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Optimization of polysaccharide extraction from Okra (*Abelmoschus esculentus*) by using response surface methodology

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ABSTRACT

Okra (*Abelmoschus esculentus*) pod is known as the polysaccharide-rich vegetable. The polysaccharides from natural sources were recently used in various purposes in food, pharmaceuticals and cosmetics formulations. In the recent study, hot water extraction of polysaccharide from okra pod was optimized by using response surface methodology (RSM). The extracted polysaccharides were determined for their percentage of yield and $ABTS^{\bullet+}$ radical scavenging activity. The optimum polysaccharide yield of 26.35% (w/w) was obtained when using extraction condition of 95°C for 5 h at pH 7. The optimum ABTS radical scavenging activity of 78.03 mg trolox equivalent antioxidant capacity (TEAC)/g was achieved when using the condition with 90°C for 3 h duration at pH 9. It can be seen that temperature was high significant to the extraction potential, while the time was not significant. The pH was also significant to the total polysaccharide content and antioxidant capacity. All experiments showed closely related to the predicted values. The percentage error of experimental polysaccharide yield and antioxidant capacity were 0.99 and 0.49%, respectively, when compare to those of predicted values. Therefore, the RSM could be applied for extraction optimization of okra polysaccharide which is a promising natural active ingredient for food, pharmaceuticals as well as cosmetics production.

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INTRODUCTION

Okra (*Abelmoschus esculentus*) is widely cultivated in the tropics, sub-tropical and temperature regions around the world including Asia, Africa and North-America. A net global production was found to be 6

million tons ha^{-1} and its total trade was more than $5 billion (Kontogiorgos et al, 2012). Okra pods are commonly used in Asia as a vegetable, food ingredients and traditional medicine for several purposes (Sengkhamparn et al., 2009). It has been reported that the immature fruit has been used in folk medicine as diurectic agent and for treatment of dental disease (Ndjouenkeu et al., 1996). The peel and seed have also been reported to have antidiabetic and antihyperlipidemic effect (Sabitha et al., 2011). Okra extracts obtained from fresh okra pod are naturally available, inexpensive and non-toxic biopolymer, which make okra an attractive resource for industrial application (Ghori et al., 2014). The polysaccharides within okra extracts are predominately pectins (Ghori et al., 2014) and recently the structure has been identified as pectic rhamnogalacturonan which contained a repeating unit of alternating α-(1-2)-linked methyl galacturonate and α-(1-4)-linked rhamnosyl residues with pentasaccharide side chain of β-(1-4)-linked galactosyl moieties or arabinosyl galactosyl moieties attached to O-4 of rhamnosyl backbone residues (Liu et al., 2018). The okra polysaccharide consists of four monosaccharide namely arabinose, galactose, rhamnose and galacturonic acid with approximate ratio of 1:18:6:6 and its molecular weight was calculated to be larger than 2.99x103 kDa (Liu et al., 2018). The polysaccharide form viscous solution that exhibit pseudoplastic behavior (Georgiadis et al., 2011)

Okra polysaccharide has been reported to have various biological activities and commercial availability. It is used as dietary for blindness, cataract and glaucoma development in type 2 diabetic patients (Moise et al., 2012). It can lower body weight and glucose level, improve glucose tolerance and decrease total serum cholesterol level in mice (Fan et al., 2013). It might be considered novel immunomodulatory because it could increase spleen index, splenocyte proliferation and cytokine secretion (Chen et al., 2016). It has the potential to serve as an adjuvant for diabetic nephropathy (Peng et al., 2016). The okra polysaccharide has been reported to be applied as drug release modifier, film former, scaffold for tissue engineering, emulsion stabilizer, anti-static agent, tablet coating agent and compressibility enhancer (Alba et al., 2013; Dimopoulou et al., 2014; Ghori et al., 2014a; Ghori et al., 2014b). Okra polysaccharide can also provide effective skin moisturization.

Okra polysaccharide has been extracted by water or aqueous buffer (Kpodo et al, 2017; Samavati, 2013; Ghori et al., 2014a; Sengkhamparn et al. 2009). Sequential extraction using a series of hot water extraction buffer and chelating agents yielded fractions consisting of pectin and highly branched rhamnogalacturonan has been revealed (Sengkhamparn et al., 2009). In the extraction processes, there are multiple independent factors affecting the extraction capacity. The possibility of interaction between the independent factors should also be considered in order to determine the optimal experimental condition. Response surface methodology (RSM) has been reported to be an effective tool for optimization of a process when the independent variables have a combined effect on the desired response (Samavati, 2013). RSM is a collection of statistical and mathematical system that has been successfully used for optimizing the process of extraction (Cui et al., 1994). Although the effect of extraction conditions on okra polysaccharides is well documented, the previous works only focus on extraction yield rather than polysaccharide content in the extract. Optimization of the extraction process with the monitoring polysaccharide content and antioxidant capacity of the extract has not been studied. Therefore, the purpose of the present study was to optimize the condition for polysaccharide extraction from *A. esculentus* using RSM and employing Box-Behnken Design (3 factors and 3 level) to study the effects of temperature, extraction time and pH on the polysaccharide content and antioxidant capacity.

MATERIALS AND METHODS

Chemicals and reagents

2,2'-azino-bis(3-ethylbenzthiazoline–6-sulphonic acid) (ABTS), and potassium persulphate were purchased from Sigma-Aldrich (USA). Sulfuric acid and phenol were purchased from Lab scan. Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was obtained from Aldrich (Steinheim, Germany). All other chemicals and solvents in this study were of analytical grade.

Plant material

Fresh okra fruits were collected from local market at Muang, Chiang Rai, Thailand during Jan 2018. After removal of the okra seeds, the pods were cut into small pieces and dried at 55°C until their weight was constant. The dried pods were pulverized and sieved through 60 mesh. The okra powder was stored at -20°C until used.

Extraction of polysaccharide from okra

The extraction of crude polysaccharide from okra pods was performed using a method modified from Samavati (2013). The polysaccharide from okra pod was extracted with hot water by using water bath shaker with 150 rpm. The residue was separated by centrifugation at 9000 rpm, 4°C for 30 min. The supernatant was further subjected to precipitation the polysaccharide by addition 3 volume of 95% ethanol. The mixture was kept at cold temperature for overnight. After centrifugation, the precipitate was lyophilized and stored at -20°C until used for further analysis.

Before studying the optimization, the okra powder to water ratio was investigated by varying between 1:10, 1:20, 1:30, 1:40 and 1:50 w/v. The okra polysaccharide was extracted by using hot water at 90°C with 4 h extraction. It was found that the ratio of sample to water of 1:40 (w/v) provided the highest yield of okra polysaccharide, therefore, this ratio was selected for the next optimization study.

Optimization of okra polysaccharide extraction

A three–level three factor Box–behnken design (BBD) was chosen to evaluate the combination effect of three independent variables: temperature, time and pH, coded as X_1, X_2 and X_3, respectively. Each independent variable had coded levels of -1, 0 and 1. For each factor, the experimental range was determined based on the results of single factor experiment (data not shown). Table 1 shows the range and center point values of the three independent variables based on the results of the above experiments.

Table 1. The levels of variables for the construction of Box–Behnken design (BBD).

Variables	Coded variables	Levels		
		-1	0	1
Temperature (°C)	X_1	85	90	95
Time (h)	X_2	3	4	5
pH	X_3	5	7	9

Determination of polysaccharide extraction yield

After freeze drying the precipitated polysaccharide, the percentage polysaccharide extraction yield (%) was calculated as follow:

$$\text{Polysaccharide extraction yield (\%) (w/w)} = \frac{\text{Dried polysaccharide powder (g)}}{\text{Okra powder weight (g)}}$$

Determination of total polysaccharide content

The total polysaccharide content of okra extract was determined by using phenol–sulfuric acid method modified from previous study of Chatchawal *et al*. (2010). Polysaccharide solution sample was mixed with 2.5 mL conc. sulfuric acid and then 0.5 mL of 5% aqueous phenol was added. The mixture was incubated at 50°C for 20 minutes and absorption at 490 nm was measured. The result was expressed as mg glucose equivalent per gram polysaccharide extract powder (mg GE/g)

ABTS radical scavenging capacity of okra polysaccharide

Antioxidant capacity of okra polysaccharide was determined by using ABTS radical scavenging assay (Bandasak et al. 2011). Trolox was used as standard. The radical form of $ABTS^{\bullet+}$ was generated by reacting the ABTS (7 mM) with potassium persulphate (2.45 mM). The mixture was allowed to stand in the dark place at room temperature for 16 h before used. When assay, the polysaccharide sample solution was mixed with ABTS solution and the mixture was incubated at room temperature for 30 min. The reaction was measured for its absorbance at 734 nm. Percentage radical inhibition was calculated as followed equation.

$$\% \text{ Inhibition} = \frac{(A_C - A_S) \times 100}{AC}$$

Where A_C is absorbance of control.

A_S is absorbance of the sample or standard trolox.

The antioxidant capacity of the okra polysaccharide was expressed as mg trolox equivalent per g polysaccharide powder (mg TEAC/g).

Statistical analysis

The response surface analysis was employed to determine the interaction and optimal level from mathematic model using the Design Expert Software (Trial Version 7.1.6, Stat-Ease, USA). A Box-Behnken experiment was employed in this regard. All the experiments were carried out in triplicate, and the results were expressed as means ± SD.

RESULTS AND DISCUSSION

Extraction of okra polysaccharide

Effect of sample to water ratio

Liquid-to-solid ratio is important factor in the process of conventional extraction. Generally, a larger solvent volume can dissolve constituents more effectively, leading to an enhancement of the extraction yield (Lee et al., 2005). However, this will induce the waste of solvent. On the contrary, lower levels of solvent will result in the lower yield because of it might not well dissolve constituents from the plant material (Valachovic et al., 2011). Therefore, the choice of a proper solvent volume is significant. In this experiment, the suitable okra powder to water ratio was evaluated. The okra polysaccharide extraction yields were different when the ratio of water and okra were varied. Under the fixed conditions of other factors, the maximum extraction yield of okra polysaccharide was observed at 1:40 w/v ratio. This ratio was used to be fixed factor in the optimization study.

Data analysis and evaluation of the model

The extraction yield of polysaccharide, total polysaccharide content and radical scavenging capacity of okra polysaccharide extraction (ABTS) were considered as dependent variable or response and for a Box–Behnken Designs with three independent variables at three levels, 17 experimental runs were required.

Test results of significance for regression coefficient of extraction yield

By employing multiple regression analysis and the data in Table 1, the predicted response *Y* for extraction yield okra polysaccharide can be obtain by the following second–order polynomial equation:

$$Y = 21.55 + 0.61X_1 + 0.16X_2 + 0.28X_3 + 0.40X_1.X_2 + 0.060X_1.X_3 + 0.24X_2.X_3 + 1.17X_1^2 + 2.19X_2^2 - 13.04X_3^2$$

This where in X_1, X_2 and X_3 are in term coded factor of test variables of temperature, time and pH, respectively. Table 2 shows the assessed with various descriptive statistics. The F-value of model was 408.90, implied that the model was significant. The determination coefficient ($R^{2)}$) was 0.9981, which indicated good agreement between experimental and prediction values of yield polysaccharide extraction. Lack of fit F-value is test for comparing lack of fit variance with pure error variance, which the variances are close to the same, the ratio will be close to 1 and it is less likely that lack of fit is significant (Muthukumar et al., 2003). Lack of fit F-value was 0.47 indicated that was not significant and relative to the pure error (p=0.7721). The 2.62% of coefficient of variation (C.V. %) was below 5%. The below 5% of C.V was clearly indicated a very high degree of precision and a good deal of reliability of the experimental values (Sun *et al.,* 2010). The model's predicted residual sum of squares (PRESS), a measure of how a particular model fits each point in design, was 7.37. The value of Pred R^2 (0.9899) was in reasonable agreement with Adj R^2 of 0.9957.

The 3D response plot in Figure 1 shows significant effect of the linear term of temperature (X_1) on extraction yield. Increase in the temperature significantly increased the extraction yield. High temperature increases the ability of water to solubilize the compounds and reduce the viscosity of the liquid solvent which is allowing better penetration of the solvent into the solid matrix (Samavati, 2013). Non-significance ($P<0.05$) of the linear term of

Table 1. The central composite experimental and test result of yield polysaccharide, total polysaccharide content and antioxidant activity.

No.	Variables			Yield of polysaccharide extraction (%)		Total polysaccharide content (mg GE/g)		Antioxidant activity (mg TEAC/g)	
	X_1	X_2	X_3	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
1	-1	-1	0	24.54	24.28	14.78	15.29	42.12	41.84
2	1	-1	0	24.96	24.99	18.20	18.40	32.86	33.23
3	-1	1	0	24.07	24.04	15.55	15.35	37.86	37.49
4	1	1	0	26.09	26.35	19.96	19.45	33.27	33.55
5	-1	0	-1	8.86	8.90	22.81	22.41	54.75	55.50
6	1	0	-1	9.96	9.71	26.79	26.70	38.24	38.34
7	-1	0	1	9.29	9.54	18.25	18.34	72.48	72.38
8	1	0	1	10.63	10.59	22.09	22.49	75.13	74.37
9	0	-1	-1	10.51	10.73	28.92	28.81	47.62	47.15
10	0	1	-1	10.35	10.34	30.52	31.12	48.41	48.03
11	0	-1	1	10.58	10.59	24.63	24.03	77.65	78.03
12	0	1	1	11.39	11.18	25.54	25.65	73.01	73.48
13	0	0	0	21.55	21.09	16.66	16.70	40.80	40.26
14	0	0	0	21.55	21.13	16.66	16.70	40.80	40.56
15	0	0	0	21.55	21.96	16.66	16.70	40.80	40.71
16	0	0	0	21.55	21.37	16.66	16.60	40.80	41.16
17	0	0	0	21.55	22.21	16.66	16.60	40.80	41.31

X_1; Temperature (°C), X_2; Time (h), X_2; pH

time (X_2) and pH (X_3) was observed which could be explained that duration time of extraction and pH were not linear correlation with the extraction yield. However, quadratic terms of time (X_2^2) and pH (X_3^3) were significant like the temperature (X_1^1) ($P<0.05$). This could be described by alteration of these variables resulting in exponential change of the extraction yield. Samavati (2013) found that extraction time was the largest effect to the extraction yield, followed by water to raw material ratio, extraction cycle and temperature, respectively. However, interaction term and quadratic terms were found to be insignificant (Samavati, 2013).

Table 2. Test results of significance for regression coefficient of yield okra polysaccharide.

Variables	Sum of squares	df	Mean Square	F value	p - value
Model	731.18	9	81.24	408.90	<0.0001
X_1	2.98	1	2.98	14.98	0.0061
X_2	0.22	1	0.22	1.09	0.3313
X_3	0.61	1	0.61	3.09	0.1222
$X_1 X_2$	0.64	1	0.64	3.22	0.1158
$X_1 X_3$	0.014	1	0.014	0.072	0.7955
$X_2 X_3$	0.24	1	0.24	1.19	0.3117
X_1^2	5.75	1	5.75	28.96	0.0010
X_2^2	20.27	1	20.27	102.01	<0.0001
X_3^2	715.53	1	715.53	3601.31	<0.0001
Residual	1.39	7	0.20		
Lack of fit	0.36	3	0.12	0.47	0.7221
Pure error	1.03	4	0.26		
Total model	732.57	16			
R^2	0.9981		Adj R^2	0.9957	
C.V.%	2.62		Pred R^2	0.9899	
PRESS	7.37		Adeq R^2	50.404	

X_1; Temperature (°C), X_2; Time (h), X_3; pH

Test results of significance for regression coefficient of radical scavenging activity of okra polysaccharide extraction (ABTS)

The predicted response *Y* for extraction yield okra polysaccharide can be obtained by the following second–order polynomial equation:

$$A = 40.80 - 3.47X_1 - 0.96X_2 + 13.66X_3 + 1.17X_1.X_2 + 4.97X_1.X_3 - 1.36X_2.X_3 - 2.90X_1^2 - 1.37X_2^2 + 22.25X_3^2$$

Table 3. Test results of significance for regression coefficient of radical scavenging (ABTS) of polysaccharide extraction.

Variables	Sum of squares	df	Mean Square	F value	p - value
Model	3796.39	9	421.82	957.66	<0.0001
X_1	96.05	1	96.05	218.06	<0.0001
X_2	7.41	1	7.41	16.83	0.0046
X_3	1491.67	1	1491.67	3386.54	<0.0001
$X_1 X_2$	5.45	1	5.45	12.38	0.0098
$X_1 X_3$	91.68	1	91.68	208.14	<0.0001
$X_2 X_3$	7.37	1	7.37	16.73	0.0046
X_1^2	35.38	1	35.38	80.32	<0.0001
X_2^2	7.95	1	7.95	18.04	0.0038
X_3^2	2083.77	1	2083.77	4730.77	<0.0001
Residual	3.08	7	0.44		
Lack of fit	2.34	3	0.78	4.17	0.1007
Pure error	0.75	4	0.19		
Total model	3799.48	16			
R^2	0.9992		Adj R^2	0.9981	
C.V.%	1.35		Pred R^2	0.9899	
PRESS	38.55		Adeq R^2	87.993	

X_1; Temperature (°C), X_2; Time (h), X_3; pH

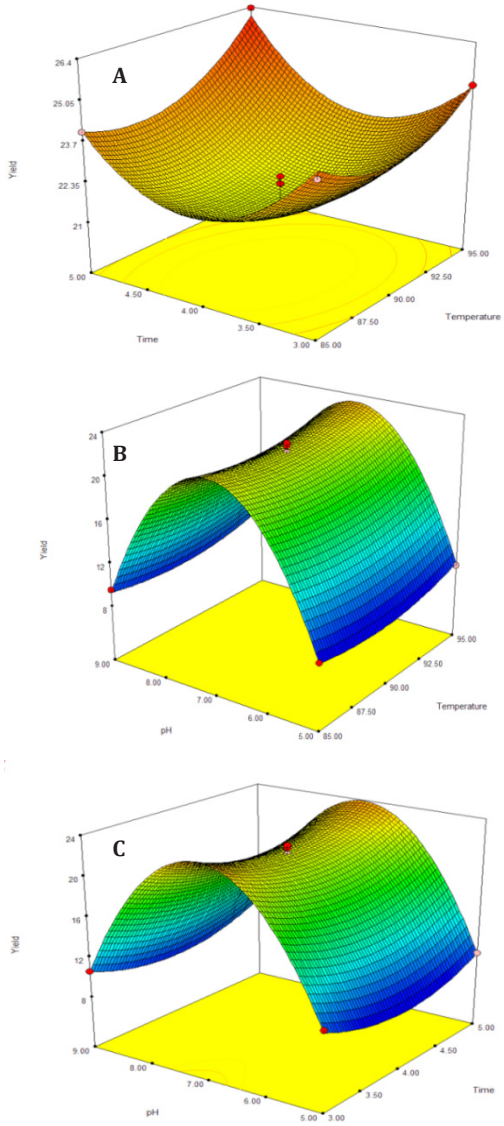

Figure 1. The 3D response surface plot and contour plot of polysaccharide extraction yield (A; temperature & time, B; temperature & pH and C; time & pH).

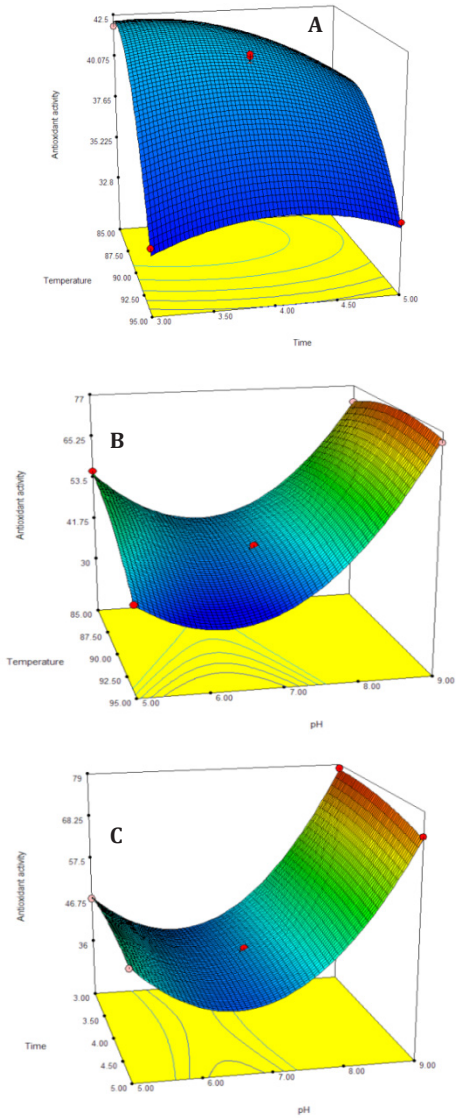

Figure 2. The 3D response surface plot and contour plot of antioxidant capacity (A; temperature & time, B; temperature & pH and C; time & pH).

This where in X_1, X_2 and X_3 are in term coded factor of test variables of temperature, time and pH, respectively. Table 3 shows the analysis of variance results for the ABTS radical scavenging activity. The F-value of model was 957.66. The determination coefficient (R^2) was 0.9992. Lack of fit F-value was 4.17 that there was not significant and relative to the pure error. The 1.35% of coefficient of variation (C.V. %) was below 5%. The model's predicted residual sum of squares (PRESS) was 38.55. The value of Pred R^2 (0.9899) was in reasonable agreement with Adj R^2 of 0.9981.

The 3D response of radical scavenging activity of okra polysaccharide was aligned on Figure 2. Higher antioxidant capacity was obtained when increased temperature. It can be seen that the linear coefficients (X_1, X_2 and X_3), a quadratic term coefficients (X_1^2, X_2^2 and X_3^2) and cross product coefficients (X_1X_2, X_1X_3 and X_2X_3) were significant, with very small P values ($P<0.05$). The three-dimensional (3D) are the graphical representation of response equation obtained from the calculated response surface using Design-Expert. Each model shows the correlation between responses and experimental levels of each variable, which relationship between two factors variable and their optimum range can be seen. It is very easy and convenient to understand the interaction between two variables and to locate their optimum range (Liu et al., 2013).

The result of analysis and response of yield polysaccharide and ABTS radical scavenging activity revealed the relationship of three factor variable that important to give the optimum condition. Temperature was the major factor that ranged between 90-95°C gave the high effectively of extraction. It can be described that temperature was a strong effect on the mass transfer rate of the water-soluble polysaccharides in the cell wall (Singthong et al., 2008). The water extraction using high water to the sample ratio at high temperature led to an increase in extraction yield of polysaccharide (Samavati, 2013). The accumulation of water in the sample leads to the binding of the water-soluble components, thus raising the extraction yield. On the other hand, the presence of a higher amount of water makes less sticky slurry, thus providing a more efficient extraction of the mucilage (Tabatabaee Amid and Mirhosseini, 2012; Samavati, 2013). Duration of extraction was not significant in this study contrasting with previous work of Samavati (2013), a longer extraction time present a positive effect of polysaccharide extract.

Increase in temperature and time of extraction condition provided the raise of antioxidant capacity. This was similar contour to the extraction yield which higher yield of polysaccharide extraction corresponded to higher ABTS radical scavenging capacity.

Polysaccharide content and structure including monosaccharide composition, molecular weight and configuration related to antioxidant activity (Tian et al., 2011). Moreover, biological activities including antioxidant activity of polysaccharide are closely related to the presence of polyanioinic charge (Xiao et al., 2012). Structure of okra polysaccharide contains galacturonic acid accounted for around 19% (Liu et al., 2018). This might contribute its antioxidant capacity. It should be noted that neutral pH gave the highest okra polysaccharide yield, but alkaline condition dramatically increased the antioxidant activity. This might be due to high pH condition altered the polysaccharide configuration and promoted the presence of anionic from acid monosaccharide resulting in rising of antioxidant capacity.

Optimization of polysaccharide extraction and verification of predictive model

As showed in Table 4, the optimum condition to provide the highest polysaccharide yield was 95°C, 5h duration and using buffer pH 7. The predicted value was 26.09% and the actual experimental data was 26.35%. Solvent extraction as water with high temperature was a factor to give high yield extraction (Carr *et al*., 2011). The optimum of antioxidant activity (ABTS) was found to be 90°C, 3 h with buffer pH 9. The predicted value was 77.65 mg TAEC/g and real experimental was 78.03 mg TAEC/g. Good agreement must exist between the values predicted using the model equations and the experimental values at the point of interest. To ensure the predicted result was not biased toward the practical value, experimental rechecking was performed using this deduced optimal condition. This condition was also used to validate experimentally and predict the values of the responses using the model equation (Samavati, 2013). In this study, there was no significant difference between experimental and predicted values, suggesting that the response model was adequate for reflecting the expected optimization.

Table 4. Predicted and experimental values of the responses at optimum conditions.

Responses	Optimum condition			Predicted	Experimental
	Temperature (°C)	Time (h)	pH		
Yield polysaccharide (%)	95	5	7	26.09	26.35
Antioxidant activity: ABTS (mg TAEC/g)	90	3	9	77.65	78.03

CONCLUSIONS

In this study, hot water extraction technique was employed to extracted okra polysaccharide by yield extraction polysaccharide and radical scavenging of okra polysaccharide extraction were perform with Box – Benhken Design and used RSM to indicate the experimental result of optimized extraction parameters. The optimized experimental of extraction yield polysaccharide was extracted at temperature 95°C, 5 h with buffer pH 7 and radical scavenging auctioning of okra polysaccharide extraction (ABTS) was extracted at 90°C, 3 h with buffer pH 9 which was agreed closely with the predicted value. The extracted okra polysaccharide possessing antioxidant capacity is promising to be used as active ingredient in cosmetic products.

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