CHEMICAL COMPOSITION AND ANTIFUNGAL EFFICACY OF STEM AND LEAF EXTRACTS OF V*itex negundo* L.

Đến tòa soạn 05-07-2024

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TÓM TẮT

THÀNH PHẦN HÓA HỌC VÀ HIỆU QUẢ KHÁNG NẤM CỦA CAO CHIẾT TỪ THÂN VÀ LÁ CÂY HOÀNG KINH (*Vitex negundo* L.)

Cây Hoàng kinh (Vitex negundo L.), là một trong những loại thảo dược quan trọng thuộc họ Verbenaceae. Mục đích của nghiên cứu này là phân lập và nhận danh các hoạt chất có hoạt tính kháng nấm của loài cây này, sau khi đã nghiên cứu về hoạt tính kháng nấm của cao chiết tổng ethanol và các cao chiết phân đoạn. Sắc ký cột của cao chiết ethyl acetate, là cao cho hoạt tính kháng nấm hiệu quả, đã phân lập và nhận danh ba hợp chất là: 4-hydroxybenzoic acid (1), sitosterol 3-O-*β*-D-glucopyranoside (2), và 3,4-dihydroxybenzoic acid (3). Hoạt tính kháng nấm của cao chiết tổng ethanol cũng như các cao chiết phân đoạn và các hợp chất phân lập được đối với hai chủng nấm Botryosphaeria dothidea và Lasiodiplodia theobromae, là nấm phổ biến gây hại cho nền nông nghiệp Việt Nam, được tiến hành bằng phương pháp gây độc môi trường. Các kết quả thu được cho thấy cao ethyl acetate và hợp chất (1), (3) đã thể hiện hoạt tính kháng nấm hiệu quả đối với chủng nấm *Lasiodiplodia theobromae* với phần trăm ức chế đạt lần lượt là 96,9%, 88,6% và 90,7% tại nồng độ 312.5 µg/mL; từ đó cho thấy rằng cây hoàng kinh (*Vitex negundo* L.) là một dược liệu tiềm năng về khả năng kháng nấm.

Từ khóa: kháng nấm, thành phần hóa học, cây Hoàng kinh.

1. INTRODUCTION

Plants and their derivatives have a long-standing history as sources of potential chemotherapeutic agents in medicine. *Vitex negundo* L., belonging to the Verbenaceae family, is extensively utilized in traditional medicine and is known for its diverse pharmacological properties, including analgesic, anti-inflammatory, anti-rheumatic, antitumor, insecticidal, antimicrobial, and antioxidant activities. This species contains bioactive compounds such as flavonoids, lignans, terpenoids, and volatile oils [1,2].

Bacterial infections are identified as a major cause of death in agriculture. The

escalating resistance of bacteria to commercial antibiotics emphasizes the critical need for discovering novel active substances with antibacterial properties effective against pathogenic bacteria [3].

In Vietnam, a few studies have examined the essential oil of *Vitex negundo* L. leaves. GC-MS analysis identified caryophyllene oxide (23.36%) and valencene (21.95%) as the main compounds in summer, while *β*-caryophyllene (40.60%) and sabinene (12.14%) dominated in winter. Another study demonstrated that the essential oil exhibited antibacterial activity against *Vibrio vulnificus* using the Ariole and Nyeche method. Furthermore, the research seeks to establish the formula and preparation process for products derived from *V. negundo* leaves, quantify their total flavonoid content, and explore their preliminary anti-inflammatory effects [4-6]. Research on the chemical composition and biological activity of extracts from this plant species remains limited. Therefore, this paper describes the antifungal activity of *V. negundo* as well as the isolation and structural determination of some compounds from the extract which have given the effective antifungal activity in order to know more the relationship between biological activity and chemical structure of compounds in this species.

2. EXPERIMENT

2.1 Chemicals and reagents

NMR spectra were recorded on a Bruker Advance 600-MHz NMR spectrometer (Bruker, Karlsruhe, Germany) at Institute of Chemistry - Vietnam Academy of Science and Technology, Hanoi, Vietnam. TLC was performed on silica gel 60 F_{254} (0.063–0.200mm, Merck, Germany). The detection of zones was carried out using a UV lamp at wavelengths of 254 nm or 365 nm, or by applying a solution of $FeCl_3$/EtOH or H_2SO_4/EtOH. Column chromatography was conducted utilizing silica gel (240-430 mesh, Merck, Germany) and silica gel (70-230 mesh, Merck, Germany).

The solvents employed in this study, including *n*-hexane, chloroform, ethyl acetate, methanol (≥ 99.0% purity), and ethanol (96%), were obtained from Chemsol Company, Vietnam.

Biological safety cabinet (Class II BSC, Esco, Indonesia), autoclave (HVE-50, Hirayama, Japan), centrifuge (Mikro 12-24, Hettich, Germany), were measured for antifungal activity.

2.2 Preparation and processing of samples

The stems and leaves of *Vitex negundo* L. were harvested in Hau Giang city and verified by Dr. Nguyen Thi Kim Hue, a biologist from the Department of Biology at the College of Natural Sciences, Can Tho University. A voucher specimen has been deposited under the number ViN122023. The samples were carefully cleaned to eliminate mud and dust, and any rotten or damaged parts were removed. The raw materials were then left to air-dry in the shade at room temperature for several days, followed by oven-drying at approximately 50°C until fully desiccated.

2.3 Extraction and isolation of the compounds

The thoroughly dried plant material (4.0 kg) was ground into powder, subsequently soaked in 96% ethanol four times (20 L each time) at ambient temperature, and filtered. The solvent was then evaporated under reduced pressure, yielding 350 g of crude extract. The extract was suspended in water and sequentially partitioned with *n*-hexane and ethyl acetate. Each solvent extraction was repeated multiple times. After vacuum evaporation, the resulting fractions yielded 60 g of *n*-hexane extract and 40 g of ethyl acetate extract.

The ethyl acetate fraction, exhibiting potent antifungal activity, underwent chromatography on a silica gel column using a gradient of *n*-hexane and ethyl acetate up to 100% ethyl acetate, followed by a gradient of ethyl acetate and methanol up to 100% methanol, resulting in the collection of eight fractions (**EAF1-8**). Fraction **EAF1** was re-chromatographed with a chloroform and methanol gradient (25:1 to 0:100, v/v). Ten fractions (EAF1.1-EAF1.10) were collected, yielding 40 mg of compound **1** from subfraction **EAF1.2**.

By using similar methods, subfraction **EAF1.3** was further separated on a silica gel CC and eluted with C: MeOH (40:1 to 0:100, v/v) to yield seven subfractions (**EAF1.3.1-7**). Compound **2** (24 mg) and compound **3** (60 mg) were obtained respectively from subfraction **EAF1.3.2** and **EAF1.3.3**.

2.4 Antifungal activity

Antifungal activity of the plant extracts and isolated compound were tested using the poisoned food techniques described by Adjou *et al.* (2012) [7]. PDA medium was prepared and the test sample was added to achieve concentrations: 312.5, 625, 1250, 2500, 5000 and 10000 µg/mL Harmful fungal strains are carved into small pieces about 6 mm in diameter. Use sterile forceps to pick up the fungal pieces and place them in the middle of petri dishes containing the extract. Mycelium diameter was read after 7 days of incubation at 28^{o}C. At the end of this period, all plates were examined for any zones of inhibition. The results were assessed by observing the zones where fungal growth was inhibited on each plate, and the diameter of each inhibition zone was measured and recorded accordingly. The percentage of inhibition of mycelial growth was calculated according to the following formula: (%) = $(1 - dt/dc) \times 100$; where dc (mm) was the mean colony diameter for the control sets and dt (mm) was the mean colony diameter for the treatment sets. The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration of an antimicrobial agent required to prevent visible growth of a microorganism after a specified period, under standardized conditions. [8, 9].

The tested fungi in this study consisted of *Botryosphaeria dothidea* and *Lasiodiplodia theobromae* which cause serious diseases in many agriculture regions in Viet Nam.

3. RESULTS AND DISCUSSION

3.1 Structure elucidation

The structures of the isolated compounds were characterized using NMR spectra and by comparing the spectral data with published literature.

*3.1.1 Compound **1***

Compound **1** was isolated in the form of a white crystalline solid, m.p. 213-215°C

^{1}H-NMR (600 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 7.90 (2H, *d*, *J* = 9.0 Hz, H-2,6); 6.84 (2H, *d*, *J* = 8.4 Hz, H-3,5).

^{13}C-NMR (150 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 170.1 (C-7); 163.3 (C-4); 133.0 (C-2,6); 122.7 (C-1); 116.0 (C-3,5).

*3.1.2 Compound **2***

Compound **2** was obtained as white powder, m.p. 276-278°C

^{1}H-NMR (600 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 5.37 (1H, *d*, *J* = 5.4 Hz, H-6); 4.42 (1H, *d*, *J* = 7.5 Hz, H-1□); 3.84 (2H, *dd*, *J* = 11.4 Hz and 2.4 Hz, H-6□); 3.73 (1H, *m*, H-5□); 3.60 (1H, *m*, H-4□); 3.57 (1H, *m*, H-3); 3.42 (1H, *m*, H-3□); 3.36 (1H, *m*, H-2□); 1.02 (3H, *s*, H-19); 0.94 (3H, *d*, *J* = 6.6 Hz, H-21); 0.85 (3H, *d*, *J* = 5.4 Hz, H-26); 0.83 (3H, *d*, *J* = 5.4 Hz, H-27); 0.82 (3H, *t*, H-29); 0.70 (3H, *s*, H-18).

^{13}C-NMR (150 MHz, CD_3OD, *δ*, ppm): 140.3 (C-5); 122.0 (C-6); 101.1 (C-1□); 79.1 (C-3); 76.4 (C-3□); 75.8 (C-5□); 73.5 (C-2□); 70.1 (C-4□); 61.7 (C-6□); 56.7 (C-14); 56.0 (C-17); 50.2 (C-9); 45.8 (C-24); 42.3 (C-13); 39.7 (C-4); 38.6 (C-12); 37.2 (C-1); 36.6 (C-10); 36.1 (C-20); 33.9 (C-22); 31.8 (C-8); 31.8 (C-7); 29.5 (C-2); 29.1 (C-25); 28.1 (C-16); 26.0 (C-23); 24.2 (C-15); 23.0 (C-28); 21.0 (C-11); 19.6 (C-26); 19.1 (C-19); 18.8 (C-27); 18.6 (C-21); 11.8 (C-29); 11.7 (C-18).

*3.1.3 Compound **3***

Compound **3** appeared as as white powder, m.p. 198-200°C

^{1}H-NMR (600 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 7.46 (1H, *d*, *J* = 1.8 Hz, H-2); 7.44 (1H, *dd*, *J* = 7.8 Hz and 1.8 Hz, H-6); 6.82 (1H, *d*, *J* = 7.8 Hz, H-5).

^{13}C-NMR (150 MHz, CD_3OD, *δ*, ppm, *J*/Hz): 170.5 (C-7); 151.4 (C-4); 146.0 (C-3); 123.9 (C-5); 123.4 (C-1); 117.8 (C-2); 115.7 (C-6).

The ^{1}H-NMR and ^{13}C-NMR spectra were used to assign the signals in the structural elucidation of the compounds (**1**–**3**).

The ^{1}H-NMR spectrum of compound (**1**) clearly confirmed the presence of an aromatic ring bearing four protons at δ_H 7,90 (2H, *d*, *J* = 9,0 Hz, H-2,6) and δ_H 6,84 (2H, *d*, *J* = 8.4 Hz, H-3,5). Their multiplicity indicated a 1,4-disubstitution pattern. The ^{13}C-NMR and DEPT- NMR spectra showed that compound (**1**) has 5 signals including one signal (δ_C 170.1) representing the presence of -COOH group. Four different signals of δ_C at 122.7, 133.0 (2 overlapped signals), 116.0 (2 overlapped signals) and 163.3 are signals of C-1, C-2, C-6, C-3, C-5 on the benzene ring, respectively and C-4 shows a signal bound to the oxygen group. The structure of 4-hydroxybenzoic acid (**1**) was confirmed by comparing its spectra with reported data. [10] (**Figure 1**).

The ^{1}H-NMR spectrum of compound (**3**) revealed two doublet signals at δ_H 6.82 (1H, *J* = 7.8 Hz) and δ_H 7.46 (1H, *J* = 1.8 Hz), as well as a doublet-doublet proton signal at δ_H 7.44 (1H, *J* = 7.8 and 1.8 Hz). The presence of these doublet-doublet patterns and their respective coupling constants aligns with the structural assignment of a trisubstituted aromatic

nucleus at positions 1, 2, and 4. The ^{13}C-NMR spectrum exhibited 7 distinct carbon signals, with DEPT-135 analysis revealing 4 quaternary carbons. Furthermore, a prominent downfield signal at δ_C 170.5 was conclusively attributed to the carbonyl carbon based on spectral analysis. Thus, the structure of compound (**3**) was determined to be 3,4-dihydroxybenzoic acid [11] (**Figure 1**).

Compound (**2**) was identified as a glycoside sterol due to its characteristic signals in NMR spectra. This compound is in accordance with a sterol aglycone and a six-carbon hexose moiety. The ^{1}H-NMR spectrum showed an olefinic proton signal at δ_H 5.37 (*d*, *J* = 5.4 Hz, H-6) and six methyl signals as follows: Me-18 at 0.70 (*s*), Me-19 at 1.02 (*s*), Me-21 at 0.94 (*d*), Me-26 at 0.85 (*d*), Me-27 at 0.83 (*d*) and Me-29 at 0.82 (*t*). The doublet at δ_H 4.42, exhibiting a coupling constant (*J*) of 7.5 Hz, is attributed to the anomeric proton. The axial-axial coupling with the H-2′ proton at this chemical shift confirms the glycoside's configuration as a *β*-glucoside. The ^{13}C-NMR spectrum revealed the presence of 35 carbon atoms in the molecule, including six carbon signals attributed to the glycosidic group, indicating a hexose moiety, and 29 carbon signals for the aglycone moiety. The spectrum displayed two olefinic signals at δ_C 140.3 and 122.0, indicating the presence of an endocyclic double bond between C-5 and C-6 of sterols. Furthermore, a deshielded oxymethine signal at δ_C

76.4 was observed for the C-3 carbon, and the anomeric carbon signal was detected at 101.1 ppm. By comparing the NMR spectral data with those reported in literature the structure of (**2**) was determined as sitosterol 3-*O*-*β*-D-glucopyranoside [12] (**Figure 1**).

Table 1. The percentage of inhibition and MIC values of extracts and isolated compounds

Extracts and isolated compounds		**The percentage of inhibition at different concentrations** (%)						**MIC (µg/mL)**
Extracts (µg/mL)		**312.5**	**625**	**1250**	**2500**	**5000**	**10000**	
Crude extract	*B. dothidea*	29.1±1,0^{e}	40.8±2,0^{d}	43.3±1.1^{d}	68.9±5,6^{c}	84.0±4,7^{b}	100,0±0,0^{a}	10000
	L. theobromae	96.8±1.6^{b}	97.7±0.2^{ab}	97.7±0.2^{ab}	98.4±1.4^{ab}	99.2±1.4^{ab}	100.0±0.0^{a}	10000
***n*-Hexane extract**	*B. dothidea*	4.0±3.6^{d}	23.8±7.6^{c}	26.3±8.3^{c}	69.9±8.4^{b}	76.4±7.0^{b}	100.0±0.0^{a}	10000
	L. theobromae	39.1±3.0^{d}	48.1±6.8^{cd}	57.2±7.1^{bc}	62.2±6.6^{bc}	69.7±6.8^{b}	100.0±0.0^{a}	10000
Ethyl acetate extract	*B. dothidea*	60.9±3.0^{e}	63.4±0.7^{d}	66.6±3.1^{d}	83.4±1.8^{c}	99.2±1.3^{b}	100.0±0.0^{a}	10000
	L. theobromae	**96.9±1.4^{b}**	**96.9±1.4^{b}**	**97.7±0.1^{ab}**	**99.2±1.4^{ab}**	**100.0±0.0^{a}**	**100.0±0.0^{a}**	**5000**
Isolated compound (µg/mL)		**312.5**	**625**	**1250**	**2500**	**5000**	**10000**	
4-hydroxybenzoic acid	*B. dothidea*	29.0±3.2^{e}	34.0±4.8^{de}	40.8±3.5^{cd}	51.5±5.9^{c}	74.0±4.9^{b}	100.0±0.0^{a}	10000
	L. theobromae	**88.6±2.3^{b}**	**92.5±1.9^{ab}**	**92.6±2.0^{ab}**	**95.4±2.3^{a}**	**96.6±2.5^{a}**	**100.0±0.0^{a}**	**10000**
3,4-dihydroxybenzoic acid	*B. dothidea*	32.3±3.3^{e}	36.2±4.5^{de}	42.7±3.3^{cd}	54.0±6.0^{c}	76.1±5.3^{b}	100.0±0.0^{a}	10000
	L. theobromae	**90.7±2.1^{b}**	**94.7±2.1^{ab}**	**94.7±2.1^{ab}**	**97.2±2.5^{a}**	**98.5±2.6^{a}**	**100.0±0.0^{a}**	**10000**
Metiram complex	*B. dothidea*	25.6±8.5^{d}	65.7±4.7^{c}	81.4±7.4^{b}	97.5±0.2^{a}	100.0±0.0^{a}	100.0±0.0^{a}	5000
	L. theobromae	17.1±4.5^{f}	25.7±2.3^{e}	46.8±5.1^{d}	57.9±1.9^{c}	79.6±3.2^{b}	100.0±0.0^{a}	10000

Note: Values are expressed as means ± SE (n=3). Different letters (a, b, c, d, e, f) within the same row indicate significant differences at the 0.05 level. Statistical analysis was performed using Minitab 16 software (ANOVA-Turkey's).

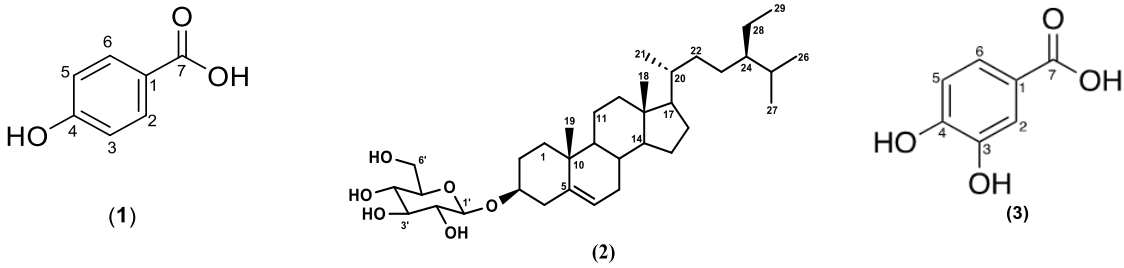

Figure 1. Structures of compounds **1-3**

Three compounds, numbered **1** to **3**, were isolated and characterized from the stems and leaves of *Vitex negundo* L., including 4-hydroxybenzoic acid (**1**), sitosterol 3-*O*-*β*-D-glucopyranoside (**2**), and 3,4-dihydroxybenzoic acid (**3**), based on NMR spectral analysis and comparison with literature data (**Figure 1**).

3.2 *In vitro* antifungal activity results

Medicinal plants are employed as alternatives for medicine to treat various health conditions. Plant extracts have shown exceptional biological activities against microorganisms [13]. In this study, extracts and isolated compounds from *Vitex negundo* L. were screened for antifungal activity against two strains of fungi including *Botryosphaeria dothidea* and *Lasiodiplodia theobromae*, which commonly occur in agriculture sectors [14,15].

Antifungal effect of tested extracts and two isolated compounds including 4-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid were tested against two selected strains of fungi *Botryosphaeria dothidea* and *Lasiodiplodia theobromae*. According to results mentioned in **Table 1**, all *Vitex negundo* L. extracts used in this study exhibited varying degrees of antifungal activity against the *Botryosphaeria dothidea* and *Lasiodiplodia theobromae* strains and increased linearly with increase in concentration of samples. Moreover, the ethyl acetate extract of *Vitex negundo* L. was the most effective among the extracts tested. In fact, this extract exhibited potent antifungal effect against *L. theobromae* with the percentage inhibition from 96.9% at the concentration of 312.5 µg/mL and get the MIC value at 5000 µg/ml. Two isolated compounds also displayed high activity against *L. theobromae* with the percentage inhibitions of 88.6% and 90.7% at 312.5 µg/mL, compared with a standard, positive control (312.5 µg/mL metiram complex) of 17.1%.

Vitex negundo L. is renowned for its diverse secondary metabolites, particularly polyphenols, which enhance its nutritional and medicinal significance. The ethyl acetate extract demonstrates notable antifungal activity against various pathogenic fungi, aligning with traditional medicinal uses of the plant. This effect is likely attributed to the presence of phenolic compounds known for their antimicrobial properties, as observed in previous research [16]. Several mechanisms have been proposed to explain the antimicrobial effects of

phenolic compounds. These mechanisms include their ability to neutralize bacterial toxins, inhibit biofilm formation, reduce adhesion of bacterial ligands to host cells, inhibit enzymes, interact with eukaryotic DNA, and disrupt microbial membranes [17].

Despite the more complex cell wall structure of fungi compared to bacteria and their differences in permeability, certain extracts have still demonstrated inhibition against fungi. In the present study, some extracts of *Vitex negundo* L. exhibit antifungal activity against tested fungi, suggesting the plant contains potential antifungal components for therapy. Traditional plants may serve as novel sources of stable, biologically active antimicrobials, supporting their integration into modern medicine. These compounds are recognized for their biological activity, thereby enhancing antifungal efficacy.

4. CONCLUSION

The findings of this study revealed that the ethyl acetate extract from the stems and leaves of *Vitex negundo* L., cultivated in Hau Giang city, led to the isolation and identification of three compounds: 4-hydroxybenzoic acid (**1**), sitosterol 3-*O*-*β*-D-glucopyranoside (**2**), and 3,4-dihydroxybenzoic acid (**3)**. The structures of these compounds have been elucidated by NMR spectroscopy and by comparison of the spectral data with published data. Furthermore, the antifungal potentials of fractioned extracts and isolated compounds were also evaluated through the poisoned food technique. The results indicated that ethyl acetate extract have the good antifungal activity and two compounds: 4-hydroxybenzoic acid (**1**) and 3,4- dihydroxybenzoic acid (**3)** exhibited the good ability of antifungal activity against *Lasiodiplodia theobromae*.

ACKNOWLEDGEMENT

This study is partially funded by Can Tho University, Code: TSV2024-03.

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