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Antifungal and antioxidant activity of marine derived *Fusarium oxysporum* extract

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Abstract

Marine drugs are employed in the management of several disorders where tolerance arises, necessitating the pursuit of novel therapeutics. Marine fungi are also producers of compounds that may serve as novel therapeutic candidates. This study examined total phenol (TPC), total flavonoid content (TFC), and the antioxidant and antifungal properties of the crude ethyl acetate (EtOAc) extract derived from the *Fusarium oxysporum* fungus isolated from the sea sponge *Petrosia ficiformis* in Hatay/Turkiye. The total phenol content of the extract was found to be 85.42±3.93 mg gallic acid (GAE)/g extract and the total flavonoid content was 50.11±8.35 mg rutin/g extract. The antioxidant capacity of the extract was measured at 0.90 mg GAE/g of extract by DPPH• assay. The antifungal activity of the *F. oxysporum* EtOAc extract was determined against five *Candida* strains. Although the Minimum Inhibitory Concentration (MIC) values obtained against the tested species were not significantly different (7.031-28.125 mg/mL), *C. parapsilosis* was found to be more susceptible to the crude extract than other tested species, followed by *C. glabrata*, *C. albicans* and *C. tropicalis*. In conclusion, it is believed that the marine fungus *F. oxysporum* obtained from the sea of Turkiye is a promising pharmaceutical candidate and that its place in the scientific field should be increased with future studies.

Keywords: Marine fungi, natural medicine, *Candida*, phenolic, flavonoid, antifungal, antioxidant

Introduction

Free radicals are associated with the development of cancer, diabetes, cardiovascular illnesses, autoimmune disorders, and neurological conditions. The identification of antioxidant compounds that inhibit free radical generation is crucial in the prevention and management of autoimmune disorders, diabetes, and various other ailments [1]. *Candida* species are pathogenic microbes prevalent in the human body that can induce mucosal, cutaneous, and systemic infections, especially in immunocompromised individuals. The escalating resistance of *Candida* species to conventional antifungal agents is constraining therapeutic alternatives [2]. Consequently, pharmaceutical research is focused on the identification of novel therapeutic candidate medications.

97.5% of the Earth's surface comprises saltwater, while 2.5% consists of freshwater. Vertebrates, invertebrates, plants, algae, phytoplankton, and microbes inhabiting extreme saltwater habitats generate unique bioactive natural products with structural and chemical characteristics frequently absent in terrestrial natural products. Marine organisms serve as a substantial source of nutraceuticals and prospective agents for the treatment of many ailments [3]. Approximately 75% of newly discovered chemicals from marine species have been extracted from invertebrates, predominantly sponges. Moreover, microbes serve as a significant source of novel metabolites [4]. Fungi are essential contributors to terrestrial and marine ecosystems, constituting a substantial segment of Earth's microbial diversity, and are omnipresent microorganisms spread throughout various habitats [5]. Marine fungi constitute a biochemically diverse assemblage of species that serve as a promising source of novel bioactive natural chemicals [6,7].

Since the 1980s, many studies have focused on natural compounds produced by sponges, algae, sea cucumbers, bacteria, fungi, and other marine organisms. As a result, a variety of products with

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distinct chemical frameworks that can inhibit *Candida* species, including strains resistant to antifungal drugs, have been found. In comparison to all other researched organisms, sponges occupy the highest classification. Terpenoids, sterols, and alkaloids in this category demonstrate diverse inhibitory effects against various *Candida* species [2].

Thus far, the fungal species *Penicillium cf. brevicompactum* Dierckx [8], *Aspergillus insuetus* [9], *Gibberella intermedia*, and *Fusarium subglutinans* [10] have been obtained from *Petrosia ficiformis* sponges, and pharmaceutical investigations have been conducted. *Fusarium* is a genus of filamentous fungi located in several environments, including aquatic systems, soil, and vegetation [11,12]. *F. oxysporum* is a facultative ascomycete fungus that frequently impacts plants and is regarded as a morphologically diverse species complex. This phytopathogenic fungus is a semi-biotrophic species including over 120 specialized forms categorized by host specialization [13,14]. Certain strains of *F. oxysporum* have been identified as possible biological control agents exhibiting activity against fungi, pests, and certain insects [15]. Moreover, numerous secondary metabolites, including alkaloids [16,17], anthranilic acid derivatives [18], flavantrones, quinones, jasmonates [19], fumonisins [20], cyclic peptides, and terpenoids, exhibiting antibacterial [21-24], cytotoxic [25,26], insecticidal [26,27], and antioxidant [28] properties, have been documented from *F. oxysporum*, recognized as a prolific source of diverse enzymes.

This study aimed to determine the total phenol and total flavonoid content of the crude ethyl acetate extract(EtOAc) of *F. oxysporum* fungus obtained from *Petrosia ficiformis* sponges collected from the Samandag Port in Hatay, and to elucidate its antioxidant and antifungal effects against 5 different *Candida* species.

Material and Methods

Since no *in vivo* or clinical trials were conducted in this study, ethical committee approval is not required.

Fungal Strain, Incubation and Extraction

The host organism, the sponge *Petrosia ficiformis*, was collected by Dr. Biologist Gözcelioğlu from a depth of 30 meters in the Port of Samandag, Hatay, Turkiye, in November 2020 (36°4'13"N 35°52'56"E). The fungus was recovered from the external part of P. ficiformis. For identification, the fungus was grown on Sabouraud 4% dextrose agar (SDA, Merck, Germany) at room temperature for a week in the darkness. The fungus was identified as *F. oxysporum* (GenBank accession number PX745138) based on ITS rDNA sequence data. The fungal strain has been maintained in the Department of Pharmacognosy, Faculty of Pharmacy, Inonu University.

The rice broth was made by combining 100 g of rice with 3.5% sea salt and 100 mL of distilled water in 1000 mL Erlenmeyer flasks. The broth was then sterilized using autoclaving. The fungal strains cultivated on Sabouraud's 4% dextrose agar medium were transferred in fragments to the rice medium in the Erlenmeyer flask. The fermentation process spanned a duration of 30 days at room temperature, within a lightless setting, devoid of any agitation.

The fungi that had completed mycelial and spore formation were subjected to the extraction process by the addition of EtOAc (3x300 mL). Following a 24-hour period of maceration, the extracts underwent filtering using a vacuum filtration method. During the subsequent phases, the fungus underwent a minimum of three cycles of 24-hour agitation, filtering, and evaporation under reduced pressure at a temperature of 40 °C until a solid residue, EtOAc extract (1.25 g) was obtained. The viscous extract was kept at +4°C until the assays.

Total Phenolic Compounds (TPC) Assay

The TPC of the extracts was determined as gallic acid equivalent utilizing the Folin-Ciocalteu method [29]. 100 µL of sample solution in methanol and 500 µL of Folin-Ciocalteu reagent were introduced into a 10 mL vessel containing 6 mL of distilled water. After one minute, 1.5 mL of 20% aqueous Na_2CO_3 was introduced, and the solution was then diluted to 10 mL with distilled water. The reagent mixture lacking the extract served as a control. Absorbance was recorded relative to a blank at 760 nm and incubated at 25°C for 2 hours prior to comparison with the gallic acid calibration curve. The TPC was quantified as gallic acid equivalent. Experiments were performed in triplicate, and the data were provided as mean values.

Total Flavonoid Compound (TFC) Assay

The TFC of the extract was determined by modifying the method utilizing in the study by Zhishen et al. (1999) [30]. Therefore, 1 mL of extract was mixed with 0.3 mL of 5% $NaNO_2$ solution. After five minutes, 0.3 mL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added. After one minute, 2 mL of 1 M NaOH solution was introduced, followed by the addition and mixing of 2.4 mL of water. Absorbance was quantified relative to a methanol blank at 510 nm. The total flavonoid content of the extracts was quantified as rutin equivalent.

Antioxidant Activity by DPPH• Assay

The DPPH• (1.1-diphenyl-2-picrylhydrazyl; TCA brand) test was performed according to a modified method [31]. The DPPH• solution was formulated as 0.1 mM using methanol (MeOH, Merck). Gallic acid (GAE; Sigma-Aldrich) was chosen as the reference compound for comparison. The stock solution of gallic acid was formulated as 1 mg/mL with MeOH and prepared as 50% dilutions. A stock solution of fungal EtOAc(Merck) extract at 6.4 mg/mL was obtained using a solvent mixture of Dimethyl sulfoxide (DMSO, Merck) and MeOH in a 1:9 ratio. Dilutions were prepared as 50% dilutions. 50 µL of sample and 150 µL of DPPH• solution were dispensed into each well of a 96-well plate. Absorbance measurements were conducted at 517 nm after a duration of 30 minutes. The trials were conducted thrice for both the standard substance and the extract. The antioxidant capacity of the extract is expressed as mg GAE/g extract based on IC_{50} values.

Antifungal Activity

Tested Strains and Media

To test the antifungal activity of the *F. oxysporum* EtOAc extract, five yeast strains were used. These strains are *Candida tropicalis*

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(ATCC 13803), *Candida krusei* (ATCC 14243), *Candida parapsilosis* (ATCC 22019), *Candida glabrata* (ATCC 2001) and *Candida albicans* (ATCC 14053), and were purchased from the American Type Culture Collection. For the activity tests, Sabouraud Broth (Biolife, Milan, Italy) and Sabouraud Agar (Chemsolute, Renningen, Germany) were used.

Determination of Antifungal Activity

The antifungal activity of the *F. oxysporum* EtOAc extract was determined using a previously reported method with minor modifications [32-34]. According to this method, the MIC values of *F. oxysporum* crude extract were determined against the tested *Candida* species. Initially, 450 mg of crude extract was mixed with 550 mg of DMSO, and the final concentration was distributed into the first wells of 200 µL microplates and diluted twofold in Sabouraud Broth. As a result of this process, the crude extract concentration ranged from 450 mg/mL to 0.879 mg/mL across the first to the tenth well. Fungal suspensions for the test were prepared to the 0.5 McFarland standard (approximately 1-1.5×106 CFU/mL), and 1 µL of the microorganism was inoculated into each well (except the negative control). The eleventh well contains only microorganisms and medium and is arranged as a positive control to verify bacterial viability. The twelfth well contains only medium and is used as a negative control to assess contamination. The microplates were incubated at 36.5 °C for 24 hours. The next day, resazurin was applied to all wells, and the plates were kept at the same temperature for approximately four more hours. Afterward, the color change in the microplates was evaluated.

Result

Total Chemical Content

All analyses were performed in triplicate. Results are expressed as mean ± standard deviation (SD). Statistical analysis of the data was performed using GraphPad Prism (v.X) software. Differences between groups were evaluated using the Mann–Whitney U test, and $p<0.05$ was considered statistically significant. The total phenolic content of the extract was determined as 85.42±3.93 mg GAE/g extract, and the total flavonoid content was 50.11±8.35 mg rutin/g extract (n=3). A statistically significant difference was not found between the total phenol and total flavonoid measurements.

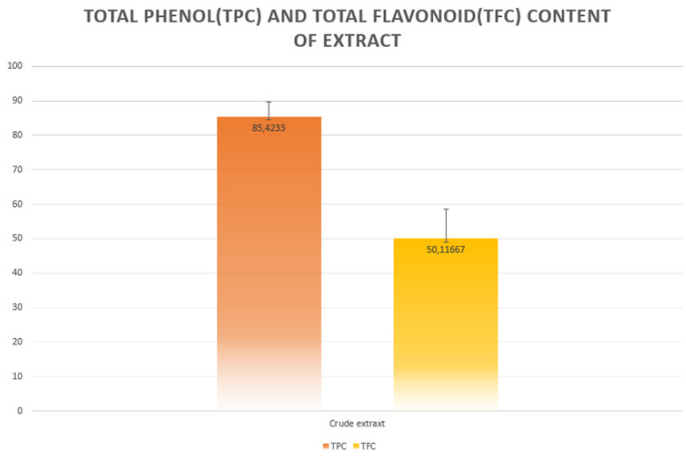

Figure 1. The TPC and TFC of *F. oxysporum* EtOAc extract

Antioxidant Activity Result

The DPPH• radical scavenging activity of the extract was quantified by the reduction in absorbance at 517 nm. Values are presented as mean ± standard deviation (n=3). The IC_{50} value was determined by formulating a regression equation derived from the percentage inhibition (Formula 1; Figure 2). The IC_{50} values of the extract and gallic acid are 2.01±0.04 mg/mL and 0.00181±0.00004 mg/mL, respectively. The antioxidant capacity of the extract was measured at 0.90 mg GAE/g of extract based on this IC_{50} value (Formula 2). Absorbance values reduce with increasing extract concentration, indicating a dose-dependent DPPH• radical scavenging activity.

Formula 1 % Inhibition = $(Absorbance_{control}-Absorbance_{sample})/Absorbance_{control}*100$

Formula 2 Antioxidant capacity = $(IC_{50}$ GAE/IC_{50} Extract)*1000

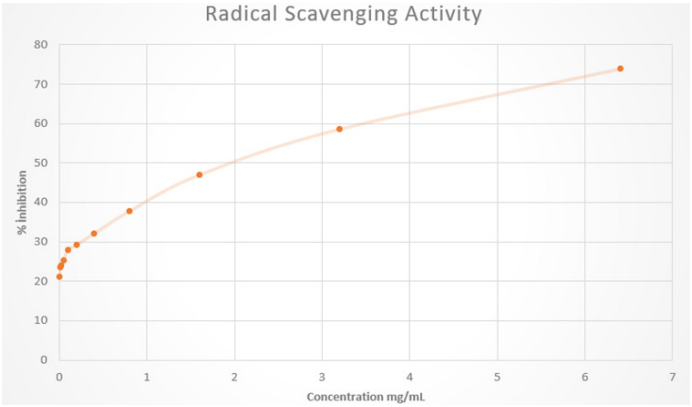

Figure 2. Radical Scavenging Activity of *F. oxysporum* EtOAc Extract

Antifungal Activity Results

Antifungal test results were evaluated based on color changes in the wells on the microplate, and MIC values were determined [34,36]. As seen in Figure 3, *C. glabrata*, *C. albicans*, and *C. tropicalis* grew from the seventh well onward. Therefore, the MIC of *F. oxysporum* crude extract against these yeast species was determined to be 14.063 mg/mL. Since *C. krusei* showed growth from the sixth well onwards, the MIC value of the crude extract against *C. krusei* was determined as 28.125 mg/mL. Moreover, since *C. parapsilosis* showed growth from the eighth well onward, the MIC against this microorganism was 7.031 mg/mL (Figure 3 and Table 1).

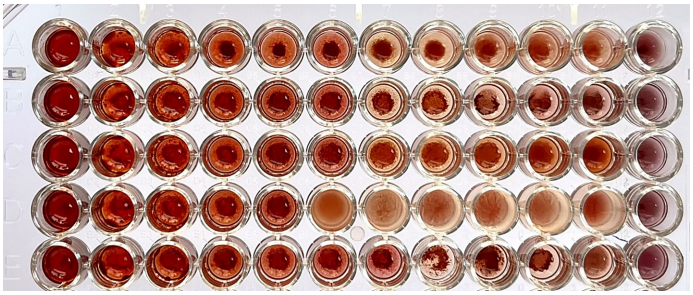

Figure 3. Antifungal activity test results of *F. oxysporum* EtOAc extract against *C. glabrata* (A), *C. albicans* (B), *C. tropicalis* (C), *C. krusei* (D), *C. parapsilosis* (E). 11th and 12th wells are the positive and negative controls, respectively

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Table 1. MIC values of *F. oxysporum* EtOAc extract against tested yeast strains

Microorganisms	*F. oxysporum* crude extract (mg/mL)
C. glabrata	14.063
C. albicans	14.063
C. tropicalis	14.063
C. krusei	28.125
C. parapsilosis	7.031

Discussion

In the discovery of new drugs, the first step in research on natural resources is the preparation of extracts. The biological activity of this extract indicates the presence of active metabolites within it. This allows for the isolation of new compounds as part of the drug candidate discovery process.

To date, only three studies have been found on marine associated *F. oxysporum*. The primary study, performed in 2015 by Chen et al., examined the antioxidant properties of extracellular polysaccharides derived from *F. oxysporum* (Genbank code JN604549). The fungus was obtained from the leaves of the Ipomoea pes-caprae (Linn.) mangrove species collected in the South China Sea. Following laboratory culturing of this fungus, a polysaccharide, Fw-1 (Glcp glucopyranose, Manp mannopyranose, Galf galactofuranose, n≈16), was isolated. The scavenging abilities of Fw-1 against hydroxyl, DPPH•, and superoxide radicals were directly proportional to the concentration (2–10 mg/mL). While the potential antioxidant activity of this polysaccharide was determined when compared to ascorbic acid, a natural antioxidant, further studies are needed to understand the mechanism of its antioxidant activity [37]. In another study researched by Nenkep et al., *F. oxysporum* (Genbank code MSA392) was obtained from marine mud collected in Suncheon Bay, Korea. The crude EtOAc extract of the fungus showed *in vitro* antimicrobial activity against methicillin-resistant and multidrug-resistant Staphylococcus aureus (MRSA and MDRSA) in primary screening. Based on this data, isolation studies were initiated, yielding oxysporisoline, a tris-anhydrotetramer of anthranilic acid (α-aminobenzoic acid), a novel antibacterial alkaloid; the 1H-indole-3-butanamide alkaloid; and two polyketides, chlamidosporol and butenolide (4-acetamido-4-hydroxy-2-butenoic acid r-lactone). The preliminary activities of the crude extract demonstrated the presence of the active compounds within it [28]. In another study conducted in 2024, five new compounds, stacylins H-K (hydroxyphenylacetic acid derivatives) and 4-(2.3-dihydroxy-3-methylbutoxy)-benzyl acetate (hydroxyphenylethanol derivative), and seven known compounds were obtained from *F. oxysporum* F0888 (GenBank code OR960654) isolated from sediments in the South China Sea. These 12 compounds were investigated for antimicrobial activity on seven types of bacteria (*S. aureus, S. epidermidis, E. coli, P. aeruginosa, V. harveyi, V. alginolyticus,* and *V. parahaemolyticus*) and one type of fungus (*C. albicans*), and for anti-inflammatory activity on RAW 264.7 macrophages by cell viability testing using the MTT assay method. Unfortunately, all of the isolated compounds were inactive [38].

Since *F. oxysporum* is a fungal microorganism, studies in the literature to date have focused on the antifungal efficacy of various substances against *F. oxysporum* [39-41]. The only antimicrobial study on *F. oxysporum* in the literature was conducted by Nenkep et al [28]. An antibacterial study of oxysporizolin (polycyclic quinazoline alkaloid) obtained from *F. oxysporum* has been conducted, but the study focuses on a single compound obtained from yeast extract and the results do not represent the entire yeast species. Therefore, this study is the first in the literature to investigate the antifungal activity of *F. oxysporum* extract.

In this study, the analysis determined that the TPC of the extract was 85.42±3.93 mg GAE/g extract, while the total flavonoid content was 50.11±8.35 mg rutin/g extract. The elevated TPC relative to the TFC indicates a substantial presence of non-flavonoid phenolic compounds, including phenolic acids, tannins, and other polyphenols. The findings suggest that the extract encompasses a wide range of secondary metabolites, notably phenolic compounds. The phenolic components may be associated with the possible biological activity of the extract. Nonetheless, the antioxidant capacity of the extract, as assessed by the DPPH• assay, was quite low (0.90 mg GAE/g extract). This mismatch indicates that the antioxidant activity of the extract is not exclusively reliant on the overall phenolic concentration but is significantly affected by the structural properties and reactivity of specific phenolic components. This preliminary study demonstrated the antifungal activity of *F. oxysporum* EtOAc extract against the tested fungal species. *C. parapsilosis* was found to be more susceptible to the crude extract than other tested species, followed by *C. glabrata C. albicans* and *C. tropicalis*. Although the MIC values obtained against the tested species were not significantly different (7.031-28.125 mg/mL), these values will guide further studies regarding the potential use of the crude extract in the pharmaceutical medical field.

Conclusion

In vitro investigations have resulted in the isolation of fungi from sponges located in Turkiye. This study, undertaken for a novel medication candidate, has demonstrated that the fungus *F. oxysporum* possesses a substantial total phenolic content, a considerable fraction of which comprises flavonoids. Despite *F. oxysporum* extract exhibiting limited antioxidant and antifungal properties, it remains a significant species for the exploration of novel chemicals. Future efforts must focus on isolating new metabolites from this marine fungus and conducting activity tests related to these compounds.

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Fungal species identification and Genbank registration processes were carried out by BMLabosis company (https://www.bmlabosis.com/).

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Ethical committee approval was unnecessary for this study.

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