

Production of some biologically active secondary metabolites from marine-derived fungus *Varicosporina ramulosa*

Atalla, M. Mabrouk.,¹ Zeinab, H. Kheiralla.,² Eman, R. Hamed.,¹ Amani, A. Youssry² and Abeer, A. Abd El Aty¹

¹ Chemistry of Natural and Microbial Products Dept. National Research Centre, Cairo, Egypt

² Botany Dept. Faculty of Girls for Arts, Science and Education, Ain Shams University
E.mail: atallam@mailcity.com , erhamed@yahoo.com

ABSTRACT

In a screening of fungal isolates associated with marine algae collected from Abou-keer, Alexandria during the four seasons of 2004, to obtain new biologically active compounds. *Varicosporina ramulosa* isolate was identified and selected as a producer of 13 compounds. Out of 13 pure compounds produced, compounds 3 and 10 were considered as antibacterial and antifungal compounds, respectively as they were active against gram positive, gram negative bacteria and a fungus. Optimization of conditions (fermentation media, incubation period, temperature, initial pH, aeration levels) which activate compounds 3 and 10 production were studied. Also the spectral properties (UV, MS, GC/MS, IR and ¹H-NMR) of the purified compounds were determined. Compound 3 suggested to be dibutyl phthalate and compound 10 may be ergosterol or one of its isomers. Biological evaluation of the two compounds towards 6 different types of tumor cell lines showed weak effect of compound 3 at different concentrations on the viable cell count of the different tumor cell lines. While compound 10 showed different activities against the viable cell count of the 6 different tumor cell lines. It kills 50% of the viable infected liver and lung cells at concentrations equal to 99.7 µg/mL, 74.9µg/mL, respectively. Compound 10 can be recommended as new anticancer compounds.

Keywords: Marine fungi, Cytotoxicity, Pharmaceuticals, Secondary metabolites, anticancer compounds, *Varicosporina ramulosa*.

INTRODUCTION

In recent years, chemical studies of culturable marine microorganisms have led to the discovery of numerous, structurally novel, biologically active secondary metabolites. Although most of these compounds were isolated from marine bacteria, a growing number of new structures have been reported from filamentous marine fungi.

Marine fungi proved to be a rich source of new biologically natural products (Cuomo *et al.*, 1995; Farooq Biabani and Laatsch, 1998; Jensen and Fenical, 2000). Because of their particular living conditions, salinity, nutrition, high 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).

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).

Belofsky *et al.* (1998) isolated four new sesquiterpenoid nitrobenzoyl esters (1-4) 9 α , 14-Dihydroxy-6 β -p-nitrobenzoyl-cinnamolide (1) from the fungus *Aspergillus versicolor*, which displayed significant cytotoxicity against

HCT-116 human colon carcinoma cells in vitro and exhibited moderately selective cytotoxicity towards a panel of renal tumor cell lines. In (1999) they isolated a new cyclic pentadepsipeptide, sansalvamide (1) from organic extracts of the mycelium of a marine fungus *Fusarium*. Sansalvamide exhibited selective in vitro cytotoxicity towards COLO 205 colon and SK-MEL-2melanoma cancer cell lines. In (2000) they isolated three new oxpin-containing natural products (1-3) and two new fumiquinazoline metabolites (4-5) from organic extracts of the culture broth and mycelia of a marine isolate of the genus *Acremonium*. Compound 1 exhibited good anti-inflammatory in a tropical RTX-induced mouse ear edema assay. Compounds 4 and 5 exhibited weak antifungal activity toward *Candida albicans* in broth microdilution assay.

Höller *et al.*, (1999) recorded the production of five macrodiolides grahamimycin A1 (6), colletotetol (7), (6R, 11R, 12R, 14R)-colletodiol (8), 9, 10-dihydro-(6R, 11S, 12S, 14S)-colletodiol (9) and 9, 10-dihydro-(6R, 11R, 12R, 14R)-colletodiol (10) together with ergosterol from marine fungus *Varicosporina ramulosa* (strain no. 195-31). Compound (9) and (10) being new fungal metabolites and showed antimicrobial activity against *E. repens*, compound

* Corresponding author

7 also showed antifungal activities against *E. repens* and *U. violacea*,

Cueto *et al.*, (2000) isolated N-Methylsalsalvamide(1), a new cytotoxic cyclic depsipeptide from extracts of cultured marine fungus, strain CNL-619, identified as a member of the genus *Fusarium*. N-Methylsalsalvamide exhibits weak *in vitro* cytotoxicity in the NCI human tumor cell line screen (GI_{50} 8.3 μ M).

Chinworrungsee *et al.*, (2001) recorded the production of three known compounds, 2-hexylidene-3-methylsuccinic acid (1), cytochalasin Q (2), and 5-carboxymellein (3), together with two new derivatives, 2-hexylidene-3-methylsuccinic acid 4-methyl ester (4) and an ophiobolane sesterterpene named halorosellinic acid (5), from culture broth of the marine fungus *Halorosellinia oceanica* BCC5149. Compounds 1-3 exhibited moderate cytotoxicity against KB and BC-1 cell lines with IC_{50} values of 1-13 μ g/ml, while compounds 2, 3, 5, and 6 showed antimalarial activity with respective IC_{50} values of 17, 4, 13, and 19 μ g/ml. Halorosellinic acid (5) possessed only weak antimycobacterial activity with the minimum inhibitory concentration of 200 μ g/ml. Jadulco *et al.*, (2001) reported the isolation of a new furan carboxylic acid, an acetyl derivative of *sumiki's acid* from the marine fungus *Cladosporium herbarum* that was found to be active against *Bacillus subtilis* and *S. aureus*. Kuznetsova *et al.*, (2001) identified the fusidic acid, a steroidal antibiotic from marine isolate of the fungus *Stilbella aciculosa*.

Bringmann *et al.*, (2003) isolated two new products, evariquinone and isoemicellin from a strain of the fungus *Emericella varicolor*. Evariquinone showed antiproliferative activity towards KB and NCL-H460 cells at a concentration of 3.16 μ g /ml. Furthermore, the fungus was found to produce the known metabolites stromemycin, shamixanthone, and 7-hydroxyemodin.

Wei *et al.*, (2004) attained two new sesterterpenes, 6-epi-ophiobolin G and 6-epi-ophiobolin N, and six known ophiobolins from the extracts of the fungus *Emericella varicolor* GF10. Ophiobolin K showed cytotoxic activity against various tumor cell lines, including adriamycin-resistant mouse leukemia cells (P388), with IC_{50} of 0.27-0.65 μ M. In (2005) he attained shimalactone A (1), a novel polyketide having bicycle [4.2.0] octadine and oxabicyclo [2.2.1] heptane units. From the same marine fungus *Emericella varicolor* GF10, Shimalactone A (1) induced neurogenesis at 10 μ g/mL against neuroblastoma Neuro 2A cells.

Lin *et al.*, (2005) isolated new compound (1), named diaporthelactone, together with two known compounds (2 and 3) from the culture of the marine fungus *Diaporthe sp.*, Compound (1) showed cytotoxic activity against KB and Raji cell lines (IC_{50} 6.25 and 5.51 μ g/mL, respectively). The two known compounds also were demonstrated to exhibit cytotoxic activities for the first time. All three compounds were assessed for antimicrobial activity. Tsukamoto *et al.*,

(2005) extracted a new ubiquitin-activating enzyme (E1) inhibitor, himeic acid A, from a culture of marine-derived fungus, *Aspergillus sp.* but in (2006) they isolated hexylitaconic acid, a new inhibitor of p53-HDM2 interaction, from a culture of marine derived fungus, *Arthrinium sp.*

Sun *et al.*, (2006) reported the production of a new cyclotetrapeptide, trichoderide A, from the marine fungus *Trichoderma reesei*. Which showed moderate cytotoxicity against human A375-S2 melanoma cell line. Xu *et al.*, (2006) isolated three new trichothecenes, named 12-hydroxyroridin E (1), roridin Q (2), and 2,3 deoxyroridin D (3) from the marine-derived fungus *Myrothecium roridum* TUF 98F42, and a new macrocyclic trichothecene, named roridin R (4) from *Myrothecium sp.* TUF 02F6 together with roridins A and H and isororidin E. The IC_{50} values of compounds 1, 2, and 4 against the murine leukemia cell line L 1210 were 0.19, 31.2, and 0.45 μ M, respectively. Compound 3 showed antiyeast activity to *Saccharomyces cerevisiae* at 1 μ g/disc.

Gu *et al.*, (2007) isolated two new cyclic peptides, microsporins A and B (7 and 8) from culture extracts of the marine-derived fungus *Microsporium cf. gypseum*. The microsporins A and B were the first cyclic tetrapeptide histone deacetylase inhibitors with significant cancer cell cytotoxicities. Microsporin A (7) showed *in vitro* cytotoxicity against human colon adenocarcinoma HCT-116 (IC_{50} 0.6 mg/mL) and a mean IC_{50} 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 (IC_{50} 8.5 mg/mL).

MATERIALS AND METHODS

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.

Biomalt broth (BIO) in g/L.

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

Malt extract broth (MS) in g/L.

Malt extract 30 g, peptone 3 g.

Potato carrot broth (KM) in g/L.

20 g of potatoes were sliced and cooked for 30 min in a suitable volume of distilled water, the left to stand, squeezed through muslin layers and the extracted decoction was collected. The same procedure was adopted for carrot. Both decoctions were pooled, mixed, and volume was adjusted to 1 liter. pH of the medium was adjusted to the desired number using 0.1 N HCL or 0.1N NaOH. The medium was distributed in 250 mL conical flasks each

containing 50 ml medium. The flasks were sterilized for 15 min at 15 lbs.

Cellulose broth (g/L)

Cellulose powder 10 g, yeast extract 1.0 g.

Glucose peptone yeast extract broth (GPY) in g/L

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

Procedure of fermentation

Biomalt medium (BIO) was used as liquid fermentation medium for obtaining secondary metabolites (Höller, 1999). 250 ml conical flasks were used each containing 50 ml of sterile medium. Each flask was inoculated with 2 discs, 10 mm. diameter, from 7 days old cultures formerly inoculated with 1ml spore suspension (10⁴ spore/mL) of the solid biomalt agar (BIO) medium. Inoculated flasks were incubated on a rotary shaker at 65 rpm, for 14 days at 22 °C.

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:-

Extraction of the mycelia

The fresh mycelium of each fungus was washed three times with sterile distilled water to remove adherent filtrate, and then plotted between folds of sterilized filter paper no 1. The plotted mycelium was crushed by silica gel 60 in mortar, extracted by ethyl acetate to obtain intracellular metabolites. Both crushing and extraction were repeated three times, left in separating funnel for 15 min to precipitate. The crude ethyl acetate was collected.

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.

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 dichloromethane: 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 366 nm respectively). The R_f values were determined and available pure compounds were used to prepare standard curves and also analyzed by using UV spectrophotometer.

Bioassay of the active compounds

The pure compounds produced were tested for anti microbial activity against a gram negative bacterium (*Escherichia coli*), a gram positive bacterium (*Bacillus subtilis*), *Candida albicans* and *Fusarium solani*.

Antibiotic assay by filter paper disc method (Ely *et al.*, 2004; 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.

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).

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 bio-assay. 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.

Measurement of potential cytotoxicity by SRB assay

Potential cytotoxicity of the compound(s) was tested using the method of SKeahan *et al.* (1990). Cells were plated in 96-multiwell plate (10^4 cells/ well) for 24 h 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 °C 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.

RESULTS AND DISCUSSION

Separation and antimicrobial investigation of the extract

The crude extract of the marine fungal genera *V. ramulosa* was spotted on precoated silica gel sheets F₂₅₄ and allowed to dry, plates were developed in the following solvent system: Dichloromethane: Methanol (95:5 v/v).

Antimicrobial investigations were done on the separated compounds as in Table 1. The results showed that the solvent system dichloromethane: methanol (95:5 v/v) separated a large number of compounds (13) present in base line, from *V. ramulosa*. The compounds were separated from each other and the most active compounds were 3 and 10. Compound 3 had broad spectra against bacteria, yeast, and fungus as antibacterial and antifungal compound. However, compound 10 showed activity against both gram positive (*B. subtilis*), gram negative (*E. coli*) and yeast (*C. albicans*).

Höller *et al.*, (1999) recorded the production of five macrodiolides grahamimycin A1 (6), colletoketol (7), (6R, 11R, 12R, 14R)-colletodiol (8), 9, 10-dihydro-(6R, 11S, 12S, 14S)-colletodiol (9) and 9, 10-dihydro-(6R, 11R, 12R, 14R)-colletodiol (10) together with ergosterol from marine fungus *V. ramulosa* (strain no. 195-31). Compound (9) and (10) being new fungal metabolites and showed antimicrobial activity against *E. repens*, compound 7 also

showed antifungal activities against *E. repens* and *U. violace*.

From these results it was recommended that the solvent system dichloromethane: methanol (95:5 v/v) was the most promising one for active compounds separation.

Physiological factors affecting compounds 3 and 10 production by *Varicosporina ramulosa*

The aim of these studies was to optimize the physiological factors enhancing maximum production of active compounds.

V. ramulosa produced 13 different compounds, extracted from culture filtrate with ethyl acetate and purified by TLC using the solvent system dichloromethane: methanol (95:5; v/v). Compounds 3 and 10 were the most active against gram positive, gram negative bacteria and fungus. They considered as anti-bacterial and anti-fungal compounds (broad spectrum).

Survey of medium favoring compounds 3 and 10 production

The aim of this experiment was to select the most favorable medium for compounds 3 and 10 production. The fungal isolate was inoculated in 5 different media i.e., BIO, MS, KM, Cellulose broth and GPY. The same procedure as mentioned in materials and methods section was adopted.

The results in Figure 1 showed that *V. ramulosa* produced variable amounts of compounds 3 and 10 on all the different media used. Data also revealed that MS medium was the most favorable for compounds 3 and 10 production (7.598 mg/L) and (17.298 mg/L) respectively, while KM medium was the lowest media for compound 3 productions (2.147 mg/L) also cellulose broth medium for compound 10 production (1.603 mg/L).

From the aforementioned results, it was clear that the marine fungus *V. ramulosa* showed different activities for compounds 3 and 10 production according to the medium composition.

These results agreed with what obtained by Höller *et al.*, (1999) from the fungus *V. ramulosa* (strain no. 195-31) when cultured on sea water and malt extract-containing medium for the production of five macrodiolides grahamimycin 6-10.

Effect of incubation period on compounds 3 and 10 production

The main goal of this experiment was to determine the optimum incubation period for *V. ramulosa* active compounds production. The fungus was grown in biomalt

medium (BIO), at pH 6.2. Inoculated flasks were incubated at 24 °C on an incubator shaker (65 rpm) for 16 days. The compounds production was determined each two days.

The results in Figure 2 showed that there was a consistent relation between incubation period and compounds 3 and 10 production. Compound 3 increased gradually at prolonged periods of incubation until reached the maximum (5.397 mg/L) after 8 days, then decreased. Compound 10 reached the maximum (7.163 mg/L) after 10 days and then decreased.

The obtained results are in agreement with those of Kuznetsova *et al.*, (1998) who incubated flasks of marine *Cladosporium sphaerospermum* for 8 days for sterols production. But lined with those of Sabu *et al.* (2000) who incubated flasks of marine *Beauveria sp.* for 5 days for L-glutaminase production. Compound 10 increased gradually by increasing incubation period until reached the maximum (7.163 mg/L) after 10 days, then decreased.

Table 1: Antibacterial and antifungal activities of the separated compounds produced by *V. ramulosa* by using the solvent system dichloromethane: methanol (95:5v/v).

Separated compounds	Diameter of inhibition zone in (mm)			
	<i>E. coli</i>	<i>B. subtilis</i>	<i>C. albicans</i>	<i>F. solani</i>
<i>V. ramulosa</i>				
1	11	14	13	0
2	0	0	8	0
3	11*	12*	14*	15*
4	0	0	0	0
5	0	0	0	10
6	0	0	0	8
7	9	7	0	8
8	9	9	7	8
9	9	9	7	9
10	8*	12*	11*	0
11	9*	0	0	0
12	9*	8	9	9
13				
	9*	8	0	9

Effect of temperature on compounds 3 and 10 production

This experiment was designed to determine the optimum temperature for fungal growth 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 adopted.

Results showed that compound 3 production increased as incubation temperature increased until reached maximum (6.225 mg/L) at 24 °C and then declined Figure 3. Compound 10 recorded maximum production (5.995 mg/L) at 26 °C.

These results agreed with Raghukumer *et al.*, (1999) who incubated culture flasks of a marine *Flavodon flavus* for lignin-modifying enzymes (LMEs) production at room temperature (25 to 27 °C), while marine *Cladosporium*

sphaerospermum produced sterols in fermentation medium at temperature of 22 °C (Kuznetsova *et al.*, 1998).

Effect of different initial pH values on compounds 3 and 10 productions

This has been done to determine the optimum pH-values of fermentation medium suitable for biologically active 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 8.0. The inoculum size and incubation conditions were as described in the previous experiments.

The results illustrated in Figure 4 showed that the initial pH of the medium suitable to enhance growth of *V. ramulosa* to produce high quantity of compound 3 (5.833 mg/L) and compound 10 (15.751 mg/L) was pH 6, but deviation to more or less pH caused decline in compounds production.

These results lined with those of Keerthi *et. al.*, (1999) who adjusted the pH of fermentation medium at 8.0, to produce L- glutaminase by *Beauveria bassiana* BTMF S10.

Effect of aeration levels on compounds 3 and 10 production

It was economically important to examine the role of aeration levels on compounds 3 and 10 production in biomalt medium (BIO). In order to maintain different levels of aeration, Erlenmeyer flasks (250 mL) were used for fungal growth and incubated on an incubator shaker at different rpm (0, 65, 100, 150, 180, 200, and 220).

After incubation period of 14 days at 24 °C and pH 6.2, production of compounds 3 and 10 by *V. ramulosa* were illustrated in Figure 5. It was observed that increasing aeration levels activate compound 10 production till reached maximum (17.002 mg/L) at 150 rpm, but reached maximum at 65 rpm for compound 3 (2.734 mg/L).

The results for compound 10 agreed with those mentioned by Suresh and Chandrasekaran (1999), who incubated flasks of *Beauveria bassiana* on a rotary shaker at 150 rpm for chitinase production.

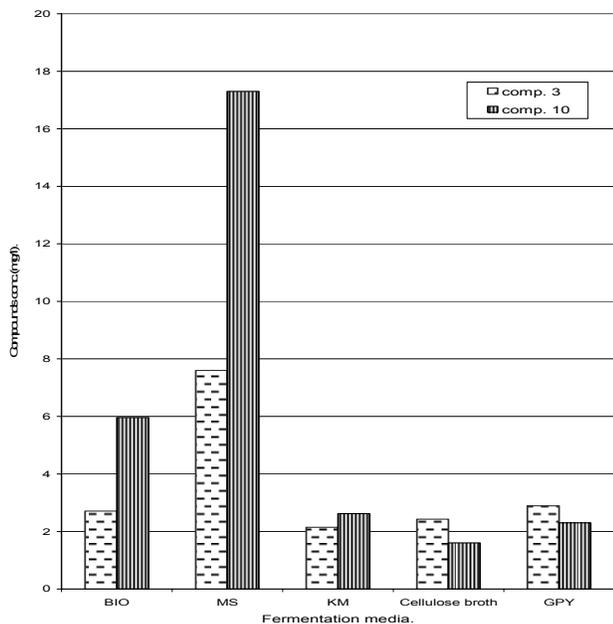


Figure 1: Effect of different fermentation media on compounds 3 and 10 production

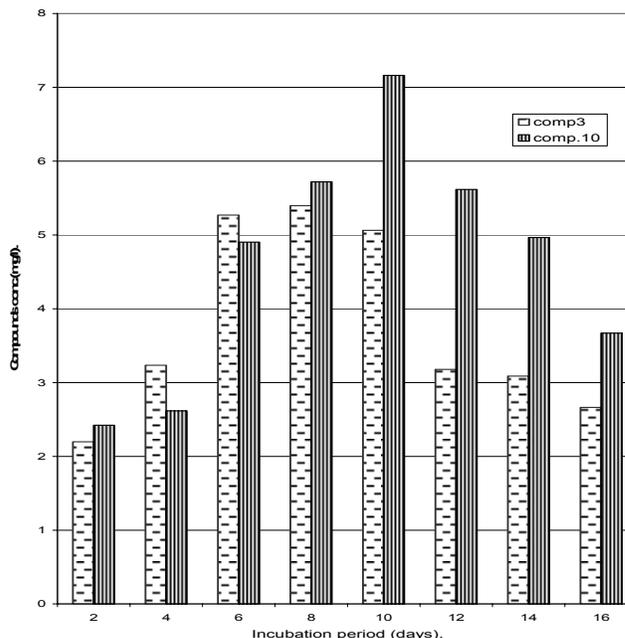


Figure 2: Effect of incubation period on compounds 3 and 10 production

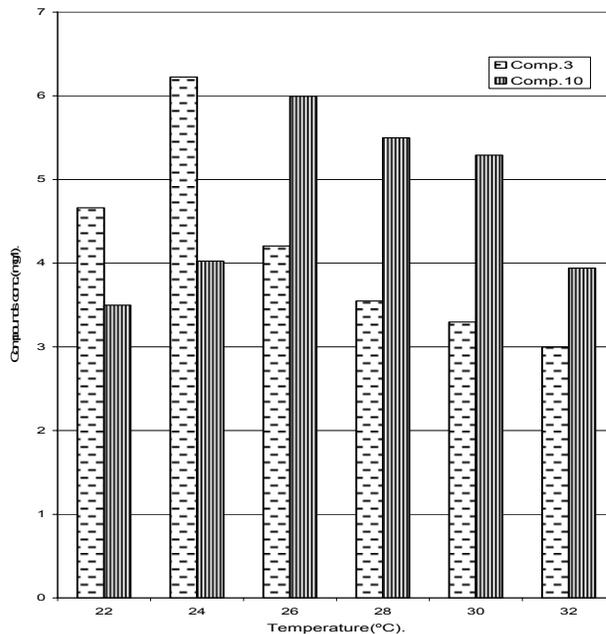


Figure 3: Effect of temperature on compounds 3 and 10 production

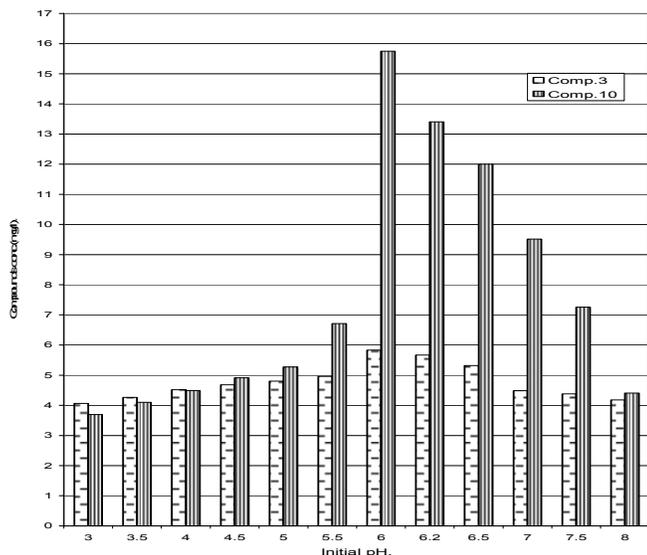


Figure 4: Effect of different initial pH-values on compounds 3 and 10 production

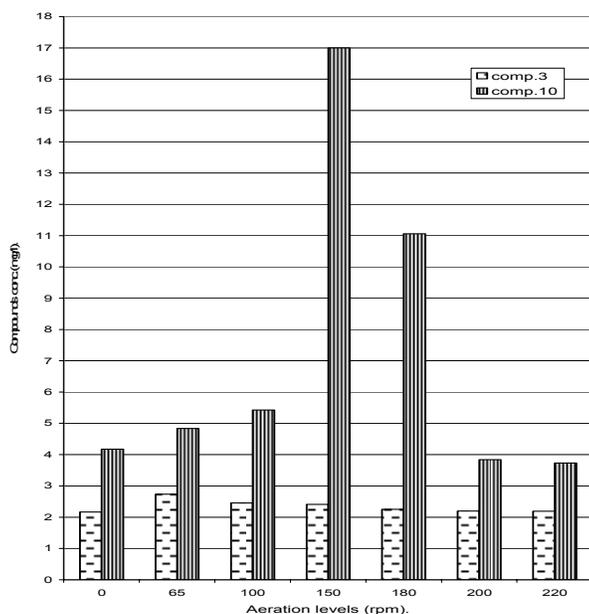


Figure 5: Effect of different aeration levels on compounds 3 and 10 production

Structural elucidation of the most active compounds (Budzikiewicz *et al.*, 1964; Williams and Fleming, 1980).

Identification of compound 3

The ultraviolet absorption spectrum of compound 3, showed absorption band at λ max. (Methanol) 260 nm.

The infrared spectrum of compound 3, showed characteristic absorption bands at 3436 cm^{-1} (OH), 2926 cm^{-1} (C-H, aliphatic), 1728 cm^{-1} (C=O, aryl carbonyl of ester), 1570 cm^{-1} and 1420 cm^{-1} (COO⁻, carboxylate ion). Note: usually water in trace amounts (Water of crystallisation), in KBr discs showed a broad band in the range $3100\text{-}3600\text{ cm}^{-1}$.

The ¹H NMR spectrum of compound 3 (DMSO), showed signals at δ 0.83 (3H, t, CH₃) and 1.0 (3H, t, CH₃) at δ 1.25-2.48 (12H, m, 6-CH₂) and 6.63-7.26 (4H, m, aromatic protons).

The mass spectrum (rel. int. %) of compound 3, showed the molecular ion at m/z 279 (M⁺+1) (13), and other fragments at 167 (40), 149 (100), 132 (5), 113 (30), 104 (10), 83 (28), 71 (34) and 55 (55). The fragments at m/z 149, 167 and 279 due to phthalic acid derivatives (dialkyl phthalate) and its molecular ion at m/z 278 corresponding to molecular formula C₁₆H₂₂O₄.

The obtained data, UV, IR, ¹H NMR and MS suggested that the isolated compound may be dibutyl phthalate Figure 6 and Table 2.

Identification of compound 10

The ultraviolet absorption spectrum of compound 10, showed absorption band at λ max. (Methanol) 274 nm.

The infrared spectrum of compound 10, showed characteristic absorption bands at 3468 cm^{-1} (OH), 2990 cm^{-1} (C-H, aliphatic), 1417 cm^{-1} (CH=CH) and 1023 cm^{-1} (C-OH, alcoholic).

The ¹H NMR spectrum of compound 10 (DMSO), showed signals at δ 0.79 (3H, CH₃), 0.84 (3H, CH₃), 0.86 (3H, CH₃), 0.87 (3H, CH₃), 0.88 (3H, CH₃) and 1.06 (3H, CH₃). Also, signals at 1.22-2.26 (19H, m), 2.49-2.67 (2H, m), 3.49-4.35 (5H, m) and at δ 8.5 (1H, OH).

The mass spectrum (rel. int. %) of compound 10, showed M⁺ at m/z 396 (5), corresponding to molecular formula C₂₈H₄₄O. It loses CH₃+ H₂O at δ 363 (3) [M⁺-(CH₃+H₂O)]. Fragment at 278 (7.5) (M⁺-C₉H₁₇), and other fragments at m/z 253 (3), 211 (4), 185 (5), 167 (32), 149 (77.5), 133 (18), 109 (23), 95 (30), 81 (47), 69 (95) and 53 (100).

The obtained data, UV, IR, ¹H NMR and MS suggested that the isolated compound may be ergosterol or one of its isomers Figure 6 and Table 2.

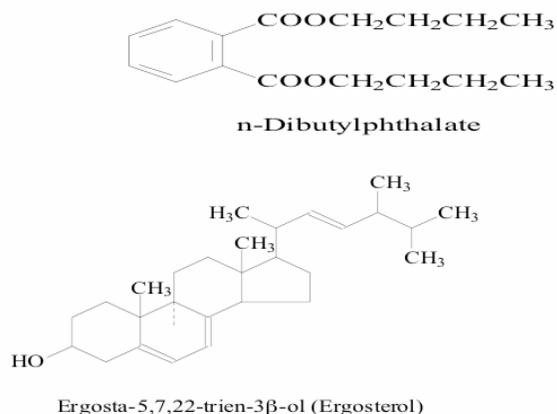


Figure 6: Structure of compound 3 and 10 produced by *V. ramulosa*

Biological evaluation of active compounds

The obtained pure compounds were evaluated for potential cytotoxicity, against 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 $\mu\text{g/mL}$ of each compound, and incubated at 37 $^{\circ}\text{C}$ in 5% CO_2 . Viable cells were counted after 48 h from incubation.

Compound 3 obtained from the fungus V. ramulosa was tested for any cytotoxic activity against the 6 tumor cell lines

From the results illustrated in Figure 7 it was clear that, compound 3 has weak effect on brain, liver, lung, cervix and colon viable infected cells and the concentration of 100 $\mu\text{g/mL}$ kills nearly 20% of the viable cells.

Also it was appeared that the maximum concentration 100 $\mu\text{g/mL}$ of compound 3 kills only 10% of the viable breast infected cells.

Compound 10 obtained from the fungus V. ramulosa was tested for any cytotoxic activity against the 6 tumor cell lines

From the results illustrated in Figure 8, it was clear that different concentrations of compound 10 have different activities against the viable cells of the 6 different tumor cell lines, where the maximum concentration 100 $\mu\text{g/mL}$ has weak effect on the viable cells of breast and colon

carcinoma cell lines 20%, it kills about 30% of the viable cervix infected cells. Also, the number of viable infected brain cells decreases gradually by increasing compound 10 concentration until reached the maximum concentration used 100 $\mu\text{g/mL}$, which kills about 40% of the viable cells, so it is considered as a moderate active compound. Increasing compound 10 concentrations was in favor of more death of viable infected liver and lung cells and reached maximum at the concentration 100 $\mu\text{g/mL}$ which kills approximately 50% of the viable cells which was in a range equal to 50-100 $\mu\text{g/mL}$. (IC_{50} =99.7 $\mu\text{g/mL}$), (IC_{50} =74.9 $\mu\text{g/mL}$), respectively.

The compound produced can be recommended as new anticancer compound. The weak effect of compound 10 on colon carcinoma cell line agree with (Abdel-Wahab *et al.*, 2007) who found that, the *Massarina* CNT-016 metabolites (1–4) showed weak activity against the human colon carcinoma cell line, HCT-116.

Mcdonald *et al.*, (1999) extracted spiroxins (1-5), DNA cleaving antitumor antibiotics from culture of a marine-derived fungus (strain LL-37H248). And also from the fungus *Beauveria bassiana* BTMF S10, Keerthi *et al.*, (1999) stated the production of extracellular L-glutaminase by alkalophilic L-glutaminase which received significant attention recently owing to its potential applications in medicine as an anticancer agent and in food industries.

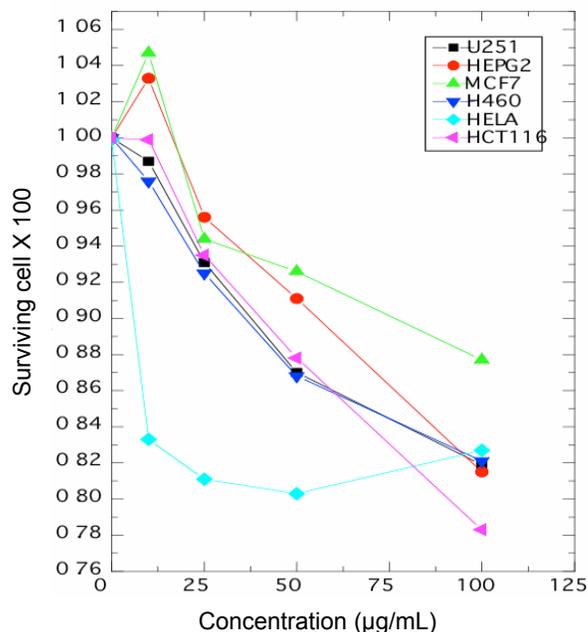


Figure 7: Effect of different concentrations of compound 3 on the viability of 6 different types of tumor cell lines

Table 2: Physical characterization of compounds 3 and 10 produced by *Varicosporina ramulosa*

Comp.	Color on TLC under UV light		Maximum absorption by UV spectrophotometer at nm.	Absorption bands by IR spectrum at (cm ⁻¹)	Mass spectrum	¹ H NMR spectrum (ppm)
	254nm	336nm				
3	-	Dark Blue	260	3436, 2926, 1728, 1570, 1420	279(13%), 167(40%), 149(100%), 132(5%), 113(30%), 104(10%), 83(28%), 71(34%), 55(55%)	0.83, 1.0, 1.25-2.48, 6.63-7.26
10	Dark	Blue Greenish	274	3468, 2990, 1417, 1023	396(5%), 363(3%), 278(7.5%), 253(3%), 211(4%), 185(5%), 167(32%), 149(77.5%), 133(18%), 109(23%), 95(30%), 81(47%), 69(95%), 53(100%)	0.79, 0.84, 0.86, 0.87, 0.88, 1.06, 1.22-2.26, 2.49-2.67, 3.49-4.35, 8.5

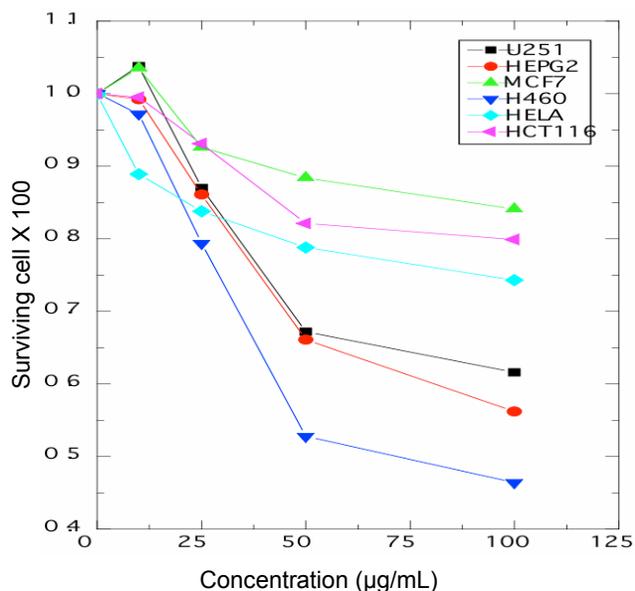


Figure 8: Effect of different concentrations of compound 10 on the viability of 6 different types of tumor cell lines

CONCLUSIONS

It can be concluded that, isolated marine fungus *V. ramulosa* showed different activities for production of compounds 3 and 10 when grown on malt extract broth medium (MS) at pH 6 on a rotary shaker at 65 r.p.m for 8-10 days at 24-26 °C respectively. Compound 3 was identified as dibutyl phthalate, while compound 10 was ergosterol or one of its isomers. The two compounds were evaluated as anticancer compounds. The results proved that compound 10 was a new anticancer compound especially against liver and lung tumor cell lines at concentration 99.7ug/mL and 74.9ug/mL, respectively.

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