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STUDY ON EXTRACTING OF *CAMELLIA RUBRIFLORA* OIL

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Abstract:

The objective of the study is to investigate the chemical composition and biological activity of the essential oil of *Camellia rubriflora* leaves in Sin Ho, Lai Chau. Using the steam distillation method to extract the essential oil. The chemical composition of the essential oil was determined by gas chromatography-mass spectrometry (GCMS). The antioxidant capacity of *Camellia rubriflora* leave essential oil was evaluated by using the DPPH free radical method. The results of the study determined that *Camellia rubriflora* essential oil has 20 components, including: esters with the highest proportion (39.62%), followed by flavonoids (34.86%), phenols (12.23%), acids (12.63%) and other compounds. *Camellia* sinensis leaf essential oil has antioxidant activity IC50 = 12.03 µg/ml. These research results provide the basis for further research and development of functional products and health care products from the chemical composition of this plant.

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TỐI ƯU HOÁ QUY TRÌNH CHIẾT XUẤT VÀ ĐÁNH GIÁ HOẠT TÍNH SINH HỌC CỦA TINH DẦU LÁ TRÀ HOA ĐỎ (*CAMELLIA RUBRIFLORA*)

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Tóm tắt

Mục tiêu của nghiên cứu nhằm khảo sát thành phần hóa học và hoạt tính sinh học của tinh dầu lá Trà hoa đỏ ở Sìn Hồ, Lai Châu. Sử dụng phương pháp chưng cất lôi cuốn hơi nước để trích ly tinh dầu. Thành phần hóa học của tinh dầu được xác định bằng phương pháp sắc ký khí khối phổ GCMS. Đánh giá khả năng kháng oxy hóa của tinh dầu lá Trà hoa đỏ bằng phương pháp sử dụng gốc tự do DPPH. Kết quả của nghiên cứu đã xác định được tinh dầu lá Trà hoa đỏ có 20 thành phần với thành phần bao gồm: este chiếm tỷ lệ cao nhất (39,62%), tiếp theo là flavonoid (34,86%), phenol (12,23%), axit (12,63%) và các hộp chất khác. Tinh dầu lá Trà hoa đỏ có hoạt tính kháng oxy hóa IC_{50} = 12,03 µg/ml. Những kết quả nghiên cứu này tạo cơ sở cho các nghiên cứu tiếp theo và phát triển các sản phẩm chức năng, sản phẩm chăm sóc sức khỏe từ thành phần hóa học của loại cây này.

1. Introduction

Vietnam has been identified as a center for the distribution of *Camellia* species. Among them, *Camellia* is a large and economically important genus within the *Theaceae* family. Out of the 55 *Camellia* species distributed in Vietnam, red-flower tea lines have attracted significant attention from scientists. *Camellia rubriflora*, also known as red-flower tea, is named after its red flowers. It was first discovered in Vietnam in 1998, at Tam Dao National Park, Vinh Phuc, by Ninh & Hakoda, and has gradually been cultivated as an ornamental plant. In some countries, red-flower tea is often used by locals for brewing tea due to its beneficial properties. Yellow-flower tea has many valuable uses, including timber production, planting as an under-canopy tree in forest belts, serving as a premium beverage, and being a valuable medicinal plant in medicine. The *Camellia* genus, including the red-flower tea, possesses valuable pharmacological properties such as antioxidant, anticancer, antibacterial, antifungal, and antiviral effects. Given these potential uses, the development and research on

red-flower tea are essential. However, there is still a lack of in-depth studies on this plant. Therefore, in this study, we will optimize the extraction process of essential oil from red-flower tea leaves in the Sin Ho, Lai Chau; concurrently, we will evaluate the biological activities of the obtained essential oil.

2. Literature Review

Vietnam is recognized as a distribution center for *Camellia* species. Among the 55 *Camellia* species distributed in Vietnam, red-flower tea lines have received considerable attention from scientists. According to the report by Le Nguyet Hai Ninh, red-flower tea species are scattered from the North to the Central Highlands of Vietnam. Four species have been identified in Vietnam: (1) Yodon red-flower tea (also known as Hong tea), discovered in Yodon National Park and named "*Camellia yodonnesiss*"; (2) Red-flower tea (*Camellia krempfii*), first reported in 1949. In Vietnam, *Camellia krempfii* is found in Khanh Hoa province near Lam Dong and Ninh Thuan; (3) Double-petaled red-flower tea (*Camellia* japonica L.), commonly known as Tra My (*Camellia japonica*), is very popular in Japan, and Tra My flowers have been imported into Vietnam; (4) Red-flower tea (*Camellia rubriflora*) in the northern mountainous region, first discovered in Tam Dao, Vinh Phuc, in 1998.

The red-flower tea (*Camellia rubriflora*) found in Vinh Phuc province is a 3-6 m tall woody plant with hairy young branches. The leaves are oblong or lance-shaped, 10-12.5 cm long, 4-5.5 cm wide, with a smooth upper surface and hairy underside along the main veins; the leaf base is wedge-shaped, and the tip pointed or sharply pointed; there are 10-11 pairs of lateral veins, and petioles are 1-1.2 cm long. The flowers are solitary at branch tips, red, with a 4-5 cm diameter; the pedicel is 0.3-0.5 cm long. Bracts and sepals are undifferentiated, numbering 9-10, without hairs. The petals are nearly round, obovate or broadly obovate, 2.5-4.5 cm long.

Currently, research on red-flower tea in northern mountainous areas is limited. Studies on red-flower tea in Vietnam have identified the presence of some important chemical components. According to Antia et al. (2022), using liquid chromatography-mass spectrometry to analyze the leaves, flowers, and seeds of red-flower tea (*Camellia japonica*) has revealed several biological compounds, such as phenolic acids, flavonoids, tannins, fatty acids, terpenoids, and other compound groups [6,7,8,10]. Notably, *C. japonica* contains quercetin-3-O-β-D-xylopyranosyl-(1 3)-O-α-L-rhamnopyranosyl-(1 6)-O-β-D-glucopyranoside, a flavonol glucoside with superior antioxidant capacity compared to common antioxidants like L-cysteine and L-ascorbic acid [9]. The biological compounds in the essential oil of red-flower tea (*Camellia japonica*) offer numerous benefits, such as antioxidant, antibacterial, anti-inflammatory, and anticancer properties.

3. Methods

3.1. Research Materials and Chemicals

Research Materials: Single-petaled northern mountainous red-flower tea (*Camellia rubriflora*) grown in Sin Ho, Lai Chau. The sample was identified by Assoc. Prof. Dr. Ha Duy Truong – Director of the Center for Training, Research on Crop and Livestock Breeding. Leaves were collected early morning, November 2023, from a six-year-old red-flower tea tree and preserved for analysis and study.

Chemicals: Food-grade ethanol, nHexane, Citric Acid, Acetic Acid, NaCl, Na2SO4, NaOH, chemicals used for extraction and analysis meet PA standards.

3.2. Experimental Design Methods

Experiment 1: Study the chemical composition of red-flower tea leaves. Use 2g of red-flower tea

leaves to determine moisture content and total ash. Use 5kg of red-flower tea leaves to analyze the total essential oil content.

Experiment 2: Study solvent-to-leaf ratio. Test solvent-to-material ratios of 1/5, 3/5, 5/5, 7/5, 9/5 (L/kg). The best formula will be selected for subsequent experiments.

Experiment 3: Study extraction time. Investigate time intervals: 30, 45, 60, 75, 90 minutes and select the best formula.

Experiment 4: Study soaking time for raw materials. Investigate soaking durations: 0, 6, 12, 18, 24 hours, with optimal conditions as previously determined.

Experiment 5: Assess antioxidant activity of the essential oil.

Experiment 6: Assess cytotoxicity of the essential oil.

Experiment 7: Evaluate antimicrobial activity of the essential oil.

3.3. Antioxidant assay

Investigation of the antioxidant capacity of *Camellia rubriflora* essential oils was tested using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) technique by Radical Scavenging Activity method (Goldschmidt, S., & Renn, K.,1922). DPPH is a free radical used to perform a screening reaction for the antioxidant activity of the studied substances. The antioxidant activity was demonstrated by reducing the color of DPPH free radicals, as determined by measuring the optical absorbance at 517 nm.

Dilute 0.1 mM DPPH solution in ethanol by dissolving 4 mg of DPPH with a sufficient amount of ethanol to dissolve DPPH. Then put in a volumetric flask and add enough ethanol to 100 ml, in a colored glass bottle. The extract of *Camellia rubriflora* essential oil with concentration of 8.68 µg / ml; 17.41 µg / ml; 26.14 µg / ml; 34.81 µg / ml; 43.41 µg / ml were used in this test. From each concentration, 1 ml was taken and reacted with 3 ml of DPPH. Samples were kept in the dark, at room temperature. After 30 minutes, measure the absorbance at 517 nm. The experiment was performed in 3 replicates.

The percentage of scavenged DPPH of extract was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{A_c - A_e}{A_c} \times 100$$

In there:

Ac: Absorbance of control reaction

Ae: Absorbance in presence of test or standard sample

The IC_{50} value of the sample, which is the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated from sample concentration and DPPH (%), using Excel software, make a regression equation of the form y = ax + b showing the correlation between DPPH (%) (y) and concentration (x). The lower absorbance of the reaction mixture indicated higher free radical activity.

3.4. Method for Evaluating Cytotoxic Activity

Procedure:

Use the MTT (3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay to evaluate the effects on the viability of cancer cells, including HepG2 liver cancer cells and MCF7 lung tumor cells. Cells are plated in 96-well plates (1.5 x 10^5 cells/ well), incubated with samples at concentrations ranging from 100 → 6.25 µg/ml for extracts or 50 → 1 µg/ml (µM) for purified compounds, each concentration repeated three times. Ellipticine or Paclitaxel (Taxol) in DMSO serves as a positive control (+). The formazan crystals are dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich), and absorbance is measured at λ = 540/720 nm on an Infinite F50 device (Tecan, Männedorf, Switzerland).

Result Calculation: Read results on an ELISA machine at 495-515nm. Calculate cell survival (CS) values based on test sample concentrations as a percentage compared to control.

$$S\% = \frac{\text{OD (mẫu)} - \text{OD (ngày 0)}}{\text{OD (DMSO)} - \text{OD (ngày 0)}} \; x\,100$$

The CS% value after calculating according to the above formula, is entered into Excel calculation to find the average % ± standard deviation of the test repeated 3 times according to Ducan's formula as follows: Standard deviation σ

$$\sigma = \sqrt{\left(\Sigma\left(xi - \bar{x}\right) \wedge 2\right) / \left(n-1\right)}$$

Samples with activity (CS < 50%) will be selected for further testing to find the IC50 value.

The IC50 value (50% Inhibitory Concentration) is the concentration of the sample at which 50% of the number of cells studied is inhibited. How to calculate the IC50 value:

Use the CS value of 10 concentration scales, based on the Table curve program according to the logarithmic value scale of the cell growth curve and the concentration of the test substance to calculate the IC50 value:

$$\frac{1}{y} = a + b \ln X$$

Where Y: concentration of test substance; X: CS value (%)

Test substances with IC50 <20 µg/ml (with crude extract, or with chemical fraction) or IC50 ≤ 4 µg/ml (with pure active substance) will be considered to have cytotoxic activity and the ability to inhibit the growth or kill cancer cells.

3.5. Microbial Resistance Survey

Procedure:

Bacterial Cultivation: Culture bacterial solution in LB liquid medium, shake at 37°C for 16-18 hours, dilute bacteria to a concentration of 10^8 cells/mL.

Prepare medium: Dissolve 42g Luria-Bertani agar with 1000 mL distilled water, sterilize at 121°C for 30 minutes, pour into plates, and allow to cool.

Spread bacteria on agar surface: Spread 100 µl bacterial solution onto the agar surface. Pierce the agar with a 8mm diameter tip or use antibiotic discs. Mark plates to avoid confusion. Drill three wells per plate; one with water (negative control), one with ampicillin (0.1g/100mL, positive control), and one with essential oil. Pipette 100 µl of diluted essential oil into the well.

Incubation: Incubate at 37°C for 12-24 hours, then observe and measure the inhibition zone diameter.

Antibacterial Zone Analysis

Measure inhibition zones to assess antibacterial activity:

Average diameter of antibacterial zone = D - d

Where D is the inhibition zone diameter, and d is the well diameter or antibiotic disc diameter.

4mm: strong activity (+++)

3-4mm: moderate activity (++)

< 3mm: weak activity (+)

0mm: no antibacterial activity (-)

3.6. Some physicochemical of Camellia rubriflora essential oils

Preliminary sensory examination of essential oils is based on the observation of exterior indications such as odor, taste, color, and transparency. This allows for a preliminary assessment of the essential oil's quality as well as the planned usage of the essential oil. Sensory assessment based on TCVN 8460: 2010.

3.7. Data satistical analysis methods

All of the tests were carried out in triplicate. The results are provided as means with standard

deviations from three separate studies. Analysis of variance (ANOVA) was used to find significant differences, which were then tested using the Duncan test at a $P < 0.05$ level. Data were analyzed by using SPSS Statistics software, version 20.0.

4. Results

4.1. Research Results on Solvent-to-Material Ratio Selection

Different solvent-to-material ratios affect the yield of essential oil obtained. Table 2 shows that the ratios of 3/1 and 9/1 (L/kg) yield the highest essential oil content. However, there is no statistically significant difference between these two formulas. In this study, a 3/1 (L/kg) solvent-to-material ratio was selected for subsequent experiments.

Table 1: The effect of water/raw material ratio on total essential oil content

No.	Water/ingredient ratio (L/kg)	Total essential oil content (%)
1	1/1	0.041[a]
2	3/1	0.064[c]
3	5/1	0.059[b]
4	7/1	0.062[bc]
5	9/1	0.063[c]

(Note: Letters in the same column indicate statistically significant differences at α = 0.05)

4.2. Research Results on Extraction Time Selection

Table 2 shows that the essential oil yield increases with longer distillation times, reaching the highest yield at 75 minutes (0.064) of distillation. However, after 90 minutes, the essential oil content slightly decreases. To save time and costs, experiments were stopped after 90 minutes. Thus, a distillation time of 75 minutes will be used in further experiments.

Table 2: Results of the effect of extraction time on total essential oil content

No.	Time (minutes)	Total essential oil content (%)
6	30	0.013[a]
7	45	0.025[b]
8	60	0.051[c]
9	75	0.064[e]
10	90	0.061[d]

(Note: Letters in the same column indicate statistically significant differences at α = 0.05)

4.3. Research Results on Soaking Time Selection

Before the distillation process of *Camellia rubriflora* leaf essential oil, the raw material was soaked for a specified period. To evaluate the effect on the yield of essential oil obtained in this study, soaking times of 0, 6, 12, 18, and 24 hours were used in the experiment. The results in Table 4 indicate that the essential oil yield was highest at 18 hours. Therefore, a soaking time of 18 hours was selected for the following experiments.

Table 3: Results of the effect of soaking time on total essential oil content

CT	Time (hours)	Total essential oil content (%)
11	0	0.057[a]
12	6	0.058[a]
13	12	0.064[b]
14	18	0.072[c]
15	24	0.063[b]

(Note: Letters in the same column indicate statistically significant differences at α = 0.05)

4.4. Optimization of the Extraction Process

Based on surveys of factors affecting distillation conditions, influential factors include soaking time, solvent-to-material ratio, and distillation time. The Box-Behnken experimental design with three variables and three levels was used for this study. Data were processed using Design-Expert 7.0 software (Stat-Ease, Inc, Minneapolis, USA), and ANOVA was used to evaluate the results.

Table 4: The Box-Behnken experimental matrix with three factors extraction time, solvent-to-material ratio, and extracttion time was applied to total essential oil yield.

No.	Factor			Essential oil content (%)
	A Soaking Time (hours)	B Water/ingredient ratio (l/kg)	C Extraction Time (minutes)	
1	12.00	1.00	75.00	0.017
2	2 .00	1.00	75.00	0.044
3	12.00	5.00	75.00	0.015
4	24.00	.00	75.00	0.054
5	12.00	3.00	60.00	0.01
6	24.00	3.00	60.00	0.015
7	12.00	3.00	90.00	0.014
8	24.00	3.00	90.00	0.065
9	18.00	1.00	60.00	0.007
10	18.00	5.00	60.00	0.025
11	18.00	1.00	90.00	.043
12	18.00	5.00	90.00	0.034
13	18.00	3.00	75.00	0.071
14	18.00	3.00	75.00	0.072
15	18.00	3.00	75.00	0.072
16	18.00	3.00	75.00	0.069
17	18.00	3.00	75.00	0.069

By applying regression analysis to the experimental data, a second-degree polynomial model representing essential oil content was obtained.

Y = + 0,071 + 0,015*A + 0,00215*B + 0,012 *C + 0,003*A*B + 0,012*A*C - 0,00675*B*C – 0,020*A2 – 0,018*B2 – 0,025*C2

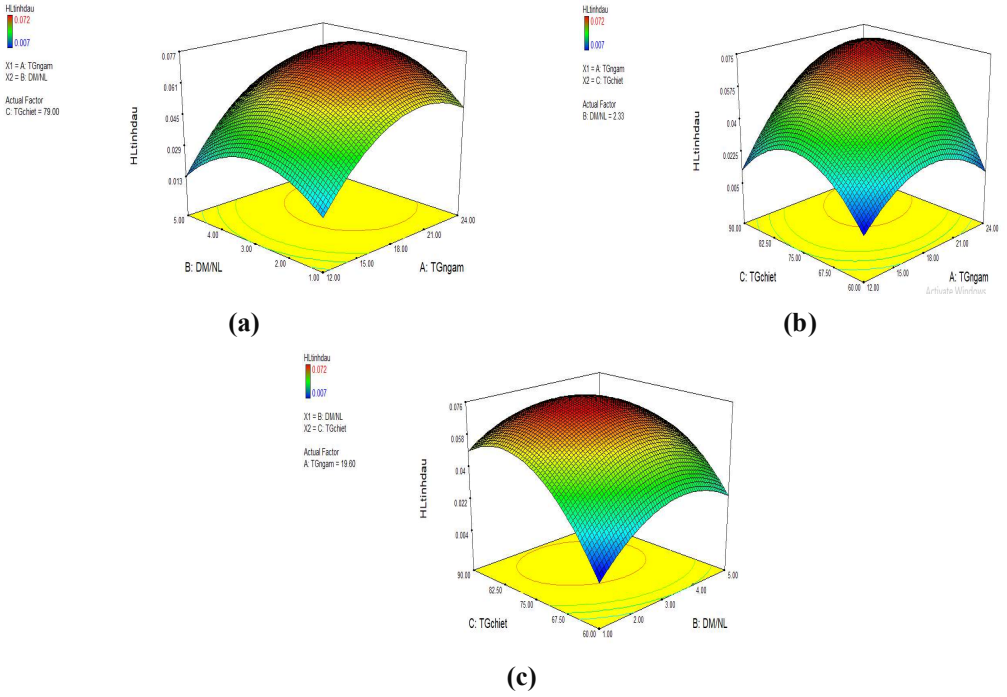
In which Y is the essential oil content obtained, the values A, B, C are the values of the factors of raw material soaking time, solvent/raw material ratio, and distillation time, respectively. ANOVA analysis is used to evaluate the model. The results of ANOVA analysis are shown below

Table 5: ANOVA Analysis of Essential Oil Extraction Model from *Camellia rubriflora* Leaves

Source	SS	DF	MS	F value	P value
Model	0.010	9	1.333E-003	248.18	< 0.0001
A	1.861E-003	1	1.861E-003	407.62	< 0.0001
B	3.613E-005	1	.613E-005	.91	0.0260
C	1.225E-003		.225E-003	268.42	<0.0001

Source	SS	DF	MS	F value	P value
AB	3.600E-005	1	.600E-005	7.89	0.0262
AC	5.290E-004	1	.290E-004	115.90	<0.0001
BC	1.823E-004	1	1.823E-004	39.93	0.0004
A^2	1.630E-003	1	.630E-003	357.10	< 0.0001
B^2	1.429E-003	1	1.429E-003	313.17	< 0.0001
C^2	2.616E-003	1	.616E-003	573.10	<0.0001
Residual	3.195E-005	7	4.564E-006		
Lack of Fit	2.275E-005	3	7.583E-006	3.30	0.1396
Pure Error	9.200E-006	4	2.300E-006		
Cor Total	0.0010	16			

Model significance and fit were tested through table analysis. Based on the ANOVA results, the model probability value (P-value < 0.0001 < 0.05) indicates that the model is suitable for explaining the results.



(a) **(b)**

(c)

Figure 1: Surface response to essential oil content
(a) Interaction model between soaking time and solvent-to-material ratio
(b) Interaction model between distillation time and soaking time
(c) Interaction model between distillation time and solvent-to-material ratio

Using the "expected function" method in Design-Expert 7 software, essential oil yield was optimized. A total of 43 solutions were found, with the best solution to maximize the target being: soaking time of 19.6 hours, solvent-to-material ratio of 2.33 (L/kg), and distillation time of 79 minutes. Under these conditions, the essential oil yield was calculated to reach 0.0731907%, showing high compatibility with experimental results. The findings are presented in Figure 2.

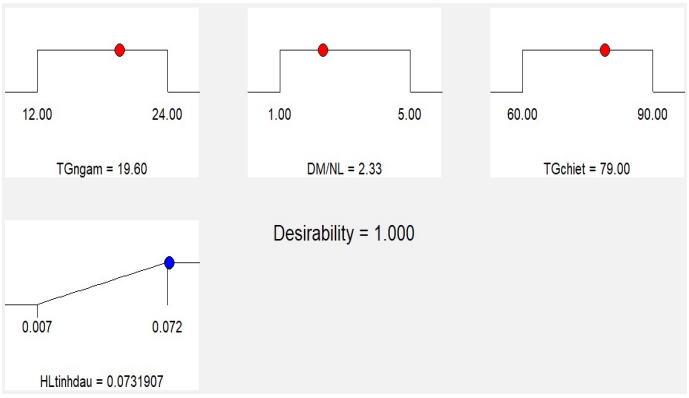


Figure 2: Expectation function and optimal conditions for essential oil content

4.5. ntioxidant assay

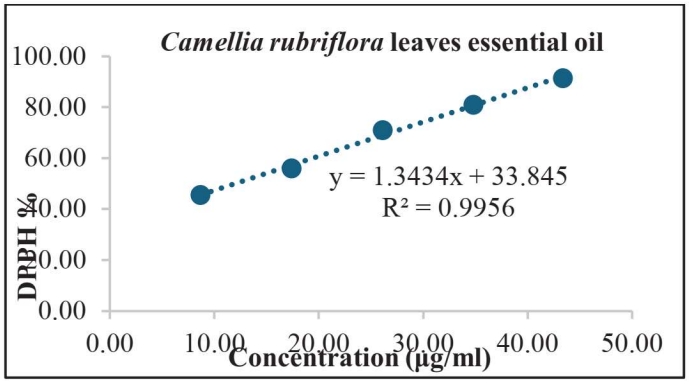


Figure 3: Correlation between free radical inhibitory activity and concentration of ascorbic acid

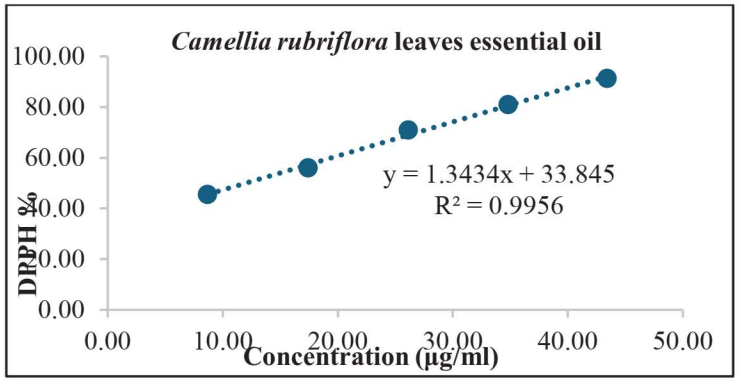


Figure 4: Correlation between free radical inhibitory activity and concentration of Camellia rubriflora

Construct an ascorbic acid standard curve based on the percentage of free inhibition and the concentration of ascorbic acid. From the equation in Figure 3, the IC50 value of ascorbic acid is deduced as: IC50 = 20.06 (µg/ml). From the equation in Figure 4, it is deduced that the essential oil of Camellia rubriflora leaves has an IC50 value of 12.03 µg/ml, which is 1.5 times lower than the IC50 value of ascorbic acid (20.06µg/ml). Thus, compared with ascorbic acid, the antioxidant activity of the essential oil is 1.5 times higher than that of ascorbic acid. Their values at different

concentrations (25-100 ppm) are higher than that of ascorbic acid (97%).

4.6. Microbial Resistance Survey

Table 6: Results of the survey on antibacterial activity of essential oils

TT	Microbiological testing	Diameter of sterile zone (A, mm)	
		ĐC	G
1	*Staphylococcus aureus* ATCC 25923	0	2 ±0,1
2	*Bacillus subtilis* VTCC-B-88	0	8 ±0,1
3	*Escherichia coli* VTCC-B-883	0	5 ± 0,1
4	*Pseudomonas aeruginosa* VTCC-B-481	0	0

The results showed that the essential oil has the ability to inhibit and resist bacteria such as Escherichia coli, Bacillus subtilis with the diameter of the antibacterial zone of the essential oil acting on each type of microorganism being different. In which, the antibacterial ability of the essential oil with Bacillus subtilis strain is stronger than Escherichia coli and Staphylococcus aureus with the results of sterile zone being 8 mm, 5 mm and 2 mm respectively.

4.7. Method for Evaluating Cytotoxic Activity

Table 7: Results of cytotoxic activity evaluation of essential oils

Sample	IC_{50} (µg/ml)	
	Hep-G2	MCF7
Camellia rubriflora essential oils	51.62	55.18

The results of evaluating the anti-cancer activity of the essential oil of red Camellia sinensis leaves in the northern mountainous region showed that the extracts had IC50 values > 20 µg/ml, so they did not show the ability to cause toxicity to liver cancer and breast cancer cell lines.

4.8. Result for determining some physicochemical of essential oils

The physicochemical characteristics of *Camellia rubriflora* essential oils are determined and presented in Table 8.

Table 8: Some physicochemical of *Camellia rubriflora* essential oils

Features	Result
Color	Light yellow
Odor	Specific smell of essential oil
Taste	Bitter, warm nature
Solubility	Insoluble in water, soluble in organic solvents such as methanol, diethyl ether, chloroform...
Density	0.917g g/ml

5. Conclusion and Discussion

The single-factor extraction conditions and optimal conditions for extracting essential oil from *Camellia rubriflora* leaves were studied, with an extraction time of 79 minutes; solvent/material ratio of 2.33/1 (1kg of withered material/2.33 liters of distilled water); Soaking for 19.6 hours, the predicted essential oil content was 0.0731907%.

The antioxidant capacity of the essential oil was determined with IC50 = 12.03 (µg /ml), proving that the essential oil has very good antioxidant capacity. *Camellia rubriflora* essential oil from the northern mountainous region has a light yellow color, a characteristic essential oil smell, and a bitter taste. The basic physical and chemical properties of the essential oil were determined.

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