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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**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: Dictytaceae, 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, antiadiabetic, and cytotoxic (Karak et al. 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-Beihnenk 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 (Elmoubariki 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. Ultrasound chamber model VGT-1860QDT, China. Extraction of temperature, time, and ethanol concentration were selected as independent variables (Table 1).
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):

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

where $Y$ is the predicted total flavonoid content (g/100 g Simplicia); $\beta_0$ is the intercept; $\beta_i$ is the linear coefficients; $\beta_{ii}$ is the quadratic coefficient; $\beta_{ij}$ 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 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):

\[
Y = 0.21 - 0.000875 X_1 + 0.009575 X_2 - 0.015 X_3 - 0.021 X_1 X_2 + 0.00715 X_1 X_3 + 0.00705 X_2 X_3 - 0.058 X_1^2 - 0.026 X_2^2 - 0.058 X_3^2
\]

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 R² 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 R² 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).

| No | Coded | Uncoded | Total flavonoid content (%) |
|----|-------|---------|----------------------------|
|    | X₁    | X₂      | X₃                          |
| 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 | 0     | 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-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  |          |
| $X_2 X_3$                    | 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%   |         |          |

**FIGURE 1.** Response surface graph (a) and 3D contour plot (b) illustrating the effect of extraction time 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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