Physiological Studies on Some Biologically Active Secondary Metabolites from Marine-Derived Fungus *Penicillium brevicompactum*

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Abstract

The fungal isolate *Penicillium brevicompactum* which isolated from the associated marine alga *Pterocladia* sp. in autumn season was able to produce 11 clear and active compounds, separated by the best solvent system dichloromethane: methanol (95:5 v/v). Compounds 4 and 9 were considered as antibacterial compounds, active against gram positive (*B. subtillis*) and gram negative (*E. coli*) bacteria. Malt extract broth medium with initial pH 4 when incubated at 28 °C in an incubator shaker at 200 rpm for 12 days were the most favorable conditions for compound 4 production (19.87 mg/l). The suitable conditions for compound 9 production (121.13 mg/L) were potato carrot broth medium, initial pH 4, incubation temperature 26 °C at 180 rpm after incubation period for 10 days. Structural elucidation of the pure compounds suggested that compound 4 may be [Di(2-ethyl hexayl) phthalate], and compound 9 may be fungisterol or one of its isomers. Pure compounds were evaluated for cytotoxicity towards 6 different types of tumor cell lines performed in Cancer Biology Department, National Cancer Institute, Cairo University. The results revealed that, the maximum concentration of compound 4(100 μg/mL) kills about 30% of lung cells. The maximum concentration of compound 9 (100 μg/mL) kills approximately 40% of the viable infected liver cells and also kills approximately 50% of the viable infected lung cells at concentration equal to 91.6 μg/mL. It can be concluded that compound 9 can be recommended as an anticancer compound.

Keywords: Marine fungi, *Penicillium brevicompactum*, Pharmaceuticals, Cytotoxicity, Anticancer compounds.

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1. Introduction

Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities (Smetanina et al., 2007; Blunt et al., 2006; Pivkin et al., 2006 and Somei et al., 2005). Natural products research is turned to marine animals and plants. A lot of structurally and pharmacologically new and interesting substances have been, for instance, isolated from algae. Compared with these organisms, marine fungi are poorly investigated, but the general increased interest in marine and estuarine habitats in the last decades has led to an increase in studies on marine fungi (Liberra and Lindequist, 1995).

Marine fungi are those that grow and sporulate in the oceans, in brackish waters and in estuarine habitats. Fungi are found in all marine environments...
from the high water line down to the deep sea. The higher marine fungi occur as parasites on plants and animals, in symbiotic associations with algae and as saprobes on dead organic materials like drift wood. They have developed unique metabolic and physiological capabilities that offer the potential for the production of biological active metabolites (Pietra, 1997).

Marine fungi have proved to be a rich source of new biologically natural products (Cuomo et al., 1995; Liberra and Lindequist, 1995; Farooq Biabani and Laatsch, 1998; Jensen and Fenical, 2000). Because of their particular living conditions, salinity, nutrition, higher pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have developed specific secondary metabolic pathways compared with terrestrial fungi (Liberra and Lindequist, 1995).

Marine microorganisms have proven to be a promising source for the production of novel antibiotic, anti tumor, and anti inflammatory agents. The marine fungi particularly those associated with marine alga, sponge, invertebrates, and sediments appear to be a rich source for secondary metabolites (Belofsky et al., 1999).

A number of antibiotics have been obtained from the culture broths of filamentous fungi to date. Recent investigations on marine filamentous fungi looking for biologically active secondary metabolites indicate the tremendous potential of them as a source of new medicines (Namikoshi et al., 2002).

From *Penicillium janczewskii*, obtained from a marine sample, two new diastereomeric quinolinones, 3S*, 4R*-di-hydroxy-4-(4′-methoxyphenyl)-3,4-dihydro-2(1H)-quinolinone (1) and 3R*, 4R*-di-hydroxy-4-(4′-methoxyphenyl)-3, 4-dihydro-2 (1H)-quinolinone (2), were identified. Along with two known alkaloids, peniprequinolone (3) and 3-methoxy-4-hydroxy-4- (4′-methoxyphenyl)-3, 4-dihydro-2(1H)-quinolinone (4) (He et al., 2005). In another study (Tsuda et al.2005) attained three pyrrolidine alkaloids, scalusamides A-C (1-3), from the cultured broth of the fungus *Penicillium citrinum*, each of 1-3 was found to be a mixture of epimers at C-7. Scalusamide A (1) exhibited antifungal and antibacterial activities. (Smetanina et al., 2007) stated the isolation of three new indole alkaloids, shearinines D, E and F, together with the known shearine A from the marine-derived strain of the fungus *Penicillium janthinellum Biourge*. Shearinines A, D and E induce apoptosis in human leukemia HL-60 cells, and shearine E also inhibits EGF-induced malignant transformation of JB6 P+ CI 41 cells in a soft agar.

2. Materials and methods

2.1. Fermentation media:

Five liquid fermentation media were used in this study. All the ingredients of these media were dissolved in 800 ml sterile sea water and 200 ml distilled water, pH was adjusted at 6.2 and sterilized at 121 °C for 15 min.

2.1.1 Biomalt broth (BI O). Shake flask culture:

Biomalt 20 g. (Sheng Wang et al., 1998).

2.1.2 Malt extract soymeal broth (MS)

Malt extract 30 g, peptone from soymeal 3 g.

2.1.3 Potato carrot broth (KM)

Cooked and sliced potatoes 20 g, cooked and sliced carrots 20 g.

2.1.4. Cellulose broth.

Cellulose powder 10 g, yeast extract 1.0 g.

2.1.5. Glucose peptone yeast extract broth (GPY).

Glucose. H2O 1.0 g, peptone from soymeal 0.5 g, yeast extract 0.1 g. (Shigemori et al., 1999; Iwamoto et al., 2001).

2.2. Procedure of fermentation:

Biomalt media (BIO) was used as liquid fermentation media for obtaining secondary metabolites (Höller, 1999). 250 ml conical flasks were used each containing 50 ml of sterile media. Each flask was inoculated with 2 discs, 10 mm. diameter, from 7 days old cultures of the solid biomalt agar (BIO) media. Inoculated flasks were incubated on a rotary shaker at 65 rpm. for 14 days at 22°C.

2.3. Extract preparation: (Belofsky et al., 1998; Höller, 1999 and Lin et al., 2000).
At the end of each growth period, inoculated flasks were collected and centrifugated. Both mycelia and filtrate were separately subjected to solvent extraction as follows:

2.3.1. Extraction of the mycelia:
The fresh mycelium of each fungus was crushed by silica gel 60 in mortar to obtain intracellular secondary metabolites, which extracted several times by ethyl acetate.

2.3.2. Extraction of the filtrate:
The filtrate of each fungus was extracted several times with ethyl acetate (v/v) in a separating funnel.

The combined ethyl acetate extracts from both mycelia and filtrate were evaporated under vacuum at 50 °C till dryness. The obtained solid material was dissolved in ethyl acetate to form the crude extract.

2.4. Determination and purification of the biologically active compounds.
Active compounds were determined by thin layer chromatography (TLC). Crude extracts in ethyl acetate were spotted 2 cm from the bottom of a precoated aluminum sheet of silica gel 60 F254 (Merk). Glass jars were saturated overnight by the solvent system dicholoromethane: methanol (95: 5) (v/v). The silica gel sheet allowed to dry and then developed in an ascending order for few hours until solvent front about 16 cm length.

Produced spots were located by their fluorescence on chromatograms under short and long wave UV light (254 and 366nm). The Rf values were determined and available pure compounds were used to prepare standard curves and also analyzed by using UV spectrophotometer.

2.5. Bioassay of the active compounds.
The pure compounds produced were tested for anti microbial activity against gram negative bacteria (Escherichia coli), gram positive bacteria (Bacillus subtilus), Candida albicans and Fusarium solani.

Antibiotic assay by filter paper disc method (Ely et al, 2004 and Petit et al., 2004) was carried out by impregnation of the compounds on filter paper discs of the same diameter (5mm).

Petri dishes containing 30 ml of growth medium (nutrient agar for bacteria and Dox media for fungi and yeast) were seeded with the test organism. Discs containing the compounds were placed on the surface of the medium at suitably spaced apart. Inhibition zones around each disc were measured in mm. after incubation at 35 °C for 24-48 h. for bacteria and yeast, and at 28-30 °C for 3-5 days for fungi.

2.6. Physical properties of biologically active compounds.
The pure compounds were subjected to the following analysis:

High resolution mass spectra (MS) and (GC/MS) were obtained on a finnigan Mat SSQ-7000 spectrometry.

The infra red spectra (IR) were obtained on the Fourier Transform infra red spectrometer (Nicolet, model 670, USA) using pellet of KBr.

Nuclear Magnetic Resonance (1H-NMR) was measured on Jeol ECA 500. The compound was dissolved in dimethyl-d6-sulphoxide (DMSO, 99.5 % deuterium).

2.7. Biological evaluation of active compounds.
Natural products may serve as lead as sources for new pharmaceuticals. Thus, the obtained pure compounds were evaluated in a diverse set of bioassay. This include specific assays, e.g. for cytotoxicity performed in cooperation with other research groups in Pharmacology unit, National Cancer Institute, Cairo University.

The biologically active compounds were tested for any cytotoxic activity against the tumor cell lines i.e., Brain tumor cell line (U251), Liver carcinoma cell line (HEPG2), Breast carcinoma cell line (MCF7), Lung carcinoma cell line (H460), Cervix carcinoma cell line (HELA) and Colon carcinoma cell line (HCT116) at drug
concentration between (0-100 ug/ml) using the SRB assay.

2.7.1. Measurement of potential cytotoxicity by SRB assay.

Potential cytotoxicity of the compound (s) was tested using the method of SKehan et al. (1990).

= Cells were plated in 96-multiwell plate (104 cells/well) for 24h. before treatment with the compound (s) to allow attachment of cells to the wall of the plate.

= Different concentrations of the compound under test (0, 10, 25, 50 and 100 ug/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose (concentration).

= Monolayer cells were incubated with the compound (s) for 48h. at 37 ºC and in atmosphere of 5% CO2.

= After incubation for 48h, cells were fixed, washed and stained with sulforhodamine B stain.

= Excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer.

= Color intensity was measured in an ELISA reader.

= The relation between surviving cells and drug conc. is plotted to get the survival curve of each tumor cell line under the effect of the specified compound.

3. Results

The fungal isolate Penicillium brevicompactum which isolated from the associated marine alga Pterocladia sp. in autumn season was able to produce high number of clear and active compounds.

Separation and antimicrobial investigation of the extracts

The crude extract of the marine fungus Penicillium brevicompactum (APT) was spotted on precoated silica gel sheet F254 and allowed to dry, plate was developed in the solvent system, Dichloromethane: Methanol (95:5 v/v).

In P. brevicompactum, 11 compounds were separated from each other, the most active compounds are 4 and 9. They considered as anti bacterial compounds, active against gram positive (B. subtilis) (10mm for compound 4 & 13mm for compound 9), and gram negative bacteria (E. coli) (11mm for compound 4 & 12mm for compound 9).

Physiological factors affecting compounds 4 & 9 production by Penicillium brevicompactum.

The aim of this study was to select the most favorable conditions i.e. (medium, incubation period, rpm, temperature, and pH value) which can help optimize biomass and active compounds production.

Penicillium brevicompactum produced 11 different compounds. The different compounds were extracted from culture filtrate with ethyl acetate and purified by TLC using the solvent systems dichloromethane: methanol (95:5v/v). Compounds 4&9 were the most active against gram positive (B.subtilis) and gram negative bacteria (E.coli), they considered antibacterial compounds.

Survey of medium favoring compounds 4&9 production

Liquid fermentation has advantages in allowing continual control of nutrients, pH and temperature. The aim of this experiment was to select the most favorable medium for compounds 4&9 production. The fungal isolate Penicillium brevicompactum was inoculated in 5 different media i.e., BIO, MS, KM, Cellulose broth and GPY with pH 6.2.

Fifty mL of the medium were placed in 250mL conical flask, the flasks were then inoculated with 2 disks 10 mm. in diameter from the edge of expanding colonies grown for 7 days and incubated for 14 days at 24ºC on rotary shaker at 65 rpm, the experiment was run in duplicate. Compounds were extracted from culture filtrate and mycelium as a crude material with ethyl acetate and purified through TLC.

Figure 1 showed that Penicillium brevicompactum produced the two compounds in all the different media but with different concentration. Data also revealed that MS medium was the most favorable for compound 4 production (17.086 mg/l) followed by BIO medium (6.242 mg/l) and GPY was the poor medium used, the results also showed that KM medium was the most
favorable medium for compound 9 production (63.051 mg/l) followed by BIO medium (51.753 mg/l) while GPY medium was the poorest one. From the aforementioned results, it was clear that the fungal isolate showed different activities for compounds 4&9 production according to the medium composition.

**Figure (1):** Effect of different fermentation media on compounds 4&9 production.

**Effect of incubation period on compounds 4&9 production**

The main goal of this experiment was to determine the optimum incubation period for *Penicillium brevicompactum* active compounds production. It was grown in biomalt medium (BIO), at pH 6.2. Flasks were incubated at 24°C on an incubator shaker 65 rpm for 16 days. The compounds production were determined each two days. Figure 2 showed that the incubation period affected greatly production of compounds 4&9. Compound 4 increased gradually by increasing incubation period until reached the maximum (10.58 mg/l) after 12 days, and then decreased. Compound 9 increased gradually by increasing incubation period until reached the maximum (21.039 mg/l) after 10 days, then decreased. The results coincided with the feedback inhibition theory.
Effect of temperature on compounds 4 & 9 production

An experiment was designed to determine the optimum temperature for fungal growth to be able to produce high quantity of compounds. BIO medium was used for growing the fungus isolate and incubated on an incubator shaker at different degrees of temperature (22, 24, 26, 28, 30 and 32 °C). The aforementioned culture conditions were adapted.

Results given showed progressive increase of compound 4 production as incubation temperature increased until reached maximum at 28 °C (22.688 mg/L) and then declined Figure 3. On the other hand compound 9 recorded maximum production at 26 °C (26.97 mg/L).

Effect of different initial pH values on compounds 4 & 9 production

The purpose of this experiment was to determine the optimum pH-values of fermentation medium suitable for compounds production. The initial pH of BIO medium was adjusted at 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.2, 6.5, 7, 7.5 and 8. The inoculum size and incubation conditions were as described in the previous experiments.

Figure 4 showed that Bio medium at pH 4 was most suitable for Penicillium brevicompactum to grow and produce high quantity of compounds 4 (8.700 mg/L) and 9 (12.889 mg/L), but more or less of pH caused decrease in compounds production.
Effect of aeration levels on compounds 4&9 production

It was economically important to examine the role of aeration levels on compounds 4&9 production in biomalt medium (BIO). In order to maintain different levels of aeration, Erlenmeyer flasks (250 mL) were used for fungal growth, incubated on an incubator shaker at different rpm (0, 65, 100, 150, 180, 200, 220).

After incubation period of 14 days at 24 °C and pH 6.2, production of compounds 4&9 by Penicillium brevicaespactum illustrated that increasing aeration levels was in favour of more compounds 4 & 9 production and reached maximum at 200 rpm for compound 4 (12.351 mg/l), and at 180 rpm for compound 9 (20.476 mg/l) (Figure 5).
The aim of this experiment was to determine the higher amount of each compound produced when the fermentation takes place according to the most favourable conditions (i.e. medium, incubation period, rpm, temperature, and pH value) in the previous studies.

Production of the biologically active compounds 4 and 9 at the favorable physiological conditions

The fungal isolate was inoculated in malt extract soy meal broth (MS) medium the favorable one, with initial pH 4, incubated at 28 °C on an incubator shaker at 200 rpm for 12 days. The results showed that the optimum production of compound 4 was 19.869 mg/L.
Production of compound 9 produced by the fungus \textit{P. brevicompactum}

After determining the favorable physiological factors affecting compound 9 production, the fungus was inoculated in potato carrot broth (KM) medium with initial pH 4 and incubated at 26 °C on an incubator shaker at 180 rpm for 10 days, the production of compound 9 obtained was 121.132 mg/L.

![Figure (5): Effect of different aeration levels on compounds 4&9 production.](image)

Figure (5): Effect of different aeration levels on compounds 4&9 production.
Structural elucidation of the most active compounds 4 and 9 (Budzikiewicz et al., 1964; Williams and Fleming, 1980).

The obtained data, UV, IR, 1H NMR and MS suggested that the isolated compounds may be [Di (2-ethyl hexyl) phthalate] and fungisterol or one of its isomers Figure 6.

Biological evaluation of active compounds

The obtained pure compounds were evaluated for potential cytotoxicity, against the 6 tumor cell lines i.e. brain tumor cell line (U251), liver carcinoma cell line (HEPG2), breast carcinoma cell line (MCF7), lung carcinoma cell line (H460), cervix carcinoma cell line (HELA) and colon carcinoma cell line (HCT116).

Each cell line was treated with serial concentrations of 10, 25, 50 and 100 μg/mL of each compound, and incubated at 37 °C in 5% CO2. Viable cells were counted after 48 h from incubation.

Compound 4 obtained from the fungus Penicillium brevicompactum was tested for any cytotoxic activity against the 6 tumor cell lines

Results illustrated in Figure 7 showed the following effects:

Different concentrations of compound 4 have approximately the same effect on the viable cells of 5 carcinoma cell lines. The maximum concentration 100 μg/mL kills only 20% of the viable cells, while killed 30% of lung carcinoma cell line. It seemed to be susceptible to the different concentrations used.
Compound 9 obtained from the fungus Penicillium brevicompactum was tested for any cytotoxic activity against the 6 tumor cell lines.

Results illustrated in Figure 8 showed the following effects:

Different concentrations of compound 9 obtained from the fungus Penicillium brevicompactum have different activities against the viable cell count of the 6 different tumor cell lines tested. Where there was weak effect of the concentrations on the viable cell count of breast and cervix carcinoma cell lines (20%), the number of viable cells in infected brain and colon decreased gradually by increasing the concentration until reached the maximum concentration 100 μg/mL which kills about 30% of the viable cells. And results recorded showed that increasing compound 9 concentrations was in favor of more death of viable infected liver cells and reached maximum at the concentration of 100 μg/mL, which kills approximately 40% of the viable cells, so it was considered as a moderate active compound.

Data also revealed that the number of viable infected lung cells decreased gradually by increasing compound 9 concentration, and the concentration in range equal to 50-100 μg/mL (IC50=91.6 μg/mL) was the most favorable for Killing approximately 50% of the viable cells.
3. Discussion

The results obtained with this selected strain demonstrated that it produced antimicrobial products, so that *Penicillium brevicompactum* (APt) was the most promising which able to produce the higher number of clear, highly active compounds.

From the marine fungus *Penicillium* sp. Lin et al. (2000) stated the isolation of penicillazine (1) a new compound with both quinolone and 4H-5,6-dihydro-1,2-oxazine ring systems. Iwamoto et. al. (2001) attained new cytochalasans, penochalasins D-H, along with chaetoglobosin O, which exhibited significant cytotoxicity against cultured P388 cells. Li et. al. (2007) stated the characterization of Penisporolides A and B, two novel compounds containing a rare spiral-lactone skeleton. Also the first member of a novel family of bioactive spiroditerpenoids, a potential allelopathic agent, (+)-Brevione A, which show several biological activities such as anti-juvenile-hormones, insecticidal and fungicidal activity, has been isolated from extracts of semi-solid fermented *Penicillium brevicompactum* (Macias et al., 2000).

Optimization of the conditions favoring production of the biologically active compounds (i.e. medium, incubation period, rpm, temperature, and pH value) was studied. Survey of medium favoring compound 4&9 productions indicated that (MS) medium was the most favorable media for compound 4 production (17.086 mg/l), but (KM) medium was the most favorable for compound 9 production (63.051mg/l),
followed by (BIO) medium. (GPY) was the lowest medium used for compound 4 and 9 production. These results lind with those of Shigemori et al. (1999) who used (GPY) a suitable medium for the production of seragakinone A from a marine-derived fungus.

From the aforementioned results, it is clear that the fungal isolate showed different activities for compounds 4&9 production according to the medium composition.

The medium contain malt extract with peptone was suitable substrates for compound 4 production. These results agree with those of Tsukamoto et al. (2004) who used medium contain malt extract and peptone for production of Aspermytin A from marine-derived fungus of the genus Aspergillus. But the rich fresh materials such as cooked and mashed potatoes& carrots were suitable substrates for compound 9 productions.

By studying the effect of incubation period on compound 4&9 productions it was found that the incubation period affects greatly production of compounds 4&9. Compound 4 increased gradually by increasing incubation period until reached the maximum (10.58mg/L) after 12 days, then decreased. Compound 9 increased gradually by increasing incubation period until reached the maximum (21.039mg/L) after 10 days, then decreased. These results agree with those of Daferner et al. (2002) who obtained zopfillamides A and B from the marine fungus Zopfiella latipes after 264 h. of fermentation. But Toske et al. (1998) used a marine fungus of the genus Aspergillus for aspergillamides A and B (1, 2) production after 13 days of incubation period.

In this study, it was observed that compound 4 production increased as incubation temperature increased and reached maximum at 28 °C (22.688 mg/L) and then declined, except for compound 9 recorded maximum production at 26 °C (26.97 mg/L). These results agreed with Tsuda et al. (2003) who found that the optimum temperature for speradine A production by a marine-derived fungus Aspergillus tamarii was 28 °C.

The optimum initial pH-values of fermentation medium suitable for compound 4 (8.700 mg/l) & compound 9 (12.889 mg/L) productions was pH 4, but increasing the pH to 4.5 caused decline in compounds production. These results agreed with those of Wang et al. (2005) who found that the optimum pH for proteases production from Aspergillus fumigatus was 4.

By studying the role of aeration (rpm) on compound 4&9 productions it was found that increasing aeration levels was in favor of more production and the maximum at 200 rpm (12.351 mg/L) for compound 4 production, but reached maximum at 180 rpm (20.476 mg/L) for compound 9. These results agreed with those of Mohapatra et al. (1998) and Raghukumar et al. (1999) who incubated flasks of fermentation medium on rotary shaker at 200 rpm to produce amylos by Mucor sp. and lignin-modifying enzymes by Flavodon flavus, respectively. But Kuznetsova et. al. (1998) used Cladosporium sphaerospermum for sterols production at 220 rpm.

The most active extracts (compound 4 and 9) in the agar diffusion assay, originated from the fungal strain Penicillium brevicompactum, were further investigated for cytotoxicity towards 6 different types of tumor cell lines. Data obtained showed that different concentrations of compound 4 produced by the fungus Penicillium brevicompactum have weak effect on the viable cell count of 5 carcinoma cell lines (20%), except Lung carcinoma cell line (30%). There was weak effect of compound 9 concentrations on the viable cell count of breast and cervix carcinoma cell lines (20%), but there was a moderate effect of compound 9 concentrations on the viable cell count of colon and brain carcinoma cell lines (30%), it was considered as a moderate active compound, where it kills approximately 40% of the viable infected liver cells and also approximately 50% of viable infected lung cells at concentration equal to 91.6 μg/mL. These results of compound 9 agreed with those of (Jang et al., 2006) who obtained a new dihydrobenzofuran derivative, awajanoran (1), from the marine-derived Acremonium sp. AWA16-1. Awajanoran (1) inhibited the growth of A549 cells, the human lung adenocarcinoma cell line, with IC50 value of 17 mg/mL. But from the marine-derived fungus Microsporum cf. gypseum (Gu et al., 2007) isolated two new cyclic peptides, microsporins A and B. Microsporin A showed in vitro cytotoxicity against human colon adenocarcinoma HCT-116 (IC50 0.6
mg/mL) and a mean IC50 value of 2.7 mM in the National Cancer Institute's diverse 60-cell line panel. Microsporin B showed reduced in vitro cytotoxicity against HCT-116 (IC50 8.5 mg/mL).

Also cultivation of the fungus Emericella nidulans yielded two new compounds, arugosins G (1) and H (2), together with the known metabolites 3-9, the indole alkaloid 7 displayed antitumor activity in a panel of 36 human tumor cell lines, exhibiting a mean IC50 value of 5.5 mg/mL in an in vitro survival and proliferation assay. Furthermore, compounds 3 and 4 showed moderate antitumor activity toward individual tumor cell lines (Kralj et al., 2006).

REFERENCES


