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DIMETHYL CARDAMONIN FROM *CLEISTOCALYX OPERCULATUS* LEAVES: OPTIMISED EXTRACTION CONDITIONS AND INHIBITIVE ACTIVITY AGAINST FOOD-POISONING BACTERIA

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ABSTRACT

In this study, the predominant phenolic compound of *Cleistocalyx operculatus* leaves harvested in Vietnam was identified as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (also called dimethyl cardamonin) by using UHPLC-DAD-HRMS. The extraction of this compound from leaves was optimised using response surface methodology. A second-order polynomial model with three important variables (ethanol concentration, temperature and extraction time) was used. A rotatable central composite design consisting of 17 experimental runs with three replicates at the center point was applied to describe the experimental data as the dimethyl cardamonin apparent content of leaves. The experimental results fit well to the model with R^2 equal to 0.9618 and without a lack of fit. The optimised conditions were as follows: 80% ethanol, 85 °C and extraction time of 22 min. These conditions were applied to produce the extract from *C. operculatus* leaves. The obtained freeze-dried extract powder exhibited inhibitive activity against five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species, with the inhibitive zone ranging from 1.83 to 9.17 mm. The antibacterial activity of the extract was dose dependent. The results indicated the potential application of *C. operculatus* leaves as a source of natural antimicrobial agents for food preservation.

1. Introduction

Cleistocalyx operculatus (synonyms: *Cleistocalyx nervosum*, *Syzygium nervosum* and *Eugenia operculata*; common name "Voi" in Vietnamese) belongs to the Myrtaceae plant family (Do, 2004) and is widely distributed in tropical areas of Southeast Asian countries (reviewed by Pham *et al*., 2020). The decoction of *C. operculatus* leaves and flower buds is a popular beverage for Vietnamese people. In addition, parts of this plant have been used in traditional Vietnamese medicine for a long time. The leaves and flower buds promoted digestion. The leaves have antibiotic activity and have been used to treat boils, sores and scabies (Do, 2004).

C. operculatus has important pharmacological activities that have been demonstrated in several studies. Extracts from the flower buds of *C. operculatus* showed cytotoxic activity against cancer cell lines, including HeLa, HL-60 and A549 (Min *et al*., 2010), SMMC-7721, 8898, HeLa, SPC-A-1, 95-D and GBC-SD (Ye *et al*., 2004), PANC-1 and MIA PACA2 (Huynh *et al*., 2019). Ethanol extract of *C. operculatus* flower buds showed

anti-inflammatory effects in an experimental lipopolysaccharide-induced sepsis mouse model (Tran *et al*., 2019). Truong *et al*. (2009) reported that the aqueous extract of *C. operculatus* flower buds showed a strong antioxidant effect and antihyperlipidemic effect through the suppression of pancreatic lipase activity in diabetic rats. Oral administration of an aqueous extract from *C. operculatus* buds to diabetic rats for 8 weeks resulted in a significant reduction in the levels of glucose, total cholesterol and triglycerides in plasma. According to Nguyen *et al*. (2017), a methanolic extract of *C. operculatus* leaves inhibited bacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus* mutans GS-5) and three multiresistant bacteria (*Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *Staphylococcus aureus* North German epidemic strain) with inhibition zone diameters ranging from 7 to 16 mm. Eighty-six phytochemical compounds have been identified in *C. operculatus* belonging to three main groups, including terpenoids, flavonoids and phloroglucinols (Pham *et al*., 2020). Among them, 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone or dimethyl cardamonin, a phenolic compound, is considered the major constituent and pharmacologically responsible of this medicinal plant (Ye *et al*., 2004; Huynh *et al*., 2019; Tran *et al*., 2019; Pham *et al*., 2020).

Food poisoning or foodborne disease is one of the main problems in public health worldwide. According to the WHO, an estimated 600 million people in the world, almost 1 in 10 people, fall ill after eating contaminated food, and 420,000 die every year. Children under 5 years of age carry 40% of the foodborne disease burden, with 125,000 deaths every year. One hundred and ten billion American dollars are lost each year in productivity and medical expenses resulting from unsafe food in low- and middle-income countries (WHO, 2020). Approximately 70% of foodborne diseases result from food contaminated with a microorganism (Hernández-Cortez *et al*., 2017). Among the microorganisms causing foodborne diseases, bacteria have the highest significance with respect to public health (Park *et al*., 2001). Bacteria frequently documented as foodborne disease agents include *Bacillus cereus*, *Escherichia coli*, *Clostridium botulinum*, *Staphylococcus aureus*, *Salmonella* and *Listeria monocytogenes* (Park *et al*., 2001; Kirk *et al*., 2015).

The objectives of this study were to identify the major phenolic compounds in *C. operculatus* leaves harvested in Vietnam and to optimise the extraction of this compound from leaves using response surface methodology (RSM). The antibacterial activity against some food poisoning microorganisms of the freeze-dried leaf extract was then evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol, formic acid, and myricetin standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile was purchased from Merck (Germany). Dimethyl sulfoxide (DMSO) was produced in Japan. Methanol, agar, yeast extract, meat extract, peptone and tryptone were obtained from China. Antibiotics were bought in pharmacies in Hanoi.

Five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species were taken from the microorganism collection of Gene Key Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology.

2.2. Sample collection

C. operculatus leaves were harvested in Hai Duong Province (Vietnam) in July 2019. All leaves were first washed with tap water and rinsed in distilled water. They were then freeze-dried at -58 °C for 3 days and ground to a fine powder with a particle size of less than 0.3 mm using a Tecator Cyclotec 1093 Sample Mill (Foss Tecator AB, Sweden). The leaf powder was stored in an airtight bag and kept at 4 °C until further use.

2.3. Identification of dimethyl cardamonin in *C. operculatus* leaves harvested in Vietnam by UHPLC-DAD-HRMS

2.3.1. Extraction of phenolic compounds from C. operculatus leaves

Due to the lack of dimethyl cardamonin standards on the market, a primary extract of *C. operculatus* leaves harvested in Vietnam was prepared and sent to the Luxembourg Institute of Science and Technology to identify the peak of dimethyl cardamonin. Briefly, 0.5 g of freeze-dried *C. operculatus* leaf powder was mixed with 10 mL of 70% ethanol and shaken for 60 min at 40 °C. The mixture was then centrifuged at 3,642 × g for 10 min at 4 °C. The supernatant was collected, and the solvent in the extract was evaporated in a R210 rotary evaporator (Buchi, Switzerland) at 40 °C/58 mbar for 30 minutes. The concentrated extract was freeze-dried, and the extract powder was sent to Luxembourg Institute of Science and Technology.

2.3.2. Identification of dimethyl cardamonin in the extract powder

Extract powder was redissolved in 70% methanol and analysed with a Waters Acquity UPLC I-class system (Milford, MA) equipped with a diode-array detector (UPLC PDA eLambda) and hyphenated to a high-resolution time-of-flight mass spectrometer (TripleTOF 6600, AB Sciex, Concord, Ontario, Canada). The separation of the 5-µL aliquot was performed in two technical replicates on a reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size, Waters). In both ionisation modes, the eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient was as follows: 0 min, 1% B; 4 min, 1% B; 16 min, 5% B; 35 min, 40% B; 45 min, 100% B; 50 min, 100% B; 53 min, 1% B; 60 min, 1% B. The flow rate was 0.5 mL/min, and the column temperature was 50 °C. The total wavelength chromatogram was first acquired between 190 and 800 nm. Analytes were then ionised with an electrospray ionisation (ESI) source. For MS1 and MS2, full HR-MS spectra between 100 and 1300 mass-to-charge ratios (m/z) and between 25 and 1300 m/z were recorded. The structure of the predominant peak was putatively identified by comparison of its UV–VIS and mass spectral data with previous literature and reference data from the PubChem Compound database (http://pubchem.ncbi.nlm.nih.gov). The molecular formula was considered valid when the mass error was below 4 ppm.

2.4. Modelisation and optimisation of dimethyl cardamonin extraction from *C. operculatus* leaves

2.4.1. Effect of the solid-to-liquid ratio on the extraction of dimethyl cardamonin

Freeze-dried *C. operculatus* leaf powder was mixed with 5 mL of 60% ethanol to have a solid-to-liquid ratio ranging from 1/5 to 1/25 and shaken for 60 min at 40 °C. The mixture was then centrifuged at 3,642 × g for 10 min at 4 °C. The supernatant was collected, and the dimethyl cardamonin content was analysed by HPLC. Extraction was performed in triplicate.

2.4.2. Modelization and optimisation of dimethyl cardamonin extraction

Response surface methodology was used to optimise the extraction of dimethyl cardamonin from freeze-dried *C. operculatus* leaf powder. A three-factor and rotatable central composite design consisting of 17 experimental runs with eight factorial points, six axial points (two axial points on the axis of each design variable at a distance of 1.68 from the design center) and three replicates at the center point (Table 1) was employed. The design variables were ethanol concentration (X_1), extraction temperature (X_2) and extraction time (X_3). The optimised solid-to-liquid ratio was determined in the previous experiment and kept at a constant value. The response was the apparent dimethyl cardamonin content of *C. operculatus* leaves. Three replicates at the central points of the design were used to allow for estimation of a pure error sum of squares.

For all runs, extractions were performed in 15 mL Falcon tubes. The extraction was terminated by centrifugation of the mixture at 3,500 × g for 10 min at 4 °C. The supernatant was collected, filtered and analysed for dimethyl cardamonin content by using an HPLC equipped

with a multiple wavelength detector (HPLC-MWD). The experimental data were fitted to the following second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} x_i x_j$$

where Y is the response, βo, βi, βii, βij are regression coefficients for intercept, linear, quadratic and interactions terms, respectively, and xi, xj are the coded values of the independent variables. The formula to convert coded values to real values and vice versa was as follows: $x_i = (X_i - X_0)/\Delta X_i$, where xi and Xi are the coded and real values of the independent variable i (i = 1, 2, and 3), respectively, X_0 is the real value of the independent variable i at the central point, and ΔX_i is the step change of X_i corresponding to a unit variation of the coded value.

The optimum conditions for dimethyl cardamonin extraction were determined by maximising the desirability with JMP 10 software. Four separate experimental extractions under optimised conditions were performed. The experimental and predicted values were then compared to validate the model.

2.5. Quantification of dimethyl cardamonin in *C. operculatus* leaves by HPLC

Quantification of dimethyl cardamonin was performed by HPLC using an Aligent system 1260 (Santa Clara, CA) equipped with G1311B-Quat pumps, G1329B auto sampler, G1330B thermostat, and G1365 MWD VL lamp. A 20 µL aliquot of the extract was injected onto a Kinetex EVO C18 column (150x4.6 mm i.d:5 µm particle size) equipped with a guard column of the same type (Phenomenex, Netherlands). The mobile phases were A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The flow rate was 1 mL/min, and the column temperature was set at 30 °C. The mobile phase gradient was as follows: 0 min, 0% B; 1 min, 25% B; 5 min, 50% B; 6 min, 70% B; 12 min, 100% B; 15 min, 100% B; 18 min, 0% B; 20 min, 0% B. Monitoring was set at 360 nm. The identification of the dimethyl cardamonin peak was previously performed by using UHPLC-DAD-HRMS at Luxembourg Institute of Science and Technology as described above. Myricetin was used as a standard, and dimethyl cardamonin content was calculated by using a five-point calibration curve y = 28.346*x - 140.98 with $R^2 = 0.9999$ (y: peak area and x: myricetin content (µg/mL)).

2.6. Evaluation of the inhibitive activity against food-poisoning bacteria of *C. operculatus* leaf extract powder

2.6.1. Preparation of C. operculatus leaf extract powder

Freeze-dried *C. operculatus* leaf powder was extracted under the optimal conditions obtained for the modelisation and optimisation experiment. The extraction was terminated by centrifugation of the mixture at 3,500 × *g* for 10 min at 4 °C. The supernatant was collected, filtered and concentrated in an R210 rotary evaporator. The concentrated extract was then freeze-dried.

2.6.2. In vitro antimicrobial activity test

The antimicrobial activity of *C. operculatus* leaf extract powder was measured by using the agar well diffusion method described in the work of Dang *et al*. (2015). First, bacteria were transferred to the corresponding liquid media and then incubated at 37 °C in a shaking incubator (200 rpm) for 12 hours. Meat-peptone-agar medium (peptone 5 g/L, NaCl 5 g/L, meat extract 5 g/L) was used for *Salmonella, E. coli* and *Vibrio parahaemolyticus,* while Luria Bertami medium (NaCl 10 g/L; tryptone 10 g/L; yeast extract 5 g/L) was used for *Staphylococcus aureus* and *Listeria monocytogenes*.

One hundred microliters of bacterial culture adjusted to a microorganism concentration of 10^{10} colony forming units per mL (CFU/mL) was spread on a Petri dish containing 25 mL of their specific media. *C. operculatus* leaf extract powder was dissolved in DMSO to make concentrations of 10, 20, 30, 40 and 50 mg/mL for the antibacterial activity test. One hundred microliters of extract solution at different concentrations was added to test wells (dimension of 8 mm), while the negative control well consisted of 100 µL DMSO and the positive control wells consisted of 300 µg/mL

antibiotics. Petri dishes were kept at 4 °C for 2 hours and then incubated at 37 °C for 20 hours. After incubation, the zone of inhibition was measured as the difference between the dimension of the inhibition zone surrounding the well and the dimension of the well (8 mm). Experiments were performed in triplicate.

2.7. Statistical analysis

Data were analysed using the statistical software SAS 9.4 (SAS Institute, Cary, NC). In the experiment determining the effect of the solid-to-liquid ratio on extraction, the dimethyl cardamonin apparent content was expressed as the mean ± standard deviation of three extraction replications. One-way analysis of variance (ANOVA) and Duncan's test were used to determine the differences among the means. p values < 0.05 were considered to be significantly different. Analysis of variance was carried out using a generalised linear model (GLM) procedure to determine the effect of the bacteria, extract concentration and their interactions on the zone of inhibition. The model configuration was $Y = a + b_1*X_1 + b_2*X_2 + b_{12}*X_1*X_2$ (Y: zone of inhibition; X_1: bacteria and X_2: extract concentration). In the RSM experiment, multiple linear regression analysis was performed by JMP 10 software (SAS Institute, Cary, NC).

3. Results and discussions

3.1. Identification of the major compound in *C. operculatus* leaf extract

The predominant peak in the HPLC chromatogram (Figure 1) had a retention time of 38.37 and exhibited molecular ions $[M-H]^+$ at m/z 299.1289 and ion $[M-H]^-$ at m/z 297.1141, with mass errors of 3.7 and -0.4 ppm, respectively, which were below our cut-off of 4 ppm.

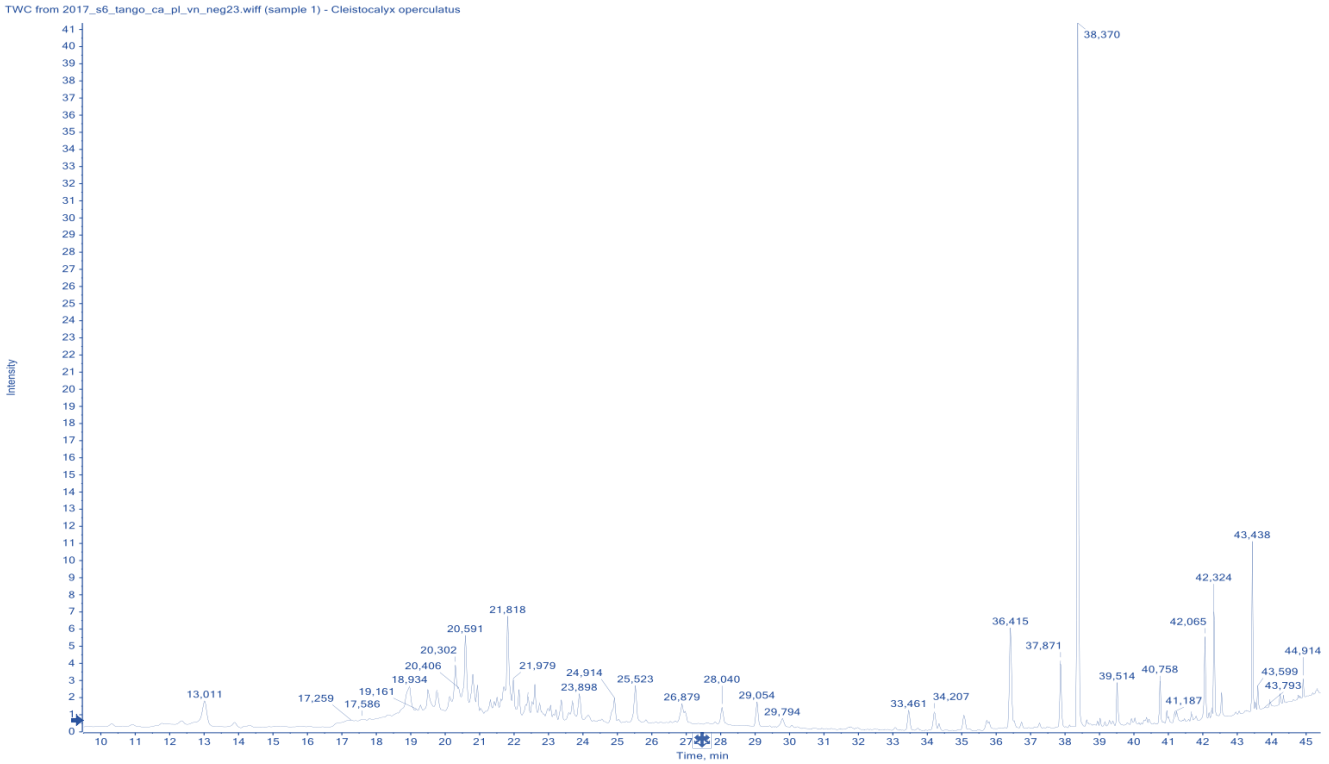


Figure 1. Total wavelength chromatogram of *C. operculatus* leaf extract**.**

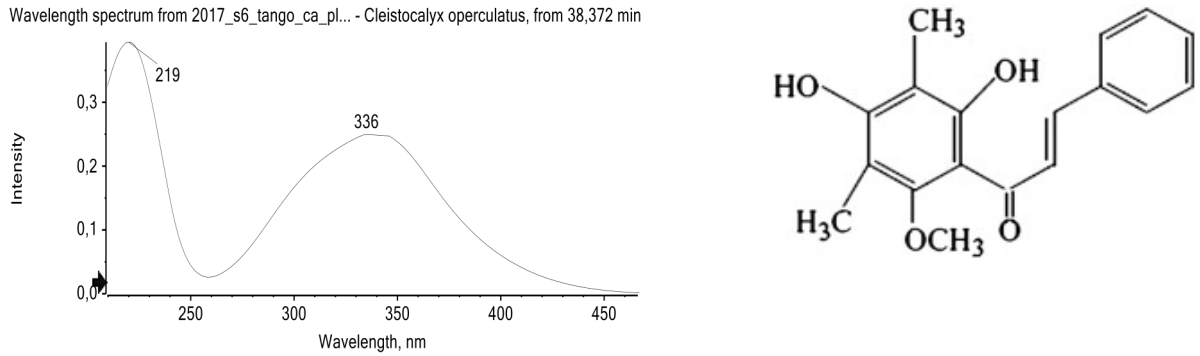


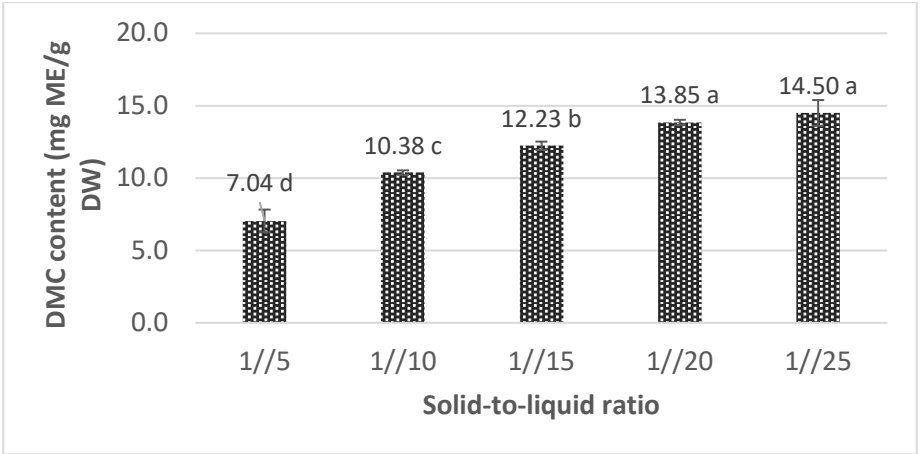
Figure 2. Absorbance spectrum and structure of dihydroxy-methoxy-dimethyl-chalcone (predominating peak) in *C. operculatus* leaf extract**.**

The molecular formula of the compound was hence inferred to be $C_{18}H_{18}O_4$. Fragmentation in positive mode (*m/z* 195.0726 (90) and 180.0442 (80)) was similar to the fragmentation pattern of protonated chalcones reported in the work of Tai *et al*. (2006) and Ye *et al*. (2004). This fragmentation has also been previously reported for 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone by Malterud *et al.* (1977). In addition, the absorbance spectrum of this peak with λ_{max} (336 nm) was very close to that described for 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone in the report of Malterud et al. (1977). All these data suggested that the predominant peak was a dihydroxy-methoxy-dimethyl-chalcone (Figure 2). 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone, also called dimethyl cardamonin, has been reported to be present in the buds and leaves of *C. operculatus* (Pham *et al*., 2020) and showed anti-inflammatory (Yu *et al*., 2015; Tran *et al.*, 2019) and antitumor activity against six established human cancer cell lines, including SMMC-7721, 8898, HeLa, SPC-A-1, 95-D and GBC-SD cells (Ye *et al*., 2004), as well as antiviral properties against H1N1, H9N2, and novel H1N1, through strong enzymatic inhibition of various virus neuraminidases (Ha *et al*., 2016).

3.2. Modelization and optimisation of dimethyl cardamonin extraction from *C. operculatus* leaves

3.2.1. Effect of the solid-to-liquid ratio

The impact of the solid-to-liquid ratio on the extraction of dimethyl cardamonin from *C. operculatus* leaves is presented in Figure 3.



** Mean ± SD (n = 3 experimental replications). Means followed by the same letter do not differ statistically using Duncan test with 5% probability.*

Figure 3. Effect of solid-to-liquid ratio on dimethyl cardamonin apparent content of *C. operculatus* leaf.

The results of the one-way analysis of variance showed that the solid-to-liquid ratio had a significant effect on the dimethyl cardamonin extraction ($p < 0.0001$). The quantity of extracted dimethyl cardamonin initially increased when the ratio varied from 1/5 to 1/20. This is consistent with mass transfer principles. The driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent to solid ratio is used (Elboughdiri, 2018). However, the dimethyl cardamonin content remained fairly constant when the ratio varied from 1/20 to 1/25. A similar effect of the solid-to-liquid ratio on extraction yield was reported for the extraction of phenolic compounds from *Inula helenium* (Wang *et al.*, 2013), *Inga edulis* leaves (Silva *et al.*, 2007), olive leaves (Elboughdiri, 2018), green coffee bean (Lai *et al*., 2018) and brewers' spent grain (Andres *et al*., 2020). A ratio of 1/20 gave a high dimethyl cardamonin content and hence was chosen for the dimethyl cardamonin extraction.

3.2.2. Modelization and optimisation of dimethyl cardamonin extraction

The experimental design of the five-level, three-variable central composite rotatable design (CCRD) and the experimental results of extraction are shown in Table 1. By applying a multiple regression analysis, the relation between the tested independent variables and the response was explained in Equation 1, in which x_i were standardized or coded variables.

$$Y = 16.56 + 5.29*x_1 + 4.01*x_2 + 0.28*x_3 + 0.33x_1* x_2 – 0.44x_1*x_3 – 0.35x_2*x_3 – 1.77x_1^2 + 1.33x_2^2 – 0.51x_3^2 \quad (1)$$

Tables 1. Rotatable central composite design setting in the coded form (x_1, x_2, x_3) and real values of the independent variables.

Run	Standard variables			Real variables			Dimethyl cardamonin apparent content (mg/g DW)
	x_1	x_2	x_3	Ethanol concentration (%)	Temperature (°C)	Time of extraction (min)	
1	1	1	1	80	85	45	23.04
2	-1	1	1	40	85	45	12.53
3	1	-1	1	80	45	45	15.66
4	-1	-1	1	40	45	45	7.04
5	1	1	-1	80	85	15	24.47
6	-1	1	-1	40	85	15	12.74
7	1	-1	-1	80	45	15	16.26
8	-1	-1	-1	40	45	15	5.31
9	1.68	0	0	93.6	65	30	21.99
10	-1.68	0	0	26.4	65	30	3.88
11	0	1.68	0	60	98.6	30	29.56
12	0	-1.68	0	60	31.4	30	13.87

13	0	0	1.68	60	65	55.2	17.81
14	0	0	-1.68	60	65	4.8	15.19
15A	0	0	0	60	65	30	17.39
15B	0	0	0	60	65	30	16.01
15C	0	0	0	60	65	30	15.81

To fit the response function and experimental data, the linear and quadratic effects of the independent variables, as well as their interactions on the response, were evaluated by analysis of variance (ANOVA), and regression coefficients were determined (Tables 2 and 3).

Table 2. Analysis of variance for the response surface quadratic model of dimethyl cardamonin apparent content of *C. operculatus* leaf.

Source	Degree of freedom	Sum of square	Mean square	F
Model	9	687.76	76.42	19.56
Error	7	27.34	3.91	$p = 0.0004$
Lack of fit	5	25.86	5.17	6.98
Pure error	2	1.48	0.74	$p = 0.1299$
Total	16	715.10		

Table 3. Parameter estimates of the predicted second-order model for the response (dimethyl cardamonin apparent content of *C. operculatus* leaf).

Term	Estimate	Standard error	t ratio	*p*
Intercept	16.5617	1.1389	14.54	<.0001
Ethanol concentration	5.2916	0.5348	9.89	<.0001
Temperature	4.0198	0.5348	7.52	0.0001
Time	0.2852	0.5348	0.53	0.6102
Ethanol concentration*Temperature	0.3338	0.6988	0.48	0.6475
Ethanol concentration*Time	-0.4438	0.6988	-0.64	0.5456
Temperature*Time	-0.3463	0.6988	-0.50	0.6354
Ethanol concentration* Ethanol concentration	-1.7719	0.5886	-3.01	0.0197
Temperature* Temperature	1.3323	0.5886	2.26	0.0580
Time*Time	-0.5115	0.5886	-0.87	0.4137

The ANOVA of the regression model showed that the model was highly significant due to a low probability value ($p < 0.0004$) (**Table 2**). The fitness of the model was judged by the coefficient of determination (R^2). In this study, the R^2 value for the regression model of the dimethyl cardamonin apparent content of *C. operculatus* leaves was 0.9618, which was close to 1, suggesting that the predicted second-order polynomial model well defined the dimethyl cardamonin extraction process from *C. operculatus* leaves and that 96.18% of the variation in the dimethyl cardamonin apparent content was attributed to the three studied

factors (Bharathi *et al.*, 2011). In addition, the lack of fit test is used to verify the adequacy of the model. The sum of squared errors (SSE) is split into two components called pure error (variation between observed and average values at X) and lack of fit (variation between average and predicted values at X). In our study, the absence of lack of fit ($p = 0.1299$) meant that the total error of the model was due to the pure error. This strengthened the reliability of the model (Table 2).

The effects of ethanol concentration, temperature and time of extraction on the apparent dimethyl cardamonin content of *C. operculatus* leaves are presented in Table 3 and Figure 4. As illustrated in Table 3, the ethanol concentration and temperature showed significant linear effects on the dimethyl cardamonin apparent content ($p < 0.0001$ and $p = 0.0001$, respectively), while the time of extraction had no effect ($p = 0.6102$). Among them, ethanol concentration appeared to be the most affecting factor of the dimethyl cardamonin extraction process from *C. operculatus* leaves because its coefficient had the highest value (5.2916). As shown in Figure 4, the quantity of extracted dimethyl cardamonin increased as the ethanol concentration increased. Similarly, higher temperatures of extraction resulted in higher dimethyl cardamonin apparent contents. Moreover, a high quantity of dimethyl cardamonin (29.56 mg/g DW) was obtained at 98.6 °C (run 11), indicating that this compound was a thermoresistant compound.

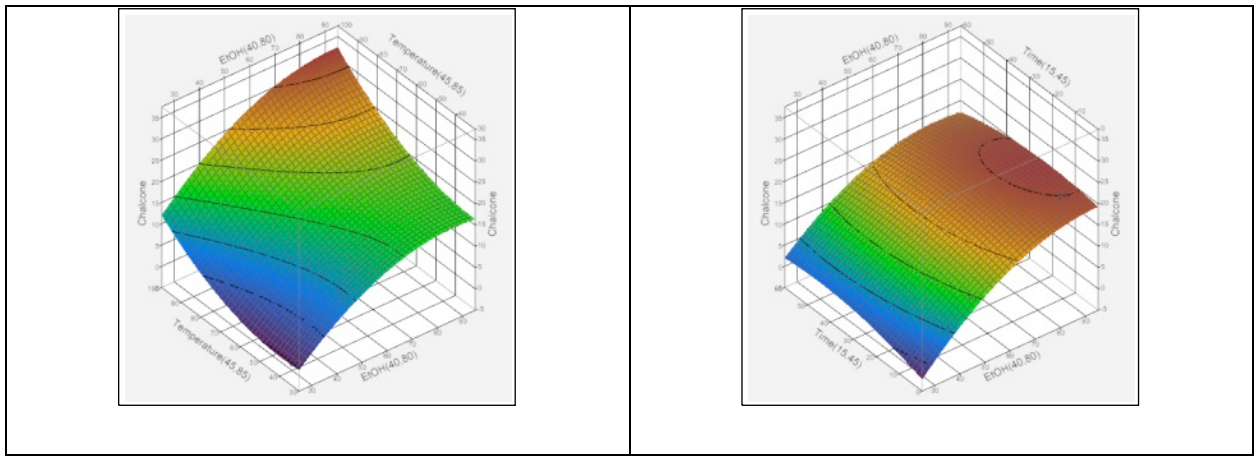


Figure 4. Response surface for dimethyl cardamonin apparent content in function of ethanol concentration, temperature and time of extraction

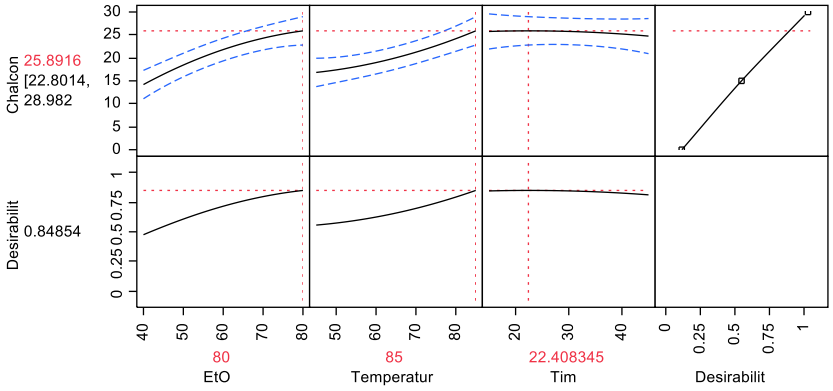


Figure 5. Ethanol concentration, temperature and time of extraction as well as predicted response at point with maximal desirability.

Concerning time extraction, this factor had a nonsignificant effect on the dimethyl cardamonin apparent content of *C. operculatus* leaves. In runs 1 and 14 (Table 1), a small increase in dimethyl cardamonin quantity (17.81 - 15.19 = 2.62 mg/g DW) was observed when the time of extraction increased from 4.8 to 55.2 minutes. Similarly, when the time of extraction increased from 4.8 (run 14) to 30 min (run 15), the dimethyl cardamonin content slightly increased (from 15.19 to 16.4 mg/g DW). This indicated that an important quantity of dimethyl cardamonin could be extracted during the first minutes of extraction. Accordingly, the maximal rates of extraction of phenolic compounds from agrimony, sage and savoury leaves and green coffee bean were found to take place during the first minutes of extraction (Kossah *et al.*, 2010; Lai *et al*., 2018).

The optimum conditions of dimethyl cardamonin extraction from *C. operculatus* leaves were acquired by using JMP 10 software. The software was set to determine the optimum desirability of the response, which was the maximum dimethyl cardamonin apparent content of the leaves. The optimum conditions were found as follows: ethanol concentration, 80%; temperature, 85 °C; and time of extraction, 22 min, as shown in Figure 5. To examine the validity of the model, extraction was performed with four replicates under these optimised conditions. The measured values (23.52, 25.50, 23.81 and 24.84 mg/g DW) lay within a 95% mean confidence interval of the predicted value (22.80 - 28.98 mg/g DW). These results confirmed the predictability of the model. The second-order polynomial model (Eq. 1) can thus be effectively applied to predict the amount of dimethyl cardamonin extracted from *C. operculatus* leaves.

3.3. *In vitro* antimicrobial activity against food-poisoning bacteria of *C. operculatus* leaf extract powder

Table 4 shows the antibacterial activities against five food poisoning bacteria of *C. operculatus* leaf extract powder measured by the agar well diffusion method. The *C. operculatus* leaf extract exhibited inhibitory activity against both gram-positive and gram-negative bacteria. The inhibition zone for the five food-poisoning bacteria ranged from 1.83 to 9.17 mm and significantly depended on the bacterial strain ($p < 0.0001$). Among the five tested bacteria, *Staphylococcus aureus* and *Listeria monocytogenes* were the most sensitive strains to *C. operculatus* leaf extract, followed by *Salmonella* and *Escherichia coli,* while *Vibrio parahaemolyticus* was the least sensitive. The extract concentration significantly affected the antimicrobial activity ($p < 0.0001$). In general, higher concentrations of leaf extract resulted in higher inhibition zones. However, the interaction between bacterial strain and extract concentration had no significant effect on the inhibitory zone ($p = 0.4567$).

Table 4. Inhibition zones (mm) of *C. operculatus* leaf extract powder against five food poisoning bacteria*.

Bacteria	Extract concentration					Antibiotic (300 µg/mL)
	10 mg/mL	20 mg/mL	30 mg/mL	40 mg/mL	50 mg/mL	
S. aureus	4.75±0.25^{aD}	6.92±0.14^{aC}	8.08±0.38^{aB}	8.33±0.76^{abAB}	9.17±0.88^{aA}	10.58±0.52 (Norfloxacin) 13.58±0.29 (Gentamicin) 1.83±0.14 (Ceftriaxone)
V. parahaemolyticus	1.83±1.04^{bC}	4.58±0.76^{bB}	6.00±0.43^{bA}	6.42±0.63^{cA}	7.17±0.14^{bA}	10.83±0.14 (Norfloxacin) 1.83±0.29 (Ceftriaxone) 13.75±0.25 (Chloramphenicol)

E. coli	4.42±0.80^{aC}	5.92±0.29^{abBC}	6.58±0.88^{abAB}	7.00±0.87^{ABbc}	7.67±1.15^{bA}	0 (Aztreonam) 14.22±0.38 (Norfloxacin) 0.92±0.29 (Amoxycillin)
Salmonella	5.33±0.58^{aB}	6.08±1.42^{abAB}	6.92±1.01^{abAB}	7.58±0.95^{abcA}	7.67±0.38^{bA}	0 (Aztreonam) 12.75±0.25 (Norfloxacin) 15.75±0.25 (Piperacillin)
L. monocytogenes	5.33±0.58^{aC}	6.50±1.32^{aBC}	6.76±1.38^{abAB}	8.67±0.80^{aA}	9.00±0.43^{aA}	13.33±0.14 (Spectinomycin) 4.58±0.38 (Gentamicin) 1.33±0.52 (Amoxycillin)

** Mean ± SD (n = 3 experimental replications). Means followed by the same lowercase letter in a column and uppercase letter in a row do not differ statistically using Duncan test with 5% probability.*

Antibacterial activity of *C. operculatus* leaf extract has not been extensively studied. In the work of Nguyen *et al.* (2017), a methanolic extract of *C. operculatus* leaves inhibited bacterial activity against Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans* GS-5) and three multiresistant bacteria (*Staphylococcus epidermidis* 847, *Staphylococcus haemolyticus* 535 and *Staphylococcus aureus* North German epidemic strain) with inhibition zone diameters ranging from 7 to 16 mm. Phytochemical screening of the extract using thin layer chromatography indicated the presence of flavonoids, which could contribute to the antibacterial activity of the extract. In our study, *C. operculatus* leaf extract inhibited the growth of five food poisoning bacteria, indicating the potential application of *C. operculatus* leaves as a source of antimicrobial agents for food preservation.

4. Conclusions

In this study, by using UHPLC-DAD-HRMS, the predominant compound in *C. operculatus* leaves harvested in Vietnam was identified as 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone, also called dimethyl cardamonin. The extract of this compound from leaves was optimised by using response surface methodology. The optimised extraction conditions were as follows: ethanol concentration of 80% and extract temperature of 85 °C for 22 min. The obtained extract powder exhibited inhibitive activity against five food poisoning bacterial strains belonging to *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes* species. The results indicated the potential application of *C. operculatus* leaf extract powder as a natural antibacterial preservative

5. References

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