ACADEMIA JOURNAL OF BIOLOGY 2018, 40(3): 45–51
DOI: 10.15625/2615-9023/v40n3.12674

SECOND METABOLITES AND ANTIOXIDANT, ANTIMICROBIAL, ANTICANCER ACTIVITIES OF *Helicteres hirsuta* ROOT EXTRACT

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ABSTRACT

Helicteres hirsuta Lour., particularly its stems and leaves have been used in Vietnamese traditional medicine to cure furuncles, dysentery, measles and flu. In this study, we examined biological activities of 5 fractions from methanol extract of *H. hirsuta* roots. The results showed that ethyl acetate fraction has the highest content of phenolic and flavonoid compounds (220.9 ± 15.67 mg GAE, gallic acid equivalents/g dry weight and 23.03 ± 0.97 mg QE, quercetin equivalents/g dry weight, respectively). Fractions from *H. hirsuta* roots exhibited antioxidant, antibacterial and anticancer activities. Ethyl acetate fraction showed the strongest antioxidant activity through 1,1diphenyl-2-picrylhydrazyl (DPPH) scavenging capacity (half-maximal inhibitory concentration, IC_{50} value of 0.117 ± 0.023 mg/ml). Ethyl acetate fraction of roots also showed the strongest reducing power (0.071 ± 0.009 mg/ml) and cytotoxicity on human KB cell lines (IC_{50}= 3.23 µg/ml). Besides, fractions from *H. hirsuta* roots had high inhibitory activity against Gram-positive bacteria namely *Staphylococcus aureus* and *Lactobacillus fermentum*. The results suggest potential exploitation of bioactive compounds from *H. hirsuta* roots especially ethyl acetate fraction and its application in medicine and pharmacy. This is the first report on second metabolite composition and biological activities of *H. hirsuta* roots.

Keywords: Helicteres hirsuta, total phenolics, antioxidant, antimicrobial activity, anticancer activity.

Citation: Diem Thi Thuy Dzung, Trinh Huyen Trang, Le Thi Khanh Linh, Dao Van Tan, Le Thi Phuong Hoa, 2018. Second metabolites and antioxidant, antimicrobial, anticancer activities of *Helicteres hirsuta* root extract. *Academia Journal of Biology*, 40(3): 45–51. https://doi.org/10.15625/2615-9023/v40n3.12674.

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Received 15 June, accepted 25 September 2018

INTRODUCTION

Being one of most biodiverse countries in the world for vertebrates and vascular plant species, Vietnam has advantages in research, exploitation and application of medicinal plants for the rising global concern for preventive healthcare and treatment. *Helicteres hirsuta* Lour. is a small tree growing mostly in Southeast Asia and north Australia. Aerial parts of *H. hirsuta*, especially leaves, have been used in folk medicine for treatment of furuncles, dysentery, measles and flu (Tran Cong Khanh, 2014), and roots for pain relief like uterine pain (Libman et al., 2006). Chin et al. (2006) reported lignans from *H. hirsuta* stems to have cytotoxic effects against a small panel of cancer cell lines. Petroleum ether and dichloromethane fractions from ethanol extract of *H. hirsuta* aerial parts exhibited cytotoxicity against Hep-G2 (hepatocellular carcinoma) cell line. Activity-guided isolation from dichloromethane fraction led to the identification of one sterol, one triterpenoid and two flavonoids (Nguyen Huu Duyen, Le Thanh Phuoc, 2016). Besides, extracts of *H. hirsuta* aerial parts showed anti-inflammatory activity through inhibition of cyclooxygenase-1 and cyclooxygenase-2. As a result, three phenolic compounds were isolated and identified from the extracts (Nguyen et al., 2017). Le Thi Hai Yen et al. (2017) demonstrated that water extract of *H. hirsuta* had analgesic and anti-inflammatory effects on mice. Moreover, Pham et al. (2015, 2017) carried out researches on drying and extraction methods for phenolics, flavonoids and antioxidant properties

of *H. hirsuta* stems and leaves. However, there have been no reports on phytochemical constituents and biological activities of *H. hirsuta* roots to date. The present research aims to evaluate second metabolite composition and antioxidant, antimicrobial, anticancer activities of various fractions from *H. hirsuta* root extract.

MATERIALS AND METHODS

Helicteres hirsuta L. roots were collected in Binh Phuoc province. Plant sample was identified by Dr. Do Huu Thu, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

KB cancer cell line (Human HeLa contaminant carcinoma cell line) and microbial strains including *Staphylococcus aureus* ATCC 13709, *Bacillus subtilis* ATCC 6633, *Lactobacillus fermentum* N4, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* 15422, *Salmonella enterica* ATCC 13076 and *Candida albicans* ATCC 10231 were obtained from the Laboratory of Applied Biochemistry, Institute of Chemistry, Vietnam Academy of Science and Technology.

Chemicals and reagents were of analytical grade and purchased from Sigma Chemicals (MO, USA) and Merck Chemicals (Darmstadt, Germany).

Sample preparation

Fresh roots were washed with distilled water to remove debris and dust, and then dried to constant weights. Dried roots were ground and extracted in absolute methanol at room temperature for 3 days and in an ultrasonic bath thereafter in three replicates of 30 min. The extracts were mixed and concentrated in a rotary evaporator at 40°C, and then lyophilized.

The crude extract was further partitioned in n-hexane, ethyl acetate, butanol, chloroform and water. The five fractions were concentrated by vacuum evaporation and freeze-dried. All the fractions were stored at (-)20°C until use.

Determination of total phenolic content

The total phenolic content of each fraction was evaluated according to the method of Waterhouse (2002) with gallic acid as the standard. The amount of total phenolics was calculated using a gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE) per g dry weight of each fraction.

Determination of total flavonoid content

The total flavonoid content was measured using the method described by Sapkota et al. (2010) with quercetin as the standard. The results were expressed in mg quercetin equivalents (QE) per gram dry weight using a quercetin standard curve.

DPPH radical scavenging activity assay

DPPH radical scavenging potential was determined according to Blois (1958) using DPPH 0.1 mM. The control was prepared with ethanol instead of extracts. Ascorbic acid was used for comparison with extracts. DPPH radical scavenging capacity was calculated using the following formula:

$$\text{DPPH scavenging capacity (\%)} = [(A_{control} - A_{sample})/(A_{control})]\times 100$$

where $A_{control}$ stands for the absorbance of the control and A_{sample} for the absorbance of the test sample. The IC_{50} value was deduced from the logarithm regression curve of scavenging capacity vs. sample concentration.

Reducing power assay

The reducing power was determined according to the method of Sapkota et al. (2010) using 0.1% ferric chloride. Increase of the absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid and quercetin were used for comparison.

Antimicrobial activity assay

The antimicrobial activity of samples was assessed using the broth dilution method of Hadacek & Greger (2000). MHB medium (Mueller-Hinton broth) was used for *S. aureus, E. coli, P. aeruginosa*; TSB (Tryptic soy broth) for *B. subtilis, L. fermentum, S. enterica* and SDB (Sabouraud -2% dextrose broth) for *C. albicans*. Each fraction was dissolved in dimethyl sulfoxide and water. Ampicillin, streptomycin and amphotericin B were used as positive controls. The IC_{50} value of antimicrobial activity was calculated from the logarithm curve of growth inhibitory percentage vs. sample concentration.

Anticancer activity assay

All the fractions were tested against KB cell lines according to the method described by Scu-

diero et al. (1988) at the Laboratory of Applied Biochemistry, Institute of Chemistry, Vietnam Academy of Science and Technology. Fractions were dissolved in dimethyl sulfoxide and diluted in water. Ellipticine was used as the positive control. IC_{50} values were estimated based on the percentage of growth inhibition.

Statistical analysis

For statistical analysis, data were analyzed using Microsoft Excell and SPSS 20.0 software. Results were expressed as mean ± standard deviation of at least 3 replicates. A level of *p*-value less than 0.05 was considered to be significant according to Dunnet's T3 test.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Phenolic compounds have been widely investigated in medicinal plants for multiple biological effects such as antioxidant, antipathogenic, anti-inflammatory, anticancer activity (Dai & Mumper, 2010; Sapkota et al., 2010; Li et al., 2015).

H. hirsuta root contained a moderate amount of phenolics (Table 1). Ethyl acetate fraction had significantly higher level of phenolic compounds, approximately 2 times higher than that of butanol fraction, 4 times than chloroform fraction and 9 times than n-hexane and water fraction. However, the flavonoid content was at low level in all *H. hirsuta* fractions examined. Ethyl acetate fraction also possessed highest amount of flavonoids, about 3-8 times as much as that of other fractions.

Total phenolics of *H. hirsuta* root fractions are much higher than those of various extracts from *H. isora* roots (0.76–0.97 mg GAE/g) (Jain et al., 2014). Phenolic content of *H. hirsuta* root ethyl acetate fraction is similar to that of crude extract and fractions from stems and leaves (Pham et al., 2017).

Table 1. Total phenolic and flavonoid content of *H. hirsuta* root fractions

Fractions	Total phenolic content (mg GAE/g)	Total flavonoid content (mg QE/g)
n-Hexane	24.19 ± 5.51^{a}	4.69 ± 1.20^{a}
Chloroform	65.87 ± 9.32^{b}	8.59 ± 2.45^{a}
Ethyl acetate	220.90 ± 15.67^{c}	23.03 ± 0.97^{b}
Butanol	121.24 ± 18.90^{d}	5.92 ± 0.42^{a}
Water	37.68 ± 8.33^{a}	3.09 ± 1.11^{c}

GAE: gallic acid equivalents, QE: quercetin equivalents, ^{a,b,c,d}: Significant difference among fractions at $p < 0.05$

Chin et al. (2006) isolated 6 phenolic compounds (lignans) from *H. hirsuta* stems, three of which showed anticancer activity against human cancer cell lines. Two flavonoids from dichloromethane fraction of *H. hirsuta* aerial parts were reported to have antioxidant, anti-inflammatory and anticancer activity (Nguyen Huu Duyen & Le Thanh Phuoc, 2016). Three phenolic compounds isolated from extracts of *H. hirsuta* aerial parts have potent anti-inflammatory activity (Nguyen et al., 2017). It is suggested that *H. hirsuta* root extract, especially ethyl acetate fraction is a potential source for bioactive compounds.

Antioxidant activity

DPPH radical scavenging activity

It was indicated that ethyl acetate fraction of *H. hirsuta* root methanol extract had strong DPPH radical scavenging capacity, roughly one fourth as strong as ascorbic acid (Table 2). IC_{50} value was not determined for n-hexane fractions due to poor scavenging capacity even at the highest experimental concentration (1.6 mg/ml). Other three fractions showed moderate to low scavenging activity. However, *H. hirsuta* root fractions exhibited stronger activity as compared to *H. isora* root extracts with only about 50–70% inhibition at the concentration of 10 mg/ml (Jain et al., 2014). All *H. hirsuta* root fractions except n-hexane and water fractions also had stronger DPPH radical scavenging activity than *H. angustifolia* root extracts (EC_{50} values of 0.69–0.73 mg/ml) (Li et al., 2015).

DPPH radical scavenging activity of *H. hirsuta* root fractions followed the order of ethyl acetate > butanol > chloroform > water > n-hexane, in the same manner as that of total

phenolic content (Table 1). High correlation (R^2 = 0.95) was observed between scavenging ability of *H. hirsuta* root fractions and their phenolic content (Fig. 1). It is likely that phenolic compounds contribute to DPPH scavenging capacity of *H. hirsuta* root fractions, especially of ethyl acetate fraction. Various biological effects are attributed to phenolic compounds, particularly antioxidant activity due to their reduction property, donating a hydrogen atom or an electron to a free radical, or delocalizing an unpaired electron (Dai & Mumper, 2010). Our result suggested potential exploitation of antioxidant compounds from ethyl acetate fraction in pharmaceutical industry.

Table 2. DPPH scavenging activity of *H. hirsuta* root fractions

Fractions and control	IC_{50} (mg/ml)
n-Hexane	> 1.6
Chloroform	0.415 ± 0.077[a]
Ethyl acetate	0.117 ± 0.023[b]
Butanol	0.252 ± 0.045[c]
Water	0.951 ± 0.054[d]
Ascorbic acid	0.026 ± 0.003

[a,b,c,d]: Significant difference among fractions at $p < 0.05$

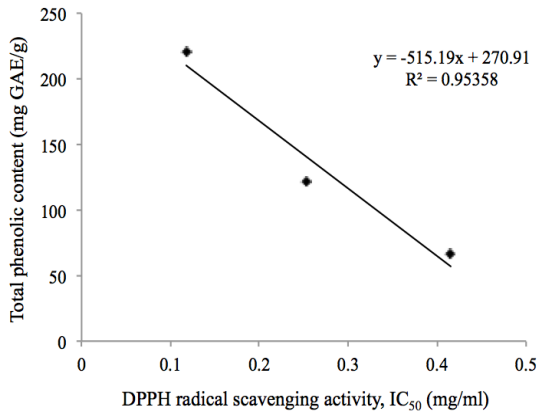

Figure 1. Correlation between DPPH scavenging activity and total phenolic content of *H. hirsuta* root fractions

Reducing power

Reducing power assay, in which the oxidative form of iron (Fe^{+3}) in ferric chloride is converted to ferrous (Fe^{+2}) by the antioxidant compounds, was also used to measure the antioxidant activity of plant extracts.

Reducing power of *H. hirsuta* root fractions (Fig. 2) is correlated relatively with scavenging activity. Ethyl acetate fraction had the highest reducing power as compared to that of the other fractions of *H. hirsuta* root extract. Its reducing power was roughly two times and four times lower than ascorbic acid and quercetin, respectively (Table 3).

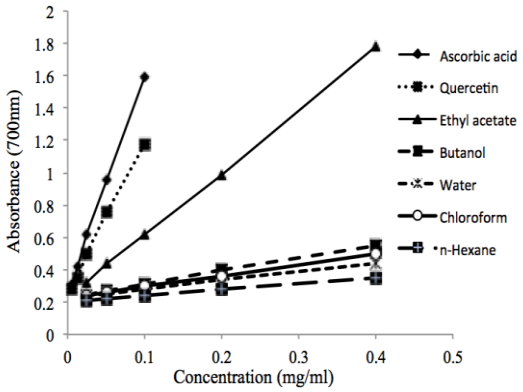

Figure 2. Reducing power of *H. hirsuta* root fractions

Table 3. Concentration of *H. hirsuta* root fractions at absorbance 0.5 compared with ascorbic acid and quercetin as standards in reducing power assay

Fractions and standards	Concentration (mg/ml)
n-Hexane	1.240 ± 0.726[a]
Chloroform	0.400 ± 0.012[b]
Ethyl acetate	0.071 ± 0.009[c]
Butanol	0.267 ± 0.053[d]
Water	0.862 ± 0.041[e]
Acid ascorbic	0.019 ± 0.001
Quercetin	0.028 ± 0.002

[a,b,c,d,e]: Significant difference among fractions at $p < 0.05$

Li et al. (2015) reported that reducing power of ethyl acetate fraction was higher than that of aqueous extract (0.13 mg/ml) and ethanol extract (0.20 mg/ml) of *H. angustifolia* roots and comparable to Trolox (0.07 mg/ml). Ferric ion-reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action.

Antimicrobial activity

Results of broth dilution assay indicated that all *H. hirsuta* root fractions exhibited no or weak

inhibition against tested Gram-negative bacterial and fungal strains at the concentration of 128 µg/ml (Table 4). There were only two Gram-positive bacterial strains, *S. aureus* and *L. fermentum*, which were affected by *H. hirsuta* root fractions. Chloroform and ethyl acetate fractions had low and moderate inhibitory effects on *S. aureus*, respectively. Butanol and n-hexane fractions showed strong inhibition on the growth of *L. fermentum*, stronger than did chloroform fraction. Water fraction had no effect on all tested microorganisms. The results suggested that *H. hirsuta* root fractions possess different profiles of antimicrobial compounds as compared to *H. hirsuta* aerial parts. According to Tran Van Tien & Vo Thi Mai Huong (2017), methanol extract of *H. hirsuta* aerial parts and its fraction exhibited inhibitory activity against both Gram-negative (*E. coli* and *S. typhi*) and Gram-positive bacteria (*S. aureus* and *Streptococcus faecalis*) in agar well diffusion method. Various extracts from *H. isora* roots showed inhibition on the growth of Gram-positive, Gram-negative bacterial strains and fungal strains, although there was no effect on *S. typhi* and low effect on *E. coli* (Venkatesh et al., 2007).

Table 4. Antimicrobial activity of *H. hirsuta* root fractions

Fractions and controls	IC_{50} (µg/ml)						
	S. aureus	*B. subtilis*	*L. fermentum*	*S. enterica*	*E. coli*	*P. aeruginosa*	*C. albicans*
n-Hexane	>128	>128	4.33	>128	>128	>128	>128
Chloroform	20.58	>128	15.24	>128	>128	>128	>128
Ethyl acetate	128	>128	>128	>128	>128	>128	>128
Butanol	>128	>128	1.55	>128	>128	>128	>128
Water	>128	>128	>128	>128	>128	>128	>128
Ampicillin	0.0099	0.0304	0.0106	0.1205	0.0704	-	-
Streptomycin	-	-	-	-	-	10.316	-
Amphotericin B	-	-	-	-	-	-	0.073

>128: no inhibitory activity at or below concentration of 128 µg/ml; (-): not experimented

Anticancer activity

Extracts and compounds from *H. hirsuta* aerial parts were reported to have significant anticancer activiy (Chin et al., 2006, Nguyen Huu Duyen & Le Thanh Phuoc, 2016). In order to characterize further the biological activity of *H. hirsuta* roots, fractions from methanol extract were tested with KB human cancer cell line. It is revealed that ethyl acetate fraction had strong cytotoxic effect on KB cell line (Table 5). n-Hexane and butanol fractions exhibited moderate activity.

Table 5. Cytotoxicity of *H. hirsuta* root fractions on KB cell line

Fractions and control	IC_{50} (µg/ml)
n-Hexane	23.08
Ethyl acetate	3.23
Butanol	64.98
Water	>128
Ellipticine	0.51

Chin et al. (2006) reported three polyphenol compounds (lignans) from *H. hirsuta* stems to have non-selective cytotoxic activity against various human cancer cell lines including LNCaP (hormone-dependent human prostate cancer), Lu1 (human lung cancer), MCF-7 (human breast cancer) cell lines and HUVEC (human umbilical vein endothelial) cell line.

The results of this study suggest the requirements of further characterization on cytotoxicity *H. hirsuta* roots on other cancer cell lines and normal cell lines for activity-guided isolation of anticancer compounds. Ethyl acetate fraction would be a promising source for its high phenolic content and strong antioxidant activity.

CONCLUSION

H. hirsuta root fractions had moderate phenolic content and low flavonoid content. They exhibited antioxidant activity through DPPH radical scavenging activity and reducing power in correlation with phenolic content. *H. hirsuta* root fractions also showed certain degree of anticancer activity against KB human cell line. The ethyl acetate fraction of *H. hirsuta* roots had significantly stronger activity than other

fractions. The present findings encourage further characterization of bioactive compounds in *H. hirsuta* roots, especially the ethyl acetate fraction, and their mechanism of actions for better application in preventive and therapeutic medicine.

Acknowledgements: The research is supported by a grant from Ministry of Education and Training of Vietnam (B2017-SPH-31).

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