Optimization of extraction of total flavonoid from leafs of *Helicteres hirsuta* Lour. based on bath ultrasound-assisted and testing some biological activities

Long Dang Thanh[1*], Le Van Thi My[2], Han Hoang Thi Ngoc[1], Hue Tong Thi[1] and Hong Hoang Thi Kim[4]
1. Institute of Biotechnology, Hue University, Phu Thuong, Hue, Thua Thien Hue, VIETNAM
2. University of Sciences, Hue University, 77 Nguyen Hue, Thua Thien Hue, VIETNAM
3. University of Education, Hue University, 32 Le Loi, Thua Thien Hue, VIETNAM
4. Biotechnology Department, College of Medicine and Pharmacy, Duy Tan University, Danang 550000, VIETNAM
*dtlong@hueuni.edu.vn

Abstract

Helicteres hirsuta Lour. *contains highly bioactive compounds that have therapeutic effects on many diseases, hence are used to make medicines from most parts of the plant. We optimize using the RSM surface response method based on Design Expert 11 software (Stat-Ease Inc, Minneapolis, USA) and analyze LC-MS/MS method and some other biochemical methods of methanol extraction from leaves of H. hirsuta L. collection Thua Thien Hue, Vietnam. The results identified the ideal parameters for extracting the greatest total flavonoids from leaves of H. hirsuta L. as follows: Times (30 minutes); methanol solvent concentration (52.2407%); ultrasonic frequency (12.4253); and material/solvent ratio (1:19.9145 (w/v)). The total flavonoid value obtained was 7.78425 (mg Catechin/g extract). The actual experiment obtained an actual value of 7.538 (mg Catechin/g extract).*

The results obtained 22 compounds of a flavonoid nature (61.111%) appearing in the purified methanol fractional extract. These compounds can inhibit DPPH free radicals at 50% with an IC_{50} value of 521.498 g/mL and they also have inhibitory activity against the growth of cancer cell lines under study with IC_{50} values ranging from 104.02 to 188.48 g/mL. The best cytotoxic effect of methanol extract from leaves of H. hirsute L. on human leukemia cell line (HL-60) with IC_{50} was 104.02 µg/mL. The results obtained indicate that leaves of H. hirsute L. have prospective uses as therapeutic herbs in antioxidant and cancer cell-inhibiting properties.

Keywords: Flavonoid, *Helicteres hirsuta* Lour., Leaf, RMS, LC-MS/MS.

Introduction

Helicteres hirsuta Lour. *(H. hirsuta* L.*)* contains highly bioactive compounds that have therapeutic effects on many diseases, hence are used to make medicines from most parts of the plant. It is used in cases of people who have often aches and pains, back pain, insomnia, blue skin and even those with a tired heart can effectively improve health[10]. Currently, different portions of *H. hirsuta* L. are frequently utilized in traditional medicine to treat liver cancer[2]. The biological activity studies of *H. hirsuta* extracts showed that they have cytotoxic activity[3]. Many studies on *H. hirsuta* L. have been conducted so far, both internationally and in Vietnam. Many studies have been done on the effects of various factors and extraction methods of active components on various portions of it[5,10,11]. Chin et al[2] extracted the active components in it from Indonesia and obtained 6 lignans while Quang et al[13] recovered 12 compounds from *H. hirsuta* L. collected in Binh Phuoc. Several significant pharmacological effects of *H. hirsuta* L. have been demonstrated including antioxidant activity[5,10], antibacterial and cytotoxic action against various cancer cell lines *in vitro*[4,11,12].

Flavonoids have drawn a lot of interest in recent years for their potential to protect against various chronic illnesses including cardiovascular disease, type II diabetes, neurodegenerative diseases and many cancers[1,9]. A study on flavonoid extraction in different parts of *H. hirsuta* L. by Jain et al[5] evaluated the flavonoid content, as well as its antioxidant activity and showed that the optimum solvent for the extraction of flavonoid molecules and antioxidants is methanol. The ideal extraction conditions were carried out in Thua Thien Hue to extract the flavonoids that are present in various parts of *H. hirsuta* L. to obtain the maximum flavonoid extract content as a foundation for testing their biological activity.

Material and Methods

Plant Materials: The leaf samples of *H. hirsuta* L. were collected at Linh Mu pagoda, Kim Long ward, Hue city, Thua Thien Hue province. The leaf sample was washed under the water faucet and air-dried in the shade. The leaves were pulverized and put through a sieve with $d \leq 1$ mm after being dried at 50°C and 10% moisture. The leaf powder was kept in polyethylene bags and placed in closed plastic containers, stored at room temperature, avoiding light and moisture.

Experimental design: Total flavonoids were extracted from leaves of *H. hirsuta* L. by bath ultrasound-assisted method at 60°C on 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 of 1:10, 1:20 and 1:30 g/mL (w/v), ultrasonic wave frequency being 10, 12, 14 Hz. The results of the previous experiment are the conditions used for the

subsequent experiments. To assess the impact of these single parameters, we used all four of the variables that have the biggest impact on the overall flavonoid content from leaf of *H. hirsuta* L. Experimental design was made according to RSM surface response method using Design Expert 11 software. With the help of regression analysis, a quadratic polynomial model displaying the total flavonoid content was created using experimental data.

Determination of total flavonoid content: The total flavonoid content in the leaf extract from *H. hirsuta* was determined according to the description of Pham et al[11]. Catechin standard flavonoid solution (sigma) was diluted to concentrations of 45, 90, 180, 360 and 720 g/mL using methanol 70%. The reaction solution was measured photometrically at 510 nm ($OD_{510\ nm}$) by UV-Vis spectrophotometer (U2900 Hitachi, Japan).

Determination of antioxidant activity: Antioxidant activity of *H. hirsuta* L. leaf extract was performed based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method as described by Long et al[6]. The extract was initially diluted into ratios of 1; 0.5; 0.25; 0.125; 0.0625 and 0.03125 mL; 0.2 mM DPPH solution was mixed in 70% ethanol and control ascorbic acid solution into concentrations 0.05, 0.67, 0.10, 0.20, 0.50 and 1 mg/mL. It is based on the standard curve equation (y = ax + b) with y = 50% to find x (x is IC_{50} to look for)[7].

***In vitro* cancer cell line culture:** Testing of cytotoxic activity on some cancer cell lines was carried out 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 three to five days, the cells were transplanted grown in 1:3 ratio and kept in CO_2 incubators at 37°C with 5% CO_2.

Cytotoxic assay: The experiment was carried out to ascertain the total cellular protein content using the optical density obtained when the protein composition of the cells was stained with Sulforhodamine B. (SRB, Sigma-Aldrich, USA). This approach was used by Monks[8].

Cancer cell line for suspension cell lines (HL-60): Tim Mosmann's histology method was used to examine the cytotoxicity of the suspension cell line (HL-60) *in vitro*. Tetrazolium salt (MTT-(3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium)) was utilized as a reagent in a colorimetric assay to assess the development of cell survival and detection abilities. The method were described by Long et al[6].

Chromatographic and Mass Spectrometry Conditions: The LC-MS/MS assays of compounds performed in methanol extract are based on the machine Exion LC™-$X500_R$ QTOF (Sciex, USA) with an electrospray ionization (ESI) source. The chromatographic separation was achieved on a Hypersil GOLD Dim. 150 x 2.1, 3µ (Thermo Scientific, USA) column and the column temperature was kept at 30°C. Mobile phases which consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) were 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 set at 0.4 mL/min and the injection volume was 2 µL. All data were analyzed by Mass Hunter workstation software (Agilent Technologies, USA).

The mass spectrometer was carried out in negative ionization multiple-reaction monitoring (MRM) mode. The source parameters were as follows: the capillary voltage set at -4500 V for negative ionization mode, TOF_{MS} with TOF start mass and TOF stop mass being 100 and 2000 (Da) respectively while that for $TOF_{MS/MS}$ 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/).

Statistical analysis: The analytical values were statistically significant with $p < 0.05$. The data were analyzed with Excel 2010, IBM SPSS Statistics 20.

Results and Discussion

Optimization of some conditions of total flavonoid extract from the leaf of *H. hirsuta* L.: Three central experiments are among the 27 conditional experiments that make up the surface method's design. The results of the total flavonoid content obtained from each experiment show that the total amount of flavonoids extracted from leaves of *H. hirsuta* L. ranged from 0.296 to 8.849 (mg Catechin/g extract) (Table 1).

These results were utilized to analyze the data in Expert software 11. Table 2 shows that the contributing factors in the model are highly significant with $p < 0.05$. The linear factor (X_4), two interacting factors (X_1X_3 and X_3X_4) and four quadratic values (X_1^2, X_2^2, X_3^2 and X_4^2) participating in the model are shown in high confidence level above 95%, except for 3 linear factors (X_1, X_2, X_3) and 4 interactions (X_1X_2, X_1X_4, X_2X_3 and X_2X_4) with $p > 0.05$, because these values have no regression significance (Table 2).

The final regression equation representing the relationship between total flavonoid content and independent variables for the Box–Behnken response surface quadratic model is obtained as follows:

$$Y = 8{,}58 - 1{,}43\ X_4 - 1{,}78\ X_1X_3 + 1{,}28\ X_3X_4 - 1{,}08\ X_1^2 - 3{,}67\ X_2^2 - 3{,}22\ X_3^2 - 2{,}43X_4^2 \quad (1)$$

where Y is total flavonoid content (mg Catechin/g extract).

Table 1
Experimental design for the matrix of four factors affecting the extraction of total flavonoid

S.N.	Expression				Y
	X_1 Time (min)	X_2 Solvent concentration (%)	X_3 Ultrasound frequency (Hz)	X_4 Ratio of raw material:solvent (w/v)	Total flavonotid content (mg Catechin/g extract)
1	50	50	10	1:10	6.114 ± 0.011
2	30	50	10	1:20	3.500 ± 0.024
3	50	30	10	1:20	2.046 ± 0.007
4	70	50	12	1:10	7.681 ± 0.134
5	50	50	14	1:30	1.680 ± 0.144
6	70	50	10	1:20	6.433 ± 0.027
7	30	50	12	1:10	6.832 ± 0.773
8	50	50	10	1:30	1.063 ± 0.007
9	50	50	12	1:20	8.694 ± 0.116
10	70	70	12	1:20	4.350 ± 0.024
11	70	50	14	1:20	1.036 ± 0.018
12	50	30	14	1:20	0.756 ± 0.015
13	50	50	14	1:10	1.595 ± 0.056
14	30	70	12	1:20	3.245 ± 0.093
15	50	50	12	1:20	8.192 ± 0.160
16	30	30	12	1:20	2.512 ± 0.022
17	50	50	12	1:20	8.849 ± 0.036
18	70	30	12	1:20	3.962 ± 0.021
19	50	70	14	1:20	4.447 ± 0.248
20	50	30	12	1:10	3.303 ± 0.042
21	30	50	14	1:20	5.223 ± 0.002
22	70	50	12	1:30	2.193 ± 0.014
23	50	70	12	1:10	3.976 ± 0.083
24	50	30	12	1:30	0.296 ± 0.087
25	30	50	12	1:30	5.740 ± 0.052
26	50	70	12	1:30	1.403 ± 0.009
27	50	70	10	1:20	1.732 ± 0.011

Table 2
Significance levels of regression coefficients

Factor	Coef	SE Coef	p-value
β_o	8.58	0.603	-
X_1	-0.1163	0.302	0.7065
X_2	0.5232	0.302	0.1083
X_3	-0.5126	0.302	0.1149
X_4	-1.43	0.302	0.0005
X_1X_2	-0.0860	0.522	0.8719
X_1X_3	-1.78	0.522	0.0052
X_1X_4	-1.10	0.522	0.0571
X_2X_3	1.00	0.522	0.0793
X_2X_4	0.1085	0.522	0.8389
X_3X_4	1.28	0.522	0.0301
X_1^2	-1.08	0.452	0.0342
X_2^2	-3.67	0.452	< 0.0001
X_3^2	-3.22	0.452	< 0.0001
X_4^2	-2.43	0.452	0.0002

The Fisher F test model result (F = 10.61) and the low probability p-value (p = 0.0001) in table 4 demonstrated the high statistical significance of the set regression equation (Table 3).

According to Xiao and Yao[15], is required when R^2 (correlation coefficient) is at least 80%. The model was built using selected univariates as shown by the model analysis results in table 3, which show that the R^2 value of the model is 92.530%; R^2 - (adj) = 82,810% and all p values show a high level of statistical significance. This shows that the model is appropriate and that there is a good degree of agreement between the experimental values and the predicted values. Additionally, the F value of Lack of Fit is used to assess the model's goodness of fit.

A good correlation model must suit the theory and the data, so a model derived with a lack of fit test (non-conformity) is not wanted because it is not statistically significant. The results in table 4 show that both F (10.90) and p (0.0869) > 0.05, so there is no statistical significance. Therefore, the fit of the model is very high (Table 3).

In conclusion, we can theoretically forecast the total flavonoid content value of the leaf extract of *H. hirsuta* L. using the regression equation. Figure 1 displays a response surface graph illustrating the relationship between the total flavonoid content of leaf extract from *H. hirsuta* L. The outcomes demonstrated that the total flavonoid extraction efficiency also increased with increasing extraction duration, ultrasonic frequency and raw material/solvent ratio. However, to a certain limit, the extraction efficiency tends to decrease if we continue to increase those factors.

According to theoretical calculations, we must conduct experiments under optimal conditions including time (30 minutes), methanol solvent concentration (52.2407%), ultrasonic frequency (12.4253) and raw material/solvent ratio (1:19.9145 (w/v)), then the total flavonoid content was determined to be 7.78425 (mg Catechin/g extract) (Figure 1). The actual experiment results showed that the total flavonoid content was not significantly lower than the theoretical calculation (7.538 mg Catechin/g extract) (Table 5).

Assessing the existence of compounds in the methanol fraction: Table 4 lists the outcomes of the high-resolution mass spectrometry analysis that identified negative ions $[M+H]^-$ with m/z values corresponding to each compound. The results of MS/MS analysis of ion fragmentation in negative ion measurement mode obtained the main m/z fragments. Time recorded retention of compounds ranged from 5.45 to 18.62 minutes at a detection wavelength of 350 nm (λ= 350 nm).

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

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P Value
Model	162.10	14	11.58	10.61	0.0001
Lack-of-Fit	12.86	10	1.29	10.90	0.0869
Pure Error	0.2358	2	0.1179		
Total	175.19	26			
R - Sq =92.530% ; R^2 - (adj) = 82.810%					

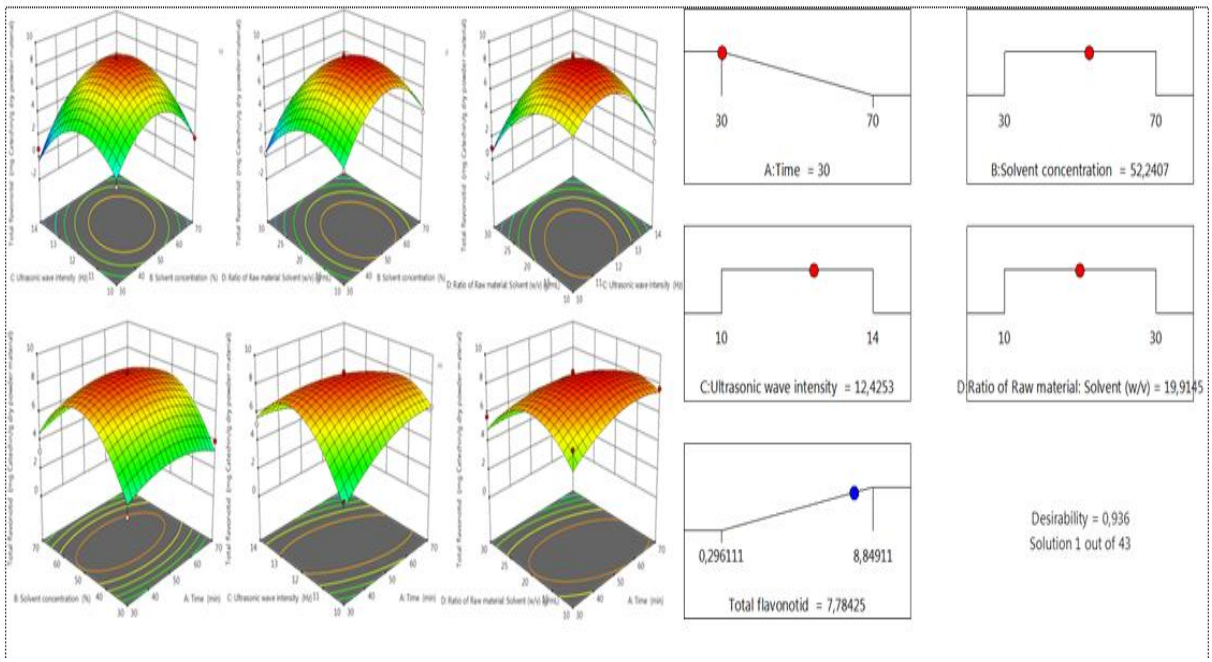

Figure 1: Expected function, response surfaces plotted in 3D and to determine ranges of conditional values for optimal responses total flavonoid content

Table 4
Results of mass spectrometry analysis by HPLC-MS/MS of the methanol extract from leaf *H. hirsute* L.

S.N.	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
7	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	17.14	669.38	Monensin*	$C_{36}H_{62}O_{11}$	688.4639; 693.4183; 689.4668; 694.4212; 690.4695	97.00
21	10.95	161.02	6-Hydroxycoumarin*	$C_9H_6O_3$	133.0274; 143.8884	98.00
22	9.95	173.01	DL-Isocitric acid lactone*	$C_6H_6O_6$	111.4331; 129.1322; 155.3224	95.10
23	6.15	153.02	3,4-Dihydroxybenzoic acid**	$C_7H_6O_4$	65.0027; 81.0335; 91.0182; 108.0206	98.50
24	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
25	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

26	8.00	163.04	trans-2-Hydroxycinnamic acid**	$C_9H_8O_3$	119.0501; 162.8392	100
27	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
28	10.95	181.05	3,4-Dihydroxyhydrocinnamic acid**	$C_9H_{10}O_4$	93.0339; 121.0281; 122.0359; 123.0433; 136.9204	100
29	12.65	179.03	4-Hydroxyphenylpyruvic acid**	$C_9H_8O_4$	109.0273; 134.0359; 135.0439	92.10
30	15.97	194.14	2,4,6-Trichlorophenol**	$C_6H_3Cl_3O$	194.9178; 158.9412	100
31	6.67	109.03	Pyrocatechol***	$C_6H_6O_2$	81.0332; 82.0372; 93.0332; 109.0274; 111.0436	95.10
32	10.34	719.16	Rosmarinic acid***	$C_{18}H_{16}O_8$	161.0239; 179.0346; 197.0448; 359.0765; 719.1612	98.00
33	10.31	179.03	6-Fluoro-4-hydroxycoumarin***	$C_9H_5FO_3$	136; 136; 187.9	100
34	10.21	144.04	2-Hydroxyquinoline****	C_9H_7NO	146.0599; 147.0627; 118.0647; 128.049; 117.0566	100
35	13.47	265.15	Dodecyl sulfate****	$C_{12}H_{26}O_4S$	103.3871	100
36	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 5
Flavonoid content and DPPH free radical scavenging activity of extract methanol from leaf of *H. hirsute* L.

Concentration of extraction (mg/mL)	Total flavonoid content (mg Catechin/g extract)	% free radical scavenging activity SC
0.03125	0.303 ± 2.253	4.802 ± 0.066
0.0625	0.502 ± 1.465	21.286 ± 0.613
0.125	0.984 ± 1.656	40.209 ± 2.296
0.25	1.273 ± 2.309	55.396 ± 2.619
0.5	1.623 ± 3.832	74.143 ± 1.583
1	7.538 ± 1.285	96.604 ± 2.007

The methanol fraction contains 36 compounds with similarity values ranging from 92.10 to 100%, according to the comparison of the compound's MS/MS spectra with the standard MS/MS data spectrum on the NIST/PubChem data spectrum bank of natural compounds, of which 22 compounds were of flavonoid nature (61,111%), 8 phenolics (22,222%), 3 polyphenols (8,333%), 3 alkaloids (8,333%) (Table 4).

Biological activities of methanol fraction from leaf of *H. hirsuta* L.

Antioxidant activity: The methanol extract taken from the leaves of *H. hirsuta* L. was diluted to different quantities, producing variable free radical scavenging activities as evidenced by the results of the evaluation of DPPH free radical scavenging activity presented in table 5 in which the maximum DPPH free radical scavenging ability was 96.604% at a concentration of 1 mg/mL (7,538 mg Catechin/g extract). The research results showed that the difference was statistically significant with $p < 0.05$ (Duncan's test). The high concentration of methanol extract with the ability to inhibit DPPH free radicals at 50% is IC_{50} = 521,498 µg/mL. This outcome is significantly low than that of ascorbic acid (0.002 ± 0.001 µg/mL) (Table 5).

Cytotoxic activity: A study on the cytotoxicity to some cancer cell lines of methanol extract segment from leaf *H. hirsute* L. *in vitro* conditions shown in table 6 shows that the ability to cause cytotoxicity on cell lines is moderate with IC_{50} values ranging from 104.02 - 188.48 µg/mL in which the best inhibitory effect is on human leukemia cell line (HL-60) with IC_{50} = 104.02 µg/mL.

However, at a concentration of methanol extract 200 µg/mL, the human leukemia cell line HL-60 (83.33%) exhibited the strongest inhibitory effect followed by the human liver cancer cell line HepG2 (77.22%), the human colon carcinoma cell line SW480 (76.08%) and the human skin cancer cell line SK-Mel-2 (73.96%). The human carcinoma cell line in the mouth (KB) and the human lung carcinoma (SK-LU-1) demonstrated the lowest inhibitory potency and are equal to 55.42% (Table 6). Research by Duyen and Phuoc[4] showed that there are two extracts showing cytotoxic activity against Hep-G2 cell line, petroleum ether (PE) and dichloromethane (DC) with 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.

Table 6
Carcinogenic effects of extract methanol from leafs *H. hirsute* L.

Con. (µg/mL)	Inhibitions of leafs *H. hirsute* extract methanol on cell lines (%)									
	MCF-7	**SK-LU-1**	**HepG2**	**Hela**	**SW480**	**MKN-7**	**KB**	**SK-Mel-2**	**LNCaP**	**HL-60**
200	57.37 ± 1.38	55.42 ± 3.31	77.22 ± 2.17	65.21 ± 3.50	76.08 ± 1.58	60.77 ± 2.01	55.42 ± 3.31	73.96 ± 2.01	70.47 ± 2.40	83.33 ± 2.47
IC_{50}	184.22 ± 3.36	188.48 ± 2.95	125.12 ± 3.46	151.28 ± 6.17	123.60 ± 4.68	168.48 ± 3.36	188.48 ± 2.95	108.54 ± 5.32	143.22 ± 4.05	104.02 ± 3.54
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; Con. = Concentration

The methanol (MeOH) fraction has not shown cytotoxic activity against Hep-G2 cell line[4]. However, according to our study, at a concentration 200 µg/mL of methanol extract from leaf *H. hirsuta* L., the human leukemia cell line HL-60 (83.33%) exhibited the strongest inhibitory effect followed by the human liver cancer cell line HepG2 (77.22%). Thuy[14] showed that the antioxidant activity of ethanol extracts (IC_{50} = 60.83 µg/mL) was higher than that of chloroform extract (IC_{50} = 74.58µg/mL). However, HepG2 hepatotoxic activity of chloroform extracts (IC_{50} = 9.17µg/ml) was stronger than that of alcohol extract (IC_{50} = 19.96 µg/mL)[14].

Conclusion

In this study, we determined the conditions optimal linear regression equation to obtain the highest total flavonoid content (Y mg Catechin/g extract) according to theoretical calculations from *H. hirsuta* L. leaves:

$$Y = 8.58 – 1.43\ X_4 – 1.78\ X_1X_3 + 1.28\ X_3X_4 – 1.08\ X_1^2 – 3.67\ X_2^2 – 3.22\ X_3^2 – 2.43X_4^2$$

Theoretical calculations show that time (30 minutes), methanol solvent concentration (52.2407%), ultrasonic frequency (12.4253) and material/solvent ratio (1:19.9145 (w/v)) are the best conditions for extracting the greatest total flavonoids from *H. hirsuta* L. leaves (7.78425 mg Catechin/g extract). The results obtained from the actual experiment are not significantly lower than the theoretical calculation (7.538 mg Catechin/g extract).

The methanol segment extract from the leaf *H. hirsuta* L. contained 36 different compounds, 22 of which were flavonoid (61.111%). High DPPH free radical scavenging activity was demonstrated by a methanolic extract from leaves of *H. hirsuta* L. (IC_{50} = 521.498 g/mL); nevertheless, inhibitory action against various cancer lines was only moderate, with an IC_{50} ranging from 104.02 to 188.48 g/mL. The best cytotoxic effect is of methanol extract from leaves of *H. hirsuta* L. on human leukemia cell line (HL-60) with IC_{50} value = 104.02 µg/mL. The results obtained indicate that leaves of *H. hirsuta* L. have prospective uses as therapeutic herbs in antioxidant and cancer cell-inhibiting properties. Future research should focus on identifying biologically active substances and their pharmacological mechanisms of action.

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References

1. Ahmed W., Ahmad M., Khan R.A. and Mustaq N., 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 **(2016)**

2. 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. and Kinghorn A.D., Cytotoxic lignans from the stems of *Helicteres hirsuta* collected in Indonesia, *Phytother. Res*., **20**, 62-65 **(2006)**

3. Didna B., Debnath S. and Harigaya Y., Naturally occurring iridoids, A review, Part 1, *Chem. Pharm. Bull.*, **55(2)**, 159-222 **(2007)**

4. Duyen N.H. and Phuoc L.T., Survey of chemical composition and toxicity activity in Hep-G2 cell of *Helicteres hirsuta* L., *Can Tho University Journal of Science*, **47a**, 93-97 **(2016)**

5. Jain A., Sinha P. and Desai N.S., Estimation of flavonoid. phenol content and antioxidant potential of Indian screw tree (*Helicteres isora* L.), *IJPSR*, **5(4)**, 1320-1330 **(2014)**

6. Long D.T., Hong H.T.K., Trang N.T.Q., Tram L.L.T., Tien N.P.T. and Hanh N.T.N., 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 **(2020)**

7. Marxen K., Heinrich K., Lippemeier S., Hintze R., Ruser A. and Hansen U.P., Determination of DPPH Radical Oxidation Caused by Methanolic Extracts of some Microalgal Species by Linear Regression Analysis of Spectrophotometric Measurements, *Sensors*, **7**, 2080-2095 **(2007)**

8. Monks A., Scudiero D., Skehan P., Shoemaker R., Paull K., Vistica D., Hose Langley C., Cronise J. P., Vaigro-Wolff A. And

Gray-Goodrich M., 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 **(1991)**

9. Noorjahan C.M. and Saranya T., Antioxidant. anticancer and molecular docking activity of tulsi plant, *Agricultural Science Digest*, **38**, 209-212 **(2018)**

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

11. Pham H.N.T., Vuong Q.V., Bowyer M.C. and Scarlett C.J., Optimum conventional extraction conditions for phenolics. flavonoids. and antioxidant capacity of *Helicteres hirsuta* lour., *J. Chemical Engineering*, **12(2)**, 332-347 **(2017)**

12. Pham H.N.T., Vuong Q.V., Bowyer M.C. and Scarlett C.J., *In vitro* anti-pancreatic cancer activity of HPLC-derived fractions from *Helicteres hirsuta* Lour., *Stem. Mol Biol Rep*., **47(2)**, 897-905 **(2020)**

13. Quang D.N., Chung T.P., Linh T.K.L., Quynh N.T., Ngoc K.D., Nhung T.H. and Dien H.P., Cytotoxic constituents from *Helicteres hirsuta* collected in Vietnam, *Natural Product Research*, **34(4)**, 1-6 **(2018)**

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

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

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