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Ultrasound-Assisted Extraction using Response Surface Methodology for Extracting Flavonoids from *Padina australis*

(Pengekstrakan Berbantu Ultrabunyi menggunakan Kaedah Gerak Balas Permukaan untuk Mengekstrak Flavonoid daripada *Padina australis*)

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ABSTRACT

Seaweed or sea macroalgae are rich in potential compounds which can be used for the treatment of disease. Padina australis *is one of the important brown macroalgae classes (Phaeophyes). One of the bioactive compounds* of P. australis *is a phenolic compound and its derivatives (flavonoid). In this research,* P. australis *was collected from Bayah Beach, Banten, Indonesia. For the extraction flavonoids from* P. australis*, ultrasound-assisted extraction (UAE) was employed. In this study, a three-level Box-Behnken design (BBD) and the response surface methodology (RSM) were employed to obtain the optimal combination of extraction conditions. The effects of several independent variables including temperature (30, 50, 70 °C), extraction time (20, 40, 60 min) and ethanol concentration (30, 50, 70%) were investigated. The result showed that RSM was an accurate and reliable method in predicting the total flavonoid content with R^2 value of 0.9935. The optimal UAE conditions for the highest yield of total flavonoid content were 49.70 °C in temperature, process time under 44.03 min, and 47.80% ethanol with 0.2162% total flavonoid content. Under the above conditions, the experimental value of total flavonoid content was 0.2144± 0.0035%. The predicted and experimental values for total flavonoid from brown algae* P. australis *were not significant differences, it indicating that the developed models are accurate. Therefore, UAE using RSM is effective for the extraction of flavonoid from* P. australis*.*

Keywords: Box-Behnken design; optimization; P. australis*; total flavonoid; ultrasound-assisted extraction*

ABSTRAK

Rumpai laut atau makroalga kaya dengan sebatian berpotensi yang dapat digunakan untuk rawatan penyakit. Padina australis *adalah salah satu kelas alga makro coklat yang penting (Phaeophyes). Salah satu sebatian bioaktif* P. australis *adalah sebatian fenolik dan turunannya (flavonoid). Dalam penyelidikan ini,* P. australis *dikumpulkan dari Pantai Bayah, Banten, Indonesia. Untuk mengekstrak flavonoid daripada* P. australis*, pengekstrakan berbantu ultrabunyi (UAE) digunakan. Reka bentuk Box-Behnken tiga tahap (BBD) dan kaedah gerak balas permukaan (RSM) digunakan untuk mendapatkan gabungan keadaan pengekstrakan yang optimum. Kesan beberapa pemboleh ubah bebas termasuk suhu (30, 50, 70 °C), masa reaksi (20, 40 60 min) dan kepekatan etanol (30, 50 70%) dikaji. Hasil kajian menunjukkan bahawa RSM adalah kaedah yang tepat dan boleh dipercayai dalam meramalkan jumlah kandungan flavonoid dengan nilai R^2 0.9935. Keadaan UAE yang optimum untuk hasil tertinggi kandungan flavonoid adalah suhu 49.70 °C, masa proses di bawah 44.03 min dan 47.80% etanol dengan kandungan flavonoid 0.2162%. Di bawah keadaan ini, nilai uji kaji flavonoid adalah 0.2144±0.0035% yang sangat sesuai dengan nilai yang diramalkan oleh model. Oleh itu UAE menggunakan RSM berkesan untuk pengekstrakan flavonoid daripada* P. australis*.*

Kata kunci: Kandungan flavonoid; pengekstrakan berbantu ultrabunyi; pengoptimuman; P. australis*; reka bentuk Box-Behnken*

INTRODUCTION

Seaweeds are rich in potential compounds and is used in pharmaceutical applications as they have interesting biological activities and contribute to the discovery of natural therapeutic agents. *Padina australis* is brown algae (Class: Phaeophyceae, Order: Dictyotales, Family: Dictyotaceae, Genus: Padina), is distributed worldwide

in tropical and temperate seas (Silberfeld et al. 2013). *P. australis* quite abundant and widespread in Bayah Beach which is located on the south island of Java. Exploration and utilization of *P. australis* from Bayah Beach are still very limited, especially of its bioactive compounds content.

P. australis contained phenolic compound and its derivatives (flavonoid), β-carotene, diadinoxanthin, diatoxanthin, fucoxanthin, chlorophyll a, chlorophyll c, and alginate (Handayani & Zuhrotun 2017; Setha et al. 2013). Flavonoid compounds are one of the bioactive compounds that are currently used in such industries as food, pharmaceutical, and medicinal industries due to their health benefits. Numerous studies have been conducted to prove flavonoids efficacy as an antioxidant, cardioprotective effects, immune system promoting, skin protective effect from UV radiation (Tungmunnithum et al. 2018), antibacterial antiviral, antiinflammatory, antiulcer, anticancer, antidiabetic, and cytotoxic (Karak 2019).

The previous study has shown that the conventional extraction of *P. australis* was macerated using different solvents i.e. methanol, ethyl acetate, or n-hexane at a ratio of 1:16 (w/v) for 48 h at room temperature under dark condition. The samples of *P. australis* were obtained from Pramuka Island, an island in the Thousand Islands archipelago, Indonesia. The result showed that the highest total phenolic content of methanol extract was 246.1 mg GAE/1000 g dry sample. The ethyl acetate and n-hexane extracts with values of 90.17 mg and 17.3 mg GAE/1000 g dry sample, respectively (Santoso et al. 2013). Variations of genotypes, growing regions, temperature, season, harvesting time, process, and storage conditions are possible to affect the phenolic profile (Sulastri et al. 2018). The total flavonoid content in *Padina* sp. which was taken from Punaga Ocean, Takalar, South Sulawesi was 2.357± 0.025% used maceration method (Ruslin et al. 2018).

Alara et al. (2018) reported that conventional methods for extracting flavonoids often need long extraction times, large amounts of solvent, and low efficiencies. Moreover, thermal processing has caused the flavonoids more unstable and easily degrade during the extraction. To increase the extraction yield, ultrasound has attracted more attention to be an economically feasible technology suitable for the extraction of thermolabile compounds. Ultrasound-assisted extraction (UAE) is simpler and faster than microwave-assisted extraction, inexpensive, and efficient or reducing the amount of solvent and energy (Altemimi et al. 2017; Chandrapala et al. 2012). The sound waves of UAE at frequencies above the range audible to humans greater than 20 kHz can be used for the extraction of bioactive compounds, including from phenolic compounds (Ma et al. 2009). Ultrasound also shows a mechanical effect, allowing greater penetration of solvent into the sample matrix, increasing the contact surface area between solid-liquid phases (Meullemiestre et al. 2015).

Box-Behnken experimental design of response surface methodology (RSM) was applied to optimize the extraction process conditions with the independent variables of material-solvent ratio, extraction time, and ethanol concentration (Zheng et al. 2016). The reason for choosing UAE in this study was ultrasound will enhance in the extraction of flavonoid compounds from *P. australis*. RSM designs will estimate an interaction and even quadratic effects, and hence give us the idea of the (local) shape of the response surface under investigation (Elmoubarki et al. 2017). In this research, the optimization of UAE conditions as temperature, time of extraction, and ethanol concentrations.

Materials and Methods

Chemicals and materials

The samples of brown algae *P. australis* were collected from Bayah Beach located in Banten Indonesia. The identification was done at Indonesian Institute of Sciences Research Centre for Oceanography. The chemicals were purchased at Sigma-Aldrich Indonesia. Ethanol (cat. no. 493511), aluminium chloride (cat. no. 206911), sodium acetate (cat. no. W302406), and quercetin (cat. no.337951).

Methods

Samples

The procedure for sample preparation was washed with tap water to remove salt, epiphytes, and sand attached to the surface of the samples, and the remaining water was dried by air in the shade on 10-days. The dried seaweeds were crushed and ground into a powder, passed through a 40-mesh sieve, and stored at room temperature (Yuguchi et al. 2016).

Extraction

The powder sample was prepared by dissolving 100 mg of air-dried powder in 10 mL of ethanol with different concentrations (30, 50, 70%). Extraction was done in an ultrasound chamber (Model VGT-1860QTD, China) using 40 kHz of frequency and 150 watts of power. ultrasonic chamber model VGT-1860QDT, China. Extraction of temperature, time, and ethanol concentration were selected as independent variables (Table 1).

TABLE 1. Variables and their levels in the response surface design

Independent variables	Symbols	Level		
		-1	0	+1
Temperature (°C)	X_1	30	50	70
Time (min)	X_2	20	40	60
Ethanol concentration (%)	X_3	30	50	70

EXPERIMENTAL DESIGN OF RSM

The experimental design used three variables and levels in the Box-Behnken design, requiring a total of 15 experiments for the optimization of extraction parameters. This design was composed of a 12 factorial design (runs 1-12), and 3 center points (runs 13-15). The experimental design is presented in Table 2. The range of extraction parameters chosen in this study was based on preliminary experiments. The influence of extraction includes extraction temperature (X_1; 30 - 70 °C), extraction time (X_2; 20 - 60 min), ethanol concentration (X_3; 30 - 70%). The range of extraction parameters was chosen in this study were based on the modification of Tatke and Rajan (2019).

This model was designed to establish the optimum experimental total flavonoid content implemented using Design Expert Software version 7.0 (Chakraborty et al. 2013). Ultrasonic wave extraction techniques have been applied for extracting flavonoids from *P. australis*. Predictive models of quadratic polynomial were chosen as the best-fitted model to demonstrate the influence of the variable and their interactions on the response variable. The mathematical model corresponding to the Box-Behnken design was (1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted total flavonoid content (g/100 g Simplicia); β_0 is the intercept; β_i is the linear coefficients; β_{ii} is the quadratic coefficient; β_{ij} is the interaction coefficient; X_i and X_j are independent variables.

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content was determined by aluminum chloride colorimetric assay adapted from Sembiring et al. (2018). After the extraction, 2 mL of liquid extract was loaded into a 10 mL volumetric flask, added 0.2 mL of aluminium chloride solution, 0.2 mL of 1M sodium acetate and 3 mL of 95% ethanol. Quercetin was used as standard (Sigma-Aldrich Indonesia cat. no.337951) with purity > 95%. The absorbance was determined using a spectrophotometer (Jasco V-730) at 431 nm after incubated for 20 min at room temperature. Total flavonoid contents were expressed as g/100 g simplicia.

Based on the total flavonoid measurement data, a quercetin calibration curve was made resulting in the equation $Y = 0.0499 x + 0.0367$ ($R^2 = 0.9999$) where y is the absorbance value and x is the quercetin content. Using the quercetin calibration curve, absorbance measurement samples were used to determine the total flavonoid contents. The standard curve used serial dilution method at 2, 4, 6, 8, and 10 ppm.

STATISTICAL ANALYSIS

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). The data analysis tool in Microsoft Excel 2019® was used to analyze the experimental results of the response surface designs. Response surface and contour plot showing the relationship between variable experiment with the response and the type of interaction between the variables tested (Sugiono et al. 2014). The adequate of the model was determined by evaluating the lack of fit, coefficient of determination (R^2), and the Fisher test value (F-value). Differences were considered significant if $p < 0.05$.

RESULTS AND DISCUSSION

After determining the best condition of a single factor, the BBD of 15 runs was applied to optimize these three independent factors and study the extraction of total flavonoid from *P. australis*. To optimize the total flavonoid content, extraction conditions were chosen in low, middle,

and upper levels. The design matrix and experimental result of total flavonoid content present in Table 2. The highest yields of total flavonoid (0.2144+0.0035%) at the extraction temperature of 50 °C, extraction time 40 min and ethanol concentration of 50%. The extraction of total flavonoids content increased with the extension of temperature, extraction time, and ethanol concentration.

By increasing in temperature, the extraction will increase both solubilities of the solute and diffusion coefficient (Uma et al. 2010). It might be due to the denaturation of the total flavonoids through a long period of ultrasonication. In this research, variables of ethanol concentration extraction affect the solubility of chemical constituents and it can extract flavonoid substances. The ethanol concentration reached a peak at 50% then increasing in the concentration makes it easier to volatile, caused the flavonoid content to decrease sharply (Widyawati et al. 2014). After multiple regression analysis of the current experimental data, the relationship between the predicted response Y and the test variables can be explained invoking the following second-order polynomial (2):

$$Y = 0.21 - 0.000875\,X_1 + 0.009575\,X_2 - 0.015\,X_3 - 0.021\,X_1X_2 + 0.00715\,X_1X_3 + 0.00705\,X_2X_3 - 0.058\,X_1^2 - 0.026\,X_2^2 - 0.058\,X_3^2 \quad (2)$$

The analysis of variance (ANOVA) is essential to test the significance of the curvature in the responses at a confidence level of 95% and adequacy of the model. The ANOVA data for the coded quadratic model for the response are reported in Table 3. The F-value obtain 85.74 and values of 'Prob > F' less than 0.05 indicate that model terms are significant. Especially larger F-value with the associated p-value (smaller than 0.05, confidence intervals) means that the experimental systems can be modeled effectively with less error (Box & Cox 1964; Kutner et al. 2004). The ANOVA showed that quadratically, *P. australis* extract significantly higher effect ($p < 0.05$) on the overall acceptability of *P. australis* whereas the lack of fit F-value of 4.46 was not significant as the p-value is > 0.05. The non-significance lack of fit suggested that the model was valid for the present study.

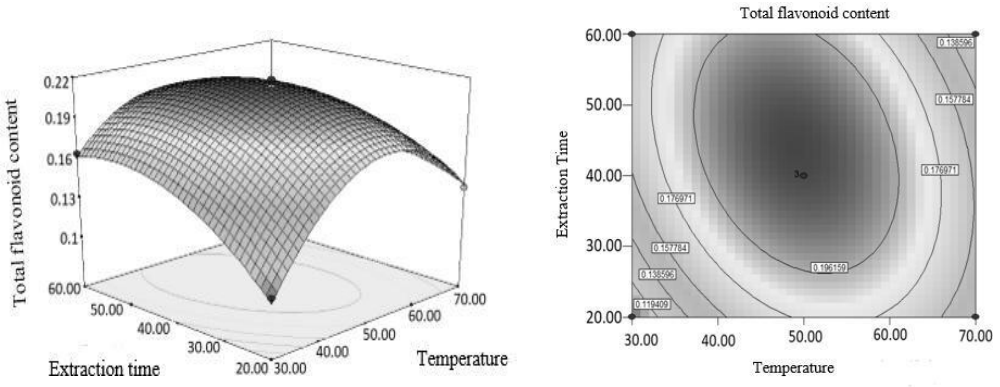
The determination coefficient R^2 of the regression quadratic model was 0.9936, suggesting that the relationship between the dependent and independent variables could be described well using this model. The adjusted R^2 was 0.9820, indicating that 98.20% of the change of responses could be explained by this model. All the results indicated that this regression quadratic model had enough resolution ability and it could fit the experimental results (Arabi & Sohrabi 2013). The effect of extraction time, temperature, and ethanol concentration on total flavonoid content was also shown in response plots (Figures 1, 2, and 3).

TABLE 2. Experimental data for the Total Flavonoid Content obtained from Box-behnken design

No	Coded			Uncoded			Total flavonoid content (%)
	X_1	X_2	X_3	X_1	X_2	X_3	Y exp
1	-1	-1	0	30	20	50	0.1039 ± 0.0005
2	-1	0	-1	30	40	30	0.1155 ± 0.0011
3	-1	0	1	30	40	70	0.0775 ± 0.0015
4	-1	1	0	30	60	50	0.1639 ± 0.0015
5	0	-1	-1	50	20	30	0.1459 ± 0.0089
6	0	-1	1	50	20	70	0.0947 ± 0.0002
7	0	0	0	50	40	50	0.2124 ± 0.0008
8	0	0	0	50	40	50	0.2184 ± 0.0047
9	0	0	0	50	40	50	0.2124 ± 0.0086
10	0	1	-1	50	60	30	0.1524 ± 0.0031
11	0	1	1	50	60	70	0.1294 ± 0.0071
12	1	-1	0	70	20	50	0.1385 ± 0.0031
13	1	0	-1	70	40	30	0.1054 ± 0.0013
14	1	0	1	70	40	70	0.0959 ± 0.0002
15	1	1	0	70	60	50	0.1139 ± 0.0020

TABLE 3. Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of squares	Df	Mean square	F- value	p-value Prob > F
Model	0.029	9	3.217E-0.003	85.74	<0.0001
X_1 - Temperature	6.498E-006	1	6.498E-006	0.17	0.6945
X_2 - Time	7.331E-004	1	7.331E-004	19.54	0.0069
X_3 – Ethanol concentration	1.849E-003	1	1.849E-003	49.27	0.0009
$X_1 X_2$	1.789E-003	1	1.789E-003	47.69	0.0010
$X_1 X_3$	2.015E-004	1	2.015E-004	5.37	0.0683
X_2X_3	1.985E-004	1	1.985E-004	5.29	0.0697
X_1	0.012	1	0.012	332.95	< 0.0001
X_2	2.525E-003	1	2.525E-003	67.31	0.0004
X_3	0.012	1	0.012	326.63	< 0.0001
Residual	1.876E-004	5	3.752E-005		
Lack of Fit	1.632E-004	3	5.439E-005	4.46	0.1887
Pure Error	2.441E-005	2	1.220E-005		
Cor Total	0.029	14			
R^2	0.9936	Adj. R^2	0.9820		
Adeq Precision	27.428	C.V. %	4.42%		



a b

FIGURE 1. Response surface graph (a) and 3D contour plot (b) illustrating the effect of extraction time and temperature on total flavonoid content

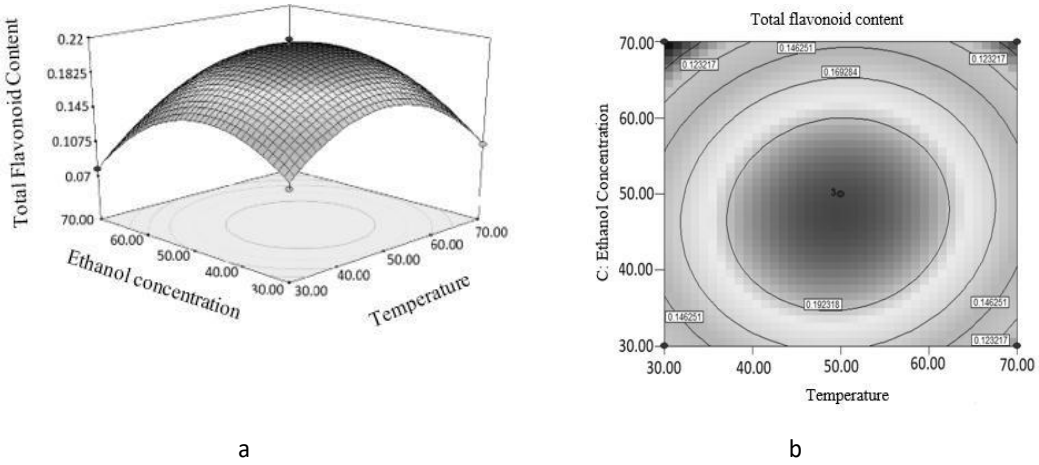


FIGURE 2. Response surface graph (a) and 3D contour plot (b) illustrating the effect of ethanol concentration and temperature of extraction and on total flavonoid content

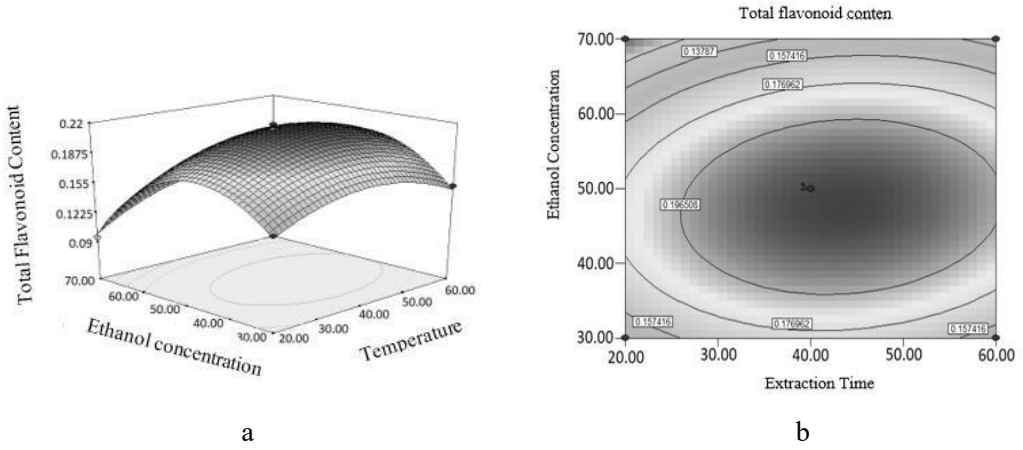


FIGURE 3. Response surface (a) and 3D contour plots (b) illustrating the effect of temperature of extraction and ethanol concentration and temperature on total flavonoid content

Optimization the process is the main objective of the experimentation to find the levels of factors that optimize response. The optimum conditions of 49.70 °C, 44.03 min, 47.80% of ethanol concentration yielded a predicted value of 0.2162% which is close to the experimental value of 0.2144 + 0.0035% with composite desirability of 1, determination coefficients of 0.9936 and 0.9820 were obtained for R^2 and adjusted R^2, respectively. The verification of experimental and predicted values was determined by absolute errors (AE). The low absolute error value (0.84%) indicates that the model can be used to predict the response value.

Conclusion

The BBD quadratic model was used to determine the interaction of temperature, extraction time, and ethanol

concentration for analyzing total flavonoid content using UAE. Results from the analysis showed that the quadratic model could express the interaction among the three factors well.

Predicted values gained from the model were 0.2162% close to those obtained from the experimental analysis, further indicating the suitability of the model. The relationship between different factors can be shown by setting up a mathematical model. According to the results, the optimum condition for temperature was 49.70 °C, reaction time was 44.03 min, and ethanol concentration was 47.80%. The related parameters test proved that brown algae *P. australis* was rich in flavonoid which obviously could be an ideal resource for nutraceuticals and could be treated as the foundation for varied further research.

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