https://doi.org/10.31925/farmacia.2019.5.5 **ORIGINAL ARTICLE**

ISOLATION, IDENTIFICATION AND BIOACTIVITY SCREENING OF TURKISH MARINE-DERIVED FUNGI

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Abstract

Marine-derived fungi are considered as a promising source for discovering new secondary metabolites with pharmaceutical potential. In this study, 18 marine-derived fungi were isolated and identified from marine invertebrates and investigated with regard to their antioxidant, antimicrobial and cytotoxic activities. DPPH, SO, NO and ABTS assays were used for monitoring free radical scavenging activity, and MTT assay was used for cytotoxic activity. For antimicrobial activity determination minimum inhibitory concentration was calculated. As a result, six *Penicillium*, five *Aspergillus*, one *Alternaria, Cladosporium*, *Malassezia, Mycosphaerella, Sporobolomyces, Talaromyces* and *Trichoderma* species were isolated from the marine invertebrate. Some of these fungal extracts such as *Aspergillus chevalieri* has shown high antioxidant and antimicrobial activities, further *Aspergillus awamori*, *Aspergillus niger* and *Penicillium brevicompactum* have shown significant cytotoxic activity against HCT-116 cells. This was the first study about habitant of marine-derived fungi of Turkey's coasts and their antioxidant, antimicrobial and cytotoxicity activities. Besides, it is also the first report about the antioxidant and cytotoxicity activities of *C. funiculosum*, *A. awamori* and *P. crustosum*. Antioxidant and antimicrobial activities of *P. rubens* were reported for the first time in this study.

Rezumat

Ciupercile de origine marină sunt considerate o sursă promiţătoare de metaboliți secundari cu potențial farmaceutic. În prezentul studiu sunt descrise 18 ciuperci, izolate și identificate din nevertebrate marine, pentru care a fost studiată activitatea antioxidantă, antimicrobiană și citotoxică. Pentru a demonstra activitatea de chelatare a radicalilor liberi, s-au folosit metodele DPPH, SO, NO și ABTS, iar pentru activitatea citotoxică, metoda MTT. Pentru activitatea microbiană, a fost calculată concentrația minină inhibitorie. Șase specii de *Penicillium*, cinci *Aspergillus*, o specie de *Alternaria*, o specie de *Cladosporium*, specii de *Malassezia*, *Mycosphaerella*, *Sporobolomyces*, *Talaromyces* și *Trichoderma* au fost incluse în studiu. *Aspergillus chevalieri* a prezentat acțiune antioxidantă și antimicrobiană, în timp ce *Aspergillus awamori*, *Aspergillus niger* și *Penicillium brevicompactum* au prezentat activitate citotoxică pe linia celulară HCT-116. Studiul de față aduce noi informații privind activitatea citotoxică și antioxidantă ale speciilor *C. funiculosum*, *A. awamori* și *P. crustosum*, precum și asupra activității antioxidante și antimicrobiene a speciei *P. rubens*.

Keywords: antimicrobial activity, antioxidant, cytotoxic activity, marine-derived fungi, *Penicillium*, *Aspergillus*

Introduction

About 70% of the earth surface is covered by seas and oceans and their different habitats with extreme pressure, changing levels of salinity and temperature give rise to an enormous source of biodiversity [1]. Marine derived microbial communities due to their extensive genetic, biochemical diversity and wide range of bioactivities including antibacterial, anti-fungal [2, 3], antidiabetic [4], anti-inflammatory [5], antiprotozoal [6], anti-tuberculosis [7], antiviral [8], antitumor, and cytotoxic activities [9] have become a leading hotspot for the discovery of new pharmaceutically active compounds. According to the literature, there are 530 fungal species in 321 genera, among them 424 strains belonging to *Ascomycota* and 94 strains belonging to anamorphic division [10]. Marine-derived fungi which usually do not cause serious damage to the hosts (Coral, sponge, tunicate and etc.) colonize the internal tissues of their hosts harmoniously and produce a wide range of secondary metabolites such as polyketides, terpenes, peptides and compounds of mixed biosynthesis [11].

Antioxidants are agents which protect cells against the deleterious effects of reactive oxygen species that cause degenerative diseases such as aging, cancer, heart dysfunction and Alzheimer's disease. Marine derived fungi are rich sources of potential anti-oxidant secondary metabolites [12, 13].

Infectious diseases claim numerous lives worldwide every year, and many of these infections are caused by multi-resistant microorganisms. According to extensive research, some natural compounds from marine-derived fungi have the potential to be used in clinical trials [14].

According to the latest global census in 2012, there was an estimate of 14.1 million new cancer cases. Some secondary metabolites from marine-derived fungi such as aldehydes, alkaloids and nitrogen-containing heterocycles, chromones, cyclohexanones have cytotoxic effects. These natural compounds and their analogues are promising in a new era of cancer treatment [15].

The intent of this study was to provide an overview of the isolation and identification of marine-derived fungi from marine invertebrates and to evaluate their antimicrobial, antioxidant and cytotoxicity activities. It is the first study about Turkish marine- derived fungi and their bioactivity.

Materials and Methods

Materials

General

1,1-Diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS), (±)-6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Trolox), ascorbic acid, quercetin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-di-phenyl-2H-terazoliumbromide (MTT), sulfanilamide, napthylethylenediamine dihydrochloride and sea salts were purchased from Sigma Aldrich, USA Sabouraud 4% dextrose agar, Muller Hinton broth, Sabouraud dextrose broth and ethyl acetate was purchased from Merck. RPMI 1640 medium, foetal bovine serum (FBS), streptomycin and glutamine were from PAA (PAsching, Austaria), HCT 116 colon cancer cells were kindly provided by Bert Vogelstein. Nutrient agar was prepared from Oxide. Biospeedy® Fungal Diversity Kit was prepared from Bioeksen, Turkey, Biorad CFX Connect was prepared from Bio-Rad Laboratories, USA. For ABI Prism 377 DNA Sequencer, Applied Biosystems, USA was used. Molecular devices Spectra MAX 190 Microplate Reader helps to get absorbance.

Isolation of marine-derived fungi

Marine samples were collected from Turkey's coast. According to the Kjer *et al*., samples (sponges, corals, tunicate, worm, alga, molluscs and annelid) were collected by scuba diving and were transferred in suitable containers in sea water and isolation process was carried out within the next hours to avoid the growth of ambient bacteria. The samples were cut to approximately 1 cm × 1 cm and were rinsed three times with sterile water to eliminate adherent surface debris and for surface sterilization it was immersed in 70% EtOH (vol/vol) for 60 - 120 s. Samples were dried in a sterile cotton cloth and transferred to a Petri dish containing Sabouraud 4% dextrose agar and artificial sea salt. Petri dishes were kept for 5 - 7 daylight at 25°C. Treated fungi were purified several times until obtaining pure fungi strain. The obtained pure fungi strains were stocked in glycerine at -80°C [16]. The sample information is shown in Table I.

Table I

Obtained fungal strains from marine species

Fungus strains	Species	Origin	Location
Alternaria alternata	*Parazoanthus axinella*	Coral	Sinop
Aspergillus awamori	*Parazoanthus axinella*	Sponge	Ayvalik
Aspergillus chevalieri	*Anthozoa sp.*	Coral	Marmara
Aspergillus costaricaensis	*Hermodice carunculata*	Worm	Cirali
Aspergillus niger	*Microcosmus vulgaris*	Tunicate	Saroz
Aspergillus terreus	*Spirorbis sp.*	Annelid	Marmara
Cladosporium funiculosum	*Clavelina lepadiformis*	Tunicate	Ayvalik
Malassezia globosa	*Halimeda tuna*	alga	Ayvalik
Mycosphaerella tassiana_1	*Bursatella leachii*	Mollusca	Seferihisar
Penicillium brevicompactum	*Aplysina aerophoba*	Sponge	Saroz
Penicillium commune	*Mytilus galloprovincialis*	Mollusca	Sinop
Penicillium crustosum	*Axinella damicornis*	Sponge	Saroz
Penicillium griseofulvum	*Eudendrium racemosum*	Coral	Saroz
Penicillium roqueforti	*Pyura momus*	Tunicate	Cirali
Penicillium rubens	*Agelas oroides*	Sponge	Ayvalik
Sporobolomyces carnicolor	*Spirorbis sp.*	Annelid	Marmara
Talaromyces brunneus	*Axinella polypoides*	Sponge	Marmara
Trichoderma harzianum	*Parazoanthusaxinella*	Sponge	Sinop

Identification of Fungi

DNA isolation was performed from fungal cultures using Fungal DNA Isolation Kit. PCR-based Biospeedy® Fungal Diversity Kit was used to determine fungal diversity after spectrophotometric quality checks of the DNA obtained. The kit contains the forward primer (TCCTCCGCTTATTGATATGC) and reverses primer (GGAAGTAAAAGTCGTAACAAGG) pair

(Schochand Conrad, 2012) and optimized qPCR (Real Time PCR) chemistry specific to the ITS1-5.8S-ITS2 region in the fungi genomic DNA. All reactions were performed using Biorad CFX Connect qPCR. The device was subjected to an optimized thermal cycle program specific to primer pair and a melting curve analysis between 65°C and 95°C to determine that only the desired product was amplified during qPCR. qPCR results were analysed in Biorad CFX Connect Software 3.0.

Sequences of the fungal amplicons were determined with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit using ABI Prism 377 DNA Sequencer. Sequence obtained for each fungal sample was analysed in Chromas software package version 1.45.

The sequences were compared with NCBI DNA database ITS regions identified from known fungal species and paired with most similar species. (BLAST). At this step, sequences with similarity ≥ 98% were considered to belong to the same species [17].

Preparation of marine fungal extracts

Pure fungi strains were cultured on a solid medium to obtain marine-derived fungi extract bioactivity as proposed by Kjer *et al*. The strains covering the surface of the inoculated Petri dishes were cut into small pieces of 1.5 cm × 1.5 cm and transferred into 2 L Erlenmeyer flasks contained sterilized rice and artificial sea salt medium, and were kept for 4 - 6 weeks at 25°C. For extraction, EtOAc was added to the culture medium containing the mycelium, the extracts were filtered and evaporated under *vacuum* to obtain EtOAc extracts. The crude extracts were kept at 4°C until use [16].

Bioactivity screening for fungal extracts

Antimicrobial activity

The stock solutions of sample extracts were prepared in dimethylsulphoxide (DMSO) at a final concentration of 512 µg/mL and were sterilized by using 0.22 µm Millipore Membrane Filter (USA). Standard micro-organisms for the antimicrobial activity studies were supplied from Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ankara University. *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 43300), *Staphylococcus epidermidis* (ATCC 35984), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (RSKK 574), *Candida albicans* (ATCC 10231) and *Candida parapsilosis* (ATCC 22019) strains were used for antimicrobial screening. As *per* the recommendations of CLSI, broth microdilution method was used for the determination of the antibacterial and antifungal activity of these samples extracts [18]. The tested two fold serial dilutions of the extracts were between 256 and 0.5 µg/mL. The sealed microplates were placed in a humid chamber and incubated at 35°C, 24 hours for the bacteria and at 25°C, 48 hours for yeasts. The lowest concentration of the extract that completely inhibited the growth of the microorganism was accepted as minimum inhibitory concentration (MIC).

Cell Viability Assay

Colon cancer cell line (HCT-116) were grown in RPMI 1640 medium supplemented with 10% foetal bovine serum, glutamine (2 mM) and 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Trypsinization was used to passage cells. Cytotoxic activity of extracts against HCT-116 colon cancer cells was performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) test. For this purpose, (2 x 10^4 cells/well) were seeded to 96 well plates and incubated for 24 h. Then methanolic extracts (0.1 - 200 µg/mL) were added to the cells and incubated for 24 h. After incubation, MTT reagent (5 mg/mL) was added to the wells and incubated for 4 h. To dissolve the formazan crystals, DMSO was added and the absorbance was measured at 550 nm using microplate reader (Thermo Scientific). GraphPad Prism 7.0 software (GraphPad Software, USA) was used to calculate the IC_{50} values [19].

DPPH Antioxidant Activity Determination

The stock solution of DPPH was prepared freshly in methanol (0.1 mM). Different concentrations of EtOAc extracts in different concentrations were added to an equal volume to methanolic DPPH solution. After 30 min at room temperature, the absorbance was recorded at 517 nm. Ascorbic acid and quercetin were used as standards [20]. Radical scavenging activity was calculated by the following formula:

$$\text{Inhibition\%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100.$$

$ABTS^{\cdot+}$ Antioxidant Activity Determination

$ABTS^{\cdot+}$ stock solution was prepared by mixing 7.4 mM $ABTS^{\cdot+}$ solution and 2.6 mM potassium per-sulfate solution, the obtained solution was allowed to react for 12 - 16 h at room temperature in the dark. The solution was then diluted by mixing $ABTS^{\cdot+}$ solution and methanol to obtain an absorbance of 0.7 ± 0.02 units at 734 nm. In a final volume of 300 µL, the reaction mixture comprised 275 µL of $ABTS^{\cdot+}$ solution were added to 25 µL of the EtOAc extract at various concentrations. Absorbances of these solutions were measured spectrophotometrically at 734 nm [21]. Radical scavenging activity was calculated by the following formula:

$$\text{Inhibition\%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100.$$

The IC_{50} were obtained through extrapolation from a regression analysis. The antioxidant activity was evaluated based on this IC_{50} value.

The antioxidant potential of the EtOAc extracts also was quantified by reference to a Trolox standard

calibration curve. The calibration curve is linear in the range 0.01 to 0.125 mM for Trolox, with an equation of y = 99.709 x + 38.707 and a correlation coefficient of $r^2 = 0.9973$.

Superoxide radical scavenging activity by alkaline DMSO method (SO)

Superoxide radical scavenging activity of the extracts were determined by alkaline DMSO method. Briefly, superoxide radical was generated in non-enzymatic system. 1 mL of alkaline DMSO (5 mM NaOH in 0.1 mL water) was added to 10 µL of NBT (1 mg/mL) and 30 µL of different concentration of extracts or standard compounds. To the reaction mixture containing solution in DMSO) to give a final volume of 140 µL. The absorbance was measured at 560 nm using a microplate reader [22].

Radical scavenging activity was calculated by the following formula:

$$\text{Inhibition\%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100.$$

The IC_{50} were obtained through extrapolation from the regression analysis. The antioxidant activity was evaluated based on this IC_{50} value.

Nitric oxide radical scavenging activity (NO)

NO radical scavenging activity of extracts was determined. Brifley, 60 µL of sodium nitroprusside (10 mM) dissolved in phosphate buffered saline was added to 60 µL of serially diluted extracts and incubated under light at room temperature for 150 min. Finally, an equal volume of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, 2.5% H_3PO_4) was added into each well in order to measure the nitrite content. Absorbance at 577 nm was measured in a microplate reader after the formation of chromofore at room temperature in 10 min [23].

Radical scavenging activity was calculated with the following formula:

$$\text{Inhibition\%} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})/ \text{Absorbance}_{\text{control}}] \times 100.$$

The IC_{50} were obtained through extrapolation from a regression analysis. The antioxidant activity was evaluated based on this IC_{50} value.

Results and Discussion

Marine species listed in Table I were collected from different coasts of Turkey. Six *Penicillium*, five *Aspergillus*, one *Alternaria*, *Cladosporium*, *Malassezia*, *Mycosphaerella*, *Sporobolomyces*, *Talaromyces* and *Trichoderma* species were isolated. EtOAc extracts were obtained and prepared from fungi used for bioactivity tests. Antioxidant activity of extracts was determined by DPPH, ABTS, NO and SO assays. According to the obtained results *A.chevalieri* and *A. terreus* showed high antioxidant activity in all four assays. On the other hand, *A. awamori*, *M. globose*, *M. tassiana*_1 and *T. harzianum* showed the lowest activity. The results are presented in Table II.

Table II

Antioxidant activity of fungal extracts

Fungi Extracts	DPPH	SO	NO	ABTS	
	IC_{50} (µg/mL) ±SD	IC_{50} (µg/mL) ±SD	IC_{50} (µg/mL) ±SD	IC_{50} (µg/mL) ±SD	TEAC (mM_{Trolox}/mg $_{Extract}$)
Alternaria alternata	6294.6 ± 2.1	3303.45 ± 1.1	3539.72 ± 0.96	2877.18 ± 1.68	0.0258 ± 1.68
Aspergillus awamori	> 20000	15811.35 ±0,99	3393.1 ± 1.98	2344.28 ± 1.6	0.0184 ± 1.6
Aspergillus chevalieri	9.04 ± 1.50	109.38 ± 3.8	250.59 ± 1.01	109.64 ± 0.73	1.17 ± 0.73
Aspergillus costaricaensis	456.0 ± 1.24	330.34 ± 1.1	397.16 ± 0.89	194.98 ± 1.76	0.3044 ± 1.76
Aspergillus niger	42.3 ± 1.56	1545,14 ± 0.99	1617.96 ± 0.79	676.08 ± 1.36	0.1804 ± 1.36
Aspergillus terreus	8.07 ± 1.12	46.66 ± 1.5	82.97 ± 1.77	56.23 ± 0.86	1.44 ± 0.86
Cladosporium funiculosum	5874.4 ± 1.36	3971.64 ± 0.87	4255.38 ± 1.54	5011.78 ± 1.62	0.0266 ± 1.62
Malassezia globosa	868.9 ± 1.73	548.23 ± 1.25	629.46 ± 0.29	741.31 ± 1.07	0.0183 ± 1.07
Mycosphaerella tassiana_1	> 20000	9098.5 ± 1.98	1172.11 ± 0.69	1548.16 ± 1.49	0.0146 ± 1.49
Penicillium brevicompactum	222.55 ± 1.09	4255.6 ± 0.77	4456.25 ± 1.2	1862.08 ± 1.46	0.023 ± 1.46
Penicillium commune	42.35 ± 1.66	370.65 ± 0.87	466.62 ± 0.98	398.1 ± 1.35	0.397 ± 1.35
Penicillium crustosum	125 ± 1.24	868.9 ± 0.14	952.73 ± 0.96	831.76 ± 1.36	0.042 ± 1.36
Penicillium griseofulvum	189.19 ± 1.80	1285.15 ± 0.89	1545.14 ± 0.39	1122.01 ± 1.49	0.105 ± 1,49
Penicillium roqueforti	126.12 ± 1.89	810.9 ± 1.5	909.85 ± 1.65	1002.11 ± 1.15	0.0808 ± 1.15
Penicillium rubens	377.49 ± 1.90	2084.3 ± 0.36	1733.68 ± 0.88	2754.22 ± 1.05	0.0272 ± 1.05
Sporobolomyces carnicolor	497.63 ± 1.49	756.78 ±0.87	931.04 ± 1.09	1479.1 ± 1.5	0.0259 ± 1.5
Talaromyces brunneus	392.78 ±1.39	706.26 ± 0.77	868.9 ± 2.11	1174.89 ± 1.42	0.21 ± 1.42
Trichoderma harzianum	601.13 ± 1.82	599.7 ± 0.98	810.9 ± 1.2	933.25 ± 1.17	0.0185 ± 1.17
Ascorbic acid	5.72 ± 0.89	8.52 ± 0.78	10.12 ± 0.19	8.21 ± 0.91	-
Quercetin	7.04 ± 0.94	10.11 ± 0.28	13.18 ± 0.15	11.1 ± 0.23	-

Cytotoxicity activity of these extracts was carried out by using the MTT assay against HCT 116 cells. As shown in Table III, *A. awamori*, *P. brevicompactum* and *A. niger* showed the highest growth inhibition effect against HCT-116 cells where camptothecin was used as a positive standard drug.

Table III

In vitro cytotoxic activity of fungal extracts

Fungi Extract	IC_{50} (µg/mL) ± SD
Alternaria alternata	28.194 ± 2.81
Aspergillus awamori	3.13 ± 0.58
Aspergillus chevalieri	32.38 ± 5.6
Aspergillus costaricaensis	12.03 ± 1.04
Aspergillus niger	4.428 ± 0.60
Aspergillus terreus	99.054 ± 7.36
Cladosporium funiculosum	157.45 ± 6.77
Malassezia globosa	102.70 ± 7.68
Mycosphaerella tassiana_1	20.002 ± 1.589
Penicillium brevicompactum	6.55 ± 1.03
Penicillium commune	182.98 ± 5.25
Penicillium crustosum	104.60 ± 7.02
Penicillium griseofulvum	19.98 ± 1.17
Penicillium roqueforti	13.075 ± 1.85
Penicillium rubens	48.46 ± 6.52
Sporobolomyces carnicolor	> 200
Talaromyces brunneus	165.12 ± 9.51
Trichoderma harzianum	53.78 ± 9.14
Camptothecin	0.305 ± 0.078

Antimicrobial activity of fungal extracts was tested against Gram positive and Gram negative bacterial strains and against yeasts; the results are presented in Table IV. *A.chevalieri*, *A. niger*, *M. tassiana_1*, *S. carnicolor* were shown to have higher antibacterial activity than the other fungi extracts when tested against Gram positive bacterial strains. On the other hand, *P. griseofulvum* showed higher antibacterial activity than the other fungal extracts tested against Gram negative bacteria. *P. roqueforti* and *P. brevicompactum* were more effective than other fungal extracts against tested yeasts.

A number of studies on antimicrobial activity of *A. awamori* as well as its secondary metabolites have been reported until now. Among the isolated compounds (1,4-Dimethoxybenzene, emodin and 3, 6-dibenzyl-piperazine-2,5-dione) only emodin was active against *Staphyloccocus aureus* and *Bacillus subtilis* (MIC: 16 µg/mL and 32 µg/mL respectively) and has also showed high antimicrobial activity against *Enterococcus faecalis* and *A. niger* (MIC: 125 and 85 µg/mL, respectively) [24]. In similar studies on the crude extract of *A. awamori* at a concentration of 200 µg/mL also showed high antimicrobial activity against *Candida rugose* and *Escherichia coli* (zone diameter: 16 and 24 mm respectively) [25]. In this study, the EtOAc extract of *A. awamori* was shown significant cytotoxic activity against HCT 116 cells (IC_{50}: 3.13 ± 0.58 µg/mL), but showed no significant antimicrobial and anti-oxidant activity.

According to the previous studies, 2-carboxymethyl-3-n-hexyl maleic acid and 2-methylene-3-hexyl-butanedioic acid which were isolated from *A. niger* have germination inhibitory effect on plants [26]. In another study, crude extract of *A. niger* showed antimicrobial activity against *Escherichia coli* and *Pseudomonas fluorescens* (18 and 6 mm zone inhibition) [27]. In our study the crude extract of *A. niger* was shown to have significant cytotoxic activity against HCT 116 cells (IC_{50}: 4.428 ± 0.60 µg/mL) and moderate antibacterial activity against Gram positive bacteria, especially against *B. subtilis* (MIC: 62.5 µg/mL). *A. niger*, besides these two bioactivities, has shown high radical scavenging activity.

According to the studies, mycophenolic acid was isolated from *P. brevicompactum* to be responsible for the antimicrobial activity of this fungi [28, 29]. The EtOAc extract of *P. revicompactum* which was found to be effective especially against *S. epidermidis* and *B. subtilis* (MIC: 31.25 µg/mL) showed moderate antioxidant and cytotoxic effect.

Previous studies show, antibacterial activity of *P. crustosum* extracts without and with β-lactamase was reported 16.6 ± 1.1 and 21 ± 1.0 mm inhibition against *Micrococcus luteus* respectively [30]. According to the literature survey, phenylpyropene A, B and C isolated from *P. griseofulvum* and these compounds showed cholesterol acyltransferase inhibitor effect [31]. In our study, *P. griseofulvum* and *P. crustosum* showed antibacterial activity, especially against *S. epidermidis* (MIC: 31.25 µg/mL) and *B.subtilis* (MIC: 31.25 µg/mL) respectively.

Aspergillus species are, widely distributed in nature and the species of this genus have a great ecological and medical importance. They are thought to be the highest producers of bioactive secondary metabolites. Up to now, there are a lot of studies on bioactive secondary metabolites. In a study by Coudhary *et al*., there were tested the bioactivities of secondary metabolites isolated from *A. terreus*. They found that 6-(4'-hydroxy-2'-methyl phenoxy) - (-) - (3R) - mellein and (3R, 4R) -6,7-dimethoxy-4-hydroxy-iminene showed significant antifungal activity against human pathogens. In the same study, 6-(4'-hydroxy-2'-methyl phenoxy) - (-) - (3R) -melle was also found to have strong antioxidant activity [32].

According to the literature survey, forty seven bioactive compounds were identified by GC-MS in the methanolic extract of *A. terreus* and volatile compounds of *A. terreus* were highly effective to suppress the growth of *Streptococcus pneumoniae* [33]. In this study, we investigated the ethyl acetate extracts of *A. terreus* in terms of antimicrobial, cytotoxic and antioxidant activity. These fungal species were found to be active only against *Candida parapsilosa* (250 µg/mL). In addition to this activity, it was found that the fungi examined showed high antioxidant activity and moderate cytotoxicity activity. Bladt *et al*., isolated chloctanspirone A from *P. rubens* this compound was shown activity against HL-60 and A-549 cell lines [34]. In our study, *P. rubens* extract has shown moderate antioxidant and cytotoxicity activity, but it was not active against tested microbial strains.

Table IV

Antimicrobial activity of fungal extracts

Extracts	MIC (µg/µL)								
	Staphylococcus aureus ATCC 25923	*Staphylococcus aureus* ATCC 43300	*Staphylococcus epidermidis* ATCC 35984	*Bacillus subtilis* ATCC 6633	*Escherichia coli* ATCC 25922	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumoniae* RSKK 574	*Candida albicans* 10231	*Candida parapsilosis* ATCC 22019
	Gram Positive				Gram Negative			Yeast	
Alternaria alternata	1000	1000	500	500	500	500	500	1000	250
Aspergillus awamori	500	500	-	-	1000	-	-	500	500
Aspergillus chevalieri	62.5	125	125	125	500	500	500	500	500
Aspergillus costaricaensis	-	-	500	-	500	-	250	500	500
Aspergillus niger	250	500	500	62.5	1000	500	1000	500	500
Aspergillus terreus	500	-	-	-	1000	500	-	1000	250
Cladosporium funiculosum	1000	1000	-	1000	1000	1000	1000	1000	-
Malassezia globosa	500	-	-	-	500	500	-	-	-
Mycosphaerella tassiana_1	250	125	125	62.5	1000	500	-	500	500
Penicillium brevicompactum	500	250	31.25	31.25	500	500	1000	125	250
Penicillium commune	250	-	-	-	1000	500	-	-	-
Penicillium crustosum	1000	500	250	31.25	1000	1000	1000	500	250
Penicillium griseofulvum	500	500	31.25	125	125	250	-	500	500
Penicillium roqueforti	1000	1000	500	250	1000	-	-	250	125
Penicillium rubens	-	-	-	1000	1000	1000	-	-	-
Sporobolomyces carnicolor	250	250	62,5	31.25	1000	500	1000	500	500
Talaromyces brunneus	1000	1000	500	-	-	1000	500	500	250
Trichoderma harzianum	-	-	-	-	1000	500	1000	250	500
Ciprofloxacin	1.25	0.625	0.078	0.078	0.009	0.625	0.039	*	*
Miconazole	*	*	*	*	*	*	*	1.56	0.78

- not active, * no experiment

Isophomenone and 3-deacetylcitreohybridonol were isolated from *P. commune*, and tested against *Bacillus subtilis*, *Candida albicans*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and methicillin resistant *Staphylococcus aureus*, but none of them displayed notable activity [35]. In the other study by *Shang et al*., xanthocillin X, which is a known antibacterial compound and three steroids, two ceramides, six aromatic compounds and three alkaloids were isolated from *P. commune*. In the bioassay, xanthocillin X displayed significant antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*, and cytotoxicity against MCF-7, HepG2, H460, Hela, Du145, and MDA-MB-231 cell lines. Meleagrin, one of the alkaloids that was isolated from *P. commune* exhibited cytotoxicity against HepG2, Hela, Du145, and MDA-MB-231 cell lines [36].

Several studies have been reported on the bioactivity of *Alternaria* species as well as their secondary metabolites. Twenty six bioactive compounds were identified in methanolic extract of *A. alternata* by GC Mass. The antimicrobial activity of the extract has shown inhibition (5.04 ± 0.29 mm) against *Klebsiella pneumoniae* [37]. In a study by Fernandes *et al*., the total phenol content (3.44 GAE/mg) and antimicrobial effect of the crude extract of *A. alternata* was examined. As a result, it was found that the extract was active against *S. aureus* and *E. coli* (MIC: 50 - 100 µg/mL and 400 - 800 µg/mL) but not against *C. albicans*. In addition, the DPPH assay showed no antioxidant effect, antitumor activity was determined by the MTT assay against HeTa cell lines (400 µg/mL) [38]. According to the previous studies, Secondary metabolites from *Alternaria* sp. cyclohexenone derivative, cyclopentenone derivative exhibited potent ABTS scavenging activities. Two xanthone derivatives and fischexanthone from *Alternaria* sp. also showed antimicrobial activity [39]. In the other study by Wang *et al.*, alternariol isolated from *A. alternata* was active against *Bacillus subtilis* (MIC_{80}: 8.6 µg/mL) and alternariol 9-methyl ether showed mild cytotoxic activity against U2OS cell lines (IC_{50}: 28.3 µM) [40]. According to the results obtained in this study, *A. alternata* extract has shown weak antimicrobial and moderate antioxidant and cytotoxicity activity.

In the study which carried out by Silvia- Hughes *et al*., *C. funiculosum* has shown antifungal activity against *Candida fragariae*, *Candida gloeosporioides* and *Candida acutatum* [41]. Brefeldin A, ethyl 2,4-

dihydroxy-5,6-dimethylbenzoate and phomopsi-lactone that was isolated by Singh *et al*., from *Cladosporium* species have shown strong antifungal activity and also *Cladosporium* sp. extract was screened for antibacterial test against *E. coli*, *B. subtilis*, *S. aureus*. The zones of inhibition ranged from 14 - 37 mm [42]. In the previous studies, T22 azaphilone and harzianopyridone isolated from *T. harzianum* showed significant antifungal activity [43, 44]. In present study *C. funiculosum* and *T. harzianum* extracts were shown weak antioxidant, antimicrobial and cytotoxicity activity.

In this study we found that, some of the marine derived-Turkish fungi extracts such as A. *awamori*, *A. niger* and *P. brevicompactum* have shown high cytotoxic activity against HCT 116 cell lines (IC_{50}: 3.13 ± 0.58, 4.428 ± 0.60 and 7.43 ± 2.1 µg/mL respectively). The highest antioxidant activity belonged to *A. chevalieri* and *A. terreus* extracts containing ftavoglaucin and this compound was reported to exert antioxidant activity [45].

Conclusions

In conclusion, in this study 18 fungi were isolated from marine species collected from Turkish coasts. According to the results of the study, six *Penicillium*, five *Aspergillus*, one *Alternaria*, *Cladosporium*, *Malassezia*, *Mycosphaerella*, *Sporobolomyces*, *Talaromyces* and *Trichoderma* species were isolated. This study is a preview study to discover more active fungi species and will serve as guidance for future studies. Some of these fungi extracts displayed significant antioxidant, antimicrobial and cytotoxicity activity. For instance, *A. chevalieri* was the most active fungi against the tested microbial organism and showed high antioxidant activity. Likewise, *A. awamori* was the most effective fungi against HTC 116 cell lines. This is the first study about habitant of marine-derived fungi of Turkey's coasts and their antioxidant, antimicrobial and cytotoxicity activity. Besides, it is also the first report about antioxidant and cytotoxicity activity of *C. funiculosum*, *A. awamori* and *P. crustosum*. In addition of these results antioxidant and antimicrobial activity of *P. rubens* was reported for the first time in this study. Consequently, these extracts originated from the marine derived fungi sources need further investigation with a view to identify the active components that could be developed as potential drugs to be used against diseases threatening human health. This type of studies may help our understanding of the fungal diversity in various marine habitats with different biological activity.

Conflict of interest

The authors declare no conflicts of interest. All the experiments were undertaken in this study comply with the current laws of the country where they were performed.

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