Marine fungi showing multifunctional activity against human pathogenic microbes and cancer

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Abstract

Multifunctional drugs have shown great promise in biomedicine. Organisms with antimicrobial and anticancer activity in combination with antioxidant activity need further research. The Red Sea and the Arabian Gulf coasts were randomly sampled to find fungi with multifunctional activity. One hundred strains (98 fungi and 2 lichenized forms) were isolated from 15 locations. One-third of the isolates inhibited clinical bacterial (Staphylococcus aureus, Bacillus subtilis, Vibrio cholerae, Salmonella typhi, S. paratyphi) and fungal pathogens (Talaromyces marneffei, Malassezia globose, Cryptococcus neoformans, Candida albicans, Aspergillus fumigatus) and four cancer cell lines (Hep G2 liver, A-549 lung, A-431 skin, MCF 7 breast cancer). Bacterial and cancer inhibition was often accompanied by a high antioxidant activity, as indicated by the principal component analysis (PCA). PCA also indicated that fungal and bacterial pathogens appeared to be inhibited mostly by different marine fungal isolates. Strains with multifunctional activity were found more from the Red Sea than from the Arabian Gulf coasts. The highest potential for multifunctional drugs were observed for Acremonium sp., Acrocalymma sp., Acrocalymma africana, Acrocalymma medicaginis (activity reported for the first time), Aspergillus sp. Cladosporium oxysporum, Emericellopsis alkaline, Microdochium sp., and Phomopsis glabrae. Lung, skin, and breast cancers were inhibited 85%–97% by Acremonium sp, while most of the isolates showed low inhibition (ca 20%). The highest antifungal activity was observed for Acremonium sp., Diaporthe hubeiensis, Lasiodiplodia theobromae, and Nannizia gypsea. One Acremonium sp. is of particular interest to offer a multifunctional drug; it displayed both antifungal and antibacterial activity combined with high antioxidant activity (DPPH scavenging 97%). A. medicaginis displayed combined antibacterial, anticancer, and antioxidant activity being of high interest. Several genera and some species included strains with both high and low biological activities pointing out the need to study several isolates to find the most efficient strains for biomedical applications.
1. Introduction

Microbial metabolites are continuously studied to solve the problem of drug resistant microbes and cancer. High expectations have been set for novel multifunctional drugs due to their high efficacy [1, 2]. Multifunctional metabolites are offered by plants, bacteria, and fungi [3, 4] of which metabolic activity is generally high in extreme environmental conditions [5]. High metabolic activities of fungi have been observed, for instance, in desert soils [6]. Marine habitat is another extreme environment studied as a source of biologically active fungi, as reviewed several times [7–11] recently.

The potential of marine fungi in biomedicine is diverse. Several marine fungal species have been shown to inhibit cancer cell growth [12–14]. The enormous potential of marine fungi to produce antibiotic compounds was reviewed recently [15, 16]. A total of 133 anti-inflammatory compounds produced by marine fungi have been reported [17]. Antifungal compounds produced by marine fungi have also been reported, although much less than antibacterial compounds [9]. Even viruses are inhibited by marine fungal metabolites [18].

Most reports show one or two biological activities at a time. However, recent advances in biomedicine support a new efficient treatment strategy; to combine antioxidants with other biomedicines [19, 20]. Therefore, the organisms offering antioxidant activity in combination with antimicrobial activity and/or cytotoxicity are of great interest. Research on organisms offering these multifunctional drugs is in its infancy and more research on potential organisms producing metabolites that could be used as novel multifunctional drugs is needed.

We aimed to find marine fungi that have high activity against pathogenic bacteria, fungi, and cancer cells in combination with high antioxidant activity. These isolates would offer multifunctional drugs for further biomedical studies. The coast of the two northernmost tropical seas, namely the Red Sea and the Arabian Gulf were sampled. Both seas provide harsh conditions. The Red Sea is one of the most saline and warmest waterbodies, and the Arabian Gulf is the hottest sea in the world [21]. Marine fungi were isolated from 15 different locations on the coasts of the seas around the Arabian Peninsula and studied for their antioxidant activity as well as their activities against both bacterial and fungal pathogens and cancer cells.

2. Materials and methods

2.1 Sample collection sites and sampling

Fifteen sites on the coasts of the Arabian Gulf (4 sites) and the Red sea (11 sites) were sampled at low tide. The sites were in Sametah (S), Jazan (S), Ras Al-Turfa (S), Farasan island (S), Qunfidah (S), AlLith (S), Jeddah (W), Rabigh (W), Yanbue (W), Umlajj (N), Alwajh (N), Hofuf (E), Dammam (E), Jubail (E), and Khafji (E) (Fig 1). One water sample (50 mL) was collected from each site, around 450 m away from the shoreline at the depth of 20 cm into a sterilized amber coloured container and transported to the laboratory of Botany and Microbiology, College of Science, King Saud University, Saudi Arabia in November 2021.

The water temperature ranges on the Red Sea coasts between 23˚C and 30˚C, salinity between 36 PSU and 38 PSU and the pH is 8.1 [22]. The respective values in the Arabian Gulf are 27˚C- 33˚C, 39.5–42 PSU and 8.2.

2.2 Isolation of fungi and pure culture preparation

The seawater samples were serially diluted (10⁴ or 10⁵), and 1 mL was spread on potato dextrose agar (PDA) plates, including antibiotic chloramphenicol 200 µg/L. Plates were incubated at 25˚C for 7 days in a laboratory incubator (Remi Lab World). Individual colonies (isolates) were selected based on their morphological characteristics. The selected isolates were sub-
Fig 1. Location map of the study area (The map is modified from Google maps).

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cultured on PDA plates and preserved for further studies. Fungal mycelia were transferred into sterile Eppendorf tubes containing 1 mL, 30% (v/v) sterile glycerol and incubated at 28˚C for 5 days and then stored at -20˚C.

2.3 Molecular identification of fungi

The DNA extraction was carried out as described by Ameen et al. [23]. Fungal mycelia were collected into a 2 mL centrifugation tube containing 500 μL of extraction buffer (25 mM EDTA, 0.5% SDS 200 mM, 250 mM NaCl, Tris-HCl, pH 7.5) and centrifuged at 13,000 g for 1 min. The supernatant was transferred into a fresh centrifugation tube followed by the addition of an equal volume of an ice-cold phenol: chloroform mixture (1:1) and centrifuged at 13,000 g for 2 min. The supernatant was collected into another fresh tube with 300 μL of chloroform, then centrifuged at 13,000 g for 2 min (repeated again) and then the supernatant was transferred into a fresh tube with 300 μL ice-cold isopropanol, gently mixed and kept in water bath at 80˚C for 30 min. The mixture was then centrifuged at 13,000 g for 5 min, the resultant pellet was collected and washed with 70% ice-cold ethanol, and resuspended in 1 mL sterile water. The yield and quality of the DNA were assessed by agarose gel electrophoresis.

The DNA was amplified using ITS1 (5’-TCCGTAGGTGAACCTGCGG-3’) and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’) primers [24] using 5 min initial denaturation (95˚C), 1 min denaturation for 35 cycles (94˚C), 30 s annealing (55˚C), 2 min extension (72˚C), and 10 min final extension (72˚C). The sequencing was completed using BigDye terminator sequence kit (Applied Biosystems) and the sequence identification was carried out using NCBI (The National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) databases (BLAST software). The alignments were completed with T-Coffee algorithm (https://www.ebi.ac.uk/, EMBL-EBI, Cambridgeshire, UK). The phylogenetic analysis was carried out using the neighbor-joining method in the MEGA 5.2 program. The sequences were deposited to GenBank. The purified marine fungi are preserved at the university storage.

2.4 Metabolite extraction

Fungal metabolites (crude extracts) were collected as described by Ameen et al. [23]. Fungi were first inoculated into conical flasks (2 L) with PDB and incubated for 30 days at 28˚C in a static condition. Culture broth was filtrated using Whatman No.1 filter paper and the filtrate was extracted with an equal volume of ethyl acetate. Then, the solution was evaporated using a rotary evaporator (Lab Tech) and dissolved in methanol/DMSO (100 μg/μL). The extract was membrane filtered (0.22 μm) for further studies. All analyses were carried out as three replicates.

2.5 Antimicrobial activity

The antibacterial test was carried out using a modified Bauer- Kirby method [25]. Five clinical human bacterial pathogens, Staphylococcus aureus (ATCC 6538), Bacillus subtilis (ATCC 6633), Vibrio cholerae (ATCC 14033), Salmonella typhi (ATCC 6539), and S. paratyphi (ATCC 9150), the microbial cultures were obtained from reference culture collection, Department of Botany & Microbiology, King Saud University, were grown on Mueller-Hinton (HiMedia) agar plates. The paper discs impregnated with the crude extracts of marine fungi (100 μg/μL) were placed on agar and the inhibition zones were measured (Equation number-1) after incubation in static conditions at 37˚C overnight. Antibiotics ampicillin and tetracycline, and fungicides gentamycin and fluconazole (5 μg/mL) were used as positive controls.

The antifungal test was carried out as described by Satika et al. [26]. Five fungal pathogens, namely Aspergillus fumigatus (ATCC 46645), Cryptococcus neoformans (ATCC 32045),
Candida albicans (ATCC 10231), Malassezia globosa (ATCC 4612), and Talaromyces marneffei (ATCC 18224), provided by the university above, were used. As above, the plates with the paper discs were incubated in static conditions at 28°C for 5 days.

2.6 Antioxidant activity analysis
The total reducing power of the crude extracts was studied as described by Oyaizu [27]. The crude extracts and controls were mixed with 0.5 mL potassium hexacyanoferrate (1%) and 0.5 mL phosphate buffer (0.2 M, pH 6.6) and incubated in a water bath (50°C) for 20 min. Then, 10% of TCA (0.5 mL) was added to end the reaction. 1 mL of the upper portion was collected in a separate tube and 0.1 mL ferric chloride solution (0.1%) and 1 mL distilled water were added. After a 10 min incubation at room temperature, the absorbance was measured at 700 nm. The crude extract without marine fungi were used as the negative control and citric acid as the positive control.

Free radical scavenging activity was studied using DPPH assay (2,2-diphenyl-1-picrylhydrazyl) as described by Gang et al. [28]. Crude extracts and controls were incubated at 37°C in darkness for 30 min and the absorbance was measured at the wavelength of 517 nm. The crude extract without marine fungi were used as the negative control and citric acid as the positive control.

The scavenging percentage was calculated using the following formula.

\[
\text{Inhibition percentage (\%) = } \left( \frac{\text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}}}{\text{OD}_{\text{Control}}} \right) \times 100
\]

where optical density (OD) is the absorbance of the sample.

2.7 Cytotoxicity analysis
The cytotoxicity of the crude extracts was studied using the 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described by [29]. Cell lines MCF-7 (breast cancer), Hep G2 (liver cancer), A-431 (skin cancer), and A549 (lung cancer) stored at the cell collection center in Riyadh (ECACC Cell lines, Merck) were used. Cells were maintained in a DMEM medium (Dulbecco’s Modified Eagle’s medium) with 10% FBS where 250 mg/L streptomycin, 100 mg/L penicillin, and 2 mM glutamine were added before the incubation at 37°C in a CO₂ incubator (5%). The cell concentration of 5 × 10⁴ cells/well was used (tissue culture grade 96 wells plate flat bottom) in 100 μL DMEM medium and incubated at 37°C for 24 h in a CO₂ incubator (5%). After the incubation, the crude extracts and control (without marine fungus) (50 μg/mL) and cisplatin (5 μg/mL) as a positive control were added to the wells and incubated for 48 h. MTT (10 μL, 0.5 mg/mL) was added to each well and incubated for 2 h at 37°C for the removal of PBS (phosphate-buffered saline). The DMSO (dimethyl sulfoxide, 100 μL) was added to each well and the absorbance was measured at 595 nm using an ELISA (enzyme-linked immunosorbent assay) reader. The inhibition percentage was calculated as above.

2.8 Data analysis
Principal component analysis using a correlation matrix was carried out for the whole dataset using FactoMineR [30] package in RStudio Desktop version 2022.02.0+443 [31]. The means of the three replicates were used making 100 observations and 16 variables altogether.
3. Results

3.1 Fungal species

One hundred isolates, including 31 marine fungal species, were identified (GenBank accession number in Table 1) and their phylogenetic tree is presented (Fig 2). The most common taxon was Cladosporium sp., isolated from almost all sites, from 13 out of 15 sampled sites. The most common Cladosporium species were C. corybiicola, C. cladosporioids and C. tenuissium isolated from five different sites. While many species were found in many sites, certain species such as Phomopsis glabrae and C. perangustum were found only in one site on the Red Sea coast. D. hubeiensis and Heydenia alpina were found only on the northern Red Sea coast.

Two isolates of the lichen Usnea, which is a symbiosis of algae and fungi, was found from the rocky shores of the sea. The species Usnea was obtained by the extraction protocol carried out and the identification of the sequence was obtained from NCBI. The species cultured for the activities measurements was an unidentified mycobiont, which belongs to Ascomycota. The mycobiont is called Usnea herein.

3.2 PCA

The two first axes of PCA explained 72% of the variation. PC1 explained 50%, and PC2 explained 22% of the variation, the eigenvalues being 8.1 and 3.5, respectively.

The loadings of the PC1-axis were relatively high (> 0.5) for all variables except for reducing power, as indicated by the loadings plot (Fig 3a). The PC1 loadings were highest for DPPH (0.85) and for all four cancer types (0.80–0.78). The inhibition of bacterial pathogens had loadings varying from 0.78 to 0.69. The inhibition of fungal pathogens had slightly lower loadings varying from 0.59 to 0.72. We interpreted that PC1 describes the general biological activity of the marine fungi, the biological activity of the isolate being higher the higher its score on PC1 (Fig 3b).

The loadings plot also indicated that the biological activity variables were separated by PC2, some having positive and some negative PC2 values (Fig 3a). PC2 was interpreted to separate the marine fungi according to their activity against either pathogenic fungi or bacteria. The latter marine isolates also had high cytotoxicity and DPPH scavenging activity, as indicated by their high negative loadings on PC2-axis. However, negative PC2 loadings were relatively low for all variables mentioned (bacteria, cancer, DPPH: 0.22–0.36). PC2 positive loadings of fungi varied between 0.63 to 0.76.

The highest positive PC1 scores (biological activity) were for the marine fungal genera Acremonium, Acrocalymma, Microdochium, Emericellopsis, Phomopsis, Aspergillus, Diaporthe, Buellia, and Cladosporium (Fig 3b). These genera had the 25 highest PC1 scores. The species identified were A. africana, A. medicaginis, E. alkalina, P. glabrae, D. hubeiensis, and C. oxyosporum. All of these strains except one Microdochium sp. and the two Aspergillus sp. were isolated from the Red Sea (Fig 3c). The highest negative PC1 scores indicating no or low biological activities were for the genera Ceratocystis, Cladosporium, Sordariomycetes, Emericellopsis, Heydenia, Fusarium, Nannizzia, and Microdochium. These isolates were collected more often from the Arabian Gulf than from the Red Sea (Fig 3c). The sampling sites within the seas were mixed and showed no grouping.

The highest positive scores on PC2 (inhibitors of fungi) were for Nannizia gypsea, Lasiodiplodia theobromae, Diaporthe hubeiensis, and Acremonium sp. (Fig 3b). The two first with the highest scores were isolated from the Arabian Gulf (Fig 3c). Other isolates having relatively high antifungal activity were Emericellopsis sp., E. phycophila, Cladosporium perangustum, C. tenuissium, and C. cladosporioids.
| Species                     | Name of isolates | GenBank Accession Number | Site   | Area    |
|-----------------------------|------------------|--------------------------|--------|---------|
| Acremonium sp.              | JEF1             | OM510389                 | Jeddah | West    |
| Acremonium sp.              | JEF2             | OM510390                 | Jeddah | West    |
| Acremonium sp.              | SF3              | OM510382                 | Sametah| South   |
| Acrocalymma sp.             | JF4              | OM514681                 | Jazan  | South   |
| Acrocalymma africana        | SF5              | OM490687                 | Sametah| South   |
| Acrocalymma medicaginis     | RAF6             | OM514682                 | Ras Al-Turfa | South |
| Aspergillus sp.             | HF7              | OM655253                 | Hofuf  | East    |
| Aspergillus sp.             | DF8              | OM655254                 | Dammam | East    |
| Aspergillus sp.             | JEF9             | OM655255                 | Jeddah | West    |
| Buellia sp.                 | HF10             | OM514685                 | Hofuf  | East    |
| Buellia lauricassiae        | HF11             | OM514686                 | Hofuf  | East    |
| Buellia lauricassiae        | HF12             | OM489216                 | Hofuf  | East    |
| Ceratocystis sp.            | DF13             | OM510400                 | Dammam | East    |
| Ceratocystis sp.            | DF14             | OM510403                 | Dammam | East    |
| Ceratocystis sp.            | JUF15            | OM510405                 | Jazan  | South   |
| Ceratocystis sp.            | SF17             | OM510411                 | Sametah| South   |
| Ceratocystis sp.            | DF18             | OM510413                 | Dammam | East    |
| Ceratocystis sp.            | DF19             | OM510414                 | Dammam | East    |
| Ceratocystis sp.            | FF20             | OM510417                 | Farasan island | South |
| Ceratocystis cerefabiensis  | QF21             | OM510402                 | Qunfidah | South |
| Ceratocystis corybicola     | SF22             | OM505092                 | Sametah| South   |
| Ceratocystis corybicola     | JF23             | OM502070                 | Sametah| South   |
| Ceratocystis corybicola     | DF24             | OM510399                 | Dammam | East    |
| Ceratocystis corybicola     | DF25             | OM510401                 | Dammam | East    |
| Ceratocystis corybicola     | DF26             | OM510404                 | Dammam | East    |
| Ceratocystis corybicola     | DF27             | OM510407                 | Dammam | East    |
| Ceratocystis corybicola     | DF28             | OM510409                 | Dammam | East    |
| Ceratocystis corybicola     | RF29             | OM510410                 | Rabigh | West    |
| Ceratocystis corybicola     | YF30             | OM510415                 | Yanbue | West    |
| Ceratocystis corybicola     | DF31             | OM510416                 | Dammam | East    |
| Ceratocystis corybicola     | DF32             | OM510418                 | Dammam | East    |
| Ceratocystis polycephrona   | AF33             | OM510406                 | ALith  | South   |
| Ceratocystis polycephrona   | JF34             | OM510412                 | Jeddah | West    |
| Cladosporium sp.            | RF35             | OM510301                 | Rabigh | West    |
| Cladosporium sp.            | SF36             | OM510303                 | Sametah| South   |
| Cladosporium sp.            | JF37             | OM510306                 | Jazan  | South   |
| Cladosporium sp.            | RAF38            | OM510308                 | Ras Al-Turfa | South |
| Cladosporium sp.            | FF39             | OM510310                 | Farasan island | South |
| Cladosporium sp.            | JEF40            | OM510311                 | Jazan  | South   |
| Cladosporium sp.            | JEF41            | OM510319                 | Jeddah | West    |
| Cladosporium sp.            | JEF42            | OM510320                 | Jeddah | West    |
| Cladosporium sp.            | JEF43            | OM510322                 | Jeddah | West    |
| Cladosporium sp.            | JEF44            | OM510328                 | Jeddah | West    |
| Cladosporium sp.            | SF45             | OM510325                 | Sametah| South   |
| Cladosporium sp.            | SF46             | OM510329                 | Sametah| South   |
| Cladosporium sp.            | HF47             | OM510331                 | Hofuf  | East    |

(Continued)
| Species                | Name of isolates | GenBank Accession Number | Site         | Area   |
|-----------------------|------------------|--------------------------|--------------|--------|
| Cladosporium cladosporioids | JF48             | OM510302                 | Jazan        | South  |
| Cladosporium cladosporioids | RAF49            | OM510309                 | Ras Al-Turfa | South  |
| Cladosporium cladosporioids | FF50             | OM510312                 | Farasan island | South |
| Cladosporium cladosporioids | QF51             | OM510327                 | Qunf udah    | South  |
| Cladosporium cladosporioids | JEF52            | OM510332                 | Jeddah       | West   |
| Cladosporium cladosporioids | JEF53            | OM510304                 | Jeddah       | West   |
| Cladosporium cladosporioids | JEF54            | OM502407                 | Jeddah       | West   |
| Cladosporium oxysporium  | JEF55            | OM510310                 | Jeddah       | West   |
| Cladosporium oxysporium  | UF56             | OM510314                 | Umlajj       | North  |
| Cladosporium oxysporium  | JF57             | OM510316                 | Jazan        | South  |
| Cladosporium oxysporium  | JF58             | OM510326                 | Jazan        | South  |
| Cladosporium oxysporium  | JF59             | OM510323                 | Jazan        | South  |
| Cladosporium oxysporium  | JUF60            | OM510333                 | Umlajj       | North  |
| Cladosporium perangustum | JEF61            | OM510307                 | Jeddah       | West   |
| Cladosporium perangustum | JEF62            | OM510315                 | Jeddah       | West   |
| Cladosporium perangustum | JEF63            | OM510324                 | Jeddah       | West   |
| Cladosporium tenuissium | KF64             | OM510305                 | Khafji       | East   |
| Cladosporium tenuissium | JEF65            | OM510317                 | Jeddah       | West   |
| Cladosporium tenuissium | UF66             | OM510321                 | Umlajj       | North  |
| Cladosporium tenuissium | JEF67            | OM510330                 | Jeddah       | West   |
| Cladosporium tenuissium | FF68             | OM510334                 | Farasan island | South |
| Diaporthe hubeiensis    | UF69             | OM459577                 | Umlajj       | North  |
| Emericellopsis sp.      | QF70             | OM510377                 | Qunf udah    | South  |
| Emericellopsis sp.      | AF71             | OM510378                 | AlLith       | South  |
| Emericellopsis sp.      | JF72             | OM510379                 | Jeddah       | West   |
| Emericellopsis sp.      | FF73             | OM510392                 | Farasan island | South |
| Emericellopsis sp.      | FF74             | OM510385                 | Farasan island | South |
| Emericellopsis sp.      | FF75             | OM510391                 | Farasan island | South |
| Emericellopsis alkalina | FF76             | OM502406                 | Farasan island | South |
| Emericellopsis alkalina | FF77             | OM510380                 | Farasan island | South |
| Emericellopsis alkalina | JF78             | OM510381                 | Jazan        | South  |
| Emericellopsis alkalina | RAF79            | OM510383                 | Ras Al-Turfa | South  |
| Emericellopsis alkalina | FF80             | OM510387                 | Farasan island | South |
| Emericellopsis phycophila | AF81            | OM510384                 | Alwajh       | North  |
| Emericellopsis phycophila | HF82            | OM510386                 | Hofuf        | East   |
| Emericellopsis phycophila | DF83            | OM510388                 | Dammam       | East   |
| Fusarium magnifereae    | JEF84            | OM487085                 | Jeddah       | West   |
| Heydenia alpina         | AF85             | OM491187                 | Alwajh       | North  |
| Lasiodiplodia theobrome | DF86             | OM510320                 | Dammam       | East   |
| Microdochium sp.        | SF87             | OM514683                 | Sametah      | South  |
| Microdochium sp.        | HF88             | OM514684                 | Hofuf        | East   |
| Microdochium sp.        | HF89             | OM489215                 | Hofuf        | East   |
| Metarhizium anisoplae   | SF90             | OM491186                 | Sametah      | South  |
| Nannizzia sp.           | DF91             | OM522070                 | Dammam       | East   |
| Nannizzia sp.           | JUF92            | OM522071                 | Jubail       | East   |
| Nannizzia gypsea        | HF93             | OM502405                 | Hofuf        | East   |
| Phomopsis glabrae       | YF94             | OM456372                 | Yanbue       | West   |

(Continued)
The highest negative PC2 scores (inhibitors of bacteria) were for the genera *Phompsis* > *Buellia* > *Aspergillus* > *Microdochium* > *Ceratocystis*. The species identified were *P. glabrae*, *B. lauricassiae*, *M. anisopliae*, and *C. polychorma*. *P. glabrae* and one *Aspergillus* sp. were isolated from the Red Sea, while the rest of the most efficient mentioned were isolated from the Arabian Gulf. The isolates of the genera *Emericellopsis* and *Microdochium* were either on the negative or positive sites of the PC2-axis.

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**Table 1.** (Continued)

| Species                      | Name of isolates | GenBank Accession Number | Site     | Area  |
|------------------------------|------------------|--------------------------|----------|-------|
| Sordariomyces sp.           | RF95             | OM456779                 | Rabigh   | West  |
| Sordariomyces glycinus      | SF96             | OM490685                 | Sametah  | South |

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Fig 2. Phylogenetic tree of marine fungi and reference sequence accession numbers constructed by using neighbour joining method in the MEGA 5.2 program.

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Fig 3. a. Loading's of variables in PCA where marine fungal metabolites were measured for their inhibitory effect against bacteria, fungi, and cancer and their DPPH scavenging activity and reducing power. b. PCA sample score plot of marine fungal species. c. PCA sample score plot of marine fungal species marked according to their sampling location on the coast of the Red Sea (R) or the Arabian Gulf (A).

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3.3 Biological activities

One-third of the extracts (33 out of 100) showed relatively high antibacterial activity (mean inhibition zone $\geq$ 20 mm), measured as the inhibition zone, when compared to antibiotics used as positive controls (mean ampicillin 16–17 mm, mean tetracycline 13–17 mm). For most of these isolates, the antibacterial activity varied among the five pathogenic bacteria; the isolates did not inhibit all pathogens to the same extent (S1 Table). The combination of high antioxidant activity and antibacterial activity was displayed by 21 isolates (Fig 4). Eleven marine fungal isolates inhibited (mean inhibition zone $\geq$ 20 mm) at least two fungal pathogens at the same level as the fungicides used (mean gentamycin 20–24 mm, mean fluconazole 20–23) (S2 Table). Antifungal activity of the four most efficient marine fungal isolates, assessed as their zone of inhibition, was two to fourfold compared to the mean of all isolates (Fig 5). Most of the isolates showed only low antifungal activity and some, in practice, no inhibition (S2 Table).

The genera Acrocalymma, Acremonium, Aspergillus, Buellia, Cladosporium, Emericellopsis, Microdochium, and Phomopsis had the highest cytotoxicity. Lung cancer cell line A549 was inhibited most (97%) by Acremonium sp. SF3 (Table 2). Skin cancer A431 and breast cancer cell line MF7 were inhibited most by Acremonium sp. JEF2, 98% and 85%, respectively. Aspergillus sp. HF7, Microdochium sp. SF87 and P. glabrae YF94 inhibited most HepG2 liver cancer cell line, 89% each. Most of the isolates showed low inhibition: the mean inhibition of all 100 marine isolates against the four cancer cell lines varied between 18% and 23% (SD 20–28). The positive control cisplatin displayed 85% inhibition.

High antioxidant activity, evaluated as DPPH scavenging activity, in combination with high antimicrobial or anticancer activity was observed for 23 marine fungal isolates (Table 3). These isolates were Acremonium sp., Acrocalymma sp., A. africana, A. medicaginis, Cladosporium oxysporium, Emericellopsis sp. E. alkalina, Microdochium sp., M. anisopliae, and P. glabrae, the DPPH scavenging % varying between 62 ± 5% for E. alkalina RAF79 and 97 ± 5% for Acremonium sp. SF3. The mean of all 100 marine fungi DPPH% was relatively low, 29% with SD of 33%. Antioxidant activity evaluated as reducing power was low for almost all marine fungi. Only six Ceratocystis sp. isolates (JUF15, KF16, SF17, DF18, DF19, FF20) showed some reducing power (21–31%).

4. Discussion

Biomedicine is looking for multifunctional drugs because antioxidants seem to improve the efficiency of the treatments of bacterial infections and cancer [32–36]. In our survey, one hundred marine fungal strains included more than 30 potential strains for biomedical applications showing antibacterial, antifungal, antioxidant, and cytotoxic activities to different extents. We chose 23 strains offering the most promising multifunctional drugs to be developed: the strains displayed a varying combination of activities (Table 3). We also raise four fungal inhibitor strains as significant although they showed no antioxidant activity. The reason is that potential antifungal drugs have been found much less than antibacterial drugs. The most potent fungal inhibitors were Acremonium sp., Diaporthe hubeiensis, Lasiodiplodia theobromae, and Nannizia gypsea. The isolate Acremonium sp. JEF1 was among the best multifunctional candidate for further studies due to its combined antifungal, antibacterial and antioxidant activity. However, this isolate had relatively low cytotoxic activity while two other Acremonium isolates had high cytotoxicity. All three Acremonium isolates deserve further studies.

The most remarkable result of the PCA was that the antibacterial activity was accompanied by the capability to inhibit cancer cells and high antioxidant activity assessed as DPPH scavenging activity. The result might be generalizable, and it should be studied further. From the practical point of view, these multifunctional isolates were, however, in the minority. The
Fig 4. Inhibition (zone of inhibition, mean and error bar for SD) of five pathogenic bacteria (in colors) by the metabolites of the marine fungi isolated from the coasts of the Red Sea and the Arabian Gulf. Mean refers to the mean of all isolates tested. Letters after the species names refer to the isolate code in Table 1.

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multifunctional potential was observed for *Acremonium* sp., three *Acrocalymma* sp. including *A. africana*, and *A. medicaginis*, three *Aspergillus* sp., *Cladosporium oxysporium*, two *E. alkalina*, two *Microdochium* sp. and *P. glabrae*. No fungal agents have been approved as anticancer drugs so far, maybe because the mechanisms in action are not understood [37]. However, many species have been shown for their potential as anticancer agents (S3 Table) [14, 38, 39], and for instance, several fungal endophytes have been shown to have anticancer activities [40–42].

Another interesting result of the PCA was that marine fungal strains were grouped according to their ability to inhibit bacteria or fungi. Based on this result, it seems that the marine fungal isolates were specialized to inhibit either bacteria or fungi. Only the genera *Acremonium*, *Emericellopsis* and *Microdochium* included both fungal or bacterial inhibitors, and thus, were not specialized. Most published studies deal with either antifungal or antibacterial activities, which is not surprising because the mechanisms of action differ for bacteria and fungi [43]. We found only some studies where both fungi and bacteria were studied. The plant endophytic fungus *Diaporthe schini* metabolites inhibited several bacteria and the fungus *Candida krusei* [44]. The plant root endophytic fungus *Trichoderma hamatum* inhibited both bacterial and fungal plant pathogens [45]. The plant species *Rumex abyssicus*, *Tagetes lucida*, and *Lallemantia royleana* have been shown to inhibit both bacteria and fungi [46–48]. *Aloe vera* plant extract inhibited several bacterial species and a few fungal species [49]. When marine fungi metabolites were reviewed by [9], most compounds inhibited bacteria. Out of 170
Phomopsis glabrae

Microdochium sp. SF87 83

Microdochium sp. HF88 89

Emericellopsis alkalina

Emericellopsis alkalina

Cladosporium oxysporium

Buellia lauricassiae

Aspergillus sp. JEF2 93

Acrocalymma medicaginis ±

Acrocalymma africana ±

Table 2. Activity (% of inhibition) against four cancer types (mean ± SD, n = 3) of the top 19 marine fungal isolates in comparison to the mean of all 100 marine fungal isolates.

| Fungi                     | Isolate | Lung     | A549 | Skin    | A-431 | Breast | MCF-7 | Liver | Hep G2 |
|---------------------------|---------|----------|------|---------|-------|--------|-------|-------|--------|
| Mean ± SD (n = 100)       | 23 ± 27 | 22 ± 28  | 18 ± 20 | 21 ± 22 |
| Acrocalymma africana     | SF5     | 58 ± 9   | 65 ± 5 | 40 ± 7  | 70 ± 7 |
| Acrocalymma medicaginis  | RAF6    | 48 ± 2   | 76 ± 3 | 52 ± 5  | 66 ± 5 |
| Acrocalymma sp.          | SF3     | 97 ± 3   | 90 ± 8 | 80 ± 3  | 67 ± 3 |
| Acrocalymma sp.          | JEF2    | 93 ± 9   | 98 ± 5 | 85 ± 9  | 69 ± 5 |
| Aspergillus sp.          | DF8     | 44 ± 2   | 48 ± 5 | 56 ± 2  | 68 ± 4 |
| Aspergillus sp.          | HF7     | 37 ± 3   | 55 ± 3 | 62 ± 3  | 89 ± 7 |
| Buellia sp.              | HF10    | 85 ± 9   | 88 ± 9 | 54 ± 6  | 68 ± 5 |
| Buellia lauricassiae     | JEF11   | 86 ± 3   | 66 ± 2 | 23 ± 4  | 45 ± 2 |
| Cladosporium oxysporium  | JEF55   | 84 ± 5   | 55 ± 8 | 50 ± 7  | 28 ± 8 |
| Cladosporium oxysporium  | UF56    | 58 ± 5   | 42 ± 4 | 35 ± 8  | 29 ± 4 |
| Cladosporium oxysporium  | JEF57   | 54 ± 3   | 39 ± 7 | 28 ± 4  | 32 ± 7 |
| Emericellopsis sp.       | JF72    | 81 ± 8   | 80 ± 5 | 63 ± 7  | 59 ± 5 |
| Emericellopsis sp.       | AF71    | 78 ± 4   | 86 ± 3 | 52 ± 5  | 60 ± 4 |
| Emericellopsis alkalina  | JF78    | 93 ± 7   | 90 ± 5 | 55 ± 3  | 62 ± 7 |
| Emericellopsis alkalina  | FF76    | 86 ± 5   | 80 ± 5 | 45 ± 6  | 52 ± 8 |
| Emericellopsis alkalina  | FF77    | 82 ± 3   | 87 ± 9 | 50 ± 9  | 58 ± 4 |
| Microdochium sp.         | HF88    | 88 ± 6   | 68 ± 2 | 35 ± 2  | 56 ± 7 |
| Microdochium sp.         | SF87    | 83 ± 4   | 45 ± 3 | 52 ± 3  | 89 ± 3 |
| Phomopsis glabrae        | YF94    | 86 ± 7   | 62 ± 9 | 73 ± 9  | 89 ± 9 |

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compounds, 26 were fungal inhibitors, of which only six metabolites inhibited both bacteria and fungi. From that perspective, our findings about fungal inhibitors were of importance. Particularly, we can raise* Acremonium *and* Acrocalymma *isolates that inhibited both bacteria and fungi. However, only one of the isolates had also high antioxidant activity.

The comparison to previous studies reveals that most of the genera we report have already been reported for their biological activities, but seldom for multifunctional activity including antioxidant activity. *Acremonium* and *Emericella* strains isolated from soils, waters, and plants have been shown to produce more than 400 different metabolites with a wide range of biological activities [50–53]. An endophytic *Acremonium* showed antifungal activity against *Pythium* sp. causing root rot [54]. The marine *Acremonium* strain has been reported for antibacterial and also for multifunctional activities including cytotoxicity [55, 56]. *Phomopsis* and *Diaporthe* have been shown to produce more than 300 bioactive metabolites, including cytotoxic and antibacterial compounds [57, 58]. *P. glabrae* was among the most active species in our tests. Previously, bioactive polyketides were identified from *Phomopsis* sp. isolated from both marine and terrestrial habitats [13, 59, 60]. *Microdochium* sp. showed antimicrobial activity, as also reported previously [61].

The results of some genera, such as *Ceratocystis* and *Fusarium*, previously reported as potential antimicrobials [62, 63], were not supported by our survey. Some *Cladosporium* isolates collected from all our sampling locations had strong bioactivities. However, most of the commonly found *Cladosporium* isolates showed only weak bioactivities. This is surprising since many *Cladosporium* strains have previously been shown to produce bioactive metabolites [15, 16]. *Cladosporium* sp. has produced almost 300 metabolic compounds with antimicrobial, anticancer, and antioxidant properties [64–66]. Our survey does not raise *Cladosporium* among the most interesting genera in the biomedical field.
The species of Emericellopsis are found in different environments and commonly in marine environments. The genus Emericellopsis is known for its bioactive properties [50]. In our study, the genus was shown to have antibacterial, antifungal, and anticancer activities. The genus has been of particular interest because it has been reported to produce antimicrobial peptides inhibiting drug-resistant organisms [67].

Emericellopsis sp. have been shown to produce several metabolites with antifungal, antibacterial and cytotoxic activities [50, 68]. Emericellopsis commonly produce peptides [67] that have been shown for their multifunctional activities [68]. For instance, the peptide emericellipsin A inhibited human pathogenic fungi and bacteria as well as cancer cells [69]. Emericellipsin A affects cell membranes, which has been suggested to be the mechanism behind the multifunctional activities [70].

A. medicaginis showed bioactivities in our tests. The species was the only one where we found no previous reports about its bioactivities. However, the species appeared to be of particular interest because of its combined antibacterial, anticancer, and antioxidant activity. The fungi A. medicaginis isolated from the southern Red Sea coast deserve further studies for
biomedical applications. Several other isolates such as Acremonium, and D. hubeiensis collected from the Red Sea as well as L. theobrome and N. gypsea from the Arabian Gulf coast deserve further studies.

Both seas are hot and saline with no remarkable difference. The greatest difference between the Red Sea and the Arabian Gulf is the depth. While the former reaches 2000 m, the latter reaches only 100 m [22]. The sea level annual cycle and the water outflow differ markedly [71]. Despite some differences, no evident difference between the seas regarding bioactive fungal metabolites was observed. Some of the species such as C. corybicola and C. tenuissium were isolated from both seas. The isolates differed in their activity regardless of the sea. Some isolates of the same species such as E. alkalina differed in their activity although they were isolated from the same sea. One southern Red Sea isolate of E. alkalina displayed low activities while four of the isolates displayed high activities. Thus, the sea seemed not to be the explaining factor in general. Many different factors affect the microbial metabolism and more isolates need to be studied. It is known that the environmental conditions modify the pathways of microbial metabolism in complex ways, and therefore, microbes in harsh conditions are of great interest in searching novel drugs and other bioactive molecules [72].

5. Conclusion
Marine fungi that were randomly collected from the sea coasts around the Arabian Peninsula showed multifunctional activities potentially valuable for biomedicine. Great differences in the bioactivities of the isolates were observed. Out of one hundred isolates collected, about one-third inhibited pathogenic bacteria, fungi, or cancer cells. All isolates of certain genera, such as Acremonium, Acrocalymma, and Aspergillus, showed some bioactivities. However, in the case of some genera, such as Cladosporium and Ceratocystis, only a few of the isolates showed remarkable bioactivities. Strains of the same species differed in their activity pointing out the need to test several isolates of the species. It appeared that certain genera were efficient fungal inhibitors while some were bacterial and cancer cell inhibitors. The isolates that had high antibacterial and anticancer activities and were accompanied by high antioxidant activity are particularly interesting in biomedical applications due to their multi-functionality. Further studies on the actual bioactive compounds present in the crude extracts are needed. Our study revealed thirty fungal strains that have the potential to produce multifunctional metabolites and offers them for further studies.

Supporting information
S1 Table. Inhibition (zone mm, mean ± SD, n = 3) of four pathogenic bacteria by antibiotics (ampicillin and tetracycline, 5 μg/mL) and marine fungal extracts. Mean ≥ 20 mm against some bacterial pathogens in bold.

S2 Table. Inhibition (zone mm, mean ± SD, n = 3) of five pathogenic fungi by fungicides (Gentamycin and Fluconazole, 5 μg/mL) and marine fungal extracts. Mean ≥ 20 mm against some fungal pathogens in bold.

S3 Table. Variable loadings of PCA for different cancer types, bacterial and fungal pathogens and antioxidant activity assays.
Author Contributions

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