Healthy, full-grown *B. angulata* fruits (without mechanical damages and microbial infections, weighing 25 - 40 g) were chosen (Mikail *et al.*, 2016). The fruits were juiced with an electric juicer after a thorough washing with tap water, followed by rinsing with distilled water and left to air dry. The following ingredients were also used; gelatine, water, sugar, and glucose syrup, and kept at a constant amount. Firstly, the gelatine was mixed with water (85 - 90°C) at ratio of 1:2 (gelatine:water) using a double-boiler method. Water, sugar, and glucose syrup were added and cooked to 100°C before being left to cool to 90°C (Jahan *et al.*, 2015). Each ingredient was slowly added while the mixture was continuously stirred. A thermometer was used to constantly monitor the temperature. Next, TH and *B. angulata* fruit juice was added based on the combinations developed by RSM (Table 1). The mixture was boiled for 5 min at 90°C with constant stirring and allowed to cool at room temperature for 15 min. The samples were then measured (around 5 g) and transferred into sachets using a kitchen weighing scale and funnel. They were then sealed using an impulse sealer, before being stored at 4°C.

Extraction of samples

The samples were weighed, and transferred

Table 1. Independent variables, their values for optimisation, and two-factor central composite design.

Standard order	Factor 1 ratio (X_1) <i>B. angulata</i>	Factor 2 ratio (X_2) TH	X_1 : <i>B. angulata</i> (g)	X_2 : <i>Trigona</i> sp. honey (TH) (g)	Response 1 (Y_1) AC (mg AAE/g)		Response 2 (Y_2) TPC (mg GAE/g)		Response 3 (Y_3) TFC (mg QE/g)	
					Exp.	Predicted	Exp.	Predicted	Exp.	Predicted
1	15.00	15.00	75.0	75.0	3.147	3.10	0.403	0.40	0.225	0.22
2	85.00	15.00	425.0	75.0	2.883	2.84	0.345	0.35	0.319	0.32
3	15.00	85.00	75.0	425.0	3.240	3.25	0.470	0.46	0.138	0.14
4	85.00	85.00	425.0	425.0	3.170	3.18	0.438	0.44	0.211	0.22
5	0.500	50.00	2.5	250.0	3.307	3.33	0.454	0.46	0.131	0.13
6	99.50	50.00	497.5	250.0	3.077	3.10	0.409	0.41	0.271	0.26
7	50.00	0.500	250.0	2.5	2.733	2.79	0.339	0.34	0.310	0.32
8	50.00	99.50	250.0	497.5	3.157	3.14	0.444	0.45	0.209	0.20
9 ^a	50.00	50.00	250.0	250.0	3.793	3.83	0.533	0.53	0.263	0.28
10 ^a	50.00	50.00	250.0	250.0	3.770	3.83	0.517	0.53	0.263	0.28
11 ^a	50.00	50.00	250.0	250.0	3.990	3.83	0.529	0.53	0.299	0.28
12 ^a	50.00	50.00	250.0	250.0	3.743	3.83	0.515	0.53	0.264	0.28
13 ^a	50.00	50.00	250.0	250.0	3.833	3.83	0.542	0.53	0.307	0.28

^a = centre point; TH = *Trigona* sp. honey; AC = antioxidant capacity; TPC = total phenolic content; TFC = total flavonoid content; AAE = ascorbic acid equivalent; GAE = gallic acid equivalent; QE = quercetin equivalent; and Exp. = Experimental.

into separate centrifuge tubes containing methanol, resulting in a final concentration of 0.25 g/mL for each sample. Extraction was carried out for 30 min using an incubator shaker set at 300 rpm, 55°C. The solutions were then filtered using a filter paper, and the filtrates were kept at 4°C. The samples were heated at 37°C for 10 min before each analysis. The extracts were used to measure the AC, TPC, TFC, FRAP, and DPPH free radical scavenging activity.

Evaluation of antioxidant properties

Antioxidant capacity through phosphomolybdenum assay

Firstly, 100 µL of the extract was mixed with 1 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). Next, the mixture was covered, and incubated at 95°C for 90 min. After it has cooled to room temperature, the absorbance was measured (695 nm) against blank using a microplate reader (VERSA max Kinetic Microplate Reader, USA). Results were expressed as mg ascorbic acid equivalence per gram of sample weight (mg AAE/g). Experiment was conducted in triplicates.

Estimation of total phenolic content (TPC)

Each sample extract (500 µL) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent, and incubated at room temperature for 5 - 10 min. Then, 2 mL of 75 g/L Na₂CO₃ was added to the solution before it was thoroughly mixed using a vortex, and incubated for 2 h at room temperature. The absorbance was measured at 760 nm against blank. The results were expressed as mg gallic acid equivalence per gram of sample weight (mg GAE/g). Experiment was conducted in triplicates.

Estimation of total flavonoid content (TFC)

The extracts (2.5 mL) were mixed with distilled water (250 µL) and 5% sodium nitrate (200 µL). The mixture was left to stand at room temperature for 5 min before 350 µL of 10% aluminium chloride was added, and incubated for 6 min. Sodium hydroxide (1 mL) was added to the mixture and absorbance was measured at 415 nm. Results were expressed as mg quercetin equivalence per gram of sample (mg QE/g). Experiment was conducted in triplicates.

Statistical method for RSM analysis

The Design Expert Version 6.0 software was used to analyse the experimental designs and the statistical analysis. To determine the regression coefficients, statistical significance of the model terms and to 'fit' the mathematical model of the experimental data, a response surface analysis and analysis of variance (ANOVA) were employed. A second order polynomial

model; which incorporates the predicted dependent variable, the constant that fixes the response at the central point of the experiment, the regression coefficients for the linear effect terms, the quadratic effect terms, and the interaction effect terms, was applied to predict the response variables. The adequacy and sufficiency of the model was predicted through the regression (R^2) and ANOVA analysis ($p < 0.05$). The relationship between the independent variables (X_1 and X_2) and the response variables (Y_1 , Y_2 , and Y_3) was demonstrated by the generated plots.

DPPH free radical scavenging activity analysis

Firstly, 1,000 µL of DPPH in methanol (100 µM) was mixed with 500 µL of the samples. The mixtures were vortexed, and left to stand in dark for 15 - 30 min before being read at 517 nm. A standard calibration curve was constructed using ascorbic acid and quercetin. To define the anti-radical activity of the samples, the amount of antioxidant that caused the reduction of the initial DPPH absorbance by 50% (EC_{50}) was also determined.

Ferric reducing ability of plasma (FRAP) analysis

FRAP reagent was prepared by mixing 200 mL of 300 mM acetate buffer (pH 3.6), 20 mL of 10 mM TPTZ solution, 20 mL of 10 mM FeCl₃·6H₂O and 25 mL of distilled water. Next, 200 µL of the extracted sample was mixed with 1.5 mL of the FRAP reagent, and incubated for 5 min at 37°C in dark conditions. Readings were obtained at 593 nm. Absorbance of the FRAP reagent was considered as blank. The results were expressed in micromoles of ascorbic acid equivalent per gram of sample (µM AAE/g sample). All measurements were in triplicates and reported as mean ± SEM.

UHPLC-MS/MS analysis of phenolic compounds

The optimised soft jelly sample, as well as the control sample, *B. angulata* fruit juice and TH were utilised in this analysis. Analysis was carried out using an ultra-high-performance liquid chromatography (UHPLC), AB Sciex 3200QTrap liquid chromatography-tandem mass spectrometry (LC-MS/MS) (AB Sciex, Toronto, Canada) coupled to a Flexar FX15 UHPLC system (Perkin Elmer, Massachusetts, USA), with AB Sciex Analyst software for data acquisition. The UHPLC system, operated using electrospray ionisation (ESI) ion source, was equipped with a binary pump, a column oven/heater, and an autosampler with a 20 µL loop. The samples were separated using a reverse-phase Zorbax C₁₈ column (150 × 2.1 mm, 5 µm; Agilent, USA). The mobile phase comprised of

Solvent A (water with 0.1% formic acid and 5 mM ammonium formate) and Solvent B (acetonitrile with 0.1% formic acid and 5 mM ammonium formate). The compounds were eluted under the following conditions; a flow rate that interchanged from 800 $\mu\text{L}/\text{min}$ to 1 mL/min, temperature set at 40°C, rapid screening at 15 min run time, linear gradient conditions from 10 to 90% B at 0.01 to 8 min, held for 2 min at 90% B, followed by a return to 10% B (the initial conditions) for 0.1 min, and re-equilibration for 5 min. Ionisation was performed in negative mode. Identification of compounds was obtained by using the full mass spectrum and its mass fragmentation spectrum. The compounds (quercetin, kaempferol, rutin, caffeic, cinnamic, coumaric, p-hydroxybenzoic, salicylic, sinapic, and vanillic acids) were calculated with the regression equations from the standard curves.

Results and discussion

Preliminary analysis through phosphomolybdenum assay

Preliminary analysis was conducted so that the results could be used to set the ratios of *B. angulata* fruit juice and TH to be incorporated into the soft jelly samples in the RSM. Overall, only certain combinations of ratios resulted in high antioxidant values as the 0:0, 25:25, 50:50, 75:75, and 100:100 ratio of *B. angulata* fruit juice and TH obtained a reading of 1.911 ± 0.028 , 2.717 ± 0.110 , 2.902 ± 0.119 , 2.687 ± 0.257 , and 2.557 ± 0.064 mg GAE/g, respectively. It could be observed that the 50:50 ratio produced the highest total antioxidant capacity. It was also noted that when the ratio was increased to more than 50, the antioxidant capacity of the samples started to decline. In brief, it was deduced that the range of ratios for *B. angulata* fruit juice and TH should be set around the ratio with the highest preliminary AC outcome, which was the 50:50 ratio. Thus, with reference to the preliminary results and with consideration towards the limits set by RSM, the range of the independent variables/factors that was computed into the software was 15 - 85 ratio for both the *B. angulata* fruit juice (X_1) and TH (X_2).

Fitting the response surface models

The experimental values obtained for the

response variables were in proximate to the predicted values (Table 1), indicating satisfactory models for each of the response variables. The experimental values of the antioxidant (Y_1), phenolic (Y_2), and flavonoid content (Y_3) were used in a multiple regression analysis to fit the second-order polynomial equations. In Table 2, the coefficients of determination (R^2), adjusted R^2 values, probability values (p), and lack-of-fit values for all the dependent variables are stated. As all the values for the lack-of-fit test were higher than $p > 0.05$, the models were deduced to be suitable in accurately predicting the variations as the lack-of-fit of the models were found to be not significant ($p > 0.05$).

Antioxidant capacity, total phenolic content, and total flavonoid content

The influence of the independent variables towards the antioxidant capacity (Y_1), total phenolic content (Y_2), and total flavonoid content (Y_3) was reported based on the significant coefficient ($p < 0.05$) of the second-order polynomial regression equations. Quadratic models were selected for all the analysis as they had the highest order polynomial and was not aliased. Moreover, the regression (p -value) was found to be significant ($p < 0.05$) with a reading of < 0.0001 for AC and TPC, while TFC showed a reading of 0.0021, meaning that there was a low chance that the model- F -value could occur due to noise. The predicted model obtained for each analysis are given in Table 2, while the graphs of the quadratic model are shown in Figure 1. From the results in Table 1, the highest readings for AC and TPC were identified at *B. angulata* fruit juice and TH ratio of 50:50, which were the centre points. In contrast, the highest flavonoid content was obtained from sample standard 2, which was a fact point (0.319 ± 0.006 mg QE/g), and standard 7, which was an axial point (0.310 ± 0.007 mg QE/g), where the ratio of *B. angulata* fruit juice and TH was 85:15 and 50:0.5, respectively (Table 1).

For AC and TPC, the effect of the various combinations of *B. angulata* fruit juice and TH was found to be significant ($p < 0.05$) for the first-order linear effect (X_1 and X_2) and second-order quadratic effect (X_1^2 and X_2^2), while the interactive effect (X_1X_2) was noted to be not significant ($p > 0.05$) (Table 2).

Table 2. Polynomial equation and statistical parameters.

Regression coefficient	2 nd order polynomial equation	R^2	R^2 (adjusted)	Regression (p -value)	Lack of fit
AC (Y_1)	$y = 3.83 - 0.082X_1 + 0.12X_2 - 0.31X_1^2 - 0.43X_2^2$	0.9758	0.9585	< 0.0001	0.8041
TPC (Y_2)	$y = 0.53 - 0.019X_1 + 0.039X_2 - 0.0457X_1^2 - 0.067X_2^2$	0.9891	0.9719	< 0.0001	0.8224
TFC (Y_3)	$y = 0.28 + 0.046X_1 + 0.042X_2 - 0.041X_1^2$	0.9454	0.9064	0.0021	0.7690

AC = antioxidant capacity; TPC = total phenolic content; and TFC = total flavonoid content.

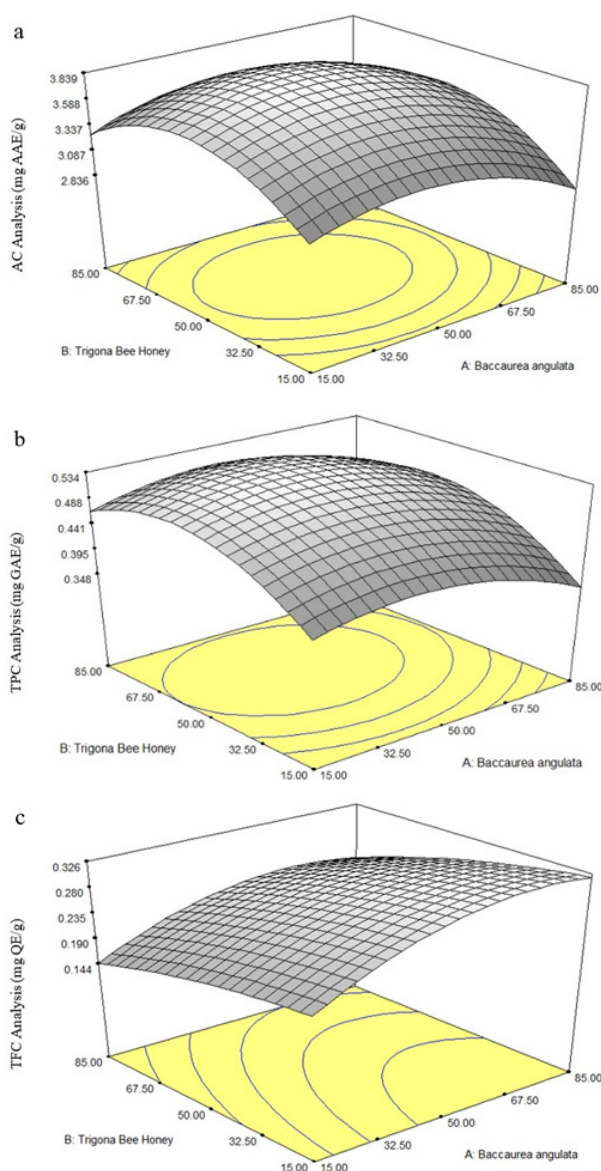


Figure 1. Response surface plot of the combined effect of *Baccaurea angulata* fruit juice and *Trigona* sp. honey on; (a) antioxidant capacity (AC), (b) total phenolic content (TPC), and (c) total flavonoid content (TFC) of the soft jelly samples.

Subsequently, the individual amount of *B. angulata* fruit juice (X_1) and TH (X_2) utilised in the production of the soft jelly had a substantial influence on the antioxidant and phenolic properties of the extracts. A difference was observed for TFC as the results were only significant ($p < 0.05$) for the first-order linear effect (X_1 and X_2) and second-order quadratic effect of X_1^2 , while the second-order quadratic effect of X_2^2 and the interactive effect (X_1X_2) were found to be not significant ($p > 0.05$) (Table 2). This signified that the presence of *B. angulata* fruit juice in the samples had a greater influence towards the flavonoid content of the samples than that of TH. The predicted R^2 for AC (0.8464), TPC (0.9719), and TFC (0.9355) were in reasonable agreement with their adjusted R^2 (0.9064, 0.9814, and 0.9585, respectively).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are very reactive groups of atoms that have the potential to damage components of living cells when excessively produced due to the presence of odd electrons, called free radicals (Di Meo *et al.*, 2016). Their presence in the human body is regulated by the antioxidant defence mechanism as continuous exposure to free radicals can contribute to fatal ailments such as cancers and cardiovascular diseases (Di Meo *et al.*, 2016). Phenolic compounds as bioactive substances are important constituents in a normal nutritional intake as they represent a large heterogeneous group of secondary plant metabolites (Altemimi *et al.*, 2017). The abundance of flavonoids available in fruits and vegetables has been reported with potential effects in managing or delaying a number of ailments such as cancers, cardiovascular diseases, inflammations, and infections (Kadian *et al.*, 2016). As plants and honey essentially constitute unending sources of natural bioactive compounds, many studies have been associated with the intake of food sources containing natural antioxidants, phenols, and polyphenols that have protective effects against various diseases (Jaganathan *et al.*, 2015; Nunes *et al.*, 2016).

A considerable amount of studies has identified antioxidants as reliable constituents for protection against different ailments such as cardiovascular diseases, inflammations, and cancers (Kadian *et al.*, 2016; Mikail *et al.*, 2016). The fruit of *B. angulata* consists of promising nutritional and physiochemical properties, which are mainly attributed to its high levels of antioxidants (Ibrahim *et al.*, 2013; Jauhari *et al.*, 2013; Ahmed *et al.*, 2015; Mikail *et al.*, 2016). A study on the fruit has indicated that high levels of phenolics could be obtained from several of its parts, ranging from 0.060 to 15.36 mg GAE/g (Ahmed *et al.*, 2015). The *B. angulata* fruit has also been reported to have high levels of flavonoids depending on its extraction process and the parts of the fruit that were tested; previous studies have shown that its flavonoid content could range from 5.82 to 37.32 mg QE/g (Ibrahim *et al.*, 2013; Ahmed *et al.*, 2015). Subsequently, with consideration to the fact that the extraction solvent used could influence the outcome of the phenolic compounds extracted (Nagendra Prasad *et al.*, 2011; Ahmed *et al.*, 2015), the results from the present work showed similar trends with those of the previous studies as the predicted model obtained reflected on the probable contribution of the *B. angulata* fruit juice with regards to the antioxidants extracted from the samples.

Honey is also known to produce a variety of antioxidants that react against cellular oxidative

damages, and are involved in the removal of free radicals (Erejuwa *et al.*, 2012; Gül and Pehlivan, 2018). Accordingly, the *Trigona* sp. honey in the samples might have also contributed to the antioxidants, phenolic, and flavonoids detected. Studies have indicated that TH, depending on its quality, can exhibit a high degree of antioxidants which makes it effective as an antiradical, antiproliferative, anti-inflammatory, or anticancer agents (Khalil *et al.*, 2011; Kek *et al.*, 2014). A study conducted by Kustiawan *et al.* (2014) suggested that TH, depending on the extraction solvent, bee species, and cell activity, can exhibit high cytotoxic activities towards human cancer cells. Flavonoids are also common constituents of honey that have the potential to prevent and regulate the formation of free radicals, which may contribute to their role as antimicrobial and anti-inflammatory agents (Khalil *et al.*, 2011; Moniruzzaman *et al.*, 2014). A study conducted by Truchado *et al.* (2011) discovered that the main flavonoid constituents in their samples of stingless bee honey were flavonoid glycosides, which could be associated with their reputed anticataract properties. Overall, based on the results, both *B. angulata* fruit juice and TH played an important role in the yield of phenolic compounds obtained from the samples.

Optimisation of responses and the model verification

To obtain the soft jelly sample with high antioxidant, phenolic, and flavonoid contents, the optimal ratios of the samples (*B. angulata* fruit juice and TH) were generated based on the combinations of goals and limits computed for each of the three response variables. The independent variables (*B. angulata* fruit juice, X_1 and TH, X_2) were also computed with specific goals that minimised the ratio/amount of *B. angulata* fruit juice and TH needed in the development of the samples while achieving the highest combination of all three analysis. Subsequently, to determine the optimum levels of independent variables with response to the variable objectives (Y_1 , Y_2 , and Y_3) desirable goals, multiple numerical optimisations were conducted. Through the CDC, an optimal combination was developed for

the responses, where the most optimised results achieved for all three analysis was calculated to be at ratio 40:40 (*B. angulata* fruit juice:TH). Accordingly, with reference to the optimised ratio generated, freshly prepared samples underwent the same set of analysis conducted earlier in the study (AC, TPC, and TFC analysis). The corresponding predicted and experimental values are listed in Table 3.

It was noted that the optimal combination produced by RSM resulted in a high degree of desirability ($p < 0.05$), signifying that the optimised model had a high potential in producing the desired outcome aimed for the model. Furthermore, both the response surface model and the optimal model indicated that only moderate amounts of *B. angulata* fruit juice and TH were needed in producing soft jelly samples with high levels of antioxidants. This was supported by several studies which suggested that although flavonoid-rich diets may lower the risk of some diseases, very high doses of certain flavonoids might have adverse effects such as interfering with the actions of common medications and causing liver toxicity (Peterson *et al.*, 2015). Therefore, it is important to accurately assess the needs of a person's diet from the perspectives of health safety. Moreover, it is more cost effective as with minimal use of *B. angulata* fruit juice and TH, optimal number of antioxidants could still be achieved.

Radical scavenging activity assays

In verifying the optimised soft jelly sample with regards to its antioxidant activities, a few radical scavenging activity assays were conducted. Based on the results, the optimised sample showed high levels of dose-dependent antioxidant activity against DPPH, where the DPPH radical scavenging activity of the optimised sample was $80.81 \pm 0.00\%$ at 500 mg/mL, and had an EC_{50} value of 124.65 ± 0.67 mg/mL (Table 3). This agrees with studies related to the *B. angulata* fruit in which substantial antiradical activities have been documented (Jauhari *et al.*, 2013; Ahmed *et al.*, 2015). *Trigona* sp. honey might also have contributed to the antiradical activity as honey has a significant radical scavenging potential

Table 3. Optimised models, their experimental and predicted response values, and radical scavenging activities.

Criteria	<i>B. angulata</i> : TH	AC (Y_1) (mg AAE/g)		TPC (Y_2) (mg GAE/g)		TFC (Y_3) (mg QE/g)		DPPH		FRAP (μ M AAE/g)
		Predicted	Obtained	Predicted	Obtained	Predicted	Obtained	% Inhibition at 500 mg/mL	EC_{50} (mg/mL)	
Optimise	40:40	3.758	3.849 ± 0.005	0.493	0.484 ± 0.006	0.274	0.255 ± 0.001	80.81 ± 0.00	124.65 ± 0.67	191.22 ± 0.00
Control	-	-	-	-	-	-	-	55.34 ± 0.17	401.79 ± 0.68	10.85 ± 0.32

TH = *Trigona* sp. honey; AC = antioxidant capacity; TPC = total phenolic content; TFC = total flavonoid content; AAE = ascorbic acid equivalent; GAE = gallic acid equivalent; and QE = quercetin equivalent.

(Khalil *et al.*, 2011; Kek *et al.*, 2014).

FRAP assay was utilised in order to measure the ability of the samples to reduce Fe³⁺ complex of Fe(TPTZ)³⁺ to Fe²⁺ complex, Fe(TPTZ)²⁺. The FRAP value of the optimised soft jelly sample was recorded at 191.22 ± 0.00 µM AAE/g) (Table 3). Jauhari *et al.* (2013) and Khalil *et al.* (2011) reported that FRAP values exhibited by indigenous fruits and honey strongly correlated with total phenolic and flavonoid contents. This is reflected by the results obtained in the present work as the optimised sample was the combination of *B. angulata* fruit juice and TH produced by the RSM with the focus of having higher outcome of antioxidant properties while using minimum amount of *B. angulata* fruit juice and TH possible. The control showed lower values as compared to the other samples in both assays, as without the presence of *B. angulata* fruit juice and TH in the control sample to contribute to its antioxidant properties, very low antiradical activity could be observed.

Identification of phenolic compounds using UHPLC-MS/MS analysis

In identifying the various phenolic compounds, the multiple reactions monitoring (MRM) was utilised as it is a highly selective and sensitive LC-MS/MS analytical mode for specific compound identification and quantification. To determine the conjugated forms of phenolic compounds in the present work, the ionisation was performed in negative mode due to its higher selectiveness and efficiency in characterising phenolic compounds (Ramirez *et al.*, 2014). Moreover, in the negative mode, the deprotonated molecules (M-H⁻) of the various compounds were also generated, thus promoting a rapid

determination of the molecular mass of a compound directly after its elution from the UHPLC column. The parent ion (M-H⁻), as well as its unique fragment (MS²) ion, were targeted and identified by the instrument. Through UHPLC-MS/MS, a total of 10 phenolic compounds were identified in all the samples, except for the control sample; three were classified as flavonoids, while seven were classified as phenolic acids (Table 4).

Even after years of its discovery, attention towards flavonoids is still at its peak due to their broad range of functions that are vastly beneficial for human health. The utmost acknowledged characteristic possessed by numerous types of antioxidants, including flavonoids, is their ability to remove free radicals and protect the body from damages caused by oxidation stress/reactions (Kadian *et al.*, 2016). In the present work, several phenolic compounds classified as flavonoids (i.e.: quercetin, kaempferol, and rutin) were detected in the optimised soft jelly sample, as well as the *B. angulata* juice and TH samples (Table 4). This was similar with previous studies where the presence of quercetin, kaempferol, and rutin have been identified in either *B. angulata* or TH (Ahmed *et al.*, 2017; Ranneh *et al.*, 2018). All the detected flavonoids eluted around the 3 - 4 min mark. It has been well documented that quercetin not only exhibits proapoptotic effects on cancer cells, but it is also known to have anti-inflammatory and antiviral activities (Ghasemzadeh and Ghasemzadeh, 2011). Several studies have also found a positive correlation between the consumption of food containing kaempferol or/and rutin and a reduced risk of developing major diseases such as cancers, diabetes, and cardiovascular diseases (Ghasemzadeh and Ghasemzadeh, 2011; Al-Dhabi *et al.*, 2015).

Table 4. Identification of flavonoids and phenolic acids using UHPLC-MS/MS analysis.

Compound identified	Standard retention time (min)	Mass spectra		Presence in samples			
		M-H ⁻ (m/z) (Parent Ion)	MS ² (m/z) ⁻ (Fragment Ion)	Optimised jelly	Control jelly	<i>B. angulata</i> fruit juice	<i>Trigona</i> sp. honey
Quercetin	4.32	300.8	151.0	/	/	/	/
Kaempferol	4.72	285.0	93.0	/	/	/	/
Rutin	3.41	609.7	300.0	/	/	/	/
Caffeic acid	2.64	178.9	135.1	/	-	/	/
Cinnamic acid	4.05	147.0	103.1	/	/	/	/
Coumaric acid	3.02	162.9	119.1	/	-	/	/
p-Hydroxybenzoic acid	3.24	136.9	93.0	/	-	/	/
Salicylic acid	2.28	137.0	137.0	/	/	/	/
Sinapic acid	3.34	223.0	121.0	/	/	/	/
Vanillic acid	2.57	167.0	123.0	/	-	/	/

‘/’ = the phenolic compound was present in the samples; ‘-’ = the phenolic compound was absent in the samples.

It is an acknowledged fact that antioxidants, including phenolic acids, can protect cells against the damaging effects of reactive oxygen species (ROS) such as superoxide and hydroxyl radicals (Kadian *et al.*, 2016). Current and past data on phenolic acids support their beneficial effects toward improving human health, which include antioxidant, antimicrobial, and anticancer activities (Anantharaju *et al.*, 2016). The phenolic acids identified in the present work for the optimised sample, *B. angulata* fruit juice and TH (Table 4) reflected on past studies where they stated that most phenolic acids such as caffeic acid, cinnamic acid, and coumaric acid can be found in many fruits and vegetables, including the *B. angulata* fruit and TH (Ahmed *et al.*, 2015; 2017; Biluca *et al.*, 2017; Ranneh *et al.*, 2018). It was noted that the control sample, which was a jelly without *B. angulata* fruit juice and TH, showed no peaks for cinnamic, coumaric, synapic, and vanillic acids (Table 4). This could be due to the lack of *B. angulata* fruit juice and TH in the control sample. In general, these results highlighted the probability that most of the antioxidants were from *B. angulata* fruit juice and TH in the samples, as well as the fact that the cooking/heating process did not cause any significant effects to the overall phenolic content of the samples (Šarić *et al.*, 2013).

Many studies have reported the benefits of plants and honey as antioxidant agents to reduce the risk of diseases. For example, quercetin is known to have anti-inflammatory benefits and have exhibited anticancer activities, while rutin is a good free radical scavenger (Ahmed *et al.*, 2015). Polyphenols such as kaempferol, quercetin, and coumaric acid have been shown to inhibit the growth of human breast and oral cancer cells (Ghasemzadeh and Ghasemzadeh, 2011). Therefore, with the acknowledged ability of polyphenols to remove free radicals and be involved in inhibiting mechanisms that might lead to the occurrence of several diseases, the consumption of the soft jelly samples with high antioxidant content might be beneficial towards improving a person's health.

Conclusion

Response surface methodology was successfully implemented for optimisation of antioxidant capacity, total phenolic content, and total flavonoid content of the soft jelly samples. Subsequently, the model equation developed by the RSM was adequate in predicting the effects of the variables, which resulted in an optimal combination being developed for the responses. The optimised high antioxidant soft jelly generated was at a 40:40 ratio of *B. angulata* fruit juice and *Trigona* sp. honey. Moreover, the antioxidant,

phenolic, and flavonoid contents of the optimised soft jelly sample appeared to have been successfully verified through its antiradical properties that were estimated using DPPH and FRAP assays. Among the antioxidants that were present in the optimised soft jelly sample, 10 polyphenols were identified through UHPLC-MS/MS. Thus, based on their rich antioxidant properties, *B. angulata* and *Trigona* sp. honey have high potentials to be used in fortifying the soft jelly samples, making them prospective food supplements due to their nutritional and health benefits. Further studies are warranted to evaluate their antioxidant activities and to discover other nutritional benefits that could be extracted from the samples.

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