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ISOLATION, TAXONOMICAL CHARACTERIZATION, ANTIMICROBIAL ACTIVITY AND CHEMICAL PROFILING OF THIRTEEN MARINE-DERIVED FUNGI FROM THE MEDITERRANEAN SEA COASTS OF EGYPT

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Abstract

In this study, the bioactive secondary metabolites obtained from thirteen marine derived fungi were tested against seven pathogens. The reported fungi were isolated from water and sediment samples collected from the Mediterranean Sea at Egyptian coasts. Taxonomically, the fungal isolates were morphologically identified, and classified into five genera: *Penicillium* (33%), *Aspergillus* (33%), *Fusarium* (7%), *Acremonium* (7%) and yeast (13%). The strains were cultivated preliminary on broth media, and the antimicrobial activities of their supernatants were investigated. The most promising five fungal isolates (Nr.8, 9, 12, 13 and 15) were subsequently cultivated on rice-solid state medium, re-visualizing their antimicrobial activity. From the antimicrobial activity test, the phytochemical profiling of the most interesting isolates (Nr.8 and 13**)** were analyzed based on GC-MS, and their bioactive secondary metabolites were tentatively identified, revealing the presence of twenty-four and twenty-eight compounds belonging to diverse structural categories, respectively. Furthermore, the last two strains were applied to different agricultural waste as solid-state media; their antimicrobial activities were studied comparatively, and the obtained bioactive secondary metabolites were tentatively assigned as well using GC-MS analysis. We target the selection of the most promising strains for production of auspicious bioactive secondary metabolites, representing as drug leads, for forthcoming full studies.

Keywords: Marine-derived Fungi, Mediterranean Sea, Taxonomy, Antimicrobial Activity, Phytochemical Profiling

1. Introduction

Oceans have provided a complacent base for biological activities on the Earth. Many biological compounds with varying degrees of action, such as anti-tumor, anti-cancer, antiproliferative, and cytotoxic as well as antibiotic properties have been isolated from marine sources [1]. Fungi constitute one of the most abundant kingdoms of living organisms on Earth, with more than 120,000 identified species up to date. The revised estimation of the species yet to be discovered lies in the extraordinary range of 2.2 to 3.8 million [23]. The ecological and economic impact of these ubiquitous eukaryotic microbes is enormous and diverse, from the role of yeasts in the fermentation industry and the production of life-saving drugs [4,5] to body infections and the toxification of foodstuffs, livestock, and crops by the mycotoxins [6,7]. Through millions of years of chemical warfare for development and defense, fungi have developed a powerful genetic and biochemical machinery [8], making them a vast source of natural products with unique structures [9,10] and with a wide

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range of biological activities, including those against bacterial and fungal infections (antibiotics) [11,12].

The diversity of fungal species and their biosynthetic gene clusters have a nearly limitless potential for metabolic variation and a great resource for drug discovery and synthetic biology [13].

In the ancient decades, fungi were believed to exist only in the terrestrial environments, but over time they were discovered in the marine habitats [14].

The marine environment is an unexplored source for isolation of new microbes (bacteria, fungi, actinomycetes, cyanobacteria and diatoms) that are potent producers of bioactive secondary metabolites, which affect particularly central nervous system (CNS), respiratory system (RS), neuromuscular system (NMS), autonomic nervous system (ANS), cardiovascular system (CVS) and gastrointestinal system (GI). Marine secondary metabolites can easily impede other microorganisms [15]. Marine fungi have proved to be a rich source of new biological natural products because of their characteristic properties with reference to temperature, nutrients, competition, and salinity, developing specific secondary metabolic pathways compared with terrestrial fungi [16,17]. Generally, 10% of all currently known biologically active natural products are produced by microbial origin [18]. Particularly, during 1981–2002, more than half of the FDA approved drugs had produced from marine life [19]; between 2006 and 2010, a total of 690 natural products were reported as being isolated from fungi in marine habitats [20], and finally there are more than 22,000 known microbial secondary metabolites about 20% by were reported by fungi [13].

In the present research work, isolation, taxonomy, and broad antimicrobial activity of thirteen diverse marine-derived fungi obtained from the Mediterranean Sea at Alexandria, Egypt, have been investigated. The most interesting five marine fungal strains were selectively cultivated on rice-solid state and their antimicrobial activities were re-visualized. From the antimicrobial activity testing point of view, the phytochemical profiling of the most promising two fungal strains (8 and 13) were analyzed based on GC-MS, and their bioactive secondary metabolites were primarily identified, and finally their optimal cultivation were performed using different solid-state media, from which their antimicrobial and phytochemical profiling (using GC-MS) were comparatively investigated. In this study, we report the isolation, taxonomic characterization, and antimicrobial activity studies as well of the 13 fungal strains, targeting the selection of the most promising and talented strains for production of auspicious bioactive secondary metabolites, representing as drug leads, for forthcoming full studies.

2. Experimental Section

2.1. Sample collection:

The source marine samples of the fungal isolates under study were collected from marine water and sediments at a depth of ~1.5 m from three different locations in Alexandria Governorate, Egypt: **a**) Easterner harbor, **b**) Abu Qir, and **c**) Max. The sediments were collected under aseptic condition in pre-sterilized polythene bags, while the water samples were collected in sterile Falcon tubes.

2.2. Fungi Isolation

0.1 gm of sediment was diluted in 9 mL of sterile sea water. Three-fold serial dilution was carried out, then cultivated on peptone glucose (dextrose) agar medium (g/L: peptone (20), dextrose (10), Agar (20)) at pH 6.5, poured into Petri-dishes (9 cm) using a spread plate technique supplemented with cidocetine to inhibit the bacterial growth, and incubated at 30°C for three days. All grown and purified fungal isolates were then cultivated in potato dextrose agar (g/l: Potato infusion form (200), Dextrose (20), agar (20), pH 4.9) and the obtained pure colonies were stored in sterile slants containing glycerol until further investigation.

2.3. Primary Screening

The fungal isolates obtained were cultivated in 250 mL Erlenmeyer flasks containing potato dextrose medium dissolved in 100 mL sea water at pH 4.9. After sterilization, each Erlenmeyer flask was inoculated with one lob of fresh fungal isolate, followed by incubation at 30°C using shaker (120RPM) for 10 days. After cultivation, 1 mL of each broth culture supernatant was centrifuged at 10000 RPM for 10 min. The obtained clear supernatants were then applied to antimicrobial activity testing against different microbial pathogens: *Escherichia coli (ATCC8739), Bordetella sp., Klebsiella pneumonia (ATCC13883), Candida albicans, Staphylococcus aureus (ATCC25923), Staphylococcus epidermidis*, and *Listeria sp.* The antimicrobial technique using well diffusion method was done according to [21]. All inoculated Petri dishes were incubated in an inverted position at 37 °C for 24 hr. he obtained inhibition zones were finally determined.

2.4. Solid State Fermentation

2.4.1. Solid state fermentation on rice medium

In 250 mL Erlenmeyer flask, 30 g commercial rice and 60 mL seawater were soaked for two hours at ambient temperature, then autoclaved for 20 min at 121°C and 1.5 bar. After sterilization, the media was

inoculated with 1 mL of prepared inoculums of the fungal isolates 8, 9, 12, 13 and 15, and then incubated at ~25 °C for 21 days. The obtained afforded fungal metabolites were extracted by soaking in methanol, followed by filtration and concentration *in vacuu*. The afforded aqueous extracts were re-extracted by ethyl acetate affording to the desired bioactive fungal metabolites.

2.4.2. Solid state fermentation on some agricultural (agrowaste) solid wastes

In alternative cultivating conditions, the selected interest fungal strains 8 and 13 were cultivated on four different solid-state media using agricultural (agrowaste) solid wastes as nutrients: Rice straw, wheat straw, rice husk and wheat bran. The served media were prepared individually in equivalent seawater volume (2 gm solid waste: 2 ml seawater) in sterilized 100 mL cupped bottles (3 bottles for each media) and then further autoclaved at same conditions aforementioned. After sterilization, the media used were inoculated with 2 mL of prepared inoculums of the fungal isolates 8 and 13, and then incubated at ~25 °C for 21 days. The obtained afforded fungal metabolites were extracted using the same extracting procedure mentioned above, resulting in the corresponding fungal metabolites.

2.4.3. Antimicrobial activity of solid- state fermentation extracts

After cultivation on rice solid media, and working up, the corresponding organic extracts of the five fungal strains (8, 9, 12, 13, 15) were applied to antimicrobial activity studies using the following test microbes: *Escherichia coli (ATCC8739), Bordetella sp., Klebsiella pneumonia (ATCC13883), Candida albicans, Staphylococcus aureus (ATCC25923) and Staphylococcus epidermidis.*

On the other hand, the potential antimicrobial activity of fungal strains 8 and 13, cultivated selectively on the mentioned above four agricultural (agrowaste) solid wastes as solid state media were tested against further test microbes with higher diversity and stronger drug resistance properties: *Staphylococcus aureus (*ATCC25923*), Escherichia coli (*ATCC8739*), Pseudomonas aeruginosa (*ATCC9027*), Bacillus subtilis (*ATCC6633*), Enterococcus Faecalis (*ATCC29212*), Klebsiella pneumonia (*ATCC13883*), Vibrio damsela, Vibrio fluvialis* and *Candida albicans.*

3. Result and Discussion

3.1. Sampling and isolation of the Marine-Derived Fungi

From three marine locations at Alexandria coasts (at depth of 1.5 m) Egypt; Easterner Harbor, Abu Qir, and Max, fourteen fungal isolates were obtained. From of the Easterner Harbor, one and six fungal strains were isolated from the water and sediment samples, respectively. On the other hand, from Abu Qir, two strains were isolated, representing one from water and the other one from sediment. Finally, from the Max coast, five strains were isolated from the water sample, while one strain was from the sediment sample. Altogether, fifteen fungal isolates were isolated. However, and according to the morphological and taxonomical characterization, they belong to fourteen isolates (Table 1).

Table 1**:** List of fungal strains isolated from three locations at Alexandria coasts of Egypt.

Sampling area	Sample Nature	Isolated Fungal code No.	Isolated fungal genus
The Easterner Harbour, Alexandria, Mediterranean Sea	Water	3	*Yeast*
	Sediment	1*	*Penicillium*
		2	*Fusarium*
		4	*Penicillium*
		5	*Aspergillus*
		6	*Aspergillus*
		7*	*Penicillium*
Abu Qir, Alexandria	Water	8	*Penicillium*
	Sediment	14	*Ascomycetes*
Max, Alexandria	Water	9	*Aspergillus*
		10	*Aspergillus*
		11	*Penicillium*
		12	*Yeast*
		13	*Aspergillus*
	Sediment	15	*Acremonium*

*****Isolates Nr.1 and 7 are mostly identical and isolated from the same sediment source.

3.2. Primary Screening and Antimicrobial Activity Studies

For surveying and visualizing the marine fungal strains isolated herein, their supernatants obtained after liquid fermentation and centrifugation were applied to antimicrobial screening against seven microbial strains classified into three Gram-negative bacteria, *E. coli (*ATCC8739*), Bordetella* sp., *Klebsiella pneumonia (*ATCC13883*)*, three Gram-positive bacteria, *S. aureus (*ATCC25923*), S. epidermidis* and *Listeria*, and the pathogenic yeast *C. albicans*. Based on this study, it has been found the following conclusions: strains 1 and 7 showed identical antimicrobial activities concluding and confirming their identical morphological taxonomy (*Penicillium* genus), showing moderate and high antimicrobial activity against the Gram-positive bacteria *S. aureus* (15 mm) and *Listeria* (20 mm), respectively. Alternatively, the fungal strains Nr. 2-6, 8, 10-13 showed high antimicrobial activity against two Gram negative and two gram-positive bacteria, namely *E. coli* (12-18 mm), *Bordetella* sp (15-16 mm), *S. aureus* (15-35 mm) and *Listeria* (17-41 mm). Unexpectedly, strains 9 and 15 showed activity ranged between moderate and high against the whole test microbes. Remarkably, it has been shown that strain 14 showed no growth and hence it has been ignored from the antimicrobial assaying reported herein (Table 2).

Table 2**:** Antimicrobial activity of the supernatant of isolated marine-derived fungi: Inhibition Zone (mm)

Fung. Isolates	***E. coli (*ATCC8739*)***	***Bordetella sp.***	***Klebisella pneumonia (*ATCC13883*)***	***C. albicans***	***S. aureus (*ATCC25923*)***	***S. epidermidis***	***Listeria***
1*	0	0	10	0	15	0	20
2	18	16	12	0	33	0	25
3	16	21	13	0	30	0	21
4	14	16	15	0	20	0	17
5	15	16	12	7	25	6	20
6	12	15	12	0	35	0	23
7	0	0	10	0	15	0	20
8	17	24	15	0	34	9	30
9	15	21	16	17	27	25	25
10	16	23	17	0	28	0	25
11	13	16	16	0	20	0	15
12	0	0	0	20	0	23	41
13	14	15	15	0	21	0	0
14**							
15	12	12	14	19	19	20	40

*Strain 1 = strain 7, **strain 14 did not show any growing in shaker culture.

Table 3:Antimicrobial activity of selected five marine-derived fungi cultivated on rice solid-state: Inhibition Zone (mm)

Fungal Isolates	***E. coli (*ATCC8739*)***	***Bordetella sp.***	***Klebsiella pneumonia (*ATCC13883*)***	***C. albicans***	***S. aureus (*ATCC25923*)***	***S. epidermidis***
8	26	29	24	24	0	25
9	0	0	0	13	0	0
12	0	0	0	12	0	0
13	30	26	27	25	0	27
15	27	26	27	27	22	2

3.3. Antimicrobial activity of the solid-state fermentation

3.3.1. Antimicrobial activity studies of the promising Fungal Strains using rice solid-state fermentation.

Due to their unusual antimicrobial activity reported through the liquid culture aforementioned (Table 3 and Figure S1)) the fungal strains Nr. 8, 9, 12, 13 and 15 were selectively applied to alternative cultivation on rice-solid state medium, aiming to further revisualize the antimicrobial activity of their obtained secondary metabolites. In accordance, it has been found that strains 8 and 13 showed high antimicrobial activity against the whole test microbes with inhibition zone of 24-29 mm except their inactivity against S. aureus. Likely, strain

15 is highly active against all test microbes (26-27 mm) even *S. aureus* (22 mm), however, it was oppositely very less active against S. epidermidis (2 mm).

3.3.2 Antimicrobial activity studies of the most promising Fungal isolates (fungal strains 8 and 13) using some agricultural wastes (AgroWaste) as solid-state media.

Due to their interest antimicrobial activity during the above studies mentioned, strains 8 and 13 were applied to further optimal cultivating conditions using four different compositions of solid-state fermentation, namely Rice straw medium (RS), Rice husk medium (RH), Wheat bran medium (WB) and Wheat straw medium (WS) to investigate their a) antimicrobial activity against nine different test microbes classified into four Gram-positive bacteria, four Gram-negative bacteria and one yeast; and b) study their chemical profile using GC-MS analysis. Based on the antimicrobial activity study, extracts of both fungal strains 8 and 13 were tested against four Gram-positive bacteria (*St. aureus* [ATCC25923], *B. subtilis* [ATCC6633], *Ent. Faecalis* [ATCC29212] and *Kleb pneumonia* [ATCC13883]), four Gram-negative bacteria (*E coli* [ATCC8739], *Ps. aeruginosa* [ATCC9027], *Vib damsela* and *Vib fluvialis*) and the pathogenic yeast *C. albicans* (Table 4). Based on this study, it has been remarked that there is a little bed variation in the antimicrobial activity for the extracts obtained from the different cultivating media, displaying the activity in the range of 11-18 mm. Remarkably, it has been shown that strain 8 was exceptionally highly active against *C. albicans* (17 mm) in case of rice husk-solid medium (RH), while it was exceptionally active against *Vib. damsel* (18 mm) in case of Wheat bran-solid medium (WB). In case of strain 13, it showed exceptional high activity (18 mm) against the Gram-negative bacteria *E coli* (ATCC8739) through its cultivation on WB medium.

Table 4**:** Antimicrobial activity of the marine-derived fungal strains 8 and 13 cultivated on rather different solid-state fermentations: Inhibition Zone (mm)

Fungal strain	Optimizing solid state*	*S. aureus (ATCC25923)*	*E. coli (ATCC8739)*	*Ps. aeruginosa (ATCC9027)*	*B. subtilis (ATCC6633)*	*Ent. Faecalis (ATCC29212)*	*Kleb. pneumonia (ATCC13883)*	*Vib. damsela*	*Vib. fluvialis*	*C. albicans*
Nr. 8	RS-8	14	0	15	14	11	12	12	13	11
	RH-8	14	0	14	0	0	15	13	11	17
	WB-8	13	0	13	13	0	15	18	0	0
	WS-8	12	15	13	13	0	12	13	13	0
Nr.13	RS-13	14	13	17	11	11	14	12	11	0
	RH-13	15	0	17	11	0	16	12	14	0
	WB-13	12	17	15	12	0	14	13	16	0
	WS-13	12	11	15	12	0	12	13	15	0

*Served nutrational media: RS = Rice straw; RH = rice husk, WB= Wheat brain, WS = Wheat straw

3.3.3 Chemical Profiling by GC-MS Analysis

The tentative identification of the produced secondary metabolites obtained from the two fungal strains 8 and 13 after their optimization using different nutritional and cultivating conditions were carried out using GC-MS analysis, assessed by comparing their mass spectra with related counterparts reported by NIST, Wiley9, Mainlib, Replib libraries [20]. Designed samples EM-8, EMRH-8, EMWB-8 and EMWS-8 are those of the optimized cultures of strain 8, meanwhile EM-13, EMRH-13, EMWB-13 and EMWS-13 are the optimized ones for strain 13. Details of identified compounds are shown in Tables S1 and S2, respectively, and their chemical structures are depicted in Figures S3-S12. Based on the diversity of the nutritional and cultivating conditions, it has been confirmed their influence on the obtained variety of secondary metabolites and their productivity.

Remarkably, it has been shown that strain 14 showed no growth and hence it has been ignored from the antimicrobial assaying reported herein (Table 2 and Figure S1).

In accordance, it has been found that strains 8 and 13 showed high antimicrobial activity against the whole test microbes with inhibition zone of 24-29 mm except their inactivity against *S. aureus* (Table 3, Figure S2). Likely, strain 15 is highly active against all test microbes (26-27 mm) even *S. aureus* (22

mm), however, it was oppositely very less active against *S. epidermidis* (2 mm). and *Kleb pneumonia* [ATCC13883]), four Gram negative bacteria (*E coli* [ATCC8739], *Ps. aeruginosa* [ATCC9027], *Vib damsela* and *Vib fluvialis*) and the pathogenic yeast *C. albicans* (Table 4). Based on this study, it has been remarked that there is a little bed variation in the antimicrobial activity for the extracts obtained from the different cultivating media, displaying the activity in the range of 11-18 mm. Remarkably, it has been shown that strain 8 was exceptionally highly active against *C. albicans* (17 mm) in case of rice husk-solid medium (RH), while it was exceptionally active against *Vib. damsel* (18 mm) in case of Wheat bran-solid medium (WB). In case of strain 13, it showed exceptional high activity (18 mm) against the Gram-negative bacteria *E coli* (ATCC8739) through its cultivation on WB medium.

According to the GC-MS analysis, strain 8 cultivated on five nutritional solid media revealed the presence of twenty four, twenty two, thirty three, seventeen and eleven diverse bioactive secondary metabolites when it was cultivated on the solid media: rice [EM8], rice husk [EMRH-8], rice straw [EMRS-8], wheat brain [EMWB-8] and wheat straw [EMWS-8], respectively. The obtained compounds are classified altogether different categories: hydrocarbons, polyketides, phenolic compounds, quinones, fatty acids/esters, terpenes, alkaloids, halo compounds, sulfur containing compounds [21-22].

On the other hand, the fungal strain 13 showed the presence of twenty-eight, thirty-four, eighteen, seventeen and twelve bioactive compounds during its cultivation on the solid media: rice [EM-13], rice husk [EMRH-13], rice straw [EMRS-13], wheat brain [EMWB-13] and wheat straw [EMWS-13], respectively. The obtained compounds are classified altogether into different categories as well: hydrocarbons, polyketides, phenolic/multi-oxygenated compounds, fatty acids/esters, terpenes, steroids, alkaloids, halo compounds and sulfur containing compounds [21-22].

4. Conclusions

The bioactive secondary metabolites obtained from thirteen marine derived fungi, isolated from the Mediterranean Sea at Egyptian coasts, were tested against seven pathogens. The fungal isolates classified into five genera: *Penicillium* (33%), *Aspergillus* (33%), *Fusarium* (7%), *Acremonium* (7%) and yeast (13%). According to antimicrobial activity visualization, the most promising five fungal isolates (Nr.8, 9, 12, 13 and 15) were re-visualized as antimicrobial agents. The phytochemical profiling of the most interest isolates (Nr.8 and 13) was done based on GC-MS, and their bioactive secondary metabolites were tentatively identified, revealing the presence of twenty-four and twenty-eight compounds belonging to diverse structural categories, respectively.

The last two strains were further applied to different agricultural waste as solid-state media; their antimicrobial activities were studied comparatively, and the obtained bioactive secondary were tentatively assigned by GC-MS analysis as well. The research team targets to select the most promising strains for production of auspicious bioactive secondary metabolites, representing as drug leads, for forthcoming full studies.

5. Conflicts of interest

There are no conflicts to declare.

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