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CHEMICAL CONSTITUENTS OF THE ETHYL ACETATE FRACTION OF THE FRUIT BODIES OF *PHELLINUS GILVUS*

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ABSTRACT

Five fungal secondary metabolites named 1,2,4,5-tetrachloro-3,6-dimethoxybenzene (**1**); ergosterol (**2**); ergosterol peroxide (**3**); (*E*)- 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**4**) and [bi-1,4-cyclohexandien-1-yl]-3,3',6,6'-tetrone, 4, 4'-dihydroxyl-2,2',5,5'-tetramethyl (**5**) were purified from the ethyl acetate fraction extract of the methanol extract fruit bodies of *Phellinus gilvus* (Schwein.) Pat. collected at Pu Mat national park, Nghe An province. Their structures were characterized by 1D, 2D NMR and GC-MS spectroscopies. Especially, the structure of compound **1** was confirmed by X-ray crystallographic analysis. This is the first report on the chemical constituents of Vietnamese *Phellinus gilvus*.

Keywords: Phellinus gilvus, metabolites, antimicrobial, cytotoxicity.

1. INTRODUCTION

It is estimated that there are totally 1.5 million mushrooms species globally, about 22000 species are found in Vietnam. They play a very important role not only for ecosystem but also for our human life. From ancient time, many mushrooms have been used in traditional medicines to treat various diseaes [1]. The mushroom *Phellinus gilvus* (Schwein.) Pat. widely grows in Asia, North America and Africa. Its major chemical constituents are anticancer polysaccharides [2, 3], together with other secondary metabolies such as antioxidant phenollic compounds [4], ergosterol peroxide [5] and triterpenoids [6]. In Viet Nam, it is found in several places such as Hoa Binh, Ninh Binh, Nghe An, Quang Nam and Lam Dong. However, chemical constituents of Vietnamese *P. gilvus* remain unknown. In the course of our investigation on the Vietnamese wood rotting mushrooms, a large amount of *Phellinus gilvus* was collected, which allowed us to study deeply its chemical constituents. This paper reports the isolation, structural elucidation and biological activities of fungal metabolites from Vietnamese *Phellinus gilvus*.

2. MATERIALS AND METHODS

2.1. Materials

The fruit bodies of *Phellinus gilvus* (Schwein.) Pat. were collected in August 2016 at Pu Mat national park, Nghe An province and identified by Assoc. Prof. Dr. Ngo Anh, Hue University. The voucher specimen (PGE 2016) was deposited at Faculty of Chemistry, Hanoi National University of Education, Hanoi, Viet Nam.

2.2. General procedure

Thin layer chromatography (TLC) was carried out on precoated Si gel GF_{254} (Merck Co., Germany) and TLC spots were viewed at 254, 302 and 366 nm and visualized by spraying with vanillin-10 % H_2SO_4 solution. Column chromatography was carried out on silica gel 60 (60-100 μM, Merck) and Sephadex LH-20. Preparative high performance liquid chromatography (HPLC) was performed on a Jasco PU-2087 instrument with a UV-2070 and RI-2031 detectors using a Waters 5 SL-II column (10.0 × 250 mm), flow rate of 1.0 ml/min. Nuclear magnetic resonance (NMR) spectra were recorded on a Brucker Avance 500 MHz. The chemical shift (δ) values are given in ppm with TMS as internal standard, coupling constant *J* - by Hz. Gas chromatography-mass spectrometry (GC-MS) was carried out on an Agilent 7890B/5977A. X-ray reflection data was measured on a Bruker D8 Advance.

2.3. Extraction and Isolation

The powder of dried fruit bodies of *P. gilvus* (2.0 kg) were extracted with methanol (5 times x 5 L) at room temperature in 45 minutes using an ultrasonic system. The solvent was removed under *vacuo* to give a crude extract (93 g), which was further partitioned between hexane, EtOAc, BuOH and water. The EtOAc extract (19 g) was applied to silica gel column chromatography, using hexane/EtOAc gradient from 50 % to 100 % EtOAc to afford eight fractions. Fr. 1 (2.01 g) was recrystallized in methanol to yield compound **1** (545.6 mg) as a white crystal. Fr. 2 (773 mg) was purified by silica gel column chromatography, eluting with hexane/EtOAc (2/1), followed by sephadex LH-20 column, using $CHCl_3$/MeOH (1/3) to give compound **2** (11.4 mg) and **3** (11.6 mg). Compound **4** (20.4 mg) was obtained from Fr. 3 (331 mg) by using silica gel column chromatography, hexane/EtOAc (1/2), then prep. HPLC, with the same solvent system. Fr. 4 (125 mg) was treated as Fr. 3 and followed by prep. HPLC, hexane/EtOAc (1/3) to give compound **5** (1.7 mg).

Compound **1**: ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 3.85 (6H, 3,6-OCH_3). ^{13}C NMR (125 MHz, DMSO-d_6): δ_C 60.9 (3,6-OCH_3), 126.9 (C-1, C-2, C-4, C-5), 150.0 (C-3, C-6).

Compound **2**: ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.63 (3H, s, H-19), 0.83 (3H, d, *J* = 7.5 Hz, H-26), 0.84 (3H, d, *J* = 7.0 Hz, H-27), 0.92 (3H, d, *J* = 6.5 Hz, H-28), 0.95 (3H, s, H-18), 1.04 (3H, d, *J* = 6.5 Hz, H-21), 3.61, (1H, m, H-3), 2.46 (1H, m, H-4), 5.16 (1H, m, H-22), 5.23 (1H, m, H-23), 5.38 (1H, m, H-7), 5.57 (1H, dd, *J*= 2.5, 5.0 Hz, H-6). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 12.1 (C-18), 16.3 (C-19), 17.6 (C-28), 20.0 (C-12), 21.1 (C-11, C-21, C-26), 21.6 (C-27), 23.0 (C-15), 32.0 (C-2), 33.1 (C-25), 38.4 (C-10), 39.1 (C-1), 40.3 (C-20), 40.4 (C-16), 40.8 (C-4), 42.9 (C-13, C-24), 46.3 (C-9), 54.6 (C-14), 55.8 (C-17), 70.5 (C-3), 116.3 (C-7), 119.6 (C-6), 132.0 (C-32), 135.6 (C-23), 139.8 (C-8), 141.3 (C-5).

Compound **3**: ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.82 (3H, d, *J* = 7.0 Hz, H-27), 0.83 (3H, d, *J* = 6.0 Hz, H-26), 0.84 (3H, s, H-18), 0.88 (3H, S, H-19), 0.91 (3H, d, *J* = 7.0 Hz, H-27), 1.00 (3H, d, *J* = 6.5 Hz, H-21), 3.96 (1H, m, H-3), 5.14 (1H, m, H-22), 5.22 (1H, m, H-23), 6.24 (1H, d, *J* = 8.0 Hz, H-6), 6.50 (1H, d, *J* = 8.0 Hz, H-7). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 12.9 (C-18), 17.6 (C-28), 18.2 (C-27), 19.7 (C-19), 20.0 (C-26), 20.7 (C-15), 20.9 (C-21), 23.4 (C-11), 28.6 (C-16), 30.1 (C-12), 33.1 (C-25), 34.7 (C-1), 37.0 (C-2, C-13), 39.4 (C-4), 39.7 (C-20), 42.8 (C-24), 44.6 (C-10), 51.1 (C-9), 51.7 (C-14), 56.2 (C-17), 66.5 (C-3), 79.4 (C-8), 82.2 (C-5), 130.8 (C-7), 132.3 (C-23), 135.2 (C-22), 135.4 (C-6).

Compound **4**: ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 2.36 (3H, s, 1-OCH_3), 6.56 (1H, d, *J* = 16.5 Hz, H-2), 6.89 (1H, d, *J* = 8.0 Hz, H-5'), 7.02 (1H, d, *J* = 8.0 Hz, H-6'), 7.13 (1H, brs, H-2'), 7.43 (1H, d, *J* = 16.5 Hz, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ_C 27.3 (1-OCH_3), 114.2 (C-2'), 115.6 (C-5'), 123.1 (C-6'), 125.1 (C-2), 127.5 (C-1'), 144.0 (C-3), 146.8 (C-3', C-4'), 199.1 (C-1).

2.4. Bioassays

Antimicrobial assay: Compounds **1** was evaluated its antimicrobial activity against seven strains (*Staphylococcus aureus*, *Bacillus subtilis*, *Lactobacillus fermentum*, *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albican*) followed the method described by Hadacek [7].

Cytotoxicity assay: Compounds **1** was tested against KB cell line (a human epidermal carcinoma) from ATCC (American Type Culture Collection) according to the method described by Scudiero [8].

3. RESULTS AND DISCUSSION

The EtOAc extract of *P. gilvus* was subjected repeatedly to silica gel and Sephadex LH-20 column chromatography, followed by preparative HPLC to yield five compounds (**1**-**5**).

Compound **1** was isolated as a white crystal. Its ^{1}H NMR spectrum showed the presence of the methoxyl group at 3.85 ppm. The ^{13}C NMR spectrum exhibited one methoxyl group (60.9 ppm) and two signals for olefinic carbons (126.9 and 150.0 ppm). These NMR signal suggested that compound **1** has a symmetic structure [9]. Furthermore, its crystals were successfully obtained and its OTEP drawing (**1b**) is described in Figure 1, that allowed us to figure out the structure of compound **1** as 1,2,4,5-tetrachloro-3,6-dimethoxybenzene [9].

Compound **2** was obtained as an amorphous powder. The ^{1}H and ^{13}C NMR spectra suggest that it possesses a steroid carbon skeleton, including three double bond and one carbon-bearing oxygen. Finally, compound **2** has identical NMR spectral data with those of ergosterol, therefore, it is determined as ergosterol [10]. Compound **3** has very similar NMR spectral data with those of compound **2**, except for the different of the signal splitting in proton spectrum as well as two more olefinic carbon signals in the ^{13}C NMR spectrum of compound **3**. Detail analysis of its proton NMR revealed that it contains two olefinic protons with a large coupling constants (H-6 and H-7, both have *J* value of 8.0 Hz). In addition, two more carbon atoms bearing oxygen at 79.4 ppm (C-8) and 82.2 ppm (C-5) as compared with that of compound **2**, suggesting the presence of a peoxide group in compound **3**. Consequently, compound **3** is found as ergosterol peroxide, which was previously purified from Philippines *Phellinus gilvus* [5].

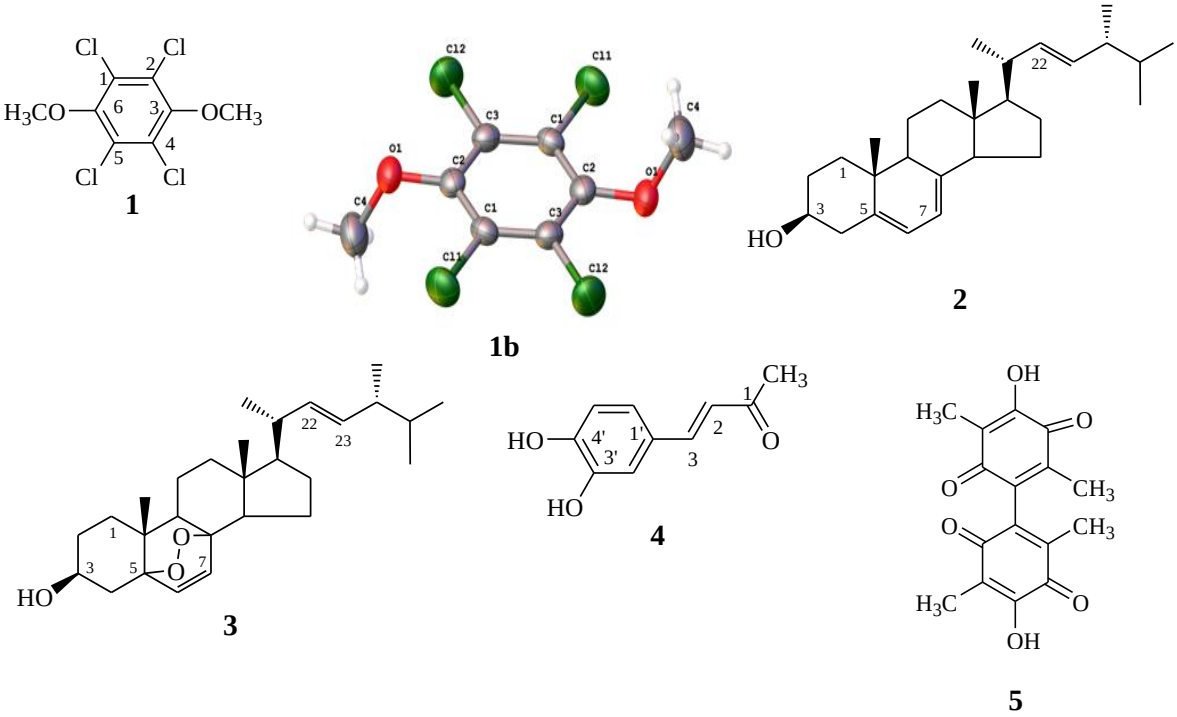

Figure 1. Structures of isolated compounds (**1**-**5**) from *P. gilvus.*

The ^{1}H NMR spectrum of compound **4** has signals for the *trans*-double bond with big coupling constant (H-2, H-3, J = 16.5 Hz) and three aromatic protons, together with one methyl group. The methyl group has HMBC correlation with C-1 (199.1 ppm), indicating the presence of CH_3CO- partital structure. This moiety is connected with the double bond $C_{2\text{-}3}$ due to HMBC correlations from H-2, H-3 and C-1. The substitution groups at 1,3,4 in the aromatic ring were deduced by the coupling constants of H-2'. H-5' and H-6'. Thus, compound **4** is characterized as (*E*)- 4-(3,4-dihydroxyphenyl)but-3-en-2-one, which was previously isolated from *Phellinus igniarius* [11]. Compound **5** was obtained with a small amount. Therefore, GC-MS spectrum was applied to find its structure. Based on the MS spectrum and in comparison with that in database with the mathching of 96 % revealed that compound **5** is [bi-1,4-cyclohexandien-1-yl]-3,3',6,6'-tetrone, 4, 4'-dihydroxyl-2,2',5,5'-tetramethyl as shown in Figure 1.

Previously, compounds which were purified from Vietnamese *Phellinus* sp. are likely to exhibit broad, non-specific activities in biological systems [12-15]. Specifically, compounds **2** and **3** exhibited moderate cytotoxicity to KB cell lines with their IC_{50} value of 10.72 and 12.12 μg/mL, respectively [16]. In addition, compound **4** was reported to have strong cytotoxicity against several cell lines, especially MCF-7 [11]. In this study, the antimicrobial toward seven strains and cytotoxicity against KB cell line of compound **1** were evaluated. However, it did not show any activity (IC_{50} > 128 μg/mL).

4. CONCLUSIONS

The EtOAc extract of Vietnamese *P. gilvus* (Schwein.) Pat. contained five secondary metabolites, such as 1,2,4,5-tetrachloro-3,6-dimethoxybenzene (**1**), ergosterol (**2**), ergosterol peroxide (**3**), (*E*)- 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**4**) and [bi-1,4-cyclohexandien-1-yl]-

3,3',6,6'-tetrone, 4, 4'-dihydroxyl-2,2',5,5'-tetramethyl (**5**). These compounds (**2-4**) might be responsible for the anticancer activity of this medicinal mushroom.

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