Optimization of microwave assisted extraction from *Rhodomyrtus tomentosa* fruits using response surface methodology

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Abstract. *Rhodomyrtus tomentosa* (Ait.) Hassk include in the horticulture products with superior bioactive compounds. The benefit of fruit parts has been used as traditional herbs in Malaysia, Vietnam, Thailand and China. In this research, bioactive compounds from *Rhodomyrtus tomentosa* were obtained by Microwave Assisted Extraction (MAE). MAE is a modern extraction method that used microwaves to enhance the effectiveness and efficiency of cell rupture. The optimum result of extraction process determined by Response Surface Methodology (RSM) toward the extract yields, total anthocyanins content and total flavonoids content. Based on ANOVA test results, there is a significant influences on each responses with p-value<0.05. The p-value of the lack of fit in each responses has an insignificant influence (p-value>0.05) which means the model can be used as an optimization predictions. The optimum conditions for MAE process were 190 seconds of extraction times and 1:19.05 (w/v) of sample to solvents ratio. The verification of optimum condition are 1.07% of extract yields, 3.78 mg/g of anthocyansins and 15.47 mg/g of flavonoids, which all of responses are in the threshold of the predicted value.

1. Introduction
Indonesia is located in a tropical region with diversity and wealth of horticultural commodities. *Rhodomyrtus tomentosa* (Ait.) Hassk is one of the horticultural commodities that contained bioactive compounds and superior properties be required as an ingredient in the pharmaceutical industry with great demand lately. The leaves, roots and fruit of *Rhodomyrtus tomentosa* have been as traditional herbs in Vietnam, China, Thailand and included 240 plants of “Neglected and Underutilized Crop Species of Vietnam, China, Thailand and Cambodia” as the Scientific Project of Agroforestry [1].

Sim (*Rhodomyrtus tomentosa*) fruits was extractad with a mixture of trifluorophosphate acid in HPLC column filled with methanol (MeOH), obtained 6 main of anthocyanin compounds, namely cyanidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside, pentunidin-3-O-glucoside, delphinidin-3-O-glucoside and pelargonidin-3-glucoside [2]. Identification of flavonoid from extract of sim fruits obtained 6 main flavonoid compounds, namely myricetin, quercetin, dihydromyricetin, kaempferol, quercetin 7,4-diglocoside and vitexin [3].

Agricultural commodities are known to be perishable, seasonable and customable, due to an advanced physiological process even though it has been released from the stem. This process is known as respiration [4]. The bad handling of fresh fruits caused degradation of bioactive compounds inside
the cells. The extraction process in the plant parts is done to obtain the desired bioactive compounds. Bioactive compounds in sim fruits was still extracted conventionally with a long time and high solvent requirements. The relatively modern extraction for sim fruit has never been carried out by previous researchers, such as the Microwaves Assisted Extraction (MAE).

MAE’s method is widely proven and more effective for the extraction of bioactive compounds from plants that are labile to high temperatures. As a modern extraction method, MAE is known with a higher extraction rate, lower solvent consumed and faster extraction time than conventional method extractions. MAE is an electromagnetic wave with a frequency threshold between 300 MHz and 300 GHz and have been widely applied to extract flavonoids with solvents. MAE also can be used with combination of solvents for polar or non-polar flavonoid compounds [5].

The optimum extraction conditions produced maximum yield extracts and bioactive compounds. The optimization of extraction is influenced by factor variables that are treated during the process. After several runs, some variable factors were determined for determining the optimum condition of extraction process. Response Surface Methodology (RSM) is an effective technique for optimizing the extraction process variables, modeling extraction parameters, validating statistical significance on independent variables and determining the optimum operating conditions [6].

This study was aimed to optimise the extraction and the yield of bioactive component form caramunting fruit.

2. Materials and Methods

2.1. Sample collection and preparation

The raw material for sim fruits was brought from Tarakan City, North Kalimantan. Sim fruit is picked in the morning. The Ripe sim fruit is marked with purple colour. Then, sim fruit packed with styrofoam box in cold conditions, and sent to Malang city by cargo sevices. When it arrived in Malang city, West Java, sim fruits were immediately brought to the laboratory to be stored in the refrigerator until handling of extraction is applied.

2.2. Chemicals and reagents

Extraction of sim fruits was used by ethanol solvent 96% (pa). The ingredients of extract yields analysis, total flavonoids content and total anthocyanins content are methanol, buffer solution pH 1 and pH 4, potassium chloride (KCl), Na acetate, HCl (pa), NaOH 45% (pa), alcohol 95% (pa), quercetion, NaNO₂ and AlCl₃.

2.3. Microwave assisted extraction

Sim fruit (10 g) was weighed and mixed with ethanol solvent according to the ratio of feed: solvent (v/w) which had been determined into a 500 mL Erlenmeyer bottle. The Erlenmeyer bottle was capped tightly and put in the microwave. The process of sim fruits extraction at 400 watts of power with extraction time in accordance to design of the research unit. The result of extracts was bright yellow, then evaporated with a rotary vacuum evaporator at 55°C for 5 minutes. Determination of the temperature is due to the degradation and instability of anthocyanins starting at 80°C [7]. The filtrate was separated from the solid by means of a centrifuge of 5000 rpm for 10 minutes at room temperature.

2.4. Determination of yields extracts

The definition of extract yields is ratio of weight from extracts with the weight of raw material in percent units. Yields are used to be an indicator of extraction process of cost efficiency. The formulation used for determining yield extracts of sim fruits using Eq. (1) as follows:

\[
yield \ extract = \frac{\text{extract weights}}{\text{raw weights}} \times 100\%
\]  

(1)

Where extract yields in percent (%), extract weights in g and raw weights in g.
2.5. Determination of total flavonoids content

The total flavonoids content in the sim fruits extracts was determined following the method described by Oriakhi et al. [8]. Quercetin (10-100 mg/mL) was used to prepare a standard calibration curve (y = 0.0004x – 0.1394, R² = 0.9362), where y is the absorbance at 420 nm and x is the sample concentration in mg/mL. Flavonoids total was expressed as mg quercetin equivalents per g of dried sample (mg/g). The flavonoids total of the extracts were calculated using Eq. (2). Ethanol was used as the netralization and the analyses were carried out in triplicate.

\[ TFC = \frac{c \times V}{m} \times 100\% \]  

(2)

Where c is the sample concentration from the calibration curve (mg/mL), V is the volume (mL) of the solvent used in the extraction and m is the weight (g) of the dried sample used [9].

2.6. Determination of total anthocyanins content

Anthocyanin totals was determined with pH-differential assay which described by Giusti et al. [10]. Firstly, the adequate factor dilution of the samples were characterised by diluting the samples with 0.025 M potassium chloride buffer pH 1.0 for a generic volume of 1 mL with the absorbency at a wavelength at the maximum absorbance (λ_{vismax}) lower than 1.2. To characterise the λ_{vismax}, a spectrum of the sample (260-710 nm) was constructed. Two dilutions of each samples were prepared until a total volume of 1 mL was reached, one with buffer pH = 1.0 and the other with 0.4 M sodium acetate buffer with pH = 4.5. These dilutions were standing for 15-45 minutes to reach an equilibrium conditions. A blank of distilled water was used to re-zero the spectrophotometer at all specified wavelengths. The final absorptions were recorded at UV_{vismax} and 700 nm. The total anthocyanins content for pigment concentration of the original sample was determined with the Eq. (3):

\[ TAC = \frac{AX \times MW \times DF \times 1000}{\varepsilon \times 1} \times 100\% \]  

(3)

Where: A = [(A_{530}\text{-}A_{700})_{pH 1.0} – (A_{530}\text{-}A_{700})_{pH 4.5}], MW presents the molecular weight of cyanidin-3-glucoside in mg, DF indicates the factor dilution of the sample in this determination and expresses the molar absorptivity. When the compositions are unknown, the pigment quantity is determined and expressed as cyanidin-3-glucoside [11].

2.7. Optimization of MAE based on RSM

The first step of RSM is finding the relationship between response y and factor x through the first-order polynomial equation and using a linear regression model, or better known as first-order model:

\[ Y = \beta + \sum_{i=1}^{k} (\beta_i X_i) \]  

(4)

The first-order experimental design that is suitable for the factor filtering phase is the 2k factorial design (Two Levels Factorial Design). Furthermore, for the second-order model, there is usually curvature and a second-order polynomial model whose quadratic function is used:

\[ Y = \beta_0 + \sum_{i=1}^{k} \beta_0 X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon \]  

(5)

Where: Y is the observation response, is in intercept, Bii is the linier coefficient, Bii is the quadratic coefficient, Bij is the treatment interaction coefficient, Xi is the treatment code for factor I, Xj is the treatment code for the factor of j and K is the number of factors tried. To determine the optimum
operating conditions in the second order, a central composite design is needed in the experimental data collection. According to Montgomery [12], a central composite design (CCD) design is a 2k factorial design or factorial, which is extended by adding observation points to the center to allow estimation of the second order (quadratic) surface parameter coefficients.

Analyses of variance (ANOVA) was used to determine the significance of each response by p-value and the predicted model was verified by conducting the experiments in triplicate under optimum extraction conditions. The number of lack of fit is used to known that the models can used to prediction of optimization [13]. The experimental design matrix is shown in Table 1 as follows.

### Table 1. Optimization of MAE based on RSM-CCD.

| Std | Extraction time (seconds) | Solvent volume (mL) | Yields extraction (%) | TAC (mg/g) | TFC (mg/g) |
|-----|---------------------------|--------------------|-----------------------|------------|------------|
| 1   | 120                       | 220.07             | 1.26                  | 3.43       | 11.77      |
| 2   | 120                       | 150                | 0.97                  | 4.01       | 13.23      |
| 3   | 60                        | 100                | 0.70                  | 3.56       | 12.64      |
| 4   | 120                       | 150                | 1.03                  | 3.73       | 15.14      |
| 5   | 35.16                     | 150                | 0.77                  | 2.89       | 10.47      |
| 6   | 60                        | 200                | 0.95                  | 3.01       | 10.90      |
| 7   | 204.84                    | 150                | 1.68                  | 4.34       | 20.12      |
| 8   | 120                       | 150                | 0.91                  | 3.67       | 14.55      |
| 9   | 180                       | 200                | 1.42                  | 4.09       | 18.38      |
| 10  | 180                       | 100                | 1.25                  | 3.83       | 16.34      |
| 11  | 120                       | 150                | 0.96                  | 3.93       | 13.40      |
| 12  | 120                       | 150                | 1.01                  | 3.79       | 14.88      |
| 13  | 120                       | 70.93              | 0.87                  | 2.91       | 10.26      |

3. Results and Discussion

#### 3.1. Identification of sim fruits

Sim fruit is known have bioactive compounds that are rich in phytochemical content, so the development in the biopharmaceutical industry is very potential and wide open. The sim fruits as research material was identified on water content, total dissolved solids, pH and colours. The results of the analysis on the characteristics of sim fruits are presented in Table 2 as follows.

### Table 2. Characteristics from sim fruits.

| Parameters                  | Average |
|-----------------------------|---------|
| Water content (%)           | 83.41   |
| Total dissolved solids (°Brix) | 8.48   |
| pH                          | 2.9     |
| Color                       |         |
| - L* (brightness)           | 13.5    |
| - a* (redness)              | 19.5    |
| - b* (yellowness)           | 10.5    |

From Table 2 above, known the sim fruits water content is quite high, namely 83.41% (b/v). high water content does not allow the sim fruit to be stored for a long time [14]. The more content of dissolved solids in a solution will make the solution thicker and more opaque, so the viscosity of the solution that contains a lot of dissolved solids is greater [15].

The results of the average pH analysis of sim fruits were 2.9 in the range of 2.8-3.1. The chemical and color forms of anthocyanin compounds depend on the pH of the solution [16]. The waiting time of
handling process affects the pH value of sim fruits during storage. Sim fruits with higher acidity will get a higher storability than pH fruits in the lower acidity. The effect of storage time on ascorbic acid content was not significantly different, but tended to decrease. This is due to delayed evaporation of water which causes the original whole cell structure to wither [17]. This affects the efforts to prevent damage by pathogenic microbes, which the sim fruit in the acid condition.

The brightness level (L) of sim fruits ranges 11-16, redness value (a) ranges 15-26 and yellowness value (b) ranges 6-15. In some areas of planting, sim fruits used as an ornamental plant, given the attractive colour of flowers, leaves and fruit. The part of the plant that can be consumed is the fruit parts that feels sweet. The unripened sim fruits has green colours and an astringent taste. The colour of the fruit flesh which ripened is turns black purplish, which indicated there is anthocyanin compounds inside and the texture of fruit is soft [18].

3.2. Process of microwave assisted extraction

The principle of heating microwave energy based on the direct effects of microwaves on material molecules. Electromagnetics energy transformation in heat energy is carried out by two mechanisms: ionic conduction and dipole rotation in solvents and materials. Both of these mechanisms take place simultaneously, which effectively changes heat energy from microwave energy. The mechanism of absorption of microwaves by materials is caused by microwave heating which takes place very quickly when compared with conventional heating [19]. Furthermore, dilation of cell walls occurred and breaks when the internal pressure cannot be retained [20].

3.3. Optimization of extraction based on RSM

The influence of significance between factor variables and each response, as well as optimization data prediction is done by Analysis of Variance (ANOVA). Furthermore, the influence of each response is used to determine the optimum model and verification data with RSM-CCD.

3.3.1. ANOVA test in each responses

ANOVA test was carried out on the extract yields, anthocyanin totals and flavonoids totals with Design Experts programs which can be seen in Table 3, as below.

| Sources                | Yield extracts | Anthocyanin totals | Flavonoid totals |
|------------------------|----------------|--------------------|------------------|
| Model                  | < 0.0001       | 0.0082             | 0.0008           |
| A-Extraction of time   | < 0.0001       | 0.0012             | < 0.0001         |
| B-Solvent of volume    | 0.0010         | 0.5261             | 0.4526           |
| AB                     | 0.5708         | 0.1204             | 0.1243           |
| A²                     | 0.0027         | 0.6131             | 0.0691           |
| B²                     | 0.2703         | 0.0182             | 0.0180           |
| Residual Lack of Fit   | 0.1453         | 0.0770             | 0.2232           |

Based on ANOVA test results, there is a significant influence on each responses with p-value<0.05. The p-value in the lack of fit of each responses has an insignificant influence (p-value>0.05) which means the model can be used as an optimization prediction. From software, the result of quadratic model for final equation in terms of actual factors toward each responses, namely yield extracts, anthocyanins total and flavonoids total gives as follows:

Extract yields = 0.613591 - 0.001473 A - 0.000259 B + 0.00003 A*A + 0.000011 B*B - 6.24167e-06 A*B

Anthocyanins total = 1.4331 - 0.000037 A + 0.025153 B - 0.000013 A*A - 0.000107 B*B + 0.000068 A*B
Flavonoids total = $4.968 - 0.054028 A + 0.119361 B + 0.000244 A^2 - 0.000503 B^2 + 0.000315 A*B$

### 3.3.2. Responses of variable factors

Ethanol solvents are used for the absorption of bioactive compounds contained in sim fruit. The cell membrane is expected to be ruptured with the extraction process of the MAE, so that the bioactive compounds inside can be absorbed by ethanol solvents. The relation between extraction times and solvent volumes on response of extract yields, anthocyanins total and flavonoids total obtained from sim fruit extraction is shown in Figure 1, as follows.

**Figure 1.** The relation of extraction times and solvent volumes toward response of yield extracts (A) response of anthocyanins total (B), response of flavonoids total (C) and desirability value (D).

Determination of optimum extraction was carried out by selecting the variable factors of the extraction time and the solvent ratio within in range. Each response of extract yields, anthocyanins total and flavonoids total were maximized. The optimal of yield extract known at 1.56% (Figure 1A), anthocyanins total at 4.32 mg/g (Figure 1B) and flavonoids total at 19.37 mg/g (Figure 1C). The optimum extraction obtained with variable factors for 190 seconds of extraction times and 1:19.05 (w/v) of sample to solvents ratio. The desirability value at 0.93 (Figure 1D).

### 3.3.3. Verification of optimum condition

The verification of the models needed to test the accuracy of the model in the application of empirical conditions. Determination of the optimum point of the extraction process is obtained from a
comparison of several response results of the analysis that has been carried out. These results are then compared with the results of optimization calculations based on the models (Table 4). Based on the results, the optimization data for the sim fruit extraction process with the MAE method has been verified.

| Table 4. Verification of optimum extraction condition. |
|-------------------------------------------------------|
| Lower predict. | Prediction | Higher predict. | Verify results | Deviation (%) |
|----------------|------------|-----------------|----------------|---------------|
| Yield extracts (%) | 0.70 | 1.10 | 1.68 | 1.07 | 2.73 |
| Flavonoids total (mg/g) | 10.26 | 15.52 | 20.12 | 15.47 | 0.32 |
| Anthocyanins total (mg/g) | 2.90 | 3.94 | 4.34 | 3.78 | 4.06 |

4. Conclusion

The Microwave Assisted Extraction was used to obtain the bioactive compound from sim fruit. Based on ANOVA test results, there is a significant influences on each responses of extraction processing with p-value<0.05. The p-value of the lack of fit in each responses has no significant influences (p-value>0.05) which means the model can be used as an optimization predictions. The optimum conditions for MAE process were 190 seconds of extraction times and 1:19.05 (w/v) of sample to solvents ratio. The verification of optimum condition were 1.07% of extract yields, 3.78 mg/g of anthocyanins and 15.47 mg/g of flavonoids, which all of responses are in the threshold of the predicted value.

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