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Optimization of Total Flavonoid Extraction From the *Helicteres hirsuta* Lour. Roots by Bath Ultrasound Assisted method and cytotoxic activities of these Flavonoids

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Helicteres hirsuta Lour.

Extract

Root

RMS

LC-MS/MS

Human leukemia cell line

ABSTRACT

This study was carried out to optimize the various approaches to analyze the effects of various variables on the total flavonoid content extraction from the roots of *Helicteres hirsuta* L. The existence of various compounds in the methanol fraction was accessed by using LC-MS/MS analysis. The results of the study identified the ideal parameters such as times (30 minutes); methanol solvent concentration (50%); ultrasonic frequency (12 Hz); and material/solvent ratio [1:30 (w/v)] for extracting the highest total flavonoids from the roots of *H. Hirsuta*. The study's results suggested that the total flavonoid value was 3.52684 (mg Catechin/g extract). The verified experiment obtained an actual value of 5.205 (mg Catechin/g extract). Further, the results of the study suggested the presence of 20 compounds of a flavonoid nature (66.667%) appearing in the purified methanol fractional extract. These compounds can inhibit DPPH free radicals at 50%, with an IC_{50} value of 536.760 g/mL, and they also have inhibitory activity on the growth of cancer cell lines with IC_{50} values ranging from 115.81 and 219.17g/mL. The human leukemia cell line (HL-60) exhibits the most significant cytotoxic response to a methanol extract from *H. hirsuta* root with an IC_{50} value of 115.81 g/mL.

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1 Introduction

Helicteres hirsuta Lour contains many bioactive compounds that have therapeutic effects on many diseases; hence it is widely used in various herbal and allopathic medicine. All parts of *H. hirsuta* are frequently used in treating people who often have aches and pains, back pain, insomnia, blue skin, and even those with a tired heart (Pham et al. 2016). Recently, different parts of *H. hirsuta* are also utilized in traditional medicine to treat liver cancer (Chin et al. 2006). Similarly, Didna et al. (2007) also established the cytotoxic activity of the *H. hirsuta* extracts.

Many studies have been conducted on the effects of various factors on extracting various active components from different parts of *H. hirsuta* (Pham et al. 2015, 2016, 2017; Jain et al. 2014). Chin et al. (2006) extracted the active components from *H. hirsuta* in Indonesia and obtained six lignans (Chin et al. 2006), while Quang et al. (2018) recovered 12 compounds from *H. hirsuta* collected from Binh Phuoc (Quang et al. 2018). Several significant pharmacological effects of *H. hirsuta* include Antioxidant (Loganayaki et al. 2013; Jain et al. 2014; Phạm et al. 2015, 2016; Hieu et al. 2019), analgesic (Yen et al. 2017), antibacterial, and cytotoxic activities against various *in vitro* cancer cell lines have been demonstrated so far (Pham et al. 2017, 2020; Duyen and Phuoc 2016).

In recent years, flavonoids have drawn much interest for their potential to protect against various chronic illnesses such as cardiovascular disease, neurodegenerative diseases, and many cancers (Vijayan and Tsou 2010; Noorjahan and Saranya 2018; Ahmed et al. 2016). A study on flavonoid extraction from different parts of *H. hirsuta* and its Antioxidant activity has been conducted by Jain et al. (2014) and Phạm et al. (2015, 2017), and reports show that the best solvent for the extraction of flavonoid molecules is methanol. The ideal extraction conditions were tested in Thua Thien Hue to extract the flavonoids in the roots of *H. Hirsuta*, to obtain the maximum flavonoid extract content as a foundation for testing their biological activity. This study was carried out to optimize based on the surface response method (RSM) by Design Expert 11 software to predict the optimization by the expected function method to select the values of the four-factor (solvent concentration, ultrasonic time, material/solvent ratio, and ultrasonic wave frequency) at which total flavonoid collection from the *H. hirsuta* roots leaves was highest.

2 Materials and methods

2.1 Materials plant

The root samples of *H. hirsuta* Lour. (*H. hirsuta* L.) were collected from the Linh Mu pagoda, Kim Long ward, Hue city, Thua Thien Hue province, washed under the water faucet and air-dried in the shade. To be employed in further experiments, the root powder was kept in Polyethylene bags, and stored at room temperature, avoiding light and moisture (Figure 1).

2.2 Experimental design

Total flavonoids were extracted from the roots of *H. Hirsuta* (d < 1 mm) by bath ultrasound-assisted method at 60^{o}C in a methanol solvent (pH = 5) at range 30, 50, and 70 (v/v), ultrasonic time of 30, 50, and 70 minutes with material/solvent ratio is 1:10, 1:20 and 1:30 g/mL (w/v), ultrasonic wave frequency is 10, 12, 14 Hz. The outcomes of the preceding experiment determine the conditions for the subsequent experiments. After surveying the single factors, all four factors that influenced the total flavonoid in *H. hirsuta* roots extract were used to evaluate the influence of these mutual influence of each pair of single factors. Using Design Expert software version 11, the RSM surface response approach was designed for the trials. A quadratic polynomial model displaying the total flavonoid was produced using the regression of analysis approach based on experimental data.

Figure 1 The root samples of *H. hirsuta* L. (A. Roots; B. Root powder)

2.3 Determination of total flavonoid content

The amount of the total flavonoid extracted from the *H. hirsuta* root was determined according to the description by Pham et al. (2017). Catechin standard flavonoid solution (sigma) was diluted to concentrations of 45, 90, 180, 360, and 720 g/mL using methanol 70%.

A series of test tubes containing 2 mL of distilled water and 0.15 mL of $NaNO_2$ was prepared, and then 0.15 mL of Catechin standard (concentrations 45, 90, 180, 360, and 720 µg/mL) was mixed into each test tube, shake well and allow to stand at room temperature for 6 min. Add 0.15 mL $AlCl_3$, shake well, and let stand for 6 min. This was followed by adding 2 mL NaOH and 0.7 mL of distilled water, shaking thoroughly, and letting stand for 15 min. The reaction solution was measured photometrically at 510 nm ($OD_{510\ nm}$) by UV-Vis (U2900 Hitachi, Japan). Each treatment was replicated three times. The results of the $OD_{510\ nm}$ value were recorded, and a calibration line was drawn using Excel 2010. The total flavonoid content from the root extract of *H. hirsuta* was calculated based on the Catechin linear regression equation utilizing the following formula:

$$M = \frac{V_1 * m_1 * n}{V_2 * m_2 * 1000} \left(^{mg}/_{g}\right) \quad (1)$$

Here: V_1: initial extract volume, m_1: total flavonoid content calculated based on Catechin standard curve (µg/mL), n: number of dilutions, V_2: sample volume used for reaction (mL), m_2: initial sample mass (g), 1000: conversion factor from mg to µg.

2.4 Determination of Antioxidant activity

The Antioxidant activity of the methanol extract of *H. Hirsuta* roots was performed based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method described by Long et al. (2020). The methanol extract was initially diluted into ratios of 1; 0.5; 0.25; 0.125; 0.0625, and 0.03125 mL; control Ascorbic acid solution into concentrations 0.05; 0.67; 0.10; 0.20; 0.50 and 1 mg/mL, and 0.2 mM DPPH solution mixed in 70% ethanol used for the reaction. Free radical scavenging of DPPH was estimated by adding 1 mL of the test sample and 1 mL DPPH (0.2 mM) into each test tube at each dilution, mixing well and allowing it to stand for 30 min in the dark, and then observations were taken at 517nm. For the control, ascorbic acid, similar steps were performed. The formula calculates the result of free radical scavenging of DPPH:

$$\%SC = \frac{OD_c - OD_m}{OD_c} \text{ x } 100 \quad (2)$$

Here: ODm: Valuesoptical density of the test sample; ODc: Valuesblank optical density

standard curve was constructed based on percent inhibition of free radical DPPH obtained at different concentrations. The IC_{50} value was calculated (at their roots extract or Ascorbic acid inhibited 50% free radical DPPH).

2.5 *In vitro* cancer cell line culture

The cytotoxic activity of isolated flavenoids was estimated against the selected cancer cell lines at the Institute of Biotechnology, Vietnam Academy of Science and Technology. The cancer cell lines were grown in monolayers in DMEM medium (Dulbecco's Modified Eagle Medium) containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate adding 10% fetal bovine serum-FBS (Gibco, Invitrogen). After 3-5 days, cancer cells were transplanted on the same media grown in a 1:3 ratio and kept in CO_2 incubators at 37°C and 5% CO_2.

2.6 Cytotoxic assay

2.6.1 Cytotoxic assay for monolayer cultured cancer cell lines

The experiment was conducted to ascertain the total cellular protein content based on the value OD (Optical Density) obtained with stained Sulforhodamine B (SRB, Sigma-Aldrich, USA). The amount of SRB bound to each protein molecule determines the observed OD value, and more cells (and consequently, protein) have a higher OD value. This approach is used by Monks et al. (1991). The method performs as described by Long et al. (2020).

2.6.2 Cytotoxicity assay to suspension cancer cell line (HL-60)

Tim Mosmann's histology method (1983) examined the suspension cell line (HL-60) cytotoxicity *in vitro* (Mosmann 1983). Tetrazolium salt was utilized as a reagent in a colorimetric assay to assess the development of cell survival and detection abilities. The active cell's mitochondria are firmly attached to the reagent's tetrazolium ring. The yellow color of MTT changed to formazan purple under the influence of the dehydrogenase enzyme in cells. The estimations method was performed as described by Long et al. (2020). The % inhibiting cell growth would be determined through the following formula:

$$\%\ \text{Alive cells} = \frac{[OD_{\text{reagent}} - OD_{\text{day 0}}]}{[OD_{\text{negativecontrol}} - OD_{\text{day 0}}]} \text{ x } 100 \quad (3)$$

$$\%\ \text{inhibited cells} = 100 - \%\ \text{alive cells} \quad (4)$$

2.7 Chromatographic and Mass Spectrometry Conditions

The methanol extract (1 µg) was diluted in 500 µL of 70% methanol and 500 µL of formic acid, vortex-mixed, and centrifuged at 14000 rpm/10 min. The supernatant (10 µL) was injected into the LC-MS/MS system for analysis.

Table 1 The levels of experimental design are based on factors

Factor	Variable	Lower level	Base level	Upper level	Interval
A: Time	X_1	30	50	70	20
B: Solventconcentration	X_2	30	50	70	20
C: Ultrasound frequency	X_3	10	12	14	2
D: Ratio of raw material:solvent	X_4	1:10	1:20	1:30	10

The LC-MS/MS assays of the methanol extract were performed for compound identification on the machine Exion LCTM-X500R QTOF (Sciex, USA) with an electrospray ionization (ESI) source. The implementation process was on a Hypersil GOLD Dim. 150x2.1, 3µ (Thermo Scientific, USA) column, at the 30°C column temperature. The mobile phase in chromatography consisted of 0.1% formic acid in water (A), and 0.1% formic acid in acetonitrile (B) was used in the following gradient elution method: 1 min: 98% (A): 2% (B); 20 min: 2% (A): 98% (B) và 25 min: 2% (A): 98% (B). The flow rate was 0.4 mL/min, and using the supernatant 2 µL was injected into the column.

The mass spectrometry was carried out in negative ionization multiple-reaction monitoring (MRM) mode. The source parameters were as follows: the capillary voltage is -4500 V, TOFMS with TOF start mass, and TOF stop mass is 100 and 2000 (Da), respectively, while that for TOFMS/MS is 50 and 2000 (Da). The Collision Gas (CAD) pressure was 7 psi. The mass spectrometer data of compounds were searched for comparison on the NIST2017 spectrum library of the American Academy of Science and Technology (https://chemdata.nist.gov/).

2.8 Statistical analysis

The experimental values are expressed as the mean of the measurements over the three experimental replications plus the standard deviation. The mean was compared using Duncan's test and the ANOVA analysis of variance. The analytical values were statistically significant, $p < 0.05$, based on Excel 2010 and IBM SPSS Statistics 20.

3 Results and discussion

3.1 Optimization of suitable conditions for total flavonoid extraction

Following research on the impact of each univariate element on total flavonoid extraction, optimization of these factors' reciprocal influence in the extraction process is necessary. This work forecasts the optimization issue by the "expected function" technique using the RSM surface method to choose the best values of four influencing parameters at maximum total flavonoid content. The regression analysis is based on experimental data collection of a quadratic polynomial model displaying the total flavonoid produced. The collected data were assessed using ANOVA.

The experiment was designed to predict the maximum total flavonoid content. The four single elements studied in this study are time, solvent concentration, material/solvent ratio, and specific ultrasonic frequency from X1 to X4 were employed as the four variables. The upper and lower-level values of every single factor are presented in table 1. The surface approach has 27 conditional trials designed, including 3 in the center. Table 2 displays each experiment's total flavonoid content findings (Table 2).

Table 2 shows that the flavonoids found in *H. Hirsuta* roots extract ranged from 2.426 to 5.768 mg (mg Catechin/g extract). Tables 3 and 4 demonstrate that the model's constituent parts exhibit a high significance level; most second-order values have $p < 0.05$. The above notation indicates that four quadratic values (X_1^2, X_2^2, X_3^2, and X_4^2) participating in all models show a high confidence level of over 95% and that the linear factors (X_1-X_4) and interactive factors (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4) do not participate in the regression equation because $p > 0.05$ (Tables 3 and 4).

The regression equation derived represents the relationship between total flavonoid with independent variables based on the Box-Behnken model as follows:

$$Y = 5.41 - 1.31\ X_1^2 - 0.8286\ X_2^2 - 0.9172\ X_3^2 - 0.6657 X_4^2 \quad (1)$$

Here: Y: Total flavonoid content (mg Catechin/g extract)

The setup regression equation has a highly significant statistical difference, as demonstrated by the Fisher F test model value (F = 3.6100) and the low probability p-value (p = 0.0158), as represented in Table 3.

Alternatively, the correlation coefficient R^2 is used to evaluate how well the model fits. According to the model analysis results in table 3, the model's R2 value collection is 80.830%; R2 - (adj) = 58.460%; and all p values exhibit high statistically significant differences. An acceptable model is required when R^2 is at least 80% (Xiao and Yao 2008). It is ideal for investigating the agreement between the actual data and the theory to produce

Table 2 Experimental design for the matrix of four factors affecting

No	Expression X_1 Time (min)	X_2 Solvent concentration (%)	X_3 Ultrasound frequency (Hz)	X_4 Ratio of raw material:solvent (w/v)	Y Total flavonotid content (mg Catechin/g extract)
1	50	50	10	1:10	3.269±0.008
2	30	50	10	1:20	3.812±0.199
3	50	30	10	1:20	3.116±0.003
4	70	50	12	1:10	4.140±0.123
5	50	50	14	1:30	3.821±0.125
6	70	50	10	1:20	4.946±0.015
7	30	50	12	1:10	3.991±0.544
8	50	50	10	1:30	3.808±0.005
9	50	50	12	1:20	2.877±0.009
10	70	70	12	1:20	3.171±0.021
11	70	50	14	1:20	3.294±0.007
12	50	30	14	1:20	3.416±0.004
13	50	50	14	1:10	3.442±0.020
14	30	70	12	1:20	3.092±0.055
15	50	50	12	1:20	3.283±0.199
16	30	30	12	1:20	3.866±0.800
17	50	50	12	1:20	3.439±0.001
18	70	30	12	1:20	2.426±0.289
19	50	70	14	1:20	3.191±0.444
20	50	30	12	1:10	3.443±0.003
21	30	50	14	1:20	2.855±0.286
22	70	50	12	1:30	3.822±0.015
23	50	70	12	1:10	5.048±0.024
24	50	30	12	1:30	3.712±0.007
25	30	50	12	1:30	5.301±0.015
26	50	70	12	1:30	5.176±0.007
27	50	70	10	1:20	5.768±0.145

results that are not statistically significant (based on the Lack of Fit test). There is no statistical significance, as evidenced by the data in table 3, where F (3.2900) and p (0.2556) values are both > 0.05. Therefore, the model created based on the variables chosen for our experiment is appropriate and exhibits excellent between the experimental and anticipated values (Table 3).

The regression equation can hypothetically predict the total flavonoid value collection from the *H. hirsuta* root. Figure 2 depicts the reciprocal effect between time, solvent concentration, material/solvent ratio, and ultrasonic wave frequency using the ultrasonic tank method and the total flavonoid of the *H. hirsuta* roots extract. Previous studies show that the total flavonoid extraction efficiency rose along with the extraction duration, ultrasonic frequency, and raw material/solvent ratio. To a certain extent, though, if we keep boosting these elements, the extraction efficiency tends to drop (Figure 2).

Table 3 Analysis of variance (ANOVA) of the regression equation

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P Value	
Model	14.3200	1	1.0200	3.6100	0.0158	significant
X_1	0.1245	1	0.1245	0.4399	0.5197	
X_2	0.0001	1	0.0001	0.0005	0.9833	
X_3	0.4506	1	0.4506	1.59	0.2311	
X_4	0.2633	1	0.2633	0.9299	0.3539	
X_1X_2	0.0580	1	0.0580	0.2048	0.6590	
X_1X_3	0.3997	1	0.3997	1.4100	0.2578	
X_1X_4	0.0074	1	0.0074	0.0262	0.8740	
X_2X_3	0.2177	1	0.2177	0.7690	0.3977	
X_2X_4	1.3300	1	1.3300	4.6800	0.0513	
X_3X_4	0.4282	1	0.4282	1.5100	0.2423	
X_1^2	9.1800	1	9.1800	32.4100	0.0001	
X_2^2	3.6600	1	3.6600	12.9300	0.0037	
X_3^2	4.4900	1	4.4900	15.8500	0.0018	
X_4^2	2.3600	12	2.3600	8.3500	0.0136	
Residual	3.4000	1	0.2831			
Lack-of-Fit	3.2000	10	0.3203	3.2900	0.2556	not significant
Pure Error	0.1947	2	0.0974			
Total	17.7200	26				

R - Sq =80.830%; R^2 - (adj) = 58.460%

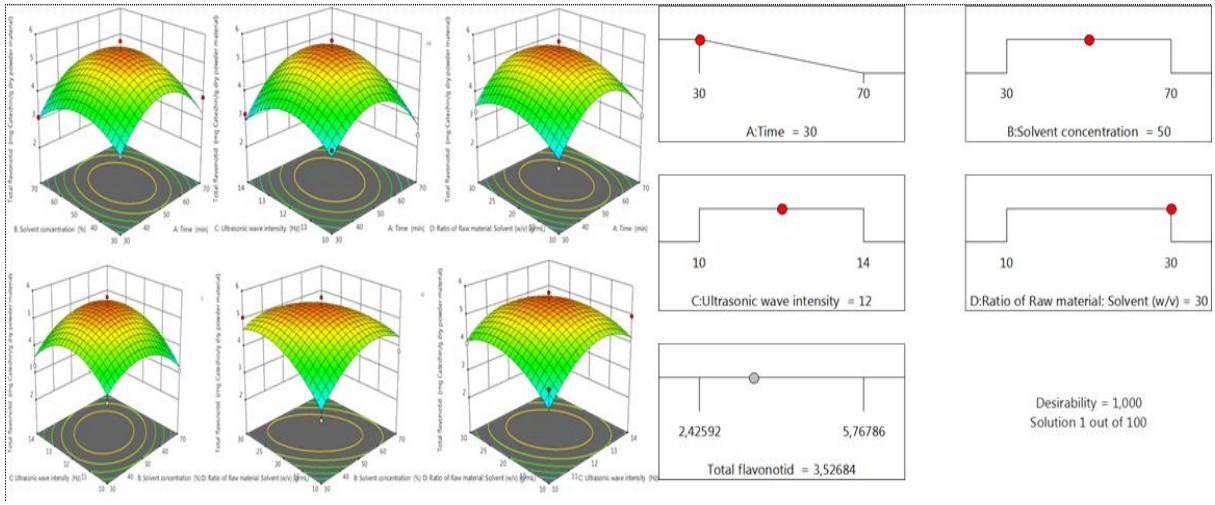

Figure 2 Expected function, response surfaces in 3D, and ranges of conditional values for optimal total flavonoid content

We needed to conduct experiments with conditions like time ultrasonic (30 minutes), methanol solvent concentration (50%), ultrasonic frequency (12 Hz), and raw material/solvent ratio (1:30 (w/v)) to obtain the total flavonoid content from the root of *H. hirsuta* under the condition as predicted by theoretical calculations. As a result, the total flavonoid content collection is 3.52684 (mg Catchin/g extract) (Figure 2). Meanwhile, the experiment's total flavonoid content under optimal conditions was 5.205 (mg Catechin/g extract), more significant than the model's theoretical calculation (Table 5). Research by Pham et al. (2017), the results indicated that the sample/solvent ratio had the most substantial impact on bioactive compounds and the Antioxidant power of *H. Hirsuta* with the optimum extraction conditions, including a temperature of 60°C, time of 35 min, the ratio of 1:100 g/mL

Table 4 Significance levels of regression coefficients

Factor	Coef	SE Coef	95% CI Low	95% CI High
β_o	5.4100	0.3072	4.7500	6.0800
X_1	0.1019	0.1536	-0.2328	0.4365
X_2	0.0033	0.1536	-0.3314	0.3379
X_3	0.1938	0.1536	-0.1409	0.5284
X_4	0.1481	0.1536	-0.1865	0.4828
X_1X_2	0.1204	0.2660	-0.4593	0.7000
X_1X_3	0.3161	0.2660	-0.2636	0.8958
X_1X_4	-0.0431	0.2660	-0.6227	0.5366
X_2X_3	0.2333	0.2660	-0.3464	0.8130
X_2X_4	-0.5758	0.2660	-1.1600	0.0038
X_3X_4	-0.3272	0.2660	-0.9069	0.2525
X_1^2	-1.3100	0.2304	-1.8100	-0.8096
X_2^2	-0.8286	0.2304	-1.3300	-0.3266
X_3^2	-0.9172	0.2304	-1.4200	-0.4152
X_4^2	-0.6657	0.2304	-1.1700	-0.1637

(sample/solvent) in 40% (v/v) methanol solvent. The highest total phenolic and flavonoid levels were 16.87 mg GAE/g and 17.55 mg CE/g, respectively (Pham et al. 2017). These results obtained two times higher flavonoids than our study when extracting on roots samples of *H. Hirsuta* collection Thua Thien Hue based on bath ultrasound-assisted method.

3.2 Assessment of the existing compounds in the methanol fraction

The results of LC-MS/MS analysis identified negative ions $[M+H]^-$ with an m/z value corresponding to each compound was recorded by high-resolution mass spectrometry analysis, and a negative ion measurement mode was obtained by LC-MS/MS analysis of ion fragmentation in negative ion measurement mode obtained the primary m/z fragmentation as presented in Table 5. The retention periods for the compounds ranged from 5.45 to 18.62 minutes at the detection wavelength of 350 nm (λ= 350 nm). The results of comparing the LC-MS/MS spectra of the compounds collected in the methanol fraction from roots of *H. hirsuta* with the standard LC-MS/MS data spectrum on the NIST/PubChem data spectrum bank with the identified natural active substances. Results of the study showed the presence of thirty compounds with similarity ranging from 95 to 100%, of which 20 compounds were of a flavonoid nature (66.667%), 7 compounds were of a nature phenolic (23.333%), 1 polyphenol compound (3.333%) and 2 alkaloids (6.667%) (Figure 3 and Table 5).

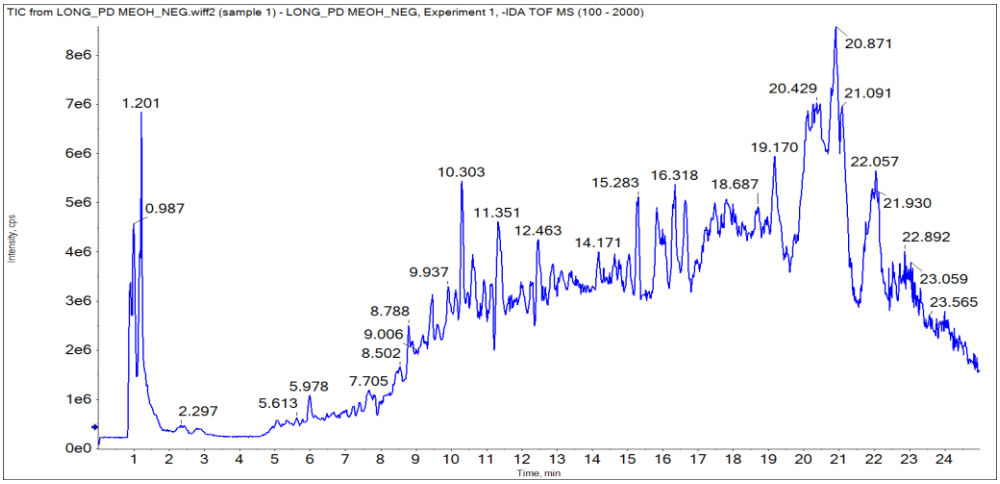

Figure 3 GC-MS spectral chromatogram analysis of segment extract methanol from the root of H. *hirsuta*

Table 5 The results analysis LC-MS/MS

No.	Retention Time	Precursor Mass [M-H]-	Library NIST/PubChem	Molecular formula	MS/MS Spectrum	Library Score
1	5.45	325.11	4'-Acetoxy-7-hydroxy-6-methoxyisoflavone*	$C_{18}H_{14}O_6$	124.0059; 195.0434; 223.0385	100
2	9.92	447.09	Luteolin 7-glucoside*	$C_{21}H_{20}O_{11}$	151.0038; 174.9570; 227.0336; 229.0477; 255.0274; 256.0350; 284.0308; 285.0386; 300.0236; 327.0463; 405.2091	100
3	9.96	285.04	Fisetin*	$C_{15}H_{10}O_6$	163.0033; 258.0412; 285.0407; 286.0445; 287.0469	97.80
4	10.06	431.10	Apigenin 7-glucoside*	$C_{21}H_{20}O_{10}$	241.1427; 268.0355; 269.0437; 311.0521	100
5	10.15	301.03	Quercetine*	$C_{15}H_{10}O_7$	121.029; 151.002; 107.011; 93.033; 139.039	98.40
6	10.36	187.10	Azelaic acid*	$C_9H_{16}O_4$	57.0339; 80.0251; 95.0495; 97.0649; 123.0806; 125.0961; 169.0837	99.60
67	10.59	461.07	Scutellarin*	$C_{21}H_{18}O_{12}$	59.0134; 85.0269; 99.0090; 113.0231; 213.0529; 241.0487; 283.0225; 284.0317; 285.0388	99.20
8	10.60	285.04	6,7,3',4'-Tetrahydroxyflavone*	$C_{15}H_{10}O_6$	183.0109; 197.0273	97.10
9	10.95	161.02	4-Hydroxycoumarin*	$C_9H_6O_3$	163.0375; 121.0275; 164.0414; 122.0315; 119.0481	95.10
10	11.16	593.13	Poncirin*	$C_{28}H_{34}O_{14}$	85.0292; 153.0197; 161.0620; 195.0300; 287.0923	100
11	11.74	315.05	6-Methoxyluteolin*	$C_{16}H_{12}O_7$	136.9884; 227.035; 228.0414; 243.0292; 300.0269;	97.30
12	12.87	299.05	Hispidulin*	$C_{16}H_{12}O_6$	79.9558; 183.0109; 239.0728	98.60
13	12.19	285.04	16.alpha.-Hydroxyestrone*	$C_{18}H_{22}O_3$	93.0341; 107.0132; 143.0486; 154.0403; 159.0446; 163.0013; 171.0430; 173.0631; 211.0387; 214.0267; 229.0492; 239.0329; 243.0277; 255.0276	98.10
14	13.57	285.05	Kaempferol*	$C_{15}H_{10}O_6$	93.0341; 107.0132; 143.0486	99.60
15	14.80	595.28	Neoeriocitrin*	$C_{27}H_{32}O_{15}$	151.0036; 135.0453; 459.115; 287.0554; 152.0076	93.70
16	16.57	297.15	Ricinoleic acid*	$C_{17}H_{14}O_5$	119,0496; 155,1060; 183,0106; 184,0186; 297,2312	97.90
17	17.32	593.27	Vitexin 4-O-glucoside*	$C_{27}H_{30}O_{15}$	78,9583; 152,9946; 241,0100; 277,2152; 315,0459; 413,2071	76.50
18	17.84	577.26	Apigenin 7-O-neohesperidoside*	$C_{27}H_{30}O_{14}$	63,9615; 71,0128; 80,9642; 85,0285; 94,9798; 101,02344	95.20
19	18.62	297.15	6,4'-Dimethoxy-7- hydroxyisoflavone*	$C_{17}H_{14}O_5$	79.9553; 119.0487; 155.9855; 170.0031; 183.0104	99.70
20	10.95	161.02	6-Hydroxycoumarin*	$C_9H_6O_3$	133.0274; 143.8884	98.00
21	6.15	153.02	3,4-Dihydroxybenzoic acid**	$C_7H_6O_4$	65.0027; 81.0335; 91.0182; 108.0206	98.50
22	7.01	181.05	p-Hydroxyphenyllactic acid**	$C_9H_{10}O_4$	72.9920; 107.0494; 119.0487; 135.0436; 134.0363; 163.0389	95.00
23	7.31	137.02	3,4-Dihydroxybenzaldehyde**	$C_7H_6O_3$	65.0027; 81.0337; 92.0259; 93.0339; 108.0207; 109.2085	99.60
24	8.00	163.04	trans-2-Hydroxycinnamic acid**	$C_9H_8O_3$	119.0501; 162.8392	100
25	10.32	359.07	(R)-rosmarinic acid**	$C_{18}H_{16}O_8$	72.9923; 96.9599; 135.0438; 179.0339; 197.0439	98.00
26	10.95	181.05	3,4-Dihydroxyhydrocinnamic acid**	$C_9H_{10}O_4$	93.0339; 121.0281; 122.0359; 123.0433; 136.9204	100
27	15.97	194.14	2,4,6-Trichlorophenol**	$C_6H_3Cl_3O$	194.9178; 158.9412	100
28	10.31	179.03	6-Fluoro-4-hydroxycoumarin***	$C_9H_5FO_3$	136; 136; 187.9	100
29	10.21	144.04	2-Hydroxyquinoline****	C_9H_7NO	146.0599; 147.0627; 118.0647; 128.049; 117.0566	100
30	13.73	293.21	Myristyl sulfate****	$C_{14}H_{30}O_4S$	293.1789; 294.1808;295.1748	100

Note: *: flavonoid/ flavone/ flavonoid glycoside; **: phenolic; ***: polyphenol; ****: alkaloid

Table 6 Flavonoid content and DPPH free radical scavenging activity of methanol extract from roots of *H. hirsuta*

Concentration of extraction (mg/mL)	Total flavonoid content (mg Catechin/g extract)	% free radical scavenging activity SC
0.03125	0.201 ± 0.514	14.692 ± 5.011
0.0625	0.397 ± 1.424	24.582 ±2.007
0.125	0.773 ± 1.710	41.308 ±2.061
0.25	1.497 ± 1.711	55.396 ±2.296
0.5	3.123 ± 3.832	73.044 ±2.318
1	5.205 ± 2.090	88.066 ±1.112

Table 7 IC_{50} values of methanol extract of roots *H. hirsute* and ascorbic acid control

Sample name	Equation	R^2	IC_{50} (µg/mL)
Extract methanol	y = 21.696ln(x) + 87.110	0.995	536.760± 0.021
Acid ascorbic	y = 26.678 ln(x) + 212.4	0.983	0.002 ± 0.001

3.3 Biological activities of methanol fraction from roots of *H. hirsuta* L.

3.3.1 Antioxidant activity

The onset of cancer and other illnesses may be influenced by cell damage brought on by free radicals. Antioxidants are recognized as compounds with the capacity to bind free radicals, thereby reducing the damage that causes the development of several diseases in people (Liu et al. 2014). The study of DPPH free radical scavenging activity yielded diverse findings in the methanol fraction from roots of *H. Hirsuta* when it was diluted to different quantities (tables 6 and 7). The examined data demonstrated statistically significant differences with $p < 0.05$ (Duncan's test), and the maximum DPPH free radical scavenging activity was reported at 1 mg/mL (5.205 mg Catechin/g extract) being 88.06% in the methanol fraction extract from the roots of *H. hirsuta* with an IC_{50} value of 536.76 g/mL. Ascorbic acid, a free radical scavenger frequently used as a standard, yielded a considerably better result than this (Tables 6 and 7).

3.3.2 Cytotoxic activity

The study on the cytotoxicity to some cancer cell lines of methanol extract of *H. hirsuta* roots *in vitro* conditions shown in table 8 revealed that the cytotoxicity to the cell lines was at the average level with IC_{50} values ranging from 115.81 – 219.17 µg/mL. The best inhibitory effect on the human leukemia cell line (HL-60) with IC_{50} = 115.81µg/mL (Table 8). However, the best inhibitory action against the human leukemia cell lines (HL-60) and human liver cancer cell line (HepG2) was observed at 79.83% (HL-60) and 79.22% (HepG2), respectively, at the concentration of 200 g/mL methanol extract. Next is Human carcinoma of the mouth cell line (KB) with inhibitory potencies of 77.46%. The human

Table 8 Effects of the *H. hirsuta* methanolic roots extract against the various cancer cell lines

Ext. Con. (µg/mL)	Inhibitory effect of *H. hirsuta* extract methanol roots extract on cell lines (%)									
	MCF-7	SK-LU-1	HepG2	Hela	SW480	MKN-7	KB	SK-Mel-2	LNCaP	HL-60
200	48.67 ± 1.12	45.62± 2.11	79.22 ± 1.37	58.31 ± 1.52	69.08 ± 1.28	62.27 ± 1.01	77.460± 2.26	65.72 ± 1.01	71.20 ± 2.62	79.83 ± 1.47
100	29.15 ± 0.94	23.45± 1.06	43.37 ± 1.27	36.52 ± 1.07	43.83 ± 1.45	30.82 ± 1.65	46.32 ± 2.50	47.15 ± 1.25	42.08 ± 2.13	64.04 ± 2.03
20	3.23 ± 0.50	11.94± 0.29	18.66 ± 1.10	12.57 ± 1.03	18.84 ± 0.17	13.02 ± 1.91	25.12 ± 1.27	12.48 ± 1.08	19.14 ± 1.75	23.72 ± 1.78
4	1.50 ± 0.42	1.68 ± 0.31	7.24 ± 0.46	1.15 ± 0.17	4.23 ± 0.71	2.11 ± 0.49	4.39 ± 1.65	2.68 ± 0.44	3.01 ± 0.82	4.13 ± 0.49
0.8	-1.42 ± 1.21	-0.64 ± 0.14	1.54 ± 0.82	-1.04 ± 0.37	2.15 ± 0.36	-0.27 ± 0.12	1.21 ± 0.09	0.47 ± 0.27	0.17 ± 0.19	0.53 ± 0.12
IC_{50}	197.86± 1.16	219.17± 1.75	119.04± 246	162.300±1.27	133.13±2.47	159.69 ±1.160	117.73±1.62	137.87±1.32	131.45±1.05	115.81±1.54
Con. (µg/mL)	Inhibitions of Ellipticine on cell lines									
	MCF-7	SK-LU-1	HepG2	Hela	SW480	MKN-7	KB	SK-Mel-2	LNCaP	HL-60
IC_{50}	0.42±0.03	0.51±0.04	0.45±0.03	0.39±0.03	0.44±0.02	0.41±0.05	0.37±0.03	0.41±0.04	0.38±0.03	0.48±0.03

Note: The concentration of Ellipticine used in the test was 10 -2-0.4-0.08 µg/mL; Ext. Con. = Extract Concentrations

breast cancer and the human lung carcinoma cell lines (SK-LU-1) displayed the lowest activity levels with inhibitory potencies of 48.67% and 45.62%, respectively (Table 8).

Research on the oxidative and cytotoxic activity of extracts from different parts of *H. Hirsuta* collected in Vietnam has been studied by many researchers. Research by Duyen and Phuoc (2016) shows that two extracts, i.e., petroleum ether (PE) and dichloromethane (DC), are showing cytotoxic activity against the Hep-G2 cell line, with percentage CS values less than 50%. Two samples with active expression were selected for further testing to find the IC_{50} value. The IC_{50} value of PE extracts was 28.29 µg/mL, and DC extracts were 30.30 µg/mL. The methanol (MeOH) fraction has not shown cytotoxic activity against the Hep-G2 cell line (Duyen and Phuoc 2016). However, according to this study, the 200 µg/mL concentration of *H. hirsuta* roots methanol extract exhibited the most substantial inhibitory effect against the human leukemia cell line HL-60 (79.83%), followed by the human liver cancer cell line HepG2 (79.22%). Thuy (2018) showed that the Antioxidant activity of ethanol extracts (IC_{50} = 60.83µg/mL) was higher than that of chloroform extract (IC_{50} = 74.58µg/mL). However, the HepG2 hepatotoxic activity of chloroform extracts (IC_{50} = 9.17µg/mL) was more potent than that of alcohol extract (IC_{50} = 19.96µg/mL). All of these results are significantly superior to the findings of this study. According to a study in Indonesia, *H. Hirsuta* can fight against cancer cells, especially liver cancer (Chin et al. 2006).

Conclusion

This study determined the optimal conditions and factors affecting the extraction of total flavonoids from *H. hirsuta* roots collected from the Thua Thien Hue province of Vietnam. The methanol solvent content (50%), ultrasonic wave frequency (12), the material/solvent ratio (1:30 (w/v)), and time ultrasonic (30 minutes) are the ideal conditions for producing the highest concentration of total flavonoids from *H. hirsuta* roots (3.52684 mg Catechin/g extract). The experiment's total flavonoid content under optimal conditions was 5.205 (mg Catechin/g extract), which was greater than the theoretical calculation of the model. The best linear regression equation to get the maximum total flavonoid content from the root of *H. hirsuta* (Y mg Catechin/g extract) is: Y = 5.41– 1.31 X12 – 0.8286 X22 – 0.9172 X32 – 0.6657X42 (1)

From the methanolic fraction extracted from the roots of *H. hirsuta* total of 30 compounds have been identified, of which 20 were flavonoid in nature (66.667%) based on the analysis of the results using the LC-MS/MS method. The DPPH free radical scavenging activity of the methanol fraction extracted from the roots of *H. hirsuta* was high (IC_{50} = 536.760 g/mL). The inhibitory effect against various cancer lines was moderate (IC_{50} fluctuated between 115.81 and 219.17 g/mL). The human leukemia cell line (HL-60) exhibits the highest cytotoxic response to a methanol extract from *H. hirsuta* root with an IC_{50} value of 115.81 g/mL.

According to the findings, the root of *H. hirsuta* may be used as a therapeutic herb for its Antioxidant and cancer cell-inhibiting properties. More research should be done to find biologically active substances and their pharmacological mechanisms of action.

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Conflict of interest

All authors declare that they have no conflict of interest

References

Ahmed, W., Ahmad, M., Khan, R. A., & Mustaq. N. (2016). Promising inhibition of krait snake's venom acetylcholinesterase by *Salix nigra* and its role as anticancer, antioxidant agent. *Indian Journal of Animal Research, 50*, 317-323.

Chin, Y. W., Jones, W. P., Rachman, I., Riswan, S., Kardono, L. B., Chai, H. B., Farnsworth, N. R., Cordell, G. A., Swanson, S. M., Cassady, J. M., & Kinghorn, A. D. (2006). Cytotoxic lignans from the stems of Helicteres hirsuta collected in Indonesia. *Phytotherapy research : PTR*, *20*(1), 62–65. https://doi.org/10.1002/ptr.1806.

Dinda, B., Debnath, S., & Harigaya, Y. (2007). Naturally occurring iridoids. A review, part 1. *Chemical & pharmaceutical bulletin*, *55*(2), 159–222. https://doi.org/10.1248/cpb.55.159

Duyen, N. H., & Phuoc, L. T. (2016). Survey of chemicalcomposition and toxicityactivity in Hep-G2 cell of *Helicteres hirsuta* L. *Can Tho University Journal of Science, 47a*, 93-97.

Hieu, L. T., Son, L. L., Vu, H. X. A., Hoa, D. T. T., &Thi, T. T. V. (2019). Antioxidant activity of extracts from *Helicteres hirsuta* Lour Fruits in ThuaThien Hue province. *Journal of Science and Technology, University of Sciences, Hue University, 14*(2), 1-9.

Jain, A., Sinha, P., & Desai, N. S. (2014). Estimation of flavonoid.phenol content and Antioxidant potential of Indian screw tree (*Helicteres isora* L.). *International Journal of Pharmaceutical Sciences and Research, 5*(4), 1320-1330.

Liu, C. M., Kao, C. L., Wu, H. M., Li, W. J., Huang, C. T., Li, H. T., & Chen, C. Y. (2014). Antioxidant and anticancer aporphine alkaloids from the leaves of Nelumbo nucifera Gaertn. cv. Rosa-

plena. *Molecules (Basel, Switzerland)*, *19*(11), 17829–17838. https://doi.org/10.3390/molecules191117829.

Loganayaki, N., Siddhuraju, P., & Manian, S. (2013). Antioxidant activity and free radical scavenging capacity of phenolic extracts from *Helicteresisora* L. and *Ceibapentandra* L. *Journal of Food Science and Technology*, *50*(4), 687-695.

Long, D. T., Hong, H. T. K., Trang, N. T. Q., Tram, L. L. T., Tien, N. P. T., & Hanh, N. T. N. (2020). Study of the Procedure for Bath Ultrasound-Assisted Extraction of Total Flavonoid from Lotus Seeds and Testing Some Biological Activities. *Indian Journal of Agricultural Research*, *54*(5), 563-570.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., & Vaigro-Wolff, A. (1991). Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *Journal of the National Cancer Institute*, *83*(11), 757–766. https://doi.org/10.1093/jnci/83.11.757.

Mosmann T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55–63. https://doi.org/10.1016/0022-1759(83)90303-4

Noorjahan, C. M., &Saranya, T. (2018). Antioxidant.anticancerand molecular docking activity of tulsi plant'. *Agricultural Science Digest*, *38*, 209-212.

Pham, H. N. T., Nguyen, V. T., Vuong, Q. V., Bowyer M. C., & Scarlett C. J. (2016). Bioactive compound yield and Antioxidant capacity of *Helicteres hirsute* Lour. stem as affected by various solvents and drying methods. *Journal of Food Process and Preservation*, *41*, e12879.

Pham, H. N. T., Nguyen, V. T., Vuong, Q. V., Bowyer, M. C., & Scarlett, C. J. (2015). Effect of extraction solvents and drying methods on the physicochemical and antioxidant properties of *Helicteres hirsuta* Lour Leaves. *Technologies*, *3*(4), 285-301.

Pham, H.N., Vuong, Q.V., Bowyer, M.C., & Scarlett, C.J. (2017). Optimum conventional extraction conditions for phenolics, flavonoids, and Antioxidant capacity of *Helicteres hirsuta* Lour. *Asia-Pacific Journal of Chemical Engineering*, *12*, 332-347..

Pham, H. N. T., Vuong, Q. V., Bowyer, M. C., & Scarlett, C. J. (2020). In vitro anti-pancreatic cancer activity of HPLC-derived fractions from Helicteres hirsuta Lour. stem. *Molecular biology reports*, *47*(2), 897–905. https://doi.org/10.1007/s11033-019-05180-0.

Quang, D. N., Pham, C. T., Le, L. T. K., Ta, Q. N., Dang, N. K., Hoang, N. T., & Pham, D. H. (2020). Cytotoxic constituents from *Helicteres hirsuta* collected in Vietnam. *Natural product research*, *34*(4), 585–589. https://doi.org/10.1080/14786419.2018.1490907.

Thuy, P. T. T. (2018). *In vitro* antioxydant and cytotoxic activities of alcohol and chloroform extract *Helicteres hirsuta* Lour. Sterculiaceae. *Journal of Science and Technology*, *4*, 90-92.

Vijayan, K., &Tsou, C. H. (2010). DNA barcoding in plants: taxonomy in a new perspective. *Current Science*, *99*, 1530-1540.

Xiao, G., & Yao H. (2008). Optimization of viscozyme L assisted extraction of oat bran protein using response surface methodology. *Food Chemistry*, *106*, 345 - 351.

Yen, L. T. H., Ha, N. T. T., Nga, N. T., Binh, T. V., &Oanh, T. T. (2017). Anti-inflammatory effects and analgesic effects of liquid extracts from *Helicteres hirsuta* Lour.in Swiss mice. *Journal of medicine and pharmacy*, *496*, 56-59.