Optimization of Microwave-Assisted Extraction Process of Callicarpa candicans (Burm. f.) Hochr Essential Oil and Its Inhibitory Properties against Some Bacteria and Cancer Cell Lines

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Abstract:

Callicarpa candicans (Burm. f.) Hochr. (Callicarpa cana L.) is a medicinal plant that is distributed mainly in the tropics and subtropics of Asia and finds a wide range of uses in traditional medicine. In this study, we attempted and optimized the microwave-assisted hydro-distillation (MAHD) process to obtain essential oil from the leaves of C. candicans. In addition, the obtained oil was analyzed for volatile composition by gas chromatography?mass spectrometry (GC-MS) and assayed for bioactivity against several bacteria and cancer cell lines. To optimize the extraction process, response surface methodology (RSM) in combination with central composite design (CCD) was adopted. Experimental design and optimization were carried out with respect to three experimental factors including the ratio of water to raw material, extraction time, and microwave power. The optimal extraction conditions were obtained as follows: water to raw material ratio of 6/1 (v/w), extraction time 42 min, and microwave power 440 W. Composition determination of the obtained C. candicans essential oil indicated the presence of predominant components including caryophyllene (10.45%), cadinene (10.28%), gurjunene (8.95%), muurolene (8.92%), selinene (7.06%), selinene (5.59%), and copaene (5.40%). In comparison with the essential oils obtained via traditional hydro-distillation method, the essential oil extracted by MAHD exhibited superior anti-proliferative activity on all tested cancer cell lines. Current results imply that the MAHD is capable of recovering biologically-active natural products of greater quantity than that recovered by the conventional distillation.

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Abstract: *Callicarpa candicans* (Burm. f.) Hochr. (*Callicarpa cana* L.) is a medicinal plant that is distributed mainly in the tropics and subtropics of Asia and finds a wide range of uses in traditional medicine. In this study, we attempted and optimized the microwave-assisted hydro-distillation (MAHD) process to obtain essential oil from the leaves of *C. candicans*. In addition, the obtained oil was analyzed for volatile composition by gas chromatography–mass spectrometry (GC-MS) and assayed for bioactivity against several bacteria and cancer cell lines. To optimize the extraction process, response surface methodology (RSM) in combination with central composite design (CCD) was adopted. Experimental design and optimization were carried out with respect to three experimental factors including the ratio of water to raw material, extraction time, and microwave power. The optimal extraction conditions were obtained as follows: water to raw material ratio of 6/1 (*v/w*), extraction time 42 min, and microwave power 440 W. Composition determination of the obtained *C. candicans* essential oil indicated the presence of predominant components including caryophyllene <b-> (10.45%), cadinene <d-> (10.28%), gurjunene <a-> (8.95%), muurolene <g-> (8.92%), selinene <a-> (7.06%), selinene <b-> (5.59%), and copaene <a-> (5.40%). In comparison with the essential oils obtained via traditional hydro-distillation method, the essential oil extracted by MAHD exhibited superior anti-proliferative activity on all tested cancer cell lines. Current results imply that the MAHD is capable of recovering biologically-active natural products of greater quantity than that recovered by the conventional distillation.

1. Introduction

The genus of *Callicarpa* consists about 140 species that are distributed mostly in Oceania and east and southeast Asia including India, Myanmar, Thailand, and Vietnam [1–3], of which, several species such as *Callicarpa candicans*, *C. americana L.*, *C. japonica*, *C. macrophylla*, and *C. longissimi* are highly valued for their essential oils, which are rich in terpenoids, phenylethanoids, volatile oils, lignans, and flavonoids. These classes have been known for a wide range of biological activities including antimicrobial, analgesic, antipyretic, anti-inflammatory, and anti-infection [2,4–7]. *C. candicans* (Burm. f.) Hochr. or *Callicarpa cana* L., popularly known as Nang Nang in Vietnamese, is typically used in folk medicine as a tonic for postpartum women, for the treatment of abdominal pain and certain liver-related diseases, and to strengthen the tendons [4,8]. Moreover, the leaf of this shrub has been widely used as a fish poison by the locals in the Caroline and Philippine Islands [9,10].

To obtain essential oils of medicinal plants at an industrial scale, conventional hydro-distillation (HD) and steam distillation are typically adopted. However, these techniques can lead to the loss of certain compounds as well as the degradation of unsaturated compounds, possibly due to heat and hydrolysis [11]. Among newer techniques devised for the extraction of thermo-sensitive compounds such as microwave-assisted hydro-distillation (MAHD) [12,13], solvent-free microwave extraction [14], and microwave hydro-diffusion and gravity [15], MAHD has been demonstrated to be particularly effective [15,16]. The method combines traditional solvent extraction with microwave to generate pressure within walls of gland cells, permitting more efficient extraction of essential oils and in turn improving yield and quality of the product [17]. The heat is generated by microwaves following two mechanisms: ion conduction and bipolar rotation. These two mechanisms generate heat in the core of the material, making the heating process much faster and more efficient. The more polar the compound is, the faster it is heated under microwave radiation, especially water. In extraction, when microwave radiation into extraction media containing polar materials and solvents, solvent molecules and polar substances will oscillate and heat up quickly, increasing the ability to dissolve substances into the solvent. In addition, the solvent is better able to dissolve the analyte when the temperature is high, whereas the surface tension and viscosity of the solvent decreases with temperature, which will improve sample wetting and penetrate the matrix. Moreover, microwaves destroy the structure of plant cell walls, facilitating the release of solutes into the environment, making extraction faster but also making the extract more impurities. The interaction of microwave with free water molecules existing in the system of glands and matrix causes the system to operate under strong dilatation and creates breakage that allows the release of essential oil from the material [18–20]. Meanwhile, conventional heating, due to heat loss resulting from conduction, may suffer from extended heating times [18]. The advantages of MAHD may include reduced solvent usage, improved extraction yield, significant reduction of extraction time and energy consumption, lower operating costs than that of HD, eco-friendliness, and energy efficiency. However, one limitation of MAHD is that the boiling temperature of the extraction solvent is increased rapidly, so care should be taken to control the device to avoid explosion [11,18–24]. As a result, MAHD has been employed for the extraction of essential oils from laurel [25], thyme [24] and rosemary [25–27]. Interestingly, the essential oil extracted from wet citrus peel waste by MAHD yielded two additional compounds in comparison to the conventional method [28].

In this study, the recovery of essential oil from the leaves of *C. candicans* by MAHD was attempted and optimized with respect to three parameters including the ratio of water to raw material, extraction time, and microwave power. Optimization of the extraction process was realized using response surface methodology (RSM) with central composite design (CCD) being utilized for experimental design. Obtained essential oils were then characterized for volatile composition using gas

chromatography–mass spectrometry (GC–MS) and compared with essential oil composition obtained from conventional HD. Finally, the biological activity of *C. candicans* essential oil, obtained by MAHD and conventional HD, were analyzed and compared regarding the anti-proliferation capacity against some human cancer cells.

2. Materials and Methods

2.1. Material Preparation

Fresh leaves of Nang Nang (*C. candicans* (Burm. f.) Hochr.), approximately 10 cm in size were collected at an altitude of 1500 m above sea level in Dai Tu district, Thai Nguyen province, Vietnam. The raw materials were washed several times with water to remove impurities then stored in desiccant bags at <10 °C.

2.2. Extraction Process

The leaves were firstly cut into small pieces or ground depending on the experiment condition. The samples were weighed and then added to a 2 L round-bottom flask containing a suitable volume of water. The flask was connected to the Clevenger apparatus, and MAHD was performed in a domestic microwave oven until no more essential oil was released. Time was measured when the microwave was turned on (model ME71A, Samsung, Ho Chi Minh City, Vietnam; microwave power range of 100–800 W, oven capacity of 23 L). Multiple experiments with different extraction durations were performed. For each condition, experiments were repeated three times. Upon completion of each extraction attempt, the crude essential oil along with some condensation products was recovered, dehydrated with Na_2SO_4 (Sigma Aldrich, St. Louis, MO, USA), and stored at 4 °C until being analyzed.

2.3. Experiment Design for Response Surface Methodology Optimization

RSM combined with the Box–Wilson CCD were employed to investigate the effect of three conditional parameters (extraction time, the ratio of water to raw material, and microwave power) on the yield of extracted essential oil [29]. Fifteen experimental attempts were designed using orthogonal design matrix with Design-Expert 7.0 software (Stat-Ease, Minneapolis, MN, USA). Results of preliminary single-factor experiments were used as inputs in CCD to determine range and central points of variables (Tables 1 and 2). To be specific, in this investigation, three parameters were varied individually with other factors being kept fixed. To confirm model validity, analysis of variance (ANOVA) was performed. Lastly, after optimal parameters had been obtained from the model, an actual experiment was performed under those conditions to compare between predicted and actual yields. Mathematically, the modeled essential oil yield could be described as a function of technology parameters as follows (1):

$$\hat{Y} = b_0 + \sum_{j=1}^{k} b_j X_j + \sum_{u,j=1}^{k} b_{uj} X_u X_j + \sum_{j=1}^{k} b_{jj} X_j^2 \tag{1}$$

where $\hat{Y}$ and X are the predicted response and independent variable, respectively; b_0 is the intercept; b_j, b_{uj}, and b_{jj} are variable coefficients.

2.4. Gas Chromatography–Mass Spectrometry (GC-MS) Analysis

To perform GC-MS analysis, gas chromatography (Agilent Technologies HP7890A, Santa Clara, CA, USA) was employed in combination with a mass spectrum detector (MSD, Agilent Technologies HP5975C, USA) and a HP5-MS column (60 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, Santa Clara, CA, USA). Temperature of the injector was configured at 250 °C and the detector was set at 280 °C. Thermal profile of the column commenced at 60 °C, then was elevated to 240 °C at the rate

of 4 °C/min. Injection of essential samples was carried out via splitting with the split ratio of 100:1 and volume of 1 μL. Parameters for MSD included ionization voltage of 70 eV, emission current of 40 mA, and acquisition scan mass range of 35–450 am under full scan. Retention time indices (RI) of essential oil constituents were determined by comparing with a reference (a homologous *n*-alkane series). MSD response was used to infer relative content of constituents without correction.

Table 1. Ranges of parameters determined by experimental design.

Independent Variables	Codes	Variable Range (Δ)	Levels				
			$-\alpha$	-1	0	+1	$+\alpha$
Extraction time (min)	A	10	27.8	30	40	50	52.2
Water to material ratio (*v/w*)	B	1	3.8	4	5	6	6.2
Microwave power (W)	C	100	278	300	400	500	522

Coefficient $\alpha = 1.215$.

Table 2. Empirical data with corresponding actual and predicted response values.

Run	A	B	C	Y(g)	
				Actual	Predicted
1	−1	−1	−1	0.523 ± 0.05	0.47
2	+1	−1	−1	0.58 ± 0.04	0.62
3	−1	+1	−1	0.618 ± 0.07	0.64
4	+1	+1	−1	0.77 ± 0.06	0.80
5	−1	−1	+1	0.572 ± 0.03	0.60
6	+1	−1	+1	0.758 ± 0.07	0.76
7	−1	+1	+1	0.776 ± 0.05	0.78
8	+1	+1	+1	0.971 ± 0.06	0.95
9	−1.215	0	0	0.693 ± 0.02	0.71
10	+1.215	0	0	0.937 ± 0.03	0.91
11	0	−1.215	0	0.739 ± 0.05	0.74
12	0	+1.215	0	0.98 ± 0.08	0.96
13	0	0	−1.215	0.802 ± 0.06	0.78
14	0	0	+1.215	0.951 ± 0.07	0.95
15	0	0	0	0.949 ± 0.04	0.97

2.5. Cytotoxicity Assays

The cytotoxicity of the obtained essential oils was assayed against three established cell lines. The cell lines Hep-G2 (ATCC HB-8065) and PC-3 (ATCC CRL-1435) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell line A-549 (Item number: 300114) was purchased from Cell Lines Service GmbH (Eppelheim, Germany). Cell lines were maintained at bioassay laboratory, Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, Vietnam. Culture media included DMEM (Dulbecco's modified Eagle's medium), EMEM (Eagle's minimum essential medium, Sigma-Aldrich, St. Louis, MO, USA), and 10% heat-inactivated fetal bovine serum (FBS). The culture was performed in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to measure cell viability [30].

2.6. Antimicrobial Activity Assays

Two Gram-negative bacterial strains, including *Escherichia coli* M42 and *Pseudomonas aeruginosa* ATCC 25923, and two gram-positive strains, including *Bacillus subtilis* ATCC27212 and *Staphylococcus aureus* ATCC12222, acted as target bacteria for determination of antimicrobial activity. Minimum inhibitory concentrations (MICs) were derived by performing assay on 96-well microplates. Inoculum size of each bacterial strain was adjusted to approximately 10^5 colony-forming units (CFU) per milliliter by diluting growth media with microorganisms. Essential oils were mixed with 5% Dimethyl sulfoxide (DMSO) to form solutions with varying concentrations. Blank controls were prepared identically with essential oils being replaced by 5% DMSO [27]. Positive controls included penicillin, streptomycin, and nystatin.

3. Results and Discussion

3.1. Single Factor Analysis

On the basis of the results of preliminary experiments, four selected parameters were initially fixed, including material size at 0.2 cm, extraction time at 30 min, water to material ratio at 5/1 (*v/w*), and microwave power at 500 W. The factors that affected the essential oil yield are shown in Figure 1. Figure 1a reveals that the amount of essential oil obtained varied with the size of the materials used. The highest amount of essential oil extracted was 0.82 grams (g) when the material size was 0.2 cm. Importantly, when the material size increased to 0.5 cm and to 0.8 cm the amount of essential oil decreased to 0.71 g and 0.5 g, respectively. This could be explained by the higher number of broken essential oil-bearing cells, which was achieved by more thorough cutting and allowed water to diffuse into the oil sacs at a higher rate. The essential oil, with the assistance of microwave, was thus pushed out to the media more quickly, resulting in higher extraction yield. However, when the size of the material was too small (0.1 cm), the amount of essential oil recovered was reduced due to the expansion of the material, which hindered the distillation process, leading to a decrease in extraction yield. On the basis of these data, a material size of 0.2 cm was selected for subsequent experiments.

As shown in Figure 1b, the ratio of water to raw material seemed to be positively proportional to essential oil yield. To be specific, as the ratio was raised from 3:1 to 6:1, the oil increased from 0.63 to 0.94 g. This was because when the volume of water increased the material absorbed water more easily, allowing more compounds to dissolve into the solvent. The addition of water promoted essential oil diffusion into the water and in turn improved extraction yield. However, at the material ratio of 7:1, a slight reduction in extraction yield was observed, which could have possibly been due to the dissolution or emulsification of the essential oil that was caused by excess water. It was found that the range of ratio from 4:1 to 6:1 was the area that gave the maximum amount of essential oil. Therefore, the ratio at 5:1 was selected as the center of the study compass to develop and conduct an experimental design in the next part. At this rate, the amount of obtained essential oil approximated the maximum value.

Effect of extraction time on essential oil yield is demonstrated in Figure 1c, indicating that a longer extraction yield was associated with improved oil yield. The peak oil yield was achieved at 0.95 g, corresponding to the time of 40 min. However, if the extraction time was raised past 40 min, essential oil yield decreased to 0.93 and 0.88 g at 50 and 60 min, respectively. This could be explained that the denaturation of certain compounds in the oil was caused by prolonged contact with high temperatures. As for the effects of microwave power, Figure 1d showed that an increase in microwave power gave a better oil extraction yield, but only at a certain energy limit. It is known that elevated temperatures generated by the movement of the molecules induced by microwave energy can affect the oil yield. Although high temperatures can decrease the surface tension and the viscosity of water and allow heat to be transferred more rapidly into the material, certain heat-labile compounds in the essential oil can be degraded, undermining the extraction yield and quality [31]. In addition, heat elevation could also

increase production cost due to higher energy consumption. Therefore, the extraction time of 40 min and microwave power of 400 W were selected as optimal conditions for subsequent optimization.

In this report, we focused on optimizing the three main technological parameters of the essential oil extraction process, which greatly influence the process: water/material ratio (*v/w*), extraction time (minute), and microwave power (W).
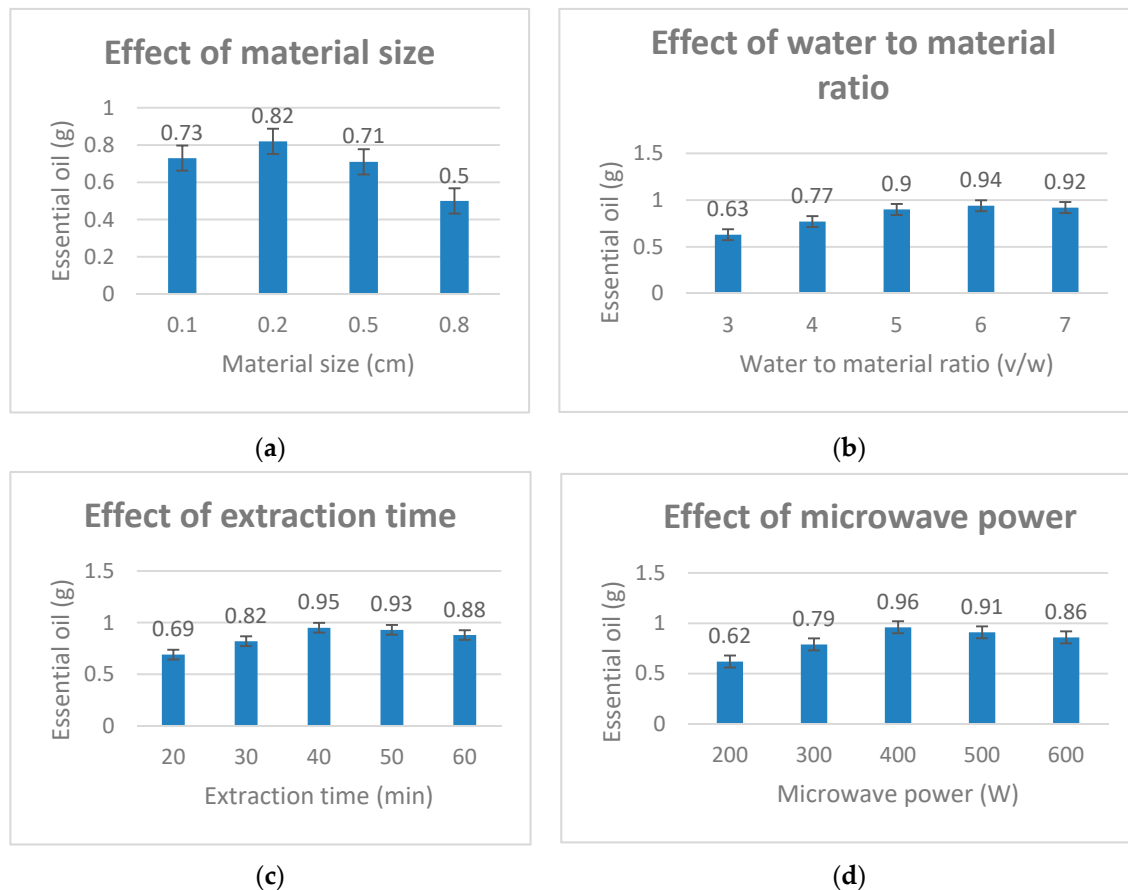
(**a**)

(**b**)

(**c**)

(**d**)

Figure 1. Single factor investigations showing effect of (**a**) size of the materials; (**b**) water-to-material ratio; (**c**) time of extraction; and (**d**) microwave power on the efficiency of essential oil extraction.

3.2. Predicted Model and Statistical Analysis

The response surfaces displayed the interaction results of the technological factors on the target function (the essential oil yield) using the MAHD process optimized by the central composite design. Extraction time (min), water to material ratio (*v/w*), and microwave power (W) were selected as independent variables. From the results of single investigation, we determined central values and ranges of the technology parameters (Table 1). The dependent variable was the essential oil yield (Y), which was determined by experiments as shown in the experimental design matrix in Table 2. After obtained optimal conditions, an actual experiment attempt was performed, and its yield was compared with predicted values to confirm model validity.

α was calculated by the following (2) mathematical formula [30]:

$$\alpha^4 + 2^k \alpha^2 - 2^{k-1}(k + 0.5\,n_0) = 0; k < 5 \tag{2}$$

with + k as the number of technological factors, and + n_0 as the number of experiments at the center of the design.

On the basis of the single factor tests, a total of 15 runs were generated and conducted. The results were then used to establish the quadratic model (Table 2). *F*-value, *p*-value, and R^2 values were then

used to evaluate model outcomes (Table 3). The ANOVA of the quadratic regression model indicated that the model was well fit and highly significant. The *F*-value of Y was 34 and the *p*-value was as low as 0.0006, indicating that the model was statistically significant (Table 3). The model coefficient of determination (R^2) was 0.9839, suggesting that most of yield variability could be explained by the experimental data (Figure 2). These data support good prediction capability of the established model in describing essential oil yield.

Table 3. ANOVA for the quadratic model.

| Source	Sum of Squares	Df	Mean Square	*F*-Value	*p*-Value	Remarks
Model	0.33	9	0.037	34	0.0006	significant
Extraction time (A)	0.07	1	0.07	64.97	0.0005	significant
Water-to-material ratio (B)	0.089	1	0.089	82	0.0003	significant
Microwave power (C)	0.052	1	0.052	48.49	0.0009	significant
AB	1.596×10^{-3}	1	1.596×10^{-3}	1.48	0.2788	not significant
AC	4.095×10^{-3}	1	4.095×10^{-3}	3.78	0.1093	not significant
BC	2.485×10^{-3}	1	2.485×10^{-3}	2.30	0.1901	not significant
A^2	0.057	1	0.057	52.83	0.0008	significant
B^2	0.031	1	0.031	28.67	0.0031	significant
C^2	0.023	1	0.023	21.38	0.0057	significant
Residual	5.410×10^{-3}	5	1.082×10^{-3}	-	-	-
Cor total	0.34	14	0.0027	-	-	-
SD	0.033	-	R^2	0.9839	-	-
Mean	0.77	-	**Adjusted R^2**	0.9550	-	-
C.V. %	4.25	-	**Predicted R^2**	0.8491	-	-
PRESS	0.051	-	**Adeq. Precision**	17.822	-	-

$p < 0.05$, significant.
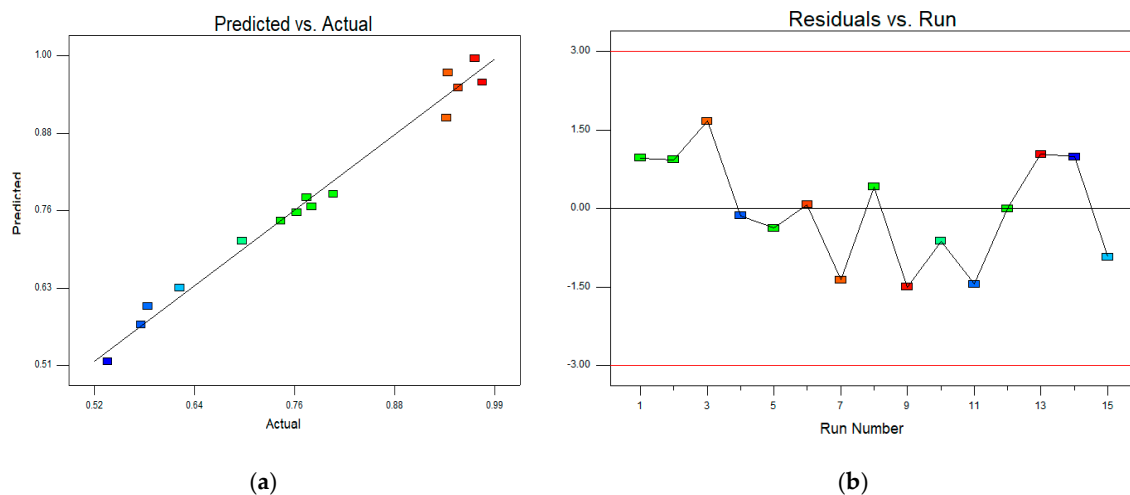
Figure 2. Plots depicting (**a**) actual responses versus predicted responses and (**b**) normalized residuals of experimental attempts.

ANOVA analysis results in Table 3 also show that the regression function Y (obtained oil content) depended on six interaction factors including three univariate interactions: A (extraction time), B (water to material ratio), C (microwave power), and three squared interactions: A2, B2, and C2. The objective function did not show the influence of double interactions. Figure 2 shows that the actual values and the predicted values according to the model were small differences and low model errors. This once again confirms the high compatibility of the model between experiment and theoretical calculation.

The regression equation for the objective function is described by the following mathematical equation (3):

$$Y = 0.97 + 0.08A + 0.09B + 0.069C - 0.11A^2 - 0.084B^2 - 0.073C^2 \tag{3}$$

Among the three univariate interaction effects A, B, C, the influence level of technological factors was ranked in the order of B > A > C, and with the three squared interaction effects, the influence level of technological factors decreased in the order of $A_2 > B_2 > C_2$.

3.3. Response Surface Analysis, Optimization, and Model Verification

The estimated quadratic model was then plotted in a three-dimensional space using Design Expert software. The *z*-axis of the plot was reserved for the predicted response (oil yield) and *x*- and *y*-axes represent two process parameters, resulting in three separate plots (Figure 3). In each plot, one parameter was fixed at the central value and other two variables were allowed to vary. Visually, all plots exhibited similar hill-shaped surfaces, which indicated the presence of a maximum response.
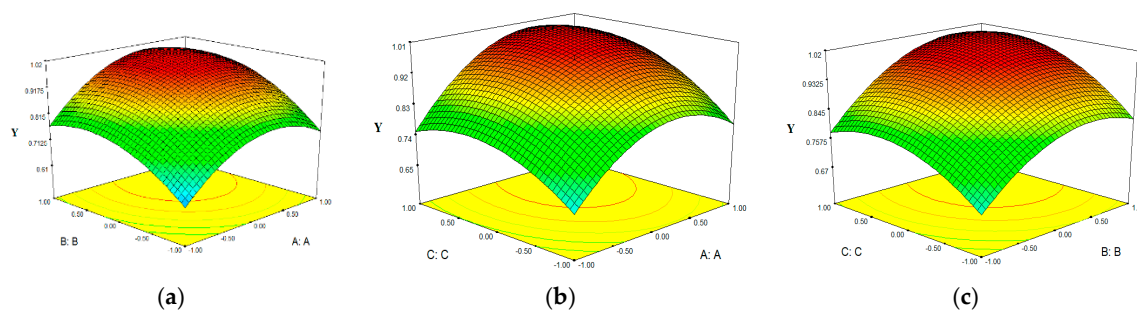

| (a) | (b) | (c) |

Figure 3. Surface plots illustrating interaction of parameters on oil yields including (**a**) effect of extraction time and water/material ratio; (**b**) effect of extraction time and microwave power; and (**c**) effect of water/material ratio and microwave power.

To determine the optimal values of the independent variables, the second-degree regressive equation was solved with respect to maximum total recovered essential oil. The importance level of the response (Y) was selected as 4. Predicted values showed that the essential oil yield attained the maximum when extraction time was 41.5 min, water/material ratio was 5.97/1 (*v/w*), and microwave power was 439 W (Table 4 and Figure 4). At optimal conditions, the predicted value and the experimental value of the amount of obtained essential oil were 1.0164 g and 1.02 ± 0.013 g, respectively. These two values were approximately identical, suggesting that the constructed model was highly compatible.

3.4. GC-MS Results

The volatile content of *C. candicans* essential oil was then analyzed by GC-MS, and the results are displayed in Table 5 and Figure 5. For comparison, when the essential oil was extracted using conventional HD, 47 compounds were obtained, accounting for 93.17% of the oil. The composition included 28 sesquiterpenes (69.84%), 12 oxidized derivatives of sesquiterpene (16.50%), 1 monosesquiterpene (0.24%), 1 oxidized derivative of monosesquiterpene (0.31%), 1 diterpenoid (0.55%), 1 benzenoid (0.13%), and 3 undetermined compounds detected at RI 1626, 1653, and 1672, accounting for 1.55%, 1.52%, and 2.53%, respectively. As shown in Table 5, a total of 46 compounds were detected in the *C. candicans* essential oil extracted by MAHD in this study, constituting 93.99% of the oil. The compounds belonged to three categories including 30 sesquiterpenes (72.35%), 15 oxidized derivatives of sesquiterpene (20.07%), and 1 diterpenoid (1.57%). Some major components of *C. candicans* essential oil extracted by MAHD were caryophyllene <b-> (10.45%), cadinene <d-> (10.28%), gurjunene <a-> (8.95%), muurolene <g-> (8.92%), selinene <a-> (7.06%), selinene <b-> (5.59%), and copaene <a-> (5.40%). In general, sesquiterpene compositions were similar between essential oils obtained by MAHD and HD. However, some quantitative differences were noticed. For example, in conventional

distillation, the gurjunene <a-> content was 21.31%, whereas the gurjunene <a-> content in MAHD was 8.95%. Another example was the caryophyllene <E-> content obtained in MAHD being 4.13% higher than that obtained in conventional HD. The MAHD method yielded fewer compounds with lower polarity, whereas more polar compounds were recovered at higher yield because the microwave mainly affects polarized compounds, rapidly increasing their temperature and allowing them to escape the cell and diffuse into the water more easily [32]. Furthermore, when extracted by MAHD, some compounds unobtainable with conventional HD were recovered, including muurola-3,5-diene <cis->, muurola-4(14),5-diene <cis->, amorphene <d->, calacorene <b->, guaiol (champacol), corocalen <a->, eudesmol <g->, and eudesmol <a->. These compounds were all either sesquiterpenes or oxidized derivatives of sesquiterpene, having great value in creating the distinct aroma of the essential oil [33].

Table 4. The values of the independent variables and real variables.

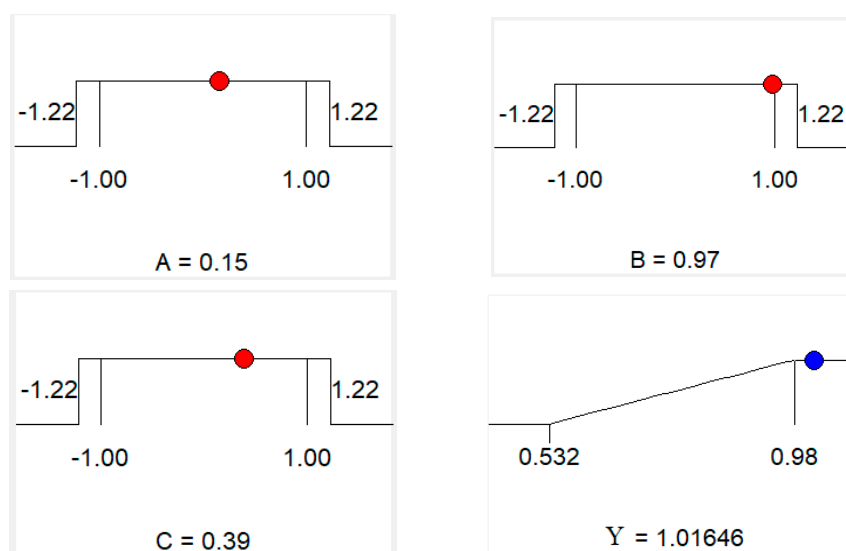| Independent Variables			Real Variables		
A	B	C	Extraction Time (min)	Water to Material Ratio (*v/w*)	Microwave Power (W)
0.15	0.97	0.39	41.5	5.97	439

Figure 4. Optimal conditions by solution of ramps.

Table 5. The chemical contents of *Callicarpa candicans* essential oil obtained by microwave-assisted hydro-distillation (MAHD). RI: retention time indices (RI); HD: hydro-distillation.

#	RI	Chemical Name	%	
			MAHD	HD
1	1348	Elemene <d->	0.18	0.27
2	1360	Cubebene <a->	0.33	0.40
3	1385	Ylangene <a->	0.62	0.70
4	1389, 1390	Copaene <a->	5.40	5.39
5	1400	Bourbonene <b->	0.15	0.17
6	1425, 1427	Gurjunene <a->	8.95	21.31
7	1437, 1438	Caryophyllene <E-> (caryophyllene <b->)	10.45	6.32

Table 5. *Cont*.

#	RI	Chemical Name	%	
			MAHD	**HD**
8	1445	Gurjunene <b-> (calarene)	1.55	0.94
9	1457	Aromadendrene	0.55	0.61
10	1466	Muurola-3,5-diene <cis->	0.24	-
11	1472	Humulene <a->	0.78	0.47
12	1476, 1488	Cadina-1(6),4-diene <trans->	0.15	0.62
13	1480	Muurola-4(14),5-diene <cis->	0.29	-
14	1491	Muurolene <g->	8.92	5.66
15	1494	Amorphene <a->	0.82	0.53
16	1498	Germacrene D	0.51	0.25
17	1505	Selinene <b->	5.59	4.23
18	1509, 1510	Amorphene <g->	1.36	1.25
19	1513, 1514	Selinene <a->	7.06	5.71
20	1518	Bisabolene <b->	0.48	0.56
21	1521	Amorphene <d->	0.43	-
22	1530, 1531	Cadinene <g->	3.15	2.17
23	1537, 1538	Cadinene <d->	10.28	7.70
24	1539	Calamenene <cis->	1.23	0.82
25	1548, 1549	Cadina-1,4-diene <trans->	0.56	0.46
26	1553	Cadinene <a->	0.61	0.46
27	1560, 1561	Calacorene <a->	0.82	0.80
28	1581	Calacorene <b->	0.19	-
29	1590	Ledol	0.70	1.30
30	1598	Spathulenol	0.44	0.43
31	1601	Axenol (gleenol)	0.33	0.21
32	1605	Caryophyllene oxide	1.56	1.25
33	1614	Guaiol (champacol)	0.22	-
34	1635	Cubenol <1,10-di-epi->	0.81	0.65
35	1639	Corocalen <a->	0.21	-
36	1647	Cubenol <1-epi>	2.09	2.51
37	1652	Eudesmol <g->	1.73	-
38	1660	Cadinol <epi-a-> (T-cadinol)	1.50	1.40
39	1661, 1662	Muurolol <epi-a-> (T-muurolol)	1.62	1.42
40	1665	Muurolol <a-> (cadinol <d->)	0.87	0.75
41	1673	Eudesmol <b->	1.50	-
42	1674, 1675	Cadinol <a->	3.03	4.17
43	1676	Eudesmol <a->	1.50	-
44	1678	Intermedeol <neo->	2.17	1.98
45	1695	Cadalene	0.49	0.50

Table 5. *Cont*.

#	RI	Chemical Name	%	
			MAHD	**HD**
46	2118, 2120	Phytol	1.57	0.55
47	984	Pinene <b->	-	0.24
48	1103	Linalool	-	0.31
49	1204	Methyl salicylate	-	0.13
50	1446	Bergamotene <a-trans->	-	0.94
51	1479	Caryophyllene <9-epi-(E)->	-	0.39
52	1541	Zonarene	-	0.21
53	1626	Unknown (109, 222, RI 1626)	-	1.55
54	1653	Unknown (161, 222, RI 1653)	-	1.52
55	1672	Unknown (162, 220, RI 1672)	-	2.53
56	1691	Caryophyllene <14-hydroxy-9-epi-(E)->	-	0.43
-	-	Total	93.99	93.17
-	-	Sesquiterpene	72.35	69.84
-	-	Oxidized derivatives of sesquiterpene	20.07	16.5
-	-	Diterpenoid	1.57	0.55
-	-	Monosesquiterpene	-	0.24
-	-	Oxidized derivative of monosesquiterpene	-	0.31
-	-	Benzenoid	-	0.13
-	-	Unknown	-	5.60
-	-	Total	93.99	93.17
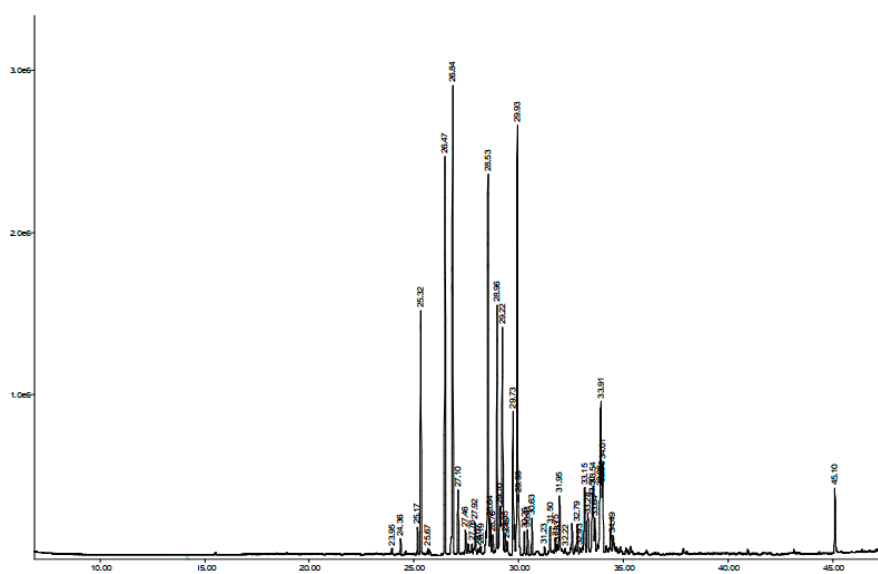
Figure 5. The chromatogram of *C. candicans* essential oil obtained by MAHD.

3.5. Biological Activity of C. candicans Essential Oil

The biological activity of *C. candicans* essential oil obtained by MAHD and HD were evaluated in terms of anti-proliferative activity on three cancer cell lines (HepG2, PC3, and A549), as well as anti-microbial activity on eight strains of fungi, yeast, and bacteria. The results showed that the dried *C. candicans* essential oil only exhibited mild activity on the Hep-G2 line (IC_{50} = 94.53 μg/mL). The essential oil extracted by conventional HD did not express any activity on all tested cell lines. In contrast, the *C. candicans* essential oil obtained by MAHD exhibited good activity on all three tested cell lines, with IC_{50} values ranging from 14.65 μg/mL (Hep-G2 line) to 56.21 μg/mL (A549 line) (Table 6). Similarly, MAHD-extracted essential oil also displayed better inhibitory activity on the fungi *Fusarium oxysporum* compared to conventional HD samples (Table 7). These data indicate that MAHD successfully recovered more important bioactive compounds in the *C. candicans* essential oil than by the HD method. In addition, the findings support that *C. candicans* essential oil obtained by MAHD has higher potential in a biological application.

Table 6. Anti-proliferation activity (IC_{50} values) of the *C. candicans* essential oil against three human cancer cell lines.

Essential Oil Sample	IC_{50} (μg/mL)		
	Hep-G2	PC3	A549
Dried *C. candicans* essential oil	94.53	>100	>100
C. candicans essential oil obtained from MAHD method	14.65	23.87	56.21
C. candicans essential oil obtained from traditional method	>100	>100	>100
Positive control (paclitaxel)	4.03 ng/mL	3.48 ng/mL	3.69 ng/mL

Table 7. Antimicrobial activity of *C. candicans* essential oil extracts.

Essential Oil Sample	MIC (µg/mL)							
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus subtillis*	*Staphylococcus aureus*	*Aspergillus niger*	*Fusarium oxysporum*	*Saccharomyces cerevisiae*	*Candida albicans*
Dried *C. candicans* essential oil	>200	>200	>200	>200	>200	>200	>200	>200
C. candicans essential oil obtained by HD	>200	>200	>200	>200	>200	200	>200	200
C. candicans essential oil obtained by MAHD	>200	>200	>200	>200	>200	100	>200	200

4. Conclusions

In this study, using RSM combined with CCD, we successfully optimized the extraction process of essential oil from the leaves of *C. candicans* with the three experimental parameters including extraction time (min), water to material ratio (*v/w*), and microwave power (W). The dependent variable studied was the essential oil yield recovered after extraction. Accordingly, the optimized conditions obtained were extraction time of 42 (min), water to material ratio of 6/1 (*v/w*), and microwave power of 440 (W). At the optimal conditions identified, the *C. candicans* essential oil yield was 1.02 ± 0.013 g. GC-MS results revealed that *C. candicans* essential oil contained large amounts of sesquiterpenes and sesquiterpene derivatives. Although the chemical profile and contents of essential oil obtained by MAHD were not significantly different from those obtained by traditional HD, only the oil extracted by MAHD exhibited the anti-proliferative activity on the tested cancer cell lines. The data demonstrate that the difference in MAHD-extracted essential oil is important and indispensable to the biological application of the oil.

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