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Isolation and Characterization of Bioactive Compounds from *Hedyotis diffusa* Willd. and Evaluation of their Antimicrobial, Antioxidant, Cytotoxic and Nitric Oxide Inhibitory Activities

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Hedyotis diffusa Willd. is traditionally used in Vietnamese and Chinese medicine for its therapeutic properties. This study aimed to explore the bioactive components of the ethyl acetate extract of *Hedyotis diffusa* Willd. and evaluate their potential antimicrobial, antioxidant and anticancer activities. The extract was collected using fractionated column chromatography and five compounds *viz*. cholestane, campesterol, stigmasterol, diosgenin and digitoxigenin were isolated and characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. The extract exhibited significant antimicrobial activity against bacteria (*Bacteroides fragilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes* at 80 mg/mL) and fungi (*Aspergillus flavus* and *Aspergillus parasiticus* at 80 mg/mL) associated with liver diseases. The extract demonstrated inhibitory activity against nitric oxide (NO) production in LPS-stimulated RAW264.7 cells at 20 µg/mL with low cell death rates 37,12% and DPPH scavenging which may be attributed to its high phenolic content. These findings suggest that *Hedyotis diffusa* Willd. extract holds promise as a therapeutic agent for liver damage and cancer, potentially through mechanisms beyond antioxidant activity.

Keywords: *Hedyotis diffusa*, Bioactive compounds, Antimicrobial activity, DPPH, Cytotoxicity, Nitric oxide inhibition.

INTRODUCTION

Hedyotis diffusa Willd. (Rubiaceae) (syn. *Oldenlandia diffusa* (Willd.) Roxb.), also known in Vietnamese as Luoi ran trang, is a plant distributed in tropical regions and warmer areas, including China, the Philippines, Malaysia, Laos, Cambodia and Vietnam. In both Vietnamese and Chinese traditional medicine, various parts of *H. diffusa* Willd., such as the aerial parts, berries, seeds, leaves, flowers, bark, roots and the whole plant, are commonly used for their heat clearing, detoxifying, dampness removing, anti-swelling, blood activating and analgesic properties [1-3]. Over 180 components have been identified in *H. diffusa* Willd., including anthraquinones, iridoids, flavonoids, triterpenes, coumarins, alkaloids and cyclotides, which have demonstrated various biological activities [4-6].

Previous studies have shown that flavonol glycosides in *H. diffusa* Willd. inhibit xanthine oxidase, cytochrome C and the thiobarbituric acid-malondialdehyde system, suggesting its potential antioxidant properties [7,8]. Cyclotides isolated from the leaves and roots have exhibited potent cytotoxicity against human prostate cancer cell lines, including PC3, DU145 and LNCaP, by preventing cell migration and invasion and reducing tumor size and weight [9]. Additionally, iridoid glycosides from *H. diffusa* Willd. extracts have shown inhibitory effects on the growth of various human tumor cell lines, such as HL-60, HeLa, HCT15, A459, HepG2, PC-3, CNE-2 and BCG-823 [10]. Furthermore, the abundant polysaccharides in *H. diffusa* Willd. have demonstrated antitumor, anti-inflammatory, antioxidant, immunity enhancing and anti-aging properties [11].

The antimicrobial properties of *H. diffusa* Willd. have also been reported, with studies indicating effectiveness against both Gram-negative and Gram-positive bacterial strains as well as fungi such as *Aspergillus niger*, *Aspergillus flavus*, *Shigella flexneri*, *Salmonella typhimurium* and *Escherichia coli* [12,13].

These findings suggest that *H. diffusa* Willd. extracts can kill or inhibit bacterial growth, making them potential candidates for antimicrobial agents. However, there is still a need for more comprehensive studies to understand the detailed mechanisms of the antibacterial and antifungal activities of *H. diffusa* Willd. and to explore its potential as an alternative treatment option amid growing concerns over antibiotic resistance.

This study aims to explore the bioactive components of *H. diffusa* Willd. extracts and their potential therapeutic applications, focusing specifically on their antimicrobial, antioxidant and anticancer properties. To achieve this, we isolated and identified the active compounds present in the extracts using chromatography techniques and structurally characterized them using mass spectrometry and NMR analysis. The antimicrobial efficacy of the extracts was assessed against a range of microbial strains using the agar disk diffusion method. In addition, the antioxidant properties were evaluated *via* DPPH radical scavenging assay, while the cytotoxicity was examined using the MTT assay and nitric oxide production inhibition assay. By systematically investigating these activities, the study seeks to provide a deeper understanding of the therapeutic potential of *H. diffusa* Willd. extracts and contribute to the development of novel natural products for combating infectious diseases, cancer and other health conditions.

EXPERIMENTAL

Plant collection: The plant samples (flowers, leaves, stems and roots) was collected from Hoai Duc district, Hanoi, Vietnam. The plant sample was identified as *H. diffusa* Willd. by Dr. Trieu Anh Trung, Faculty of Biology, Hanoi National University of Education. All parts of the plant were washed, dried at 40 ºC for 24 h and then ground into a powder.

Extract preparation and fraction collection: The powder (3 kg) was soaked in ethyl acetate for 3 days at room temperature. The content was filtered through cotton and then evaporated under reduced pressure to yield a crude extract, *H. diffusa* (207.20 g). A part of the *H. diffusa* extract (80.00 g) was separated using silica gel column chromatography, eluting with a gradient system of *n*-hexane:ethyl acetate (20:1-0:1 % v/v) to provide 7 fractions (HD1-HD7). Similarly, 4 fractions (HD2.1-HD2.4) of HD2 were obtained after silica gel column chromatography (CC) with *n*-hexane:ethyl acetate (7:1-0:1, % v/v). They were further fractionated to isolate the active compounds. Fraction HD2.2 was purified by silica gel CC eluting with *n*-hexane:acetone 4:1 to give compound **1** (10.00 mg). Fraction HD2.3 was purified by silica gel CC eluting with *n*-hexane:-acetone 2:1 (v/v) to give compound **2** (12.00 mg). Fraction HD2.4 was purified by silica gel CC eluting with *n*-hexane: ethyl acetate 1:1 to give compound **3** (15.00 mg). Fraction HD3 were separated with silica gel CC eluting with *n*-hexane: ethyl acetate 4:1 (v/v) to give compound **4** (9.00 mg) and compound **5** (11.00 mg).

Cholestane (1): White powder. ESI-MS (positive-ion): *m/z* 372.6 $[M + H]^+$ ($C_{27}H_{48}$). ^1H NMR ($CDCl_3$, 500 MHz) δ ppm: 1.58 (2H, m, H-1), 1.53 (2H, m, H-2), 1.43 (2H, m, H-3), 1.67 (2H, m, H-4), 1.19 (1H, brs, H-5), 1.71 (2H, m, H-6), 1.63 (2H, m, H-7), 1.14 (1H, m, H-8), 1.07 (1H, ddd, *J* = 13.3, 4.5, 1.8 Hz, H-9), 1.65 (2H, d, *J* = 13.0 Hz, H-11), 1.56 (2H, m, H-12), 1.14 (1H, m, H-14), 1.65 (2H, m, H-15), 1.92 (2H, m, H-16), 1.13 (1H, m, H-17), 0.84 (3H, s, H-18), 0.81 (3H, s, H-19), 1.30 (1H, m, H-20), 0.88 (3H, d, *J* = 6.8 Hz, H-21), 1.20 (2H, m, H-22), 1.31 (2H, m, H-23), 1.19 (2H, m, H-24), 1.46 (1H, m, H-25), 0.86 (3H, d, *J* = 6.8 Hz, H-26), 0.78 (3H, d, *J* = 6.8 Hz, H-27). ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 39.7 (C-1), 25.6 (C-2), 27.3 (C-3), 34.5 (C-4), 47.2 (C-5), 29.1 (C-6), 34.6 (C-7), 37.1 (C-8), 54.8 (C-9), 35.2 (C-10), 23.7 (C-11), 43.2 (C-12), 45.1 (C-13), 56.6 (C-14), 23.2 (C-15), 28.9 (C-16), 57.1 (C-17), 13.7 (C-18), 13.1 (C-19), 36.9 (C-20), 20.2 (C-21), 38.0 (C-22), 24.6 (C-23), 38.1 (C-24), 28.1 (C-25), 23.1 (C-26), 22.7 (C-27).

Campesterol (2): White powder. ESI-MS (positive-ion): *m/z* 400.6 $[M + H]^+$ ($C_{28}H_{48}O$). ^1H NMR ($CDCl_3$, 500 MHz) δ ppm: 1.32 (2H, m, H-1), 1.55 (2H, m, H-2), 3.55 (1H, dd, *J* = 11.0, 3.8 Hz, H-3), 2.22 (2H, m, H-4), 5.26 (1H, m, H-6), 2.20 (2H, t, *J* = 3.6 Hz, H-7), 1.30 (1H, m, H-8), 1.22 (1H, m, H-9); 1.62 (2H, m, H-11), 1.33 (2H, t, *J* =3.8 Hz, H-12), 1.04 (1H, m, H-14), 1.88 (2H, m, H-15), 1.92 (2H, m, H-16), 1.20 (1H, m, H-17), 0.85 (3H, s, H-18), 0.95 (3H, s, H-19), 1.50 (1H, m, H-20), 1.01 (3H, d, *J* = 6.8 Hz, H-21), 1.19 (2H, m, H-22), 1.19 (2H, m, H-23), 1.35 (1H, m, H-24), 0.98 (3H, s, H-25), 1.41 (1H, m, H-26), 0.83 (3H, d, *J* = 6.5 Hz, H-27), 1.01 (3H, d, *J* = 6.5 Hz, H-28), 1.58 (1H, s, 3-OH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 37.2 (C-1), 31.5 (C-2), 72.0 (C-3), 42.0 (C-4), 140.2 (C-5), 130.8 (C-6), 31.0 (C-7), 30.5 (C-8), 51.2 (C-9), 37.9 (C-10), 22.0 (C-11), 41.9 (C-12), 44.9 (C-13), 58.1 (C-14), 26.3 (C-15), 25.9 (C-16), 58.6 (C-17), 14.0 (C-18), 19.3 (C-19), 35.9 (C-20), 19.4 (C-21), 33.6 (C-22), 32.4 (C-23), 39.5 (C-24), 17.4 (C-25), 33.2 (C-26), 20.7 (C-27), 21.9 (C-28).

Stigmasterol (3): White crystals. ESI-MS (positive-ion): *m/z* 412.7 $[M + H]^+$ ($C_{29}H_{48}O$). ^1H NMR ($CDCl_3$, 500 MHz): δ (ppm): 1.33 (2H, m, H-1), 1.60 (2H, m, H-2), 3.33 (1H, dd, *J* = 11.0, 3.8 Hz, H-3), 2.01 (2H, m, H-4), 5.38 (1H, brd, H-6), 2.19 (2H, t, *J* = 3.8 Hz, H-7), 1.30 (1H, m, H-8), 1.11 (1H, m, H-9), 1.38 (2H, m, H-11), 1.56 (2H, m, H-12), 1.15 (1H, m, H-14), 1.65 (2H, m, H-15), 1.90 (2H, m, H-16), 1.31 (1H, m, H-17), 0.71 (3H, s, H-18), 0.88 (3H, s, H-19), 2.08 (1H, m, H-20), 1.11 (3H, d, *J* = 7.0 Hz, H-21), 5.15 (1H, dd, *J* = 8.5, 15.0 Hz, H-22), 5.02 (1H, dd, *J* = 8.5, 15.0 Hz, H-23), 1.88 (1H, m, H-24), 1.64 (1H, m, H-25), 0.81 (3H, d, *J* = 7.0 Hz, H-26), 0.68 (3H, d, *J* = 9.0 Hz, H-27), 1.44 (2H, m, H-28), 0,84 (3H, t, *J* = 8.5 Hz, H-29), 1.55 (1H, s, 3-OH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 34.1 (C-1), 27.4 (C-2), 71.8 (C-3), 42.3 (C-4), 140.8 (C-5), 121.7 (C-6), 30.5 (C-7), 35.7 (C-8), 53.9 (C-9), 35.2 (C-10), 21.1 (C-11), 39.7 (C-12), 42.9 (C-13), 56.8 (C-14), 24.3 (C-15), 28.6 (C-16), 56.4 (C-17), 12.3 (C-18), 18.9 (C-19), 41.1 (C-20), 20.5 (C-21), 138.3 (C-22), 129.5 (C-23), 51.6 (C-24), 32.0 (C-25), 21.1 (C-26), 19.0 (C-27), 25.9 (C-28), 13.0 (C-29).

Diosgenin (4): White powder. ESI-MS (positive-ion): *m/z* 414.6 $[M + H]^+$ ($C_{27}H_{42}O_3$). ^1H NMR ($CDCl_3$, 500 MHz) δ ppm: 1.31 (2H, m, H-1), 1.52 (2H, m, H-2), 3.53 (1H, dd, *J* = 11.0, 3.8 Hz, H-3), 2.29 (2H, m, H-4), 5.27 (1H, brd, H-6), 2.94 (2H, t, *J* = 4.0 Hz, H-7), 1.47 (1H, m, H-8), 1.22 (1H, m, H-9), 1.63 (2H, m, H-11), 1.36 (2H, m, H-12), 1.20 (1H, m, H-14),

1.46 (2H, m, H-15), 3.55 (1H, q, *J* = 15.4, 7.5 Hz, H-16), 2.01 (1H, m, H-17), 0.90 (3H, s, H-18), 0.93 (3H, s, H-19), 1.88 (1H, m, H-20), 0.88 (3H, d, *J* = 7.5 Hz, H-21), 1.02 (2H, m, H-23), 1.78 (2H, m, H-24), 1.80 (1H, m, H-25), 4.01 (2H, t, *J* = 11.0 Hz, H-26), 0.95 (3H, d, *J* = 7.0 Hz, H-27), 1.85 (1H, s, 3-OH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 40.0 (C-1), 32.4 (C-2), 68.9 (C-3), 45.0 (C-4), 151.0 (C-5), 131.1 (C-6), 28.7 (C-7), 41.4 (C-8), 60.0 (C-9), 40.1 (C-10), 30.5 (C-11), 45.9 (C-12), 39.9 (C-13), 60.1 (C-14), 32.8 (C-15), 83.0 (C-16), 68.6 (C-17), 18.5 (C-18), 20.3 (C-19), 50.7 (C-20), 16.9 (C-21), 101.9 (C-22), 31.3 (C-23), 26.5 (C-24), 31.1 (C-25), 73.2 (C-26), 18.0 (C-27).

Digitoxigenin (5): White powder. ESI-MS (positive-ion): *m/z* 374.5 $[M + H]^+$ ($C_{23}H_{34}O_4$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ ppm: 1.40 (2H, m, H-1), 1.71 (2H, m, H-2), 4.01 (1H, dd, *J* = 11.5, 4.0 Hz, H-3), 1.66 (2H, m, H-4), 1.53 (2H, m, H-5), 1.63 (2H, m, H-6), 1.84 (2H, m, H-7), 1.22 (1H, m, H-8), 1.58 (1H, m, H-9), 1.38 (2H, m, H-11), 1.31 (2H, m, H-12), 1.23 (2H, m, H-15), 1.83 (2H, m, H-16), 1.89 (1H, t, *J* = 8.5 Hz, H-17), 1.32 (3H, s, H-18), 0.81 (3H, s, H-19), 4.92 (2H, d, *J* = 16.0 Hz, H-21), 5.93 (1H, s, H-22), 1.55 (1H, s, 3-OH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ ppm: 33.6 (C-1), 29.7 (C-2), 70.2 (C-3), 36.1 (C-4), 40.0 (C-5), 27.1 (C-6), 30.3 (C-7), 37.7 (C-8), 45.1 (C-9), 35.5 (C-10), 20.8 (C-11), 42.8 (C-12), 47,4 (C-13), 79.2 (C-14), 33.6 (C-15), 26.2 (C-16), 51.0 (C-17), 14.4 (C-18), 12.9 (C-19), 174.8 (C-20), 73.6 (C-21), 118.1 (C-22), 177.7 (C-23).

Antimicrobial assay: The antibacterial and antifungal activities of *H. diffusa* extract were investigated by the agar disk diffusion method [14,15]. In this study, the bacterial strains, including Gram-negative bacteria *Escherichia coli*, *Bacteroides fragilis*, *Klebsiella pneumoniae*, *Salmonella typhi* and Gram-positive bacteria *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus* and the fungal strains *Aspergillus flavus*, *Aspergillus parasiticus* were used.

The bacterial strains were grown in Mueller-Hinton agar plates which includes 2% glucose whereas the fungi were grown in Sabouraud dextrose agar plates. The volume of each microorganism was 1 mL per petri dish. The crude extract was used for inhibition screening of the bacterial and fungal strains, which were prepared at the concentration scale of 20.00, 40.00, 60.00, 80.00 mg/mL in 10% DMSO. The test solutions used were 10% DMSO as the negative control, gentamicin 10 (µg/mL) and ketoconazole 10 (µg/mL) as the positive control.

The sterile media agar plates were seeded with bacterial and fungal strains at 37 ºC for 3 h. The wells were 8.0 mm in diameter. The experimental solutions (50 µL) were added to the wells. Each type of bacterial and fungal strains was examined on an agar plate, including the positive and negative control wells. The agar plates were incubated at 37 ºC for 24 h for bacterial strains and at 28 ºC for 48 h for fungal strains. The microorganism species inhibition ability of *H. diffusa* Willd. extract was determined by measuring the sizes of inhibitory zones on the agar surface around the disks and values < 8.0 mm were considered not active against microorganisms (Table-1).

DPPH radical scavenging assay: The antioxidant activity of *H. diffusa* Willd extract was determined the scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical as described earlier [16]. A solution of DPPH 300 mM in MeOH was prepared. The *H. diffusa* Willd. extract were dissolved in DMSO to prepare the concentrations range from 2 to 256 µg/mL. The sample: 10 µL of a different concentration of crude extract sample was added 190 µL DPPH. The reaction mixture was incubated at 37 ºC for 30 min. The absorbance of the mixture was measured at λ = 517 nm. The control reaction was 10 µL DMSO 0.5% mixed with 190 µL DPPH. Ascorbic acid was used as the comparison standard. The percent of DPPH radical scavenging activity was calculated by following equation:

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_{\text{conrol}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

Cell culture: Raw264.7 cell lines were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic antimycotic solution (streptomycin and penicillin) at 37 ºC in a humidified incubator under 5% CO_2.

Cytotoxicity assay: The cytotoxicity of extract was determined by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay [17]. Cells were seeded in a 96-well plate at a density of 2×10^5 cells/well and allowed to adhere overnight. Then, the cell was exposed to with various concentrations of extract ranging from 0.032 to 100 µg/mL for 24 h. MTT was added to a final concentration of 0.5 mg/mL and continuously incubated for 4 h at 37 ºC. DMSO 150 mL was added to dissolve the formazan crystals after removing the medium. Doxorubicin in a concentration range of 0.032 to 100 µg/mL were used as the positive control and the cells treated with a diluted solution of DMSO (1.0%) were used as the negative control. The absorbance was measured at 570 nm using an ELISA reader. The cell death (%) was determined by the following formula:

$$\text{Cell death } (\%) = 100 - \left(\frac{A_{\text{treated cell}} - A_{\text{positive control}}}{A_{\text{negative control}} - A_{\text{positive control}}} \right) \times 100$$

***In vitro* nitric oxide assay:** The NO production inhibitory activity was conducted by following the method as described by Giang *et al.* [17]. Raw264.7 cells were activated with 1 µg/mL LPS (lipopolysaccharide) and tested for anti-inflammatory ability using concentrations of 0.032 to 100 µg/mL of *H. diffusa* extract, for 24 h incubated, 37 ºC, 5% CO_2. Then, 50 µL Griess reagent was added to the wells and continued incubation for 10 min. The absorbance was measured at 570 nm by using a microplate reader. The negative and positive control were described in the previous experiment. The nitrite content was calculated from $NaNO_2$ standard curve. The ability of NO inhibition of a sample was calculated by the following formula:

$$\text{Inhibition } (\%) = 100\% - \left(\frac{\text{Conc. of NO}_{\text{sample}}}{\text{Conc. of NO}_{\text{negative control}}} \right) \times 100$$

RESULTS AND DISCUSSION

The ethyl acetate extract of *H. diffusa* Willd. was separated by using column chromatography and eluting with the stepwise gradient solvent system of increasing polarity (*n*-hexane:ethyl

acetate, v/v), a total of 7 fractions were obtained. Five compounds were isolated from the chromatographic fractions and their structures were determined on the basis of mass spectroscopy and NMR analysis as cholestane (**1**), campesterol (**2**), stigmasterol (**3**), diosgenin (**4**), digitoxigenin (**5**) (Fig. 1). The NMR data were in agreement with the reference values [18-22].

The organic solvent was completely removed from the extract using a vacuum rotary evaporator until the weight of the extract remained constant. Therefore, the ethyl acetate solvent is guaranteed not to be present in the extract, so the solvent did not influence the forming of the inhibition zones in the antimicrobial test.

Liver abscess is a disease with 80% being caused by bacteria and 10% being caused by fungi. If not promptly diagnosed and treated, liver abscesses can increase the risk of liver cancer [23-26]. The main bacterial culprits include intestinal and biliary tract bacteria such as *E. coli*, *Staphylococcus*, *Klebsiella*, *Streptococcus*, *Enterococcus* and *Bacteroides* [27].

The *H. diffusa* Willd. extract showed a significant potential to inhibit several bacterial and fungal strains involved in liver diseases. Interestingly, the extract showed strongest inhibition effects on several microorganisms including *Bacteroides fragilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes* at 80 mg/mL of crude extract, the inhibition zone diameters ranged from 14.9-15.9 mm, positive control zones ranged between 31.0-35.00 mm. The weaker effects of this extract for *E. coli*, *S. typhi* and *B. cereus*, the inhibition zone diameters ranged from 9.8-10.5 mm (Table-1). According to the recorded health statistics in China, Taiwan and other Asian countries which has similar climatic conditions to Vietnam, the liver abscess patients caused by *K. pneumoniae* 12.3% [28]. These results suggested that the ethyl acetate extract of *H.*

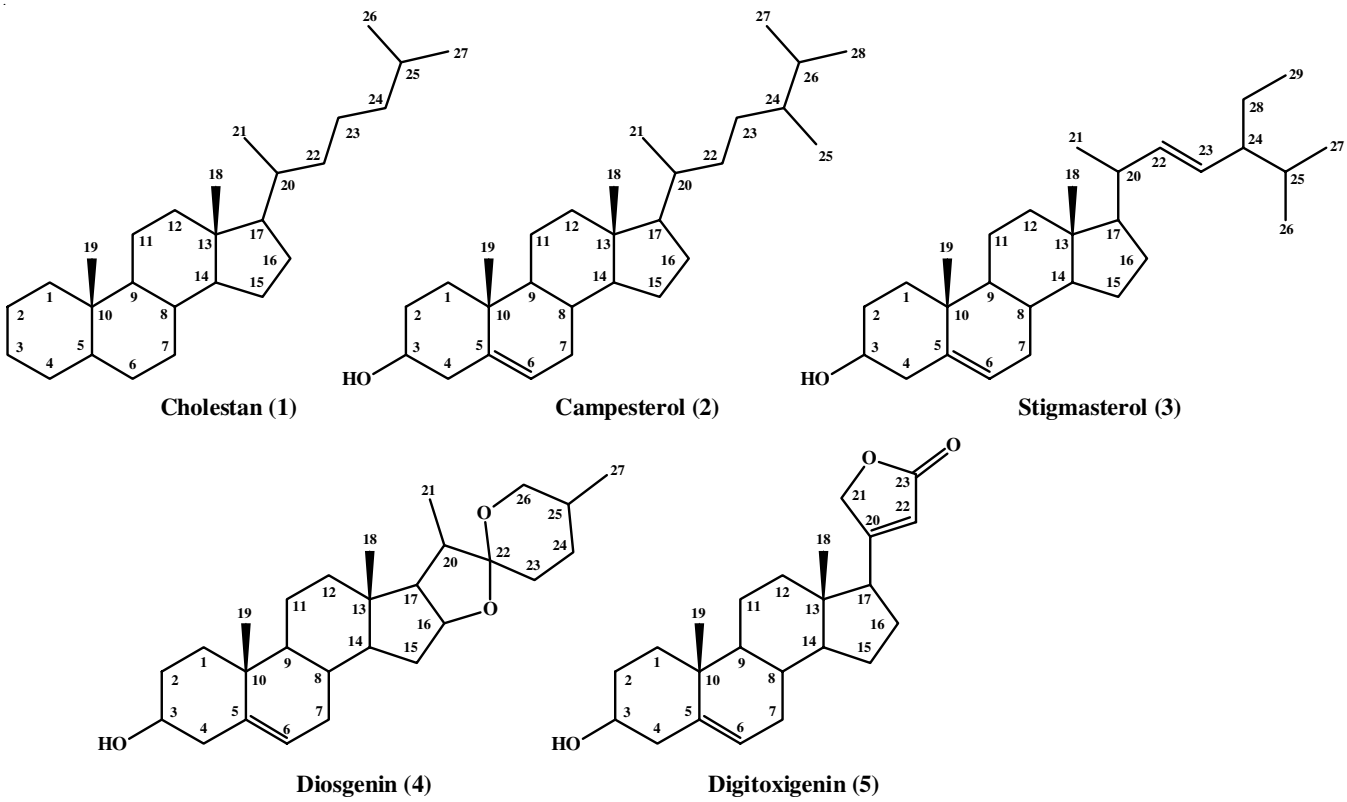

Fig. 1. Structure of compounds **1-5** isolated from the ethyl acetate extract of *Hedyotis diffusa* Willd.

TABLE-1
THE INHIBITION ZONE DIAMETER OF *H. diffusa* EXTRACT AGAINST MICROORGANISMS

	Diameter of inhibition zone (mm)					
	Concentration (mg/mL)				Ketoconazole (10 mg/mL)	Gentamicin (10 mg/mL)
	20	40	60	80		
Escherichia coli	Not detected	8.0	9.3	9.8		34.0
Bacteroides fragilis	Not detected	9.7	11.6	14.9		35.0
Klebsiella pneumoniae	Not detected	10.1	12.1	15.5		35.0
Salmonella typhi	Not detected	8.0	9.8	10.5		32.0
Staphylococcus aureus	Not detected	10.9	13.2	15.9		35.0
Streptococcus pyogenes	Not detected	10.7	12.1	13.8		35.0
Bacillus cereus	Not detected	8.0	8.8	10.1		31.0
Aspergillus flavus	Not detected	10.3	14.1	16.0	25.0	
Aspergillus parasiticus	Not detected	10.2	13.8	16.0	21.0	

diffusa Willd. has a potential ability to protect the liver through the inhibition of the causal agents related to liver damage.

In the fungi tested, the *H. diffusa* Willd. extract demonstrated high activity against for *A. flavus* and *A. parasiticus* with inhibition zone diameters expressed 16.00 mm for both, with ketoconazole as the standard at 10 µg/mL showing the control zone of 21.00-25.00 mm (Table-1). *A. flavus* and *A. parasiticus* often contaminate food and feed which mainly produce aflatoxin, a kind of dangerous mycotoxin [29]. Aflatoxin B1 is known for its carcinogenic, teratogenic, mutagenic and immunosuppressive properties. Consequently, the International Agency for Research on Cancer (IARC) has classified it as a human carcinogen [30]. Overall, the results indicate that *Hedyotis diffusa* Willd. extract holds significant potential for development as a therapeutic agent in the treatment of liver damage and cancer.

The radical scavenging ability of the crude extracts and fractions of *H. diffusa* Willd. were measured using the DPPH scavenging assay (Fig. 2). The results showed that the most of the fractions were able to scavenge DPPH radicals. However, the scavenge activity of the fractions showed a concentration dependent manner from 2 to 64 µg/mL. The increase of concentration of fractions into 256 µg/mL did not show a significant difference compared to those of 64 µg/mL (Fig. 2).

Fractions HD3, HD4, HD5, HD6 and HD7 showed a stronger radical scavenging ability than the fractions HD1 and HD2 (Fig. 2). However, most of fractions did not show strong radical scavenging activity compared to the standard ascorbic acid, except the fraction HD6 at the concentration of 256 µg/mL. Interestingly, the fractions obtained from the experiments showed a low DPPH activity compared to the standard, but the crude extract showed a strong ability to kill the cancer cells (Fig. 3). This suggests that the anticancer activity of the crude extract may be attributed to mechanisms other than antioxidant activity, potentially involving direct cytotoxic compounds or other bioactive constituents.

To investigate the ability of kill the cancer cells and anti-inflammation of the crude extracts we have used cytotoxicity and nitric oxide assays. Nitric oxide (NO) is essential for regulating functions like blood pressure and immune response [31, 32]. However, excessive NO can cause tissue damage and is linked to inflammatory diseases such as atherosclerosis and hypertension. This has driven research toward natural antioxidants that inhibit NO production in treating chronic inflammation [33]. Bacterial lipopolysaccharides (LPS) activate murine macrophage RAW264.7 cells, a suitable model for assessing NO production inhibition.

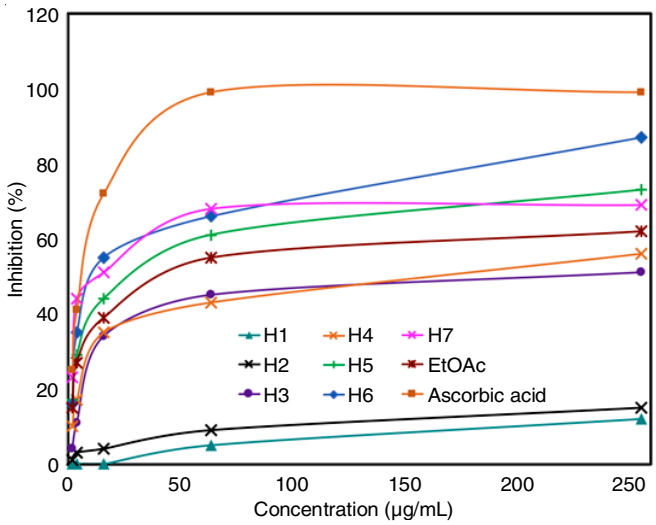

Fig. 2. DPPH scavenging activity of the crude extracts from *H. diffusa* Willd. The data showed % inhibition of different concentration of *H. diffusa* extract and fractions

The results showed that the ability to kill the cells of the crude extract was similar to the standard doxorubicin in a concentration dependent manner, except at the 20 µg/mL (Fig. 3a). At this concentration, specific compounds within the *H. diffusa* Willd. extract may exhibit differential activity, where certain components that promote cell survival or inhibit cytotoxicity become more prominent, unlike the consistent action seen with doxorubicin. Additionally, the components of the extract might interact synergistically or antagonistically at this concentration, leading to a reduced overall cytotoxic effect, which does not occur with the uniform mechanism of a single compound like

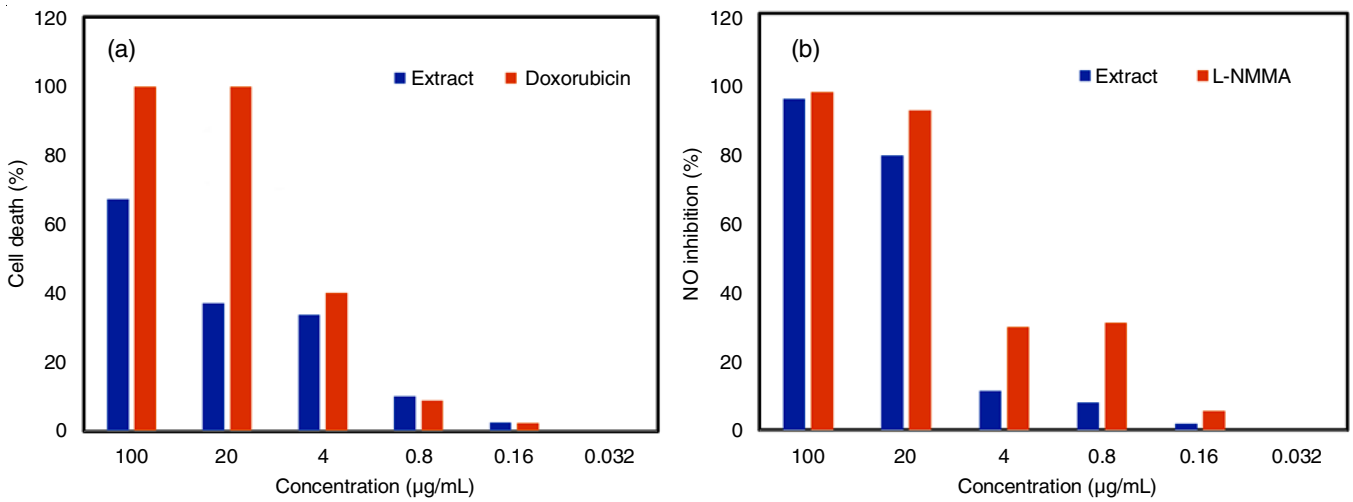

Fig. 3. Percentage of cell death (a) and *in vitro* nitric oxide assay (b) using crude extract of *H. diffusa* Willd.

doxorubicin. The crude extract showed a significant ability to inhibit NO production compared to the standard L-NMMA at the concentrations of 0.16 to 100 µg/mL, except 0.032 µg/mL, measured *via* the Griess assay. The highest nitric oxide inhibitory activity value was observed at 100 µg/mL with 92.35% but 67.23% cell death. Concentrations of 0.16-4 µg/mL showed high cell viability ranging from 97.47-66.23% however poor inhibition of NO production with 1.98-11.31%. The sample concentration of 20 µg/mL for the nitrite assay was evaluated as over 75% inhibition, 0.86-fold less than the L-NMMA standard and toxic to RAW264.7 cells with 37.12% (Fig. 3a-b).

Conclusion

In this work, five compounds *viz*. cholestane, campesterol, stigmasterol, diosgenin and digitoxigenin were successfully extracted and identified from the ethyl acetate extract of *Hedyotis diffusa* Willd. The extract demonstrated significant antimicrobial activity, particularly against bacterial strains associated with liver abscesses and fungal strains like *Aspergillus flavus* and *Aspergillus parasiticus*, which are known for producing the carcinogenic aflatoxin B1. The extract also demonstrated inhibitory activity against nitric oxide (NO) production in LPS-stimulated RAW264.7 cells, an effect that is likely linked to its high total phenolic content. Phenolic compounds are known inhibitors of NO and peroxynitrite production, contributing to the anti-inflammatory and anticancer properties observed [34]. Despite its relatively low antioxidant activity in the DPPH assay, the NO inhibition suggest that the therapeutic effects of *H. diffusa* Willd. extract may be attributed to mechanisms involving direct cytotoxicity and modulation of inflammatory pathways. These findings highlight the potential of *H. diffusa* Willd. extract as a source of novel therapeutic agents for managing liver diseases and cancer, warranting further investigation and development.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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