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Optimization of Combined Microwave and Ultrasound Extractions of Cannabinoids Compounds from *Cannabis indica* L. (Blueberry Cultivar) Using Response Surface Methodology

Rossakornpat Hirunyasiri[1], Natnakorn Punamorntarakul[1], Raweeroj Jintawiwat[1], Tanachai Pankasemsuk[1], Kriangsak Phramphun[1], Rien Loveemongkol[2], Supakiat Supasin[1] and Arthitaya Kawee-ai[1*]
[1] *Division of Cannabis and Medicinal Plants for Local Development, Graduate School, Payap University, Chiang Mai 50000, Thailand*
[2] *International College, Payap University, Chiang Mai 50000, Thailand*
*E-mail: kaweeai@gmail.com

Abstract

Microwave and ultrasound are novel technologies that are widely used for extracting bioactive compounds from plant materials. In this study, combined extraction techniques, microwave and ultrasound, were applied to extract cannabinoids, cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC), from *Cannabis indica* L. (Blueberry cultivar). Three independent variables including solid to liquid ratio (X1, A: 1:10–1:30 w/v), microwave extraction time (X2, B: 5–20 min), and ultrasound extraction time (X3, C: 10–30 min) were optimized using central composite design (CCD). The experimental data obtained was fitted to a second-order polynomial equation using multiple regression analysis and additionally analyzed using appropriate statistical methods (analysis of variance, ANOVA). The optimum conditions were determined according to the solid to liquid ratio (X1, A) of 1:22 (w/v), microwave extraction time (X2, B) of 5 min and ultrasound extraction time (X3, C) of 14 min. Under these conditions, the experimental CBD and THC content was 0.298±0.001 mg/g dry weight and 91.35±0.35 mg/g dry weight, respectively. The experimentally-achieved values were in accordance with those estimated by the CCD model, suggesting the applicability of the utilized model and the favorable result of CCD's application in the optimization of the combined microwave and ultrasound extraction.

Keywords: Green Technology, Cannabinoids, Ultrasound, Microwave

1. Introduction

Cannabis (*Cannabis* ssp.) is a herbaceous plant of the Cannabaceae family. It has been utilized for medical, therapeutic, and spiritual purposes. Cannabis was made legal for medical and recreational use in Thailand on February 18, 2019 (1). It contains many biologically active compounds such as cannabinoids, terpenoids, alkaloids, and quinones (2). Δ9-tetrahydrocannabinol (THC) is the most prominent cannabinoid in cannabis that receives attention because of its psychoactive properties, as well as analgesic, anti-inflammatory, appetite stimulant, and antiemetic properties (3). Regular use by teenagers under the age of 30, however, result in cognitive impairments and neurodevelopmental abnormalities (4). Cannabidiol (CBD) can control THC's euphoric effects and has antipsychotic, neuroprotective, anticancer, antidiabetic, and other properties such as lowering the anxiety caused by fear or decreasing the cigarette intake of tobacco

smokers (5). Despite the promise of these natural biomolecules and their prospective use in industrial areas of cannabis, developing more effective techniques for their recovery remains a challenge. *Cannabis indica* L. (Blueberry cultivar) is a hybrid cultivar of a Purple Thai, Highland Thai, and Afghani (6). This variety contains a high range of THC content by 14-25% (7). The Blueberry cultivar is one of the promising cultivars for medicinal uses (8).

Several research studies have been conducted to enhance the extraction of these chemical compounds using conventional, ultrasound, or microwave-assisted extraction techniques (9-11). Microwave and ultrasonic extractions are more efficient techniques than other extraction procedures (9, 10). They are rapid, easy, safe, and ecologically beneficial technologies due to less solvent usage and a lower energy consumption (12). Microwave irradiation creates an electric and magnetic field, generating high-frequency motion and dipole rotation. In contrast to typical conductive heating, it may swiftly transmit energy directly to the reactants and raise the internal and exterior temperatures virtually simultaneously. Ultrasound is a sound wave with a frequency greater than 20 kHz. Acoustic cavitation and acoustic streaming are the two fundamental phenomena of every ultrasonic process. Ultrasound waves are made up of compression and expansion cycles. When an ultrasonic wave passes through a liquid, it exerts positive pressure during the compression cycle and negative pressure during the expansion cycle. During the period of negative pressure, the molecules are drawn apart from one another and as a result, cavities occur in the liquid. These holes form and expand throughout several cycles. Finally, the cavities implode (13). As a result, combining these two extraction procedures might be a beneficial tool for improving the extraction of cannabinoids.

It is necessary to consider the influences of the affecting factors of each technique. The work of Addo et al. (9) reported that the sample-to-solvent ratio, extraction time, extraction temperature, and duty cycle influenced the ultrasound- and microwave-assisted extraction of cannabis extracts. Using a microwave power of 1,000 W and a temperature of 60°C resulted in a 111.4% increase in maximal cannabinoids production. The microwave-assisted ethanol extraction of cannabis is where the yield of CBD and THC is dependent on the ethanol concentration and solid-to-liquid ratio (14). Agarwal et al. (15) found that solvent concentration and extraction time have an influence on Cannabis sativa L. extraction by ultrasound-assisted extraction. Thus, statistical experiments are required to create a significant model of the diverse variables while doing the fewest number of experiments possible. Central composite design (CCD) and response surface methodology (RSM) are fast and adaptable approaches that give adequate data for simulating multivariable systems while avoiding experimental mistakes and greatly lowering the number of tests required (16).

Therefore, the main objective of this study was to extract bioactive compounds (CBD and THC) using a combination of these two extraction methods from the inflorescences of *C. indica* L. (Blueberry cultivar). The evaluation was done by analyzing the influence of factors such as the solid-to-liquid ratio, microwave extraction time, and ultrasound extraction time using a response surface methodology. This work is expected to encourage the exploration of the potentially useful compounds present in *C. indica* L. (Blueberry cultivar).

2. Materials and Methods

2.1 Raw material and preparation

The inflorescences of *C. indica* L. (Blueberry cultivar) were manually collected in May 2023 in Chiangmai, Thailand. They were dried and stored in a glass vial at ambient temperature until further used (15). Prior to use, the cannabis was decarboxylated in an oven at 150°C for 10 min (17). Then, the dried samples were ground into fine powder (200 mesh) and stored in a bag under vacuum in a cool and dry place until use.

2.2. Experimental design

In this study, central composite design (CCD) was used to extract CBD and THC from Blueberry cultivar by optimizing the influence of factors such as the solid-to-liquid ratio, microwave extraction time, and ultrasound extraction time of the combination of microwave and ultrasound. The design consisted of 16 randomized runs with two replicates at the central point, as presented in Table 1.

Table 1 Central composite design of the combination of microwave and ultrasound extractions.

No.	Factors		
	Ratio (X_1, A)	Microwave time (X_2, B)	Ultrasonic time (X_3, C)
1	1:10	5	10
2	1:30	5	10
3	1:10	20	10
4	1:30	20	10
5	1:10	5	30
6	1:30	5	30
7	1:10	20	30
8	1:30	20	30
9	1:3.2	12.5	20
10	1:36.8	12.5	20
11	1:20	0	20
12	1:20	25.1	20
13	1:20	12.5	3.2
14	1:20	12.5	36.8
15	1:20	12.5	20
16	1:20	12.5	20

2.3. Extraction procedure

In total, 5 g of ground *C. indica* L. was weighed and inserted into a microwave machine tube. Ethanol was used as an extraction solvent. The ratio of *C. indica* L. and ethanol used was according to the experimental design (Table 1). Firstly, the extractions were performed in a microwave digestion system (ETHOS UP, Milestone srl, Bergamo, Italy). The temperature and power were set at 60°C and 1000 W, respectively (9). After the microwave extraction conditions selected were reached, the samples were transferred to do an ultrasonic extraction using an ultrasonic bath system (Crest ultrasonics, Bangkok, Thailand) with a fixed frequency of 40 kHz, setting the extraction temperature at 60°C (9). After extraction, the samples were centrifuged (5804 R centrifuge, Eppendorf, Hamburg, Germany) at 5,000 rpm for 5 min and then transferred into vials for further analysis. The diagram of the combination of microwave and ultrasound extraction is presented in Figure 1.

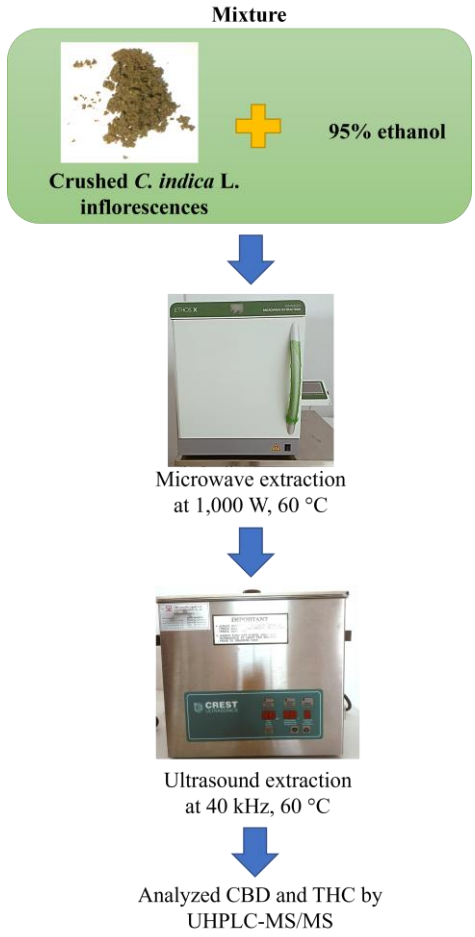

Figure 1 The schematic diagram of the combination of microwave and ultrasound extraction used in this study.

2.4. Liquid chromatography analysis of the cannabinoid content

The extract samples were filtered using a 0.45 µm nylon filter (Part No. 5191-5909, Agilent Technologies, USA) before injection into an ultra-high performance liquid chromatography-tandem mass spectrometry device (UHPLC-MS/MS) (PerkinElmer, MA,

USA). The CBD and Δ9-THC content was determined using Quasar SPP C18 column (100 mm × 2.1 mm, 2.6 µm-PerkinElmer, Buckinghamshire, UK) at 30°C with a flow rate set at 0.2 mL/min and with an injection volume of 3 µL. The modification gradient program for mobile phase A (0.1% formic acid in a water) and mobile phase B (0.1% formic acid in a methanol) was adjusted at 0–18-min, 90% B; 18.0–19.8-min, 95% B; and 19.8–20.0-min, 75% B (3).

2.5. Statistical analysis

All experimental values were done in triplicate and presented using mean ± standard derivation. The experimental data was fitted to the second-order polynomial model (Equation (1)) to obtain the regression coefficients (b) using Design-Expert software version 13 (Stat-Ease Inc., Minneapolis, MN, USA). The generalized second-order polynomial model used in the response surface analysis is presented as in Eq. (1).

$$Y = y_0 + \sum_{i=0}^{n=3} y_i X_i + \sum_{i=0}^{n=3} y_{ii} X_i^2 + \sum_{i=j=1}^{n=3} y_{ij} X_i X_j \quad (1)$$

where Y is the predicted dependent variable (response variable) to be modelled, y_0 is an intercept; y_i, y_{ij}, and y_{ii} are the coefficients of the linear, quadratic, and interactive terms; X_i and X_j are the independent variables; and n is the number of factors analyzed. The parametric estimation responses were collected in the form of CBD and THC content.

3. Results & Discussion

3.1 Cannabinoid identification

Figure 2 presents the UHPLC fingerprinting of *C. indica* L. (Blueberry cultivar) according to the ethanolic extracts .The predominate cannabinoid compound is Δ9-THC at a retention time of 12.70 min. Backes (7) revealed that the Blueberry variety was approximately 14-16% Δ9-THC, whereas CBD presented at a retention time of 7.64 min.

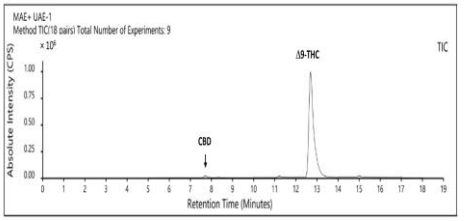

Figure 2 UHPLC chromatogram of *C. indica* L. (Blueberry cultivar) extracted by a combination of microwave and ultrasound extraction.

3.2. CCD optimization for cannabinoid extraction

In this study, the CCD was used in the screening experiment to determine the most important factor that produced the highest content for both CBD and THC. The experimental and predicted CBD and THC content are shown in Table 2, while adequacy and fitness were evaluated using ANOVA (Table 3). Using multiple regression analysis, the polynomial model for an empirical relationship between the content of CBD and THC and the test variables is expressed as a code unit according to Eq. (2) and Eq. (3), respectively.

Based on Table 2, the results indicate that the highest CBD content (0.350±0.018 mg/g) was achieved at 1:20 for ratio, 0 min of microwave extraction, and 20 min of ultrasound extraction, (the run no. 11), followed by run no. 12 (A =1:20, B = 25.1 and C = 20), which presented with a content of CBD of 0.293±0.018 mg/g. This indicates that microwave had no influence on CBD content, which is consistent with the quadratic regression model equation 2. The data from Table 3 also reveals that microwave extraction time had no significant effect on CBD ($p = 0.3471$). Drinić et al. (14) proved that ethanol concentration and solid-to-liquid ratio had a highly significant influence on microwave extraction. They found that 47% of the ethanol concentration and 1:5 of the solid-to-

liquid ratio gave the maximum CBD for *C. sativa*, cultivar Helena (14). Microwave extraction time had no influence on CBD content, which is consistent with our results, as presented in Table 3.

The highest concentration of THC (105.460±5.433 mg/g) was obtained with extraction conditions of a 1:30 of ratio, 20 min of microwave extraction, and 30 min of ultrasound extraction (run no. 8). The second-best condition for the extraction of THC (98.087±2.675 mg/g) was a 1:36.8 ratio, 12.5 min of microwave extraction, and 20 min of ultrasound extraction (run no. 10). Thus, a higher solid to liquid ratio had a positive effect on the content of THC, associated with p-value < 0.0001 for the solid to liquid ratio of THC as presented in Table 3. The increase of the solid to liquid ratio increased the amount of solvent diffusion into cannabis, so the concentration of the cannabinoids was improved (9, 18).

Table 2 Experimental and predicted values of CBD and THC content from the Blueberry cultivar following a combination of microwave and ultrasound extraction.

No.	Factors			CBD content (mg/g)		THC content (mg/g)	
	Ratio (A)	M time (B)	U time (C)	Predicted	Experimental	Predicted	Experimental
1	1:10	5	10	0.147	0.143±0.080	72.29	74.528±0.885
2	1:30	5	10	0.206	0.193±0.037	91.04	90.476±5.322
3	1:10	20	10	0.201	0.217±0.018	71.61	74.047±7.934
4	1:30	20	10	0.156	0.154±0.004	82.52	82.581±7.064
5	1:10	5	30	0.128	0.118±0.073	51.08	53.619±1.086
6	1:30	5	30	0.152	0.107±0.019	93.52	93.680±3.031
7	1:10	20	30	0.150	0.150±0.013	70.50	73.656±2.659
8	1:30	20	30	0.054	0.045±0.017	105.11	105.46±5.433
9	1:3.2	12.5	20	0.043	0.036±0.010	52.01	47.085±0.070
10	1:36.8	12.5	20	0.012	0.038±0.039	96.84	98.087±2.675
11	1:20	0	20	0.322	0.350±0.018	81.00	79.632±4.622
12	1:20	25.1	20	0.301	0.293±0.018	90.09	87.758±1.581
13	1:20	12.5	3.2	0.152	0.148±0.119	79.97	78.734±0.608
14	1:20	12.5	36.8	0.051	0.074±0.059	81.13	78.682±0.939
15	1:20	12.5	20	0.147	0.138±0.011	87.38	86.546±1.656
16	1:20	12.5	20	0.147	0.153±0.021	87.38	88.888±0.379

The experimental values expressed as mean ± standard deviation (n=3).
M time is microwave extraction time and U time is ultrasound extraction time.

The ANOVA results for the effect of the extraction parameters on the CBD and THC content (Table 3) demonstrate that the model was highly significant (p = 0.0009 and p = 0.0002). The value of the determination coefficient (R^2) was 0.9664 for CBD and 0.9786 for THC, indicating a good agreement between the experimental and predicted values which explains 96.64% and 97.86% of the variability of the responses, respectively. Lack-of-fit measured the failure of the model to represent the data in the experimental domain at points not included in the regression. In this study, C, AB, A^2, and B^2 are significant model terms for CBD, while A, B, AC, BC, and A^2 are significant model terms for THC.

The full quadratic regression model represents the predicted CBD (Eq. 2) and THC (Eq. 3) of *C. indica* L. (Y): solid to liquid ratio (A), microwave extraction time (B), and ultrasound extraction time (C). Model Eq (2) in terms of the coded factors shows that the positive coefficients for CBD are A, C, and B^2, which are the high levels for the factors, while the negative coefficients are B, AB, AC, BC, A^2, and C^2. The interaction of AB, AC, BC are the lowest levels of the factors. The positive coefficients for THC are A and AC.

Table 3 Statistical analysis (ANOVA) of the central composite design.

Compound	Source	SS	df	MS	F-value	*p*-value	
CBD	Model	0.1082	9	0.0120	19.16	0.0009	significant
	A	0.0012	1	0.0012	1.86	0.2220	
R^2=0.9664	B	0.0007	1	0.0007	1.04	0.3471	
	C	0.0124	1	0.0124	19.80	0.0043	
	AB	0.0053	1	0.0053	8.42	0.0273	
	AC	0.0013	1	0.0013	2.09	0.1985	
	BC	0.0005	1	0.0005	0.8262	0.3984	
	A^2	0.0167	1	0.0167	26.58	0.0021	
	B^2	0.0314	1	0.0314	50.09	0.0004	
	C^2	0.0025	1	0.0025	3.93	0.0948	
	Residual	0.0038	6	0.0006			
	Lack of Fit	0.0037	5	0.0007	6.49	0.2891	not significant
	Pure Error	0.0001	1	0.0001			
THC	Model	3266.52	9	362.95	30.45	0.0002	significant
	A	2428.37	1	2428.37	203.76	< 0.0001	
R^2=0.9786	B	101.00	1	101.00	8.47	0.0269	
	C	1.62	1	1.62	0.1356	0.7253	
	AB	30.69	1	30.69	2.58	0.1597	
	AC	280.64	1	280.64	23.55	0.0028	
	BC	201.93	1	201.93	16.94	0.0062	
	A^2	194.99	1	194.99	16.36	0.0068	
	B^2	4.00	1	4.00	0.3353	0.5836	
	C^2	54.24	1	54.24	4.55	0.0769	
	Residual	71.51	6	11.92			
	Lack of Fit	68.76	5	13.75	5.01	0.3261	not significant
	Pure Error	2.74	1	2.74			

$$CBD = +0.002018 + 0.022899A - 0.017999B + 0.007410C - 0.000343AB - 0.000128AC - 0.000107BC - 0.000424A^2 + 0.001043B^2 - 0.000163C^2 \quad (2)$$

$$THC = +59.49908 + 2.31180A - 0.160096B - 1.01927C - 0.026115AB + 0.059229AC + 0.066988BC - 0.045897A^2 - 0.011760B^2 - 0.024206C^2 \quad (3)$$

Figures 3 and 4 illustrate the 3D surface plots for the CBD and THC content as a function of the solid to liquid ratio and microwave extraction time (A), solid to liquid ratio and ultrasound extraction time (B), and microwave extraction time and ultrasound extraction time (C). Based on the response of the 3D surface plot, it was observed that the CBD concentration increased with an increase in the solid to liquid ratio but decreased with an increase in microwave extraction time and ultrasound extraction time (Figure 3A-3C). An increased solid-to-liquid ratio improved the efficiency of the solvent diffusing into the plant matrix in both microwave and ultrasonic systems, resulting in an increased cannabinoid concentration (9). A longer extraction time by microwave caused the degradation of the thermolabile constituents (19).

As shown in Figures 4A and 4B, the presence of THC in the extractrants increased with an increase in the solid to liquid ratio. This finding was in agreement with the work of Addo et al. (9) on increasing the cannabinoid concentration of the extracts by increasing the sample-to-solvent ratio. Figure 4C displays the effect of microwave extraction time (B) and ultrasound extraction time (C). The continual rise in THC corresponded to an increase in microwave extraction time (B) and ultrasound extraction time (C). This was due to cavitation bubbles as part of the ultrasound extraction and volumetric heating properties as in the microwave extraction (9, 14, 15).

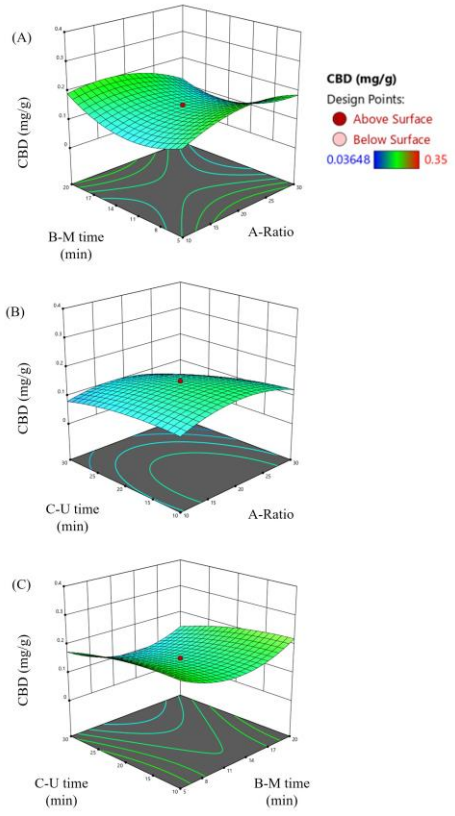

Figure 3 3D surface presented the correlation of factors AB (A), AC (B), and BC (C) on the CBD extracted by a combination of microwave and ultrasound extraction methods.

As can be seen in these results, the simultaneous application of microwave and ultrasound had a significant effect on the content of THC. To our knowledge, this is the first study focusing on the simultaneous application of microwave and ultrasound for the purpose of extracting high-value compounds from cannabis. Wu et al. (20) demonstrated that a combination of microwave and ultrasound was able to be used to degrade phenolic compounds as the sono-generated radicals, in conjunction with the rapid thermal effect of microwaves, have a significant effect when degrading polar chemicals.

By solving the inverse matrix (from Eq. (2) and Eq. (3)), the optimal values of the variables affecting the extraction of CBD and Δ9-THC given by the software were a solid to liquid ratio of 1:22.54, microwave extraction time of 5 min, and ultrasound extraction time of 14.31 min, with a desirability of 0.774. Desirability showed a good acceptable value for the optimization of the combination technology (21). Regarding operating convenience, the optimal extraction parameters were a solid to liquid ratio of 1:22, microwave extraction time of 5 min, and ultrasound extraction time of 14 min with predicted values for CBD and THC of 0.224 mg/g and 88.60 mg/g, respectively. Triplicate experiments were performed under the determined conditions, and the concentration of CBD and THC (0.298±0.001 mg/g and 91.35±0.35 mg/g) was in agreement with the predicted value, indicating that the model was adequate for use as a cannabinoid extraction process.

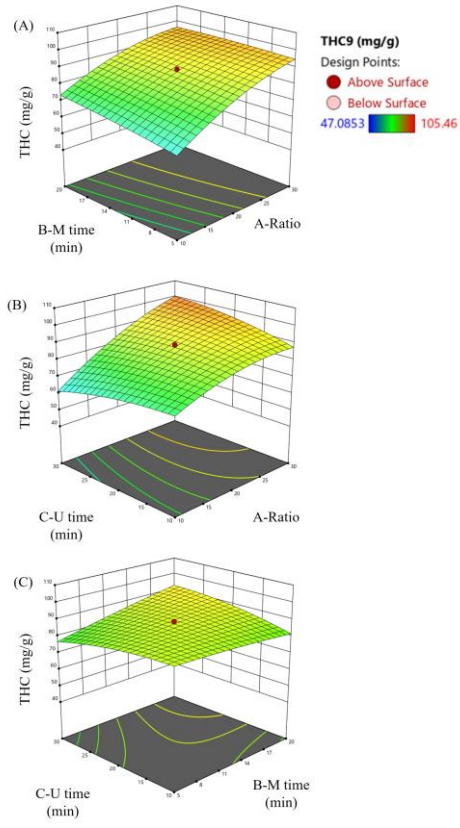

Figure 4 3D surface presented the correlation of factors AB (A), AC (B), and BC (C) on the THC extracted by a combination of microwave and ultrasound extraction.

These results reveal that the time of extraction significantly influenced the yield of CBD and THC from the Blueberry cultivar. The optimal concentrations were obtained following 5 min of microwave extraction and 14 min of ultrasound. This finding agrees with the results of Lou et al. (22), namely that the simultaneous application of microwave and ultrasound reduced the extraction time.

4. Conclusion

Microwave and ultrasound are both typical extraction procedures utilized for the recovery of bioactive substances from plants. In this study, the optimal condition of the combination of microwave and ultrasound extraction determined by CCD with the highest concentrations of CBD and THC produced at an optimized solid to liquid ratio of 1:22, microwave extraction time of 5 min, and ultrasound extraction time of 14 min. The three factors of solid to liquid ratio, microwave extraction time, and ultrasound extraction time have a significant influence on CBD and THC recovery. Thus, the present study provides a green extraction process to produce CBD and THC from *C. indica* L. (Blueberry cultivar). Finally, this study is the initial step for the combination of microwave and ultrasound extraction methods to obtain cannabinoid substances from cannabis. More applications including microwave power, microwave temperature, and ultrasound temperature need to be further developed.

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Declaration of conflicting interests

The authors declared that they have no conflicts of interest in the research, authorship, and this article's publication.

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