Box-Behnken experimental design for extraction optimization of cytotoxic activity from *Curcuma aeruginosa* rhizome

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**ABSTRACT**
*C. aeruginosa* is the common name of temu hitam in Indonesia, and the rhizome parts of the plant have several pharmacological activities. Generally, pharmacological activities are associated with bioactive content in the extract of medicinal plants. Several factors can influence the bioactive extraction from medicinal plants such as solvent types, extraction time, extraction technique, and liquid-to-solid ratio. In this research, the extraction factors for extraction yield and cytotoxic activity of *C. aeruginosa* rhizome were optimized using the Box-Behnken experimental design. Effect of ethanol concentration, the ratio of liquid to solid, and extraction time for the maceration process was studied. The cytotoxic activity was determined by the brine shrimp lethality test. The optimum value that maximizes the extraction yield was 70% ethanol, 300:15 ml/g liquid to solid ratio, and 1-day extraction time. The optimum value that maximizes the cytotoxic activity was 70% ethanol, 150:15 ml/g liquid to solid ratio, and 2-day extraction time. The predicted extraction yield and cytotoxic activity at these projected values are 14.78% and 78.26 mg/l, respectively. In this model, Adeq Precision (10.35 and 4.16), R-Squared (0.86 and 0.79), and F-value (7.92 and 2.04) is rational to fit the model for extraction yield and cytotoxic activity from *C. aeruginosa* rhizome.

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**INTRODUCTION**
*Curcuma aeruginosa* Roxb. (*C. aeruginosa*) is a medicinal plant that is generally known in Indonesia as temu hitam, and widely utilized in traditional medicine. The *C. aeruginosa* rhizome is used in folk medicine, including the treatment of rheumatic, asthma, enteritis, stomach pain, obesity, increase appetite, and obesity (*Nurcholis et al., 016a*). Numerous works have reported the biological activity of *C. aeruginosa*, including its antimicrobial (*Kamazeri et al., Akarchariya et al., 2017*), anticancer (*Fitria et al., 2019*), antioxidant (*Nurcholis et al., 016b, 2017*), skin lightening, hair-growth (*Srivilai et al., 2017*), anti-dengue (*Moektiwardoyo et al., 2014*), anti-androgenic (*Suphrom et al., 2012*) and uterine relaxant (*Thaina et al., 2009*) properties. Previous works have founded the presence of various metabolites such as germacrene, camphor (*Akarchariya et al., 2017*), curcuminoid (*Nurcholis et al., 016a, 2019*), cycloislongifolene, 8,9-dihydro formyl, dihydrocostunolide (*Kamazeri et al., 2012*), terpenoids (*Simohand Zainal, 2015*), and sesquiterpenes (*Takano et al., 1995; Suphrom et al., 2012; Awin et al., 2019*) in the *C. aeruginosa* extract. Several reports have reported that the metabolites content in medicinal...
plant extract associated with the pharmacological activities (Mosbah et al., 2018; Chinnadurai et al., 2019; Bistgani and Sefidkon, 2019). Thus, the development of rhizome extraction is needed to produce rhizome extract from C. aeruginosa with high pharmacological properties.

The extraction is the crucial step for the recovery of metabolite compounds from medicinal plants. Various factors can affect the extraction effectiveness and extract yields such as solvent types (Qomaliyah et al., 2019), extraction time (Soós et al., 2019), extraction technique (Hmidani et al., 2019), and solvent-to-solid ratio (Sajid et al., 2019). The evaluate interaction in its extraction factors is proportionately important to enhance the extract yield. Response surface methodology, called RSM, is one alternative statistical method useful for improving complex extraction procedures. Several works have successfully reported for optimizing the extraction yield using RSM in the medicinal plant (Belwal et al., 2016; Pandey et al., 2018). Therefore, the study aims to optimize the extraction parameters, i.e., solid-to-liquid ratio, extraction time, and ethanol concentration in C. aeruginosa rhizome using RSM. Also, extraction yield and cytotoxicity for potency pharmacology studied at maximum conditions are also investigated.

MATERIALS AND METHODS

Experimental

Plant material

The dry rhizome sample of C. aeruginosa was collected from Tropical Biopharmacca Research Center, Bogor Agricultural University, Indonesia, in February 2019.

Extraction process and determination of cytotoxic activity

The powdered rhizome sample (15 g) of C. aeruginosa were macerated with varying volume (75 – 300 ml) of ethanol at a varying concentration (50 – 96%) in 500 ml elementary flask. These samples were mixed and macerated with a shaker at 235 rpm for different time extraction (1 – 3 days). The solid-to-liquid proportion, ethanol concentration, and time extraction were based on an experimental design created with response surface methodology and Box-Beihken design using Design-Expert version 11 (State Ease, Inc.). After extraction, the solution was filtered using Whatman no. 5 filter paper and then evaporated at 50 °C using a rotary evaporator (BUCHI, R-250, Switzerland). Based on the extracted content, the extraction yield was determined (% w/w). The extract directly applied to investigate the cytotoxic activity. Cytotoxic activity was performed according to the brine shrimp lethality test by (Meyer et al., 1982).

Experimental design

RSM and Box-Beihken design were used to evaluate the effect of extraction factors for obtaining the greatest extraction yield and cytotoxic activity with the maceration method from C. aeruginosa rhizome. The extraction parameter includes, ethanol concentration (%), liquid-to-solid ratio (ml/g), and time extraction (day). These variables were used for the optimization of extraction yield and cytotoxicity responses. The Box-Beihken experimental design consists of 15 experimental runs with the three replications at design center points of 150:15 ml/g liquid-to-solid ratio, 70% ethanol concentration, 2-day time extraction. (Table 1) showed the Box-Beihken experimental design and response of the extraction process. Optimization of the extraction process was performed using Design-Expert version 11.0 (Stat-Ease, Inc).

RESULTS AND DISCUSSION

Model fitting

The two-factor interaction and quadratic were suggested as a model statistic for extraction yield and cytotoxicity responses, respectively (Table 2). In these models, the R² value was 0.8559 of extraction yield and 0.7859 of cytotoxic activity responses. This study showed an R² value of less than 0.9, which indicated that the low tolerability of the model (Koocheki et al., 2009). (Table 3) presents the analysis of variance (ANOVA) employed in the 2FI and quadratic models for extraction yield and cytotoxic activity responses, respectively. The F values of 7.92 and 2.04 for extraction yield and cytotoxic activity, respectively, indicated the extraction yield model was significant but not significant for cytotoxicity response. The significant model indicated that the extraction parameters had a considerable influence on extraction yields but not effect cytotoxic activity (Tan et al., 2016). This term, the extraction yield is significantly affected by the liquid-to-solid ratio (A), time extraction (C), and interaction between liquid-to-solid ratio and time extraction (AC). The second order of liquid-to-solid ratio was significantly affected by the cytotoxicity response. The Adeq Precision was 10.35 and 4.16 for extraction yield and cytotoxicity response, respectively. Because the Adeq Precision more than 4.0, this result indicated that the model was rationally acceptable to navigate the design optimize (Shahinuzzaman et al., 2019). (Figure 1) presents the predicted vs. actual value for extraction yield and cyto-
Figure 1: Predicted value vs. actual value for (a) extraction yield and (b) cytotoxic activity

Figure 2: Response surface 3D plots displaying the influence of (a) ethanol vs. liquid-to-solid ratio, (b) time vs. liquid-to-solid ratio, and (c) time vs ethanol in the extraction parameters for extraction yield responses

Figure 3: Response surface 3D plots displaying the influence of (a) ethanol vs. liquid-to-solid ratio, (b) time vs. liquid-to-solid ratio, and (c) time vs ethanol in the extraction parameters for cytotoxic activity responses
Table 1: The extraction yield and cytotoxic activity responses with Box-Behnken experimental design from extraction process

| Run | Liquid-to-solid ratio (ml/g) | Ethanol (%) | Time (Day) | Extraction yield (%) | Cytotoxic activity (mg/l) |
|-----|-----------------------------|-------------|------------|----------------------|--------------------------|
| 1   | 150:15                      | 70          | 2          | 10.13                | 59.62                    |
| 2   | 150:15                      | 70          | 2          | 9.86                 | 70.58                    |
| 3   | 75:15                       | 96          | 2          | 8.52                 | 113.65                   |
| 4   | 75:15                       | 70          | 1          | 8.73                 | 239.90                   |
| 5   | 150:15                      | 50          | 1          | 9.55                 | 180.72                   |
| 6   | 150:15                      | 50          | 3          | 7.15                 | 109.77                   |
| 7   | 150:15                      | 96          | 1          | 11.78                | 270.67                   |
| 8   | 300:15                      | 70          | 3          | 7.89                 | 211.93                   |
| 9   | 150:15                      | 70          | 2          | 12.98                | 104.58                   |
| 10  | 300:15                      | 70          | 1          | 20.63                | 169.08                   |
| 11  | 150:15                      | 96          | 3          | 10.82                | 99.27                    |
| 12  | 300:15                      | 50          | 2          | 15.14                | 409.61                   |
| 13  | 75:15                       | 70          | 3          | 9.79                 | 160.52                   |
| 14  | 75:15                       | 50          | 2          | 6.29                 | 326.62                   |
| 15  | 300:15                      | 96          | 2          | 15.46                | 371.00                   |

Table 2: Model summary statistics for extraction yield and cytotoxic activity

| Extraction yield (%) | Source     | SD       | $R^2$     | $R^2_{Adj}$ | $R^2_{Pre}$ | Comment |
|----------------------|------------|----------|-----------|-------------|-------------|---------|
| Linear               | 0.0133     | 0.3027   | 0.5016    | 0.1914      |             |         |
| 2FI                  | 1.89       | 0.8559   | 0.7479    | 0.3587      | Suggested   |         |
| Quadratic            | 0.5973     | 0.4088   | 0.7144    | -0.2174     |             |         |
| Cubic                | 0.4088     |          | 0.7890    |             | Aliased     |         |

| Cytotoxic activity   | Source     | SD       | $R^2$     | $R^2_{Adj}$ | $R^2_{Pre}$ | Comment |
|----------------------|------------|----------|-----------|-------------|-------------|---------|
| Linear               | 0.5859     | 0.0341   | -0.0752   | -0.5704     |             |         |
| 2FI                  | 0.8344     | 0.0253   | -0.3352   | -2.0595     |             |         |
| Quadratic            | 85.17      | 0.7859   | 0.4006    | -2.3360     | Suggested   |         |
| Cubic                | 0.0451     | 0.9546   |           |             | Alased      |         |

toxicity, respectively.

**Extraction yield**

As presented in (Figure 2), liquid-to-solid ratio, ethanol concentration and extraction time were understood in the ranges of 75:15 – 300:15 ml/g, 50 – 96%, and 1 – 3 days for extraction yield response. Extraction yield was ranged 6.29% to 20.63% with maximum yield on the solid-to-liquid ratio of 300:15 (ml/g), ethanol concentration of 70%, and time extraction of 2 days (Table 1). At the constant extraction time (2 days), the maximum extraction yield (15.46%) was found at the solid-to-liquid ratio of 300:15 (ml/g) and ethanol concentration of 96% (Figure 2a). (Figure 2b) illustrates the influence of liquid-to-solid ratio and extraction time on the extraction yields. The response surface 3D plot was generated with the ethanol concentration fixed at 73%. The highest extraction yield (20.63%) was obtained at a liquid-to-solid ratio of 300:15 (ml/g) at the 1-day maceration process. The variation of extraction yield with extraction time and ethanol concentration at constant liquid-to-solid ratio (187.5:15 ml/g) is presented in (Figure 2c). The highest extraction yield was approximately 12.98% at an ethanol concentration of 73% and 2-day extraction. This work shows that the extraction yield increased linearly with extraction variables of ethanol concentration and liquid-to-solid ratio.

**Cytotoxic activity**
Table 3: ANOVA for response surface 2FI model for extraction yield and quadratic model for cytotoxic activity

| Source | Sum of Squares | df | Mean Square | F-value | p-value | Status |
|--------|----------------|----|-------------|---------|---------|--------|
| Model  | 169.39         | 6  | 28.23       | 7.92    | 0.0051  | significant |
| A      | 83.13          | 1  | 83.13       | 23.32   | 0.0013  |         |
| B      | 8.92           | 1  | 8.92        | 2.50    | 0.1522  |         |
| C      | 28.36          | 1  | 28.36       | 7.96    | 0.0225  |         |
| AB     | 0.9063         | 1  | 0.9063      | 0.2543  | 0.6277  |         |
| AC     | 47.56          | 1  | 47.56       | 13.34   | 0.0065  |         |
| BC     | 0.5146         | 1  | 0.5146      | 0.1444  | 0.7139  |         |
| Residual | 28.51     | 8  | 3.56        | 1.26    | 0.5056  | not significant |
| Lack of Fit | 22.55 | 6  | 3.76        | 1.26    | 0.5056  | not significant |
| Pure Error | 5.97    | 2  | 2.98        |         |         |         |
| Cor Total | 197.91  | 14 |             |         |         |         |

| Source | Sum of Squares | df | Mean Square | F-value | p-value | Status |
|--------|----------------|----|-------------|---------|---------|--------|
| Model  | 1.332E+05      | 9  | 14795.66    | 2.04    | 0.2238  | not significant |
| A      | 12874.19       | 1  | 12874.19    | 1.77    | 0.2403  |         |
| B      | 3703.69        | 1  | 3703.69     | 0.5106  | 0.5069  |         |
| C      | 9721.55        | 1  | 9721.55     | 1.34    | 0.2993  |         |
| AB     | 7600.21        | 1  | 7600.21     | 1.05    | 0.3530  |         |
| AC     | 3735.39        | 1  | 3735.39     | 0.5149  | 0.5051  |         |
| BC     | 2522.45        | 1  | 2522.45     | 0.3477  | 0.5810  |         |
| A²     | 61068.41       | 1  | 61068.41    | 8.42    | 0.0337  |         |
| B²     | 35718.68       | 1  | 35718.68    | 4.92    | 0.0772  |         |
| C²     | 488.87         | 1  | 488.87      | 0.0674  | 0.8055  |         |
| Residual | 36271.00      | 5  | 7254.20     |         |         |         |
| Lack of Fit | 35171.96      | 3  | 11723.99    | 21.33   | 0.0451  | significant |
| Pure Error | 1099.04       | 2  | 549.52      |         |         |         |
| Cor Total | 1.694E+05     | 14 |             |         |         |         |

A = Liquid-to-solid ratio (ml/g); B= Ethanol (%); C = Time (Day)

The effect of liquid-to-solid ratio, ethanol concentration, and extraction times on the cytotoxic activity was shown in (Figure 3). Cytotoxic activity ($LC_{50}$ value) was ranged 59.62 mg/l to 409.61 mg/l. The highest cytotoxicity (lower value of $LC_{50}$) was obtained at a liquid-to-solid ratio of 150:15 (ml/g), 70% ethanol, and 2-days extraction time. Because $LC_{50}$ < 1000 mg/l, all extracts were potency showed as the anticancer agent (Meyer et al., 1982). Cytotoxic activity under different ethanol concentrations and the liquid-to-solid ratio at a constant time of 2-days is seen in (Figure 3a). The lowest $LC_{50}$ value, highest cytotoxicity (113.65 mg/l), was obtained at a liquid-to-solid ratio of 75:15 ml/g and 96% ethanol. (Figure 3b) represents the interaction between ethanol concentration and extraction times on cytotoxic activity. The maximum cytotoxicity (59.62 mg/l) was observed at extraction situations 187.5:15 (ml/g) of liquid-to-solid ratio and 2-days extraction at a fixed of ethanol concentration (73%). (Figure 3c) depicts the cytotoxic activity with respect to ethanol concentration and extraction time at the liquid-to-solid ratio of 187.5:15 ml/g. The highest cytotoxic activity (59.62 mg/l) was recorded at 73% ethanol and 2-days extraction time.
CONCLUSIONS

RSM with Box-Behnken experimental design was effectively used to optimize the extraction factors from *C. aeruginosa* rhizome. The ethanol concentration, liquid-to-solid ratio, and extraction time were important parameters affecting the extraction yield and cytotoxic activity. For the extraction yield (20.63%), the maximize combination of parameters was 70% ethanol, 300:15 ml/g liquid to solid ratio, and 1-day extraction time. In cytotoxic activity (59.62 mg/l), the maximize combination of parameters was 70% ethanol, 150:15 ml/g liquid-to-solid ratio and 2-day extraction time.

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