Jurnal Jamu Indonesia (2023) 8(2): 57–69
ISSN 2407-7178
eISSN 2407-7763

Research Article
DOI: https://doi.org/10.29244/jji.v8i2.328

Optimization of Cardamom Fruit (*Amomum compactum*) Extraction Focused on Total Terpenoid Extraction and Cytotoxic Activity Using Response Surface Methodology

Optimasi Ekstraksi Buah Kapulaga (*Amomum compactum*) Difokuskan Pada Ekstraksi Total Terpenoid Dan Aktivitas Sitotoksik Menggunakan Metode Permukaan Respon

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Received March 02, 2023; **Accepted** June 15, 2023; **Available online** July 04, 2023

ABSTRACT

Cardamom is one of the medicinal plants called perennial herbs from the Zingiberaceae family. Cardamom, with the scientific name *Amomum compactum*, contains various phytochemical compounds such as terpenoids, flavonoids, phenolics, tannins, sterols, and proteins that are pharmacologically useful as an anticancer, antioxidant, and have cytotoxic activity. Testing the total content of terpenoid compounds that affect the cytotoxic activity of *A. compactum* fruit using Box-Behnken Design with three independent variables (solvent ratio, ethanol concentration, and extraction time) has never been studied before. Thus, this study aimed to obtain the extraction optimization conditions on the total terpenoid content and cytotoxic activity using *Artemia salina* Leach larvae with the response surface method, namely Box-Behnken Design with Design Expert 13.0 application. Determination of the total terpenoid content of the *A. compactum* fruit extract was carried out based on the standard ursalic acid, and the cytotoxic activity was carried out using the Brine Shrimp Lethality Test (BSLT) method. The maximum total terpenoid obtained was 4.8970 mg UAE/g DW, and the minimum IC50 value was 38.8813 ppm. Optimal conditions for the extraction of *A. compactum* fruit obtained a combination of 1:8 mL/g solvent ratio, 50% ethanol concentration, and extraction time of 1 day with a desirability value of 0.772. Furthermore, the optimal extraction solution results from the Box-Behnken Design were verified and analyzed using a One-Sample T-Test. Using the Box-Behnken Design extraction optimization method, the content of terpenoid bioactive compounds with cytotoxic activity from *A. compactum* fruit can be optimally obtained.

Keywords: *Amomum compactum,* Box-Behnken Design, Cytotoxicity, Terpenoid

ABSTRAK

Kapulaga merupakan salah satu tumbuhan obat yang disebut dengan herba abadi berasal dari famili *Zingiberaceae*. Kapulaga dengan nama ilmiah *Amomum compactum* diketahui memiliki berbagai kandungan senyawa fitokimia seperti terpenoid, flavonoid, fenolik, tanin, sterol dan protein yang bermanfaat secara farmakologis sebagai antikanker, antioksidan serta memiliki aktivitas sitotoksik. Pengujian total kandungan senyawa terpenpoid yang berpengaruh terhadap aktivitas sitotoksik dari buah *A. compactum* menggunakan Desain *Box-Behnken* dengan tiga variabel independen (rasio pelarut, konsentrasi etanol dan waktu ekstraksi) belum pernah diteliti sebelumnya. Sehingga, penelitian ini bertujuan untuk memperoleh kondisi optimasi ekstraksi (rasio pelarut, konsentrasi etanol, dan waktu ekstraksi) terhadap kandungan total terpenoid serta aktivitas sitotoksik menggunakan larva *Artemia salina* Leach dengan metode permukaan respon yaitu Desain *Box-Behnken*. Aplikasi *Design Expert*

13.0 digunakan untuk membuat rancangan awal percobaan dengan menggunakan Desain *Box-Behnken*, penentuan kadar total terpenoid ekstrak buah *A. compactum* dilakukan berdasarkan standar asam ursalat dan aktivitas sitotoksik dilakukan berdasarkan metode Brine Shrimp Lethality Test (BSLT). Total terpenoid maksimum diperoleh sebesar 4.8970 mg UAE/g DW dan nilai IC50 yang paling minimum diperoleh sebesar 38.8813 ppm. Kondisi optimasi ekstraksi buah *A. compactum* diperoleh kombinasi rasio pelarut 1:8 mL/g, konsentrasi etanol 50%, dan waktu ekstraksi 1 hari dengan nilai desirbility 0.772. Selanjutnya, hasil solusi optimal ekstraksi dari *Box-Behnken Design* diverivikasi serta dianalisis menggunakan *One-Sample T-Test*, maka dapat disimpulkan bahwa dengan menggunakan metode optimasi ekstraksi Desain *Box-Behnken*, kandungan senyawa bioaktif terpenoid yang memiliki aktivitas sitotoksik dari buah *A. compactum* dapat diperoleh secara optimal.

Kata kunci: *Amomum compactum,* Desain *Box-Behnken,* Sitotoksisitas; Terpenoid

INTRODUCTION

Medicinal plants have an important role in being used as an alternative treatment for chronic and acute disorders. It is known that based on previous findings, more than 300 plants have been shown to have potential in the field of pharmacology that has medicinal properties. One of the plants that can be used as medicine and have the potential for therapy is cardamom. Cardamom is not only used in the pharmaceutical field as a medicine but also widely used in the food and cosmetic industries (Jena et al., 2021). Cardamom, known by the scientific name *Amomum compactum*, comes from the genus Zingiberaceae, which is known to be a plant species containing various phytochemical compounds such as alkaloids, carbohydrates, phenolics, flavonoids, diarylheptanoids, and proteins (Ivanović et al., 2021), where the parts of this plant, such as fruit, leaves, stems, and the rhizome, are known to have various pharmacological activities such as antioxidant, antimicrobial, anti-inflammatory, anticancer, and antidiarrheal (Alam et al., 2021). Most of the phytochemical constituents in this plant consist of flavonoid, phenolic, sterol, lipid, and terpenoid compounds (Alam et al., 2021).

Terpenoid secondary metabolite compounds in cardamom are one of the constituent compounds that have an important role in pharmacology, especially for cytotoxic activity. This cardamom fruit, based on research by Moulai-Hacene et al. (2020) contains various phytochemicals, one of which is terpenoids with anti-inflammatory, anti-proliferative, pro-apoptotic, and antioxidant activities that underlie the anticancer mechanism in cardamom fruit. Based on the research of Ashokkumar et al. (2021), the results of phytochemical screening of green cardamom essential oil obtained many oxygenated monoterpenes such as terpinyl acetate, terpineol, and 1,8-cineole, which have pharmacologic activities as antioxidants, gastroprotective, anti-inflammatory, and antiviral. Furthermore, the pharmaceutical activity of these oxygenated monoterpenes was also associated with aromatic compounds having other stimulant activities such as astringent, diuretic, and carminative (Al-Zereini et al., 2022). Terpenoid compounds are commonly found in plants, animals, and fungi consisting of two or more isoprene units (Campos-Xolalpa et al., 2018). This terpenoid compound consists of an ionone or chalcone framework, which has various pharmacological properties, one of which is a cytotoxic agent and anticancer (Lima et al., 2019). The lactone group in terpenoid compounds causes these compounds to have high bioactivity, including cytotoxic, anti-inflammatory, antimicrobial, anticancer, and antimalarial activities. The use of drugs or the therapeutic use of cytotoxic compounds has various side effects. One way to reduce the side effects of drugs is to use natural ingredients, such as the use of medicinal plants. The content of terpenoid compounds containing lactone groups is known to have cytotoxic activity in both healthy and tumor cells (Surowiak et al., 2021).

One way to obtain secondary metabolite compounds from medicinal plants with cytotoxic activity, such as terpenoids, is through the extraction process. Extraction, which is influenced by several factors such as solid-liquid ratio (Sajid et al., 2019), type of solvent (Qomaliyah et al., 2019), extraction time (Soós et al., 2019), is an independent variable that must be considered and differentiates it from conventional extraction, which does not focus on these independent variables. The disadvantages of this conventional extraction method are the use of too many solvents and long extraction times (Rasul, 2018). One way that can be used to optimize the extraction process to produce the desired bioactive compounds is to use an extraction optimization design that focuses on several independent variables. The extraction optimization used in this study is the Box-Behnken Design which has been widely used for

extraction optimization in the pharmaceutical, food, bioprocess, agrochemical, and industrial fields (Aziz & Aziz, 2018). Box-Behnken Design is an instrument used to determine the effect of several different parameters or variables in an experiment (Ahmad et al., 2020). This Box-Behnken Design is one of the extraction optimization methods from the response surface method. The response surface method is a mathematical and statistical technique used to optimize the process of a complex experiment (Aziz & Aziz, 2018). This response surface method is also used to determine the effect of the desired response with some quantitative experimental factor or a series of variables (Mercurio et al., 2020).

Based on research, Khoiriyah et al. (2020) stated that the optimization of extraction using Box-Behnken Design from the rhizome of *C. aeruginosa* with variable ethanol concentration, liquid-solid ratio, and extraction time was known to have a significant influence on the extraction yield and cytotoxic activity. Testing the cytotoxic activity of *A. compactum* fruit related to the content of terpenoid bioactive compounds using the Box-Behnken Design has never been studied before. Thus, this study aimed to obtain the extraction optimization conditions (solvent ratio, ethanol concentration, and extraction time) on the total terpenoid content and cytotoxic activity using *Artemia salina* Leach larvae with the response surface method, namely Box-Behnken Design. This research hopes that it will provide ideas and information about the benefits of *A. compactum* fruit that can be used commercially, especially in the health sector, which is developed pharmacologically as an alternative natural medicine for future studies.

METHODS

1. Plant sample preparation

This research was started by preparing the *A. compactum* fruit samples obtained from the Tropical Biopharmaceutical Research Center, IPB University, Indonesia. The *A. compactum* fruit was washed using clean water and dried using an oven at 45°C for 2 days and 1 night. After the sample was dry, the next step was milled and filtered with a 100 mesh sieve size to obtain *A. compactum* fruit simplicia powder, which is ready to be extracted for processing analysis needs.

2. Box-Behnken Design and extraction

There are three independent factors or variables evaluated in this study, namely the solvent ratio (A; 1:5 g/mL, 1:10 g/mL, and 1:15 g/mL), ethanol concentration (B; 50%, 70 %, and 96%), and extraction time (C; 1 day, 2 days, and 3 days). Box-Behnken Design, these three factors (A, B, C) at three levels (-1, 0, +1) with variable code solvent ratio A, ethanol concentration B, and extraction time C (**Table 1**) were used for the optimization of process variable capacity. Total terpenoids and cytotoxicity of *A. compactum* fruit extract. The design consisted of 15 experiments (**Table 2**).

Table 1. Code of three independent variables Box-Behnken Design with three levels, namely low (-1), medium or in the range (0), and high (+1).

Variable	Variable Code		
	-1	0	+1
Solvent ratio (g/mL) (A)	1:5	1:10	1:15
Ethanol concentration (%) (B)	50	70	96
Time extraction (day) (C)	1	2	3

Note: The codes for these 3 variables and the various levels of variable codes are obtained by entering the data into Box-Behnken Design.

Table 2. The experimental design was Box-Behnken Design with three independent variables: solvent ratio (A), ethanol concentration (B), and extraction time (C).

No test	Variable code		
	Solvent ratio (g/mL) (A)	Ethanol concentration (%) (B)	Time extraction (day) (C)
1	1:10	50	3
2	1:10	96	3
3	1:10	50	1
4	1:15	96	2
5	1:5	70	3
6	1:10	70	2
7	1:5	50	2
8	1:5	70	1
9	1:10	70	2
10	1:15	70	3
11	1:5	96	2
12	1:15	50	2
13	1:10	96	1
14	1:15	70	1
15	1:10	70	2

Note: The ratio of solvents with three levels of testing, namely (A; 1:5 g/mL, 1:10 g/mL, and 1:15 g/mL), ethanol concentration (B; 50%, 70 %, and 96%), and extraction time (C; 1 day, 2 days, and 3 days)

The extraction process of *A. compactum* fruit, which is rich in terpenoid secondary metabolite compounds, was carried out by the maceration method using simplicia weighing 2 g with the provisions based on the variables in **Table 1** with combinations according to **Table 2**.

3. Total terpenoid content (TTC)

Analysis of the total terpenoid content was carried out based on Biswas et al. (2021) with modifications. A total of 200 L of extract solution in ethanol (0.2 mg/mL) was mixed with 100 L of reagent prepared by mixing 1 mL of perchloric acid and 300 L of glacial acetic acid solution. A total of 5 mL of glacial acetic acid was added to it. Then the absorbance was measured using a microplate reader (Epoch BioTek, USA) at a wavelength of 548 nm, and the results were expressed as ursalic equivalent (UE) in mg/g fruit-based on dry weight (DW). The standard used is ursalic acid with a 0-500 ppm concentration.

4. Cytotoxic activity

The cytotoxic activity of each extract of *A. compactum* was analyzed and evaluated using the Brine Shrimp Lethality Test (BSLT) method based on the study of Kamanja & John (2018) with modifications. This test begins by placing the *Artemia salina* Leach shrimp eggs into a beaker, then adding seawater (made by mixing 1 tablespoon of fish salt in 1.5 L of water), and putting the aerator into the beaker to create bubbles in the seawater. Then, please turn on the light to incubate the eggs into larvae for 2 x 24 hours. After the eggs became larvae, the sample extract was prepared by dissolving in 10% dimethyl sulfoxide (DMSO) and adding seawater to make the final concentrations of 0 (control), 10, 50, 100, 500, and 1000 ppm. The test was carried out by inserting 10 shrimp larvae into a test plate that had been added to a mixture of seawater and sample extracts according to the concentration. Dead larvae will be counted and observed after the larvae are mixed with extract and seawater and incubated for 24 hours. Then, the calculation of the LC50 value based on the percentage of larval death if it does not move for 10 seconds is calculated using Microsoft Excel with probit analysis. Each test was repeated 3 times.

5. Statistic analysis

A statistical analysis of this optimization design was carried out based on Lin et al. (2020), and modifications were made. The experimental design in each experiment was designed for three trials, with the average value of each test used as the result of the observed response. In Design Expert 13.0, the significance of the mathematical model can be known by using analysis of variance (ANOVA). Analysis of variance ANOVA was used to determine the coefficients of linear, quadratic, and interaction regression. Response surface plots were obtained for each interaction. Furthermore, the model's suitability was confirmed by comparing the predicted and experimental values with the R^2 value. The desirability value obtained from the program is used to determine the optimal conditions that are considered the best for determining total terpenoids (TTC), and cytotoxicity activity is verified.

6. Model verification

A comparison of the predicted value with the actual value is carried out to assess the adequacy and suitability of the model used. The observed response calculations were analyzed using a One-Sample T-test.

RESULT AND DISCUSSION

1. Optimization of extraction by Response Surface Method (RSM)

1.1 Fitting the RSM models

Box-Behnken Design analyzed the optimization of extraction as a first step for the recovery of terpenoid compounds that have a cytotoxic activity to investigate how the effect of solvent ratio, ethanol concentration, and extraction time on the total content of terpenoid compounds and the cytotoxic activity of *A. compactum* fruit. This RSM was used to determine how the three independent variables (solvent ratio, ethanol concentration, and extraction time) resulted in the total terpenoid content and cytotoxic activity using *Artemia salina* Leach larvae with the BSLT method. **Table 3** shows the results of the responses of the three independent variables of solvent ratio, ethanol concentration, and extraction time to the total terpenoid content and cytotoxic activity.

Table 3. Box-Behnken Design experimental design with three independent variables and experimental data on total terpenoid levels (TTC) and cytotoxic activity

No	Variable			Response	
	Solvent ratio (g/mL)	Ethanol concentration (%)	Time extraction (hari)	TTC (mg UAE/g DW)	BSLT (mg/L)
1	1:10	50	3	1.4440	62.0984
2	1:10	96	3	1.6090	52.6893
3	1:10	50	1	0.9487	63.0957
4	1:15	96	2	1.1203	76.3728
5	1:5	70	3	3.3790	66.8667
6	1:10	70	2	4.8440	94.1566
7	1:5	50	2	2.5123	38.8813
8	1:5	70	1	1.8640	52.2402
9	1:10	70	2	1.7070	122.915
10	1:15	70	3	1.9773	154.225
11	1:5	96	2	1.8803	61.9636
12	1:15	50	2	0.7828	117.707
13	1:10	96	1	1.8695	95.3768
14	1:15	70	1	0.8520	150.207
15	1:10	70	2	1.8070	154.225

Note: Solvent ratio, ethanol concentration, and extraction time consist of three levels, namely solvent ratio 1:5, 1:10, and 1:15 g/mL; ethanol concentrations 50, 70, and 90%; and extraction time of 1, 2, and 3 days.

The modeling carried out correlates with the experimental results obtained. The experimental value obtained was based on the response variable for the total terpenoid content, which ranged from 0.7828-4.8440 mg UAE/g DW. Meanwhile, the cytotoxic activity of *A. compactum* extract ranged from 52.2402-154.225 mg/L.

Multiple regression analysis on actual data from three independent variables measured by two responses, namely total terpenoid content and cytotoxic activity. The polynomial equation is used to express the response due to the responses of the three independent variables shown in **Table 4**.

Table 4. Polynomial equations and 2FI response surface analysis for the two responses were tested for total terpenoid content (TTC) and cytotoxic activity.

Test	Model	Equation
Terpenoid content	2FI	$Y = 9.996285 + 0.075537A - 0.118954B - 3.99200C + 6.96345E\text{-}06AB + 0.015095AC + 0.047542BC$
Cytotoxic activity	Linear	$Y = 46.42582 + 6.18125A - 0.0775002B - 7.04372C$

Note: Y is the predicted response; A is the independent variable solvent ratio (g/mL); B is the independent variable of ethanol concentration (%), and C is the extraction time (days).

The model generated from the total terpenoid content shows a 2FI regression model, while a linear regression model is obtained for the cytotoxic activity. The polynomial equations generated from the three independent variables and the two responses of the extraction of *A. compactum* show an empirical and reciprocal relationship between the independent variables and the responses or parameters being tested (Roy & Rahaman, 2018). The ANOVA analysis, including the F value to show the dependent variable, the coefficient of determination (R^2), and the regression coefficient, are presented in **Table 5**. The mathematical model installed for selecting the model on TTC and this cytotoxic activity was with a 95% confidence interval. The analytical model in **Table 5** consists of R^2, AdjR^2, F-value, and p-value. The value of R^2 is used to evaluate the accuracy of the model. In contrast, the value of AdjR^2 is used to compare the experimental value with the theoretical value.

Table 5. Summary statistical model of TTC and cytotoxic activity.

Source	TTC	Cytotoxic activity
Model	2FI	Linear
p-value	0.3511	0.0995
f-value	1.31	2.67
R^2	0.4961	0.4211
Adjusted R^2	0.1182	0.2632

Note: TTC (Total Terpenoid Content), the coefficient of determination (R^2), and the regression coefficient. AdjR^2 is used to compare the experimental value with the theoretical value. ANOVA statistical analysis was used in this research.

This study obtained that the AdjR^2 value for TTC was 0.1182, and the cytotoxic activity was 0.2632 with a small p-value of 0.05, indicating an adequate model and the results obtained were significant (Ahmad et al., 2020). Based on the p-value for the three independent variables, only the solvent ratio, which has a *p-value* <0.05, shows a significant linear effect (**Table 5**). Meanwhile, for the independent variables of ethanol concentration and extraction time, the *p-value* $>$ 0.05 means that it does not significantly impact the response being tested (Lin et al., 2020).

1.2 Response surface analysis of TTC

Response surface analysis of total terpenoid content (TTC) with independent variables (solvent ratio, ethanol concentration, and extraction time) was significantly less effective than *A. compactum* fruit extract. From the measurement results of the total terpenoid test in **Table 5**, the maximum total terpenoid is obtained with a solvent ratio of 1:10 g/mL, an ethanol concentration of 70%, and a 2-day extraction time of 4.8440 mg UAE/g DW. Based on the analysis of variance ANOVA, it is known that the total terpenoid content (TTC) was obtained by the 2FI model with *p-value* 0.3511, *f-value* 1.31, R^2 0.4961, and AdjR^2 0.1182. Based on the polynomial equations in **Table 4**, the analysis of the total terpenoid content using the 2FI model shows that the effect of solvent ratio (A), solvent ratio and ethanol concentration (AB), solvent ratio, and extraction time (AC), as well as ethanol concentration and extraction time (BC), marked with a positive sign, indicates that the response increases when influenced by these independent variables. This explains a combination effect of the independent variables on the total terpenoid content obtained. This result is supported by the research of Truong et al. (2019), which states that the level of solvent polarity supports the extraction efficiency for the recovery of secondary metabolites such as terpenoids. In the analysis of the results of the total terpenoid content of the results of this study, the maximum content was obtained at a solvent ratio of 70%, indicating that due to the 30% water content of the solvent used, the polarity of the solvent was higher so that the maximum TTC content was obtained.

The total terpenoid content of *A. compactum* fruit is presented in **Figure 1**, showing that the highest total terpenoids based on **Figures 1a** and **1b** explained with a solvent ratio of 1:10, and **Figures 1a** and **1c** with an ethanol concentration of 70% with a content of 30% water to increase the polarity of the solvent and be able to dissolve the metabolite compounds more maximally, the highest terpenoid content was obtained, which was 4.8440 mg UAE/g DW. The higher the ethanol concentration, the higher the terpenoid content should be. Ethanol with a concentration of 99% will extract higher terpenoids than distilled water extract. Ethanol solvent with a concentration of 50-70% extracted better glycosides, polyacetylene, sterols, polyphenols, tannins, flavonols, terpenoids, and alkaloids. This is because the lower the ethanol concentration, the more water it contains, so the polarity of the solvent will be higher than the absolute solvent. Solvents with high polarity will be able to extract metabolites more easily and more widely (Hikmawanti et al., 2021).

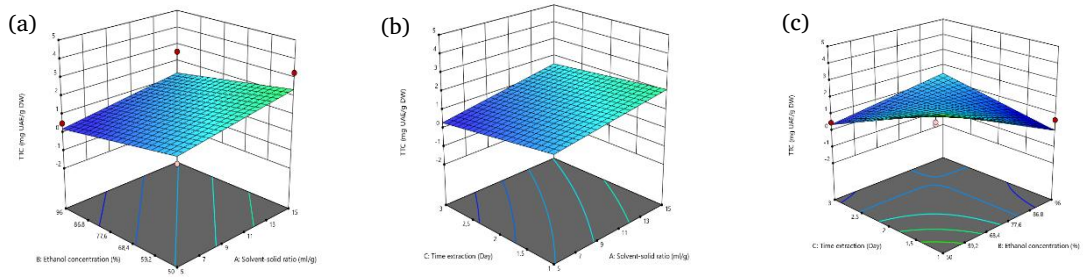

Figure 1. 3D response surface graph of the total terpenoid content (TTC) with three independent variables: (a) the relationship between solvent ratio and ethanol concentration, (b) solvent ratio with extraction time, and (c) ethanol concentration with extraction time.

1.3 Response surface analysis of cytotoxic activity

Analysis of the response surface of the cytotoxic activity of *A. compactum* fruit with solvent ratio, ethanol concentration, and extraction time as independent variables used to see the effect of these three variables on the response of its cytotoxic activity significantly showed effective results. Terpenoids are secondary metabolites that have

pharmacological activity as cytotoxic agents. Sesquiterpene lactone is a terpenoid derivative used as an effective cytotoxic agent and has been shown to have anticancer activity in several human cancer cell lines (Alam et al., 2021). The cytotoxic activity of *A. compactum* fruit was determined using the LC50 value. Several studies have revealed that the extract of *A. compactum* contains various phytochemical compounds that can act as anti-inflammatory, antiproliferative, pro-apoptotic, and antioxidant activities (Elguindy et al., 2016). From **Table 3**, it is known that the best cytotoxic activity is the one that has an LC50 value < 200 ppm, then the cytotoxic activity is obtained at a combination of 1:5 g/mL solvent ratio, 50% ethanol concentration, and extraction time of 2 days with an LC50 value of 38.8813 mg/L. Based on the analysis of variance ANOVA, known cytotoxic activity obtained a linear model with *p-value* 0.0995, *f-value* 2.67, R^2 0.4211, and AdjR^2 0.2632. Based on the polynomial equations in **Table 4**, the analysis of cytotoxic activity with a linear model shows that the effect of the solvent ratio (A) is indicated by a positive sign indicating that the response increases when influenced by the solvent ratio variable. The ratio of solvent that shows the optimal cytotoxic activity is 1:5 g/mL with a solvent concentration of 50%. The smaller the solvent ratio, the more concentrated the solution will be, and the lower the ethanol concentration, the more the polarity of the solvent will increase to optimize the metabolite compounds that act as cytotoxic agents, especially terpenoids. Based on research by Abarca-Vargas et al. (2016), it is stated that bioactive compounds extracted using organic solvents such as ethanol, ether, hexane, and benzene will obtain extraction results with higher content of metabolite compounds. Then, extraction using ethanol with increasing extraction time did not significantly affect cytotoxicity.

The cytotoxic activity of *A. compactum* fruit is presented in **Figure 2**, which shows that the maximum cytotoxic activity is at a combination of a solvent ratio of 1:5 g/mL (**Figures 2a and 2b**), and a 50% ethanol concentration (**Figures 2a and 2c**) obtained a minimum LC50 value of 38.8813 mg/L. The smaller the solvent ratio and the lower the ethanol concentration will increase the cytotoxic activity of an extract. In contrast, the extraction time plays a role in the recovery of phytochemical compounds depending on what phenolic compounds are contained in the plant (Mahmud et al., 2019). The interaction between a low solvent ratio and low ethanol concentration could increase cytotoxic activity and showed that the effect of the two independent variables dramatically increased the cytotoxic properties of *A. compactum* fruit extract.

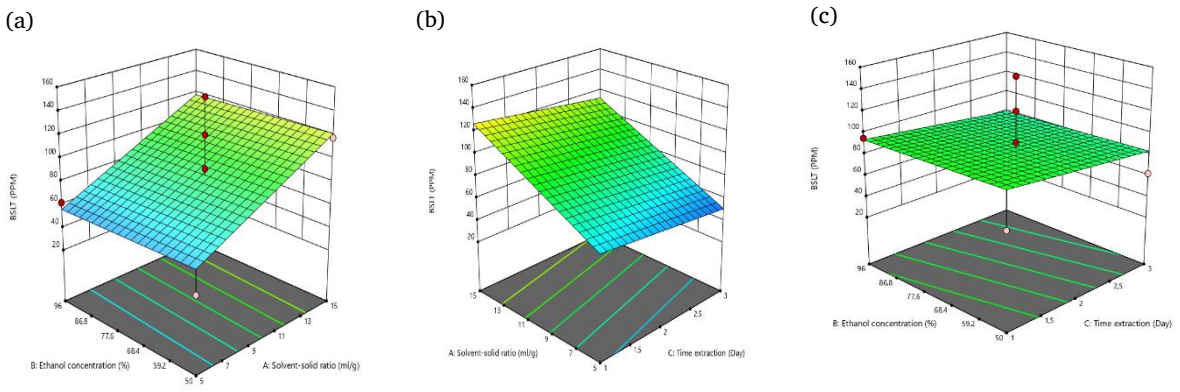

Figure 2. 3D response surface graph of cytotoxic activity with three independent variables: (a) the relationship between solvent ratio and ethanol concentration, (b) solvent ratio with extraction time, and (c) ethanol concentration with extraction time.

2. Single-factor experimental analysis

2.1 Influence of solvent ratio on TTC and cytotoxic activity

The solvent ratio is the first independent variable that affects the total terpenoid content and cytotoxic activity of *A. compactum* fruit. The best and optimum extraction time was determined to obtain the output of the content of terpenoid compounds (TTC) as compounds that influence cytotoxic activity. **Figure 3a** shows that the total terpenoid content (TTC) increased with a higher solvent ratio from 1:5 g/mL to 1:15 g/mL. At a solvent ratio of 1:15 g/mL, a value of 1.82957 mg UAE/gDW was obtained. Likewise, **Figure 4a** on the determination of cytotoxic activity

shows the maximum LC50 value obtained at a higher solvent ratio of 1:15 g/mL of 144,187 mg/L (ppm). From **Figure 3a**, it is known that a high solvent ratio can optimize the recovery of terpenoid compounds that have optimal cytotoxic activity. The ratio of the solvent used will affect the efficiency of the extracted metabolites because most of the components of the plant sample to be extracted diffuse more effectively at a high solvent ratio (Wang et al., 2016).

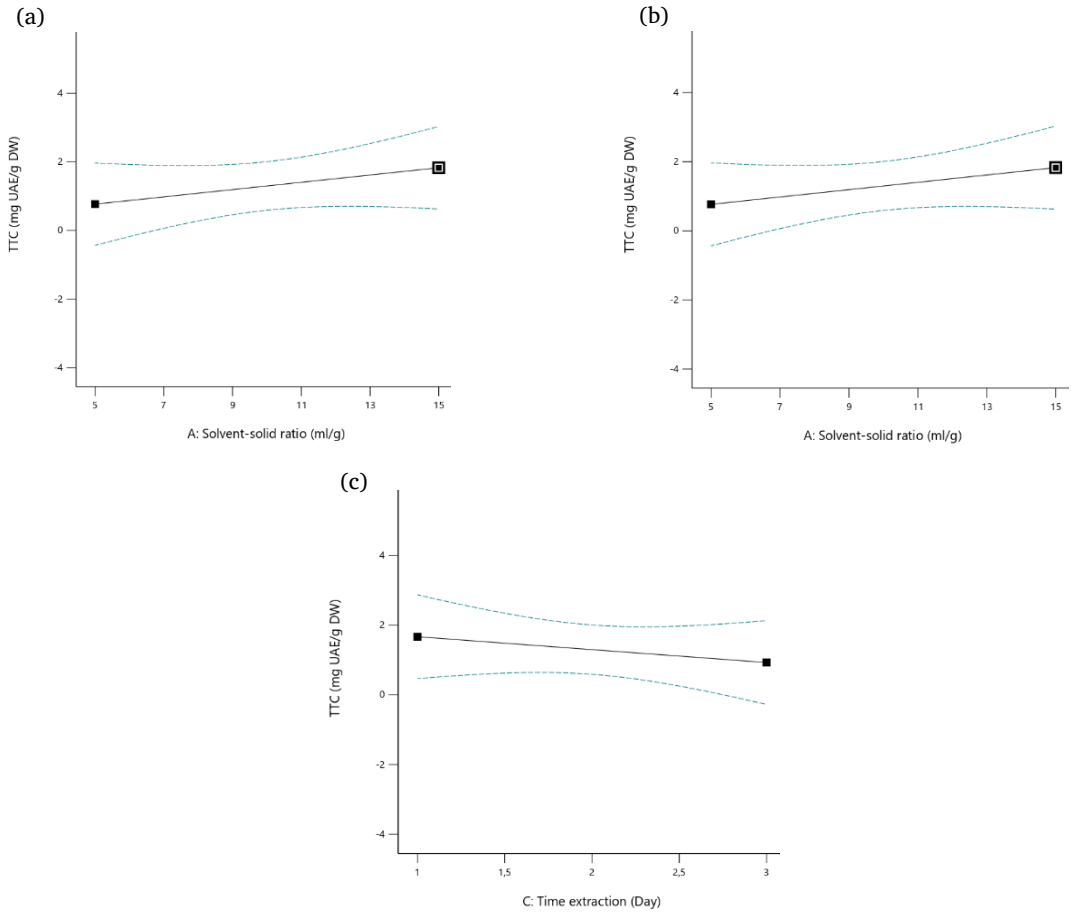

Figure 3. Effect of extraction parameters on TTC parameters. (a) Solvent ratio (70% ethanol concentration, extraction time 2 days), (b) ethanol concentration (1:10 g/mL solvent ratio, and 2 days extraction time), (c) extraction time (solvent ratio 1:10 g/mL, 70% ethanol concentration).

2.2 Influence of ethanol concentration on TTC and cytotoxic activity

The second independent variable that affects TTC and cytotoxic activity in ethanol concentration. Ethanol concentration is one of the independent variables that have an important influence on the cytotoxic activity and recovery of terpenoid compounds. **Figure 3b** shows that the highest total terpenoid content is at a 50% ethanol concentration of 1.8464 mg UAE/g DW and the total terpenoid content decreases at a higher ethanol concentration of 96%. In terms of cytotoxic activity, it is known that with 70% ethanol concentration (**Figure 4b**), the maximum LC50 value is 122,915 mg/L (ppm).

This ethanol concentration will affect the solubility of the sample because of its polarity. Samples extracted with polar solvents will be able to produce more optimal secondary metabolites. The lower concentration of ethanol will allow the polyphenol compounds to recover due to the reduced dielectric constant of the solvent, which causes an increase in the diffusion of the solubility of the compound (Lin et al., 2020). This is also in line with the results of research by (Che Sulaiman et al., 2017), which stated that similar to the extraction of compounds that have antioxidant activity, other polyphenolic compounds such as terpenoids also have good solubility in binary solvents or aqueous alcohol mixtures. The same thing was also revealed by Ismail & Chua (2020), which stated that the use of 70% ethanol caused the glycosides in terpenoids to remain in the aqueous phase, and terpenoids containing ester bonds would have good solubility in strong polar solvents such as ethanol 30. %. For

extracting biomolecules from plants, the selection of type and concentration of the solvent is determined based on the polarity of the desired solute. Solvents with the same polarity as the solute will optimally recover the desired metabolite (Altemimi et al., 2017).

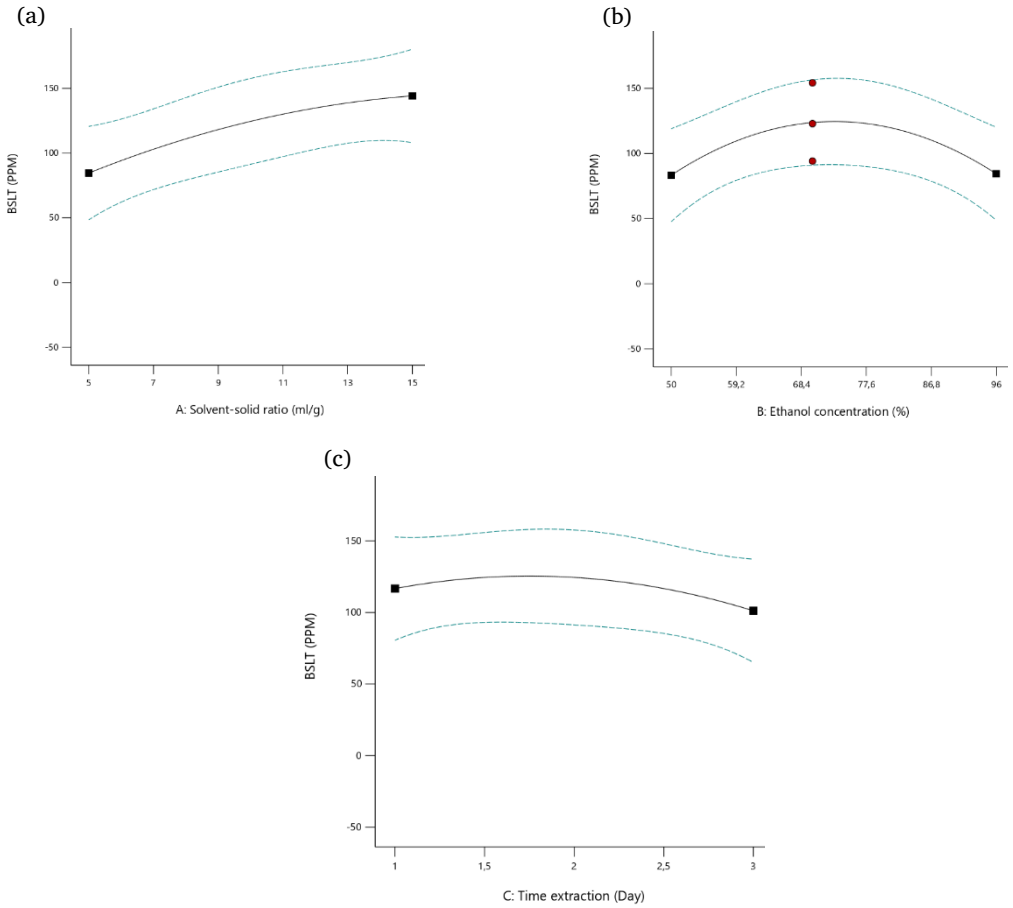

Figure 4. Effect of extraction parameters on cytotoxic activity. (a) Solvent ratio (50% ethanol concentration, extraction time 2 days), (b) ethanol concentration (1:15 g/mL solvent ratio, and 2 days extraction time), (c) extraction time (solvent ratio 1:5 g/mL, 50% ethanol concentration).

2.3 Influence of extraction time on TTC and cytotoxic activity

Extraction time is an independent variable that affects the response to total terpenoid content (TTC) and cytotoxic activity. In addition to playing an important role in the recovery of terpenoid secondary metabolites, extraction time is one of the important parameters for the recovery of polyphenol metabolites. In polyphenol compounds, the effect of the sample being too long in the solvent will allow the solubility process to increase. However, it is also explained that the extraction time required for each plant varies depending on the compound content in the plant itself (Mahmud et al., 2019). **Figure 3c** shows that the highest total terpenoid content was obtained at 1 day extraction time with a maximum TTC value of 1,66947 mg UAE/g DW. The total terpenoid content decreased with increasing extraction time. For the cytotoxic activity of *A. compactum* fruit extract, the highest LC50 value was obtained, which was also at a 1-day extraction time of 116,721 mg/L (ppm) (**Figure 4c**). Based on **Figures 3c** and **4c**, it can be seen that the more terpenoid content obtained from the extraction process, which affects the cytotoxic activity, was obtained with a short extraction time of 1 day. Lin et al., (2020) stated that the extraction time showed an important effect on the recovery of the content of polyphenol compounds and would increase significantly with increasing solvent ratio.

2.4 Optimum condition and its validation

The optimum conditions used to obtain the extract of *A. compactum* fruit will produce maximum extraction results from the two responses tested, namely the total terpenoid content (TTC) and cytotoxic activity with three independent variables: solvent ratio, ethanol concentration, and extraction

time. The best combination from Box-Behnken Design resulted in the maximum extraction formula of *A. compactum* fruit, which was 58 selected combinations with the highest desirability value in **Figure 5**, namely 0.772. The desirability value is used to determine the degree of accuracy of the optimal solution results. A desirability value close to 1 will indicate the higher the accuracy of an optimization model. Under optimal conditions, the model validation and the observed response value follow the predicted results (Mang et al., 2015).

The best combination from Box-Behnken Design was obtained with a 1:8 solvent ratio, 50% ethanol concentration, and 1 Day extraction time. Furthermore, this best combination was verified on the same *A. compactum* fruit sample but extracted with the selected combination according to **Table 6**.

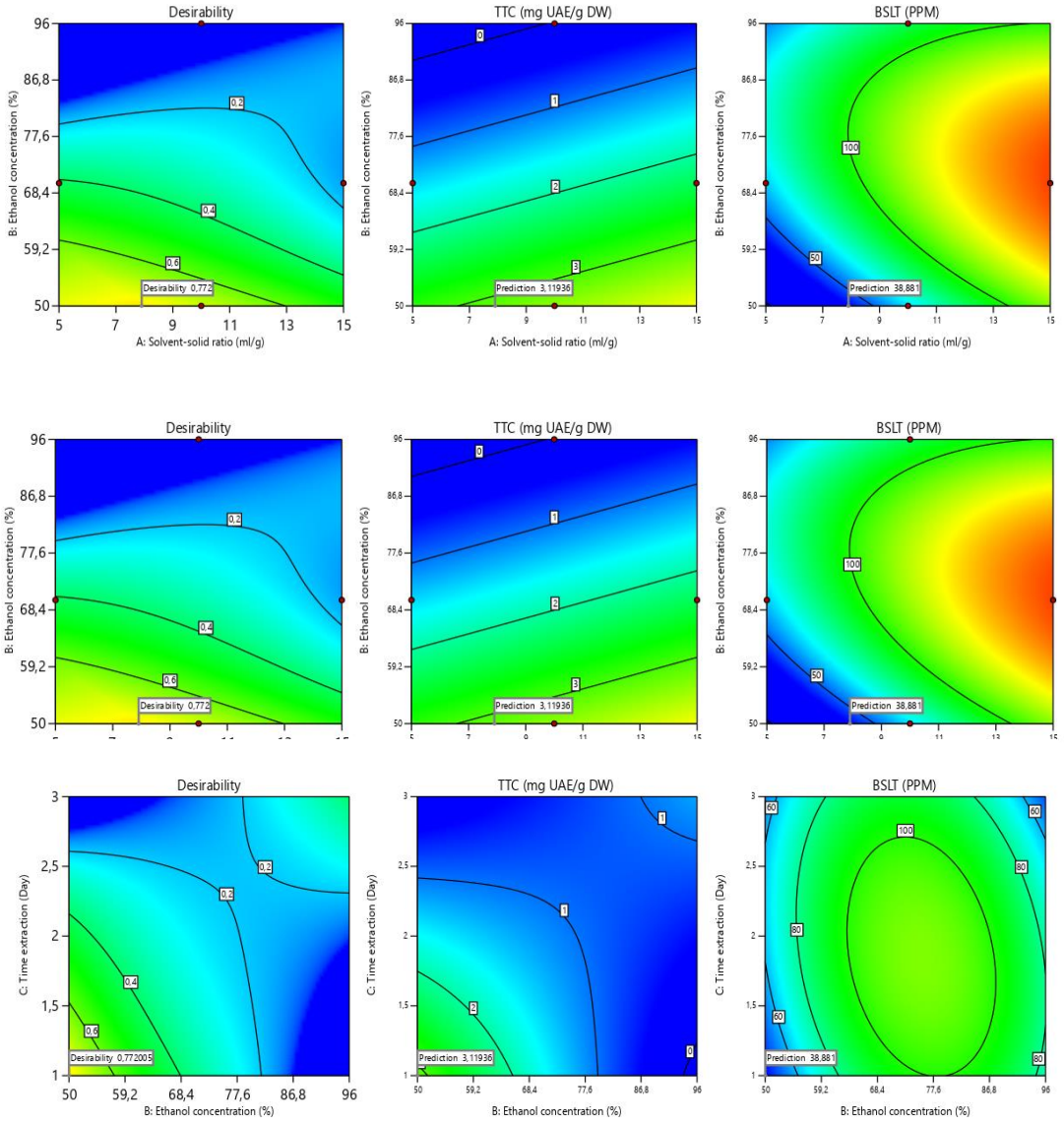

Figure 5. The contour plot of the maximum formula with a desirability value of 0.772 total terpenoid content (TTC) and cytotoxic activity based on independent variables: solvent ratio (ethanol concentration 70%, extraction time 2 days), ethanol concentration (solvent ratio 1:10 g/mL, and time extraction 2 days), extraction time (solvent ratio 1:10 g/mL, 70% ethanol concentration). Figure (a) is the relationship between the effect of the solvent ratio and the ethanol concentration, (b) the effect of the solvent ratio on the extraction time, and (c) the effect of the ethanol concentration on the extraction time. The optimum formula was obtained with a combination of a 1:8 g/mL solvent ratio, 50% ethanol concentration, and an extraction time of 1 day.

Table 6. Experimental data validated the predicted value of total terpenoid content (TTC) and cytotoxic activity using the BSLT method at optimal extraction conditions.

	A	B	C	TTC	BSLT
Prediction	8	50%	1	3.119	38.881
Actual value	8	50%	1	2.0190	140.142
p-value				0.052	0.1625
Prediction	8	50%	1	3.119	38.881

Note: The solvent ratio is denoted by the letter A, the ethanol concentration B, and the extraction time C, consists of three levels, namely the solvent ratio 1:5, 1:10, and 1:15 g/mL; ethanol concentrations: 50, 70, and 90%, and extraction time of 1, 2, and 3 days.

The verification results in **Table 6** were analyzed based on the p-value in the One-Sample T-Test analysis. This p-value is the value of a model selected based on comparing the actual value with the predicted value. Based on the *One-Sample T-Test* analysis results, the *p-value* > 0.05 means that the predicted value from the optimization results of Box-Behnken Design follows the verification results. Verification analysis based on *p-value* results indicates that the prediction results match the actual data obtained or are not significantly different (Greenland et al., 2016).

CONCLUSION

Cardamom fruit (*A. compactum*) in this study was first extracted using an extraction optimization method with three independent variables, namely solvent ratio, ethanol concentration, and extraction time using the Box-Behnken Design, which proved effective in optimizing the extraction parameters that influence the extraction yield, where the optimal extraction conditions obtained a combination of 1:8 g/mL solvent ratio, 50% ethanol concentration, and extraction time of 1 day with a desirability value of 0.772 close to 1, it can be concluded that the extraction model designed with three independent variables proved effective in obtaining the total terpenoid content. (TTC) which has a maximum effect on the cytotoxic activity of the fruit extract of *A. compactum*. The effect of independent variables on the extraction and recovery of terpenoid compounds for cytotoxic activity was stated to be significant and effective in the verification results analyzed by the *One-Sample T-Test*. The *p-value* > 0.05 obtained can conclude that the optimization accuracy is high, so the optimization model is declared effective.

ACKNOWLEDGMENT

The authors thank and express gratitude to the PDUPT research program of IPB University (1925/IT3.L1/PN/2021) for their support in completing this research.

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Citation Format: Juliana, D., Priosoeryanto, B. P., & Nurcholis, W. (2023). Optimization of Cardamom Fruit (*Amomum compactum*) Extraction Focused on Total Terpenoid Extraction and Cytotoxic Activity Using Response Surface Methodology. *Jurnal Jamu Indonesia, 8*(2), 57–69. https://doi.org/10.29244/jji.v8i2.328