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RESEARCH ARTICLE

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Steroid Components of Marine-Derived Fungal Strain *Penicillium levitum* N33.2 and Their Biological Activities

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ABSTRACT

Genus *Penicillium* comprising the most important and extensively studied fungi has been well-known as a rich source of secondary metabolites. Our study aimed to analyze and investigate biological activities, including *in vitro* anti-cancer, anti-inflammatory and anti-diabetic properties, of metabolites from a marine-derived fungus belonging to *P. levitum*. The chemical compounds in the culture broth of *P. levitum* strain N33.2 were extracted with ethyl acetate. Followingly, chemical analysis of the extract leaded to the isolation of three ergostane-type steroid components, namely cerevisterol **(1)**, ergosterol peroxide **(2)**, and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol **(3)**. Among these, **(3)** was the most potent cytotoxic against human cancer cell lines Hep-G2, A549 and MCF-7 with IC_{50} values of 2.89, 18.51, and 16.47 µg/mL, respectively, while the compound **(1)** showed no significant effect against tested cancer cells. Anti-inflammatory properties of purified compounds were evaluated based on NO-production in LPS-induced murine RAW264.7 macrophages. As a result, tested compounds performed diverse inhibitory effects on NO production by the macrophages, with the most significant inhibition rate of 81.37 ± 1.35% at 25 µg/mL by the compound **(2)**. Interestingly, compounds **(2)** and **(3)** exhibited inhibitory activities against pancreatic lipase and α-glucosidase enzymes *in vitro* assays. Our study brought out new data concerning the chemical properties and biological activities of isolated steroids from a *P. levitum* fungus.

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1. Introduction

Secondary metabolites produced by ascomycetes with diverse chemical structures and rich biological activities have attracted attention from researchers for numbers of decades. Many of these have been found to be essential and massively applied in medicine, food and agriculture, especially those from plant-inhabiting fungi. The plant-associated ascomycetes are hypothesized to evolve through adaptation to the unique habitat of photosphere with limited nutrient availability, UV radiations and plant's defensive metabolites, and have been well known for their production of pharmaceutically valuable bioactive compounds [1,2]. Representatives of these are *Taxomyces andreanae*, *Trichoderma atroviride* and *Cladosporium cladosporioides*, which produce taxol [3], camptothecin [4], and cholinesterase inhibitor huperzine A [5], respectively.

In recent years, aquatic plant-derived fungi have proved themselves to be an interesting study object due to their unique living conditions in comparison to those from terrestrial origins [6–8]. Seagrasses are principally monocotyledons of four families (Posidoniaceae, Zosteraceae, Hydrocharitaceae and Cymodoceaceae) with permanent residence in marine environments [9]. Most studied seagrass-derived fungi are *Fusarium* sp. PSU-ES73 isolated from *Thalassia hemprichii*, *Bipolaris* sp. PSU-ES64 from *Halophila ovalis* and *Pestalotiopsis* sp. PSU-ES194 from *Enhalus acoroides*, with the production of a β-resorcylic macrolide, phthalide, as well as meroterpenoid, isocoumarin, and phenol derivatives [7,10,11]. Despite the prosperous potential in exploiting novel compounds, few studies have been conducted on seagrass-derived ascomycetes.

The *Penicillium* genus belongs to the Trichocomaceae family, comprising almost 500 identified species [12] and is one of the most historical and common filamentous fungi with broad distribution in many ecosystems, both in terrestrial and marine environments. Fungi of the genus are producers of currently used

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popular and effective pharmaceutical agents, including antibiotics and metabolic diseases treating drugs [13,14]. Structures of secondary metabolites from *Penicillium* fungi are mainly attributed to alkaloids, fatty acids, macrolides, peptides, phenylpropanoids, polysaccharides, polyketides and terpenoids [15]. Of these, fungal steroids have been known to exhibit interesting biopharmaceutical activities, including cytotoxic, antimicrobial, antioxidant, anti-inflammatory and enzyme inhibitory activities [16]. The frequently mentioned mycosterols have emerged as valuable and potential ingredients for health-promoting purposes. Meanwhile, the occurrence of the steroids originating from *Penicillium* sp. has been mentioned but sparsely studied in detail. As part of our investigation on secondary metabolites from microbial symbionts of seagrasses in Vietnam, fungal strain *P. levitum* N33.2 was isolated, liquid fermented and chemical constituents from its culture broth's extract were structurally elucidated as well as biologically assayed. Hereins, we report the characteristics of the marine fungus and its steroidal metabolites in considerations of pharmaceutical activities.

2. Materials and methods

2.1. Isolation of the fungal strain

The fungal material was originated from the leaf of seagrass *Enhalus acoroides* (Hydrocharitaceae, Alismatales) obtained at Nhatrang bay, Khanhhoa, Vietnam (N 12°59.243', E 109°21.965') and symbolized as N33.2. The isolation of N33.2 from the aquatic plant was carried out following the method described previously by [7], whereby surface sterilization by 10% ethanol and 3% sodium hypochlorite in combination with incubation on potato dextrose-seawater agar (PDA- SW) were applied. The fungus was genetically identified by BLASTn analysis of the ITS sequence using the universal eukaryotic primers ITS1 and ITS4 [17,18] and morphologically characterized following [19].

2.2. Fermentation and crude extraction

The seagrass-derived fungus was grown on potato dextrose agar (HiMedia, India) at 30 °C for 5 days. The fungal mycelia were then harvested through scrapping and inoculated into Erlenmeyer flasks containing 200 mL liquid medium of potato dextrose in sea water and incubated for 15 days (30 °C, constant agitation 110 rpm). The broth (103 L total) was separated from mycelia by filtration (pore size 38 µm) and extracted with equal volumes of ethyl acetate (Xilong Scientific, China), followed by rotary vacuum evaporation (45 °C, 40 rpm) to generate crude extract (10.9 g).

2.3. Isolation of chemical compounds

The fungal crude extract (hereinafter referred to as EtOAc-CR) was loaded onto a Diaion-HP20 column and fractionated by gradient elution of methanol and water (3:7,7:3,1:0) to obtain 3 fractions, namely N33A1.1-3. Fraction N33A1.3 (5.42 g) was subjected to column chromatography (CC) using a gradient solvent system of n-hexane/acetone (40:1–0:1, v/v) to yield 4 subfractions A1-4. Insoluble components in subfraction A1 were washed and purified, yielding compound **(1)** (10.0 mg). Subfraction A3 was further chromatographed on silica gel, eluted with n-hexane/ethyl acetate (2.5:1, v/v), and then purified to obtain compound **(2)** (7.5 mg). Subfraction A4 was subjected to CC eluting with n-hexane/ethyl acetate (3:1, v/v), followed by stepwise CC with dichloromethane/ethyl acetate (3:1, v/v) elution to provide compound **(3)** (3.0 mg).

2.4. Structural characterization

Mass (MS) and nuclear magnetic resonance (NMR) spectra (^{1}H-NMR, ^{13}C-NMR, DEPT, HSQC, HMBC, COSY and NOESY) data were measured on a Bruker Ascend 600 NMR spectrometer (Bruker, USA) to elucidate the structures of purified compounds. ^{1}H- and ^{13}C-NMR spectra were recorded at 500 MHz and 125 MHz, respectively, with tetramethylsilane (TMS) as an internal standard. Chemical shifts were expressed in δ (ppm) and coupling constants (*J*) in Hz.

2.5. Cell culture conditions

Murine macrophage RAW 264.7 cells (ATCC no. TIB-71), Vero cells (ATCC no. CCL-81.4), and three human cancer cell lines, including Hep-G2 (Human hepatocellular carcinoma, ATCC no. HB-8065), A549 (Human lung carcinoma, ECACC no. 86012804) and MCF-7 (Human breast adenocarcinoma, ATCC no. HTB-22) were obtained from the American Type Culture Collection (ATCC, USA) and European Collection of Authenticated Cell Cultures (ECACC). While the human cancer cell lines and Vero cells were used to evaluate cytotoxicity, RAW 264.7 cell line was employed to determine the anti-inflammatory effect of compounds **(1)-(3)**. All lines were maintained in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA), 100 units/mL penicillin and 100 mg/mL streptomycin (Merck, Germany) in

a humidified 5% CO_2 incubator (Esco, Singapore) at 37 °C, with medium change every 2–3 days depending on cell lines.

2.6. Cytotoxic assay

Cell viability of cancer cells, Vero cells, and RAW 264.7 macrophages in exposure to test compounds was evaluated by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [20]. In brief, seeded cells ($5x10^4$ cells per well in 96-well plates) at the exponential growth phase were treated with serial diluted concentrations of test compound (50-25-12.5-6.25 µg/mL) and 1 µg/mL lipopolysaccharide (LPS, Sigma-Aldrich, Germany). After 36 h, MTT (Sigma-Aldrich, Germany) solution (0.5 mg/mL) was added to each well and incubated (4 h, 37 °C, 5% CO_2). Formed formazan blue crystals in cells were dissolved in DMSO for detection at 570 nm (Tecan F150 microplate reader, Switzerland).

2.7. Nitrite assay

RAW 264.7 macrophages were seeded in 24-well plates at a density of $2x10^5$ cells per well in DMEM supplemented with 10% FBS for exponentially growing. When cells reached about 70% confluence, the culture was replaced with 1% FBS medium for serum starvation, then incubated with test compounds at non-cytotoxic concentrations (37 °C, 5% CO_2, 24 h). After being stimulated by 1 µg/mL LPS (Sigma-Aldrich, USA) for 24 h, the induced nitric oxide (NO) production in cell culture was quantified basing on Griess reaction [21]. Briefly, cell-free supernatants were pipetted into 96-well plates and incubated at 30 °C for 20 min with Griess agent (1% sulfanilamide and 0.1% N-1-naphtylethylenediamine dihydrochloride in 2.5% H_3PO_4) (Sigma-Aldrich, USA), followed by optical density detection at 550 nm (Tecan F150 microplate reader, Switzerland). Sodium nitrite (Merck, Germany) at varying concentrations was used for standard calibration and determination of NO inhibition.

2.8. Lipase inhibitory assay

The investigation of *in vitro* lipase inhibition was performed in 96-well plates using porcine pancreatic lipase (Type II) (PPL, Sigma-Aldrich, USA) and substrate *p*-nitrophenyl palmitate (*p*NPP, Sigma-Aldrich, USA) according to the described method [22] with modifications. Briefly, a reaction mixture containing 0.1 mg of PPL, 6.66 µM of *p*NPP in ethanol/acetonitrile (2:1, v/v) and test compound in Tris-HCl buffer (0.061 M, pH 8.5) was en masse incubated (37 °C, 25 min). After the addition of ethanol to stop the reaction, the conversion rates of substrate hydrolysis in the existence of test compounds were monitored by absorbance changes at 405 nm (Tecan F150 microplate reader, Switzerland).

2.9. Alpha-glucosidase inhibitory assay

The α-glucosidase inhibitory activity was determined by quantification of hydrolyzed product *p*-nitrophenol as mentioned by [23] with minor modifications. For the *in vitro* assay, test compounds were pre-incubated with α-glucosidase from *Saccharomyces cerevisiae* (0.5 U/mL, Sigma-Aldrich, USA) (37 °C, 10 min). Thereafter substrate *p*-nitrophenyl-α-D-glucopyranoside (*p*NPG, Merck, Germany) was added to the mixture until final concentration of 10 mM and further incubated (37 °C, 30 min). The enzymatic reaction was terminated by adding 100 µL of 0.2 N sodium carbonate solution (Merck, Germany). The α-glucosidase inhibition activity was determined as the reduced proportional percentage of absorbance at 405 nm (Tecan F150 microplate reader, Switzerland) of reaction mixture incubated with test compound to control.

2.10. Statistical analysis

Tests were performed in triplicate and results are expressed as mean ± standard deviation (SD). Depending on biological activity assays, samples were analyzed at three to five different concentrations. The half maximal inhibitory concentration (IC_{50}) was estimated by plotting inhibition percentages against the test compound's concentrations using the computer software TableCurve 2D version 5.1 (Systat Software, USA).

3. Results

3.1. Characterization of the fungal isolate

According to the analyzed BLASTn result, the fungal ITS sequence (543 bp) matched absolutely to those of *Penicillium* species (100% coverage, 100% identity), including *P. javanicum* strain CMV003F5 (GenBank: MK450698.1), *Penicillium* sp. strain JRO19040853 (GenBank: MN486544.1), *Penicillium* sp. strain FGZ3-2 (GenBank: MK399697.1) and *P. levitum* strain B3056 (GenBank: MK204504.1). The fungal isolate could be assumed as a *Penicillium* fungus.

On Czapek agar medium, the fungal isolate N33.2 appeared with creamy rugose mycelium and the color was changed to brown at maturity. After 10 days of incubation at 25 °C, the fungal colonies

reached an averaged diameter of 3.5 to 4 mm with the formation of colorless to red-brown exudates. Under the microscope, the presence of both sexual reproductive structures including spherical mature cleistothecium (80–170 µm in diameter), asci (7–9 µm in diameter), and elliptic ascospores (3.5–4.5 × 3.0–4.0 µm), and structures of asexual state such as divaricated conidiophores with numerous subterminal branches and elliptic conidia (3.0–5.5 × 3.0–4.0 µm) were observed (Figure 1).

Taken together, the above characteristics indicated that the fungus belongs to species *P. levitum* (Raper & Fennell) Stolk and Scott [24]. The ITS sequence of the fungal isolate was deposited in the National Center for Biotechnology Information as *P. levitum* strain N33.2 (GenBank: OR150485.1).

3.2. Structures of the fungal metabolites

Compound (1)

Compound **(1)** was isolated as a white amorphous powder. The mass spectrum from ESI-MS data gave the [M + H]+ at m/z 431, while the ^{13}C-NMR and DEPT spectra revealed the signal of 28 carbons, including 6 methyl groups, 7 methylenes, 11 methines and 4 quaternary carbons. The molecular formula of compound **(1)** was deduced as $C_{28}H_{46}O_3$ ($M = 430$).

The ^{1}H-NMR spectrum of compound **(1)** revealed 6 methyl groups at δ_H 0.54 (3H, s, H-18), 0.90 (3H, s, H-19), 0.99 (3H, d, $J = 5.5$ Hz, H-21), 0.80 (3H, d, $J = 6.0$ Hz, H-26), 0.81 (3H, d, $J = 6.0$ Hz, H-27) and 0.89 (3H, d, $J = 6.0$ Hz, H-28), and the presence of 3 olefinic protons at δ_H 5.08 (1H, d, $J = 2.0$, H-7), 5.17 (1H, dd, $J = 6.0$, 12.5 Hz, H- 22), 5.23 (1H, dd, $J = 6.0$, 12.5 Hz, H-23).

Further data by HMBC uncovered the correlations from H-18 (δ_H 0.54 Hz) (δ_H 5.34) to C-12 (δ_C 39.0), C-13 (δ_C 42.9) and C-14 (δ_C 54.1), from H-19 to C-1 (δ_C 32.4), C-5 (δ_C 74.4) and C-10 (δ_C 36.6); from H-21 (δ_H 0.99 Hz) to C-20 (δ_C 39.9)/C-22 (δ_C 131.3)/C-17 (δ_C 55,2); from H-28 to C23/C-24/c-25; from H26/H-27 to C-26/27/C-25, suggesting the presence of methyl groups at C-10, C-13, C-20, C-24 and C-25. In addition, correlations were observed on HMBC, affirming positions of methylene and methine groups at δ_C 32.4 (C-1), 31.1 (C-2), 40.1 (C-4), 21.2 (C-11), 39.0 (C-12), 22.5 (C-15), 27.6 (C-16), 65.9 (C-3), 72.0 (C-6), 119.4 (C-7), 42.2 (9), 54.1 (C-14), 55.2 (C-17), 39.9 (C-20), 131.3 (C-22), 135.3 (C-23) and 32.4 (C-25).

The integration of the obtained spectra analysis indicated an ergostane molecular skeleton structure of compound **(1)**. Besides, these data appeared identical to the published literature [25], confirming the structure of compound **(1)** as cerevisterol (Figure 2).

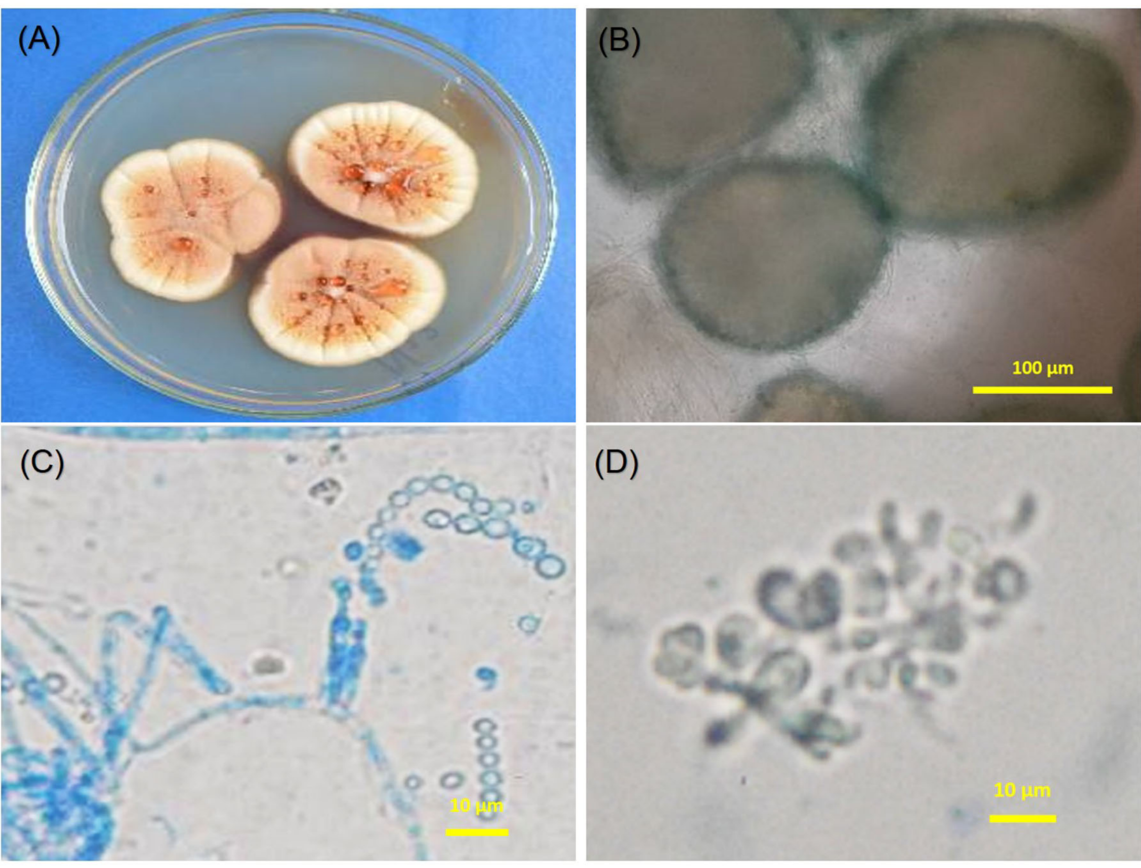

Figure 1. Morphological characteristics of *Penicillium* isolate N33.2. (A)- morphology of colonies on CZ agar plate. (B)- cleistothecium (sexual reproductive structure). (C) Conidiophores and conidia. (D) Asci and ascospores.

Figure 2. Structure of compounds (1), (2) and (3).

Compound (2)

Compound **(2)** was obtained in the form of a white amorphous powder. The MS data gave the [M + H]+ at *m/z* 427, while the ^{13}C-NMR and HSQC spectra revealed the signal of 28 carbons, including 6 methyl, 7 methylene, 11 methine groups and 4 quaternary carbons, suggesting the molecular formula of compound **(2)** as $C_{28}H_{44}O_3$ ($M = 428$).

The ^{1}H-NMR spectrum of compound **(2)** revealed the existence of 6 methyl groups at δ_H 0.81 (3H, s, H-18), 0.82 (3H, d, H-26), 0.83 (3H, d, H-27), 0.88 (3H, s, H- 19), 0.91 (3H, d, $J = 5.5$ Hz, H-28) and 1.00 (3H, d, $J = 5.5$ Hz, H-21), two olefinic protons of –CH = CH– bond at δ_H 5.15 (1H, dd, $J = 7.0$, 13.0 Hz, H-22)/5.21 (1H, dd, $J = 7.0$, 13.0 Hz, H-23) and δ_H 6.24 (1H, d, $J = 7.0$ Hz, H-6)/6.50 (1H, d, $J = 7.0$ Hz, H-7), and one proton of oximethine group at δ_H 3.96 (1H, m, H-3).

The HSQC spectrum of compound **(2)** showed signals of 6 methyl groups at δ_C 12.8 (C-18), 18.1 (C-19), 20.8 (C-21), 19.6 (C-26), 19.9 (C-27) and 17.5(C-28), two pairs of double bonded carbons (–CH = CH–) at δ_C 135.4 (C-6)/130.7 (C-7) and 135.2 (C-22)/132.3 (C-23). Further analysis of HSQC spectrum revealed signals of an oximethine group at δ_C 66.4 (C-3) and a peroxide linkage at δ_C 82.1 (C-5), 79.4 (C-8).

The achieved data of compound **(2)** were found identical to the previously reported spectral literature (Krzyzkowski et al. 2009), supporting the structural determination of compound **(2)** as ergosterol peroxide (Figure 2).

Compound (3)

Compound **(3)** was isolated as a white amorphous powder. The determination of [M + H]+ at *m/z* 431 from ESI-MS data, together with the signal of 28 carbons by analyzing ^{13}C-NMR spectrum, proposed compound **(3)**' molecular formula as $C_{28}H_{46}O_3$ ($M = 430$).

The ^{1}H-NMR spectral data indicated the existence of 6 methyl groups at δ_H 0.75 (3H, s, H-18), 0.90 (3H, s, H-19), 0.99 (3H, d, $J = 6.5$ Hz, H-21), 0.80 (3H, d, $J = 6.5$ Hz, H-26), 0.81 (3H, d, $J = 6.5$ Hz, H-27) and 0.89 (3H, d, $J = 7.0$ Hz, H-30) of compound **(3)**. Besides, the spectrum showed signals of three olefinic protons at δ_H 5.08 (1H, br s, H-7), 5.17 (1H, dd, $J = 7.0$, 15.0 Hz, H-22) and 5.23 (1H, dd, $J = 7.0$, 15.0 Hz, H-23), as well as two protons of oximethine group at δ_H 4.48 and 4.24 ppm. Combination of compound **(3)**'s ^{13}C-NMR and DEPT spectra demonstrated the existence of 6 methyl, 7 methylene, 11 methine and 4 quaternary carbons. Further nuclear magnetic resonance

spectral analysis of 3 supported for the determination of methyl groups at δ_C 12.0 (C-18), 17.6 (C-19), 19.4 (C-21), 19.7 (C-26), 20.3 (C-27) and 17.2 (C-28). Likewise, positions of methylene and methine groups were identified at δ_C 32.4 (C-1), 31.1 (C-2), 40.1 (C-4), 21.3 (C-11), 39.0 (C-12), 22.5 (C-15), 27.7 (C-16), 65.9 (C-3), 72.1 (C-6), 119.4 (C-7), 42.4 (9), 54.1 (14), 55.3 (C-17), 39.9 (C-20), 131.3 (C-22), 135.3 (C-23) and 32.4 (C-25). Four quaternary carbons were at δ_C 74.4 (C-5), 139,6 (C-8), 36.5 (C-10) and 42.9 (C-13).

Similar to those of compounds **(1)** and **(2)**, the spectral data of **(3)** were indicative of an ergostane structure, which entirely shared identities to the published literature by [26]. Thus, the structure of (3β,5α,22E)-ergosta-6,8(14),22-triene- 3,5-diol was proposed for compound **(3)** (Figure 2).

3.3. Biological activities

Cytotoxicity

Three isolated compounds **(1)** (cerevisterol), **(2)** (ergosterol peroxide) and **(3)** [(3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol)] from the culture broth of *P. levitum* strain N33.2 were tested for cytotoxic activity against three human cancer cell lines, Hep-G2, A549 and MCF-7, and Vero cells (monkey kidney epithelial cells). The results are summarized in Table 1.

Among the three, compound **(1)** did not show any cytotoxicity whereas compound **(3)** exhibited the most potent cytotoxicity against human cancer cells (Hep-G2, A549 and MCF-7), while compound **(2)** appeared to be less toxic to tested lines (IC_{50} values at 16.22, 22.48 and 27.11 µg/mL, respectively). It was noted that compound **(3)** showed a lighter negative effect to Vero cells' viability (IC_{50} at 10.40 µg/mL, 24.19 µM equivalent) in comparison to 2 (IC_{50} at 4.93 µg/mL, 11.52 µM equivalent).

The result appeared to be contradictory to earlier report [27] concerning weak cytotoxic acitivity of ergosterol peroxide (IC_{50} 52.4–99.39 µM) and moderate activity of cerevisterol (IC_{50} 32.4–41.5 µM) from endophytic fungus *Fusarium equiseti* against MCF-7, MDA-MB-231 and Caco-2 cancer cells. Ergosterol peroxide were claimed to be cytotoxic to various cancer cell lines [28]. The presence of the peroxide bridge was assumed to be the main cause of cytotoxicity due to its inducibility of apoptosis. Our results brought out new update on cytotoxic activity of the fungi-derived compound, as well as the diversity in bioactivities of fungal steroid constituents.

Table 1. Inhibitory concentrations of isolated compounds against Hep-G2, A549, MCF-7 and Vero cells lines.

No	Samples	IC_{50} (µg/mL)			
		Hep-G2	A549	MCF-7	Vero
1	Ellipticine	1.26	1.38	2.69	8.61
2	EtOAc-CE	5.85	7.16	12.96	19.74
3	Compound **(1)**	–	–	–	–
4	Compound **(2)**	16.22	22.48	27.11	14.93
5	Compound **(3)**	2.89	18.51	16.47	18.40

Anti-inflammatory activity

Nitric oxide (NO) is considered to be a pro-inflammatory mediator inducing inflammation in abnormal situations [29], and thus chemical compounds with inhibitory effect on NO production are potential anti-inflammatory drugs. By measuring NO content in culture supernatants of LPS-stimulated RAW 264.7 cells, the *in vitro* anti-inflammatory activities of compounds **(1)**-**(3)** were evaluated. As shown in Table 2, at test concentrations retaining cell survival rate >60%, all three compounds alleviated NO production with inhibition rates ranging from 14.45 ± 1.50 to 81.37 ± 1.35%. Among these, compound **(1)** (cerevisterol) showed no significant negative effect on cell viability of RAW 264.7 macrophages at the highest investigated concentration of 50 µg/mL (survival rate of 98.40 ± 1.73%), while alleviating NO production in a concentration dependent manner. The half-maximal inhibitory concentrations of compounds **(1)**, **(2)** and **(3)** were determined to be 25.45 (59.19 µM equivalent), 2.85 (6.66 µM equivalent) and 2.79 µg/mL (6.49 µM equivalent), respectively. Although compounds **(2)** (ergosterol peroxide) and **(3)** [(3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol] exhibited NO inhibitory activities at lower IC_{50} values, further investigational assessments should be carefully contemplated due to their potent cytotoxicity.

The present study demonstrated the prominent ability of fungi-derived cerevisterol, ergosterol peroxide and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol in suppressing NO production in LPS-stimulated RAW 264.7 macrophages, which is corresponded to previous report concerning anti-inflammatory property of cerevisterol from endophytic fungi *Fusarium solani* [30], but contrary to formerly reported (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol from *Hericium erinaceus* [31].

Lipase inhibitory activity

Owing to the role of pancreatic lipase as a key component of dietary fat digestion [32], the inhibition of such enzyme has become a target in the screening of fat absorption and obesity-controlling drugs. By means of *in vitro* assaying, two out of three steroid compounds isolated from fungal strain *P. levitum* N33, i.e., compounds **(2)** and **(3)**, were identified to exhibit moderate activity against PPL in a concentration-dependent manner (Table 3).

Table 2. Nitric oxide inhibitory activity of test compounds on RAW 264.7 macrophages.

Sample's name[a]	Test concentration (μg/mL)	NO inhibition[b] (%)	Cell survival[b] (%)	NO half-maximal inhibitory concentration (IC_{50}, μg/mL)
(-) control	–	0	100	–
(+) control	0.81	86.93 ± 0.96	71.80 ± 0.51	0.62
Compound **(1)**	50	54.25 ± 1.27	98.40 ± 1.73	25.45
	25	48.83 ± 1.43	96.78 ± 0.63	
	5	30.92 ± 1.04	97.69 ± 1.20	
	1	14.45 ± 1.50	98.05 ± 0.67	
Compound **(2)**	50	–	2.18 ± 0.68	2.85
	25	81.37 ± 1.35	60.87 ± 2.21	
	5	56.69 ± 1.29	83.99 ± 1.05	
	1	27.97 ± 0.84	98.08 ± 1.26	
Compound **(3)**	50	–	1.70 ± 0.27	2.79
	25	71.36 ± 1.38	82.31 ± 0.78	
	5	55.24 ± 1.20	93.86 ± 1.33	
	1	32.79 ± 0.73	93.19 ± 0.49	

[a](-) control: DMSO 0.5%; (+) control: Cardamonin 3 μM.
[b]Data represent the mean ± standard deviation of three independent wells;.

Table 3. Inhibitory effects of test compounds against pancreatic lipase.

Sample's name	Test concentration (μg/mL)	Enzyme inhibition (%)
(+) control[a]	50	**92.15 ± 1.68**
EtOAc-CE	2000	14.12 ± 0.72
	1000	5.90 ± 0.89
	500	1.04 ± 0.57
Compound (1)	500	0
Compound (2)	500	**25.85 ± 1.24**
	250	**11.67 ± 2.13**
	125	**2.54 ± 1.60**
Compound (3)	500	**23.77 ± 2.31**
	250	**12.08 ± 0.95**
	125	**1.29 ± 1.11**

[a]Orlistat (Merck, Germany) was employed as a positive sample.

To the best of our knowledge, the effects of fungal steroids ergosterol peroxide **(2)** and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol **(3)** on lipase have not been reported. Although the inhibition of PPL by compounds **(2)** and **(3)** remains limited with IC_{50} values greater than 500 μg/mL, the results are nonetheless significant and deserved to be documented, supporting evidence for the development of alternative health-promoting agents from mycosterols [33].

Alpha-glucosidase inhibitory activity

As revealed by spectrometrical analysis, compounds **(2)** (ergosterol peroxide) and **(3)** [(3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol] isolated from strain N33.2 were found as α-glucosidase inhibitors with inhibition rates greater than 50% at concentration of 400 μg/mL, whereas compound **(1)** showed 12.69 ± 1.06% of inhibition, which indicates a low activity (Figure 3). IC_{50} values of 21.89 (51.14 μM equivalent) and 25.81 μg/mL (60.02 μM equivalent) were obtained for compounds **(2)** and **(3)**, respectively, indicating more potent inhibitory activity against alpha-glucosidase than acarbose (IC_{50} = 235.56 μg/mL).

The α-glucosidase inhibitory property of fungal ergosterol peroxide has been investigated and appeared to be inconsistent, from negative [34] to very weak [35]. Contrary to these results, ergosterol peroxide in our study was first reported as a potent yeast α-glucosidase inhibitor. This inconsistence may partly be due to applied test methods of studies as well as the origins of producing organisms.

4. Discussion

In marine environment, *Penicillium* spp. are commonly found as symbionts of multicellular flora and fauna, mainly sponges, algae and corals [36]. Similar to other aerobic organoheterotrophs, these fungi inhabit more frequently in the phyllosphere of seagrasses [37]. These fungal symbionts were hypothesized to originate from terrestrial environments with development of adaptive characteristics to typical saline aquatic conditions [38]. The present *P. levitum* strain N33.2 was isolated from the seagrass *E. acoroides* by surface sterilization method, thus possibly a fungal endophyte of the marine plant. This is the first report mentioning the existence of *P. levitum* species as a symbiont of seagrasses.

Regardless of specific characteristics of the marine phyllosphere-derived ascomycetes, three sterols were isolated for the first time as an ensemble from culture broth of a *Penicillium* strain. Structurally, isolated compounds **(1)**-**(3)** are all 3β-sterols and C28 steroids, consisting of ergostane skeleton with 3β-hydroxy group and double bond at C-22/23-position. Ergostane-type steroids are quite commonly found in fungal extracts, with 5α,8α-endoperoxides, such as ergosterol peroxide and 9,11- dehydroergosterol peroxide, are the most typical representatives [16,28]. Their abundances were hypothesized to be due to the transformation of ergosterol – an essential and the major component of the mycelial membrane [39]. In the present study, three steroid compounds including cerevisterol, ergosterol peroxide and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol were firstly isolated from filamentous fungus *P. levitum*. Although the composition of these might be dependent on the physiological state of the fungal biomass as well as culturing parameters, their

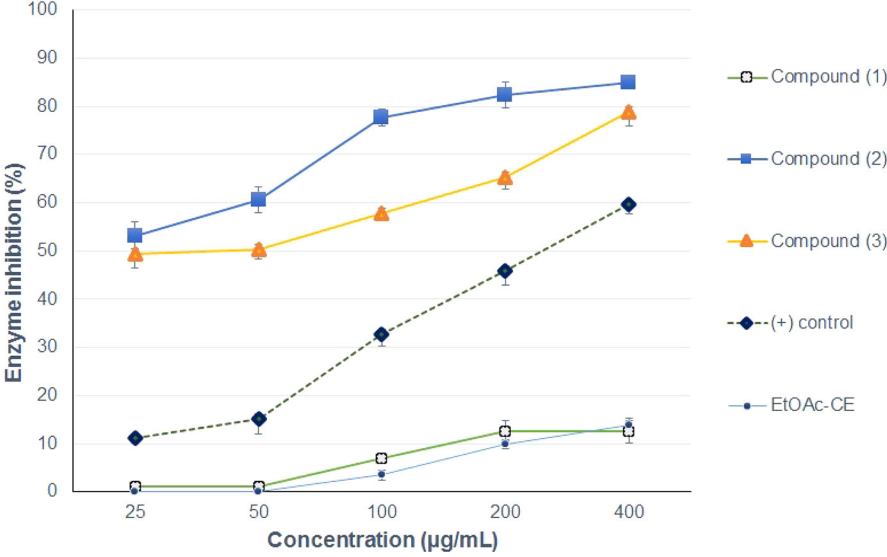

Figure 3. Inhibitory effects of compounds (1)–(3) against α-glucosidase.
* Acarbose (Merck, Germany) was employed as a positive sample.

occurrence and distribution are apparently species-specific and thus could further be utilized as bioindicators for biochemical phylogenetic approaches.

Most notably, while cerevisterol and ergosterol peroxide were frequently isolated from almost all clades of kingdom fungi [40–42], the existence of (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol in microbial extracts has not been earlier mentioned. Particularly, the substance has once been published as a component of medicinal mushroom *Hericium erinaceum* but significance in biological activities was not recorded [31]. As other fungal ergostatriene metabolites, the biosynthesis of (3β,5α,22E)-ergosta-6,8(14), 22-triene-3,5-diol by *P. levitum* strain N33.2 might be involved in the transformation of accumulated ergosterol, especially when fungal mycelia being harvested at the early stationary phase of growth [43]. Therefore, further studies on metabolism, producing conditions and extracting optimization for compounds 1-3 by fungal strain N33.2 are recommended.

Contrary to similarities in chemical structure, the properties of three isolated fungal steroids deemed to be versatile. The selectivity of these on cell viability, anti-inflammatory and enzyme inhibitory activities is attributed to either the presence of peroxide bridge and hydroxyl groups or the position of double bonds in the tetracyclic skeleton of steroid molecules.

Our present study investigated cytotoxicity, anti-inflammatory and enzyme-inhibiting activities of cerevisterol, ergosterol peroxide and (3β,5α,22E)-ergosta- 6,8(14),22-triene-3,5-diol isolated from *P. levitum* strain N33. Given the findings, fungal components with potential use as therapeutic agents in related civilization diseases, including cancer, obesity and diabetes, were highlighted. As such, the production of ergosterol peroxide and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol by certain fungal taxa of *Penicillium* spp. should be extensively investigated.

Conclusion

In conclusion, our study brought out data concerning the biological and chemical profile of a marine-derived *Penicillium levitum* strain N33. From the culture broth of the fungus, three steroids were isolated and identified as major components, namely cerevisterol, ergosterol peroxide and (3β,5α,22E)-ergosta-6,8(14),22-triene-3,5-diol. These findings contributed data to fulfill the gap in biochemistry of *Penicillium* species. Besides, the results of our study gave rise to new updates for current knowledge on the structures and biological activities of filamentous fungal constituents, especially those of mycosteroids.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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