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Application of Box-Behnken Design for the Extraction of *Padina australis*

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ABSTRACT. Optimization extraction of the brown algae *Padina australis* using the Box-Behnken design has been carried out. Box-Behnken design in relation to Response Surface Methodology analysis was conducted with four experimental factors (i.e., solvent concentration, temperature, extraction time, and sample to solvents ratio) towards the responses of yield antioxidant, anti-tyrosinase, anti-glycation, total phenolic content, and fucoxanthin content, completing with 29 running experiments. *P. australis* extraction's optimum condition was acquired at 79.99% solvent concentration, 18.48 hours extraction time, 44.50°C temperature, and 1:9 ratio powders and solvents. The optimum condition provided a 7.30% extraction yield, 43.94% antioxidant activity, 86.83% anti-tyrosinase, 98.06% anti-glycation, 9.53 mg GAE/g total phenolic content, and 347.55 μg/g fucoxanthin content. Respond Surface Methodology analysis with the Box-Behnken design succeeded in making the appropriate model for producing the optimum *P. australis* extract.

Keywords: Antiglycation, antioxidant, box Behnken design, *Padina australis,* tyrosinase inhibitor

INTRODUCTION

Seaweeds have been the subject of many studies and are widely used in the cosmetics industry due to the chemical constituents and unique properties. Brown seaweed is a source of economic value metabolites such as carotenoids, laminarin, alginate, fucoidan, mannitol, phlorotannin, vitamins, and macro and microelements (Demirel et al., (2012); Pereira (2018). Brown seaweed has the potential to be developed as a cosmetic ingredient with antioxidant, anti-inflammatory, antiallergy, UV protection, whitening agents, and matrix metalloproteinase (MMP) inhibitors (Jeon et al., 2012).

Seaweeds in Indonesia have become a potential export commodity, especially in the form of dried seaweed, while seaweed-derived products for domestic use depend mostly on import. Their further processing is essential to increase economic value, such as the production of seaweed extract for cosmetics (Indarwati et al., 2015). Based on their pigment, seaweeds are classified into three groups: brown seaweeds (Phaeophyceae), red seaweeds (Rhodophyceae), and green seaweeds (Chlorophyceae). Among these seaweeds, brown and red seaweeds have more economic value (Lee et al., 2017).

Padina australis that included in the *Phaeophyta* which is abundant along Indonesia's coastal waters. This seaweed has been reported to have interesting bioactivity. *P. australis* extract was previously reported to contain some secondary metabolites, such as alkaloid, flavonoid, terpenoid, saponin, phenol-hydroquinone, and tannin (Sachindra et al., 2007). Among the 20 species of seaweed tested from Indonesia waters, *P. australis* had the highest DPPH antioxidant activity, total phenolic, and fucoxanthin content (Nursid et al., 2016). *P. australis* has efficacy as a reducer of reactive oxygen species (ROS) that can be developed as a mitochondria-targeted antioxidant in the treatment of depression (Subermaniam et al., 2020). Other study showed that *P. australis* had interesting antiadipogenic and pro-adipolytic activity so that it has the potential to be developed in antiobesity therapy (Jaswir et al., 2017). Phytochemical constituents in the extract mainly fucoxanthin and phenolic compounds act as an antioxidant, antiobesity, antiinflammation, and anticancer (D'Orazio et al., 2012). *P. australis* and *Euchma cottonii* seaweed were formulated as a sunscreen (Nurjanah et al. (2020).

The extraction of fucoxanthin from *P. australis* can be easily carried out, depending on the solvent used.

Methanol is considered the most favorable solvent for fucoxanthin extraction (Limantara & Heriyanto, 2011), but its use is hindered with high toxicity; thus, methanol is used in the extraction process for producing cosmetic products is considered unsafe. Therefore, ethanol is applied in this present work due to being less toxic, making it more applicable for further use in cosmetic products. Brown seaweed that was extracted under various conditions resulted variety of yield and quality (Shannon & Abu-Ghannam, 2018). Hence, it is necessary to optimize the extraction process, such as temperature, concentration, time, and solvent to brown seaweed ratio for achieving the best yield and quality of *P. australis* extract. Temperature of extraction, concentration of solvent, extraction time, and solvent ratio will affect the extraction yield and quality of extract, especially when using the maceration technique as we use in this study (Nawaz et al., 2020).

The optimization was performed according to the response surface methodology with the Box-Behnken design. Response Surface Methodology (RSM) is a collection of statistical tools and process optimization of design products for optimization (Myers et al., 2004). The Box Behnken is a non-factorial experimental design in which each experiment involves the mean value of each factor (Yin & Dang 2008). The Box Behnken design was chosen because it requires less processing than other experimental designs so it is more suitable for use with four variables. This research aims to determine the most desirable condition to extract the active component from *P. australis*. The desirable condition should produce an extract with high yield, total phenol, fucoxanthin content, antioxidants, antityrosinase, and antiglycation activities.

EXPERIMENTAL SECTION

Brown seaweeds were collected from Binuangeun, Lebak District, Banten Province, Indonesia, in September 2018. Identification was conducted at the Center for Oceanographic Research, Indonesian Institute of Sciences, Jakarta. The samples were washed thoroughly using freshwater, immediately preserved on ice inside the cool box, and stored in -10°C shortly after arriving at the laboratory for subsequent analysis. Chemicals used included ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tyrosinase, aminoguanidine, Bovine Serum Albumin (BSA), kojic acid, ascorbic acid, L-tyrosine, glucose, fructose, phosphate buffer, Folin Ciocalteu, Na_2CO_3, gallic acid, fucoxanthin standard, dimethyl sulfoxide.

Experimental Design

In this study, the factors were solvent concentration, temperature, extraction time, and sample to solvent ratio. The minimum and the maximum level of each factor were shown in **Table 1**. Based on Box Behnken design, there were 29 experimental runs in which consisted of 24 runs of the mean factors and a centroid with five replications. Supplementary 1 shows the 29 combinations of experimental runs.

The quadratic model is usually used for optimization (Box et al., 1978). The model involves the linear effects, the quadratic effect, and the interaction of two factors. The model is defined as

$$Y_i = \beta_0 + \sum_{i=0}^{4} \beta_i X_i + \sum_{j=0}^{4} \beta_{ii} X_i^2 + \sum_{i=0}^{4} \sum_{j=0}^{4} \beta_{ij} X_i X_j + \varepsilon_i \quad (1)$$

where Y is the measured response associated with each factor level combination; β_0 is an intercept; β_i is regression coefficients computed from the observed experimental values of Y; and X_i is the coded levels of the factors. The terms X_iX_j and X_i^2 represent the interaction and quadratic terms, respectively. β_{ii} is the coefficient of the quadratic effect, whereas β_{ij} is the coefficient of the interaction effect of two factors. The experimental data were fitted to a second-order polynomial model as Equation (1). The model was evaluated by ANOVA, lack of fit, and coefficient determination (R^2).

Extraction of Seaweeds

Seaweeds were dried using a drier for a day at 40 °C and subsequently pulverized using a blender. The dried seaweed (50 g) was extracted using ethanol then macerated at different times and temperature levels (**Table 2**). As presented in **Table 1**, the levels of each variable were determined according to Box Behnken, resulting in 29 treatments, as shown in **Table 2**. After maceration, filtrate and residue were separated using Whatman 42 filter paper. The filtrate was evaporated using a vacuum rotary evaporator.

Determination of Antioxidant Activity

Antioxidant activity was determined by the DPPH method (Batubara, Mitsunaga, & Ohashi, 2009). One mg sample was dissolved in 1 mL methanol then taken (100 μL) and mixed with 100 μL of DPPH (125 μM). The mixture was poured into 96 microwell plates, incubated for 30 min, and analyzed for absorbance at 512 nm in a microplate reader (Thermo Fisher Multiskan™ GO). The positive control was used, i.e., ascorbic acid, while methanol was used as a blank solution.

$$\%\text{Inhibition} = \left[1 - \frac{(\text{A sample} - \text{A1})}{(\text{A0} - \text{A1})}\right] \times 100\%$$

Ao = Absorbance control negative
A1 = Absorbance control Positive
A sample = Absorbance sample

Determination of Tyrosinase Inhibition

The tyrosinase inhibition of extract was determined based on a method as described before (Batubara & Adfa, 2013). The extract (1.0 mg) was dissolved with 20 - 50 μL of DMSO and then added with buffer phosphate 50 mM (pH 6.5) until the volume was 1000 μL. The sample solution (70 μL) was transferred into a 96 microwell plate, added with 30 μL of tyrosinase (Sigma, 333 U/mL in buffer phosphate), and incubated for 5 min. Subsequently, 110 μL of a

substrate (L-tyrosine 2 mM) was added and incubated at 37°C for 30 min. Absorbance was determined at a wavelength of 492 nm using a Microplate reader (Biotek Instrumen 800™ TS Absorbance Reader). Kojic acid was used as a positive control.

$$\%\text{Inhibition} = [\frac{(\text{A blank- Asample})}{(\text{Ablanko})}] \times 100\%$$

Ablank = Absorbance negative control or blank
A sample = Absorbance sample

Determination of Antiglycation Activity

Antiglycation activity was analyzed by method that described previously (Ariansyah, Batubara, Lestari, & Egra, 2019). The extract was dissolved in distilled water and made to a concentration of 1.0 mg/mL. Test solutions were prepared, i.e. solution A (200 µL of buffer phosphate 200 mM (pH 7.4), 80 µL of BSA 20 mg/mL, 40 µL of glucose 235 mM, and 40 µL of fructose 235 mM), solution B (200 µL of buffer phosphate 200 mM (pH 7.4) and 80 µL of BSA 20 mg/mL), solution C (200 µL of buffer phosphate 200 mM (pH 7.4), 80 µL of BSA 20 mg/mL, 40 µL of glucose 235 mM, 40 µL of fructose 235 mM, and 80 µL of extract/aminoguanidine), and solution D (200 µL of buffer phosphate 200 mM (pH 7.4), 80 µL of BSA 20 mg/ml, and 80 µL of extract/aminoguanidine). These solutions were incubated at 60°C for 40 h, and each solution (100 µL) was then transferred into a 96 microwell plate. The glycated BSA was then measured using a fluorometer (FLUOstar Omega Multi-Mode Microplate Reader) at an excitation wavelength of 370 nm and an emission of 440 nm. Aminoguanidine was applied as a positive control.

$$\%\text{Inhibition} = [\frac{(A - A0)}{(B - B0)} \times 100\%]$$

A = intensity of sample solution
A_0 = intensity of corrected sample solution
B = intensity of control solution
B_0 = intensity of corrected control solution

Quantification of Total Phenol

The total phenolic content (TPC) was determinated by a modified Folin-Ciocalteu method as described in the previous report (Premakumara, Abeysekera, Ratnasooriya, Chandrasekharan, & Bentota, 2013). Gallic acid was used as a reference compound. The TPC was estimated from an equation derived from a gallic acid standard curve. Results were expressed as milligram gallic acid equivalents (GAE)/g extract.

Quantification of Fucoxanthin

Fucoxanthin quantification was analyzed by HPLC as described previously (Nursid & Noviendri, 2017). The sample was eluted using water and acetonitrile with a gradient system for 30 min at a flow rate of 0.2 mL/min using C18 column (Phenomenex 2.0 x 250 mm) and photodiode array detector (PDA). The extract (1.0 mg/ml) was injected into the HPLC (Shimadzu) via autosampler. The fucoxanthin was quantified by using standard curve of fucoxanthin.

Optimization using RSM Analysis

The optimal condition of response was achieved based on the model in Equation (1). The experimental responses included yield, antioxidant, antiglycation, antityrosinase, total phenol, and fucoxanthin concentration. The quadratic model was implemented for all responses. The experimental data were analyzed by software MINITAB. The optimum condition of all responses was based on the desirability value ranging from 0 to 1.0 (least to most desirable, respectively). Ultimately, the optimum condition of extraction by model was verified.

RESULTS AND DISCUSSION

Based on the data in **Table 2**, statistically, the quadratic model could explain the TI and TPC (see **Table 3**). **Table 3** shows the estimation of the regression coefficients and the goodness of fits of the model. The ANOVA test for TI and TPC were significant at α=5% and the ANOVA test for lack of fit test were not significant. On contrary, the ANOVA test for Yield and AG was not significant but the ANOVA test of lack of fit were significant. It is because there were unusual observations on Yield and AG. An unusual observation is that the difference between the actual response and the prediction response is large. Observation number 1 (one) and 5 (five) in **Table 2** were unusual observations on Yield. The response predictions of those observations were higher than the experimental observation. Furthermore, observation number 1 (one) was also the unusual observation on AG. Unlike Yield, the response prediction of the observation was lower than the actual response.

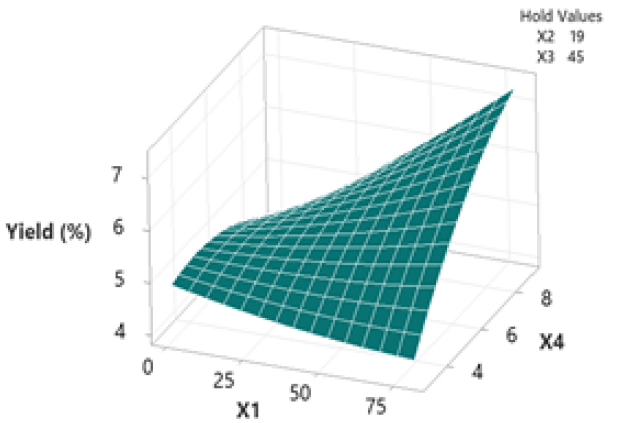
Table 1. Level of factors for Box Behnken design experiment

Independent variable	Level		
	-1	0	+1
Solvent concentration	0	40	80
Temperature	25	35	45
Extraction time	8	16	24
Sample-to-solvent ratio	1:3	1:6	1:9

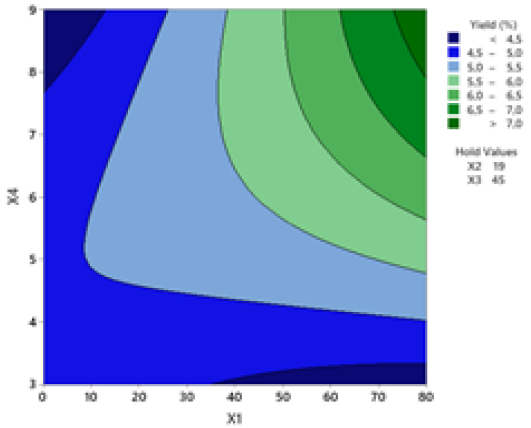
Table 2. Designed experiment by using the Box Behnken design

Run	Variable				Response					
	Concentration (%)	Time (h)	Temperature (°C)	Ratio powder & solvent (g/mL)	Yield (%)	Anti-oxidant activity (AOC) (%)	Tyrosinase inhibitor (TI) (%)	Anti-glycation (AG) (%)	Total phenolic content (TPC) (mg GAE/g)	Fucoxanthin content (FC) (μg/g)
1	0	16	35	1:9	3.36	40.65	22.55	60.36	2.64	5.04
2	0	8	35	1:6	5.20	42.92	23.82	65.55	3.07	2.62
3	80	16	35	1:9	**6.86**	**71.40**	**76.54**	**88.81**	**7.57**	**358.74**
4	40	24	25	1:6	4.33	53.35	47.34	76.54	3.83	4.61
5	40	24	45	1:6	3.85	54.63	36.80	78.16	5.62	9.78
6	40	8	35	1:3	4.02	38.91	28.30	64.15	2.73	16.29
7	40	16	25	1:3	5.83	44.46	35.08	75.01	5.28	38.16
8	80	16	25	1:6	4.94	35.61	61.78	61.15	4.23	165.91
9	80	16	45	1:6	**7.00**	53.35	**86.83**	81.24	4.20	358.90
10	0	16	25	1:6	5.33	52.95	30.54	54.81	3.82	9.79
11	80	8	35	1:6	5.04	39.43	61.72	79.99	6.29	120.48
12	0	24	35	1:6	5.44	47.77	20.57	64.75	3.73	11.51
13	40	16	35	1:6	5.81	54.20	26.96	71.16	6.22	50.10
14	40	24	35	1:3	4.70	**67.62**	33.92	64.28	4.69	5.29
15	40	8	45	1:6	4.45	17.31	20.82	**97.21**	4.93	197.30
16	80	24	35	1:6	4.86	63.73	51.05	44.13	5.86	**296.36**
17	40	16	35	1:6	5.71	55.52	35.33	73.91	6.75	50.14
18	40	16	45	1:9	5.70	40.76	41.60	80.30	**7.54**	9.51
19	40	8	25	1:6	5.80	37.25	42.42	87.76	4.76	41.32
20	40	16	35	1:6	5.35	53.79	32.90	70.55	6.03	50.11
21	0	16	35	1:3	5.61	34.22	16.86	70.32	5.96	5.45
22	0	16	45	1:6	5.66	21.67	19.74	50.39	3.93	5.25
23	40	16	35	1:6	5.83	51.47	35.14	72.59	6.61	50.12
24	40	16	25	1:9	4.59	**69.55**	35.52	42.19	5.43	10.27
25	40	16	35	1:6	5.92	56.10	23.57	75.61	7.21	54.40
26	80	16	35	1:3	5.16	57.42	62.62	47.29	3.25	75.84
27	40	8	35	1:9	5.54	40.73	39.03	59.51	7.37	6.33
28	40	24	35	1:9	4.54	49.32	44.79	73.43	5.96	4.10
29	40	16	45	1:3	4.39	38.03	41.91	61.81	3.57	3.98

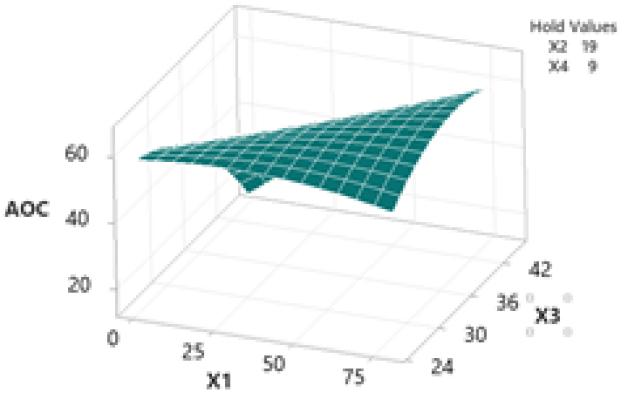
Note: Note: GAE is gallic acid equivalents



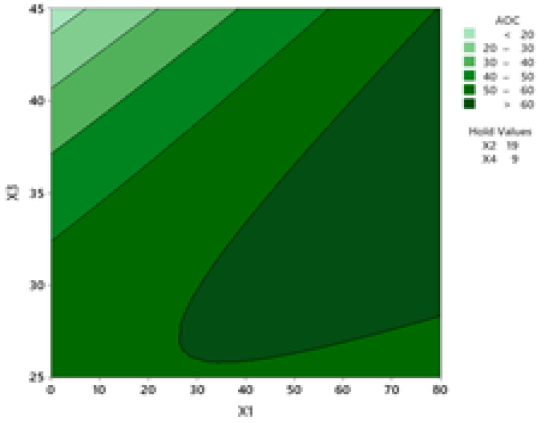
1a. The response surface of Yield by X_1 and X_4



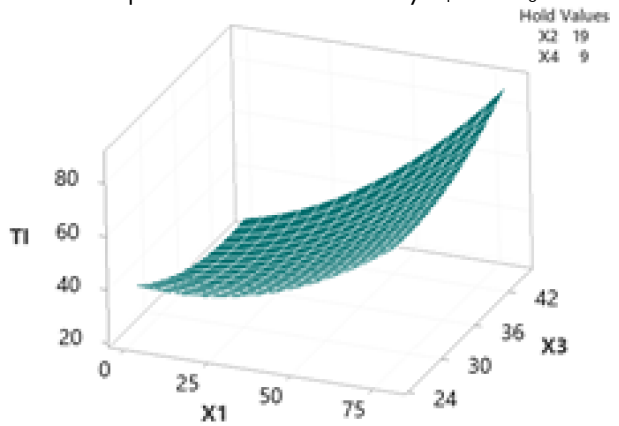
1b. The contour plot of Yield (%) by X_1 and X_4



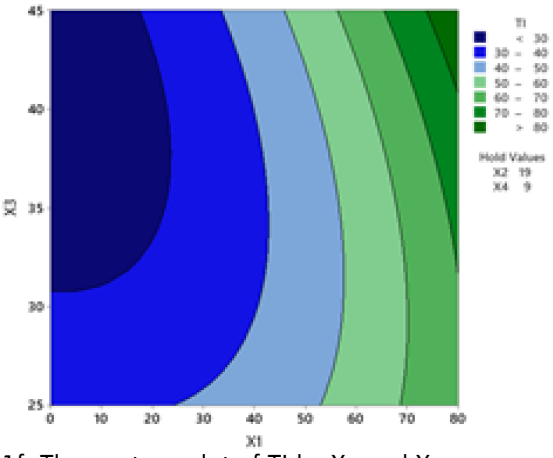
1c. The response surface of AOC by X_1 and X_3



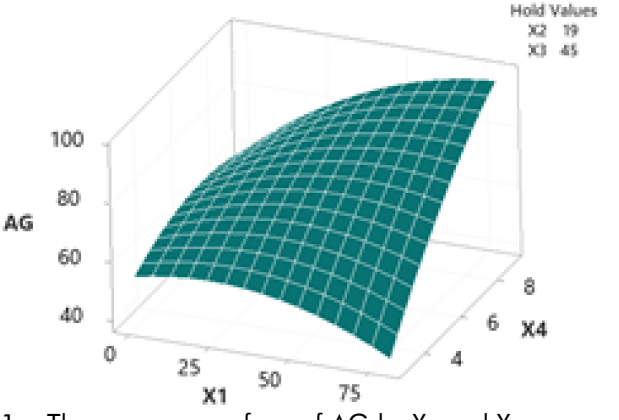
1d. The contour plot of AOC by X_1 and X_3



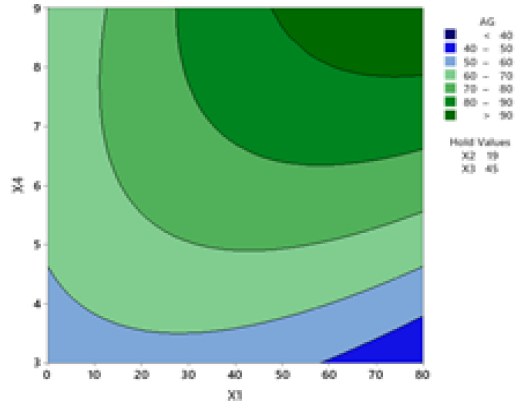
1e. The response surface of TI by X_1 and X_3



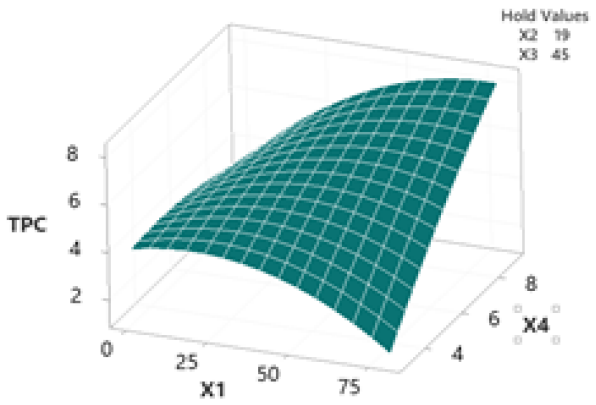
1f. The contour plot of TI by X_1 and X_3



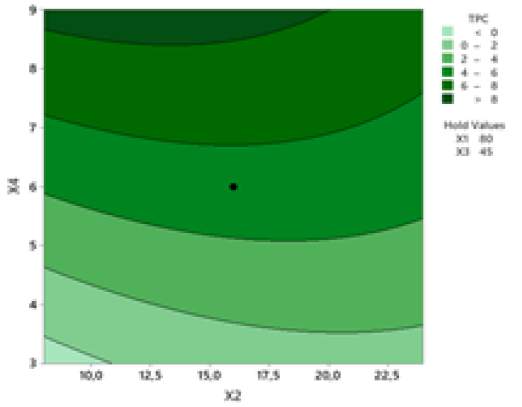
1g. The response surface of AG by X_1 and X_4



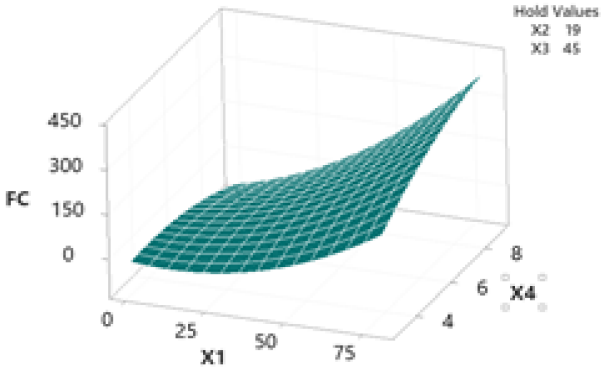
1h. The contour plot of AG by X_1 and X_4



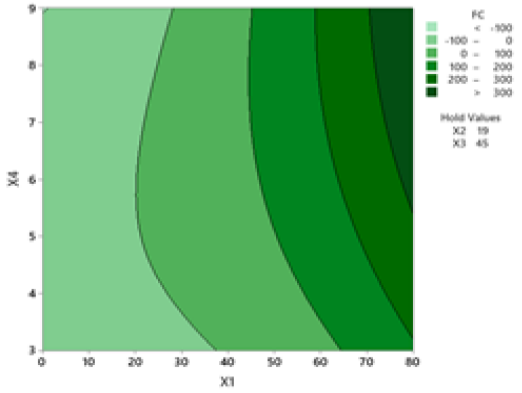
1i. The response surface of TPC by X_1 and X_4



1j. The contour plot of TPC by X_1 and X_4



1k. The response surface of FC by X_1 and X_4



1l. The contour plot of FC by X_1 and X_4

Figure 1. Response surface (3D) and the contour plot of yield (%), antioxidant activity (AOC), tyrosinase inhibitor (TI), antiglycation activity (AG), total phenolic content (TPC), and fucoxanthin content (FC).

Table 3. Regression coefficient, coefficient of determination (R2) and the F-test value from ANOVA table of the predicted second-order polynomial models for producing *P. australis* extract

Term	Yield	AOC	TI	AG	TPC	FC
Constant	5.724*	54.20*	30.78*	72.76*	6.564*	51.0
X1	0.272	6.73*	22.21*	3.04	0.688*	111.4*
X2	-0.194	9.99*	1.53	-4.41	0.045	-4.4
X3	0.019	-5.62*	-0.41	4.30	0.204	26.2
X4	0.073	2.65	3.45	1.81	0.919*	20.7
X1*X1	0.104	-3.34	10.51*	-8.16	-1.307*	78.0*
X2*X2	-0.732*	-4.35	0.01	2.67	-0.772*	-9.4
X3*X3	-0.220	-8.61*	6.23*	1.56	-0.983*	7.9
X4*X4	-0.416	0.66	3.53	-5.83	-0.378	-31.6
X1*X2	-0.105	4.86	-1.85	-8.76	-0.272	41.7
X1*X3	0.433	12.26*	8.96*	6.13	-0.033	49.4
X1*X4	0.987*	1.89	2.06	12.87*	1.910*	70.8*
X2*X3	0.218	5.30	2.76	-1.96	0.405	-37.7
X2*X4	-0.420	-5.03	0.03	3.45	-0.843*	2.2
X3*X4	0.638	-5.59	-0.19	12.83	0.955*	8.4
F value (model)	13.226	3853*	7369.66*	3235.91	54.258*	261257*
F value (lack of fit)	5.5629*	744.57*	809.03	1679.51*	7.4136	47565*
R^2	69.66%	83.57%	88.90%	65.61%	86.77%	84.59%
Unusual obs	1 & 5	21		1		15

*significant at α=5%

The ANOVA and lack of test on AOC and FC were different compared for other responses. Both ANOVA were significant at α=5%. This is because of an unusual observation on AOC and FC, respectively. The predicted response of AOC was higher than the actual response. On contrary, the predicted response of FC was lower than the actual observation. In general, the model could be accepted because the coefficient determination of the model was up to 80% except for yield and AG.

Due to there were four factors, the 3D response surface and the contour plot were drawn based on the significant factors of the interaction factors in **Table 3**. **Figure 1** shows the response surface plots and the contour plots of each responses. The low values of response show in blue colour meanwhile the high values shows in green colour. Dark green colour represents the highest values of the response.

The symbol X_1 represents solvent concentration (%), X_2 represents time (hour), X_3 represents temperature (°C), and X_4 represents sample-to-solvent ratio. The effects of terms in the quadratic model of each response would be discussed one by one. The yields were affected by the quadratic terms of X_2 and the interaction between X_1*X_4. Meanwhile other factors were not statistically significant. **Figure 1a** shows that the yield will increase when solvent concentration and sample-to-solvent ratio is increase. Corresponding with **Figure 1a**, the contour plot in **Figure 1b** shows that the highest yield achieved when X_1 = 80 and X_3 = 45, meanwhile X_2 = 19 and X_4 = 9.

Increasing the concentration of ethanol will suppress the polarity of the solvent and make the solvent stronger to extract nonpolar compounds from materials (Shadmani et al. 2004). Solvents with lower polarity can further degrade the brown seaweed cell walls, allowing easier extraction of the compounds from within the cell walls to the solvent, which is called the soluble-like principle (Tiwari et al., 2011). Increasing the movement of ethanol molecules and also encouraging pore swelling on the surface of dry matter, which makes the solvent quickly penetrate the cell (Taherzadeh & Karimi 2007).

The DPPH test (Supplementary 1) showed that the lowest and the highest value of antioxidant activity were 17.31% (observation 5) and 71.40% (observation 3), respectively. The highest antioxidant was attributed to Run 3, i.e., ethanol 80%, time 16 h, temperature 35°C, and sample-to-solvent ratio 1:9. Only temperature, which had a quadratic effect meanwhile solvent concentration and time had a linear effect. Besides, the solvent to sample ratio did not affect the response antioxidant activity. Antioxidant activity increased in solvent concentration and time. **Figure 1c** shows the response surface of X_1 and X_3. The effect of temperature was quadratic whereas the effect of solvent concentration was linear. The contour plot in **Figure 1d** shows the highest values of AOC is a part of ellipsoid.

In contrast, an increase in temperature caused a reduction in antioxidant activity. Some compounds could be degraded in higher temperature (Casagrande et al, 2018). One of the antioxidant compounds in *P. australis* is fucoxanthin (Nursid et al., 2016). Increasing temperature caused degradation of fucoxanthin (Zhao et al, 2019). **Figure 2b** showed the response of the interaction between solvent concentration and temperature; meanwhile, other factors are constant. Solvent concentration and extraction time contributed to the rise of antioxidant activity.

The results showed that the highest antityrosinase activity reached 86.83%, as shown by extract under Run 9, i.e., ethanol 80%, time 16 h, temperature 45 °C, and the ratio of 1:6. Meanwhile, the lowest value of an antityrosinase activity is 16.86 %. **Table 3** demonstrated the effect of X_1 and X_3 was quadratic and also there was interaction effect between X_1 and X_3. **Figure 1e** and **Figure 1f** shows the response surface and the contour plot of TI, respectively. The TI increases when X_1 and X_3 increase while other factors held constant. **Table 3** exhibited that the highest antiglycation activity was 97.21%, as contributed by Run 3, i.e., ethanol 80%, time 16 h, temperature 35°C, and the ratio of 1:9. This condition also produced more potent antioxidant activity. It suggests that free radicals accelerate glycation; thus, antioxidative compounds neutralize them, leading to the formation of advance glycation end product called AGEs (Povichit et al., 2010).

The only significant model term for anti-replication is the interaction between X_1 and X_4, whereas the other terms in the model are not statistically significant. This is shown in **Table 3**. **Figure 1g** shows that the interaction between X_1 and X_4. AG increases when X_1 and X_4 increase. Figure 1h shows that the highest AG values are around X_1 = 80 and X_4 = 9, while X_2 = 19 and X_3 = 45. The model shows that the antiglycation activity increases with increasing solvent concentration, solvent and time interactions and time and temperature interactions. On the other hand, the anti-glycation activity decreases with increasing extraction time and temperature.

The total phenolic content of an extract is reported as Gallic Acid Equivalent (GAE), presented in Supplementary 1. Similarly, phenol's highest content was found at Run 3, reaching up to 7.57 mg GAE/g. Brown algae consist of various phenolic compounds, such as gallic acid, catechin, epicatechin, and phlorotannins (Machu et al, 2015). The treatment also exerted the highest antioxidant and antiglycation activity. The phenolic compound and fucoxanthin well known have antioxidant properties (Balboa et al., 2013).

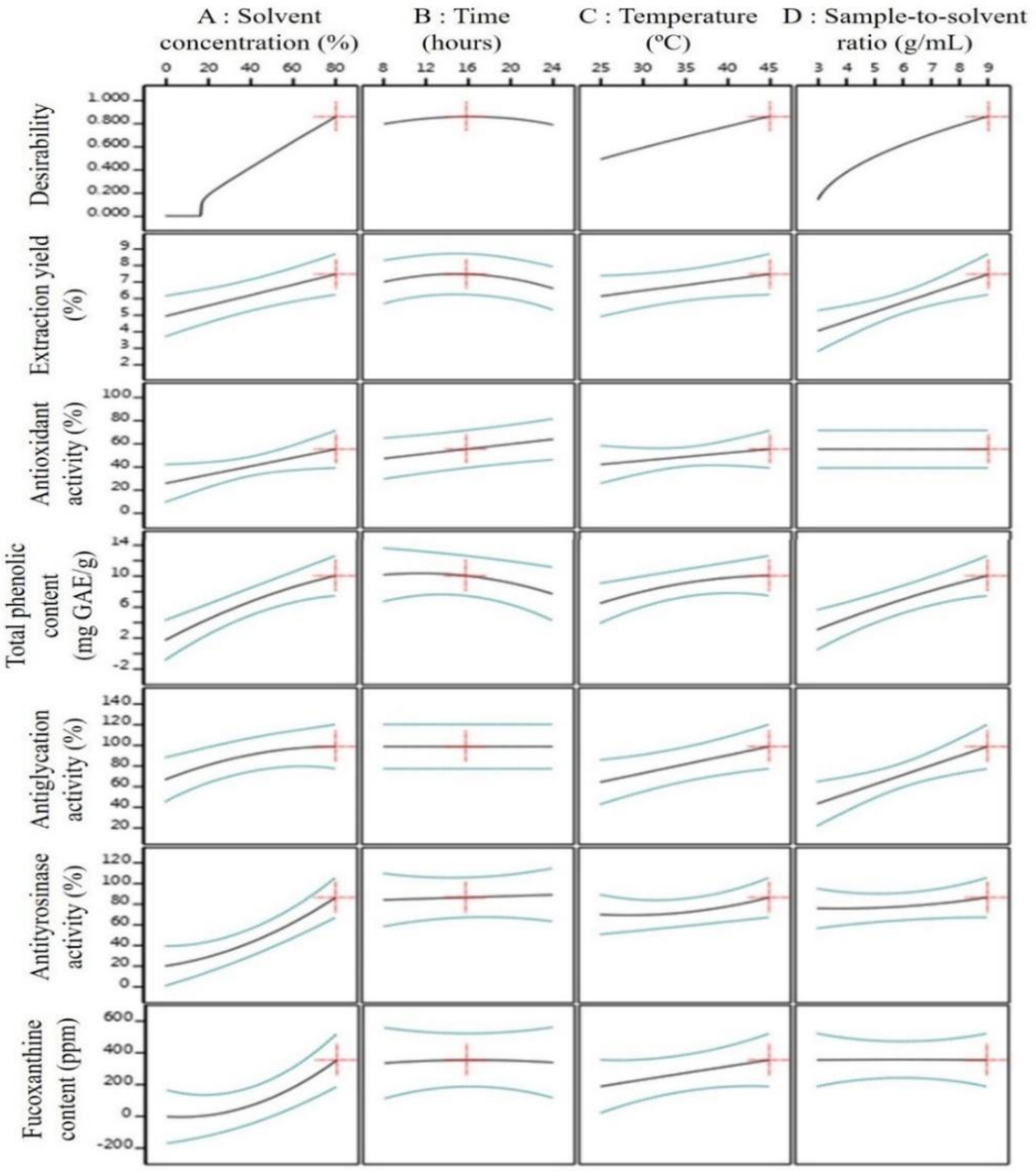


Figure 2. Profiling plot of all responses

Table 3 showed that the total phenolic content response was adequately fitted by the quadratic model. The effect of X_1, X_2, X_3 was quadratic meanwhile the effect of X_4 was linear. However, the interaction between X_4 and X_2 and X_3 was significant at $\alpha = 5\%$. Unlike other plots of response surface, Figure 1k showed that total phenol was higher as the increase in solvent but decreased in sample-to-solvent ratio and their interaction. The contour plot in **Figure 1l** shows that the high value of total phenol achieved when $X_2 = 8$ and $X_4 = 9$, while other factors constant.

Our results agree with the report of Sapitri et al. (2019) which give maximum phenolic content on ethanol 40% extraction while their research range concentration of ethanol is from 0 to 80%. Different results was stated that the higher solvent concentration could raise phenolic compounds' solubility, thereby increasing extraction time due to cell walls' degradation and enhancing the phenolic compound's extractability (Diantika, Sutan, & Yulianingsih, 2014). Different results could be happened since pervious results is used coffee beans to extract. Additionally, the previous results is only based on two factors; solvent concentration and extraction time against antioxidant activity, while our experiments used 4 factors. However, the increase in temperature especially more than 45 °C needs to be considered, since excessive temperature adversely damaged the materials studied.

The highest fucoxanthin level was obtained at Run 9, i.e., ethanol 80%, time 16 h, temperature 45 °C and ratio of 1: 6. **Table 3** shows that the effect of X_1 is quadratic. In interaction effect between X_1 and X_4 was also significant. **Figure 1k** shows the surface response

plot between X_1 and X_4 of FC. The plot clarified the effect of X_1 and X_4.

Fucoxanthin, a carotenoid compound, is an intracellular compound in algae that protected by the cell wall, plasma membrane, and chloroplast membrane. The presence of these layers can inhibit the rate of carotenoid mass transfer during the extraction process (Poojary et al. 2016), so those appropriate techniques are needed to extract carotenoids (including fucoxanthin) from brown seaweed. The enhancement of fucoxanthin content was dependent majorly on the solvent used. Methanol and ethanol are better than acetonitrile, DMSO, and acetone to extract fucoxanthin. It is due to a common fact that methanol and ethanol constitute organic solvents capable of extracting polar carotenoid such as fucoxanthin possessing OH groups (Limantara & Heriyanto, 2011).

The optimal condition that produces the optimal responses were determined by optimization for all responses. The optimal responses are indicated by the value of desirability close to 1. As depicted in Figure 2, Box-Behnken design suggested process condition with the highest desirability (0.7926), i.e., solvent concentration 79.99%, time 18.48 h, temperature 44.50°C, the ratio of (1:8.9). That suggested condition was predicted to produce *P. australis* extract with a 7.30% extraction yield, 43.94% antioxidant activity, 98.06% antiglycation activity, 9.53 mg GAE/g total phenol content, 86.83% antityrosinase activity, and 347.55 μg/g fucoxanthin content. The value of desirability is 0.7926, which means that the condition will produce products with characteristics that are by the optimization target of 79.26%. Overall, the results of this study are comparable to those of Hassan, Pham & Nguyen (2021) who used samples of *P. australis* from Vietnamese waters. By using ultrasound-assisted extraction, it was found that the time and the ratio of the sample to the solvent were significant factors in the extraction of *P.australis*. Optimal extraction conditions were determined at ultrasonic temperature of 60°C, ultrasonic time of 60 minutes, solvent concentration of 60% (v/v) aqueous ethanol and sample-solvent ratio of 1 g/100 mL.

Verification of the optimized formula was carried out in triplicate under the optimal conditions: solvent concentration 79.99%, time 18.48 h, temperature 44.50°C, and sample-to-solvent ratio 1:8.9; this selected treatment could result in yield 8.32%, antioxidant activity 56.70%, antityrosinase activity 88.52%, antiglycation activity 98.96%, total phenol 10 mg GAE/g, and fucoxanthin content 378 μg/g. Verification aims to compare the predicted and experimental results by the percentage. The different percentage of each response variable sequentially or CV was 14%, 29%, 1 %, 5%, 2%, and 9%. It was around 10% on average. The CV of Yield and AOC were large because the existing unusual observations in the experimental data. The same reason was also for AG and FC. This result indicated that the model predicted by Box-Behnken design could be used to predict the values of those responses.

CONCLUSION

In this study, the most desirable condition for the extraction of *P. australis* was found using the response surface method and evaluated by Box Behnken design. The results showed a selected treatment of solvent concentration 79.99%, time 18.48 h, temperature 44.50°C, and sample-to-solvent ratio 1:8.9 were resulting in high yield, total phenol, and fucoxanthin content, as well as high activity as antioxidant, antityrosinase, and antiglycation. Based on the experimental test at the verification stage, there is no significant differences between the experimental values and the prediction values.

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