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Propolis extract with high antioxidant capabilities: Optimisation of sonication-assisted extraction parameters using Response Surface Methodology (RSM)

Nur Diyana, A.[1] , Koh, S. P.[1*], Mazlan, M. T.[2] and Chin, N. L.[2]

[1]Food Science and Technology Research Centre, MARDI Headquarters, 43400 Serdang, Selangor, Malaysia
[2]Department of Process and Food Engineering, Faculty of Engineering, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract

Propolis is a complex compound consisting of flavonoids, phenolics, terpenes, aromatic compounds, volatile oils and resin which contribute to different pharmacological properties. This study aims to optimise sonication-assisted extraction parameters in the production of bioactive metabolites with high antioxidant properties from propolis of stingless bees (*Heterotrigona itama)* using response surface methodology (RSM) comprising of three independent variables (amplitude 30 – 70%, cycle 0.3 – 0.7 unit and time, 20 – 40 min) with five levels. Antioxidant activities were analysed using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay, ferric reducing antioxidant power (FRAP) assay and total phenolic content (TPC). Comparison between the predicted and experimental value from central composite rotatable design (CCRD) optimisation procedures showed the best fitting model was a reduced cubic modified model with a good correlation with R square of 94.2%, 97% and 93.3% for DPPH, FRAP and TPC responses, respectively. The optimum sonication parameters for the production of propolis extract with the highest antioxidant activities (based on a combination of DPPH, FRAP and TPC responses) were determined with the amplitude of 52.95%, cycle of 0.52 unit and time of 31.86 min.

Keywords: Stingless bee, *Heterotrigona itama*, green technology, response surface methodology, antioxidant

Introduction

In Malaysia, apiculture development is booming rapidly in the agriculture industry due to their honey production. Malaysia with its abundant natural resources has a conducive environment to sustain the honey industry. In addition to honey, stingless bee produce several by-products from their beehives, such as propolis and beebread. The bee products and their by-products can be converted into value-added and functional health food. Compared to honey, little information is known about propolis from stingless bees. As such, our local farmers have neglected to harvest the propolis due to insufficient awareness of its economic value. Moreover, many literally unaware of the usefulness of propolis and there is a lack of scientific evidence to support the health claims of propolis.

Propolis displays a brownish-black colour with a soft, pliable and very sticky texture but turns solid and brittle at cold temperatures (Sforcin 2016). Propolis known as bee glue, acts as a defensive substance to protect the stingless bee colony from enemies. It is derived from natural products collected by the stingless bee from plant materials like resin and sap varied by bee species and geographical region (Usman et al. 2016). Stingless bees can provide a large number of propolis per hive compared to the honey bees (Nazir et al. 2018). For example, a stingless bee's hive can generate almost 150 g of propolis at a certain harvesting time. It is also reported to have a myriad of beneficial health claims such as antioxidant (Cao et al. 2018), antimicrobe (Molnar et al. 2017; Santos et al. 2017), antiinflammatory (Santos et al. 2017; Guzman-Gutierrez et al. 2018) and anti-cancer (Kustiawan et al. 2015; Bartolomeu et al. 2016) effects. These activities were contributed by the presence of its biologically active compounds, such as phenolic compounds (flavonoids, phenolic acids, and their esters), terpenoids and steroids (Huang et al. 2014). Numerous studies reported on the capability of high antioxidant components in propolis when compared to bee products (Ismail et al. 2018; Ahmad et al. 2019). However, there

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Authors' full names: Nur Diyana Alyas, Koh Soo Peng, Mohammad Tawfiq Mazlan and Chin Nyuk Ling
Corresponding author: karenkoh@mardi.gov.my

is a limitation on the antioxidant properties of specific bee species because of their variability in geographical areas and stingless bee species.

The solvent extraction is commonly used propolis extraction method. Ethanol and methanol have been extensively used to extract antioxidant compounds from various plant materials. Methanol extraction was more effective by recovering the highest amount of phenolic compounds (Chatha et al. 2006). However, methanol also possesses few drawbacks regarding safety issues and harm to food production. In this study, we performed a sonication extraction method using ultrasound waves to aid the solvent extraction of propolis. Ultrasound provide high reproducibility, lessen solvent usage, yielded high extract purity and is safer than conventional extraction methods (Chemat et al. 2017). Hence, this rapid and straightforward sonication extraction method was applied to extract the stingless bee propolis with a high yield of antioxidant activity. These findings will generate awareness on the importance of the propolis and assist our local apiaries industry to further manage its self-sufficiency level in the future.

The research aims to identify the optimal sonication process parameters using RSM to obtain propolis extract with the maximum antioxidant activity. In addition, the assays of ferric reducing power (FRAP), DPPH free radicals scavenging activity and total phenolic content (TPC) were performed to determine the antioxidant activities of sonication extracted propolis.

Materials and methods

Preparation of sample

Heterotrigona itama's propolis was collected from a local farm located in Shah Alam, Selangor. The propolis was kept at –20 °C and became brittle before being cut into small pieces. The sample was held in the bottle and placed in the freezer at –20 °C for further analysis (Mazlan 2018).

Optimisation by response surface methodology (RSM)

A response surface methodology (RSM) comprising of three independent variables (amplitude 30 – 70%, cycle 0.3 – 0.7 unit and sonication time, 20 – 40 min) with five levels were employed. Antioxidant activities were analysed using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay, ferric reducing antioxidant power (FRAP) assay and total phenolic content (TPC). The fractional factorial design consisted of eight factorial points, six axial points and six centre points. The complete design of 20 experimental points was carried out in random order. The regression polynomial equations have described the effects of three independent variables on antioxidant activities. Comparison between predicted and experimental values from central composite rotatable design (CCRD) was analysed before optimisation procedures. Design Expert (6.0.6, Stat-Ease Inc) software was used for the experimental design and statistical analysis. Three-dimensional surface response contour plots were generated by holding one constant variable at the central point and varying two variables in the observed range (Mazlan 2018).

Sonication-assisted extraction

Sonication was performed using an ultrasonic sonicator (ultrasonic processor UP400S). Approximately 2 g of sliced raw propolis were weighed in a conical flask and mixed with 100 ml of 70% ethanol. The mixture was placed in the sonication extractor with the sonication probe immersed at 1 cm in-depth into the mixture. The mixture was sonicated at a specific amplitude, cycle and sonication time according to the process parameter set by RSM experimental design. The extract was filtered through a Whatman No. 1 filter paper to remove residue and the filtrate was stored in the freezer at –20°C for antioxidant analysis.

Antioxidant activity

Determination of 1,1-diphenyl-2-picryl-2hydrazil (DPPH) free radical scavenging activity

The DPPH free radical scavenging activity was measured as described in Koh et al. (2012)'s procedure with some modifications. First, the propolis extract of 150 µL was mixed with a freshly prepared 2850 µL of DPPH methanolic solution and vortexed. The mixture was then incubated in dark conditions for 30 min at room temperature. The absorbance was read at 515 nm. Gallic acid was employed as the standard to set up the DPPH calibration curve.

Determination of ferric reducing antioxidant power (FRAP)

The experiments were conducted according to Koh et al. (2012), with minor modifications. Aliquots of 150 µL of propolis extract were mixed with 2850 µL of freshly prepared FRAP reagent. The mixture was incubated in dark conditions for 30 min at room temperature before the FRAP values were measured by comparing the absorbance change of blue coloured ferrous-tripyridyltriazine complex at 593 nm. The results were determined from the Ferrous sulphate standard calibration curve.

Total phenolic content

Total phenolic content of the ethanolic extract was determined using Folin Ciocalteu colourimetric method with a slight modification. First, propolis aliquots (1 mL) were mixed with 5 mL Folin Ciocalteu reagent and incubated at room temperature for 5 min. Subsequently, approximately 4 mL of 7.5% sodium carbonate solution was added and vortexed. Next, the mixture was incubated

in the dark for 2 hours before measuring the absorbance at 765 nm against the reagent blank. Finally, the amount of total phenolic content of propolis extract was expressed as Gallic acid equivalents (mg GAE/g extract) from a calibration curve.

Results and discussion

Amplitude, cycle and time were identified as the critical factors affecting the antioxidant activities of propolis using the sonication extraction method. In this study, we employed the RSM to explore the relationship between three independent variables (amplitude, cycle and time) to optimize the antioxidant activities of propolis extract (DPPH free radical scavenging, FRAP and TPC responses) from the sonication process parameters. Evaluating such qualities remains a fascinating and valuable undertaking, especially when looking for promising natural antioxidant sources. According to Milojkovic-Opsenica et al. (2016), the principal constituents of propolis are derived from flavones, flavonols, flavanone and dihydro flavonoids, as well as phenylpropanoid derivatives. Propolis has been described to possess a wide range of biological activities such as antioxidant, antibacterial, antifungal, antiviral, antiparasitic, anti-cancer, anti-inflammatory, antiulcer and antidiabetic due to the presence of these bioactive compounds (Pobiega et al. 2019). In this experiment, three independent variables were selected; amplitude 30 – 70%; cycle 0.3 – 0.7 unit; time 20 – 40 mins and the specified responses were DPPH free radical scavenging activity, FRAP and TPC. A set of 20 randomised experimental design was determined based on the CCRD as shown in *Table 1*. The antioxidant activities of DPPH free radical scavenging, FRAP and TPC were carried out according to the variables proposed by the CCRD design. The centre points of experimental runs produced propolis extract significantly higher antioxidant results than other runs.

The 3D surface plots were developed using the fitted reduced cubic modified model by constantly holding one independent variable at a particular value and changing two other variables to investigate the relationship among variable factors. *Figure 1* shows the response surface plot of the effect of independent variables; amplitude, cycle and time on antioxidant activities responses. The ability of antioxidants to scavenge DPPH radicals is linked to their ability to donate hydrogen (Pyrzynska and Pekal 2013). The response surface plot of amplitude and cycle interaction with the time constant at 31.89 min noted that the DPPH free radical scavenging activity increased with the amplitude increment from 30 to 60% (*Figure 1a*). Meanwhile, the interaction of amplitude and time with the cycle constant at 0.52 towards DPPH free radical scavenging activity was displayed in *Figure 1b*. A dome shape interaction was observed in these two plots. In this case, DPPH free radical scavenging activities were lower at shorter sonication time and showed higher DPPH

Table 1. Effect of sonication parameters on antioxidant activities of the *H. itama* propolis extract

Run	Type	Independent variables			Responses		
		A: Amplitude	B: Cycle	C: Time	DPPH (mg GAE/g)	FRAP (mM $FeSO_4$/g)	TPC (mg GAE/g)
1	Center	50.00	0.50	30.00	3.24 ± 0.17	1000.53 ± 41.32	12.84 ± 1.36
2	Center	50.00	0.50	30.00	3.25 ± 0.04	1065.70 ± 9.10	13.13 ± 1.02
3	Center	50.00	0.50	30.00	3.20 ± 0.05	1036.86 ± 54.03	14.20 ± 0.30
4	Center	50.00	0.50	30.00	3.26 ± 0.07	836.30 ± 37.57	13.63 ± 2.14
5	Center	50.00	0.50	30.00	3.36 ± 0.03	1099.76 ± 76.02	12.91 ± 2.54
6	Center	50.00	0.50	30.00	3.40 ± 0.04	1023.11 ± 5.64	13.65 ± 0.80
7	Axial	50.00	0.84	30.00	2.80 ± 0.04	430.04 ± 69.72	10.92 ± 1.11
8	Axial	50.00	0.50	13.18	2.61 ± 0.05	522.78 ± 28.77	10.68 ± 1.13
9	Axial	50.00	0.16	30.00	2.77 ± 0.09	237.28 ± 49.79	5.27 ± 0.07
10	Axial	50.00	0.50	46.82	2.79 ± 0.06	632.01 ± 13.80	12.03 ± 0.31
11	Axial	16.36	0.50	30.00	2.54 ± 0.04	437.48 ± 8.46	8.93 ± 1.77
12	Axial	83.64	0.50	30.00	2.60 ± 0.12	572.74 ± 2.90	11.04 ± 1.08
13	Fact	30.00	0.70	20.00	2.51 ± 0.14	469.92 ± 80.81	8.77 ± 0.93
14	Fact	70.00	0.70	20.00	2.55 ± 0.02	551.02 ± 46.52	10.41 ± 0.92
15	Fact	30.00	0.30	20.00	2.09 ± 0.06	200.87 ± 23.84	7.28 ± 0.00
16	Fact	70.00	0.30	40.00	2.63 ± 0.05	484. 20 ± 76.97	11.12 ± 0.85
17	Fact	30.00	0.30	40.00	2.41 ± 0.07	432.11 ± 46.83	6.96 ± 1.16
18	Fact	70.00	0.70	40.00	2.53 ± 0.04	483.55 ± 4.97	10.39 ± 1.19
19	Fact	70.00	0.30	20.00	2.42 ± 0.08	397.56 ± 9.11	7.74 ± 1.37
20	Fact	30.00	0.70	40.00	2.74 ± 0.19	476.19 ± 52.37	9.84 ± 1.54

antioxidant activity when the sonication time increased. *Figure 1c* shows the response surface plot of amplitude and cycle interaction with the time constant at 30 mins. The FRAP test is regarded as one of the essential elements in evaluating antioxidants in natural resources. The presence of reductants, which break free radical chains by donating a hydrogen atom, is usually connected with the existence of reducing power (Rahman et al. 2015). The antioxidant activities of FRAP were increased when the amplitude was increased from 30 – 50%. Higher FRAP activities of 982.618 mM $FeSO_4$/g were observed at the amplitude of 50% at a shorter extraction time of 25 mins, as shown in *Figure 1d*. As apparent from *Figure 1e*, higher activities of TPC (12.6987 mg GAE/g) were observed at 0.50 cycle and 40 amplitude in response surface plot of amplitude and cycle interaction with the time constant at 30 mins. The response surface plot of cycle and time interaction with the amplitude constant at 50% is exhibited in *Figure 1f*. This result is consistent with the previous findings by Golmahi and Elhamirad (2021) who reported a lengthy ultrasonic time reduces total phenolic compounds due to the entry of impurities into the solvent. A study by Ghafoor et al. (2009) reported that the extraction time increment might increase the diffusion of the substances to the solvent. Another Oldoni et al. (2015) study also reported the mass transported to the solvent was dependent on the extraction duration and temperature. Mass transfer increases with time until the maximum yield of extraction is attained while temperature improves extraction by increasing the diffusion rate. However, we need to be cautious as high temperatures can impair antioxidant activity (Golmahi and Elhamirad 2021). Comparison between the predicted and experimental value from the CCRD optimisation procedures showed the best fitting model was a reduced cubic modified model with a good correlation with R square of 94.20%, 97.00% and 93.30% for DPPH free radical scavenging, FRAP and TPC responses, respectively. Generally, propolis can be regarded as a great source of antioxidants and sonicated propolis extract appears to be a promising natural source of antioxidants.

To predict the maximal value of DPPH free radical scavenging activity, TPC and FRAP value of sonicated propolis extract in which the variables were set at the targeted range of amplitude (30 – 70 %), cycle (0.3 – 0.7 unit) and time (20 – 40 min), an optimal process parameter was generated from optimisation model software with amplitude (52.95%), cycle (0.52 unit) and time (31.86 min). This model was verified by running triplicate analysis and validated the experimental value with predicted value as generated by RSM under optimal process parameter condition. *Table 2* summarises the model verification of optimal process parameter of sonicated propolis extract with the accuracy percentage of DPPH free radical scavenging activity, TPC and FRAP value were 61.89%, 89.90% and 75.58%, respectively. This phenomenon indicated that different sonication process parameter may extract bioactive compounds at different level which indirectly may affect the antioxidant activity of DPPH free radical scavenging activity, TPC and FRAP due to different antioxidant mechanism action of bioactive compounds. Therefore, it creates uncertainty to optimize the process parameter in order to get the maximal antioxidant activity of DPPH scavenging activity, TPC and FRAP value at the same time, particularly for DPPH free radicals scavenging activity which undergone different antioxidant mechanism of action. This finding indicated that optimization modelling of process parameter is more suitable for predicting yield response under the same antioxidant mode of action. Nevertheless, the model verification has achieved the prediction accuracy of 75.50% and 89.90% for TPC and FRAP value under optimal process parameter.

Conclusion

The optimum process parameter for the propolis extraction method consisting of sonication amplitude, cycle, and time, was significantly determined by CCRD & RSM modelling. The sonication extraction methods significantly assisted into obtaining high antioxidant activities extracts from *H. itama* propolis. Based on the RSM modelling analyses, the optimised antioxidant activities of propolis extract were obtained with the process parameter of the sonication technique set at the amplitude of 52.95%, cycle of 0.52 unit and time of 31.86 min. Sonication extraction is believed to assist and simplify the extraction method as well as accelerate the extraction processing time of propolis.

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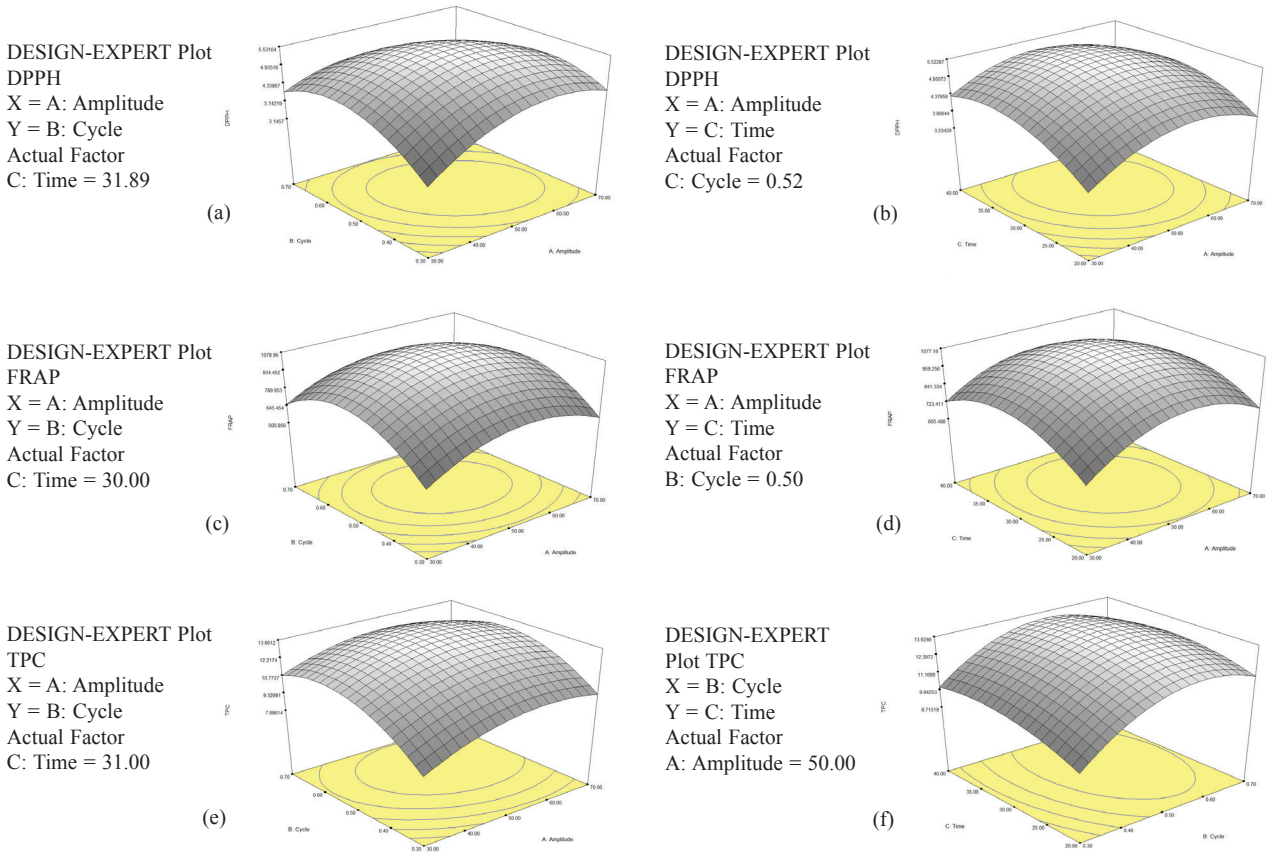

Figure 1. Response surface plots showing the effect of independent variables; amplitude, cycle and time on antioxidant activities responses:
a) The interaction effect of sonication amplitude and cycle on DPPH free radical scavenging,
b) The interaction effect of sonication amplitude and time on DPPH free radical scavenging,
c) The interaction effect of sonication amplitude and cycle on FRAP,
d) The interaction effect of sonication amplitude and time on FRAP,
e) The interaction effect of sonication amplitude and cycle on TPC and
f) The interaction effect of sonication cycle and time on TPC.

Table 2. Model verification of optimised process parameter on sonicated propolis extract

Optimised process parameter	Antioxidant activity	Predicted value	Experimental value	Accuracy
Amplitude (52.95%) Cycle (0.52 unit) Time (31.86 min)	1,1-diphenyl-2-picryl-2hydrazil (DPPH) free radical scavenging activity	5.51mg GAE/g	3.41 mg GAE/g ± 0.06	61.89 %
	Ferric reducing antioxidant power (FRAP)	1015.62 mM $FeSO_4$/g	767.56 mM $FeSO_4$/g ± 28.89	75.58 %
	Total phenolic acid (TPC)	13.66 mg GAE/g	12.28 mg GAE/g ± 1.18	89.90 %

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