

CHEMICAL PROFILES AND ANTIBACTERIAL, ANTIOXIDANT, CYTOTOXIC ACTIVITIES OF ACETONE EXTRACT FROM LEAVES OF *HELICTERES HIRSUTA* LOUR.

NHAT TAM LE¹, HONG THIEN VAN¹, NGOC TRUC PHUONG NGUYEN¹, VAN SON LE², VAN HAI CHU³, HUYNH ANH VU TRUONG³, QUOC HUNG NGUYEN³, HOANG DUNG NGUYEN⁴, NGOC NAM TRINH⁵, TAN VIET PHAM¹

¹*Institute of Biotechnology and Food technology, Industrial University of Ho Chi Minh City*

²*Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng Ward, Xuyen Moc District, Ba Ria-Vung Tau Province*

³*Center of Analytical Services and Experimentation HCMC*

⁴*Institute of Tropical Biology, Vietnam Academy of Science and Technology*

⁵*Office of Science Management and International Affairs, Industrial University of Ho Chi Minh City
phamtanviet@iuh.edu.vn*

Abstract: The present study, twenty-five chemical compounds of acetone extract from the *Helicteres hirsuta* leaves have been investigated for the first time using GC–MS, in which neophytadiene, palmitic acid methyl ester, palmitic acid, phytol, linolenic acid and octadecanoic acid were identified as the major constituents. By using disk–diffusion assay, the results showed that the extract was resistant against 6 studied bacterial strains, including *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Bacillus cereus* and *Staphylococcus aureus*. Furthermore, the DPPH and MTT methods were used to identify the antioxidant and cytotoxic activities of the studied extract. As a result, the cytotoxicity of the extract against the Hepatoma (Hep-G2) cell line was indicated with the IC₅₀ of 381.8 ppm whereas this value was 1.223 mg/mL in DPPH inhibition.

Keywords: *Helicteres hirsuta*; GC/MS; antibacterial, antioxidant, cytotoxic activities; acetone extract.

1. INTRODUCTION

Helicteres L., the large genus belonging to Malvaceae family, includes approximately 60 species distributed in tropical regions of Asia and America [1, 2] Nine species are known from Vietnam, including *H. angustifolia*, *H. daknongensis*, *H. elongate*, *H. lannata*, *H. isora*, *H. lanceolate*, *H. poilanei*, *H. viscida* and *H. hirsute* [3]. Many species of the *Helicteres* genus are remedies used in Vietnam and other countries traditional medicine as anti-inflammatory agents, vermifuges and tonic agents. [3]

Helicteres hirsuta is a small shrub growing mostly in Australia and Southeast Asia [4]. This species was recorded in some regions of Southern Vietnam, including Binh Phuoc, Kien Giang, Khanh Hoa and Ba Ria-Vung Tau Province [3, 4]. The chemical compounds and bioactivities of various extracts which were isolated from the different solvents of this species have been reported by previous studies [3, 5-8]. To date, the chemical profiles and biological activities of acetone extract of *H. hirsuta* leaves are limited, however. In this study, the chemical constituents, antibacterial, antioxidant and cytotoxic activities of the acetone extract of *H. hirsuta* leaves were investigated for the first time.

2. METHODS AND MATERIALS

2.1 Plant

Specimens of *Helicteres hirsuta* were collected from Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng ward, Xuyen Moc District, Ba Ria-Vung Tau Province, Vietnam (10°31'32.2"N 107°28'46.1"E, July 2, 2020) (Figure 1). The voucher specimen (VS Le 512) was deposited at the herbarium of Binh Chau-Phuoc Buu Nature Reserve.

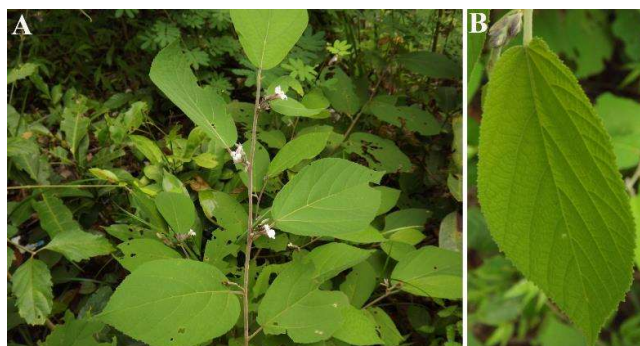


Figure 1. *Helicteres hirsuta*. A. The species in habitat, B. Leaf

2.2 Bacterial strains

Six bacterial strains were used to investigate the antibacterial activity of the acetone extract from the leaves of *H. hirsuta*, including *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella enteritidis* (ATCC 13976), *Salmonella typhimurium* (ATCC 13311), *Bacillus cereus* (ATCC 11774) and *Staphylococcus aureus* (ATCC 25923). These strains were preserved in 20% glycerol solution at 20°C and cultivated in Luria-Bertani broth at 37°C for 24 h to activate prior to antibacterial activity assay.

2.3 Extraction procedures

The fresh *H. hirsuta* leaves were sliced into small pieces and modestly dried at 50°C until constant weight. The specimens were pulverized into powder. 100g of the dried powders were subsequently macerated in 250 mL of acetone 99% solution at room temperature for 72 hours. The Whatman paper was used to filter the extract. The extraction from the dried powders was repeated twice to collect the filtrate. The filtrate was condensed under vacuum pressure at 60°C to obtain the brown extract. The brown extract was in a syrup form and it was used for further experiments [9].

2.4 Gas chromatography/mass spectrometry (GC/MS) analysis

The GS/MS assay was conducted on the TRACE 1310 Gas Chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) combined with ISQ 7000 single quadrupole mass spectrometer. This process used the DB-5MS 30m, 0.25mm, 0.25µm column and the carrier gas was helium with the column flow rate of 1.2 mL/min. Sample was injected into the system with the inlet temperature of 250°C, the split ratio of 30:1, the splitless mode of 1 min and the split flow of 36 mL/min. The oven temperature was initially set at 80°C and hold for 5 min. The temperature was then programmed to increase up to 280°C at the rate 20C/min and hold for 10 min. The electron ionization mode and the ion source temperature used were 70 eV and 250°C, respectively. The mass scan range was of 29-650 m/z. To identify the chemical constituents of the studied samples, the internal library, including NIST 2017 library and the Wiley 8th edition libraries were used.

2.5 Antibacterial activity assay

The antibacterial activity assay was conducted by disc diffusion method following the CLSI guideline [10]. Mueller Hinton Broth was used to culture the six bacterial strains until the turbidity of the cultures reached 0.5 Mc Farland Standard. Mueller Hinton agar plate was inoculated by spreading with 0.1 mL of the bacterial culture. The paper discs were impregnated with 10 µL of acetone extracts. The Gentamycin containing discs (Nam Khoa BioTek Company, Vietnam) (10 µg/mL) were used as positive controls whereas the DMSO solution was used as the negative controls. Then the plates were incubated at 37°C for 24 hours, and the diameters of the inhibition zones of extract against tested bacteria were recorded.

Three biological replicates were used for the experiment and the results were expressed as as mean ± standard deviation (SD). The differences between means groups were calculated by Fisher's least significant difference (LSD) procedure using Statgraphics Centurion XV software (Statpoint Technologies Inc, Virginia, USA) with $p < 0.05$.

2.6 Determination of antioxidant activity of extract

The method described by Thaipong et al. was used to identify the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the extract. The 100 µL of the studied extract was added to 100 µL of DPPH solution (300 µM). The mixture was then slightly shaken and placed in dark 30 min at 37°C. The absorbance of the solution was later recorded at 517 nm and the DPPH radical scavenging activity (DPPH_{RSA}) of the extract was calculated according to the following formula:

$$\text{DPPH}_{\text{RSA}} (\%) = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}} \times 100\%$$

where $\text{Abs}_{\text{control}}$ is the absorbance of the DPPH radical in methanol and $\text{Abs}_{\text{sample}}$ is the absorbance of the DPPH radical solution mixed with the sample extract [11].

2.7 Cytotoxicity assay

Hep-G2 cell line (ATCC HB-8065), USA) was used to investigate the cytotoxicity of the acetone extract from the leaves of *H. hirsuta* following MTT method [12]. The assay was conducted in 96 well – plate format, in which Hep-G2 cell line (10^4 cells/well) was grown in RPMI 1640 medium (Gibco Invitrogen, USA) containing 10% heat-inactivated fetal bovine serum (Sigma Aldrich, USA), 100 U/mL penicillin (Sigma Aldrich, USA) and 100 $\mu\text{g/mL}$ streptomycin (Sigma Aldrich, USA) at 37°C and 5% CO_2 . DMSO was the solvent of tested compounds. The compounds were stepwise diluted to the final concentration of 0–200 $\mu\text{g/mL}$ after 24 h of incubation. Cisplatin was used as a positive control. Three biological replicates were used for the experiment. IC_{50} values (concentration of the compound which have 50% inhibition on the cell growth) was recorded.

3. RESULTS AND DISCUSSION

3.1 Chemical composition

The chemical constituents of acetone extract from *H. hirsuta* leaves were presented in Table 1, in which a total of 25 compounds have been identified. According to gas chromatogram (Figure 2), six compounds, including neophytadiene, palmitic acid methyl ester, palmitic acid, phytol, linolenic acid and octadecanoic acid which stated in retention time values 12.69, 13.14, 13.33, 14.08, 14.20 and 14.30, respectively are the major constituents.

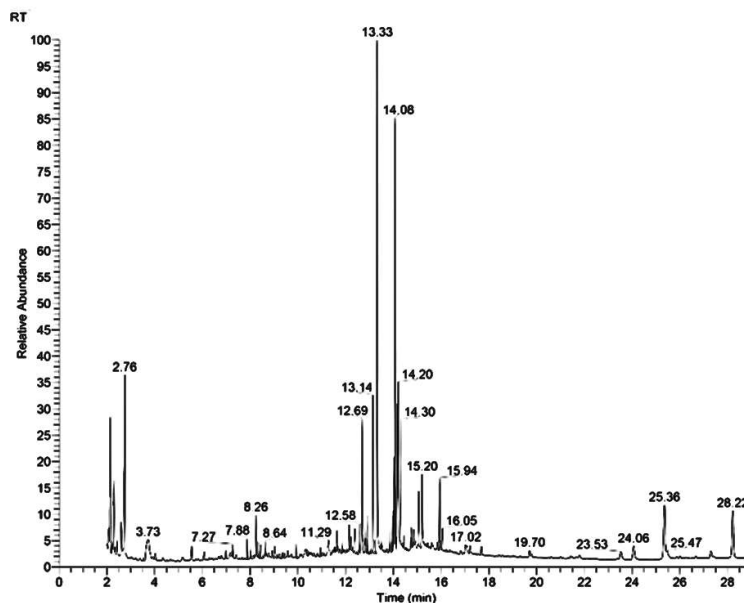


Figure 2. Gas chromatogram of the acetone extract from *H. hirsuta* leaves

The biological activities of six main constituents presented in the acetone extract of *H. hirsuta* leaves in present study have been documented in previous studies. For instance, neophytadiene was known to have antimicrobial and anti-inflammatory agent as well as a plant metabolite and an algal metabolite [13-15]. Palmitic acid methyl ester is a compound which has the ability to inhibit Kupffer cells, the resident macrophages in the liver regulating inflammatory processes by secretion of TNF α and NO [16]. Furthermore, this constituent also exhibits anti-fibrotic effects [17] as well as acts as potent vasodilator released in retina and myometrium [18-20]. Agoramoorthy et al. (2007) showed that palmitic acid, a major compound which isolated from leaves of *Excoecaria agallocha* has a role as antibacterial and antifungal agent [21]. Phytol, an abundant compound in nature, is known to have the antinociceptive, antibacterial, antioxidant, cytotoxic activities as well as the anxiolytic and anticonvulsant effect [22]. Linolenic acid, a

fatty acid found mostly in plant, has a role as anti-inflammatory agent [23]. Furthermore, Jerah et al. demonstrated that octadecanoic acid isolated from *Cymbopogon nardus* could inhibit against measles virus and Vero cell line [24].

Table 1. Chemical constituents of acetone extract from the leaves of *H. hirsuta*

No.	Retention Time	Compounds	Molecular Formula
1	2.15	2-Pentanone, 4-hydroxy-4-methyl-	C ₆ H ₁₂ O ₂
2	2.30	1-Methoxy-2-propyl acetate	C ₆ H ₁₂ O ₃
3	2.76	Ethanol, 2-butoxy-	C ₆ H ₁₄ O ₂
4	3.73	Glycerin	C ₃ H ₈ O ₃
5	6.07	Maltol	C ₆ H ₆ O ₃
6	6.99	Isophorone	C ₉ H ₁₄ O
7	7.27	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C ₆ H ₈ O ₄
8	8.02	12-Heptadecyn-1-ol	C ₁₇ H ₃₂ O
9	8.24	5-Hydroxymethylfurfural	C ₆ H ₆ O ₃
10	8.36	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	C ₇ H ₉ NO ₂
11	8.44	1,2,3-Propanetriol, 1-acetate	C ₅ H ₁₀ O ₄
12	8.64	1H-Pyrrole-2,5-dione, 3-ethenyl-4-methyl-	C ₇ H ₇ NO ₂
13	11.64	9,12,15-Octadecatrienoic acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	C ₂₁ H ₃₆ O ₄
14	12.15	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	C ₁₀ H ₁₂ O ₃
15	12.38	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7a-tetrahydrobenzofuran-2(4H)-one	C ₁₁ H ₁₆ O ₃
16	12.69	Neophytadiene	C ₂₀ H ₃₈
17	12.92	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	C ₂₀ H ₄₀ O
18	13.14	Palmitic acid methyl ester	C ₁₇ H ₃₄ O ₂
19	13.33	Palmitic acid	C ₁₆ H ₃₂ O ₂
20	14.08	Phytol	C ₂₀ H ₄₀ O
21	14.17	9(E), 11(E)-Conjugated linoleic acid	C ₁₈ H ₃₂ O ₂
22	14.20	Linolenic acid	C ₁₈ H ₃₀ O ₂
23	14.30	Octadecanoic acid	C ₁₈ H ₃₆ O ₂
24	15.94	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C ₁₉ H ₃₈ O ₄
25	28.22	Glutinol	C ₃₀ H ₅₀ O

3.2 Antibacterial activity of extract

The present study showed that the acetone extract from the leaves of *H. hirsuta* could inhibit against 6 bacterial strains (Table 2). Among them, the extract exhibited the strongest antibacterial activity against *S. aureus* with the diameter of inhibition zone about 14.2±0.8mm, followed by *S. typhimurium* (13.7±0.8mm), *P. aeruginosa* (13.5±1.3mm), *S. enteritidis* (12.1±1.3mm), *E. coli* (11.3±1.0mm) and *B. cereus* (9.1±0.3mm). According to the results which were presented in Table 2, the inhibition zones of the extract against 3 studied bacteria, including *P. aeruginosa*, *S. enteritidis* and *S. aureus* were larger than those of positive control. The findings, therefore, suggest the potential application of acetone extract of leaves as the antimicrobial agent.

Table 2. Inhibition zone of acetone extract isolated from *H. hirsuta* leaves against six bacterial strains

Tested bacteria	Growth inhibition zone (mm)	
	Studied sample	Positive control
<i>Bacillus cereus</i>	9.3±0.3 ^a	18.3±0.6 ^b
<i>Escherichia coli</i>	11.3±1.0 ^a	13.5±0.5 ^b
<i>Pseudomonas aeruginosa</i>	13.5±1.3 ^b	10.0±1.0 ^a
<i>Salmonella enteritidis</i>	12.1±1.3 ^b	9.2±0.3 ^a
<i>Salmonella typhimurium</i>	13.7±0.8 ^a	13.5±0.9 ^a
<i>Staphylococcus aureus</i>	14.2±0.8 ^b	10.7±0.8 ^b

(^{a, b} superscript letters indicate statistically different values, P < 0.05)

The previous study showed that the extracts isolated from the various fractions of *H. hirsuta* roots, including chloroform, ethyl acetate could inhibit the growth of *S. aureus*, whereas n-hexan, butanol fractions could inhibit the growth of *Lactobacillus fermentum* and no inhibitory was observed with *E. coli*, *P. aeruginosa*, *S. enteritica* [8]. In another study, Tran and Vo demonstrated that the methanolic extract of *H. hirsuta* aerial parts as well as its fractions exhibited the inhibition of the growth against 4 pathogenic bacteria such as *E. coli*, *S. typhimurium*, *S. aureus* and *Streptococcus faecalis* [25]. The study results showed that there were differences in the antibacterial activity of each *H. hirsuta* parts as well as the solvent used to extract compounds present in *H. hirsuta*. In addition, the inhibition of all 6 tested bacterial strains by acetone extract in this study showed the effective extraction of antibacterial compounds from *H. hirsuta*.

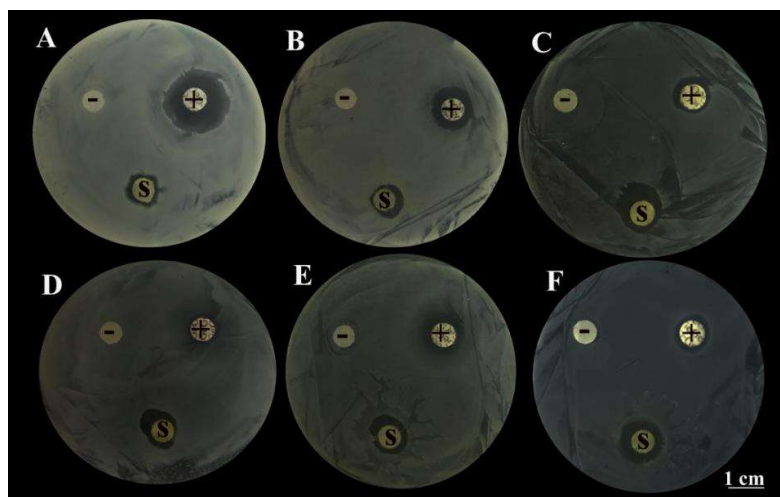


Figure 3. Antibacterial activity of acetone extract from *H. hirsuta* leaves against 6 bacterial strains. A. *B. cereus*, B. *E. coli*, C. *P. aeruginosa*, D. *S. enteritidis*, E. *S. typhimurium*, F. *S. aureus*. (-) Negative control with sterilized distilled water, (+) Positive control with discs containing gentamicin.

3.3 Antioxidant and cytotoxic activity of extract

The antioxidant activity of the acetone extract depended on the extract concentrations. An increase in DPPH_{RSA} could be controlled by the increase in extract concentrations. The DPPH radical inhibition achieved over 72% at the maximum concentration of extract (2.5 mg/mL). The IC₅₀ value of the extract was approximately 1.223 mg/mL. The antioxidant activity of the various fractions isolated from *H. hirsuta* root has been identified by one previous study. Accordingly, five fractions, including n-hexan, chloroform, ethyl acetate, butanol and water showed the antioxidant activity through DPPH scavenging capacity which the IC₅₀ values were >1.6 mg/mL, 0.415 mg/mL, 0.117 mg/mL, 0.252 mg/mL, 0.951 mg/mL, respectively [8]. Pham et al. demonstrated that the antioxidant capacity of methanol and water extracts of dried *H. hirsuta* leaves depended on the different drying conditions. The results showed that the IC₅₀ values of hot-air drying, low-temperature-air drying, Infrared drying and vacuum drying methods of methanol extracts were 3.28 mg/g, 3.35 mg/g, 3.01 g/mg and 4.73 mg/g, respectively whereas these values of water extract were 9.33 mg/g, 5.09 mg/g, 2.46 mg/g, 13.45 mg/g, respectively [26].

One human tumour cell line, hepatoma (Hep-G2), was inhibited by the acetone extract of *H. hirsuta* leaves. The results showed the IC₅₀ value of the studied extract was 381.8ppm, which causes 50% cell death. As mentioned above, the cytotoxic activity of the acetone extract isolated from the *H. hirsuta* leaves have not been elucidated yet. Some previous studies, however, showed the various solvents as well as fractions. For instance, Diem et al. showed the cytotoxicity of *H. hirsute* root fractions on KB cell line. Accordingly, three fractions, including n-hexan, ethyl acetate, butanol had cytotoxic effect on KB human cancer line which IC₅₀ values were 23.08 µg/ml, 3.23 µg/ml and 64.98 µg/mL, respectively [8]. In another study, Nguyen and Le demonstrated that petroleum ether, dichloromethane extracts isolated from *H. hirsuta* root were able to inhibit HepG2 cell line which IC₅₀ values were 28.29 µg/mL and 30.3 µg/mL, respectively [5]. Quang et al. proved the cytotoxic activity of the chemical constituents isolated from methanol extracts of leaves, stems and roots of *H. hirsute*. The results showed that five cancer lines, including SK-LU-1, HepG2, Hela,

SK-Mel-2 and AGS were killed by five compounds such as 3-*O-trans* caffeoylbetulinic acid, betulinic acid methyl ester, betulinic acid, lupeol and 5,8-dihydroxy-7,4-*O*-dimethoxyflavone [6]. Similarly, Nguyen et al. showed that the chemical compounds, including 3 β -*O*-acetylbetulinic acid; 4,4'-sulfinylbis(2-(*tert*-butyl)-5-methylphenol); 7-*O*-methylisoscuteallarein; 7,4'-di-*O*-methylisoscuteallarein, stigmasterol and β -sitosterol isolated from n-hexan, methylene chloride, ethyl acetate and methanol of *H. hirsute* aerial parts could inhibit four human cancer cell lines such as leukemia (CCRF-CEM), breast (MDA-MB-231), colon (HCT116) and glioblastoma (U251) [7].

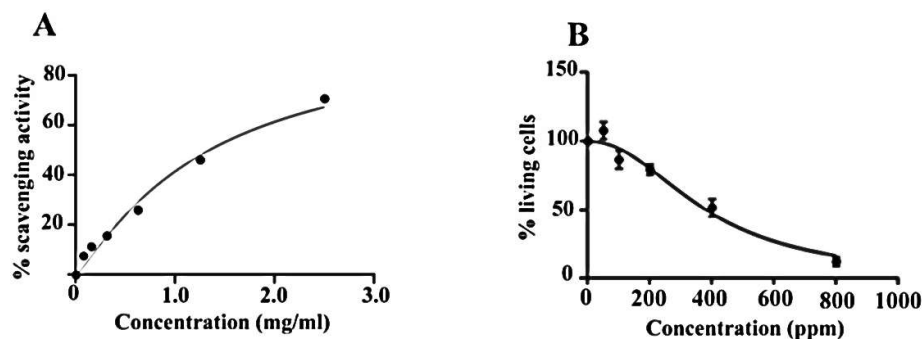


Figure 4. Radical scavenging activity (A) and cytotoxic activity (B) of the acetone extract from *H. hirsuta* leaves. Previous research has shown that extracting the plant in various solvents such as acetone, ethanol, and ethylacetate has biological activity. Former researchers utilized hazardous solvents, such as chloroform and methanol, to get the bioactive fraction in their pharmacological investigations, thus these solvents should be suggested. Plant extracts with a limited level of solvents are not acceptable materials to employ in the formulation of goods, particularly those for human consumption. As a result, customers choose food-grade solvents such as acetone, ethanol, and ethylacetate. [27, 28]

4. CONCLUSION

The present study identified twenty-five chemical compounds of acetone extract from the *Helicteres hirsuta* leaves. The studied extract was able to inhibit the growth of *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. typhimurium*, *B. cereus*, and *S. aureus*. Moreover, the extract also identified to have the antioxidant and cytotoxic activities in which Hepatoma (Hep-G2) cell line was killed with the IC₅₀ of 381.8 ppm whereas this value was 1.223 mg/mL in DPPH inhibition. The results obtained in the study have shown the effective bioactivity of acetone extract from the *H. hirsuta* leaves and are the basis for its application in the prevention and treatment of diseases in the future.

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**THÀNH PHẦN HÓA HỌC VÀ HOẠT TÍNH KHÁNG KHUẨN, KHÁNG OXY HÓA,
GÂY ĐỘC TẾ BÀO CỦA DỊCH CHIẾT ACETONE TỪ LÁ
HELICTERES HIRSUTA LOUR.**

Tóm tắt: Trong nghiên cứu hiện tại, 25 hợp chất hóa học của chiết xuất aceton từ lá *Helicteres hirsuta* đã được phân tích lần đầu tiên bằng phương pháp GC – MS, trong đó các hợp chất neophytadiene, metyl este của acid palmitic, acid palmitic, phytol, acid linolenic và acid octadecanoic đã được xác định là các thành phần chính. Bằng phương pháp khuếch tán đĩa thạch, chiết xuất aceton từ lá *Helicteres hirsuta* có khả năng ức chế sự phát triển của 6 chủng vi khuẩn kiểm nghiệm, bao gồm *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Bacillus cereus* và *Staphylococcus aureus*. Hơn nữa, các phương pháp DPPH và MTT đã được sử dụng để xác định các hoạt tính chống oxy hóa và gây độc tế bào của chiết xuất. Kết quả cho thấy chiết xuất có khả năng gây độc dòng tế bào ung thư gan (Hep-G2) với IC₅₀ là 381,8 ppm trong khi giá trị này là 1,223 mg/mL trong thử nghiệm ức chế DPPH.

Từ khóa: *Helicteres hirsuta*; GC/MS; hoạt tính kháng khuẩn, kháng oxy hóa, gây độc tế bào; cao chiết acetone.

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