



Optimization of the extraction process for extract yields, total flavonoid content, radical scavenging activity and cytotoxicity of *Curcuma aeruginosa* RoxB. rhizome

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ABSTRACT

The rhizome of *Curcuma aeruginosa* RoxB. (RCA) is used traditionally for several diseases. The flavonoid compounds contained in the RCA that responsible for biological activities. In this study, ethanol, water, and acetone had been used as solvent extraction of RCA and applied the simplex-centroid design to optimize the extraction process. The radical scavenging activity and total flavonoid content were investigated using in vitro assays, while cytotoxicity was evaluated by brine shrimp lethality test. ANOVA suggested a linear model for all responses. The ethanol: acetone extract had the highest extract yield with a value of 25.15%, and this result showed no significant with acetone extract at $p < 0.05$ (22.86%). The ethanol extracts confirmed the higher degree of total flavonoid content and cytotoxicity with a value of 17.97 mg QE/g and 140 $\mu\text{g/ml}$, respectively. The ethanol: water extract, ethanol extract, and water extract (IC_{50} , 100-500 $\mu\text{g/ml}$) showed moderate radical scavenging activity compared with ascorbic acid (IC_{50} , 7.19 $\mu\text{g/ml}$). Regarding the correlation analysis, the negative correlation was weakly observed between flavonoid content and IC_{50} of free radical scavenging activity, which indicates that the flavonoid in RCA contributes not significantly to the antioxidant activity. Introductory studies on the extraction process of the advanced flavonoid extracts exhibited a scavenging and cytotoxic effect of the ethanol extract on *C. aeruginosa* rhizome.

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INTRODUCTION

Curcuma aeruginosa Roxb. belongs to Zingiberaceae family, popularly known as temu ireng or temu hitam in Indonesia. It is an indigenous traditional medicinal plant used to relieve enteritis, asthma, stomach pain, rheumatic problems, serve as an anthelmintic, increase appetite, and prevent obesity. Traditionally, the rhizome of *C. aeruginosa* (RCA) was part of the plant used in medicine. Previous research reported that RCA has antioxidant potential, antibacterial, as well as anti-HIV and anticancer based on preliminary cytotoxicity study (Azmir *et al.*, 2013; Leite *et al.*, 2014; Garcia-castello *et al.*, 2015; Simoh and Zainal, 2015). This

high pharmacological potential is related to the content of secondary metabolite compounds of RCA, including flavonoid. The antioxidant compound of RCA was reported to be a trimethylsilyl (TMS) derivative, a flavonoid compound. The TMS derivative was soluble in semi polar-polar solvent (Simoh and Zainal, 2015).

The crucial and significant step which is responsible for enriching secondary metabolites and high potent of biological activity is the extraction process (Azmir et al., 2013). A proper extraction approach considers the superior solvent or combination of the solvent with a handy method (Leite et al., 2014). Therefore, it is necessary to conduct a systematic optimization process of flavonoid extraction from RCA to improve efficiency and minimize the number of experiments to find out the type of solvent most suitable for flavonoid extraction. The optimization process was carried out using the statistical model to obtain satisfactory results.

Mixture design, one of the statistical model, is widely used to optimize mixture proportion in many product development areas such as in food industry (Safaralie et al., 2010), in pharmaceutical industry (Valleri et al., 2004; Furlanetto et al., 2011) and in the process to find out solvent with characteristic enriching secondary metabolite (Garcia-castello et al., 2015). This approach goals to look into how responses are affected by the variation in the component's proportions (Cornell, 2011). The simplex centroid model was conducted to optimization solvent extraction on RCA based on extract yield, total flavonoid content, antioxidant activity, and cytotoxicity.

MATERIALS AND METHODS

Plant material

The *C. aeruginosa* rhizomes were obtained from the Tropical Biopharmaca Research Centre, Bogor Agriculture University, Indonesia in February 2018. The rhizomes were first dried in an oven at 45 until three days, then ground and passed through an 80-mesh sieve and subjected to the extraction process.

Chemicals and reagents

The quercetin compound standard was from Wako Pure Chemical Industries. Ascorbate acid and DPPH (2,2-diphenyl-1-picrylhydrazyl) were from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals were of analytical grade.

Extraction procedure

The extraction process was conducted by the maceration technique. RCA is extracted by two sessions

dynamic maceration 130 rpm. First, maceration for three days was used n-hexane to deflate nonpolar compound that contains in RCA. Second, after drying, maceration for 24 hours has used a mixture of three solvent: ethanol (A), water (B), and acetone (C) in a proportion of 4.5% (m/v) (w/v), through simplex centroid design. Figure 1 illustrates the proportion of solvents in specificity. All extracts were evaporated, and the extraction process was conducted in triplicate.

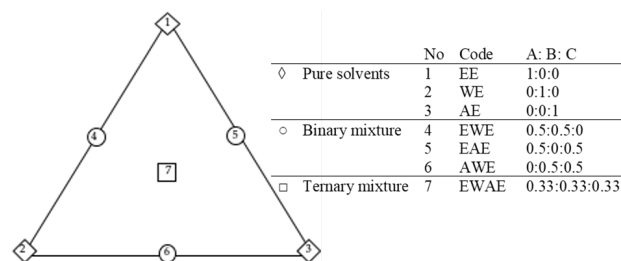


Figure 1: Arrangement of simplex-centroid design with three component A (ethanol), B (water), C (acetone) as well as solvent mixture ratios in each essay (Code)

Total flavonoid content

Total flavonoid content was determined the use of aluminum chloride approach (Nawaz et al., 2006). Each extract was diluted with methanol, the preparation process was continued with vortex 3 minutes, and the diluted extract stayed overnight, then filtered. The filtrate was used to investigate the total flavonoid content. The calibration curve was prepared using quercetin. Quercetin was dilution with methanol (25-200 $\mu\text{g/ml}$). The diluted extract or quercetin (10 μl) was combined with 60 μl methanol, 10 μl of 10% (w/v) aluminum chloride solution, 10 μl of 1 mM potassium acetate solution and 110 μl of distilled water in 96-well microplate. After incubation process 30 minutes at room temperature, the maximum absorbance of the mixture was measured at 415 nm using Elisa Reader (Epoch BioTek®). Total flavonoid content was expressed as milligram quercetin equivalent per gram extract (mg QE/g).

Radical scavenging assay

The radical scavenging capacity of every extracts was measured using the DPPH scavenging method (Vongsak et al., 2013). Stock solution of DPPH was prepared, 2.5 mg DPPH was diluted with ethanol up to 50 ml. The extracts were prepared through dilution process with ethanol (25-400 $\mu\text{g/ml}$) and helped by sonication 15 minutes to perfectly dissolve extract, placed 100 μl of sample solution to the 96 well plate, then 100 μl of DPPH (125 μM) solution was combined to the solutions

and incubation in the dark place for 30 minutes at room temperature. The absorbance was read at 517 nm with ELISA Reader (Epoch BioTek®). Negative control of sample 100 µl of sample solution was added by 100 µl ethanol. As a sample blank, 100 µl ethanol was combined by 100 µl DPPH solution and the negative control of blank consisted of ethanol 200 µl. DPPH radical-scavenging activity (DRSA) was determined as:

$$\%DRSA = \frac{Acn - As}{Acn} \times 100$$

Where Acn is the negative control absorbance and As is the absorbance of the extract solution. The half-maximal inhibitory concentration (IC₅₀) value or extract concentration essential to inhibit 50% of DPPH effect, used to be decided via plotting the %DRSA as a function of the sample concentration.

Cytotoxicity

The cytotoxic activity of every extract was evaluated by using the brine shrimp lethality test (Meyer et al., 1982). In brief, the extract was prepared through dissolving in 10% dimethyl sulfoxide (Merck, Germany) and including to the seawater to make final concentrations of 10, 50, 100, 500 and 1000 µg/ml. A total of 10 brine shrimps were entered into every vial containing seawater and extracts of samples at different concentrations. A control was run containing 10% dimethyl sulfoxide. After 24 hours of exposure, the percentage mortality was recorded, and the outcomes were subjected to SPSS 16.0 for LC₅₀ calculation.

Experimental Design

Experiments were designed and analyzed using the software Design Expert 11.0 statistical software (Stat-Ease Inc., Minneapolis, USA). The significant different responses were evaluated with the Duncan test at *p* < 0.05, and the correlation analyses performed with Pearson correlation using SPSS 25.0.

RESULTS AND DISCUSSION

Extraction Yield

The extraction yield in this study was evaluated based on the solvent ability to extract phytochemical metabolites from RCA, including the targeted secondary metabolite, flavonoid. The solubility of flavonoid in a solvent is affected by the solvent polarity and chemical structure of secondary metabolites (Biesaga, 2011). In the present study, polar and semi-polar solvents, as well as their mixture, were employed. The extract yields response of solvent ethanol (EE), water (WE), acetone (AE) and binary solvent ethanol: water (EWE), ethanol: acetone (EAE), acetone: water (AWE), and also ternary

solvent ethanol: water: acetone (EWAE) from RCA samples presented in Figure 2. Significant differences in extract yield among solvents employed are presented in Figure 2. Figure 3 presents the impact of the interplay of ethanol (A), water (B), and acetone (C) on the extraction yield of RCA. In this study, EAE (25.15%) shows higher extraction yield followed by AE, EWE, EWAE, EE, AWE, and WE, respectively. The extract yield of EAE shown different significant at *p* < 0.05 between extracts sample except with AE (22.86%). Linear model gave a satisfactory value for the determination coefficient (*r*²= 0.8232 and *p*-value < 0,05 (0.035)), significant (Table 1). All coefficients, except EE (A), were significant at *p* < 0.05 (Equation 1). Phenolics, including flavonoids, are less extracted with water (Ghasemzadeh et al., 2011) (Wijekoon et al., 2011). Similarly, the present study of linear trials showed that extraction with water resulted in the lowest yield (15.01%, w/w). Then, amongst the binary interaction, the presence of water solvent decreased the extracting strength of acetone, but not the ethanol. Aqueous ethanol (1:1) can increase the solubility of the metabolite compound than water or ethanol alone (Do et al., 2014). The ternary solvent illustrated the synergistic effect among the solvents. The solubility of flavonoids, as indicated by the yield of extraction, is higher in acetone and ethanol (Ferreira and Pinho, 2012).

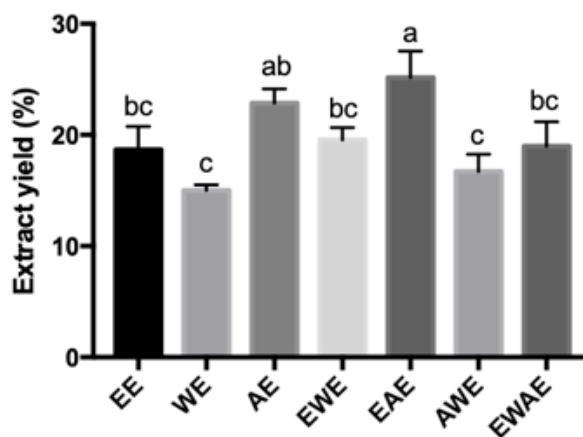


Figure 2: The extract yield percentage response of ethanol extract (EE), water extract (WE), acetone extract (AE), ethanol: water extract (EWE), ethanol: acetone extract (EAE), acetone: water extract (AWE), and ethanol: water: acetone extract (EWAE)

Values are mean of triplicate determination ± standard error. ^{a-c}Different superscripted letter indicates a significant difference between sample extracts (*p* < 0.05).

$$Extract\ yield = 25.65A + 13.76B + 22.28C \quad (1)$$

Table 1: ANOVA for the linear model of yield extraction

Source	Sum of Squares	df	Mean Square	F-value	-value	
Model	63.68	2	31.84	12.65	0.0345	significant
¹ Linear Mixture	63.68	2	31.84	12.65	0.0345	
Residual	7.55	3	2.52			
Cor Total	71.23	5				

¹ Inference for linear mixtures uses Type I sums of squares

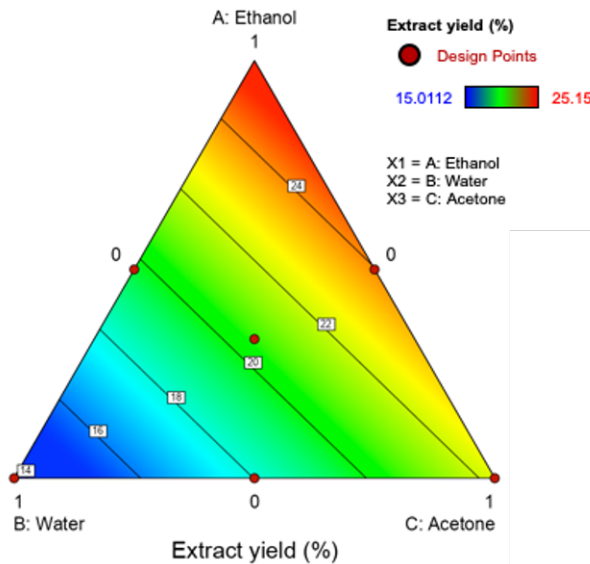


Figure 3: The contour plot of the effects of solvents three variables of ethanol (A), water (B), and acetone (C) to the extract yield percentage

Total Flavonoid Content

Curcuma, the Zingiberaceae family, is one of the biggest genera which contain high flavonoid compounds. RCA is one of them and was further confirmed in this study. A standard curve ($y = 0.0043x + 0.0063$, $r^2 = 0.996$) for determination of total flavonoid content was obtained by measuring optical density of standard solution of quercetin. The total flavonoid content was estimated based on this standard curve, and the results are presented in Figure 4. The total flavonoid varied from 6.46 to 17.94 mg QE/g (Figure 4) with the impact of the interaction solvent extraction on the flavonoid content showed in Figure 5. The total flavonoid content was once found to be extensively greater in ethanol extract (EE, 17.97 mg QE/g) at $p < 0.05$ in contrast with different solvents. Table 2 demonstrates the results of the fitting linear models for the total flavonoid content response. The result ANOVA shows that the contribution of the linear model was not significant, but this model suggested with Mean Square value of 15.12. The final equation for total

flavonoid content is $(2) Total\ flavonoid\ content = 15.02A + 10.96B + 8.10C \quad (2)$

A: ethanol; B: water; C: acetone

The total flavonoid content is positively and linearly influence by ethanol (A), water (B), and acetone (C), respectively. Data shows that ethanol was extracted useful of flavonoid compound in RCA compared to other solvents. Similarly, previous reports showed that ethanol effectively for flavonoid extraction compared to water, methanol, and acetone (Do et al., 2014). This result suggests that the extraction of flavonoid compounds on RCA is not efficient using water solvent. Generally, the flavonoid compounds were limited solubility in water, but more soluble in ethanol or acetone (Wang et al., 2009). The flavonoid of RCA is more soluble in the solvent extraction with medium polarity compared to polar (water) or nonpolar (acetone). Although extraction using ethanol: acetone (EAE) combination resulted in the highest yield of phytochemical compounds, but it did not give the most top of total flavonoid content. This result might be due to the higher level of other secondary metabolites apart from flavonoid such as polyphenol group or other compounds, which are soluble in a semipolar organic solvent such as EAE.

Values are mean of triplicate determination \pm standard error. ^{a-c}Different superscripted letter indicates a significant difference between sample extracts ($p < 0.05$).

Antioxidant Activity

The 2,2-Diphenyl-1-picryl-hydrazil (DPPH) assay was used for evaluating the radical scavenging activity. DPPH acts as a stable free radical. Antioxidant compounds from the RCA extract react with DPPH, the DPPH initially has a deep purple colour but turns to colourless diphenyl-picrylhydrazine after the reaction with RCA. The decolouration shows the scavenging potentials of the radical scavenging activity samples (Abdille et al., 2005). The DPPH radical scavenging activity of RCA extracts are shown in Figure 6. Radical scavenging activity used to be expressed in IC₅₀, the quantity of antioxidant substance required to reduce the radical impact of

Table 2: ANOVA for the linear model of total flavonoid content

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	30.23	2	15.12	1.36	0.3536	not significant
¹ Linear Mixture	30.23	2	15.12	1.36	0.3536	
Residual	44.36	4	11.09			
Cor Total	74.59	6				

¹ Inference for linear mixtures uses Type I sum of squares

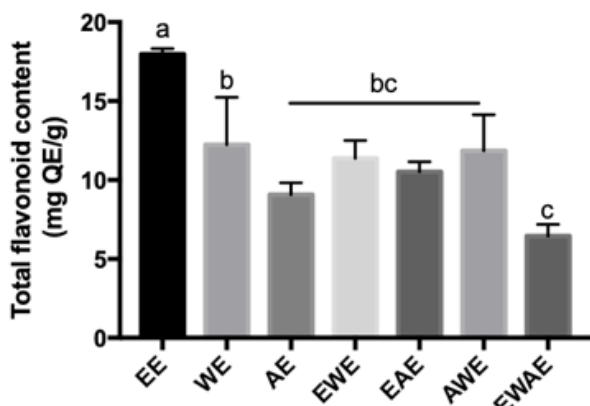


Figure 4: The total flavonoid content response of ethanol extract (EE), water extract (WE), acetone extract (AE), ethanol: water extract (EWE), ethanol: acetone extract (EAE), acetone: water extract (AWE), and ethanol: water: acetone extract (EWAE)

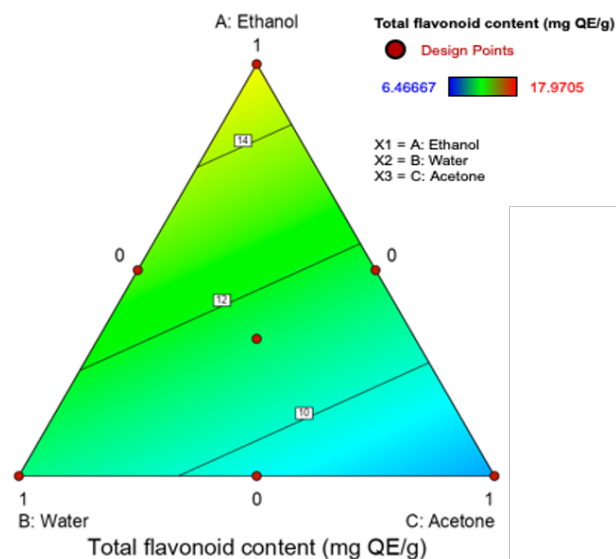


Figure 5: The contour plot of the effects of solvents three variables of ethanol (A), water (B), and acetone (C) to the total flavonoid content

DPPH concentration by 50%. The lower IC₅₀ value shows higher antioxidant activity. Significant variations were observed in radical scavenging activity among different extracts, the IC₅₀ value ranging from 276.12 to 600.91 μg/mL (Figure 6 and Figure 7). The present study employed ascorbic acid (IC₅₀ = 7.19 μg/ml) as a standard antioxidant. The IC₅₀ values indicate the free radical scavenging activity, which can be divided into three groups: (a) strong with IC₅₀ < 100 μg/ml; (b) moderate with IC₅₀, 100-500 μg/ml; and (c) weak with IC₅₀ > 500 μg/ml (Bi et al., 2016). Based on this categorization, the extracts of EWE, EE, WE showed moderate antioxidant activity, while the extracts of EWAE, AE, EAE, AWE showed weak antioxidant activity.

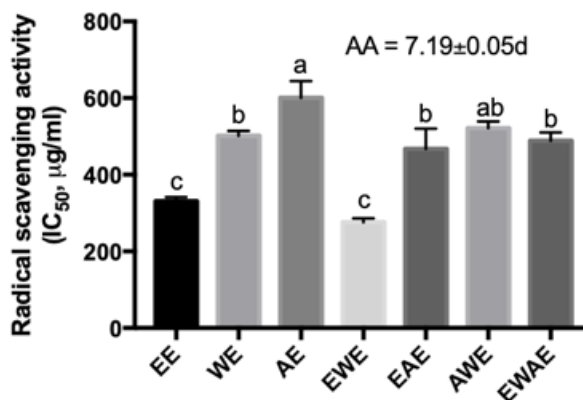


Figure 6: Theradical scavenging activity response of ethanol extract (EE), water extract (WE), acetone extract (AE), ethanol: water extract (EWE), ethanol: acetone extract (EAE), acetone: water extract (AWE), and ethanol: water: acetone extract (EWAE). AA = Ascorbic acid

Values are mean of triplicate determination ± standard error. ^{a-d} Different superscripted letter indicates a significant difference between sample extracts ($p < 0.05$).

ANOVA suggested a linear model (Table 3) for radical scavenging activity response, with a sequential R-Squared of 0.792 and p -value < 0.05. The results of the radical scavenging activity of extracts are presented as a mathematical model (Equation 3) and in

the contour plot (Figure 6). In Equation 3, ethanol had a significant positive impact on the extraction of RCA with radical scavenging activity; however, combining ethanol with acetone (EAE) improved the extract yield. As seen in Figure 5 and Figure 6, the lowest IC₅₀ values have been got with ethanol and its binary mixture, as well as the mixture of ethanol: water (EWE). These effects have been in accord with previous reports which confirmed that aqueous-ethanol had extraction of flavonoid compounds successfully (Munhoz et al., 2014; Alara et al., 2017).

$$\text{Radical scavenging activity (IC}_{50}\text{)} = 312.24A + 450.02B + 626.23C \quad (3)$$

A: ethanol; B: water; C: acetone

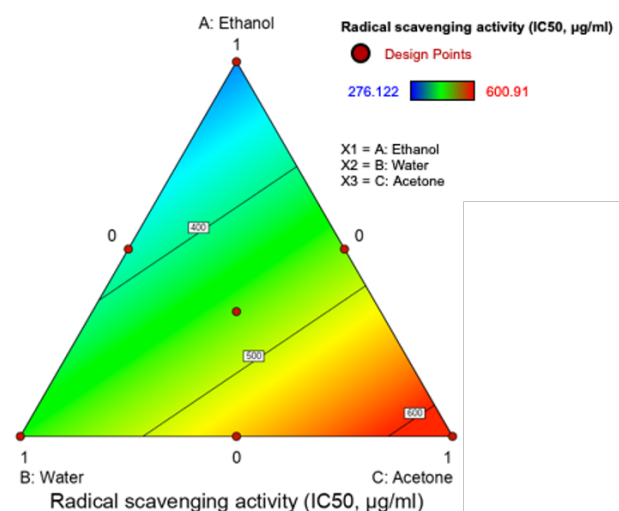


Figure 7: The contour plot of the effects of solvents three variables of ethanol (A), water(B), and acetone (C) to the radical scavenging activity

Cytotoxic Activity

Flavonoids are used for cytotoxic activity of various diseases (Marzouk, 2016; Boutennoun et al., 2017). Thus, cytotoxic activity was carried out to evaluate the optimum solvent in flavonoid extracting on RCA. The brine shrimp lethality test (BSLT) was used to investigate the cytotoxic impact of RCA extracts. The results of BSLT, in a value of LC₅₀, were shown in Figure 8. All extracts were found to be toxic with LC₅₀ < 1000 mg/ml (Supraja et al., 2018). These results indicated the presence of potent cytotoxicity as anticancer on the RCA extracts (Nurcholis et al., 2016a, 2018). Ethanol extract (EE, LC₅₀ of 140.00 µg/ml) showed a significant cytotoxic effect compared with other extracts (Figure 8 and Figure 9) except with the acetone extract (AE, LC₅₀ of 199.63 µg/ml) at *p* < 0.05. Table 4 presents the results for fitting linear models of cytotoxic activity on RCA extracts. The result of ANOVA shows that the contribution of the linear model for cytotoxicity was significant. The fit-

ted linear model for cytotoxicity in coded variables is given in Equation 4. In this case, factor B (water) and C (acetone) were a significant model term, but factor A (ethanol) was not significant. Figure 9 demonstrated the mathematical model in the ternary diagram for the cytotoxic response on different solvents extraction.

$$\text{Cytotoxicity } ([LC]_{50}) = 139.83 + 370.69B + 57.20C \quad (4)$$

B: water; C: acetone

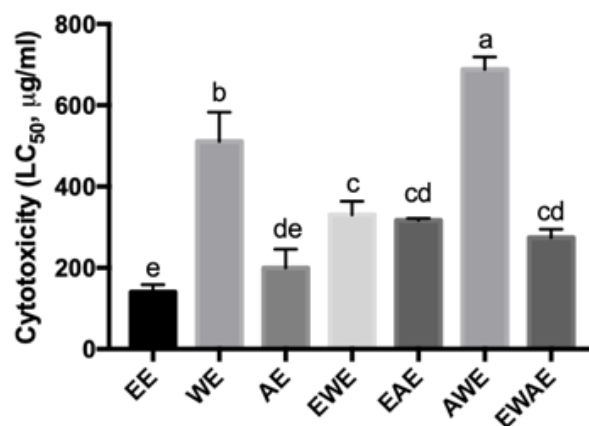


Figure 8: The cytotoxic activity response of ethanol extract (EE), water extract (WE), acetone extract (AE), ethanol: water extract (EWE), ethanol: acetone extract (EAE), acetone: water extract (AWE), and ethanol: water: acetone extract (EWAE)

Values are mean of triplicate determination ± standard error. ^{a-e} Different superscripted letter indicates a significant difference between sample extracts (*p* < 0.05).

Optimization result

The ethanol (1:0:0) was selected as an optimize solvent as significantly fitted suitable with criteria maximum effect of yield extract and total flavonoid, a minimum result of antioxidant and cytotoxic activity through the value of IC₅₀ and LC₅₀, respectively. The desirability of this selected solvent was 0.902. The overlay plot of all response is depicted in Figure 10.

Correlation all of the biological activity

Some biological activities, such as antioxidant and cytotoxicity, correlated with the phytochemical content of the plant (Pham et al., 2018). Therefore, in this study associated extract yield and total flavonoid content with biological activities of radical scavenging activity and cytotoxicity were evaluated. Extract yield and total flavonoid content were correlated with IC₅₀ of radical scavenging activity and

Table 3: ANOVA for the linear model of antioxidant activity

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	68458.28	2	34229.14	10.48	0.0443
¹ Linear Mixture	68458.28	2	34229.14	10.48	0.0443
Residual	9802.53	3	3267.51		
Cor Total	78260.82	5			

¹ Inference for linear mixtures uses Type I sum of squares

Table 4: ANOVA for the linear model of cytotoxic activity

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	81115.50	2	40557.75	886.90	0.0011
B-Water	68707.27	1	68707.27	1502.45	0.0007
C-Acetone	1794.01	1	1794.01	39.23	0.0246
Residual	91.46	2	45.73		
Cor Total	81206.96	4			

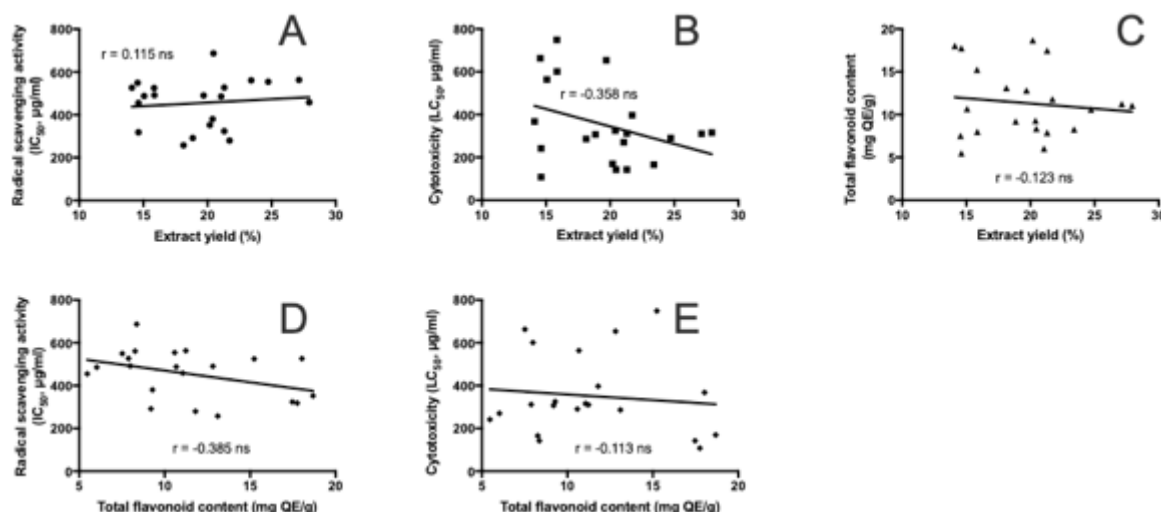


Figure 11: The Pearson correlation between extract yield and radical scavenging activity (A), cytotoxicity (B), and total flavonoid content (C), and also total flavonoid content with radical scavenging activity (D) and cytotoxicity (E) in the extracts obtained by the simplex-mixture design of RCA

LC₅₀ of cytotoxicity using simple regression analysis and Pearson correlation (Figure 11). All correlation parameters showed not significant at $p < 0.05$. The relationship between extract yield with radical scavenging activity and cytotoxicity were positive ($r: 0.115$) and negative ($r: -0.358$), respectively. In the case of total flavonoid content, this parameter correlated negatively with radical scavenging activity ($r: -0.358$) and cytotoxicity ($r: -0.113$). This result demonstrated that correlation extract yield or total flavonoid content was weakly correlated with radical scavenging activity and cytotoxicity on RCA extracts. These results were in accord with our previous reports which showed a lowly correlation in

the ethanolic extract from 20 *C. aeruginosa* accessions (Nurcholis *et al.*, 2016b). Therefore, not all the metabolites extracted in RCA corresponded to flavonoids compound. Maybe some of the metabolites in RCA, not only flavonoids, were obtained that had antioxidant and cytotoxic activities. The results are supported the previous study, which showed that the essential oils from the *C. aeruginosa* possessed cytotoxic activity against bacteria (Kamazeri *et al.*, 2012; Akarchariya *et al.*, 2017). Another study demonstrated that the *C. aeruginosa* rhizome contained various metabolite such as sesquiterpenes and terpenoids (Suphrom *et al.*, 2012; Simoh and Zainal, 2015). Recently, some studies reported that

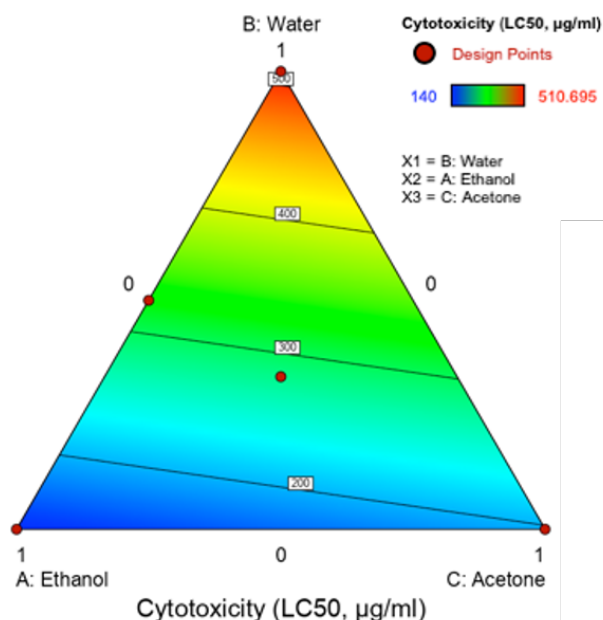


Figure 9: The contour plot of the effects of solvents three variables of ethanol (A), water(B), and acetone (C) to the cytotoxic activity

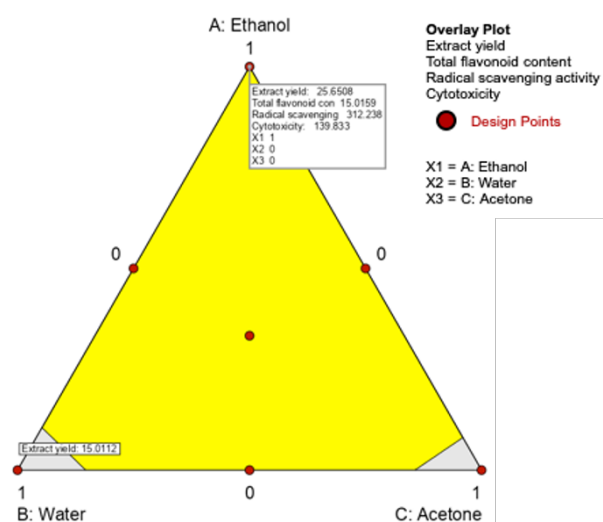


Figure 10: The overlay plot of the responses for extract yield, total flavonoid content, radical scavenging activity, and cytotoxicity from optimized solvent extraction of RCA

the sesquiterpene and terpenoids identified as a bioactive that responsible for antioxidant and cytotoxic activities (Babaei et al., 2018). These results indicate that the sesquiterpene and terpenoids compounds as major metabolite were contained in the *C. aeruginosa* rhizome which possible efficacy for the antioxidant and cytotoxicity. Therefore, further metabolite investigation needs to be done in the *C. aeruginosa* rhizome that responsible for antioxidant and cytotoxic activities.

CONCLUSION

The extraction of total flavonoid compounds with radical scavenging and cytotoxic activities from *C. aeruginosa* rhizome was optimized using the simplex-centroid design which showed that the ethanol was most effective than acetone, water, and their mixtures. The extract was found to have moderate antioxidant and cytotoxic activities. Therefore, isolate and identify bioactive were needed to be an investigation in the *C. aeruginosa* rhizome that responsible as antioxidant and cytotoxic activities.

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Conflict of interest

The authors have no conflict of interest to declare.

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