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Research Article

Secondary metabolites of a marine-derived *Penicillium ochrochloron*

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Abstract

Extremophilic fungi have received considerable attention recently as new promising sources of biologically active compounds with potential pharmaceutical applications. This study investigated the secondary metabolites of a marine-derived *Penicillium ochrochloron* isolated from underwater sea sand collected from the North Sea in St. Peter-Ording, Germany. Standard techniques were used for fungal isolation, taxonomic identification, fermentation, extraction, and isolation of fungal secondary metabolites. Chromatographic separation and spectroscopic analyses of the fungal secondary metabolites yielded eight compounds: talumarin A (1), aspergillumarin A (2), andrastin A (3), clavatol (4), 3-acetylphenol (5), methyl 2,5-dihydro-4-hydroxy-5-oxo-3-phenyl-2-furanpropanoate (6), emodin (7) and 2-chloroemodin (8). After co-cultivation with *Bacillus subtilis*, the fungus was induced to express (-)-striatisporolide A (9). Compound 1 was evaluated for antibacterial activity against *Staphylococcus aureus, Acinetobacter baumannii, Mycobacterium smegmatis,* and *M. tuberculosis,* as well as cytotoxicity against THP-1 cells. The compound, however, was not cytotoxic to THP-1 cells and had no antibacterial activity against the microorganisms tested. The compounds isolated from *P. ochrochloron* in this study are well-known compounds with a wide range of beneficial biological properties that can be explored for pharmaceutical, agricultural, or industrial applications. This study highlights the bioprospecting potential of marine fungi and confirms co-cultivation as a useful strategy for the discovery of new natural products.

Keywords: drug discovery; marine fungus; natural products; *Penicillium ochrochloron*; secondary metabolites

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Introduction

The frequent re-isolation of known metabolites from fungi has turned the interest of natural products chemists to hitherto less investigated ecological niches such as arctic glaciers, deep-sea hydrothermal vents or hypersaline lakes (Wilson and Brimble, 2009; Liu *et al.*, 2016). Fungi that live at elevated temperature, acidic or alkaline pH, high pressure, high salt concentration and /or low nutrient concentrations are called extremophiles. These fungi have developed unique metabolic mechanisms to produce bioactive secondary metabolites as a response to environmental stress (Satyanarayana *et al.*, 2005; Ma *et al.*, 2010).

Marine fungi are distinct from their terrestrial and freshwater counterparts, both in their taxonomy, morphology and adaptation to an aquatic habitat. When compared to the thousands of fungal species known from terrestrial environments, only a few have been described for oceans and estuaries which represent the largest part of the earth's surface. Some of these fungi, however, may occur in both seawater and freshwater or terrestrial habitats (Jones, 2000; Gomes *et al.*, 2008).

These marine species represent a huge potential for new natural products and an increased number of new metabolites have become known over the past years, while much of the hidden potential still needs to be uncovered. Though bioactivities of secondary metabolites from marine fungi reveal interesting levels for several clinically relevant targets, they are not well represented in the pipelines of drugs and none of them currently is on the market. However, in recent years, an increasing number of new natural products have been characterized from marine fungi and there is no doubt that they produce a large number of interesting secondary metabolites, which often show pharmaceutically relevant bioactivities and may be candidates for the development of new drugs (Imhoff *et al.*, 2011; Imhoff, 2016).

The prospects of extremophilic fungi as new promising sources for biologically active compounds with potential pharmaceutical applications inspired this current study.

Materials and Methods

Fungal isolation and identification

Sea sand collected from underwater at the North Sea in St. Peter-Ording, Germany was inoculated in malt extract agar plates and incubated at 22 °C for 3-5 days. The observed fungal growths were subcultured onto freshly prepared agar plates to obtain pure cultures. One of the fungi was identified as *Penicillium ochrochloron* according to the molecular identification protocol of DNA amplification and sequencing of the internal transcribed spacer (ITS) region previously described (Kjer *et al.*, 2010). The DNA sequence data was deposited in the NCBI database (GenBank) with accession number MN475865.

Fermentation and co-cultivation

Solid state fermentation of *P. ochrochloron* was carried out by cultivating the fungus in 1 L Erlenmeyer flask containing sterile solid rice medium (100 g of rice + 110 mL of distilled water, autoclaved at 121 °C at 15 psi for 20 min) under static conditions at 22 °C for 14 days (Eze *et al.*, 2018). Also, co-cultivation experiments were performed by growing the fungus in I L Erlenmeyer flasks containing rice medium to which broth solution of freshly grown *Bacillus subtilis* was added (Moussa *et al.*, 2020).

Extraction and isolation of metabolites

After fermentation, the fungal secondary metabolites were extracted with ethyl acetate (EtOAc) and then concentrated under reduced pressure. UV-guided column chromatographic separation of the crude EtOAc axenic extract using silica gel and Sephadex LH-20 as stationary phases followed by purification with semi-preparative reversed-phase HPLC yielded compounds (1–8). Also, chromatographic separation of the co-culture extract afforded compound 9. Initially, the crude axenic fungal extract was subjected to VLC with silica

using gradient mixtures of *n*-hexane (*n*-Hex) and EtOAc to yield 4 fractions PO1 – PO4. Fraction PO2 [*n*-Hex:EtOAc (80:20) was divided into two parts by dissolving in methanol (MeOH) to yield MeOH-soluble and MeOH-insoluble parts. The MeOH-soluble part was subjected to a Sephadex LH-20 column using 100% MeOH as eluent to obtain 7 sub-fractions (PO21 to PO27). Sub-fraction PO23 was purified by semi-preparative HPLC using MeOH:H_2O (30:70 to 70:30) to give compound **2** (code: PO232; 9.2 mg). Sub-fraction PO24 was purified by semi-preparative HPLC using MeOH-H_2O (10:90 to 55:45) to give compound **4** (code: PO241; 1.4 mg), compound **5** (code: PO242; 1.0 mg), compound **6** (code: PO243; 2.0 mg). Sub-fraction PO27 was purified by semi-preparative HPLC using MeOH-H_2O (65:35 to 95:5) to give compound **7** (code: PO271; 1.0 mg) and compound **8** (code: PO272; 2.1 mg). Fraction PO4 was subjected to Sephadex LH-20 column using 100% MeOH as eluent to obtain 8 sub-fractions (PO41 to PO48). Sub-fractions PO43 and PO42 were then subjected to semi-preparative HPLC using MeOH-H_2O (70:30 to 90:10) to yield compound **1** (PO432; 6.2 mg) and compound **3** (codes: PO42211 and RP(MeOH)-70%A-E; 13.3 mg) respectively. The crude extract (1.5 g) resulting from the co-cultivation experiment was subjected to VLC in silica gel with gradient mixtures of *n*-Hex and EtOAc. One of the fractions [*n*-Hex:EtOAc (70:30); 110 mg] was subjected to Sephadex LH-20 column using 100% MeOH to yield 14 sub-fractions (70%C-Hex-Seph-1 to 70%C-Hex-Seph-14). Semi-preparative HPLC of sub-fraction 70%C-Hex-Seph-4 using MeOH-H_2O (70:30 to 95:5) yielded compound 9 (code: 70%C-Hex-Seph-4-D; 21 mg).

General experimental procedures

NMR measurements of the isolated compounds were carried out either in deuterated MeOH, dimethyl sulfoxide (DMSO) or chloroform ($CHCl_3$) using a Bruker Avance DMX 300 and 600 spectrometers (Bruker BioSpin, Germany). The NMR spectra were referenced relative to the residual solvent signals. For mass spectral analysis, ESI-MS and HR-ESIMS were measured with a UHR-QTOF maXis 4G mass spectrometer (Bruker Daltonik, Germany). Analytical HPLC analysis was carried out using a Dionex P580 system coupled to a P580A LPG pump and a photodiode array detector (UVD340s, Dionex Softron, Germany). The HPLC instrument consists of a separation column (125 x 4 mm) prefilled with Eurosphere-10 C18 (Knauer, Germany) with MeOH and H_2O mixtures as the gradient solvent system. Semi-preparative HPLC was performed using a Merck-Hitachi HPLC System comprising of a UV detector (L-7400), pump (L-7100), and a Eurosphere column (100 C18, 300 × 8 mm, Knauer, Germany). Gradient MeOH-H_2O mixtures were used as the mobile phase at a flow rate of 5.0 mL/min. Vacuum liquid chromatography was carried out using silica gel 60 (230-400 mesh, Merck, Germany) and C_{18}-reversed phase (RP) silica gel (230-400 mesh, Merck, Germany). Open column chromatography was carried out using Sephadex LH-20 (Sigma-Aldrich, Germany). Pre-coated TLC plates (silica gel 60 F254, 20×20 cm, 0.25 mm thick, Merck, Germany) were used to monitor fractions under UV detection (Camag UV cabinet, Germany) at 254 and 366 nm. The optical rotation of compounds was measured in MeOH using a P-2000 polarimeter (Jasco, Germany). Distilled solvents were used for column chromatography and spectral-grade solvents were used for spectroscopic measurements.

Antimicrobial assay

The antibacterial activity of compound **1** was determined at concentrations of 100 to 0.049 μM against *Staphylococcus aureus* (ATCC 29213) and *Acinetobacter baumannii* (BAA 1605) using the Clinical and Laboratory Standards Institute (CLSI) broth micro-dilution method in Müller Hinton broth (CLSI, 2012). Ciprofloxacin (100 to 0.049 μg/mL) and DMSO (100%, v/v) were used as positive and negative controls respectively. Also, the antimycobacterial activity of the compound against *Mycobacterium smegmatis* (mc^2-155) and *M. tuberculosis* (H37Rv) was evaluated using the method described by Daletos *et al.* (2015). Cultures of the mycobacterial cells previously grown aerobically at 37 °C, 5% CO_2 and 85% humidity in Middlebrook 7H9 media supplemented with 0.5% (v/v) glycerol, 0.05% (v/v) Tyloxapol, and 10% (v/v) ADS enrichment (5%, w/v, bovine serum albumin fraction V; 2%, w/v, glucose; 0.85%, w/v, sodium chloride) were standardized (OD 600 nm ~0.08) and then seeded in a 96-well round-bottom microtiter plate at 1×10^5 cells per well and

incubated with test substances in a total volume of 100 µL for 5 days. Rifampicin (100 to 0.049 µg/mL) and DMSO (100%, v/v) were used as positive and negative controls respectively. To determine the viability of the cells, 10 µL of a 100 µg/mL resazurin solution (Sigma-Aldrich) was added into each well and the plates were incubated for ca. 8 h. Then cells were fixed at room temperature for 30 min after addition of formalin (5% v/v, final concentration), and fluorescence was measured using a TECAN microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to the sterile medium (0% growth) and DMSO-treated (100% growth) controls.

Cytotoxicity assay

Cytotoxicity study on compound **1** was conducted using the THP-1 cell line (human monocytic leukemia cell line) as previously described (Meier *et al.*, 2019). The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2 for 5 days. Afterwards, the cells were suspended and adjusted to a density of 1×10^6 cells/mL. Cells were then seeded into a 96-well flat-bottom plate in a total volume of 100 µL containing 2-fold serial dilutions of the tested compound in a concentration ranging from 100 to 0.049 µM. Cycloheximide (100 to 0.049 µg/mL) was used as positive control. After 48 h incubation at 37 °C in a humidified atmosphere of 5% CO_2, 10 µL resazurin solution (100 µg/mL) was added to each well and incubated for a further 4 h. Afterwards, fluorescence was quantified using a Tecan Infinite 200pro microplate reader (excitation 540 nm, emission 590 nm). Residual growth was calculated relative to non-inoculated (0% growth) and DMSO – treated (100% growth) controls, respectively.

Results

Chromatographic separation and spectroscopic analyses of the fungal extract resulted in the isolation of eight compounds 1-8. Co-culture of *P. ochrochloron* with *B. subtilis* resulted in the expression of compound 9 previously undetected in extract of the fungal axenic culture. Structures of the isolated compounds are shown in Figures 1 and 2.

Isolated compounds

Talumarin A (1)

Compound **1** exhibited $[\alpha]_D^{20}$= -49 (*c* = 0.8, MeOH), and UV (MeOH) λ_{max} 206.7, 247.2, and 313.7 characteristic of dihydroisocoumarin nucleus. The molecular formula was deduced as $C_{12}H_{12}O_6$ based on the prominent pseudomolecular ion peak at m/z 253.0706 $[M+H]^+$ in the HRESIMS spectrum. Results of the ^{1}H-NMR and 2-D NMR (600 MHz, MeOH-d_4) spectroscopic analyses are confirmed by a previous report (Küppers *et al.*, 2017).

Aspergillumarin A (**2**)

UV (MeOH) λ_{max} of compound **2** were at 239.6, 255.1 and 308.8 nm. The molecular formula was deduced as $C_{14}H_{16}O_4$ on the basis of the $[M+H]^+$ signal at m/z 249.4 (calculated as 248.4) in the ESIMS. Results of the ^{1}H-NMR (300 MHz, MeOH-d_4; 600 MHz, $CHCl_3$-*d*) spectroscopic analyses are confirmed by previous reports (Qi *et al.*, 2013; Luo *et al.*, 2019).

Andrastin A (**3**)

The molecular formula of compound **3** was deduced as $C_{28}H_{38}O_7$ on the basis of the $[M - H]^+$ signal at m/z 485.4 (calculated as 486.4) in the ESIMS. UV (MeOH) λ_{max} were at 201.4 and 262.1 nm. Results of the ^{1}H-NMR (300 MHz, MeOH-d_4) spectroscopic analyses are consistent with previous reports (Overy *et al.*, 2005; O'Brien *et al.*, 2006; Rojas-Aedo *et al.*, 2018).

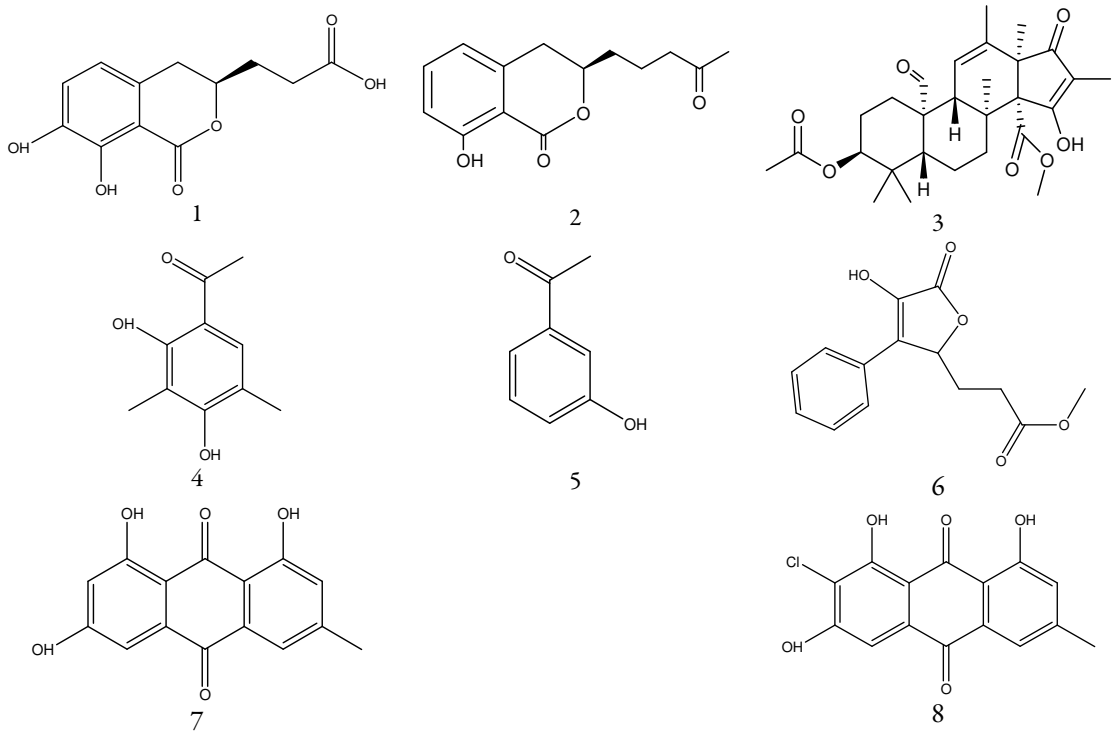

Figure 1. Compounds isolated from the fermentation extract of *P. ochrochloron*

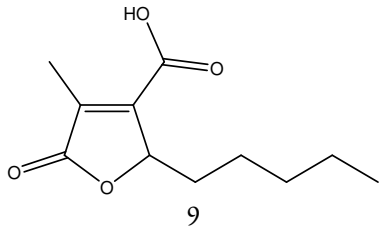

Figure 2. Compound isolated after co-cultivating *P. ochrochloron* with *B. subtilis*

Bioassays

At the concentrations analysed (100-0.049 μM), compound **1** was not active against *S. aureus*, *A. baumannii*, *M. smegmatis* and *M. tuberculosis*, and did not show cytotoxicity to THP-1 cells.

Clavatol (**4**)

UV (MeOH) λ_{max} of compound **4** were at 216.3, 286.2 and 328.4 nm. The molecular formula was deduced as $C_{10}H_{12}O_3$ on the basis of the $[M+H]^+$ signal at 181.3m/z (calculated as 180) in the ESIMS. Results of the ^{1}H-NMR (600 MHz, MeOH-d_4) spectroscopic analyses are confirmed by previous reports (da Silva and Rodrigues-Fo, 2010; da Silva *et al.*, 2013).

3-Acetylphenol, also known as 3-hydroxyacetophenone (**5**)

UV (MeOH) λ_{max} of compound **5** were at 216.9, 251.0, and 308.9 nm. The molecular formula was deduced as $C_8H_8O_2$ on the basis of the $[M - H]^+$ signal at m/z 137.1 (calculated as 136) in the ESIMS. Results of the ^{1}H-NMR (300 MHz, MeOH-d_4) spectroscopic analyses are confirmed by a previous report (Reio, 1959).

Methyl 2,5-dihydro-4-hydroxy-5-oxo-3-phenyl-2-furanpropanoate (6)

The molecular formula of compound **6** was deduced as $C_{14}H_{14}O_5$ on the basis of the $[M+H]^+$ and [M+Na] signals at m/z 263.3 and 285.3 respectively (calculated as 262.3) in the ESIMS. Results of the ^{1}H-NMR (300 MHz, MeOH-d_4) spectroscopic analyses are consistent with the report of Ancheeva *et al.* (2017).

Emodin (**7**)

The molecular formula of compound **7** was deduced as $C_{15}H_{10}O_5$ on the basis of the $[M-H]^+$ signal at m/z 269.3 (calculated as 270.3) in the ESIMS. UV (MeOH) λ_{max} were at 220.8, 266.5, and 288.0 nm. Results of the ^{1}H-NMR (300 MHz, DMSO-d_6) spectroscopic analyses are confirmed by previous reports (Frisvad, 1989; Wang *et al.*, 2014).

2-chloroemodin, also known as 7-chloroemodin (8)

UV (MeOH) λ_{max} of compound **8** were at 221.8, 272.4, and 433.9 nm. The molecular formula was deduced as $C_{15}H_9ClO_5$ on the basis of the $[M+H]^+$ signal at m/z 305.0213 (calculated as 304.0133) in the HRESIMS. Results of the ^{1}H-NMR (300 MHz, DMSO-d_6) spectroscopic analyses are confirmed by previous reports (Frisvad *et al.*, 2009).

(-)-Striatisporolide A (9)

This compound was produced by *P. ochrochloron* after co-cultivation with *B. subtilis*. Compound **9** exhibited $[\alpha]_D^{20} = -25$ ($c = 0.20$, MeOH), and UV (MeOH) λ_{max} was at 229.5 nm. The molecular formula was deduced as $C_{11}H_{16}O_4$ on the basis of the $[M-H]^+$ signal at m/z 211.1 (calculated 212.1) in the ESIMS. Results of the ^{1}H-NMR (300 MHz, MeOH-d_4) spectroscopic analyses are confirmed by a previous report (Stewart *et al.*, 2005).

Discussion

Fungi, which are well known for producing many novel chemicals that are directly used as drugs or function as lead structures for synthetic modifications, are among the most important groups of eukaryotic organisms that are being explored for generation of novel therapeutic molecules (Yadav *et al.*, 2018). The marine-derived fungi, especially the *Penicillium* spp., are rich sources of chemically diverse natural products with a broad range of biological activities. Different halophilic and halotolerant species of the genera *Penicillium* have been reported from saline habitats such as saline soils, hypersaline regions, saline lakes, etc (Butinar *et al.*, 2011; Yadav *et al.*, 2018).

The compounds isolated from *P. ochrochloron* in this study are well-known compounds, and except for compounds 1 and 8, they have all been previously reported from *Penicillium* species.

Talumarin A (1), a dihydroisocoumarin derivative, was first identified from a marine-derived fungus *Talaromyces rugulosus* associated with the Mediterranean sponge *Axinella cannabina* (Küppers *et al.*, 2017). Since our study records the second isolation of talumarin A, as well as its first isolation from *Penicillium* spp., the compound was selected for cytotoxicity and antimicrobial assays. However, talumarin A was not cytotoxic to THP-1 cells and exhibited no antimicrobial activity against the test microorganisms.

Another dihydroisocoumarin derivative, aspergillumarin A (2), has also been reported from several marine-derived fungi including *Penicillium* sp., *Aspergillus* sp. and *Talaromyces rugulosus* (Li *et al.*, 2012; Qi

et al., 2013; Küppers *et al.*, 2017; Luo *et al.*, 2019). This compound is known to possess antibacterial properties (Li *et al.*, 2012; Qi *et al.*, 2013; Yadav *et al.*, 2014).

Andrastin A (3) has been reported from several *Penicillium* species including *P. roqueforti, P. paneum*, *P. carneum* and *P. albocoremium* (Uchida *et al.*, 1996; Nielsen *et al.*, 2005; Overy *et al.*, 2005; O'Brien *et al.*, 2006; Rojas-Aedo *et al.*, 2018). Andrastins are meroterpenoids known for inhibiting protein farnesyltransferase (Matsuda and Abe, 2016).

Clavatol (4) a polyketide metabolite produced has been reported from *P. griseoroseum,* an endophyte of *Coffea arabica* (da Silva and Rodrigues-Fo, 2010; da Silva *et al.*, 2013). This compound is known to inhibit many plant pathogens (Zhang *et al.*, 2008; da Silva *et al.*, 2013).

3-Acetylphenol (5), also known as 3-hydroxyacetophenone, has been reported from a *Penicillium* species (Reio, 1959). Methyl 2,5-dihydro-4-hydroxy-5-oxo-3-phenyl-2-furanpropanoate (6) was first reported from an endophytic fungus *Chaetomium* sp. co-cultured with non-viable (autoclaved) cells of *P. aeruginosa* (Ancheeva *et al.*, 2017).

The anthraquinone, emodin (7) is a well-known plant compound, as well as a mycotoxin produced by certain species of *Penicillium* and *Aspergillus* (Frisvad, 1989; Nagashima *et al.*, 2002; Ismaiel *et al.*, 2016). This compound has been reported from *P. oxalicum, P. brunneum, P. isiandicum,* and *P. tardum* including strains from marine environments (Frisvad, 1989; Wang *et al.*, 2014; Ngan *et al.*, 2017). Emodin is reported to have several diverse biological properties including purgative, anti-inflammatory, antioxidant, antiparasitic, anticancer, and antimicrobial (Chang *et al.*, 1996; Lu *et al.*, 2008; Ismaiel *et al.*, 2016). The chlorinated anthraquinone, 2-chloroemodin (8) is a known metabolite of lichens (Nakano *et al.*, 1972; Cohen *et al.*, 1996; Caro *et al.,* 2012), and which has also been reported from *Aspergillus fumigatus* (Frisvad *et al.*, 2009). Our study is the first report of 2-chloroemodin from the genus *Penicillium*.

(-)-Striatisporolide A (9), a butenolide derivative has been previously isolated from *Penicillium striatisporum* and *P. janthinellum* (Stewart *et al.*, 2005; Liu *et al.*, 2016). Striatisporolide A is reported to possess antimicrobial properties (Deska and Bäckvall, 2009; Sheng *et al.*, 2019).

Fungi from hitherto less investigated ecological niches like arctic glaciers, deep-sea hydrothermal vents or hypersaline lakes have been attracting considerable attention in recent years as new promising sources for biologically active compounds. This study reveals the potentials of marine environments as host to fungi that express important biological active compounds. These marine fungi hold key of possibilities to the discovery of novel molecules for pharmaceutical, agricultural and applications. With the promising potentials of fungi in the area of novel drug discovery, and in the hope of achieving an effective natural products discovery process through systematic application of co-cultivation (mixed fermentation) of unrelated microbes, and as previously shown (Moussa *et al.*, 2019; Abdel-Wahab *et al.*, 2019), this study confirms co-cultivation as an interesting strategy for the discovery of new natural products.

Conclusions

This study described the isolation of eight compounds (talumarin A, aspergillumarin A, andrastin A, clavatol, 3-acetylphenol, methyl 2,5-dihydro-4-hydroxy-5-oxo-3-phenyl-2-furanpropanoate, emodin and 2-chloroemodin) from the fermentation extract of a marine-derived fungus *Penicillium ochrochloron.* Also, (-)-striatisporolide A, a known butenolide metabolite, was produced by the fungus after co-cultivation with *B. subtilis.* The findings of this study highlight the potential of marine fungi in the discovery of biomolecules with pharmaceutical, agricultural, and industrial applications, as well as the prospects of co-cultivation as an interesting approach for the discovery of new natural products.

Authors' Contributions

Funding acquisition: P.M.E.; investigation, methodology, data curation and analysis: P.M.E., Y.G., Y.L. and Lv.G.; writing - original draft: P.M.E.; writing - review and editing, visualization and validation: P.M.E., C.P.E., C.O.E., and F.B.C.O; conceptualization, supervision and research resources: P.P. and R.K. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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