Antimicrobial Agents Produced by Marine *Aspergillus terreus* var. *africanus* Against Some Virulent Fish Pathogens

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Abstract Screening of fungal isolates collected from different locations of Alexandria coast, Egypt, was carried out to obtain new biologically active metabolites against some virulent fish pathogens (*Edwardsiella tarda*, *Aeromonas hydrophila*, *Vibrio ordalli* and *Vibrio anguillarum*). Among 26 fungal isolates, *Aspergillus terreus* var. *africanus* was identified as the most potent isolate. Production of the bioactive material was optimized using response surface methodology including fermentation media, incubation period, temperature, pH, and thermo-stability. Spectral properties of the gas chromatography/mass spectrum of the ethyl acetate crude extract were determined. Partially purified components of the crude extract were chromatographically separated and bio-assayed. Out of ten separated compounds, five were with considerable antibacterial agent. The bio-toxicity of crude showed a slight toxicity against the brine shrimp *Artemia salina* ($LC_{50} = 1,500 \mu\text{g/l}$). Antibacterial activity of the crude was compared with some known standard antibiotics and found to be superior over many where its MIC against some pathogen reached $1 \mu\text{g/ml}$.

Keywords Marine fungi · Antimicrobial agents · Fish pathogens · Response surface · GC/MS · Bio-toxicity

Introduction

In recent years, it was sought to draw marine microbial diversity into the area of drug discovery [6]. Marine fungi have long been recognized as potential source of novel and biologically potent metabolites [26]. Fungi belonging to *Aspergillus* genera are one of the major contributors to the antimicrobial metabolites of fungal origin [3].

Marine *Aspergillus fumigatus* CY018 ethyl acetate extract afforded two new metabolites which subjected to in vitro bioactive assays against three pathogenic fungi *Candida albicans*, *Tricophyton rubrum* and *Aspergillus niger* [15]. New antibacterial have been isolated from the broth of a marine isolate of the *Aspergillus* sp. showed activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* [19]. Another two new antibacterial agents were isolated from a marine *Aspergillus* sp. strain 05F16, active against *S. aureus* and *Escherichia coli* [34]. Three new alkaloids were obtained from the ethyl acetate extract of a marine-derived fungal strain, *Aspergillus sydowi* PFW1-13 which display significant antimicrobial activities against *E. coli*, *Bacillus subtilis*, and *Micrococcus lysodeikticus* [35]. The ethyl acetate extract of the fungal strain WZ-4-11 of *Aspergillus carbonarius* showed, also, antimycobacterial activities against *Mycobacterium tuberculosis* H37Rv [36]. A recent report by Wu et al. [33] showed two new compounds from *Aspergillus* sp. (MF-93) considered as anti-viral active components. Terrestrial species of *Aspergillus terreus* known for its antibacterial activities against *S. aureus*, *Enterococcus faecalis*, *B. subtilis*, *Pseudomonas aeruginosa* and *E. coli* [24].

This report focuses on the isolation and separation of new antimicrobial agents from culture medium of marine fungus *A. terreus* var. *africanus* against some virulent fish

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pathogens. Also ethyl acetate crude extract from the isolated fungus was applied on thin layer chromatography plate for compounds separation. Characterization was carried out using gas chromatography/mass spectrum spectral analysis.

Materials and Methods

Fungal Isolation and Cultivation

13 fungal strains were isolated from different marine sites; Alexandria, Egypt and screened according to their ability to produce antimicrobial agents against some virulent fish pathogens (*Edwardsiella tarda*, *Aeromonas hydrophila*, *Vibrio ordalii* and *Vibrio anguillarum*).

Fungal isolates were grown on neopeptone-glucose rose Bengal agar containing (g/l seawater): neopeptone (5.0), glucose (10.0), 3.5 ml rose Bengal solution (2.0 g/100 ml distilled water) and agar (20.0). The pH must be adjusted at 7.3. Separately, a solution of tetracycline was prepared (1.0 g water soluble antibiotics/150 ml) and sterilized by filtration then 0.5 ml added to 100 ml melted basal agar at 45°C. 100 ml of broth medium was inoculated with the fungal isolates. This followed by incubation at 30°C on a rotary shaker at 120 rpm for 10 day.

Identification of the Potent Isolate

The potent *A. terreus* var. *africanus* were identified according to the Regional Center for Mycology and Biotechnology (RCMB), El-Azhar University, Egypt, Data Base Management System for Aspergilli Identification. Colonies on Czapek-agar grew somewhat restrictedly attaining a diameter of 3.3 cm in 7 days, mycelium at first bright yellow shades, deep colonies buff at maturity. Sclerotium-like bodies were found. Conidiophores were 4.9 µm in diameter. Vesicle was 15 × 11 µm in diameter. Sterigmata were biserial with primary structure (7.0 × 2.2 µm) and secondary structure (5.3 × 2.0 µm). Conidia globose were 2.5 µm

Antimicrobial Activity

Screening for the bioactivities was carried out using a Tryptic Soya Agar (TSA) plates and the disc diffusion assay. 400 µl of 24 h pre-cultured tested pathogen (10⁴ CFU/ml) was mixed well using 25 ml of a sterile molten media at 45°C, then pour plates to solidify. The cultivated fungi were filtered, and then sterile discs (5 mm in diameter) were immersed in the obtained free mycelia balls filtrate and placed on the prepared plates. The plates were incubated at 30°C for 24–48 h. Minimal inhibitory

concentrations in µg/ml (MIC) were measured. Moreover, the effect of three adjusted temperatures (28, 30 and 45°C) and three different pH values (6, 7 and 8) were carried out with three replications for each factor and measured in µg/ml.

Thermo-stability Test

The filtrate of the precultured *A. terreus* was divided into five portions and exposed separately to different temperature degrees (30, 40, 60, 80 and 100°C) for 30 min. The thermo-stability was assessed as a function of their bioactivity against the tested pathogens [17].

Extraction

For the extraction process, 300 ml of three polar solvents (ethyl acetate, diethyl ether and butanol) and three non-polar solvents (gasoline, n-hexane, and petroleum ether 40) were tested. The organic layer was collected and concentrated using a rotary evaporator [1]. The bioactivity against the four tested pathogens was detected.

Toxicity

The toxicity bioassay was carried out according to Meyer et al. [18] using the brine shrimp *Artemia salina*. Two hundred mg ethyl acetate-crude extract were dissolved in 2 ml DMSO. Different concentrations of the crude (100, 200, 500, 1,000, 1,500, 2,000, 4,000 and 6,000 µg/ml) were prepared, then 10 ml of sterile brackish water was added in 20 ml glass vials. Ten *A. salina* nauplii were transferred to each vial. The number of the viable biomarker was counted after 24 h of application. The percentage of mortality and the half lethal concentration (LC₅₀) were determined using the probit analysis method [23].

Compared with Antibiotics Used in Fish Therapy

Three formulations of antibiotic-loaded discs gentamicin, streptomycin and lincolin were used to test the inhibition of the tested organisms using the same routine laboratory disc diffusion method to comparing with the bioactivity of the selected isolates [27].

Determination of the Biologically Active Compounds

The selected ethyl acetate crude extract was fractionated using silica gel thin layer chromatography plates. Different mobile phases were prepared separately: acetone, acetone: ethyl acetate (1:2), acetone: ethyl acetate. (2:1), methanol, methanol: ethyl acetate (1:2), methanol: ethyl acetate (2:1), and ethyl acetate. The silica gel plates were performed in

size of 20 × 20 cm and a thickness of 0.25 mm using 60GF₂₅₄ Merck. Then the crude was applied as a spot and left to complete separation using the previously prepared mobile phases. The plates stood for solvent evaporation and the *R_f* of resulted colored and uncolored spots were recorded using an ordinary and UV lamps. Separated compounds were further examined by column chromatography using ethyl acetate as eluting solvent. The collected samples were evaporated, weighed and dissolved in ethyl acetate to be applied on inoculated agar plate. The bioactivity of the five collected samples was checked up against the four pathogens using the disc diffusion assay.

Spectral Analysis Using GC–MS

Analysis was conducted using an SHIMADZU gas chromatography/mass spectrum -QP5050A with CLASS 5000 as software. MS conditions were as follows: Detector mass spectrometer voltage 70 eV and its source temperature was 300°C. The injector temperature was 280°C. The column was performed with length 30 cm × 0.53 mm ID, coating thickness film 1.5 µm (J&WSCIENTIFIC). Temperature program was carried out as follow: 40°C (0.5 min) –150°C (1 min) at 10°C/min –250°C (5 min) at 5°C/min –270°C (5 min) at 3.5°C/min. Carried gas was HELIUM and ionization mode EI. The components were identified by comparing their retention times to those of authentic samples of WILEY MASS SPECTRAL DATA BASE Library (16).

Results and Discussion

Marine fungi are considered a remarkable source of biologically active natural products with new chemical structures [3, 8]. Therefore, it is of interest to develop different approaches for the discovery of marine fungal species that produce biologically active secondary metabolites. Facultative marine strains such as *Aspergillus*, *Penicillium*, *Verticillium* and *Phoma* are frequently isolated and produce bioactive extracts and natural products of structural novelty [5, 31]. Therefore the present study was performed to isolate some local marine fungal strains capable of producing antimicrobial agent(s) against some bacterial fish pathogens. Fungal isolate from Abou Quir bay showed highest value against *E. tarda*, (8 µg/ml) and broader spectrum observed against *V. ordalli*, *V. angularum* and *A. hydrophila* (16, 32 and 48 µg/ml, respectively).

Antimicrobial activity measured after 2, 3, 4, 5, 6, 7, 8, 9 and 10 days. There was a consistent relation between incubation period and bioactive compounds production. The inhibition zone from 3rd to 7th day should show increase in zone from 15 to 30 mm. After the 7th day, a

Fig. 1 Effect of incubation period on the productivity of *A. terreus* against fish tested organisms

gradual decrease of the antibacterial activity was observed. The loss in activity was evidently determined after 10 days of incubation (Fig. 1). The obtained results are in agreement with those of Mabrouk et al. [16] who showed that the marine isolate *Varicosporina ramulosa* inhibition zones were increased gradually until reached the maximum 15 mm after 8 days, then decreased. Moreover, Kuznetsova et al. [12] who found that the maximum production of bioactive compounds from marine *Cladosporium sphaerospermum* took 8 days. But lined with those of Sabu et al. [25] who incubated flasks of marine *Beauveria* sp. for 5 days for the maximum bioactivity.

Effect of pH and Temperature on the Productivity

Different pH values and temperature were experimentally tested. It was found that the optimum pH and temperature reached at 7 and 28°C showed the highest value of MIC at 4, 12, 24 and 48 µg/ml. The tendency of *A. terreus* bioactivity was found to increase in acidic instead of the alkaline condition against the four tested pathogens. Similarly, the initial pH of the medium suitable to enhance marine *V. ramulosa* for highest bioactivity (5.8 µg/ml) was pH 6 [16]. Gasparetti et al. [7] showed the oxidase highest activity of *Aspergillus oryzae* within an acidic and neutral pH range, having an optimum at pH 5.6. On the other hand, Lectin activity by *Aspergillus terricola* was stable within the pH range of 7.0–10.5 [28].

The maximum bioactivity was reached at 5.9 µg/ml against *E. tarda* at temperature range from 25–30°C. In addition, *A. terreus* bioactivities recorded as 14.9 and 26.5 µg/ml against *V. ordalli* and *V. anguillarum* respectively, showed a wide tolerance of temperature above 30°C. Comparing with these results, the optimum temperature for the marine *V. ramulosa* was ranged from 24–26°C with maximum productivity of 6.2 µg/ml [17]. Vishwanatha et al. [30] showed the culture flasks of *A. oryzae* MTCC 5341 for maximum acid protease production at

30°C, while, marine fungus *Aspergillus ustus* MSF3 showed antimicrobial activity in fermentation medium at temperature of 20°C [11].

Thermo-stability of the Antibacterial Substances

Sixty degree centigrade showed the greatest loss of activity of cell-free supernatant, and then complete loss was detected at 80°C. The antimicrobial agent was active at the lower temperature. Supernatant samples kept at 28°C maintained a higher activity compared to samples present at 60, 80 and 100°C (Data not shown). Singh et al. [28] showed remarkable thermostability of lectin produced by *A. terreicola* remained unaffected upon incubation at 70°C for 2.5 h. Another report by Gasparetti et al. [7] showed highest activity of catechol oxidase from *A. oryzae* with good thermostability up to 60°C. A novel antifungal peptide produced by *Aspergillus clavatus* was enriched in the supernatant after heat treatment at 70°C [29].

Toxicity

The toxicity experiment was carried out using *A. salina* as biomarker and different crude concentrations (ranging from 100 to 6,000 µg/ml). The mortality percent was estimated. The results indicated that the crude had a low toxicity effect with maximum concentration of 1,500 µg/ml after 24 h. Where, the LC₅₀ of this crude was estimated to be 3.18 ppm (Table 1). Ethyl acetate extract of marine *A. terreus* was found to be LC₅₀ = 32 µg/ml for brine shrimp lethality [13]. Another marine microorganisms extracts showed lethality toward *A. salina* at LC₅₀ < 1,000 µg/ml with an increased of lethal activity at exposition time [20].

The tested drugs were successful examined against the four pathogens to compare their activities with that obtained from *A. terreus*. The highest MICs of crude were

Table 1 Biotoxicity of different *A. terreus* crude extract concentrations using *A. salina*

Crude concentrations (µg/ml)	Log (crude concentration) (ppm)	Mortality % after 24 h Brine shrimp (<i>A. salina</i>)
100	2.00	10
200	2.30	15
500	2.70	25
1,000	3.00	45
1,500	3.18	50
2,000	3.30	65
4,000	3.60	80
6,000	3.78	100

Fig. 2 Comparison between the antibacterial activity and some commercial antibiotics used in fish therapy

recorded at 1, 4, 16 and 16 µg/ml against the four pathogens, respectively (Fig. 2).

Chromatographic Analysis

Thin layer chromatography of the crude using ethyl acetate led to separation of five spots. Three spots were yellowish brown at R_f 1, 0.45 and 0.30, and one pink spot at 0.22, while the only UV spot was shown at 0.08. Using column chromatography, the bioactivity of the collected samples found to be positive against the four pathogens. The most active one was spot number 2 with highest MIC ranged from 4–16 µg/ml. However, the obtained values for the other compounds are less than those of the crude so there is some sort of synergy among components of the crude (Table 2). Accordingly it is recommendable to use the crude as it is for the treatment.

Gas chromatography/mass spectrum analysis of the ethyl acetate crude extract showed the presence of 10 compounds (Fig. 3). The main active five detected compounds (based on comparison with standards in Wiley data base) were most similar to: (1) 1,2-Benzene dicarboxylic acid, dioctyl ester, (2) octadecanoic acid, (3) isobutyl acetate, (4) acetic acid, 2-propenyl ester, (5) propane-2-methyl with match quality % of 92, 72, 91, 89, 90, respectively (Fig. 4). Recently, different marine *Aspergillus*

Table 2 Bioactivity of the crude and the five major compounds against the three tested pathogens

Resulted spots	MIC (µg/ml)			
	<i>E. tarda</i>	<i>V. ordalli</i>	<i>V. anguillarum</i>	<i>A. hydrophila</i>
1	16 ± 0.8	32 ± 1.6	64 ± 3.2	48 ± 0.2
2	4 ± 0.2	8 ± 0.4	8 ± 0.4	16 ± 0.8
3	16 ± 0.8	24 ± 1.2	16 ± 0.8	48 ± 0.2
4	12 ± 0.6	16 ± 0.8	12 ± 0.6	32 ± 1.6
5	16 ± 0.8	32 ± 1.6	32 ± 1.6	48 ± 0.2

* Standard error of data at 5%

Fig. 3 Gas chromatogram of crude extracts showing the five major compounds at different retention times

Fig. 4 Mass-spectrum of 1,2-Benzene dicarboxylic acid, dioctyl ester (a), octadecanoic acid (b), isobutyl acetate (c), acetic acid, 2propenyl ester (d) and propane-2- methyl (e)

sp have been recognized for new biologically active secondary metabolites production [2, 3, 10]. Two new 5-Hydroxy-2-pyrone derivatives isolated from a marine *Aspergillus flavus* [14]. Other cerebroside analogues obtained from marine *Aspergillus flavipes* [9] and two new cytotoxic quinone type compounds from *Aspergillus variegator* have been isolated by Wang et al. [32]. Moreover, Wu et al. [33] were isolated new asperxanthone and

asperbiphenyl from the marine *Aspergillus* sp. and Parvatkar et al. [21] showed the Aspernolides A and B, butenolides obtained from a marine *A. terreus* have cytotoxic activity. A recent report by Choi et al. [4] showed the effect of diketopiperazine disulfides produced by marine *Aspergillus* sp. KMD 901.

The diversity of the natural products from marine fungi clearly demonstrates that there are potentials for transferring

some of these compounds into clinical trials for future development of anti-infective drugs. One of the challenges in future will be the large scale production of these compounds to meet the demand for clinical trials and drug development [22].

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